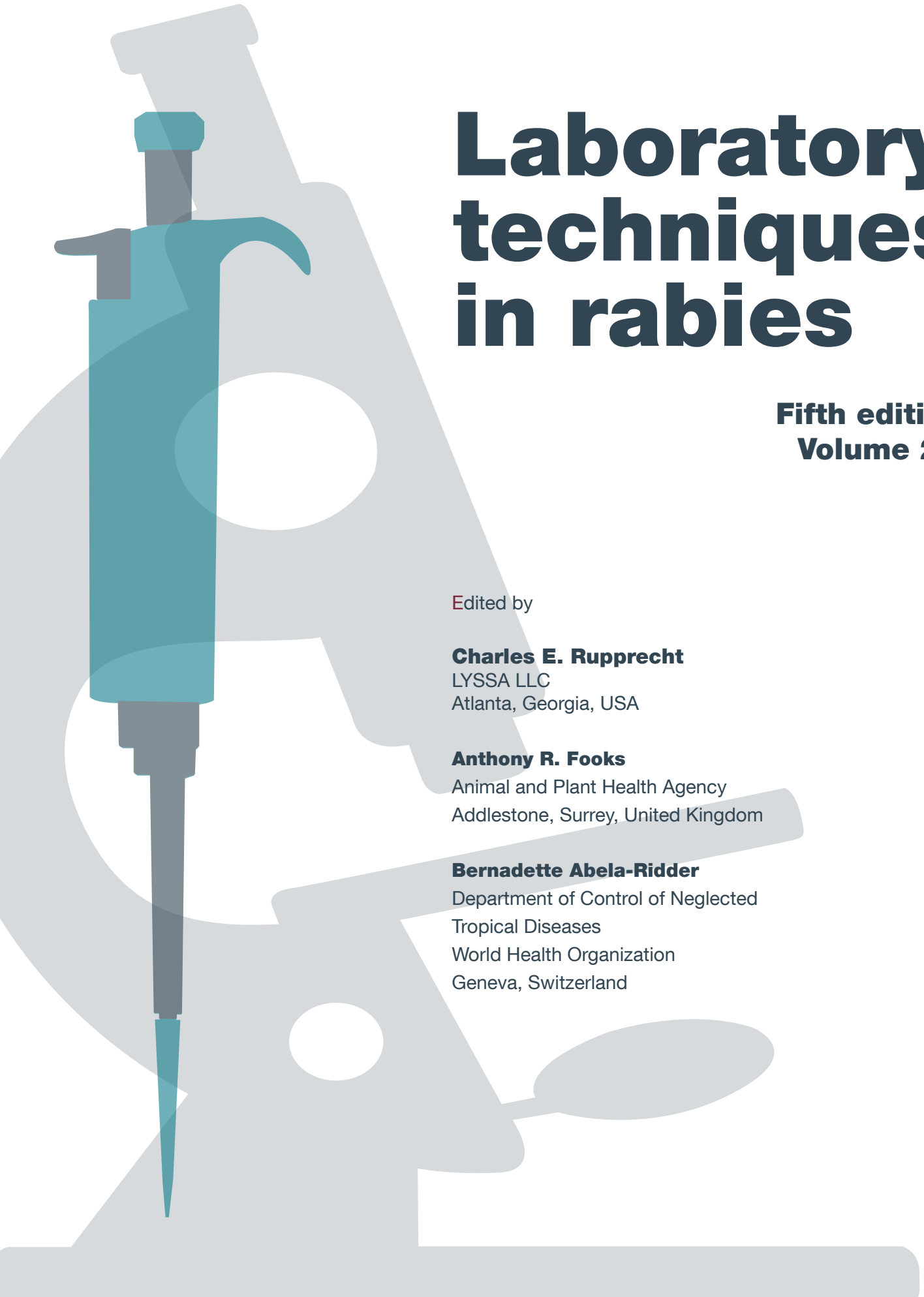

Laboratory techniques in rabies

Fifth edition
Volume 2

Edited by

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Contents

Laboratory techniques in rabies
Foreword

For more than 5000 years, humans have lived in fear of a bite from a rabid animal, so much so that the first written account of rabies, in the 23rd century BC, set the penalty for an owner's dog biting another individual at "two-thirds of a mine of silver", or about a half-day's work. Today, our focus is more on preventing rabies and advocating for its elimination, rather than imposing penalties, and our understanding of the virus has greatly improved since the 23rd century BC.

The Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE) and the World Health Organization (WHO) have prioritized action against rabies and, together with Member countries, have set a goal of zero rabies deaths by 2030. Diagnostics are crucial in attaining this goal.

New laboratory techniques and advancements in science have yielded better diagnostic techniques and control strategies to aid the more than 3 billion people, mainly children, in Asia and Africa who are threatened by the virus every day. Rabies is a preventable disease, yet despite the availability of efficacious and affordable vaccines, more than 60 000 people worldwide die agonizing deaths every year from the disease.

No diagnostic tests are available to detect the rabies virus before the onset of clinical disease, and further research on diagnostic techniques in the field of rabies is therefore paramount. The impact of suitable laboratory capacity on surveillance and elimination of the disease worldwide is evident.

The OIE's Manual of diagnostic tests and vaccines for terrestrial animals provides internationally agreed standards for the production and control of validated veterinary diagnostic methods and vaccines for use in animals. The fourth edition of WHO's Laboratory techniques in rabies has been a guiding reference for many rabies laboratories. The first edition (1954) stated that "rabies research is far from static" and, since its publication more than 60 years ago, OIE and WHO have worked to evaluate subsequent advancements in laboratory techniques in rabies. This fifth edition provides insight into validated methods recommended for use in diagnostic laboratories, but it also includes research. While not currently applicable to all settings, these research methods may stimulate the development of improved techniques for diagnosis of rabies in the future. Improved diagnostics will strengthen surveillance of the disease, leading to enhanced control of rabies where it is most needed.

Food and Agriculture Organization of the United Nations

WORLD ORGANISATION FOR ANIMAL HEALTH

World Health Organization

Laboratory techniques in rabies
Preface

Rabies has an enormous impact on both agriculture and conservation biology, but its greatest burden is undeniably on public health. As such, routine methods for rapid risk assessment after human exposures to rabies as well as applications for laboratory-based surveillance, production of biologicals and management of this infectious disease are critical. Given its mandate to improve human health and control disease among its Member States, WHO has led the production of this fifth edition of *Laboratory techniques in rabies*.

During the more than 60 years that have elapsed since the first edition was published, methods of viral diagnosis, characterization of pathogens and production of biologicals have advanced. At that time, only a single etiological agent was recognized as causing rabies. Detection of Negri bodies was the standard for diagnosis. Nerve tissue-based vaccines were the norm. Combination use of vaccines and rabies immunoglobulins in human prophylaxis was not standard. Global elimination of canine rabies was merely a dream. Rabies in wildlife was managed via population reduction. All of that has changed for the better.

In the ensuing decades, further advancements in detection, prevention and control of lyssaviruses have been monitored by regular meetings of WHO experts, international research groups and countries in which rabies is endemic. The second edition of the manual was published in 1966, the third in 1973 and the fourth in 1996. The late Martin Kaplan and Hilary Koprowski were instrumental in editing the previous editions, as was input on the fourth edition by François-Xavier Meslin, now retired from WHO. Initial plans for preparation of this edition were made in 2016 and its contents were discussed at the WHO Expert meeting on rabies (Bangkok, Thailand) and modified in response.

This fifth edition of *Laboratory techniques in rabies* contains 44 detailed chapters written by more than 85 authors from Africa, the Americas and Eurasia. The text was peer reviewed by Dr Matthias Schnell, Head of the WHO Collaborating Centre for Neurovirology; Professor Thiravat Hemachudha, Head of the WHO Collaborating Center for Research and Training on Viral Zoonoses; and Dr Asefa Deressa, Team Leader of Zoonoses Research at the Ethiopian Public Health Institute. The manual focuses on the basic methods for detection of lyssavirus antigens, antibodies and nucleic acids and the relevance of their use under different operating conditions, from the basic to the advanced. The chapters on older, less sensitive techniques used to detect Negri bodies have been removed, as have those chapters on methods of vaccine production given the progress made in the commercial use of tissue culture products in human and veterinary medicine. Recommendations for the preparations of antibodies by homologous or heterologous production have been replaced by newer methods in an effort to promote a next generation of less expensive and more readily available immunoglobulins in the future. Other basic chapters have been retained and updated and more than a dozen added. Each of the protocols described are prescriptive and should be followed point by point in the laboratory.

We gratefully acknowledge the collaboration of the many eminent scholars who contributed to the current volume, and look forward to the publication of the next edition as continued advances in the field are made.

List of abbreviations and acronyms used in this manual

3Rs	"Replacement, Reduction and Refinement" of laboratory animal testing
AALAS	American Association for Laboratory Animal Science
Ab	antibody
ABLV	Australian bat lyssavirus
ACD	acid citrate dextrose
ACIP	Advisory Committee on Immunization Practices
ACS	American Chemical Society
AEC	3-Amino-9-ethylcarbazole
Ag	antigen
ANSM	Agence Nationale de Sécurité du Médicament et des produits de santé
AMA	African Medicines Agency
AP	alkaline phosphatase
APS	ammonium persulphate
ARAV	Aravan virus
ATCC	American Type Culture Collection
AVMA	American Veterinary Medical Association
BBLV	Bokeloh bat lyssavirus
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BEEM	better equipment for electron microscopy
BHK	baby hamster kidney
bnAbs	broadly neutralizing antibodies
bp	base pair
BP	British Pharmacopeia
BPL	β-propiolactone
BRP	Biological Reference Preparation
BSA	bovine serum albumin
BSC	biosafety cabinet
BSL	biosafety level
CCID	cell culture infectious dose
CDC	United States Centers for Disease Control and Prevention
cDNA	complementary deoxyribonucleic acid
CER	chicken embryo-related
CFIA	Canadian Food Inspection Agency

CHAPS	3-(3-cholamidopropyl) dimethylammonium 1-propanesulfonate
CHO	Chinese Hamster Ovary cells
CIE	counter immunoelectrophoresis
CLRW	clinical laboratory reagent water
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebrospinal fluid
Ct	Cycle threshold
CVS	challenge virus standard strain
ddNTP	dideoxynucleotide
DDSA	dodecenyl succinic anhydride
dNTP	deoxynucleosidetriphosphate
DEAE	diethylaminoethyl
Defra	Department for Environment, Food and Rural Affairs
DEPC	diethylpyrocarbonate
DFAT	direct fluorescent antibody test
DH20	distilled water
DIG	digoxigenin
DMEM10	Dulbecco's minimum essential medium with 10% fetal calf serum
DMP30	tris dimethylaminomethyl phenol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide-tri phosphate
DPX	mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate) and xylene
DRIT	direct rapid immunohistochemistry test
dsDNA	double stranded DNA
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	dithiothreitol
DUVV	Duvenhage virus
EBLV-1	European bat lyssavirus, type 1
EBLV-2	European bat lyssavirus, type 2
ED50	50% end-point
EDQM	European Directorate for the Quality of Medicines
EDTA	ethylenediaminetetraacetic acid
EIU	equivalent international units
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EMEM	Eagle's minimum essential medium
EPAA	European Partnership for Alternatives to Animal Testing
ERA	Evelyn Rokitniki Abelseth strain
ERIG	equine rabies immunoglobulin

ESI	electrospray ionization
EtBr	ethidium bromide
EVAg	European Virus Archive Global
Fabs	antigen-binding fragments
FACS	fluorescence-activated cell sorting
FAVN	fluorescent antibody virus neutralization test
FBS	fetal bovine serum
FCA	Freund's Complete Adjuvant
FCS	fetal calf serum
FFID	fluorescent focus infectious dose
FFPE	formalin-fixed, paraffin-embedded
FIA	Freund's Incomplete Adjuvant
FIMT	fluorescence inhibition microtest
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
FRET	Fluorescence Resonance Energy Transfer
FTA	Flinders Technology Associates
G	glycoprotein
GBLV	Gannoruwa bat lyssavirus
GFP	green fluorescent protein
GM	genetically modified
GMEM	Glasgow Minimum Essential Medium
GMP	Good Manufacturing Practices
gRNA	genomic RNA
HBO	mercury luminance unforced cooling lamp
HDCV	human diploid cell vaccine
H&E	hematoxylin and eosin
HEK	human embryonic kidney
HEP	high egg passage strain
HEPES	hydroxyethyl piperazine ethane sulfonic acid
HIV	human immunodeficiency virus
hn	hemi-nested
HPLC	high-performance liquid chromatography
HRIG	human rabies immunoglobulin
HRP	horse radish peroxidase
IAA	iodoacetamide
IACUC	Institutional Animal Care and Use Program
IBCMP	integrated bite case management program
IC (i.c.)	intracerebral
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods

ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICTV	International Committee on Taxonomy of Viruses
IEF	isoelectric focusing
IFA	indirect fluorescent antibody test
IgG	immunoglobulin G
IgY	immunoglobulin Y
IMAC	immobilized metal affinity chromatography
IPTG	Isopropyl-ß-D-1-thiogalactopyranoside
ISH	in situ hybridization
IIA	immunoperoxidase inhibition assay
ICTV	International Committee on the Taxonomy of Viruses
IHC	immunohistochemistry
IFA	indirect fluorescent antibody
IIF	indirect immunofluorescence
IKOV	Ikoma lyssavirus
i.m.	intramuscular
i.p.	intraperitoneal
IPC	in-process control
IRIT	indirect rapid immunohistochemistry test
IRKV	Irkut virus
IS	indicator serum
ISH	in situ hybridization
IU	international unit
KHUV	Khujand virus
L	" large " protein (i.e. the viral RNA-dependent polymerase)
lacZ	structural gene for ß-galactosidase
LAMP	loop-mediated isothermal amplification
LB	Luria-Bertani broth
LBV	Lagos bat virus
LC	liquid chromatography
LD_{50}	50% lethal dose
LED	light-emitting diode
LEP	low egg passage strain
LFA	lateral flow assay
LFD	lateral flow devices
LIMC	low and middle-income countries
LLEBV	Lleida bat lyssavirus
M	matrix protein
MAb	monoclonal antibody
MALDI	matrix-assisted laser desorption/ionization
MCIE	modified CIE

MEM	modified Eagle's medium
MES	2-(N-morpholino) ethanesulfonic acid buffer
MIT	mouse inoculation test
MLV	murine leukaemia virus
MNA	murine neuroblastoma cell
MNT	mouse neutralization test
MOI	multiplicity of infection
MOKV	Mokola virus
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
M/Z	mass to charge ratio
N	nucleoprotein
NA	numerical aperture
NAA	nucleic acid amplification
nAb	neutralizing antibody
nAChR	nicotinic acetylcholine receptor
NASBA	nucleic acid sequence based amplification
NBT	nitro blue tetrazolium
NC	negative control
NC	nucleocapsid
NCBI	National Center for Biotechnology Information
NDDR	National Donor Referral Registry
NGS	next generation sequencing
NIBSC	National Institute for Biological Standards and Control
NIH	National Institutes of Health (USA)
NMDA	N-Methyl-D-aspartic acid
NMRI	Naval Medical Research Institute
NS	negative serum
NSS	non-specific staining
NTC	no template control
OCT	optimal cutting temperature
OD	optical density
OMCL	Official Medicine Control Laboratories
OIE	World Organisation for Animal Health
ORF	open reading frame
P	phosphoprotein
PAHO	Pan American Health Organization
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PC	positive control

PCEC	purified chick embryo cell vaccine
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethyleneimine
PEP	post-exposure prophylaxis
Ph Eur	European Pharmacopeia
pI	isoelectric point
PNA	pseudotype neutralization assay
PPE	personal protective equipment
PPHS	passive public health surveillance
PMF	peptide mass fingerprinting
PT	proficiency test or testing
PTFE	polytetrafluoroethylene
PTV	pseudotyped viruses
PV	Pasteur virus
PVRV	purified vero cell rabies vaccine
QA	quality assurance
QC	quality control
RABV	rabies virus
RER	rough endoplasmic reticulum
RF	rheumatoid factors
RFFIT	rapid fluorescent focus inhibition test
rG-F	recombinant G truncated protein
RIDT	rapid immunochromatographic diagnostic test
RITM	Research Institute of Tropical Medicine
RLU	relative light unit
RIG	rabies immunoglobulin
rN	recombinant nucleoprotein
RNA	ribonucleic acid
RNP	ribonucleoprotein
rP	recombinant P protein
RP	relative potency
RPMI	Roswell Park Memorial Institute medium
rRNA	ribosomal RNA
RS	reference serum
RTCIT	rabies tissue culture infection test
RT-LAMP	reverse transcriptase loop-mediated isothermal amplification
RT-PCR	reverse transcriptase polymerase chain reaction
RVNA	rabies virus neutralizing antibodies
SAD	Street Alabama Dufferin strain
scFv	single-chain antibody fragment
SD	standard deviation

SDS	sodium dodecyl sulfate
SE-HPLC	size exclusion high-pressure liquid chromatography
SFIMT	simplified fluorescence inhibition microtest
SHIBV	Shimoni bat virus
SMB	suckling mouse brain
SNP	single nucleotide polymorphism
SPA	serological potency assay
SPRI	solid phase reversible immobilization [beads]
SRIG	standard rabies immunoglobulin
SSC	saline-sodium citrate
ssRNA	single stranded RNA
SYBR	Synergy Brand
Taq	thermostable DNA polymerase via bacterium (Thermus aquaticus)
TBS	tris-buffered saline
T-DNA	transfer DNA
TC	tissue culture
TCID	tissue culture infectious dose
Thyb	probe hybridization temperature
TPBS	Tween phosphate buffered saline
TBE	tris borate EDTA buffer
TE	tris EDTA buffer
TEA	triethanolamine
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylenediamine
TLR3	Toll-like receptor-3
TOF	time-of-flight
TRIS	tris(Hydroxymethyl)aminomethane
TS	samples such as sera or plasma to be tested
TST	Tris-buffered saline and Tween 20
USP	United States Pharmacopeia
UTR	untranslated region
UV	ultraviolet
VEP	viral envelope protein
VH	heavy-chain variable regions
VHH	variable llama-derived heavy-chain antibody fragments
VL	light chain variable regions
VNA	virus-neutralizing antibody
WCBV	West Caucasian bat virus
WHO	World Health Organization
WRS	street rabies virus
WTA	whole-transcription amplification
YM	yeast minimal

Part 5. Demonstration of viral nucleic acids and sequences

Chapter 27
Conventional pan-lyssavirus reverse transcriptase polymerase chain reaction

Introduction

Next to techniques aimed at the detection of lyssavirus antigens, such as the direct fluorescent antibody test (DFAT; see Chapter 11), the direct rapid immunohistochemistry test (DRIT; see Chapter 12), the rapid immunochromatographic test (RIDT; see Chapter 17), the rabies tissue culture infection test (RTCIT; see Chapter 9) and the mouse inoculation test (MIT; see Chapter 8) *(1–5)*, methods based on the detection of lyssavirus nucleic acids are becoming more widely accepted for the diagnosis of rabies in quality-assured laboratories *(6–8)*. Among such molecular techniques the polymerase chain reaction (PCR), developed in the 1980s *(9)* and first used for rabies diagnosis and typing in 1991 *(10–11)*, has revolutionized diagnosis not only of rabies but also of many viral, bacterial, parasitological and fungal pathogens.

PCR is an in vitro laboratory technique used to detect, among others, target DNA sequences of infectious agents in tissues, as well as in secretions or excretions of infected animals and humans. It involves exponential amplification of the target using a thermostable DNA polymerase (*Taq* polymerase) using short oligonucleotide sequences called “primers” to select the portion of the genome to be amplified. Specific alternating temperature profiles of the sample are applied to help a DNA replication enzyme rapidly copy the target DNA sequence. Depending on the interval between selected forward and reverse primers, PCR products (amplicons) of different sizes can afterwards be made visible by agarose gel electrophoresis. The use of PCR in lyssavirus diagnostics benefits also from the downstream application of Sanger sequencing to type the lyssavirus amplified in confirmed cases.

Lyssaviruses are negative stranded RNA viruses *(12)*. Therefore, before PCR amplification, the RNA is first reverse transcribed into complementary DNA (cDNA) using reverse transcription (RT), resulting in a variant of PCR referred to as reverse transcriptase polymerase chain reaction (RT-PCR). The amplification of lyssavirus RNA using RT-PCR can be achieved as either a one-step or a two-step reaction. While in the first approach, the entire reaction from cDNA synthesis to PCR amplification takes place in a single tube, in the latter the reverse transcription reaction and PCR amplification are performed in separate tubes. There are advantages to both approaches. The one-step approach reduces the number of manipulations required, thereby reducing costs and risk of cross-contamination, whereas the two-step approach generates cDNA in the first step, which can then be available for parallel testing. The latter is particularly useful if only limited quantities of RNA are available and screening for multiple pathogens is required.

Various conventional gel-based RT-PCR protocols for the diagnostic detection of rabies virus (RABV) and the generic detection of lyssaviruses have been published targeting the lyssavirus genes or intergenic regions resulting in amplicons of various sizes *(6, 7)*. The sensitivity of gel-based RT-PCR amplification can be increased by second-round RT-PCR using internal annealing sites of either one (hemi-nested-PCR) or two (nested-PCR) primers or by using specific hybridizing probes in PCR-ELISA *(8, 13–14)*. Since primers were selected from conserved regions of the genome, most assays amplify parts of the nucleoprotein (N) and polymerase (L) genes of lyssaviruses as earlier proposed *(15, 16)*. Conventional lyssavirus species-specific or generic RT-PCRs can be used both for diagnostic purposes and for characterization of lyssaviruses *(15)*.

Preparatory work and procedures

Samples and controls

Diagnostic specimens subjected to testing by RT-PCR may include brain tissue (for diagnosis of human and animal rabies) and additionally skin biopsies, saliva and cerebrospinal fluid (CSF) for human rabies diagnosis. Particular care must be taken for the optimum selection, collection, shipment and storage of such biological specimens since they can impact the test results. The use of validated positive (PC) and negative (NC) controls or in-process controls is required and should be subjected to the same procedures as test samples. PCR NC (sometimes referred to as “no template control” or “NTC”) could either be water or uninfected brain material and confirms the absence of contamination during the PCR process. PCR PC from a known lyssavirus-positive brain tissue confirms that the PCR has worked in the event of the diagnostic samples being negative. Anomalous control results indicate a test failure possibly due to incorrect formulation of the reagents or a failure of equipment. Preferably, laboratory strains or non-autochthonous PCs (that do not occur in the region) should be used, so that sequence analysis can be used to rule out cross-contamination of the PC into the test samples. The PC must be used at a concentration 1 log higher than the known limit of detection of the PCR assay. Using very high levels of PC would prevent laboratories from detecting fluctuations/trends within the assay that could prevent lower viral loads from being detected, and will increase the risk of cross-contamination. Every new batch of PC must be tested and confirmed as fit for purpose. For example, laboratories could test a 10-fold serial dilution series of each new stock of PC RNA (ranging from 100 ng/µL to 1 fg/µL, 10^{-1}–10^{-7}) and calibrate the working concentration of RNA accordingly.

RNA extraction

Obtaining high-quality nucleic acid from the sample is the first and most important step for any molecular assay. Proper handling (e.g. on ice and using gloves) and use of RNase-free materials will prevent the introduction of RNase and eliminate degradation of RNA. Viral RNA from diagnostic specimens such as brain tissue, skin biopsies, saliva and CSF can be extracted by using commercial column-based extraction kits (e.g. RNeasy Mini Kit) or guanidinium isothiocyanate-phenol-chloroform-based extraction methods (e.g. TRIzol), following the manufac-

turer's recommendations. An alternative approach employs paramagnetic beads with a nucleic acid binding surface that are used to bind RNA following lysis. Beads with bound RNA are captured on magnets and the supernatant containing cell debris and other contaminants is removed with washes. When using in-house or commercial nucleic acid extraction reagents, safety and sensitivity should be assessed locally. For skin biopsies, a preliminary step of lysis using proteinase-K is required before extraction (for example incubation, after dissociation with sterile scissors, at 37 °C for 3 h under gentle agitation in 180 µL of ALT tissue lysis buffer and 20 µL of proteinase K) *(17)*.

It is recommended to include a validation of the extraction step for each sample, based on the parallel detection of endogenous control, such as RNA from housekeeping gene (e.g. 18S ribosomal RNA or ß-actin), in one-step (running in parallel a specific RT-PCR assay) or two-step approach (see following point). The housekeeping PCR can be used to confirm the presence of RNA in samples derived from tissue with low cellular content. This validation provides confidence that a lyssavirus negative result by RT-PCR is a true negative and not the result of a failure of RNA extraction or a failure in the extraction process *(18)*. In addition, host material can be analysed for species confirmation or co-evolutionary studies (e.g. mitochondrial cytochrome B analysis).

An NC (water or non-infected tissue) must be included during the extraction step. However, the use of PC is not recommended during extraction, especially when endogenous controls are used, and to avoid any cross-contamination during this initial step.

After extraction, store RNA samples at below –70 ºC until use. Because of the high sensitivity of any RT-PCR, great care must be taken to ensure that any contamination is excluded. Record all test details onto a worksheet to ensure traceability. Obtained RNA may be quantified if required. Dilution of RNA can be performed, if necessary and according to the assay which will be used. For the highly sensitive pan-lyssavirus N gene RT-PCR detailed below, the extracted RNA is diluted 1:10 before being quantified (e.g. by NanoDrop). The original RNA is then diluted to 1µg/µL. For the L gene protocol described below, dilution of RNA is not required, although a final quantity of 1.5 µg per reaction is preferable.

Reverse transcription of viral RNA

If a two-step RT-PCR is applied, synthesis of cDNA from the extracted viral RNA is required. In contrast to targeted RT using specific lyssavirus-derived primers, the cDNA generated using random hexamers can be used in various virus or host related downstream applications (Table 27.1). The cDNA can be stored at –20 ºC until required. This facilitates greater flexibility to detect a range of viral pathogens and also enables detection of host housekeeping genes (18S ribosomal RNA or ß-actin, etc) to be employed to check the efficiency of the RNA extraction. A combination of specific lyssavirus RT primer (e.g. JW12) and random hexamers may also be used to reverse transcribe the RNA.

Table 27.1. Primers used in the N gene hemi-nested pan-lyssavirus RT-PCR

Primer	Direction	Sequence (5'- 3')	Position[a]	Details
JW12	F	ATGTAACACCYCTACAATG	55–73	7.5 pmol/µL (first round) 3.5 pmol/µL (second round)
JW6 UNI	R	ARTTVGCRCACATYTTRTG	660–641	7.5 pmol/µL (first round)
JW 10 UNI	R	GTCATYARWGTRTGRTGYTC	636–617	3.5 pmol/µL (second round)

[a] Position according to the reference Pasteur virus genome (M13125)

F, forward; R, reverse; RT-PCR, reverse transcriptase polymerase chain reaction

Thermal cycler

Commercial companies offer a complete range of thermal cyclers (also called PCR machines or DNA amplifiers) that meet the requirements of all applications. Some thermal cyclers provide multiple blocks in one housing unit (dual or three-block thermal cyclers), allowing several different PCR reactions to be carried out simultaneously. Heated lids prevent condensation of water from the reaction mixtures. Although the difference in performance of contemporary well-maintained thermal cyclers is generally limited, a validation is recommended when changing the type of thermal cycler. In a rabies diagnostic laboratory, thermal cyclers should be calibrated and regularly serviced similar to other critical equipment such as pipettes for which accuracy/precision should also be qualified and routinely checked. In addition, storage equipment (refrigerators and freezers) must be monitored to detect out of range temperature levels and lapses in temperature that may affect sample or reagent quality. Calibration, servicing and temperature monitoring are mandatory if the test is accredited to ISO17025 standard.

RT-PCR methodology

This procedure describes the generic amplification of lyssavirus RNA or cDNA for both diagnostic and research purposes, using two examples of hemi-nested RT-PCR (hnRT-PCR).

The first assay targets the N gene and is modified from a published protocol *(13)*. The universal primers JW12 (forward) and JW6UNI (reverse) of the pan-lyssavirus RT-PCR detect a 606 base pair (bp) region of the N gene from all ICTV (International Committee on Taxonomy of Virusus) recognized and novel lyssavirus species in the first round PCR. The reverse primers JW10UNI, which lie within the sequence of the first round PCR product, are used in conjunction with the first-round primer JW12 in the second round of the hnRT-PCR (Fig. 27.1, Table 27.1). Molecular grade water needs to be added to a final volume of 50 µL.

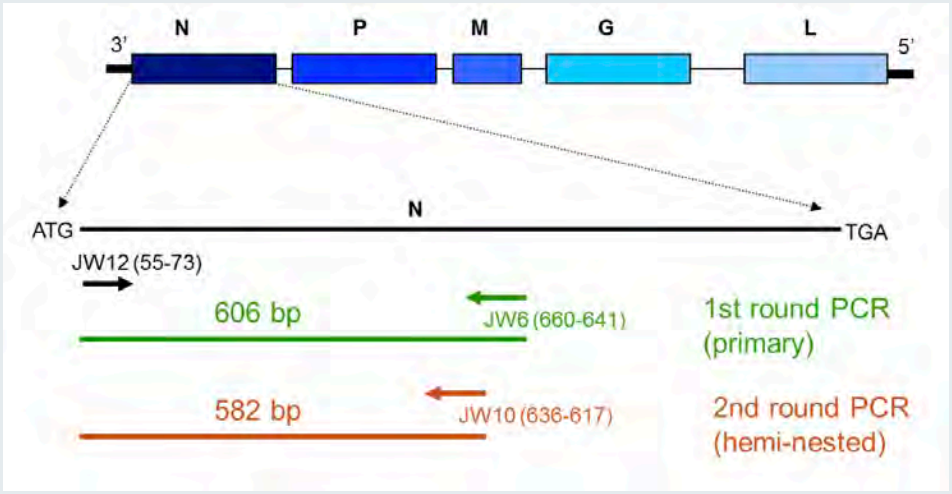

Fig. 27.1. Schematic diagram of the lyssavirus genome and the region targeted by the N gene pan-lyssavirus hemi-nested RT-PCR primers (see Table 27.1)

The second assay targets a conserved region among block III of the L gene *(16)* and has been previously validated for the postmortem and antemortem diagnosis of human rabies on one of the largest cohorts of rabid patients *(17)*. The first round is performed using the primers PVO5m (forward) and PVO9 (reverse), which amplify a 319 bp amplicon, whereas the second round uses the same forward primer (PVO5m) and the reverse primer PVO8, leading to a final amplification of a 249 pb region (Table 27.2). As previously indicated, it is strongly recommended to perform a parallel assay for the detection of the partial ß-actin mRNA in each sample (amplicon of 488 bp), to assess the quality of the RNA template and to validate the extraction process, using primers b-Taq1 and b-Taq2 (Table 27.2 *(19)*).

Table 27.2. Primers used in the hemi-nested pan-lyssavirus RT-PCR targeted L gene and the endogenous control ß-actin mRNA

Target	Primer	Direction	Sequence (5'- 3')	Position	Quantity per reaction
L gene	PVO5m	F	ATGACAGACAAYYTGAACAA	7170[a]	10 pmol (first and second round)
L gene	PVO9	R	TGACCATTCCARCARGTNG	7489[a]	10 pmol (first round)
L gene	PVO8	R	GGTCTGATCTRTCWGARYAATA	7419[a]	10 pmol (second round)
ß-actin	b-Taq1	F	TCACCCACACTGTGCCCATCTACGA	2206[b]	10 pmol
ß-actin	b-Taq2	R	CAGCGGAACCGCTCATTGCCAATGG	2500[b]	10 pmol

[a] Position according to the reference Pasteur virus genome (M13125)
[b] Position according to the human ß-actin gene (E00829)

F, forward; R, reverse; RT-PCR, reverse transcriptase polymerase chain reaction

For PCR, different "ready to use" commercial kits are available, although reagents can also be purchased separately. For this chapter the kits and methods detailed below have been validated, although in-house validation should be completed before using the assay on diagnostic samples. Alternative reagents and kits are available and can be used after appropriate validation to ensure optimal sensitivity and specificity. Sequence analysis should be used to further confirm the specificity of the PCR products. Preferably, a four-room system, e.g. clean room (master mix), template room (addition of RNA template), PCR room (thermal cycling) and amplicon room (gel electrophoresis), should be used to avoid cross-contamination.

1. N gene hnRT-PCR

First round RT-PCR (JW6UNI/JW12)

In the clean room

1. Wipe bench or surface of PCR cabinet/workstation with an appropriate disinfectant prior to use. If available, switch on the ultraviolet (UV) light for 10 min. Obtain the required test reagents from the –20 °C freezer. Ensure the enzyme mix is kept on ice. The remaining reagents can be thawed at room temperature.
2. Put the required number of 0.2 mL tubes in a rack and label the tubes clearly with sample identification and denote this is the first-round reaction by labelling (e.g. with "6/12"). Label the PCR negative (e.g. as "NC") or "NTC" (no template control) and the PCR positive control (e.g. as "PC") or "CVS", challenge virus strain (i.e. CVS RNA that is known to be positive).
3. Prepare a JW6UNI/JW12 reaction master mix using the One Step RT-PCR kit (Qiagen; Catalogue number 210212) (Table 27.2). Keep all reagents on ice, thaw and vortex before using. An NC (without template RNA) and a PC must be included in every test run. Allow for pipetting variation by preparing a sufficient volume of master mix at least one reaction greater than required.
4. Vortex the prepared master mix thoroughly, centrifuge and dispense 49 µL into each of the 0.2 ml tubes. Close the lids.
5. Transfer the sealed tubes to the ice/cool block in the template room on a disposable tray. Once a tray has been removed it must not be returned to the clean room without decontamination using an appropriate disinfectant.

In the template room – addition of template

1. Wipe bench top with an appropriate disinfectant prior to use.
2. Thaw samples and control RNA (PC and NC) on ice.
3. Add 1 µL of test RNA (e.g. at concentration of 1 µg/µL for TRIzol extracted samples) below the surface of its allocated master mix tube and mix gently. Discard the tip directly into a pot containing cleaning agent (e.g. Decon90) after use to minimize cross-contamination. Repeat this process until all samples and controls have been added to their allocated tubes.
4. Press the lids down by hand and seal firmly.

5. Transfer the sealed tubes to the PCR machine and cycle as detailed (Table 27.3). In-house validation of cycling parameters is essential to ensure optimisation for local PCR machines.

Table 27.3. First-round JW6UNI/JW12 reagent master mix (using [Qiagen] One Step RT-PCR kit)

Reagent	Volume per reaction (µL)
Molecular grade water	29.0
5× buffer	10.0
dNTPs (10 mmol)	2.0
JW12 (7.5 pmol/µL)	3.0
JW6UNI (7.5 pmol/µL)	3.0
One-step RT-PCR enzyme mix	2.0
Total	**49**

RT-PCR, reverse transcriptase polymerase chain reaction

Second-round RT-PCR (JW10UNI/JW12)

Where no amplicon is generated on the first-round reaction, a second-round, hemi-nested reaction should be performed. This will provide further confidence in a negative result. The second-round assay may also be employed to increase the specificity of the assay.

In the clean room

1. Wipe the bench with an appropriate disinfectant prior to use, then prepare the PCR workstation by opening the doors and wipin the cabinet surface with an appropriate disinfectant. Place an ice bucket (small), discard pot (containing an appropriate cleaning agent), suitable pipette and tips within the station and close the doors. If available, switch on the UV light for 10 min.
2. Obtain the required reagents from the –20°C freezer and thaw at room temperature.
3. Put the required number of 0.2 mL tubes in a rack and label the tubes clearly with sample identification and denote that this is the second-round reaction by labelling (e.g. “10/12”). Label the PCR negative as “NC2” or “NTC2”. This additional NC must be included in every second round PCR experiment to confirm the master mix is not contaminated.
4. Prepare a JW10UNI/JW12 reaction master mix using the HotStarTaq kit [Qiagen] as detailed [see catalogue number 203443] as detailed (Table 27.4).
5. Thaw and vortex all reagents before using. Allow for pipetting variation by preparing a volume of master mix at least one reaction greater than required.

6. Vortex the prepared master mix thoroughly, centrifuge and dispense 49 µL into each of the 0.2 mL tubes. Seal the tubes.
7. Transfer the sealed tubes to the template room on a disposable tray. Once a tray has been removed it must not be returned to the clean room without decontamination using an appropriate disinfectant.

Table 27.4. Hemi-nested RT-PCR first round cycling parameters

Temperature	Time	Cycles
50 °C	30 min	1
95 °C	15 min	1
94 °C	30 s	45
45 °C	45 s	
50 °C	15 s	
72 °C	1 min	
72 °C	**7 min**	**1**
4 °C	∞	**n/a**

RT-PCR, reverse transcriptase polymerase chain reaction

In the template room – addition of template

1. To reduce cross-contamination, the template may be added within a PCR workstation.
2. To prepare, open the doors of the PCR workstation and wipe the cabinet surface with an appropriate disinfectant. Place an ice bucket (small), discard pot (containing an appropriate cleaning agent, suitable pipette and tips within the station and close the doors. Switch on the UV light for 10 min.
3. Add 1 µL of first-round PCR product below the surface of the prepared second round master mix to minimize aerosols, then mix gently. Discard the tip directly into an appropriate cleaning agent after use. Ensure the lid of the PCR tube is sealed firmly. Repeat this step until all first-round PCR products and the second-round NTC have been added to its allocated second-round master mix tube. Change gloves regularly and at suitable points to avoid cross-contamination.
4. If using the PCR workstation, after removing the samples from the cabinet, empty the ice, remove the disinfectant pot, then switch on the UV light for 10 min. Record the required detail in the relevant PCR workstation workbook.

5. Cycle on the PCR machine using the following second round cycling parameters (Table 27.5). In-house validation of cycling parameters is essential to ensure optimization for local PCR machines.

   Note: Batch master mixes can be prepared so that ready-made mixes are available for hnRT-PCR reactions. These batches can be quality controlled when prepared, allowing for better quality assurance and standardization. This is the routine way of preparing master mixes for diagnostic purposes. These master mixes are stable for at least 1 year when stored appropriately at –20 °C.

Table 27.5. Second-round JW10UNI/JW12 master mix (using [Qiagen] HotStarTaq kit)

Reagent	Volume per reaction (µL)
Molecular grade water	22.0
HotStarTaq master mix (2×)	25.0
JW12 (3.5 pmol/µL)	1.0
JW10UNI (3.5 pmol/µL)	1.0
Total	**49**

Analyses of RT-PCR products by electrophoresis on agarose gels

Nucleic acids are negatively charged and can be separated on the basis of size in an agarose gel under the influence of an electric current. Historically, ethidium bromide (EtBr) has been used to stain nucleic acids in agarose gels and detection under UV light. More recently, the safer SYBR-based options have been preferred over the use of the carcinogen EtBr.

- Prepare a 1% agarose gel. Add Et Br (final concentration 0.01%) or alternatively 5 µL of SYBR Safe solution [Life Technologies Ltd] per 100 mL of gel.
- Pour gel into the cast, select a suitably-sized well former (comb), according to the volume of sample being loaded and the number of wells required, and place into the cast before the gel sets. Leave the gel to solidify for at least 30 min.
- DNA size markers (1 kb or 100 bp ladder) are diluted in TE or TBE buffer and 50 µL aliquots are stored in a –20 °C freezer. These DNA size markers must be mixed with loading buffer prior to loading. Generally, 5 µL of ladder is used.
- To enable loading of a PCR product (DNA sample) it must be mixed with a suitable volume of gel loading buffer, e.g. blue/orange 6x. For diagnostic purposes, 5 µL of PCR product and 1 µL of loading dye is generally used per sample.
- Load the samples and DNA marker into the wells and separate the samples for approximately 45 min–1 h (120 volts).
- Remove the gel and allow excess buffer to drain off. Place the gel in a tray and carry over to the UV transilluminator.
- A positive PCR result is observed in the form of a bright band of the expected size of 606 bp (first round) and 582 bp (second round).

2. L gene hnRT-PCR

As the overall process for this technique remains mostly similar to the N gene-based hnRT-PCR previously described and to the general considerations requested when performing PCR *(20)*, only specific parameters inherent to this technique will be described in this section, as well as major variations or modification steps, such as the reverse transcription of this two-step technique. In each series, positive (positive RNA) and negative controls (negative RNA and/or RNase-DNase free water) should be included.

Reverse transcription step

A total of 6 µL of extracted RNA is used for cDNA synthesis and add to the first mix reaction described in Table 27.6. A pre-incubation of RNA template with pd$(N)_6$ random primers is performed for 10 min at 65 °C in a heat-block, following with an incubation for 90 min at 42 °C in a heat-block after addition of the second mix solution (Table 27.6).

Table 27.6. Hemi-nested RT-PCR second-round cycling parameters

Temperature	Time	Cycles
95 °C	15 min	1
94 °C	30 s	35
45 °C	10 s	
50 °C	15 s	
72 °C	1 min	
72 °C	**7 min**	**1**
4 °C	∞	**n/a**

RT-PCR, reverse transcriptase polymerase chain reaction

Mix preparations and cycling parameters

This technique is relatively simple because the mix preparation is similar for both rounds of PCR used for lyssaviruses detection as well as for the PCR dedicated to the detection of the endogenous control ß-actin mRNA (with the exception of the primers used) (Table 27.7). In addition, cycling parameters are also identical for all these PCR (Tables 27.8–9). Similarly to each step of the process, positive (positive RNA) and negative (negative cDNA and/or RNase–DNase-free water) should be included in each series and each round of PCR.

Revelation of the amplification products after PCR is done by electrophoresis on agarose gels as previously described.

Table 27.7. Reverse transcription mix for the L gene-based hnRT-PCR

Step	Reagent	Volume per reaction (µL)
Mix 1: pre-incubation (10 minutes at 65°C)	pd(N)6 random primers (200 µg/mL) [Roche Diagnostics]	2
	RNase-DNase free water	2
	RNA template	6
	Total	**10**
Mix 2: incubation (90 minutes at 42 °C)	5X first-strand buffer (Invitrogen, provided with the reverse transcriptase)	6
	0.1 mol DTT (Invitrogen, provided with the reverse transcriptase)	2
	dNTP mix (10 mmol) [Eurobio]	2
	RNasin (40U/µL) [Promega]	2
	Superscript II RT (200 U/µL) (Invitrogen)	1
	RNase-DNase free water	7
	Total	**20**
Final volume		**30**

Table 27.8. L gene-based hnRT-PCR mix preparations

Reagent	Volume per reaction (µL)
10X PCR buffer II (provided with the *Taq* polymerase)	5
$MgCl_2$ (25 mmol) (provided with the *Taq* polymerase)	2.5
dNTP Mix (10 mmol) (provided with the *Taq* polymerase)	1
Forward primer[a] (10 µmol)	1
Reverse primer[a] (10 µmol)	1
Ampli*Taq* DNA Polymerase[b] (5 U/µL) [Applied Biosystems]	0.4
RNase–DNase-free water	37.1
Total	**48**

[a] The primers used are PVO5m (forward)/POV9 (reverse) and PVO5m (forward)/PVO8 (reverse) for the first and the second rounds of PCR for lyssavirus detection, respectively, and b-taq 1/b-taq2 for -actin mRNA detection.

[b] The enzyme can also be replaced by BioTaq DNA Polymerase [Bioline], after adjusting the volume of enzyme to 0.2 µL and the volume of MgCl2 to 1.25 µL.

Table 27.9. L gene-based hnRT-PCR cycling parameters

Cycling step	Temperature	Time	Number of cycles
Initial denaturation	94 °C	3 minutes	1
Amplification	94 °C	30 seconds	35
	56 °C	45 seconds	
	72 °C	40 seconds	
Final elongation	72 °C	3 minutes	1
Pending	16 °C	∞	n/a

Discussion

Various lyssavirus species-specific and generic RT-PCRs have their merit for the specific purpose they were developed *(6–8)*. This chapter describes two examples of conventional gel-based pan-lyssavirus hemi-nested RT-PCR assays with primers that target the N gene or L gene. The N gene Hn RT-PCR is one of the most widely used and has been validated to detect all recognized and putative lyssavirus species known to exist to-date at an annealing temperature of 45 ºC *(13, 21–23)*. Any alteration of annealing temperature may cause detection of the rare phylogroup 2 or 3 lyssaviruses to be impaired (24). Furthermore, this generic gel-based RT-PCR *(13)* gave the most accurate results in an international ring trial compared to other published conventional lyssavirus PCRs included in the study *(25)*.

Both assays have successfully detected all RABV isolates and other lyssavirus species tested in the framework of animal rabies diagnosis activity in a national and WHO collaborating centre for rabies, haven proven efficacy for ante-mortem and post mortem human rabies diagnosis and have performed well in successive international proficiency tests *(6, 8, 26)*.

Despite the highest level of sensitivity and their ever increasing important role in many countries, the use of molecular assays, including RT-PCR, for routine post-mortem diagnosis of lyssaviruses is currently not recommended if brain tissue is available, especially for animal rabies *(27, 28)*. Without standardization, very stringent quality control and sufficient experience and expertise such tests run the risk of high levels of false positive or false negative results *(29)*.

Nevertheless, if strict quality control procedures are applied those techniques can be used for epidemiological surveys in wildlife. In such a case, however, a positive RT-PCR result preferably requires a positive result in one of the routine diagnostic tests (DFAT, RTCIT) if it is to be officially declared to the OIE, particularly in a previously “rabies-free” region or host.

However, RT-PCR is commonly applied for postmortem and in particular antemortem diagnosis of human rabies (6, 8, 28). However, negative results do not rule out infection, especially for excretions (virus intermittently shed) or for atypical or paralytic forms of rabies for which the sensitivity remains lower than for the classical encephalitic form (28).

In the event of routine diagnostic tests (DFAT, RTCIT) being inapplicable or inappropriate, RT-PCR may be used for diagnostic purposes. In particular, when the quality of the material submitted is suboptimal, RT-PCR has been shown to superior to the conventional assays (30). In confirmed cases, RT-PCR is useful as an additional diagnostic tool for virus characterization to determine the source of infection and molecular phylogeny (29). However, only sufficiently validated RT-PCR in terms of sensitivity (genome copies), specificity, repeatability and robustness should be applied. Considering ongoing and future developments, the quality of commercial column-based RNA extraction and RT-PCR kits should be frequently checked to allow for highest sensitivity of the gel-based RT-PCR, and verification of the ability of detection of any new lyssavirus species should be recommended, if possible.

Quality control procedures should include strict precautions to avoid carryover contaminations as described (20) and to verify the intrinsic performance of the assay. In this way, participation to international proficiency tests is highly recommended. However, hemi- and nested RT-PCRs are especially prone to the risk of carryover or cross-contamination. To ensure confidence in positive diagnostic results, subsequent analysis by Sanger sequencing is recommended. Therefore, and whenever possible, it is also advisable to employ RT-qPCR for routine screening of samples, which considerably reduces the risks of cross contamination.

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Chapter 28
Rabies real-time reverse transcriptase polymerase chain reaction

Introduction

Nucleic acid-based detection of rabies viruses (and other lyssaviruses) is now routinely employed in diagnostic laboratories. Validated assays including conventional (gel-based) reverse transcriptase polymerase chain reaction (RT-PCR; see Chapter 27) and real-time RT-PCR have long been established in quality-assured settings. In contrast to conventional RT-PCR, the real-time assay combines the amplification and detection within a closed tube system; thus, real-time PCR platforms offer a more rapid and reliable indication of the presence of lyssavirus RNA in suspect samples. The thermal cyclers detect the fluorescence emitted by the exponentially increasing amplicon (PCR product).

Two approaches to detect the amplicons are commonly employed, the choice of which will depend upon the requirements of the diagnostic or surveillance system. One approach is to add a DNA intercalating dye (fluorochrome) to the reaction mix. Intercalating fluorochromes (such as SYBR Green or ResoLight) bind to double-stranded (ds) DNA during PCR. Bound fluorochromes emit fluorescence which is detectable by the thermal cycler at each cycle, thus allowing DNA concentrations to be quantified. Because such dyes may also bind to nonspecific PCR products, a melting curve analysis must be undertaken at the end of the programme to confirm the specificity of the test result.

The second approach utilizes hydrolysis probes (such as TaqMan probes). These probes make use of the Fluorescence Resonance Energy Transfer (FRET) whereby a quencher molecule at the 3'-end of the specifically designed oligonucleotide probe quenches the fluorescence emitted by the fluorophore covalently attached to the 5'-end. As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals which would otherwise be excited by the thermal cycler's light source. When the probe binds to its target region during amplification, the exonuclease activity of the polymerase leads to the dissociation of fluorophore and quencher, allowing the resulting fluorescence to be emitted and measured. Several quenchers and fluorophores are available and should be selected based on the thermal cycler's specifications. Although the probe system is inherently more specific than intercalating dyes, designing a probe which can detect all lyssaviruses within the small amplicon can be challenging.

In comparison with conventional PCR assays, both approaches to real-time PCR are closed tube systems, thus reducing the possibility of cross-contamination and false–positive results. The real-time PCR assays are usually more rapid due to the smaller amplicon sizes and eliminate the need for gel electrophoresis confirmation or subsequent rounds of amplification. When test samples are run alongside a serial dilution of positive control RNA, the amount of viral RNA present

in a sample can be quantitated. The probe-based assays also enable multiple target detection and virus typing, if using specific and appropriate probes coupled to different fluorophores.

Many real-time RT-PCR assays have been reported for lyssavirus detection, some of which were selectively developed for the individual virus strain or virus lineage *(1)*, while others were developed to detect a wider range of rabies strains and/or lyssavirus species *(2–6)*. All published assays will have limitations as regards their diagnostic range due to the restricted field samples available when validating the assays. Therefore, it is essential to consider the lyssavirus lineages and strains in each region when establishing this methodology. Indeed, emerging or novel virus strains may render the highly specific probe-based assays ineffective *(7–9)*. The choice of the SYBR Green versus the probe RT-PCR will depend on the intended application. For a laboratory conducting surveillance on brain material and expecting high numbers of negative samples, the use of the cheaper SYBR Green real-time PCR would be recommended. The SYBR Green approach would also be optimal when conducting scanning surveillance where novel or divergent lyssaviruses may be present which would otherwise be undetected by restricted probe-based assays. Whereas the more specific and sensitive probe-based assays may be preferable in a laboratory, conducting lyssavirus diagnosis on suspect samples which are expected to have a restricted lyssavirus species range and contain low viral loads may require rapid typing.

This chapter describes two pan-lyssavirus real-time RT-PCR assays. The first approach is the SYBR Green assay, a well-established and broadly applicable quantitative real-time PCR which employs SYBR Green and the pan-lyssavirus nucleoprotein (N) gene primers, JW12 and N165-146. These primers were first designed and validated for a differential TaqMan-based assay, then subsequently utilized in a SYBR Green assay *(2, 10)*. The second real-time PCR assay is the multiplex probe based LN34 assay, which uses a combination of degenerate primers and probes to achieve high sensitivity and specificity *(4)*.

Both assays have been shown to detect a wide range of RABV variants and other lyssaviruses *(2, 4, 10, 11)* and both have been successfully applied for both antemortem and postmortem diagnosis.

Methods

Viral RNA extraction

Extract the RNA following the instructions given in Chapter 27 on conventional gel-based PCRs and store RNA samples at –80 °C until use. As previously covered for conventional PCR and due to the high sensitivity of the real-time PCR, great care must be taken to ensure that any cross-contamination is excluded. Validation of the extraction step may be performed by using exogenous controls (e.g. synthetic eGFP RNA) spiked directly into each sample during the early phase (i.e. after addition of TRIzol) or by detecting host nucleic acid via a housekeeping assay (Table 28.1).

Table 28.1. PCR assays and their relative primer and probe positions

	PCR assay	Primer/Probe	Sequence (5'-3')	Position[a]	Reference
SYBR® Green pan-lyssavirus	N-gene PCR	JW12	ATGTAACACCYCTACAATG	55-73	This chapter; *(10)*
		N165-146	GCAGGGTAYTTRTACTCATA	165-146	
	L-gene PCR	Pan-Lyssa-7531F	TTCTTCGCTYTRATGTCWTGGAA	7074-7096	*(15)*
		Pan-Lyssa-7749R	ATGRTTGTTCCACTTYTCATARTC	7292-7269	
	N-gene PCR	RV-Q-RT-P	ACGCTTAACAACMARAYC	1-18	*(14)*
		RV-NP-Q-Forward	CAAGATGTGTGCYAAYTGGAG	644-663	
		RV-NP-Q-Reverse	AGCCCTGGTTCGAACATTCT	881-900	
	L-gene PCR	Taq5long forward	TATGAGAAATGGAACAAYCAYCA	7272–7294	*(5)*
		Taq16revlong reverse	GATTTTTGAAAGAACTCATGKGTYC	7366–7390	
Pan-lyssavirus probe-based assays	LN34[c]	Probe LN34[b]	FAM-AA+C+ACCY+C+T+ACA+A+TGGA-BHQ1	59 - 75	This chapter; *(4)*
		Probe LN34lago[b]	FAM-AA +C +ACTA +C +T +ACA +A +TGGA-BHQ1	59 - 75	
		Primer forward1	ACGCTTAACAACCAGATCAAAGAA	1 - 24	
		Primer forward2	ACGCTTAACAACAAAATCADAGAAG	1 - 25	
		Primer reverse	CMGGGTAYTTRTAYTCATAYTGRTC	140 - 164	
	R13 MP	JW12	ATGTAACACCYCTACAATG	55-73	*(2)* modified
		N165-146	GCAGGGTAYTTRTACTCATA	165-146	
		LysGT1-FAM	FAM-ACAAGATTGTATTCAAAGTCAATAATCAG-BHQ1	81-109	
		LysGT5-HEX	HEX-AACARGGTTGTTTTYAAGGTCCATAA-BHQ1	80-105	
		LysGT6-Cy5	Cy5-ACARAATTGTCTTCAARGTCCATAATCAG-BHQ3	81-109	
		BBLV-1TEX	TEX-CTCTGACAAGATTGTCTTCAAAGTC-BHQ2	76-101	
Lyssavirus-specific probe-based assays	R14 MP RABV	RV-N-196-F	GATCCTGATGAYGTATGTTCCTA	266-288	*(8)*
		RV-N-283-R	RGATTCCGTAGCTRGTCCA	353-335	
		RabGT1-B-FAM	FAM-CAGCAATGCAGTTYTTTGAGGGGAC-BHQ1	297-321	
	R14 MP EBLV-1	EBLV1-353F	GCTCAAACRGGAGGTCAAGA	431-450	*(15)*
		EBLV1-440R	AGACARAGAAGAAGTCCWACCA	510-489	
		EBLV1-392HEX	HEX-ACCCTACRACACCTGAACATGCATCT-BHQ1	462-487	
	R14 MP EBLV-2	EBLV2-42F	RGTGTCTGTAAARCCAGAAG	112-131	*(16)* modified
		EBLV2-173R	GACAGAATRGACTTATAAGCTCT	243-221	
		EBLV2 N Probe	Cy5-TCGGAAAAAACCCAGCATAACCCT-BHQ2	175-198	
	R14 MP BBLV	BBLV-2F	CCTTGGTRAACATTCAGAGAACG	390-412	*(17)* modified
		BBLV-2R	GGCCACAGTTGGATCCCTTG	475-456	
		BBLV-2TEX_as	TEX-TCCTCCGGTCAAGGCCCARTTGCC-BHQ2	422-445	

Table 38.1. continued

Lyssavirus-specific probe-based assays (continued)	Pan-RABV	Taq3long Primer forward	ATGAGAAGTGGAAYAAYCATCA	7273–7294	*(5)*
		Taq17revlong Primer reverse	GATCTGTCTGAATAATAGAYCCARG	7390–7414	
		RABV4 Probe	FAM-AACACYTGATCBAGKACAGARAAYACATC-TAMRA	7314–7342	
		RABV5 Probe	FAM-AGRGTGTTTTCYAGRACWCAYGAGTTTTTYCA-TAMRA	7353–7384	
Internal controls	eGFP	EGFP1-F	GACCACTACCAGCAGAACAC		*(5, 18)*
		EGFP2-R	GAACTCCAGCAGGACCATG		
		EGFP-Probe 1	FAM-AGCACCCAGTCCGCCCTGAGCA-BHQ1		
	ß-actin SYBR® Green primers	BatRat intronic	CGA-TGA-AGA-TCA-AGA-TCA-TTG		This chapter; *(2, 10)*
		BatRat reverse	AAG-CAT-TTG-CGG-TGG-AC		
	ß-actin	ACT-1005-F	CAGCACAATGAAGATCAAGATCATC		*(19)*
		ACT-1135-R	CGGACTCATCGTACTCCTGCTT		
		ACT-1081-HEX	HEX-TCGCTGTCCACCTTCCAGCAGATGT-BHQ1		
	ß-actin	ß-actin probe	(HEX)-TCCACCTTCCAGCAGATGTGGATCA-(BHQ1)		This chapter; *(4)*
		ß-actin forward	CGATGAAGATCAAGATCATTGC		
		ß-actin reverse	AAGCATTTGCGGTGGAC		

[a] The primer and probe positions are given relative to the *Lyssavirus* full genome.

[b] LNA-modified bases are indicated by a plus preceding the base in the sequence (e.g. +A, +G, +C, +T).

[c] The pan-lyssavirus LN34 assay has a mixed probe of Probe LN34 and Probe LN34lago in a ratio of 2:1 respectively; the forward primer is a 1:1 mixture of Primer forward1 and Primer forward.

Pan-lyssavirus real-time RT-PCRs

Materials and equipment

- Several thermal cyclers are available that allow real-time detection of fluorescence. The difference in cycler performance when using established manufacturers is generally limited; nevertheless, assays must be validated or optimized when moved to a new thermal cycler. Test reagents should be optimized for the individual thermal cyclers of different companies. For example, the CFX96 quantitative PCR system (Bio-Rad Laboratories Inc., Hercules, USA) should be used with the respective Bio-Rad 96-well PCR plates. For real-time RT-PCR, numerous test kits are commercially available. In the protocols below, two kits have been validated but others can also be used after appropriate validation. When developing and/or establishing real-time RT-PCR assays in the laboratory, test parameters must be validated locally. For example, the limit of detection must be identified by evaluating the sensitivity of the assay using a 10-fold serial dilution series of stock RNA (e.g. 1 µg/µL over a range of 1 µg–1 pg for viral RNA stocks extracted from positive brain). Additionally, the specificity of the assay must be confirmed using an appropriate and locally relevant panel of positive and negative samples.
- MicroAmp reaction plate base (or equivalent plate/strip/tube holder)
- 96-well PCR plates, non-skirted or strips of 8 or 12 PCR-tubes suitable for the chosen real-time machine
- optically clear flat cap strips (strips of 8 or 12 lids) or optical adhesive covers, applicator and compression pad
- range of pipettes and nuclease-free barrier tips, capable of dispensing 0.5–1000 µL
- Eppendorf tubes or bijous of relevant size for preparation of the master mix
- ice buckets and ice or equivalent method of keeping reagents cool during setup
- vortex mixer
- bench-top micro-centrifuge
- molecular biology reagent grade water
- appropriate cleansing agent to minimize cross-contamination
- permanent marker pen
- gloves and a laboratory coat must be worn at all times.

Preparation of master mix

The master mix contains all the components for the reverse transcription (if required) and amplification. Sufficient master mix should be prepared to be able to analyse all the samples plus no template control(s) and positive control(s). Master mixes can be made up fresh for the test to be run or in batches; if locally validated, they can be frozen for up to one year in suitably sized aliquots.

Test specimens

RNA extracted from postmortem brain tissue (optimally brainstem and/or cerebellum) of the animal or human being tested for the presence of lyssaviruses. RNA from human antemortem specimens (including serial saliva specimens, skin biopsies, hair follicles, cerebrospinal fluid, urine and serum) may also be tested. All test samples should be run at least in duplicate (ideally triplicate), and multiple internal controls (see below) for each of the primer/probe sets should be included in each run. All controls must be included alongside test samples in every test run to ensure the test is performing within expected parameters.

Internal controls

Positive controls. Each newly prepared batch of positive control RNA must be validated and calibrated to ensure it is consistently within the expected cycle threshold (Ct) range. The expected range will be defined by the laboratory according to the technique employed and will be used to confirm the validity of each test run. Working aliquots must be prepared to prevent multiple freeze–thaw cycles from stock batches, stored at –80 °C and should not be used if the Ct value is out of range.

Negative controls. Molecular-grade water and/or negative RNA sample are added to the master mix (no template control) to confirm that the reagents are free from contaminants.

Additional controls. The efficacy of nucleic acid extraction and/or functionality of amplification can be tested using heterologous internal control systems or endogenous housekeeping genes (Table 28.1). The use of endogenous housekeeping genes or the direct spiking of internal controls into the suspect samples ensures that all steps (extraction, reverse transcription and amplification) are analysed under the same conditions and in the same matrix as the lyssavirus screening. Spiking of exogenous internal control could also overcome the lack of detection of endogenous housekeeping genes in some biological samples due to low level of cells, such as in CSF (in the context of antemortem rabies diagnosis in humans).

Protocol

1. Wear clean gloves (not previously worn when handling extracted RNA or PCR products) when setting up assays.
2. Change or decontaminate gloves whenever you suspect they are contaminated.
3. Keep reagent tubes and reactions capped as much as possible.
4. Before setting up assays and after handling extracted RNA or PCR products, clean equipment and laboratory benches with one of the acceptable surface decontaminants listed under reagents.

   Use aerosol barrier (filter) pipette tips.

Summary of outcomes and overall result

Test result	Internal control	Overall result
Negative	Negative	Invalid: repeat extraction and/or testing
Negative	Positive	Negative result reported
Positive	Positive	Positive result reported[a]
Positive	Negative	Repeat and/or run on a gel[a]

[a] If confirmed positive, follow up may be advised with additional tests and/or sequencing (see Chapter 29 on Sanger sequencing and Chapter 31 on next generation sequencing).

The SYBR Green assay

The procedure detailed here is an example that uses the same primers as the differential TaqMan assay described previously *(2)*. The use of a universal SYBR Green one-step RT-PCR kit for the detection of lyssavirus species from clinical specimens has been demonstrated to be both highly sensitive and specific for lyssavirus RNA. Furthermore, by using SYBR Green as the detection system it is able to detect all lyssaviruses (including highly divergent WCBV, IKOV and LLEBV) based on the pan-lyssavirus primer specificity. This method includes a separate RT-PCR assay containing SYBR Green for amplification of the internal housekeeping control, ß-actin, used as a template control for RNA extraction.

Using a universal SYBR Green one-step RT-PCR kit, cDNA synthesis and PCR amplification are carried out in a single tube. SYBR Green, a cyanine dye, binds to dsDNA during the amplification and the resulting DNA-dye complex absorbs blue light and emits green light. As the target amplicon accumulates with increasing PCR cycles, increasing dye is bound and gives greater levels of fluorescence. SYBR Green assays have two major phases: amplification and dissociation. The amplification phase corresponds to the PCR portion of the assay, and results in the generation of dsDNA. In the dissociation phase, the dsDNA product is melted into single-stranded DNA by a stepwise increase in temperature, with fluorescence data being collected at each temperature step. This dissociation phase gives an indication of the amplicon size. The likelihood of false–positive reactions in quality-assured laboratories is negligible.

Specific reagents and biologicals

- Lyssavirus-specific primers (HPLC purified) diluted to 20 µmol:
  - JW12 RT/PCR primer 5’-ATG-TAA-CAC-CYC-TAC-AAT-G-3’
  - N165-146 PCR primer 5’-GCA-GGG-TAY-TTR-TAC-TCA-TA-3’
- Multispecies ß-actin primers (HPLC purified) diluted to 20 µmol:
  - BatRat ß-actin intronic primer 5’-CGA-TGA-AGA-TCA-AGA-TCA-TTG-3’
  - BatRat ß-actin reverse primer 5’-AAG-CAT-TTG-CGG-TGG-AC-3’
- Bio-Rad iTaq™ Universal SYBR® Green One-Step RT-PCR Kit (catalogue number 172-5150/1)

Test procedure

In the clean room or UV cabinet

1. Wipe the bench with an appropriate disinfectant before use, then prepare the PCR workstation by opening the doors and wiping the cabinet surface with an appropriate disinfectant (to remove residual nucleic acids). Place an ice bucket (small), discard pot (containing an appropriate disinfectant), suitable pipette and tips within the station and close the doors. Switch on UV light for 10 min.
2. Obtain the required reagents from the –20 °C freezer. Ensure the enzyme mix is kept on ice; the remaining reagents can be thawed at room temperature.
3. Put the required number of 0.2 mL strips into an appropriate holder (or a 96-well plate if there are multiple samples).
4. Prepare a reaction master mix as below and keep all reagents on ice. Allow for pipetting variation by preparing at least an extra two reaction mixes (e.g. if you are running five reactions in total, prepare master mix for seven reactions).

Reagent	µL/reaction
Molecular-grade water	7.55
2× universal SYBR Green reaction mix	10
JW12 (Forward) [20 µmol]	0.6
N165-146 (Reverse) [20 µmol]	0.6
iTaq RT enzyme mix	0.25
Total per reaction	**19**

5. Prepare a reaction master mix for the ß-actin mRNA which assesses the quality of the extracted RNA. The SYBR Green assay for ß-actin must be positive to have confidence that RNA was isolated from the starting material.

Reagent	µL/reaction
Molecular-grade water	7.55
2× Universal SYBR Green reaction mix	10
BatRatAct intronic (Forward) [20 µmol]	0.6
BatRatAct reverse (Reverse) [20 µmol]	0.6
iTaq RT enzyme mix	0.25
Total per reaction	**19**

Vortex the prepared master mixes and aliquot 19 µL into each of the relevant wells of a 96-well plate, 8-well or 12-well strips (according to the plate set up sheet).

In the template room or UV cabinet – addition of template

1. Wipe the bench with an appropriate cleanser prior to use, then prepare the PCR workstation by opening the doors and wiping the cabinet surface with an appropriate disinfectant. Place an ice bucket (small), discard pot (containing an appropriate disinfectant), suitable pipette and tips within the station and close the doors. Switch on UV light for 10 min.
2. Thaw samples and control RNA on ice.

3. Add 1µL of test RNA (where possible, at a concentration of 1 µg/µL for TRIzol extracted tissue samples) below the surface of its allocated master mix tube and mix gently. Discard the tip directly into disinfectant after use. Repeat this process until all samples and controls have been added to their allocated tubes.
4. Press the lids down by hand and seal firmly. Number the strip lids according to your plate layout for orientation.
5. Transfer the PCR plate or strips to the real-time machine for thermal cycling.

Setting up the real-time thermal cycler (this may vary depending on the machine used)

1. Load the plate or strips into the machine ensuring that they are orientated correctly. Check that all the lids are firmly sealed, and then close the machine's plate cover and door.
2. Ensure that "SYBR" is selected as mode of the detection.
3. Label the wells with sample information (e.g. specimen number).
4. Label the positive and negative controls.
5. Set up the thermal profile as follows:

Stage	Cycles	Temperature	Time	Data collection
Reverse transcription	1	50 °C	10 min	
RT inactivation/initial denaturation	1	95 °C	5 min	
Amplification	40	95 °C	10 s	
		60 °C	30 s	End-point
Dissociation curve analysis	1	95 °C	1 min	
		55 °C	1 min	
		55–95 °C	10 s	All points

6. Start the reaction. Save the appropriately labelled run in the relevant folder as necessary.

Analysis of results

1. Once the run has finished, highlight the wells for which you want to collect data for analysis.
2. The amplification plot screen shows a plot of cycles versus fluorescence for an individual collection point on which data have been gathered. Positive samples will have exponential ramps followed by plateau and a Ct value, which should be interpreted alongside the positive and negative controls. Negative samples will have a flat amplification plot and no Ct values. The Ct value is calculated automatically by the software on completion of the run.
3. The lower the Ct value, the more amplicon produced (i.e. the level of fluores-

cence crossed the threshold earlier in the run) and therefore the more viral RNA (i.e. the assay is semi-quantitative).

4. As SYBR Green binds to any dsDNA, a thorough analysis of the dissociation curve must be performed to determine the specificity of the PCR products and monitor the presence of primer-dimers or contaminants that might be contributing to the fluorescent signal.
5. Generally, populations with a temperature of ≥ 80 °C correspond to the larger PCR products and can usually be assigned as specific DNA products. These dissociation curves usually produce a narrow well-defined peak which can be compared to the positive control. DNA products displaying melting temperatures of < 75 °C corresponding to non-specific DNA products.
6. The melting temperature will peak for RABV amplicons around 79 °C. The melting temperature for ß-actin is around 86 °C. Any positive sample will have a melting temperature peak the same temperature as the positive control. Any samples with different melting temperatures are not considered positive.
7. In case of an inconclusive assay, an agarose gel can be run to confirm the presence or absence of an amplicon the same size as the positive control (approximate size 100 bp).

The LN34 assay

The multiplex probe-based LN34 assay is a real-time RT-PCR assay which uses a combination of degenerate primers and probes to achieve superior coverage of the lyssavirus genus while maintaining sensitivity and specificity *(4)*. The primers and probes of the LN34 assay target the highly conserved non-coding leader region and part of the N gene coding sequence of the lyssavirus genome to maintain assay robustness. The probe sequences overlap with the highly conserved JW12 sequences and are further modified by locked nucleotides to increase their melting temperature and meet the requirements for an optimal real-time RT-PCR assay. The LN34 assay is able to eliminate the nonspecific amplifications from the nonspecific binding of the degenerated primers and/or primer dimers and allow a standardized diagnostic interpretation based on the Ct values. The diagnostic algorithm of the LN34 assay reduces the uncertainties in rabies testing and makes the adaptation of LN34 assay easier once a laboratory has the capacity for real-time PCR. The LN34 assay has the built-in artificial positive control which can be used to monitor the quality of the assay in a laboratory and to compare diagnostic results directly among different laboratories and different regions. It produces a 165 bp amplicon which can be sequenced for a rapid genetic typing of a positive result. In the near future, the LN34 assay and the host RNA ß-actin assay could be combined into a single tube, two-colour test to further reduce costs and diagnostic errors.

Specific reagents and biologicals

- Reagents (light sensitive: store in the dark at –20 °C)
  - LN34 assay primer and probe set (Table 28.1)
  - ß-actin assay primer and probe set (Table 28.1)

- Reagents (non-light sensitive)
  - Ag-Path ID One-Step RT-PCR Kit [Life Technologies, Catalogue no. 4387391]
  - Store at –20 °C; refer to manufacturer's instructions for expiration information.

Test procedure

Each RNA sample must be run in triplicate and be tested using the LN34 assay and a ß-actin control assay. Three no template control wells and three known positives for each of the primer or probe sets should be included in each run. Reaction assay mixtures (master mixes) containing all components except templates should be made as cocktails and dispensed into the 96-well reaction plate, 8-well strips or 12-well strips. Extracted RNA or water should then be added to test and control reactions, respectively.

1. Prepare separate master mixes for LN34 and ß-actin assays using the table below.

Reagent	µL/reaction
Water	6.5
2× RT buffer	12.5
25×RT-PCR enzyme mix	1
Forward primer [10 µmol]	1
Reverse primer [10 µmol]	1
Probe [5 µmol]	1
RNA	2
Total per reaction	**23**

2. Load 23 µL of master mix into the assigned wells in an appropriate plate or tubes for your real-time PCR instrument.
3. Set up the NTC reactions by pipetting 2 µL of PCR-grade water into all the NTC wells.
4. Briefly vortex and centrifuge the tubes containing the RNA samples.
5. Set up the extracted RNA sample reactions.
   a. Pipette 2 µL of the first sample into all the wells labelled for that sample.
   b. If sample volume is < 2 µL, add PCR-grade water to the reaction tube to bring the total reaction volume up to 25 µL.
6. Repeat step 2 for the remaining samples and the positive controls.
7. After the addition of the last sample or control, peel off the protective covering of the optical adhesive cover and place it over the wells, being sure to cover all the wells.
8. Ensure bubbles are removed by tapping the plate or by centrifuging the plate at 500 × *g* for 1 min at room temperature.

   Place sealed tubes or plate into a real-time PCR instrument that can detect FAM and VIC. Set to the conditions below or optimize to local real-time PCR machine as follows:

ViiA7 – Standard mode	Cycles	Temperature	Time	Data collection
Reverse transcription	1	50 °C	30 min	
RT inactivation/initial denaturation	1	95 °C	10 min	
Amplification	45	95 °C	1 s	
		56 °C	20 s	End-point
ABI7500 – Standard mode				
Reverse transcription	1	50 °C	30 min	
RT inactivation/initial denaturation	1	95 °C	10 min	
Amplification	45	95 °C	15 s	
		56 °C	30 s	End-point
ABI7500 Fast/FastDX – Fast mode				
Reverse transcription	1	50 °C	30 min	
RT inactivation/initial denaturation	1	95 °C	10 min	
Amplification	45	95 °C	3 s	
		56 °C	30 s	End-point

Interpretation of results

1. Allow the instrument to analyse the results automatically; any manual threshold or baseline adjustments need to be recorded and explained.

2. Record DFAT results (antigen intensity and distribution) as well as Ct values for the ß-actin and LN34 assays for each sample and control, as follows:

Brain sample result guidance			
Assay	Ct value	Result	Action required
ß-actin	Earlier than 33	Positive	No action required; 99.2% of 2248 samples tested in a preliminary cohort exhibited an average ß-actin Ct value < 33.
	33–45	Inconclusive	Possible inhibition or insufficient sample. Repeat or additional testing required.
	Not detected	Fail	Insufficient sample or failed extraction. Repeat or additional testing required.
LN34	Earlier than 35	Positive	No action required; 99.6% of 678 FAT-positive samples tested in a preliminary cohort exhibited LN34 Ct value < 35.
	35–45	Inconclusive	Repeat or additional testing required. Share the tissue with National Reference Laboratory for confirmatory testing.
	Not detected	Negative or Inconclusive	Negative if ß-actin ≤ 33 and > 0. Inconclusive if ß-actin is > 33 or 0.

3. A RABV-positive sample extracted from properly collected and stored brain tissue is expected to have a Ct value less than 35 cycles for the LN34 assay. Samples that amplify after 35 cycles with the LN34 assay with ß-actin Ct values < 20 may indicate potential problems with the assay, sample, or extraction, particularly sample contamination if positive rabies samples were processed on the same day.

4. A synthetic LN34 positive control RNA template (107 bases) can be generated using an in vitro T7 transcription kit for normalization purposes (oLPC-rabies3-4: GCA CAG GGT ACT TGT ACT CAT ACT GAT CTG AAT CCA TTG TAG AGG TGT TAG AGC ACG ACA GGT TTC CCG ACT GGA TCT TTC TTT GAT CTG GTT AAG CGT TCG CCC TAT AGT GAG TCG TAT TAC A) *(4)*.

5. The LN34 Ct value of the positive control should be between 25 and 28. A triplicate set up is recommended. If the positive control sample does not produce growth curves that cross the threshold line, invalidate the run and repeat the assay with stricter adherence to the guidelines outlined above.

6. For test samples, if only one of the three triplicates amplifies, the result is considered invalid. Two of the three replicates should amplify with a Ct value earlier than 35 for LN34 or 33 for ß-actin for a valid positive result for RNA extracted from brain tissue. Repeat extraction or testing may be indicated.

7. LN34 sample Ct values should not be considered independent of their ß-actin results. All clinical specimens should exhibit ß-actin curves that cross the

threshold line, thus indicating the presence of host RNA. ß-actin Ct values determine the quality, suitability and potential inhibition of the sample being tested. Some samples may fail to exhibit ß-actin growth curves due to low concentration in the original clinical specimen. Failure to detect ß-actin in any clinical sample may indicate:

- improper extraction of RNA from clinical materials resulting in loss of RNA or carryover of PCR inhibitors from clinical specimens;
- improper assay set up and execution;
- inadequate clinical sample; and/or
- reagent or equipment malfunction.

8. A Ct value of 33–45 in the ß-actin assay may indicate PCR inhibition. Where LN34 is negative and ß-actin Ct is between 33 and 45, dilute samples 10-fold and repeat.
9. Samples where less than one half of replicates exhibited Ct values > 39 and the reminder of the replicates showed no amplification can be considered negative for LN34.
10. The NTC reactions for LN34 and ß-actin probe or primer sets should not exhibit amplification curves that cross the threshold line. If either of these NTC reactions exhibit amplification curves that cross the threshold line, specimen contamination may be indicated. Invalidate the run and repeat the assay with stricter adherence to the guidelines outlined above.
11. This assay does not differentiate between lyssaviruses.

Discussion

Real-time RT-PCR has become a standard confirmatory technique for the post-mortem diagnosis of rabies in humans and animals in many quality-assured rabies reference laboratories. This technique, and other validated molecular methods, are increasingly relied upon as the first line approach for the antemortem diagnosis of human rabies, as DFAT and RTCIT are often inappropriate. Initial concerns regarding molecular assays, including cross-contamination and false–positive results, seem to have been largely resolved by following various preventive measures and quality control systems. Indeed, in a ring-trial in Europe, the performance of real-time PCR gave more concordant results than virus isolation in cell culture *(12)*. A recent pilot programme involving 15 laboratories from the USA, Canada, Europe, Philippines, Chile, Haiti, Georgia and India tested approximately 3000 suspected animal samples using both LN34 assay and DFAT testing and detected more than 1000 positive samples successfully. Compared with the DFAT testing, the LN34 assay produced no false–negative results, one possible false–positive result with a Ct value near the cut-off value and reduced more than 80% of indeterminate results from DFAT testing. Based on the adapted diagnostic algorithm, the LN34 assay achieved 99.31% diagnostic specificity and 99.87% diagnostic sensitivity. Similarly, the JW12/N165 based SYBR Green RT-PCR assay has been validated using hundreds of clinical specimens (human and animal) and was successfully used by 56% of laboratories participating in the real-time RT-PCR

annual (EURL) coordinated proficiency ring-trials (2013–2015), demonstrating 100% concordance with the DFAT and hnRT-PCR results including non-RABV lyssavirus samples.

The two methods described in this chapter are examples of pan-lyssavirus diagnostic real-time RT-PCRs validated for diagnosis of both human and animal rabies. Other established and well-validated assays, both SYBR and probe-based, have been reported, mainly targeting the relatively conserved nucleoprotein or polymerase genes (some examples are shown in Table 28.1) and offer possibilities for laboratories to select, optimize and introduce this technique depending on their specific needs. In settings where the diversity of lyssaviruses is either variable or unknown and for surveillance schemes (such as preliminary bat surveillance) when novel virus variants or even novel lyssavirus species may be discovered, pan lyssavirus assays are highly recommended as RABV-specific based assays may yield false–negative results.

In large-scale surveillance schemes which are likely to yield significant numbers of negative samples, the probe-based assays may be prohibitively expensive relative to the less expensive SYBR® Green assays at this time, but an expansion of production may reduce the costs of probe-based assays considerably. To increase specificity, the melting curve analysis represents an essential parameter to avoid a false–positive result due to the formation of primer dimers. In addition, sequencing of the amplicon can give further confirmation.

The highly specific probe-based assays offer advantages over the SYBR Green assay when circulating virus variants are known or where rapid lyssavirus typing is desired *(2)*. However, a combination of the two approaches is often employed in reference laboratories. For example, a dual combined approach employing two L-gene based real time assays (a pan-RABV probe-based assay and a pan-lyssavirus SYBR Green assay) has recently been established and validated using a large cohort of animal and human rabies infected material *(5)*; see Table 28.1 for primer/probe details.

For the samples with a low level of RABV RNA, such as antemortem samples or samples stored or transported under suboptimal conditions and that cannot be diagnosed by other methods, the real-time RT-PCR assay is especially useful due to its superior sensitivity *(4)*. However, as for other molecular techniques, additional care is required when applying the real-time RT-PCR for the antemortem diagnosis of human rabies compared with postmortem diagnosis on brain tissue. Indeed, the viral load is often much lower in antemortem specimens, potentially below the threshold of detection of the technique, especially when the viral strain is genetically distant from the designed probes or primers. Testing multiple or serial samples and combining different molecular techniques can be useful for such antemortem diagnosis (see **Overview of antemortem and postmortem tests for diagnosis of human rabies in Chapter 5**). The real-time RT-PCR assay is able to test samples stored in the RNA stabilization buffers, or spotted on FTA cards and nitrocellulose membrane of lateral flow devices (**see relevant chapter on the rapid immunochromatographic diagnostic test in this manual**) *(13)*, which reduce the burden of sample collection, transportation and storage for rabies diagnostics and surveillance. This is particularly important in rural areas or developing countries which often have limited resources and lack experienced laboratorians.

Relative quantitation is possible using either SYBR Green or probe-based assays to estimate comparative viral loads in clinical specimens *(2, 4, 14)*. Many primer (and probe) sets have been published (some of which are outlined in Table 28.1) which can be modified and validated locally, to establish an optimal technique for their own particular needs. When established, laboratories should regularly check the performance of their assays by using internal controls and by taking part in national and/or international ring-trials. Because all assays should be fit-for-purpose in the individual laboratory, it is not advisable to recommend one specific assay. Rather, if laboratories want to use real time RT-PCR as a confirmatory test, additional to DFAT, they need to show congruent results when testing a panel of RABVs adapted to their epidemiological setting or other lyssaviruses that represents the global diversity. While positive real-time RT-PCR results may result in prompt action concerning the provision of post-exposure prophylaxis (PEP), where possible they should be followed up using classical virological techniques, i.e. DFAT or virus isolation, to allow for future characterization and support case notification to OIE and WHO.

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Chapter 29
Sanger sequencing of lyssaviruses

Introduction

Sanger dideoxy terminator sequencing was developed by Fred Sanger and colleagues in 1977 *(1)*. The method is based on the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during in vitro DNA replication. Sanger sequencing has become the most widely used sequencing method during the past three decades. For large-scale, automated genome analyses, it has more recently been replaced by next-generation sequencing methods. However, the Sanger method remains in widespread use, particularly when partial genome characterization is sufficient for virus typing or for smaller-scale genome projects. The Sanger method is also the optimal approach for obtaining long contiguous DNA sequence reads (> 500 nucleotides).

Since reverse transcriptase polymerase chain reaction (RT-PCR) was first used to amplify lyssavirus RNA in the early 1990s, a wide array of molecular-based diagnostic assays has been established *(2,3)*. Sanger sequencing enables the rapid, reliable and relatively inexpensive virus typing of lyssavirus PCR products or amplicons compared with conventional monoclonal antibody typing *(4)*. Within just a few years, the genetic typing of lyssaviruses became routine, quickly followed by an explosion of published molecular epidemiological and phylogenetic studies. A specific genomic region has not been prescribed or standardized for lyssavirus molecular typing; however, a considerable amount of data has been published for partial and complete nucleoprotein gene sequences and this region has been shown to be sufficiently discriminatory for virus typing and evolutionary studies *(5–8)*.

Complete genome sequences have been obtained for a number of lyssaviruses via Sanger sequencing of overlapping cloned PCR products or long-distance PCR products, sometimes referred to as “walking the genome” *(9–12)*. The multiple primer sets employed are often available from the authors on request or are supplied as supplementary data *(12).*

Sanger sequencing, also referred to as dideoxy sequencing or chain termination, is based on the use of fluorescently labelled ddNTPs in addition to the deoxynucleosidetriphosphates (dNTPs) found in DNA. Modern day automated sequencing employs an approach called “dye-terminator sequencing”. Each of the chain terminator ddNTPs is labelled with a different fluorescent dye, each of which emit light at different wavelengths. The four labelled ddNTPs (ddATP, ddGTP, ddCTP, or ddTTP) are added with the four normal dNTPs and the DNA polymerase. Before the DNA can be sequenced, it is heat denatured into single strands. Next, a primer is annealed to one of the template strands. The primer is specifically constructed so that its 3’ end is located next to the DNA sequence of interest. Following rounds of template DNA extension from the bound primer

(sequencing reaction), the resulting DNA fragments all start from the same primer site but are terminated by the incorporation of the ddNTP at different sites along the template sequence, thus generating fragments of different sizes that can be separated by gel electrophoresis. Unincorporated dye terminators must be completely removed before the samples can be analysed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with the base calling software. Hence prior to loading the sequence reactions onto the automated sequencers, they are purified to remove excess ddNTPs. Automated DNA sequencers perform capillary electrophoresis for size separation and detection. They record the incorporation of the labelled ddNTPs as fluorescent peak trace chromatograms.

Methods

The following describes the protocols followed when using the ABI 3130xl, 16 capillary and ABI3730, 48 capillary Genetic Analyser instruments. Other machines and reagents are also available for Sanger sequencing.

The ABI3730 and ABI3130 Genetic Analysers are fully automated capillary sequencers. They are capable of determining the nucleotide sequence of any given DNA sample and can also be used to estimate the sizes of DNA fragments that have been prepared with ABI dye-labelled reagents. The ABI 3130xl, 16 capillary Genetic Analyser can run 2 x 96 well plates; the ABI 3730, 48 capillary Genetic Analyser can run up to 16 96 well plates. Both machines can utilize capillary arrays of different lengths depending on what samples are being processed.

Each set of samples, containing labelled DNA fragments, is automatically denatured and then separated by capillary electrophoresis. The replaceable medium (polymer) is automatically replaced in the capillaries after each separation. Detection is facilitated by laser-induced fluorescence in four spectral channels. The four-channel raw data sets generated by each of the capillaries are automatically processed to produce high-quality base sequences or fragment lists after separation.

Preparing PCR amplicons for the ABI genetic analysers

After a PCR product is generated from a lyssavirus-positive sample it needs to be purified then sequenced using florescence-based terminator cycle sequencing. The following describes the protocol to be followed when using the ABI Big Dye Terminator ready reaction kit. The kit works by providing a ready mix which contains the enzyme, dNTPs, magnesium chloride, buffer and dye terminators (ddNTPs) all at the appropriate quantities. Purified amplicon DNA and one primer are added to the mix and cycled to obtain dye terminated products which can be precipitated and analysed on the ABI genetic analysers.

- Sequencing must be performed in both the reverse and forward direction (i.e. using 3’ and 5’ directed primers) so consensus (complementary) sequence can be obtained.

- Sequencing in each direction should be performed, at least, in duplicate prior to publication of the sequence to reduce the likelihood of errors.
- If cloned PCR products are sequenced, ensure at least five independent clones are sequenced and use the consensus sequence for publication to ensure minor variants are not used as the representative sequence.

Purifying the PCR product

After visualizing the PCR product by agarose gel electrophoresis, the remaining PCR product should be purified prior to sequencing. A number of commercial kits are available for the purification of PCR products (e.g. QiaQuick PCR Purification kit [Qiagen]). If the PCR product band appears weak on the gel, it can be loaded and extracted directly from the gel using a commercially available kit (e.g. MinElute gel extraction kit [Qiagen]) which will enable the product to be eluted in as little as 10µL elution buffer and thereby increase the concentration. For large-scale purification (96-well plates), automated purification may be preferable (e.g. HTS PCR 96-well purification system [Millipore Multiscreen] or AMPure clean up system [Beckman Coulter Agencourt]).

Ensure fresh tips are used between samples to avoid cross-contamination.

1. Elute purified PCR product from column in 10–50µL elution buffer depending on the amount of product and column used.
2. Quantify the DNA concentration using spectrometers (e.g. plate readers [NanoDrop or POLARstar Galaxy]).

BigDye terminator cycle sequencing reactions

On ice, add the purified amplicon DNA (approximately 50–100 ng – usually 5µL) to the appropriate wells on a 96-well plate. If plasmid DNA is being used, pre-heat the DNA at 96 ˚C for 1 min and keep on ice. If many primers are being used for sequencing (forward, reverse, etc.), make a master mix without the primer and add primers to appropriate wells at this stage. See below as an example for calculating the amount of DNA to be added.

PCR products: (PCR product length/100) x 2) / DNA conc (ng/µL)

Plasmids: 100/DNA concentration (ng/µL)

1. Make a master mix using the volumes shown below. The amount of water can be amended to allow for variation in the amount of DNA product added, ensuring final volume is 20 µL. The primer employed will depend on the template sequence to be analysed (examples given below).

Sequencing mix volume added per reaction

DNA	5.0 µL
Molecular-grade water	6.0 µL
BigDye sequencing mix	4.0 µL
Buffer	4.0 µL
Primer (3.2pmol/µL)	1.0 µL
Total volume	20.0 µL

Examples of primers (often the primers used for amplification)

Gene	Forward primer	Reverse primer
Nucleoprotein, 1st round	JW12	JW6UNI
Nucleoprotein, 2nd round	JW12	JW10UNI
Glycoprotein	GP01 (GT1)	GP02 (GT1)
Plasmid PCR II	M13–20	M13

2. Cover the plate with a foil-sealing lid and centrifuge briefly to collect liquid at the bottom of the wells if necessary.
3. Transfer the plate to a PCR machine and run on the suitable ABI sequencing programme; these can be modified to suit primers if necessary (see below).

Cycling conditions Big Dye sequencing reactions

96 °C	10 s
50 ˚C	5 s x 25
60 ˚C	4 min
4 ˚C	hold

Ethanol precipitation of sequencing reactions

The purification of the sequencing reactions facilitates the removal of unincorporated labelled ddNTPs which would interfere with base calling. Once cycling is complete, remove the plate from the PCR machine and clean up the sequencing products. It is recommended that the ethanol/EDTA/NaOAc clean-up method is used. While ethanol/EDTA can be used, the smallest PCR fragments may not be precipitated.

Note: Isopropanol precipitation is NOT recommended.

1. Add 2 µL 125 mmol EDTA to each well.
2. Add 2 µL 3 mol sodium acetate to each well.
3. Add 50 µL of 100% ethanol to each well.
4. Seal plate, vortex briefly and leave for 15 min at room temperature.
5. Centrifuge the plate at 3000 r/min for 45 min.
6. Invert plate over sink and shake three times to remove supernatant.
7. Rinse each well with 70 µL 70% ethanol.

8. Centrifuge the plate at 3000 r/min for 15 min.
9. Invert plate over sink and shake three times to remove supernatant.
10. Keeping the plate inverted, place it on a piece of paper towel and centrifuge upside down for 15–30 s at 300 r/min.
11. Air dry for 15–10 min.
12. Seal and label the 96-well plate.

Preparing 96-well sample plate(s) for the genetic analysers

Spin samples down if necessary, remove sealing film.

Loading 96-well sample plate(s)

The Genetic Analysers have a safety feature which means that no movement will occur inside the machine when the door is open. The machines will not work and the buttons on the front of the machines will not function unless the door is properly closed.

For the 3130xl. Press the button on the front of the machine labelled "TRAY". Once the tray has stopped moving you can open the door. Inside, the machine has two positions for holding plates. Position A is on the left and position B is on the right. The plate assembly will only fit in one way with column 1 furthest away from the door due to a notch in the base plate of the plate assembly. Place the plate assembly into position. Close the door and wait to see the green light appear on the front of the machine, which indicates it is properly loaded.

For the 3730. Open the stacker drawer of the Genetic Analyser, then open the In Stack door. Place the plate assemblies into the stacker with the plate(s) orientated so that the notched corner of the plate assembly is at the rear right corner of the stacker. Up to 16 plates can be placed in the "In Stack". Close the In Stack door and then the stacker draw.

Note: The In Stack is at the front of the stacker drawer with the "Out Stack" at the rear. Plates to be run are taken from the bottom of the stack and will then be at the top of the Out Stack once they have been run.

Checking run reagents

Check the polymer and buffers are within expiry dates.

Check there is sufficient volume of polymer in the bottle for the run. Check for bubbles in the tubes (and small tank above) leading from the POP-7. If bubbles are present, an error could occur and your run will stop. To check for bubbles, switch on the light and look inside. Remember to turn off light once checked.

Starting the run on the 3130xl

In the Data collection program on the PC, select from the left-hand list 3130xl/ Run Scheduler in Plate View. Files which show "Processed" next to them are completed runs. Files which show "Pending" next to them are ready to be run.

When you locate your file, it should be Pending.

You will see on the right of the screen two rectangles representing the two plate positions inside the machine. The colour of the rectangles means:

Grey – no plate(s) in holder.

Yellow – unlinked plate(s) in holder.

Green – linked plate(s) in holder.

Click once on the position that relates to your plate, position A (left) or position B (right). It should turn green. The Plate Record will now have A or B to the left of the name indicating the plate position that it is linked to.

To start the run, click on the green arrow at the top left-hand corner of the window.

A dialog box will appear telling you that you are about to start processing your plate. Click OK.

Once the run is finished, check that the run has proceeded correctly and analysed sequence data are available.

Starting the run on the 3730

In the Data collection program on the PC, select from the left-hand list 3730/ Run Scheduler in Plate View. Find the Plate Record file for the plate to be run first and highlight the file.

Click on Add – this will add it to the list in the Input Stack dialog box with Status as Pending. Keep Adding plate records in the same order as the order they will be run, until all those to be run are in the In Stack dialog box.

Plates can be added or removed during instrument operation.

Click on Done to close the Add Plates to In Stack dialog box.

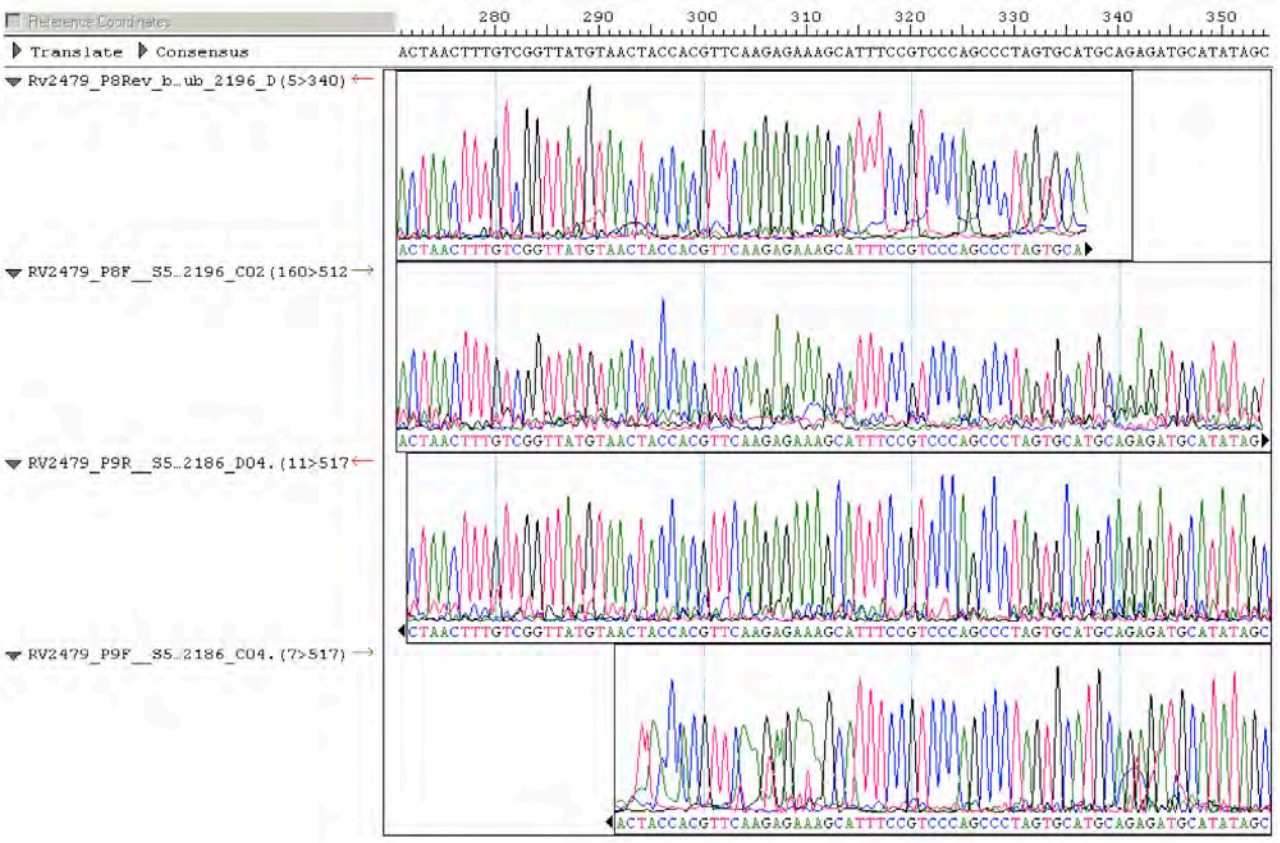
Note: The Plate Record at the bottom of the list in the In Stack dialog box is marked “1” and will be run first, etc.

If the sequencer is running, nothing further needs doing. If the sequencer is not running: to start your run, click on the green arrow at the top left hand corner of the window. A dialog box will appear telling you that you are about to start processing your plate. Click OK.

Once the run is finished, check that the run has proceeded correctly and analysed sequence data are available.

Analysing output data

The raw data will be generated in different formats depending on the sequencer used, but in general you would expect to obtain a chromatogram or trace from which you can derive the sequence (.seq or .fas file). From an ABI sequencer you would expect “.ab1” or “.abi” file formats (Fig. 29.1) whereas from a Beckman



By courtesy of the Animal and Plant Health Agency, Addlestone, Surrey, United Kingdom

Fig. 29.1. Sanger sequencing of overlapping long-distance PCR amplicons (forward and reverse) facilitate genome sequencing via "Walking the genome". The four ABI traces of four overlapping PCR products (RV2479, 1993 EBLV-2 M. daubentonii from Switzerland) yield contiguous sequences which are superimposed (aligned) to generate a consensus sequence (top frame) determined using the Seqman Programme (DNASTAR Lasergene 10)

sequencer you may expect a ".scf" file format.

A number of software packages exist for analysing the data and generating consensus sequences from the replicate forward and reverse outputs (e.g. SeqMan in DNASTAR Lasergene). If the sequences are of good quality they will form a contiguous sequence (called a contig for short). The forward and reverse contiguous sequences are aligned to generate consensus sequences (Fig. 29.1).

If the consensus sequence saved is more than the required length it can be trimmed to the correct length by opening in a sequence editing programme (e.g. SeqMan or EditSeq in Lasergene 10 or MEGA www.megasoftware.net).

Discussion

Sanger sequencing is still beneficial to rabies diagnostic laboratories for confirming PCR-positive material, for determining the source of an outbreak or for understanding the molecular evolution of emerging viruses. Sequencing PCR products can now be achieved relatively inexpensively; the average sequencing run costs less than US $8.

However, sequencing from PCR products may be error prone, particularly

when PCR products are first cloned to improve yield or study viral heterogeneity. Hence, the material from which the genome sequence is derived must be reported when publishing viral genomes. In addition to single nucleotide polymorphisms (SNPs) which may occur naturally following passage of the virus in vitro or in vivo, errors or biases can be introduced into the generated sequences during the PCR or cloning processes *(13)*. Multiple sequence anomalies have already been demonstrated between institutes due to differences in sequencing approaches or sample handling *(14)*. Next-generation sequencing will highlight similar sequencing anomalies in published data and has the potential to indicate the presence and prevalence of viral heterogeneity (quasispecies) in the original infected material. Viral sequences which represent only a minor proportion of the viral pool may be amplified during PCR, cloning or passage and therefore be misrepresented as the consensus sequence by Sanger sequencing. PCR sequence biases may be reduced by performing replicate sequencing reactions from replicate PCR reactions rather than relying on a single PCR test. Where possible, viral sequences should be derived from the original host rather than using multiple in vitro or in vivo passaged material.

All viral sequences must be published, particularly if included in a peer reviewed journal (e.g. NCBI Nucleotide http://www.ncbi.nlm.nih.gov/nucleotide/). For future submissions, we strongly recommend using the most recent version of the standardized sequence submission software, Sequin, obtained from the NCBI website (www.ncbi.nlm.nih.gov/projects/Sequin/), which will harmonize sequence submissions and encourage submitting laboratories to include all essential data, including collection date, virus species, host species and country of isolation.

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Chapter 30
The FTA sampling method for collecting, storing brain material and identification of lyssaviruses

Introduction

The Flinders Technology Associates (FTA) cards or FTA® (e.g. FTA Gene Guard System) is a commercial paper-based system designed to fix and store nucleic acids – DNA and RNA – directly on contact from fresh tissues pressed into the pre-treated paper. The cards that are impregnated with a chaotropic agent under a patented chemical formula lyse cell membranes and denature proteins on contact. Infectious pathogens on contact with such cards are rendered inactive, allowing a shipment of samples at ambient temperature through normal mail routes *(1–3)*. The ability of the FTA paper to inactivate the infectivity of lyssaviruses when stored on the paper for 2 h at room temperature has been demonstrated previously *(4)*. Cell inoculation tests were undertaken to assess the inactivation of rabies virus (RABV) in the FTA Guard System by testing elutes from the filter paper on neuroblastoma cells. Negative results of the cell culture inoculation test performed on 50 µL of elutes from the paper confirmed the inactivation of five tested lyssavirus species, placed onto the paper and treated after drying for 2 h at room temperature.

FTA cards have been demonstrated to preserve nucleic acids within the fibre matrix, allowing molecular characterization of RABV isolates *(4,5)*. The protocol for sampling, storage and shipment of rabies suspect brain samples impregnated on FTA cards is described.

Methods

Reagents

- indicating FTA card [GE HealthCare Life Sciences]
- dessicant pack (silica gel)
- sterile PBS 1x, pH 7.3
- RNase-free water
- 0.2 mL PCR tubes and sterile tubes (15 mL, 2 mL, 1.5 mL)
- pipettes and sterile tips

Protocol

Precautions must be taken to avoid cross-contamination and false–positive results by strictly following the typical routine precautions in PCR protocols in this manual. Always wear laboratory clothing when handling biological samples and FTA cards and regularly change gloves to avoid contamination of samples.

Preparation of FTA stabilized suspect samples

1. Homogenize 1 g of suspect brain tissue in 5 mL of PBS 1x in a 15 mL sterile conical centrifuge tube.
2. Centrifuge for 15 min at 2000 x *g*.
3. Remove 40 µL of clarified supernatant for its application on the FTA card.
4. Lift the cover of the card (Fig. 30.1) to expose the white sample areas.
5. Drop 40 µL of clarified supernatant evenly onto the spot, within the sample area, in a concentric circular motion.
6. Dry the card impregnated with the sample at room temperature for 2 h.
7. When the card is completely dry, put the impregnated FTA card within a sealable protective pouch with desiccant packs to adsorb moisture.
8. Store the impregnated cards in a cool dry environment at –20 °C until use.
9. As the cards inactivate infectious pathogens and as samples containing nucleic acids are not covered by the regulations on the transport of dangerous goods, the cards can be shipped at ambient temperature through normal mail routes.

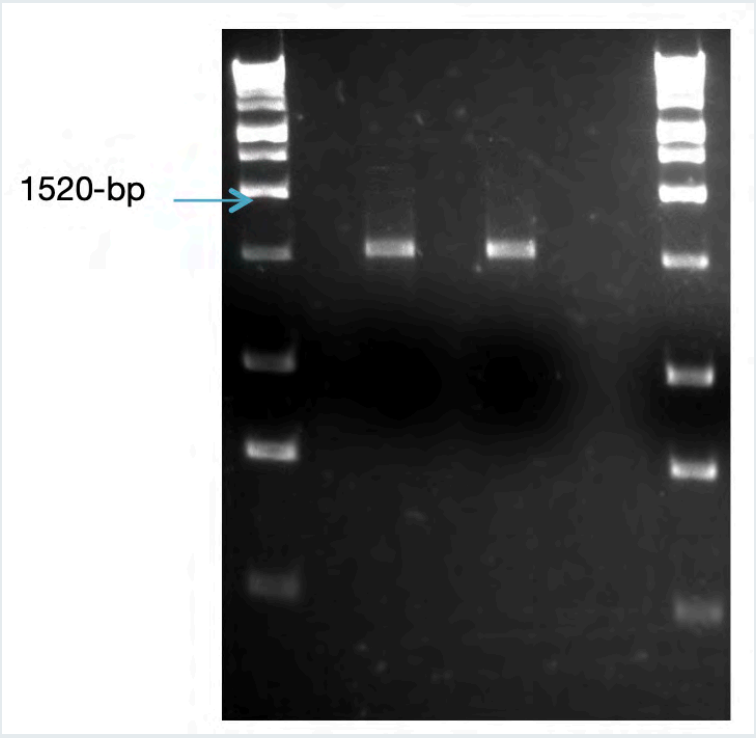
Preparation of an FTA disc for RNA extraction

1. Cut half of the dried spot with a scalpel, then cut it with sterile scissors into little pieces of 2 mm x 2 mm.
2. Place the little pieces in a 2 mL DNase or RNase-free tube.
3. Add 500 µL of PBS 1x. Vortex thoroughly for 10 s.
4. Incubate for 2 h at 4 °C.
5. Centrifuge for 3 min at 20 000 x *g*.
6. Remove the eluate by pipetting and transfer it to a new DNase or RNase-free tube; store the tube at 4 °C until RNA extraction, as described in the manual, depending upon the specific test.

A representative agarose gel of the amplicons (1520-bp) produced by nested RT-PCR using two RABV isolates with, respectively, rabies primers JW12 and PVN8 followed by M13-JW12 and M13-PVN8bis, is shown in Fig. 30.2.

By courtesy of ANSES, Malzéville, France

Fig. 30.1. Sample Whatman FTA® cards
FTA, Flinders Technology Associates



By courtesy of ANSES, Malzéville, France

Fig. 30.2. Example of the amplification of the full nucleoprotein gene from two FTA® stabilized rabies virus samples; the RT-PCR was performed on 5 µL of viral RNA with primers JW12 and PVN8 followed by a second round of amplification with M13-JW12 and M13-PVN8bis, giving an amplicon of 1520-bp

bp, base pair; RT-PCR, reverse transcriptase polymerase chain reaction

Discussion

The chemical reagents impregnated in the FTA cards inactivate most pathogens and nucleases. The ability of the cards to inactivate the infectivity of RABV when stored on the paper for 2 h at room temperature has been demonstrated, as described *(4)*. These cards have been demonstrated to preserve RABV RNA within the fibre matrix by conventional RT-PCR *(6–8)*.

Specimens for rabies diagnosis should be shipped within the UN3373 classification (category B) with triple packaging to avoid any exposure hazards; for the RABV culture, category A transport practices should be applied (UN2814 classification). To achieve reliable diagnostic results, the specimen should be preserved by freezing during transport to the laboratory for rabies diagnosis. The result of RABV inactivation is that the stabilized FTA sample is no longer infectious and, subsequently, it can be shipped through normal mail routes for research studies.

The advantages of FTA sampling have been demonstrated for the molecular characterization of RABV-infected samples with the amplification of partial nucleoprotein gene by conventional RT-PCR *(4, 6, 8)* as well as for the detection of numerous other infectious viruses, including Newcastle disease virus *(3)*, infectious bronchitis virus *(9, 10)* or avian influenza virus *(11)*.

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Chapter 31
Application of next generation sequencing to rabies virus and other lyssaviruses

Introduction

Next generation sequencing (NGS) has revolutionized the ability to determine nucleic acid sequence by offering the unprecedented capacity to parallelize the sequencing reaction, allowing the generation of thousands-to-many-millions of short sequence fragments 70–800 base pairs (bp) in length (so-called "reads"), each reflecting individual input molecules. In contrast to the Sanger-based method, NGS instruments conduct sequencing and detection simultaneously, without the need for cloning steps or electrophoresis, the base interrogation being performed cyclically and in parallel. Since the advent of NGS, different platform formats have been developed, based on various approaches which have been extensively described elsewhere *(1–5)*.

Briefly, two different main categories exist: short-read and long-read sequencing. Short-read sequencing includes two main strategies, namely sequencing by ligation (for example ABI SOLiD-Life Technologies) and sequencing by synthesis (for example Illumina, Ion Torrent [Life Technologies] and Roche 454 [Roche Diagnostics]) platforms; the latter is no longer commercially available. This short-read sequencing category requires a clonal amplification step of the library to be able to obtain sufficient signal intensity for detection. The long-read category, which does not require any amplification steps, mainly corresponds to single-molecule sequencing platforms (i.e. Helicos [Helicos Biosciences], which is no longer commercially available, PacBio [Pacific Biosciences] and MinION/GridION systems [Oxford Nanopore]).

Similar to Sanger sequencing in its time, NGS methods have revolutionized biological research in various fields, including virology, and their impact is evident in the areas of genome sequencing, evolution, ecology, discovery and transcriptomics *(6)*. Application of these approaches is emerging in the field of lyssaviruses, and appears promising. Indeed, NGS enables researchers to obtain full-length genome sequences of rabies virus (RABV) or other lyssaviruses with low time and high cost effectiveness. This represents a major improvement at the level of the molecular characterization of each individual viral strain previously detected by reference diagnosis methods, including new lyssavirus species, but also to reach the highest level of resolution and robustness for any (spatiotemporal) phylogenetic analysis, compared with those traditionally performed on a limited region of the virus genome. The massive number of sequence reads obtained for an individual isolate also offers the opportunity to investigate the viral intrinsic diversity (viral heterogeneity) previously hidden behind the consensus sequence *(7)*. As this is a rapidly developing field, it is important to keep in mind that multiple variations of protocols to obtain full-length genome sequences of RABV or other lyssaviruses exist and that NGS methodologies are continuously evolving.

This chapter describes the main steps required to obtain genome sequences by NGS from sample preparation to bioinformatics analysis, illustrated with examples of protocols or methodologies which have been demonstrated to be effective for lyssaviruses.

The Illumina-based sequencing remains the most widely used NGS sequencing approach in the area of virology, including in the rabies field. Consequently, this chapter will mostly focus on this methodology, although most of the sections can be transposable to other sequencing platforms.

Methodology

Preparation of samples

The preparation of samples is a key step influencing the quality of the genome sequence which will be generated after NGS analysis. This section proposes different protocols that are independent of whether specific or unspecific amplification is subsequently performed.

These protocols can be applied to clinical samples such as human and animal brain tissues, human saliva or skin biopsies, or from viruses propagated in cell lines. They can also be applied to inactivated viral RNA material such as FTA cards or LFD strips.

Protocol based on specific amplicon amplification

This protocol is based on the use of primers which overlap and cover nearly the full-length genome of RABV (or other lyssavirus) isolates *(8)*. The choice and the design of the primers as well as the number of primer pairs necessary will vary according to the isolates to be sequenced. Generally, six primer pairs are sufficient to cover the nearly complete genome (except the extremities), with amplicon lengths ranging from approximately 1500 to 2500 bp (base pairs). The primers can be degenerated to cover as much as possible the genetic diversity within the respective lyssavirus species, or within the corresponding specific phylogenetic clades or lineages. This approach is most effective when highly related viruses are being analysed, for example analysis of a rabies virus incursion or of viruses from the same geographical area, as the need for primer optimization will be less likely *(9)*.

RNA extraction

The RNA extraction step is based on the use of TRIzol, following the manufacturer's recommendations (also presented in Chapter 27 on conventional RT-PCR). For skin biopsies, a preliminary step of lysis using proteinase-K is required before extraction. Extracted RNA are resuspended in 50 µL of RNase–DNase-free water for all samples.

Generation of cDNA

Step	Reagent	Volume per reaction (µL)
Mix 1: pre-incubation (10 min at 70 °C), then place on ice at least 5 min before adding mix 2	$pd(N)_6$ random primers (200 µg/mL) [Roche Diagnostics]	2
	RNase–DNase-free water	2
	RNA template	6
	Total	**10**
Mix 2: incubation (10 min at 25 °C then 90 min at 50–55 °C followed by 5 min at 95 °C)	5X first-strand buffer	6
	0.1 M DTT	2
	dNTP mix (10 mmol) [Eurobio]	2
	RNasin (40 U/µL) [Promega]	2
	Superscript III RT (200 U/µL)	1
	RNase–DNase-free water	7
	Total	**20**
Final volume		**30**

In this protocol, this step is performed with SuperScript® III First-Strand Synthesis System for RT-PCR kit [Invitrogen], although various other commercial kits are available. All reagents are provided with the kit unless otherwise specified.

Prepare the master mix as follows:

The cDNA samples can be used directly for PCR amplification or conserved at –20 °C for long-term storage.

PCR amplification

This step requires the use of a high-fidelity proofreading DNA polymerase (e.g. Phusion High-Fidelity DNA Polymerase [Finnzymes] in this protocol) to minimize the introduction of errors during the amplification process. Nested PCR should be avoided for the same reason. All reagents are provided with the kit unless otherwise specified.

Prepare the following master mix

Reagent	Volume per reaction (µL)
5X Phusion® HF buffer	10
10 mmol dNTPs (Thermo Scientific)	1
Forward primer (10 mmol)	2.5
Reverse primer (10 mmol)	2.5
Phusion® DNA polymerase	0.5
RNase-DNase free water	31.5
Total	**48**

Add 2 µL of cDNA and run the amplification with the following cycling parameters:

Cycling step	Temperature	Time	Number of cycles
Initial denaturation	98 °C	30 s	1
Amplification	98 °C	10 s	Determined by primers used
	Determined by primers used	30 s	
	72 °C	To be adapted	
Final elongation	72 °C	7 min	1
Pending	16 °C	∞	n/a

The temperature of the annealing step as well as the time of elongation and the number of cycles must be adjusted according to the primers selected.

Purification and preparation of amplicons

Visualization of the amplified products after PCR is performed by electrophoresis on agarose gel (1% generally, according to the size of amplicons). Verify the size of the observed band compared with the expected size of the amplicon using appropriate DNA size markers.

Individually purify each amplicon directly for the electrophoresis gel using an appropriate gel purification kit (such as NucleoSpin Gel and PCR clean-up kit [Macherey Nagel]) according to the manufacturer's recommendations.

Quantify the purified amplicon using a fluorescence-based approach (e.g. Quant-iT PicoGreen dsDNA Assay Kit [Invitrogen]), according to the manufacturer's recommendations.

Pool all amplicons from the sample with equimolar proportions to obtain at least 1 ng of dsDNA and either use to prepare the NGS library, or conserve for long term storage at –20 °C.

Protocol based on unbiased non-specific amplification

Different protocols are available for the nonspecific amplification of genetic material. The protocol described below is based on the use of the whole-transcription amplification (WTA) protocol (QuantiTect Whole Transcriptome kit [Qiagen]) as previously described *(10–12).* This kit uses phi29 polymerase to generate large quantity of dsDNA from a low quantity of RNA. This protocol was successfully applied for the generation of complete genome sequences of EBLV-1 lyssaviruses.

RNA extraction

After resuspension of the RNA pellet in 50 µL of RNase–DNase-free water, a purification step is performed (using RNeasy® mini kit [Qiagen]) and the corresponding cleaned-up protocol. Purified RNA is eluted in a final volume of 30 µL of RNase–DNase-free water.

cDNA synthesis

The final volume of cDNA is 20 µL.

Unbiased nonspecific amplification

Prepare the following master mix 1 on ice, add 10 µL of cDNA and incubate 22 °C for 2 h:

Reagent	Volume per reaction (µL)
Ligation buffer	6
Ligation reaction	2
Ligation enzyme 1	1
Ligation enzyme 2	1
Total	10
Final (with cDNA)	**20**

Then prepare the following master mix 1 on ice, add all the previous 20 µL of ligated cDNA from mix 1 and incubate at 30 °C for 8 h, then at 95 °C for 5 min:

Reagent	Volume per reaction (µL)
REPLI-g midi reaction buffer	29
REPLI-g midi DNA polymerase	1
Total	30
Final (with cDNA)	**50**

Store at 4 °C for short-term storage or at –20 °C for long-term storage.

Protocol based on host nucleic-acid depletion without amplification steps

Different protocols are available for the preparation of RNA for next generation sequencing without the use of specific or non-specific amplification steps. Instead, these protocols are based on the removal of host nucleic acid using enzymatic depletion of host DNA and ribosomal RNA (rRNA). These protocols have been validated to the full-length genome sequencing of lyssaviruses directly from clinical or murine propagated samples (brain), and on cell culture propagated viruses *(13)*, or even from other matrices such as FTA cards *(14)*.

RNA extraction and host DNA depletion

As previously described above (and presented also in Chapter 27 on conventional RT-PCR), extraction of total RNA can be performed using guanidinium isothiocyanate-phenol-chloroform-based extraction methods (e.g. TRIzol) or commercial column-based extraction kits (e.g. RNeasy Mini Kit [Qiagen]) following manufacturer's recommendations, depending on the initial biological material. However, it is essential when sequencing samples that have not been selectively amplified that carrier RNA is not used in any part of the process. In addition, both of these approaches can be combined, as described:

1. Start total RNA extraction using TRIzol and follow the manufacturer's recommendations until the phase separation step (obtained after addition of chloroform and centrifugation).
2. Collect the upper aqueous phase in a separated tube and add an equal volume of 100–70% ethanol.
3. Continue the extraction using columns from the RNeasy Mini Kit through the loading of the sample (aqueous phase supplemented with 70–100% ethanol) onto the membrane of the column, then follow the manufacturer's recommendations (split the volume and proceed to successive loading and centrifugation steps to ensure to load all the sample). In this protocol, choose to perform an on-column DNase I treatment (using RNase-free DNase set [Qiagen]) to eliminate as much as possible host DNA (after an incubation of 15 min at room temperature). Eluate the RNA in a final volume of 30 µL nuclease (RNase–DNase)-free water.

rRNA depletion

Different protocols are available for the specific depletion of rRNA. This depletion can be performed with a 5'-phosphate-dependent exonuclease that specifically targets single stranded RNA with a 5' monophosphate, including large rRNA such as 18S and 28S (e.g. Terminator™ 5'-Phosphate-Dependent Exonuclease [Epicentre Biotechnologies] *(13)*). This approach is not limited to host species. Another strategy uses a selective RNase H-based digestion to deplete unwanted RNA (including poly(rA) carrier and ribosomal RNA) from the viral RNA sample, based on specific oligonucleotides targeting rRNA. This protocol can be performed using commercial kits (e.g. NEBNext® rRNA Depletion Kit [New England Biolabs]), although they had been initially designed for a limited number of specific mammalian species (generally human, rat or mouse); however, in-house implementation of this protocol can be done *(15)*, and specifically adapted to other animal hosts. Other commercial kits (e.g. Ribo-Zero-Gold (Epidemiology) Kit [Illumina] for rRNA depletion) are based on specific oligonucleotides coated on magnetic beads, hence avoiding the use of enzymes. The latter kit covers a wide range of application beyond the three initially designed species (human, mouse and rat), at least in silico (including dog) or after being used in large studies *(16)*. The main drawbacks of all these commercial kits are the high cost per reaction as well as the relative specificity of host species of such reagents. These latter approaches have been successfully used with RNA viruses *(15)* and are under investigation with lyssaviruses. However, only the protocol based on the Terminator™ 5'-Phosphate-Dependent Exonuclease already used for lyssaviruses will be described here.

Prepare the following master mix and incubate at 30 °C for 60 min. All reagents are provided with the kit unless otherwise specified.

Reagent	Volume per reaction (µL)
RNA template	30
Buffer A	3.5
Terminator (1 U/µL)	1
RNAsin® ribonuclease inhibitor (20–40 U/µL) [Promega]	0.5
Total	**35**

Clean up using RNA solid phase reversible immobilization (SPRI) beads (Agencourt RNAClean XP SPRI beads [Beckman Coulter Genomics]) following the manufacturer's recommendations or using the RNeasy Mini Kit without DNase digestion. Elute with 11 µL or 30 µL of RNase–DNase-free water, respectively.

cDNA synthesis

Use a volume of 8 µL of DNA–rRNA depleted RNA, to obtain a final volume of cDNA of 20 µL.

Second-strand synthesis *(15)*

This step can be also directly included in the library step, depending on the protocol used.

Prepare the following master mix on ice:

Reagent	Volume per reaction (µL)
RNase–DNase-free water	43
10x second-strand reaction buffer [New England Biolabs]	8
10 mmol dNTP mix [Eurobio]	3
E. coli DNA Ligase (10 U/µL) [New England Biolabs]	1
E. coli DNA Polymerase I (10 U/µL) [New England Biolabs]	4
E. coli RNase H (2 U/µL) [New England Biolabs]	1
1st strand reaction	20
Total volume	**80**

1. Add all the volume of cDNA (20 µL), vortex gently and centrifuge at 280 x *g* at room temperature for 1 min. Incubate for 2 h at –16 °C (keep lid at 25 °C) without allowing the temperature to rise above 16 °C.
2. Place the tubes on ice and inactivate reaction by adding 5 µL of 0.5 mol EDTA, mix gently and centrifuge at 280 x *g* at RT for 1 min.

3. Clean up using DNA SPRI beads (Agencourt AMPure XP SPRI beads [Beckman Coulter Genomics]) following the manufacturer's recommendations. Elute in a small volume (e.g. 9 µL RNase–DNAse-free water).

Preparation of libraries

The choice of the library preparation depends on several parameters, including the choice of NGS platform and the amount of input of nucleic acid available. These aspects have been extensively described elsewhere *(17, 18)* and can also be found from the respective manufacturers. However, the main general steps are mostly similar and correspond to: (i) fragmenting and/or sizing the target nucleic acid to a desired length (mechanically or enzymatically-based); (ii) converting the target to dsDNA (this step can also be performed upstream, as described previously); (iii) attaching oligonucleotide adapters (to the ends of target fragments) that contain the necessary elements for immobilization on a solid surface and sequencing; (iv) amplification of the library; and (v) quantifying the final library product for sequencing. The level of multiplexing (i.e. the number of different tagged samples sequencing simultaneously) is also dependant on the library preparation. Depending on the choice in library preparation and in NGS platform, the format of the data generated after sequencing will vary, in terms of read length (generally from 100 bp to 350 bp [for Illumina technology]) and number of sequence reads (from several thousand to millions) and therefore in terms of sequence depth and percentage of genome coverage (see below).

From a practical point of view, this preparation is mainly based on commercial kits according to the manufacturer's recommendations and adapted to the respective sequencing platform, bearing in mind the technologies are regularly being updated and improved. In the field of rabies, several different protocols have been described; the most recent is associated with the Illumina technology *(8, 13, 19)*, but will not be detailed in this chapter. However, optimization of library preparation may be necessary for DNA fragmentation and minimization of purification steps in order to reduce sample loss and prevent cross-contamination.

Bioinformatics analysis

The bioinformatics process to obtain RABV and other lyssavirus genome consensus sequences is general to those used for other viruses and is broadly similar whatever the NGS platform used. The first step of this process is the cleaning of raw sequence (reads) data using adapted and validated parameters of quality control. Then the construction of the viral consensus sequence is performed, based on the mapping of the high-quality reads against a selected reference sequence. This consensus sequence is finally controlled and validated after a second mapping or a de novo assembling step.

Cleaning of raw sequence data

Raw sequence reads need to be pre-processed before use for subsequent genome sequence reconstruction in order to select only high-quality reads after filtering. Various parameters must be taken into account and adjusted for this process, all of which are relatively common irrespective of the NGS platforms considered. The first step is to eliminate adapter sequences (used during library preparation) and (if using amplicon-based sequencing) primer sequences. The other parameters are primarily based on the quality (through the Phred quality

score) associated with each individual base, and on the length of individual reads. For example, bases at either end of each read with a Phred quality score below a selected value are trimmed, and reads with length of less than another selected value after these processing steps are discarded, as well as those containing more than a specific proportion of low Phred quality score bases. Various workflows for this pre-processing step are available (free of access or combined with specific software) and can be found in the literature *(8, 19)*.

Consensus sequence reconstruction

After high-quality filtering of sequence reads, the reconstruction of the viral consensus sequence is based on a mapping approach. The principle is to try to position each read, according to specific parameters, to a reference full-length genome sequence which is as similar as possible to the expected sequence. In the field of lyssaviruses, the choice of this reference sequence could rely on the source of the virus isolate, i.e. its principal animal reservoir and the availability of already published complete genomes, etc. After this mapping step, a first consensus sequence is obtained and will serve as the new reference sequence for a second mapping step, using all the filtered reads, to obtain a final refined consensus sequence *(8)*. Alternatively, the mapped reads can be used to perform a de novo assembly to generate a consensus sequence which can then be used as the new reference sequence for a last mapping step using all the filtered reads to generate the final consensus sequence *(19)*. The majority nucleotide (> 50%) at each position, provided there is sufficient coverage, is generally used to generate the consensus sequence. Various mapper and de novo assembler programs exist and are described elsewhere; they must be tested and adapted before use. A minimum mean coverage is necessary to obtain a reliable consensus genome sequence (see discussion section below), and sequence assembly should be visually inspected using appropriate software to evaluate the coverage along the genome sequence, at the level of each individual base.

Additional information can also be obtained from the data generated by NGS sequencing. In particular, and depending on the average coverage (the “depth” of the sequence or the number of reads covering each nucleotide position), these NGS technologies offer the possibility (i) to determine the minority single nucleotide polymorphism (SNP) at each nucleotide position, after a specific percentage cut-off has been defined, and (ii) to explore the intrinsic diversity for each isolate *(9, 20)*. Such analysis requires a coverage rate of hundreds to thousands of reads per base, as the highest resolution and reliability in terms of intrinsic diversity is associated with the greatest coverage obtained.

For viruses that are divergent from known viruses (or where a full reference genome is not available) a difference approach is required, as described *(21, 22)*. In such cases, after high-quality filtering of raw data, host sequences should be removed by mapping to a suitable host genome. Subsequently, the remaining non-host reads can be used for de novo assembly, and BLAST is used to find assembled contigs that correspond to viral genomes. Mapping can then be used as described above to ensure the accuracy of the consensus sequence.

Discussion

This chapter presents several examples of different protocols already used and validated for the preparation of RABV and other lyssaviruses for NGS sequencing, and summarizes how to analyse the output data and generate a consensus genome sequence, mainly based on Illumina technology (which is currently the most widely used NGS platform). Special focus is given to the preparation of samples for NGS sequencing using three different strategies each with their own pros and cons.

The first strategy, the amplicon-based protocol, provides homogeneous, deep coverage of the genome sequence of each sample, because the genetic material sequenced is exclusively viral. This approach is best adapted to obtain the consensus genome sequence relatively similar to a known sequence. The high depth of coverage permits the investigation of the intrinsic diversity at a low level. However, this must be interpreted with care as the amplification may have skewed the relative abundance of the minority variants, and errors may have been introduced by the amplification step, although they can be compensated for or minimized with the use of a high proof-reading DNA polymerase. The main disadvantage of this method is the cost, both in the researcher's time (in continued designing and optimizing of the primers) and in the reagents used.

The second strategy is based on an unbiased nonspecific amplification of the extracted RNA, using for example a whole transcriptome amplification. The main advantage of this approach is to obtain a huge quantity of genetic material, especially when working with precious and quantity-limited biological materials (e.g. with human CSF samples or saliva swabs), adapting to sequencing protocols requesting a large quantity of dsDNA or when additional tests are requested on starting material (e.g. RT-qPCR). In addition, because this protocol is nonspecific it can be used for any lyssaviruses or indeed any virus. Similar to the previous protocol, one of the main disadvantages, although unlikely, is the potential introduction of errors during the amplification steps. Furthermore, as the amplification is nonspecific, the amount of viral RNA remains proportionally low in relation to host RNA.

The third strategy does not rely on any amplification but on the depletion of host RNA to increase the proportion of viral or host RNA in the samples to be submitted. This method addresses the disadvantages of the other approaches as there is no amplification, and it can be used to sequence any lyssavirus or virus. Depletion of host nucleic material is utilized to increase the proportion of viral RNA present, but will mean that there is generally limited input material. This can be mitigated by using a sequencing library kit that requires minimal input material (e.g. commercially available [Illumina NexteraXT]). However, in clinical tissue samples the percentage of reads that are viral is still low (usually $< 5\%$), but a reliable consensus sequence can be obtained even with a relatively low coverage (see below). The low coverage occasionally results in an incomplete genome sequence, and can be less suitable for investigating the genetic intrinsic diversity, especially at a low level.

Despite their differences, all protocols have been used to obtain the complete genome sequence of RABV and other lyssavirus isolates, or at least the complete coding regions (the genome extremities with the leader and the trailer regions

often absent or having low coverage for all of these approaches). However, the quality and the reliability of the consensus sequence obtained remain associated with the average depth of the coverage as well as with the number of reads per nucleotide position (this number varying along the genome sequence). In this context, it is essential at least to visualize and inspect the depth of coverage along the genome, particularly through adapted software and at least through a coverage graph, as well as to evaluate the proportion of each per nucleotide position (especially for positions or regions associated with very low coverage). For novel viruses, Sanger sequencing of PCR amplicons spanning low coverage regions, followed by remapping of NGS reads with the corrected consensus, may be required to confirm the sequence.

However, it is very important to bear in mind that the methods used in NGS sequencing are constantly evolving, whether at the level of platforms, the preparation of samples or the tools applied for data analysis. Before implementing this sequencing approach, it is necessary to ensure that the choice of the methodology is cost effective and adapted to the needs and expected outcomes and to ensure that all steps have been validated, to be confident in the data generated. In parallel, remaining vigilant to the development of the methodology is recommended.

The recent advent of NGS sequencing technologies has revolutionized various fields of biological sciences, including microbiology and virology in particular. One of the major impacts in the field of virology is observed with molecular epidemiological analysis. Indeed, NGS platforms offer the possibility to obtain rapidly and cost effectively the complete genome sequences of any virus compared with previous methods based on Sanger sequencing. In this context, NGS sequencing allows a complete molecular characterization of new isolates or lyssavirus species based on the complete genome sequence *(21, 22)* essential for the official validation of new virus species, and represents now a useful tool for the quality control of viral vaccine strains found in live vaccines strains used for rabies vaccination in wildlife *(7, 23).* In parallel, the dramatic increase of genetic information available for each isolate enables more accurate and precise molecular epidemiological and phylogenetic analysis.

Various recent examples illustrate this progress, especially with the 2014–2015 outbreak of Ebola virus infection in West Africa *(24–26).* In the rabies field, NGS has also been utilized to obtain full-length genome sequences which, combined with associated epidemiological data (such as animal host, location and date of collection), already allows refined details to be obtained using phylogenomic analysis on the evolutionary history of lyssaviruses *(27)*, as well as large-scale analysis of RABV *(8)* for a specific phylogenetic lineage *(19)* or for a specific geographical location. In addition, molecular analysis of complete genome sequences, at the level of the consensus sequence or at the level of the intra-host genetic diversity, provides important data to understand the cross-species transmission and mechanisms of new host adaptation frequently observed with RABV *(8, 9)*.

As the cost associated with NGS sequencing is reducing and protocols are becoming standardized, it is now evident that the widespread use of this sequencing approach will significantly improve our capacity to understand the drivers of transmission *(28)*, providing important data for the prevention and control of rabies.

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Chapter 32
Reverse transcriptase loop-mediated isothermal amplification system for the detection of rabies virus

Introduction

Innovative loop-mediated isothermal amplification (LAMP) *(1,2)* provides a rapid, simple, sensitive and inexpensive method for amplifying specific DNA sequences. The technique was first demonstrated in 2000 *(3)*. LAMP depends on the autocycling of strand-displacement DNA synthesis conducted by *Bst* DNA polymerase. This reaction proceeds without denaturation of DNA templates *(4)* and thus can be performed at an isothermal temperature. It provides high amplification efficiency with DNA being amplified 10^9–10^{10} times in 15–60 min. The amplified products consist of a series of stem-looped DNA in various lengths *(5)*. The results can be determined by visual inspection of the turbidity due to precipitation of white magnesium pyrophosphate, a byproduct of DNA synthesis *(6)*, or by visual and ultraviolet (UV) inspection of DNA amplification with a fluorescent dye *(7)*.

For the amplification of target RNA, the RT (reverse transcriptase) LAMP method can synthesize cDNA from target RNA and the LAMP technology applied to amplify the resultant cDNA. After mixing and incubating at a constant temperature between 60 °C and 65 °C, amplification and detection can be carried out in a single step. The RT-LAMP method has potential application for detection of pathogens and has been developed for the diagnosis of many RNA viral diseases. For rabies virus (RABV), several examples of the application of this method have been reported *(8–13)*.

Materials

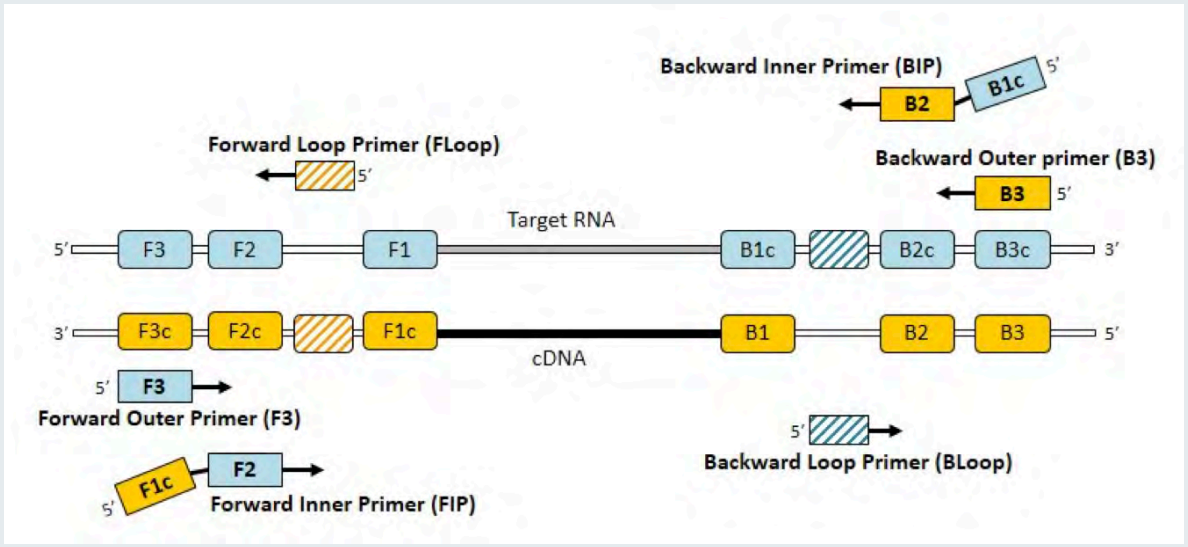
Reagents

- AMV reverse transcriptase [New England Biolabs]
- *Bst* DNA polymerase [New England Biolabs]
- Betaine [Sigma]
- 10×ThermoPol II (Mg-free) reaction buffer [New England Biolabs]
- dNTPs (100 mmol)
- $MgSO_4$
- Purified primers (HPLC or Cartridge purification grade)

These reagents, as a mixture that excludes the specific primers to be used, are commercially available [as Loopamp RNA Amplification Kit (RT-LAMP) from EikenChemical *(5)*].

Primer set for RT-LAMP

To assess the applicability of RT-LAMP, a primer set can be designed by using PrimerExplorer V4 software *(14)* or LAMP designer software *(15)*. The primer sets for RABV detection used in the previous studies are shown in Table 32.1. The concept of primer design for effective gene amplification and elongation reactions can be explored by an accessible animation on the Eiken GENOME SITE *(5)*. The standard set of four primers (F3, B3, FIP, BIP) consisting of two outer primers and two inner primers is basically used for the gene amplification in RT-LAMP (Fig. 32.1). The outer primers are referred to as the forward outer primer (F3) and the backward outer primer (B3). The two inner primers are referred to as the forward inner primer (FIP: F1c+F2) and the backward inner primer (BIP: B1c+B2). The use of the primers through OPC (oligonucleotide purification cartridge) or HPLC (high-performance liquid chromatography) purification is advisable. Two further loop-binding primers (FLoop and BLoop) have been optionally added to increase the rate of strand displacement and synthesis.



By courtesy of Takuya Itou, Wanda Markotter and Louis Nel; partially adapted with permission from the Eiken GENOME SITE, Eiken Chemical

RT-LAMP, reverse transcriptase loop-mediated isothermal amplification

Fig. 32.1. Schematic diagram of RT-LAMP primers showing the position of the six primers spanning the target gene

The inner primers FIP (BIP) are composed of F2 (B2) and F1c (B1c). The outer primers are at the region of F3 and B3. The loop primers Floop and Bloop are designed between F1c (B1c) and F2c (B2c). The U (Uracil) on target RNA sequence will be transcribed into T (thymine) for primer design.

Table 32.1. Details of oligonucleotide primers designed to detect rabies virus using RT-LAMP

Primer	Sequence (5'–3')	Position	Target strain	Reference
C-F3	ACATGTCCGGAAGACT	250–265	CVS-11	*(8)*
C-B3	CAGACTCAGGAGAAGACC	424–441		
C-BLP	GGCATGGAATTGACAAGGGACC	373–394		
C-FIP	ACTAGAGAGTTTGGGGTGA-GGACCAGCTATGGAATCC	308–326+266–283		
C-BIP	ACGGGAATTGGGCTCTGAC-CTAAAGATGCATGTTCAG	350–368+403–421		
P-F3	ACATGCCCTGAAGATT	250–265	Philippines dog	*(8)*
P-B3	AAGACTCAGGAGAAGACC	424–441		
P-BLP	GGTAGGAGCTGACAAGGGACC	373–394		
P-FIP	ACAAGGGAATCAGGGGTGA-GGACTAGCTATGGGATCT	308–326+266–283		
P-BIP	AAGGAAATTGGGCTCTGAC-CTAAAGACGCATGTTCTG	350–368+403–421		
F3	GCCCCCGACTTAAACAAAGC	118–137	PV (Cosmopolitan lineage and vampire bat lineage)	*(9)*
B3	TTCCCCTCTACATCAGTACG	319–338		
FIP	ACTGCATTGCTGCTGCCAAGTA-GCATGAACGCCGCCAAAC	199–220+158–175		
BIP	TGTCCGGAAGACTGGACCAGCT-ACAAGAGAATCTGGGGTGAT	235–256+289–308		
FLoop	GGAGCATACATCATCAGGATCNA	176–198		
BLoop	ATGGAATCCTGATTGCACGAMA	257–278		
Rab1F3	AGCCCCCGACTTAAACAAAG*	–#	Cosmopolitan lineage	*(10)*
Rab1B3	CTGTCAGAGCCCAATTTCCT*	–		
Rab1FIP	GCATTGCTGCTGCCAAGTAGGATTTTCAGGCATGAATGCAGCCA*	–		
Rab1BIP	CGTGTCCAGAAGACTGGACCAGTTTTATTTCCACCAGAGAATCC*	–		
Rab1FLOOP	ACATACATCATCAGGATCAAGT*	–		
Rab1BLOOP	CTATGGAATCTTGATCGCACG*	–		
Rab4F3	GCCCCCGATTTGAACAA*	–	Arctic lineage	*(10)*
Rab4B3	GGGAATTGGGCTTTGACG*	–		
Rab4FIP	ACTGCATCGCAGCTGCTAAGTAGGATTTTCAGGCTTGAATGCTGCCAA*	–		
Rab4BIP	CATGTCCTGAAGACTGGACCAGTTTTATCTCCACAAGAGAATCTGGGGT*	–		
Rab4FLOOP	ACATACATCAGGATCAAGC*	–		
Rab4BLOOP	CTATGGGATCTTGATTGCAAG*	–		
F3	GAAAAGGAGACAAGATCACC	363–382	PV (Africa 1b lineage)	*(11)*
B3	CCGGTGTTTTGTCCTGAT	528–545		
FIP	CCTTGTCAGCTCCATGCCTCCCGGACTCTCTAGTGGAAAT	383–460		
BIP	ACCCCACTGTCTCTGAGCATTGCTCAACCTATACAGACTCA	461–524		
CVSF3	AGCCCCCGACTTGAACAAAG	–	CVS	*(12)*
CVSB3	CTGTCAGAGCCCAATTCCCG	–		
CVSFIP	GCATTGCTGCTGCCAAGTAGGATTTTCAGGCATGAATGCCGCCAA	–		
CVSBIP	CATGTCCGGAAGACTGGACCAGTTTTATCTCCACTAGAGAGTTTGG	–		
CVSFLOOP	GCATACATCCGGATCAAGT	–		
CVSBLOOP	CTATGGAATCCTGATTGCACG	–		
Deg-F3	YCCWGATGATGTRTGYTCCTA	268–288	Indian RABV strains	*(13)*
Deg-B3	AGTTRCCRGTGTTYTGYC	534–551		
Deg-FIP	TATYTCYACMAGAGAATCYGGR+GAYTGGACCAGCTAYGGR	382–403+332–349		
Deg-BIP	GACNGGAGGAATGGARYTRAC+ACTCAARAGAAGACRACTAA	436–456+488–508		
Deg-LF	RTCYCCTTTYCKTGCRATCAR	353–373		
Deg-LB	CCACTGTYYCYGAGCATG	465–482		

*1 The combination of these 12 primers was examined and deemed feasible for use via RT-LAMP. See reference *10* for the optimal concentration of each primer.

No information

Equipment

- For real-time turbidity detection
  - Loopamp Realtime Turbidimeter [Eiken Chemical]
- For visual and real-time fluorescence detections
  - Incubator (temperature accuracy within ± 0.5 °C) and fluorescence scanner (e.g. ordinary real-time PCR detection system or ESEQuant Tube Scanner [Qiagen])
  - Heat block (for termination of the LAMP reaction)
  - UV transilluminator (wavelength at 240–260 nm or 350–370 nm)

Several additional methods for detection are reviewed by Zhang and colleagues *(16)*.

Biologicals and sample preparation

For detection, brain samples are used. Viral RNA extraction of RABV from brain tissue is undertaken as described in Chapter 27 on RT-PCR. Commercial extraction kits (such as the QIAamp Viral RNA Kit [Qiagen] and TRIzol [Life Technologies or Invitrogen]) are available.

Methods

Preparation of master mix

1. After frozen reagents are thawed at room temperature, prepare the following master mix on ice. Once the reagents are thawed, keep them on ice.

Composition of master mix[a]

Reagents		Amount/final concentration
10×ThermoPol II (Mg-free) reaction buffer		2.5 µL
dNTPs		0.5–1.0 mmol each
$MgSO_4$		8 mmol
Betaine (Sigma)		1 mol
Bst DNA polymerase		8–16 units
AMV reverse transcriptase (or equivalent enzyme)		0.2–1.0 U
Primer:	FIP	40 pmol
	BIP	40 pmol
	FLoop[b]	20 pmol
	BLoop[b]	20 pmol
	F3	5 pmol
	B3	5 pmol
RNase–DNase-free sterile water		X µL (ad q.s.)
		Total 20.0 µL/tube

[a] Master mix reagents excluding primers are replaced by reaction mix and enzyme mix in Loopamp RNA Amplification Kit (RT-LAMP).

[b] These loop primers are optional and their use is merely to accelerate the LAMP reaction.

2. For real-time fluorescence detection, add a fluorescent dye such as 1 µL of fluorescent detection reagent [Eiken Chemical] or 0.4 µmol of SYTO-9 green fluorescent dye [Life Technologies] and maintain the total mixture amount of 20 µL.

3. After dispensing, gently tap the tubes for a few times, or mix the solution by repeatedly inversing the tube, or mix thrice by vortexing for 1 s. After mixing well, centrifuge the tubes for a few seconds. Avoid too much vortexing because of enzyme inactivation. The master mix should be prepared immediately before use.

Mixing of master mix and sample (on ice)

1. Dispense 20 µL of the master mix into a micro tube.

2. Add 5 µL of extracted sample RNA to the master mix; the volume should be 25 µL in total. For the negative control reaction, use 5 µL of water instead of sample RNA. Thoroughly mix the solution by pipetting or tapping the tube with the cap closed and then spin down. Take care not to create air bubbles when mixing.

Amplification reaction

1. Place the reaction tubes in a turbidimeter, fluorescence scanner or the incubator, and incubate at 60–65 °C for 30–60 min. (The reaction condition must be optimized for specific primers sets.)

2. Inactivate the enzyme and terminate the reaction by incubating the mixture for 2 min at 95 °C.

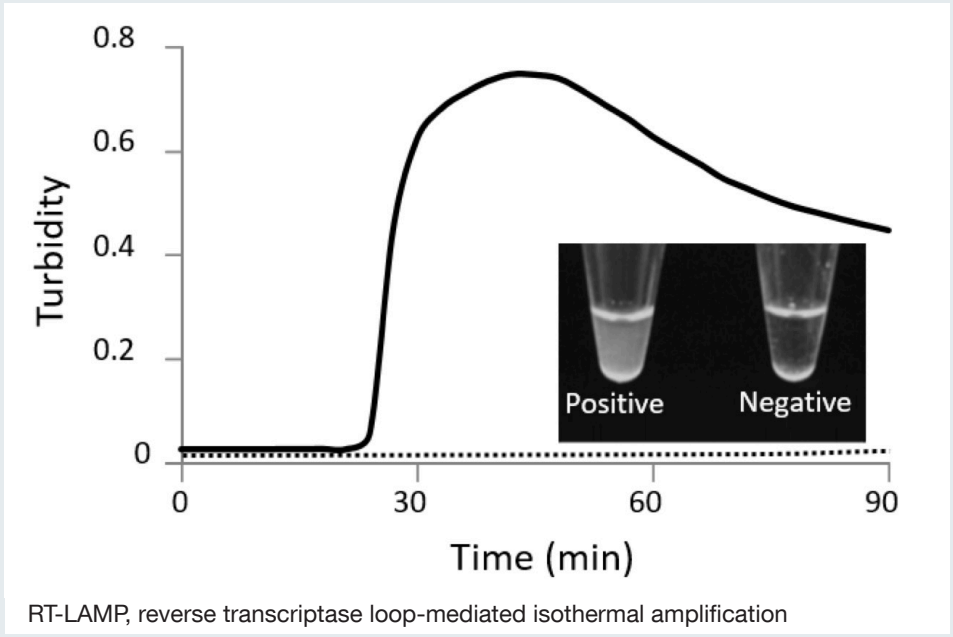
Detection and interpretation of results

Turbidity detection

The turbidity of magnesium pyrophosphate, a byproduct of the LAMP reaction, is formed in proportion to the amount of amplified products. Since LAMP can yield extremely high amounts of amplified products, white turbidity can be visually observed. Real-time turbidity detection can be conducted with the turbidimeter (e.g. Loopamp Realtime Turbidimeter). An example is shown in Fig. 32.2.

Fluorescence detection

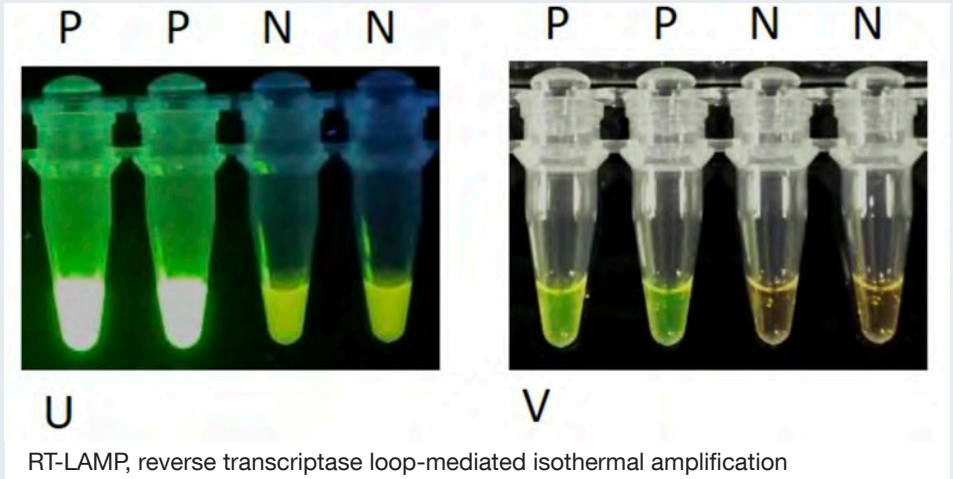
Visual fluorescence detection can be achieved by the addition of 2 µL of 10–100-fold diluted SYBR Green I [Lonza] to the reaction tube after the amplification reaction (Fig. 32.3). An ultraviolet transilluminator and protective goggles are required. The fluorescence of samples should be evaluated by comparison with the positive and negative controls. The incubation can be done in commercially available incubators or in the Loopamp Realtime Turbidimeter [Eiken Chemical]. Real-time fluorescence detection can be conducted with a fluorescence scanner (ordinary real-time PCR detection system or ESEQuant Tube Scanner [Qiagen]).



By courtesy of the University of Pretoria, South Africa

Fig. 32.2. Real-time monitoring of the representative results of the RT-LAMP assay

The solid line shows the RT-LAMP reaction of a RABV-positive sample. The dotted line shows the reaction of a negative sample. The inset shows representative tubes after the RT-LAMP reaction. A positive reaction is represented by the formation of magnesium pyrophosphate, a white precipitate by-product.



By courtesy of the University of Pretoria, South Africa

Fig. 32.3. Visual inspection with SYBR Green I after RT-LAMP

The panels U and V indicate the results under ultraviolet and visible lights, respectively.

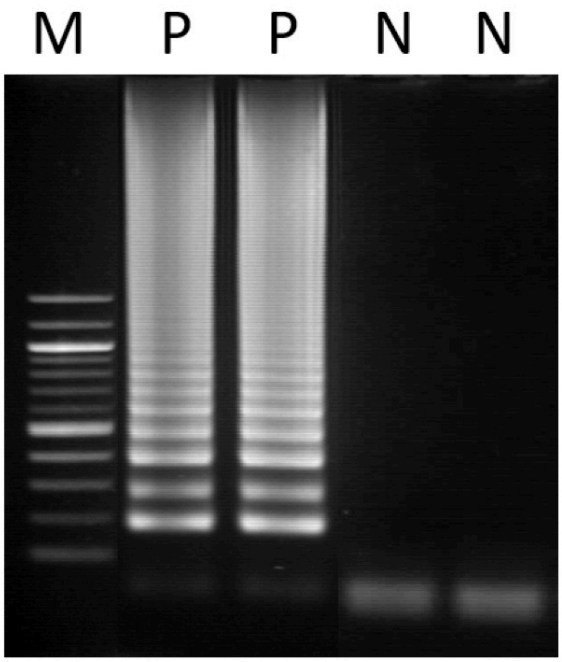
P, RABV-positive sample; N, negative control

Electrophoresis

To avoid contamination, extra care should be taken when handling the amplification products during the electrophoresis process.

The reaction solution is analysed on a 2% agarose gel (1–2 µL.)

A typical ladder pattern can be observed after electrophoresis (Fig. 32.4), as the amplified products consist of various sizes of inverted repeats of the target sequence on the same strands.



By courtesy of the University of Pretoria, South Africa

Fig. 32.4. Agarose gel electrophoresis of RT-LAMP product

M, 100 bp (base pair) ladder marker; P, RABV-positive sample; N, negative control

Validation of the specificity of LAMP reaction

To validate whether the amplified product is derived from the target region, sequencing or restriction, enzyme digestion of the amplicon can be undertaken.

Discussion

The RT-LAMP technology has remarkably high amplification efficiency, achieving highly sensitive detection of specific nucleotide sequences in about 1 h. Its sensitivity is 10–1000 times higher than that of conventional RT-PCR *(8, 9)* and equivalent to real-time RT-PCR *(12).* Thus, the RT-LAMP is a promising technology for simple and rapid genetic detection of RABV. The LAMP reactions also have high specificity and can discriminate slight differences in the sequences of the target genes because the length of binding sites complementary to LAMP primers are longer than that of PCR primers. Hence, the design of LAMP primers with high sensitivity and specificity is crucial to the success of LAMP analysis. The LAMP primer sets in previous studies using RT-LAMP for RABV detection all targeted the nucleoprotein gene, which is relatively conserved among RABV variants.

Saitou and colleagues reported a risk for false–negative results in RT-LAMP when applied to RABV isolates from different parts of the world, depending on the primers selected *(9).* In most geographical areas, multiple genetic lineages of RABV exist, and the development of primer sets based on DNA sequencing data is recommended. Ultimately, universal RT-LAMP primer sets which can detect all lyssavirus species worldwide will be ideal. Hayman and colleagues demonstrated that RT-LAMP successfully detects multiple lineages of African RABV by using a combination of two sets of LAMP primers, a total of 12 primers *(10)*. Additional evaluations using these primer sets or improved primers are necessary for the development of universal sets.

The RT-LAMP is prone to the same challenges as other molecular detection techniques, such as cross-contamination during the opening of the tubes due to the high amplification efficiency. To avoid false positive–results by contamination, the samples and reagents should be prepared in dedicated areas; the use of multiple negative controls between samples is recommended.

The RT-LAMP technique is inexpensive and can be performed without the need for a thermal cycler. Results can also be detected by a portable scanner (e.g. ESEQuant Tube Scanner [Qiagen]) and adapted to an easy-to-use lateral flow device format for visual detection of LAMP products *(10)*. Therefore, the RT-LAMP can be useful in low resource settings where rabies incidence is usually the highest.

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Chapter 33
Detection of lyssavirus nucleic acids by in situ hybridization

Introduction

Gold standard methods for the detection of rabies virus (RABV) and other lyssaviruses, such as the direct fluorescent antibody test (DFAT; see Chapter 11), or current molecular methods, such as reverse transcriptase polymerase chain reaction (RT-PCR; see Chapter 27), require the use of fresh or fresh frozen samples, and their sensitivity may be impaired by autolytic and putrefactive changes *(1).* Formalin fixation is widely and routinely used to preserve tissues for histopathology. On occasions when maintaining a suitable cold chain or accessing diagnostic laboratories with specialized equipment is a challenge, formalin fixation provides an alternative method for maintaining sample integrity for downstream testing. However, the performance of DFAT and molecular methods for RABV detection is less optimal in formalin-fixed and in formalin-fixed, paraffin-embedded (FFPE) tissues due to formalin-induced cross linking, RNase activation and RNA fragmentation, or both *(2,3).*

Alternative histopathological methods for the detection of RABV in FFPE samples have been developed, including immunohistochemistry and in situ hybridization (ISH). ISH allows the detection of specific nucleic acid sequences in morphologically preserved cells and tissues, and the visualization of messenger RNA (mRNA) and genomic RNA (gRNA) of lyssaviruses at cellular and subcellular level for diagnosis, virus typing and viral pathogenesis studies.

Originally, radioactive-labelled RNA probes were used for the detection of nucleocapsid protein mRNA and gRNA in the central nervous system of RABV-infected mice *(3)*. Radioactive probes were also used for the detection of RNA encoding all five RABV proteins in mice and human brains *(5)* and to evaluate the effect of autolysis up to 72 h in the detection of virus RNA by ISH *(6)*. Jackson and Rintoul showed that autolysis resulted in a noticeable progressive reduction of the ISH signal, less marked for the detection of RABV antigens. The detection of RABV mRNA in mouse brains using digoxigenin (DIG)-labelled RNA probes demonstrated the advantages of this method over the use radioactive probes *(7)*. DIG-labelled probes were used subsequently to detect RABV RNA in experimentally infected mouse and human brains *(8, 9)*. DIG-labelled probes have also been designed and used on FFPE tissues to discriminate and type RABV, based on P gene sequences and allowing for retrospective typing *(10)*. Such use has also differentiated RABV, European bat lyssavirus type 1 (EBLV-1) and type 2 (EBLV-2), targeting the N gene *(11)*. Fluorescent in situ hybridization (FISH) methods using biotinylated oligonucleotide probes on fixed RABV-infected cell cultures have shown that all viral RNAs (genome, antigenome and mRNA) are present in the inclusion bodies or Negri body-like structures developed in infected cells, indicating that viral transcription and replication occur in these structures *(12)*. Using

a similar method, Nikolic et al. have shown that viral mRNAs, but not gRNA, are selectively transported from Negri bodies to the stress granules induced by RABV infection, demonstrating the direct application of ISH to the study of subcellular changes and viral pathogenesis *(13)*.

The ISH protocol described in this chapter utilizes commercially sourced oligonucleotide probes. The probes can be tailored to differentiate between specific lyssaviruses (e.g. RABV, EBLV-1 or EBLV-2) or can be designed for cross-species detection. Therefore, identification of genomic regions with the adequate genetic diversity is fundamental for probe design and consequently the success of the technique. Oligonucleotides are stable, not degraded by RNase, and their small size improves tissue and cell penetration as well as target detection in FFPE tissues, where the process of tissue fixation has a detrimental effect on the length of nucleic acid sequences. The probes used for the protocol described herein are labelled with digoxigenin (DIG) and will detect viral genomic RNA. They were designed to detect the nucleoprotein gene of selected virus isolates with species specificity; their sequences are shown in Table 33.1. CVS was used as a template for the RABV probe, while RV20, isolated from a serotine bat (*Eptesicus serotinus*) in Denmark during 1986, and RV1332, isolated from a Daubenton's bat (*Myotis daubentonii*) in the UK during 2002, were used for the EBLV-1 and EBLV-2 probes, respectively *(14)*.

Table 33.1. Sequences of DIG-labelled probes used for lyssavirus ISH detection in FFPE tissues

Species	Nucleotide sequence
RABV	5'-GGATGCCGACAAGATTGTGTTCAAAGTCAATAATCAGGTGGTCTCTTTGAAGCC-3'
EBLV-1	5'-CGTCTGCTCTTATTTAGCTGGAGCCATGGTCTTGTTTGAGGGCATCTGCCCGG-3'
EBLV-2	5'-CCCTTGGAAAAGCTCCGGACCTGAACAGAGCTTATAAGTCCATTCTGTCCGG-3'

DIG, digoxigenin; EBLV-1, European bat lyssavirus type 1, EBLV-2, European bat lyssavirus type 2; ISH, in situ hybridization; RABV, rabies virus

Protocol

Generation of control material and test samples

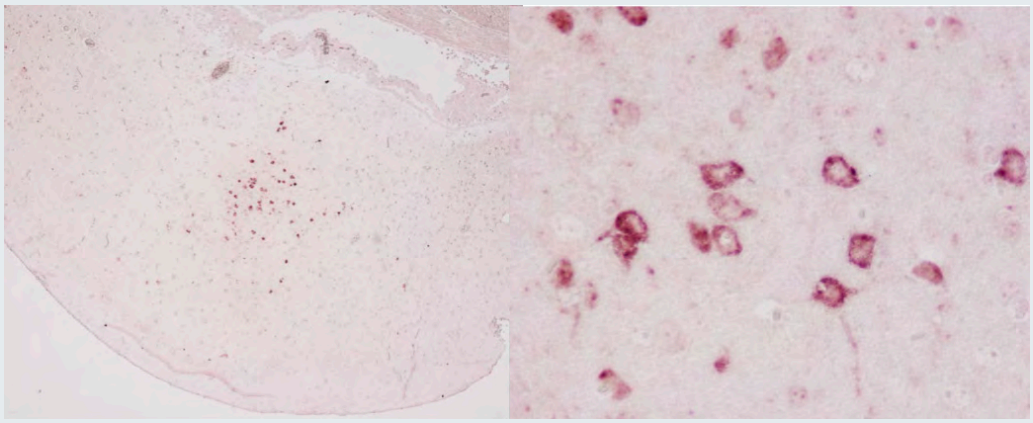
Positive control material is generated by inoculating mice intracerebrally with 10% suspensions of RABV-infected brain tissue. With presentation of clinical signs, the animal is euthanized, and the brain is removed and fixed in 10% neutral buffered formalin at room temperature for a minimum of 48 h. A proportion of the brain can be removed prior to fixation for confirmatory testing using another standard method (e.g. DFAT or RT-PCR). Similar fixation protocols will be required for the test samples. When the histological processing of the tissues is conducted at a lower containment level, a validation of the inactivation of the fixed samples may be required. Virus present in samples up to 1 cm in thickness is inactivated after 24 h. The fixed tissue is then processed through graded alcohol and a clearing agent before being embedded in paraffin wax using standard histological protocols. Negative control material can be generated in the same way using mice that have not been inoculated with RABV suspensions.

Section preparation

1. Cut embedded brain tissue at a thickness < 5 µm using a microtome, float the section on a water bath containing water treated with diethylpyrocarbonate (DEPC) and mount onto a Superfrost charged slide.
2. Improved adherence of the tissue section to the charged slide can be achieved by incubating at 60 °C for 30 min.

In situ hybridization

The ISH protocol is subdivided into three sections: (i) slide preparation and permeabilization, (ii) probe hybridization and (iii) probe detection (Fig. 33.1). Each section includes temperature dependent steps. All steps of the process are to be undertaken at room temperature unless otherwise indicated.



By courtesy of the Animal and Plant Health Agency, Addlestone, Surrey, UK

Original magnification 100x (left) and 400x (right)
ISH, in situ hybridization; RABV, rabies virus

Fig. 33.1. Specific ISH labelling of the neuronal cell body in the piriform cortex of mice intracerebrally inoculated with challenge virus standard RABV using the RABV probe labelled with digoxigenin (purple colouration)

Slide preparation

1. Use two 6-min changes in Xylene to deparaffinize the tissue sections, clear with two 6-min changes of 100% ethanol and rehydrate with 6-min incubations in each of the following: 70% ethanol, 50% ethanol and DEPC-treated distilled water.
2. Following two, 5-min washes in DEPC and phosphate buffered saline (PBS), transfer the slides to 4% paraformaldehyde in 0.1 mol phosphate buffer for 10 min.
3. After a further two, 5-min washes in DEPC–PBS, immerse the slides in a 0.1 mol triethanolamine (TEA) buffer containing 0.25% acetic anhydride for 5 min.

4. Add additional acetic anhydride to the 0.1 mol TEA buffer containing 0.25% acetic anhydride to make a final 0.1 mol TEA buffer containing 0.5% acetic anhydride and incubate the slides for a further 5 min.
5. Wash the slides in 2x saline-sodium citrate (SSC) buffer for 3 min before transferring them to a DEPC–TEA buffer containing proteinase K (10 µg/mL) and incubate for 30 min at 37 °C.
6. To quench residual proteinase K activity, immerse the slides in DEPC–PBS containing 2% glycine for 60 s and then wash in PBS for 5 min.

Probe hybridization

1. Pre-heat hybridization buffer to 37 °C before use.
2. Lay slides out on staining trays and rinse them twice with PBS, allowing the PBS to remain on the slides for 5 min between each rinse.
3. Drain excess buffer from the slides, apply hybridization buffer, cover the tissue section on the slide with plastic paraffin film (trimmed to size) and incubate for 2 h at 37 °C.
4. Rinse with 2x SSC to remove the plastic paraffin film and hybridization buffer and then wash slides in 2x SSC for 5 min.
5. To prepare the probe, vortex oligonucleotide probe stock for 60 s, add probe to the hybridization buffer to achieve a 200 ng/mL concentration, mix by inverting the probe and buffer several times and apply < 200 µL to each section. Cover the tissue section on the slide with plastic paraffin film (trimmed to size) and incubate for a minimum of 18 h at the calculated hybridization temperature.

Optimal probe hybridization temperature (T_{hyb}) can be calculated using the following formula: T_{hyb} = 24.21+0.41(%GC)–500/length of probe, based on the assumption that hybridization buffer contains 4 x SSC and a formamide concentration of 50%.

Probe detection

1. Pre-warm 0.5 and 1x SSC post-hybridization washes. These washes should be between 5 °C and 20 °C warmer than the hybridization temperature of the probe.
2. Rinse with 1x SSC to remove plastic paraffin film and hybridization buffer from the slide, immerse slides in 1x SSC wash buffer pre-heated to wash temperature and incubate at the wash temperature for 15 min.
3. Discard 1x SSC and re-fill immersion trough with pre-heated 1x SSC wash buffer and incubate slides for a further 15 min at the wash temperature.
4. Following two, 15-min immersions in 0.5x SSC wash buffer (at wash temperature), transfer the slides to 0.5x SSC wash buffer and incubate at room temperature for 10 min.
5. Transfer slides to tris-buffered saline (TBS) and wash sections three times for 5 min each.

6. Drain off TBS and apply blocking solution comprised of 0.1% Triton X-100 and 1% normal sheep serum diluted in TBS for 30 min.
7. Drain off blocking solution and apply (< 200 µL per slide) anti-DIG antibody conjugated to alkaline phosphatase diluted 1/100 in blocking solution for a minimum of 4 h.
8. Wash in three, 5-min changes of TBS and apply (200 µL per slide) NBT/BCIP (dissolved in distilled water supplemented with 10 µL of 1 mol levamisole solution per 10 mL). Development time variable and the reaction should be monitored regularly under a microscope. Minimum development time is 20 min.
9. Rinse slides in tap water to stop the reaction and counterstain with nuclear fast red for 10 min.
10. Wash slides in running tap water for 10 min, transfer to distilled water and then mount the sections using an aqueous mountant.

Interpretation of results

To ensure the fidelity of the results, negative and positive control material must be included in the assay along with the test sample. Technique controls, which include the omission of the probe or the substitution of the lyssavirus probe with a nonsense or non-lyssavirus specific sequence of the same length, should also be included. RABV-positive control material must demonstrate purple-labelled intracytoplasmic inclusions, ranging in size from fine granular particles to large inclusion bodies, within the neuron perikarya. Labelling of a similar nature and location must not be present in the negative control material nor on tests on positive control material, where the lyssavirus-specific probe has been omitted or replaced with a nonsense probe. The amount of lyssavirus-specific labelling within the sample will vary depending upon the level of infection. Positive sections can be scored based on the intensity of labelling using well established scoring procedures (e.g. 1+ weak to 3+ strong).

Laboratory optimization

The success of this protocol is directly dependent upon the ability of the probe to access the target sequence. Proteinase K actively breaks down the extensive protein framework cross-linking proteins in the tissue, which is produced during fixation, and increases accessibility to the target sequence. Therefore, manipulation of the enzyme concentration (generally 5–20 µg/mL) and the length and temperature of incubation can help optimize probe access and ultimately influence the final ISH signal intensity. The conditions described in this protocol are optimal for our laboratory. However, optimization may be required when performing this assay in other laboratories due to differences in the length and type of fixation.

Critical parameters, troubleshooting and precautions

The impact of RNase is reduced through the use of oligonucleotide probes; however, care should still be taken in the preparation of reagents and equipment used for this assay. Laboratory glassware should be sterilized or treated for the potential contamination with RNase. Use sterile slides and plastic paraffin film where possible. Reagents must be prepared with either nuclease-free or DEPC-treated water. Good laboratory practice should be maintained at all times to minimize the introduction of contaminants (e.g. by the wearing of gloves). Oligonucleotide probes are robust and are suitable for long-term storage. For example, reconstituted probes from one of the commercial suppliers can be stored at 25 °C for 3 months and at –20 °C for up to 3 years. Preparation and aliquoting on receipt for long-term storage will also minimize the number of freeze–thaw cycles, which can degrade the probe, affecting ISH signal. Several parameters can be modified if labelling is not produced to expected levels. The concentration and duration of proteinase K treatment can be altered to counter the effects (i.e. diminished or lack of labelling) of an extended or shortened tissue fixation. The length of probe hybridization can be extended (< 40 h) if labelling is suboptimal. The intensity of the labelling colour product can also be increased or reduced by extending or shortening the NBT–BCIP development period. Increased nonspecific labelling or background staining can be reduced by increasing the probe hybridization temperature or reducing the duration of NBT–BCIP development.

Alternative materials and/or methods

The use of commercial synthetic oligonucleotides in ISH enables a greater flexibility in the design and choice of visualization methods. A biotin or fluorescent tag can be incorporated into the probe in place of the DIG label allowing for the visualization of multiple target sequences by confocal microscopy.

The use of oligonucleotide probes also means that even in situations where there is difficulty in producing a consensus probe for all lyssavirus species, the capacity exists to commercially order interspecies consensus probes and apply a probe cocktail. Rapid advances have been made with ISH methodology over recent years. The emergence of kit-based systems for both the bench and for automated staining systems and commercially available probe design and preparation services have substantially reduced the labour requirements and turnaround times for testing using an ISH assay. These advances have also simplified the optimization process to a single parameter, reducing the lead-in time for technique development and validation.

Time considerations

This ISH methodology as described is labour intensive and does not lend itself to a rapid diagnostic testing turnaround in this format. Lead-in time for tissue processing to wax, once tissue is fixed, is approximately 24 h. Slide preparation requires 3–4 h, pre-hybridization steps require 2–3 h, probe hybridization

requires an overnight incubation (> 18 h), post-hybridization washes and detection with anti-DIG antibody-AP conjugate requires a minimum of 6–7 h, although the anti-DIG AP conjugate is routinely left on overnight to enable the monitoring of development the next day. Finally, development of the ISH signal and slide mounting requires 2–3 h. To incorporate the overnight incubations, approximately 5 days are required to complete this assay.

Limitations

This technique has been validated on FFPE material, where the fixation period is variable (5 days up to several months). Application of this method to FFPE tissues and material prepared in other fixatives would require optimization. The size of the oligonucleotide probes used (approximately 50 base pairs) counteract the issue of extended fixation periods to some degree. However, the fixation time should be minimized, where possible, to reduce the impact on the ISH signal.

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Annex

Equipment

- items for an automated tissue processor for paraffin embedding of fixed samples
- microtome for sectioning FFPE tissues
- slide staining trays
- vortex mixer, magnetic stirrer, water bath, micro centrifuge
- incubator (room temperature up to approximately 80 °C)
- observation light microscope (low power) to monitor colour change during development
- light microscope (with camera)
- reagents
- Superfrost plus charged slides
- Xylene
- ethanol (molecular grade)
- diethyl pyrocarbonate (DEPC)
- paraformaldehyde and buffer (0.1 mol phosphate buffer)
- proteinase K
- glycine
- phosphate buffered saline (PBS)
- acetic anhydride
- saline-sodium citrate (SSC) buffer in various concentrations
- hybridization buffer (dextran sulfate, Formamide, PolyA, ssDNA, tRNA, DTT (1 M solution), 50x Denhardts)
- anti-DIG fab fragment antibody
- M triethanolamine buffer (0.1 mol TEA buffer in distilled water)
- plastic paraffin film
- NBT/BCIP substrate-chromogen
- aqueous mountant
- tris-buffered saline (TBS, 100 mmol Tris HCl, 150 mmol NaCl, pH 7.6)
- 1x tris-EDTA buffer (TE buffer)

Laboratory animals

Mice would be required for the generation of known positive and negative control material. Field cases could be used for controls, once the technique is optimized.

Chapter 34
Rapid diagnosis and genetic typing of rabies virus and other lyssaviruses using SYBR Green RT-PCR and pyrosequencing assays

Introduction

Rabies is a major public health problem in Asia and Africa, offering diagnostic challenges *(1–3)*. Within North America, more than 100 000 suspected rabid animal samples are tested annually *(4)*. Canine rabies viruses (RABV) are responsible for most human deaths in the developing world *(5)*. Lyssaviruses include at least 15 different species with significant diversity in genome sequences, in which the sequence similarity among RABV can be as low as 80% of the nucleoprotein (N) gene sequences. Other lyssaviruses are more divergent. The sequence similarity of the N gene are in the range of 68–79% among different lyssavirus species *(6)*. The sequence divergence among RABV and other lyssaviruses makes the diagnosis challenging. Highly sensitive and specific assays and sequencing of suspected samples are needed to confirm diagnostic results with confidence.

WHO and OIE have defined the direct fluorescent antibody test (DFAT) as the gold standard for rabies diagnosis of postmortem samples (see Chapter 11). The DFAT is a rapid and sensitive method for rabies diagnosis, but its accuracy depends on the quality of brain tissue, availability of high-quality anti-rabies diagnostic conjugates, accessibility to a fluorescence microscope and, most importantly, an experienced diagnostician *(7)*.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays have been used for rabies diagnosis for decades. A recently developed pan-lyssavirus real-time TaqMan RT-PCR assay, LN34, is able to detect RABV or other lyssaviruses *(6)*. SYBR Green-based real-time RT-PCR assays have also demonstrated superior sensitivity and broad specificity in rabies diagnosis. However, the rabies SYBR RT-PCR assays may use degenerated primers or short primers for the PCR amplification and be inclined to produce nonspecific PCR products or primer dimers which can lead to false–positive results, as SYBR Green binds to double-stranded DNA nonspecifically *(8)*.

Despite rapid advances in next-generation sequencing technology, the Sanger sequencing method is still used widely for the routine analysis of suspect samples to confirm diagnostic RT-PCR results or to determine the source of infection based on the genetic typing results (see Chapter 29). Sequences from positive samples are critical for the investigation and control of outbreaks as well as the rapid identification of lyssavirus infections. Normally, a Sanger protocol takes up to 12–24 h to generate sequences from a RT-PCR amplicon. Alternatively, a pyrosequencing method can be used for the diagnosis and genetic typing of suspected RABV samples by directly sequencing the RT-PCR amplicon. The pyrosequencing method generates short sequences, but the method is fast and very sensitive *(9)*.

By combining these techniques, we take advantage of the superior sensitivity and broad specificity of the SYBR Green RT-PCR assay in rabies sample testing and the rapid sequencing ability of pyrosequencing technology. This protocol produces both real-time RT-PCR data followed by sequencing confirmation of the diagnostic results within 4 h. This protocol can be used for the diagnosis of rabies postmortem and antemortem samples, including saliva, nuchal skin samples and paraffin-embedded samples. This method is of particular use for a rabies reference centre to perform additional diagnostic confirmations, rapid genetic typing of positive samples and monitoring the emergence of novel RABV variants or other lyssaviruses.

Technique

This protocol uses a one-step SYBR Green RT-PCR assay (LN12) to amplify a suspected RABV sample, followed by a pyrosequencing method (PyroLN12) to confirm the positive LN12 assay result. The forward and reverse primers of the LN12 assay target two of most conserved regions of RABV and other lyssavirus genomes. The forward primer overlaps with previously known LYS001 primer sequences, and the reverse primer overlaps with previously known JW12 primer sequences *(10, 11).* Those primer selections allow minimal primer degenerations and are able to amplify all known lyssaviruses as the primer sequences are highly conserved. The amplicon size is about 70 base pairs, which improve the assay's sensitivity compared with previously published assays *(12, 13)*. Our validation results show that the LN12 assay is more sensitive than those of the TaqMan-based pan-lyssavirus real-time RT-PCR assay LN34 *(6)*.

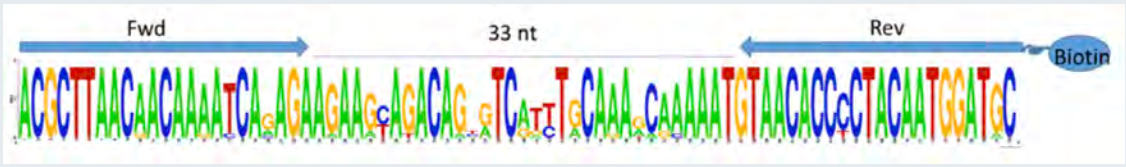
The advantage of pyrosequencing is that the sequencing reading starts at the first base after the sequencing primer and the sequencing results are generated in real time. Although the amplicon sequences of the assay LN12 are only 33 bases after the primer sequences, those 33 bases sequences are highly diverged and generate specific typing results among different lyssavirus species and major RABV variants. The PyroLN12 takes < 2 h to complete. Those pyrosequencing results may not be suitable for detailed phylogenetic analysis of the samples, especially among closely related RABV variants, but the sequences are specific for diagnostic confirmation and differentiation of RABV from other lyssaviruses and among major RABV variants, especially combined with clinical and animal contact information.

The PyroLN12 is able to generate sequences for weak or difficult RABV samples. The optimization process of the protocol shows that the pyrosequencing method produces clear sequencing reads from the amplicons with cycle threshold (Ct) values ≥ 35 (< 100 copy of RABV RNA) from the LN12 assay. This protocol has been optimized in the nucleotide dispensation order and template quantities in the pyrosequencing process to improve the sequencing signal and sequencing length.

Standard procedure

Amplicon generation using the LN12 assay

The LN12 assay is recommended to run on the real-time PCR instrument ABI ViiA7 and ABI 7500 [Applied Biosystems, Cat # 4453535 and 4406984]. Other real-time instruments may also be used following the recommended running conditions. Both forward (Fwd) and reverse primers (Rev) contain degenerate nucleotides (using the nomenclature of IUPAC). The Fwd sequences are: Fwd1, 5'-ACG CTT AAC RAC AAA ATC ARA GA-3', Fwd2, 5'-ACG CTT AAC AAR ATC AGA GA-3'; and the Rev sequences are: Rev1, 5'-Biotin-GCA TCC ATT GTA GGR GTG TTA C-3', and Rev2, 5'-Biotin-GCA TCC ATT GTA GGG GTG TTR C-3'. The Fwd1 and Fwd2 or Rev1 and Rev2 are mixed in an equal molar ratio. The Rev primers have biotin labels at the 5' end and are used to generate a single-stranded DNA template for pyrosequencing (Fig. 34.1).



Fwd, forward; Rev, reverse

Fig 34.1. Primer design for the LN12

The logograph was constructed using 12 highly diverged RABV sequences (*14*). The small letters indicate the variable positions among RABV sequences. The Fwd primer is also used as the pyrosequencing primer and the biotinylated Rev primer is used for the purification of single-stranded template.

Equipment and reagents

- Invitrogen Superscript III Platinum SYBR Green One-Step qRT-PCR kit [catalogue number 11736051]
- high-purity nuclease-free water
- Fwd and Rev primers with stock concentration of 10 µmol
- RNase Away
- RABV samples to be tested
- positive control (historic RABV-positive RNA)
- negative control, no template control (NTC), nuclease-free water
- biosafety cabinets
- ABI ViiA7
- QuantStudio Real-Time PCR Software V1.2 [ABI]
- MicroAmp 96-well fast PCR plate [catalogue number 4346906]
- MicroAmp optical adhesive film [ABI 4311971]
- small benchtop centrifuge
- pipettes

Reaction setup for the LN12

1. Keep all reagents on ice after thawing. Reagents are returned to storage at –20 ˚C after use.
2. Use primer aliquots (40 µL) to minimize possible contamination. Discard the primers after 10 times of use.
3. Use separate biosafety cabinets for PCR setup and adding RNA samples. The bench surfaces of biosafety cabinets and pipettes are treated by spraying RNase Away before and after use to eliminate possible RNase contamination.
4. Prepare a reaction master mix as shown in Table 34.1. All samples are run in duplicate.
5. Gently mix the master mix. Spin briefly to collect all the liquid to the bottom of tube.
6. Dispense 18.0 µl of master mix to each reaction well of a MicroAmp 96-well fast PCR plate.
7. Transfer the PCR plate into the other biosafety cabinet designated exclusively for RNA work.
8. Thaw RNA samples and mix well.
9. Use 2.0 µL of extracted RNA per reaction.
10. Seal the plate.
11. Spin the 96-well PCR plate for 1.0 min and place it into an ABI ViiA7 thermocycler.

Table 34.1. Preparation of master mix for the LN12 assay

Components	Volume per reaction (µL)	Volume of master mix[a] = number of reactions[b] x volume per reaction
2X SYBR Green reaction mix	10	
Forward primer (10 µmol)	0.4	
Reverse primer (10 µmol)	0.4	
ROX reference dye (50 µmol)[c]	0.04	
SuperScript III RT/Platinum Taq Mix	0.5	
Nuclease-free water	6.66	

[a] Prepare 10% extra volume.

[b] All samples are run in duplicate, including positive and negative controls.

[c] Different instruments require different ROX concentration as background reference dye, so adjust ROX volume accordingly. For example, ViiA 7 and ABI 7500 need 30–50 nmol ROX while ABI PCR StepOnePlus needs 300–500 nmol ROX.

PCR program setup using QuantStudio™ Real-Time PCR software V1.2

QuantStudio Real-Time PCR software V1.2 was used on the ViiA7 system. A similar software was used on the ABI 7500 system. Choice selections are highlighted in bold for easy illustration.

Open the software and at the home screen:

1. In the **Set UP panel**, click **Experiment Setup**.
2. The **Experiment Menu** panel contains steps for program setup and result analysis.
    a. Fill the boxes of **Experiment Name**, **User Name** and **Comments.**
    b. Check the appropriate options for **Experiment Property**.
3. Click **Define** to access the next screen.
    a. Keep **Target 1** in **Targets Name**; pick **SYBR Green** for **Reporter**, **None** for **Quencher.**
    b. In **Samples panel**, click **New** to add samples, and enter sample names.
    c. Choose **ROX** for **Passive Reference.**
4. Click **Assign** to access the next screen.
5. Assign reaction wells with sample names.
6. Select all reaction wells and assign them with **Target 1**.
7. Click **Run Method** to access the Rum Method screen
8. Set 20 µL for **Reaction Volume per Well**.
9. Set the thermal cycle profile under the **Graphical View** tab as in Table 34.2.
10. Click **Run**. Choose the ViiA7 instrument and save your run in a desired folder.
11. After the run is completed, the positive samples are sequenced or stored at –20 ˚C for future use.

Table 34.2. The thermoprofile for the LN12

Temperature ˚C	Time	Comments
50 ˚C	5 min	Reverse transcription
	3 min	
95 ˚C	3 s	PCR amplification
60 ˚C	20 s	40 cycles
95 ˚C	15 s	Melting curve (optional)
60 ˚C	1 min	
95 ˚C	15 s	
50 ˚C	5 min	

Run analysis

When the run is completed, choose **Automatic Threshold** in the **Analysis setting**. Click the **Analyse** tab above the **Plate Layout** panel to analyse the run data.

In the **Analysis** tab, check out each the following analysis.

1. **Amplification Plot**: pick **ΔRn Vs Cycle plot** and **linear** model. Expected results would be:
    a. The plots of positive samples should have the amplification curve in sigmoid with exponential phase.
    b. For a sample with a weak amplification and high Ct values, the Multi-component Plot may be used to check the increases of fluorescence for a true amplification.
2. **Melt curve** (optional): most RABV-positive samples have a single melting curve peak between 75 °C and 77.5 °C.
3. Samples that display Ct values will be processed for pyrosequencing.

For more details of run analysis and troubleshooting, refer to the *Getting started guides: Applied Biosystems ViiA™ 7 Real-Time PCR System (English)*, Booklet 2, *Running standard curve experiments* (https://www.thermofisher.com/).

Exporting the run

Run data and results can be exported by clicking **Export in the navigation panel**.

1. Check all the boxes including **Sample Setup, Raw Data, Amplification, Multi-component, Results** and **Melt Curve Raw**.
2. Chose a location and a file name for the exported file.
3. Saved file can be opened in Microsoft Excel for further analysis.

Pyrosequencing of the LN12 assay amplicon

Equipment and reagents

- PyroMark Q24 Advance Instrument [catalogue number 9002270]
- PyroMark Q24 Vacuum Workstation (110V) [catalogue number 9001516]
- PyroMark Q24 Cartridge [catalogue number 97902]
- Q24 Plate (100) [catalogue number 979201]
- PyroMark Q24 Advanced Reagents kit [catalogue number 970902]
- PyroMark Denaturation Solution (500 ml) [catalogue number 979007]
- 10x PyroMark wash buffer concentrate (200 mL) [catalogue number 979008]
- sequencing primer: Fwd1 for RABV sequencing, or Fwd1 and Fwd2mixture for other lyssaviruses; primers are prepared at a concentration of 10 µmol

- Streptavidin Sepharose High Performance beads (6% highly cross-liked Agarose) [GE Healthcare 17-5113-01]
- 70% ethanol
- 96-well PCR plate [Phenix research products, MPS-499]
- 96-well PCR plate seal [Phenix research products, LMT-SEAL-EX]
- highly purified water [Milli-Q 18.2 MΩ x cm or equivalent]
- horizontal shaker
- heat block
- marker
- timer
- PyroMark Q24 Advanced 3.0.0 (software)

Set up of the PyroLN12 assay

Pyrosequencing requires a method that matches the method number on the cartridge. The method in this protocol is 13 and can be downloaded from the Qiagen website (https://www.qiagen.com/us/resources/technologies/pyrosequencing-resource-center/managing-instrument-methods/pyromark-q24-advanced/). The dispensation order and cycle of dNTP for the RABV SEQ assay were optimized; the dNTP is dispensed in the order of AACGT for 18 cycles and the method is saved as 18 (AACGT).

1. Save the method file on a computer that has the PyroMark Q24 Advanced 3.0.0 software installed on it.
2. Import the method file into the software by clicking **Tools → Instrument Methods,** click **Import**.
3. Find the saved Method 13, then click **Open**.
4. Close the **Import** window when the method file appears in it.
5. Click **File** in the toolbar and select **New Assay → SEQ Assay**.
6. In the **SEQ Assay Setup** screen, click **Setup,** enter 18(AACGT) in the **Dispensation Order** panel.
7. Leave other **Settings** and **Analysis Parameters** in default.
8. Save the assay file 18 (AACGT) on the computer.
9. Import the assay file 18 (AACGT) into the **Shortcuts** folder on the **SEQ Assay Setup** screen.

Preparations at the beginning of the experiment

1. Bring the washing buffers and the denaturing buffer to room temperature from a 4 °C refrigerator.
2. Dilute 5.0 mL of 10x washing buffer with 45 mL of highly purified water to make a 1x working concentration.
3. Turn on the PyroMark Q24 Advanced Instrument.
4. Power on a heat block with a preset of 80 °C for later use in step 3.2.8.

Immobilizing the PCR amplicon

1. Mix Streptavidin Sepharose beads by inversion. Do not vortex.
2. Prepare the master mix in a tube as in Table 34.3.

Table 34.3. Preparation of master mix for DNA immobilization

Components	Volume per reaction (µL)	Volume of master mix[a] = number of reactions[b] x volume per reaction
PyroMark binding buffer	40	
Sepharose bead	1.0	
Water	29	
Total volume	70	

[a] Prepare 10% extra volume.

3. Distribute 70 µL master mix per well in a 96-well plate.
4. Transfer 10 µL PCR product (use one to third dilution if Ct < 20 to improve pyrosequencing read quality) into a reaction well containing the immobilizing buffer. Seal the PCR plate.
5. Shake the plate at 1400 r/min for 10 min.

Run set up and loading reagents into the PyroMark Q24 cartridge

1. Click **New Run → SEQ assay**.
2. In the **Run Setup** screen, select Method 013 from the dropdown list of **Instrument Method.**
3. Enter run info into the **Run Note** panel.
4. In the **Plate Setup** panel, choose wells for your samples and enter sample names.
5. Highlight all reaction wells, apply the assay file **18 (AACGT)**.
6. Click **Tools → Pre Run Information**, write down the volumes of each reagent, Enzyme mix, Substrate mix and four nucleotides **A, T, G, and C**.

7. Place the Cartridge on the bench with the label facing you.

   Note: Make sure the cartridge is dry at the time of use.

8. Load the volume of each reagent into its compartment.

   Caution: Make sure no air bubbles form inside the cartridge compartment during loading.

9. Tap the cartridge gently on the bench a few times to help settle reagents in the bottom of the cartridge.
10. Open the instrument lid and cartridge gate, insert the filled cartridge with the label facing out, and close the cartridge gate.
11. Save the run setup from the computer into a flash drive.
12. Insert the flash drive into the PyroMark Q24 advanced instrument and the setup file will be used during the sequencing run.

Preparation of the sequencing primer of PyroLN12

The Fwd primer of the assay LN12 is used as the sequencing primer.

1. Prepare a sequencing primer stock at 10 µmol concentration. The working concentration of the sequencing primer is 0.375 µmol.
2. Dilute the sequencing primer using the PyroMark advanced annealing buffer to make a sufficient amount.
3. Mix and spin briefly to collect all the liquid to the bottom of tube.
4. Dispense 20 µL diluted sequencing primer to each well of a Q24 plate according to the run setup.

Purification of template DNA

1. Switch on the PyroMark Q24 Vacuum pump.
2. Turn on the vacuum tool.
3. Prime the Filter Probes with 40 mL of highly pure water.
4. Place the PCR plate from Step 3.2.4 and the primer-filled Q24 plates on the vacuum station.
5. Slowly lower the filter probes of the vacuum tool into the PCR plate to capture the beads containing immobilized template. Hold the vacuum tool in the place for 15 s. Raise the vacuum tool and check for any PCR leftover in the wells of the 96-well PCR plate.

Note: Beads sediment quickly, and capturing should take place immediately after the plate is set on the holder of the vacuum work station. If more than ONE min has elapsed, agitate the PCR plate again for one min.

6. Place the vacuum tool into the trough containing 40 mL 70% ethanol. Flush the filter probes for 5 s.
7. Transfer the vacuum tool into the trough containing 40 mL denaturation solution. Flush the filter probes for 5 s.
8. Transfer the vacuum tool into the trough containing 40 mL wash buffer. Flush the filter probes for 10 s.
9. Raise the vacuum tool up and back beyond 90° vertical for 5 s to drain any liquid in the filter probes.

10. Switch off the vacuum tool, then turn off the vacuum. Detach the vacuum tool from the vacuum tubing.
11. Align the vacuum tool with the PyroMark Q24 plate and lower the filter probes into the diluted sequencing primer. Gently shake beads off the filter probes.
12. Reattach the vacuum tool to the vacuum, and power on the vacuum and switch on the vacuum tool.
13. Flush the filter probes in highly pure water for 10 s. Place the vacuum tool in the parking trough.
14. Turn off the vacuum pump.
15. Set up the pyrosequencing reactions following the steps in Table 34.4.

Table 34.4. Reaction setup for the PyroLN12

Reagents	Volume per reaction	Master mix[a] = reaction number x volume per reaction
Sequencing primer Fwd1 (10 µmol)[b]	0.75 µL	
PyroMark advanced annealing buffer	19.25 µL	
Single-stranded DNA (the Sepharose beads from Step 3.2.7)	Released in the wells with diluted primer Fwd1	
Enzyme/substrate mix	To be dispensed during sequencing	Prepared in Step 3.2.5
Nucleotides	To be dispensed during sequencing	Prepared in Step 3.2.5

[a] Prepare 10% extra volume.

[b] Sequence primer is diluted in the annealing buffer. Beads with captured DNA are released in the diluted primer solution.

Primer annealing

1. Transfer the Q24 plate containing the sequencing primer and DNA template to the heat block and heat it at 80 °C for 5 min.
2. Transfer the hot plate holder together with the Q24 plate from the heating block to the PyroMark Q24 advanced instrument.
3. Immediately place the Q24 plate into the PyroMark Q24 advance instrument. Ensure that the plate-holding frame is closed.

Note: The time from removing the hot plate holder to placing the Q24 plate into the PyroMark Q24 advanced instrument should not exceed 30 s.

Start the run

1. Select **Run** at the screen of PyroMark Q24 advance instrument.
2. Pick the run file from Step 3.2.5.
3. Click **Run** to start the reactions.

Clean-up after the run

1. Open the PyroMark Q24 advanced instrument lid.
2. Take out and trash the Q24 plate.
3. Take out the cartridge. Discard the remaining solution.

4. Wash and “milk” it three times using high-purity water.
5. Watch the straight downright water jet during the “milking” process. Place the cartridge in a box for air drying and storage.
6. Turn off the instrument.

   Note: Discard the cartridge when usage reaches 30 runs, the cartridge is blocked or the water jet is not straight.

Result analysis

1. Run result is stored automatically on the flash drive. Insert the drive into a computer with installed PyroMark Q24 Advanced software.
2. Open the software. Click **File → Open** the run file.
3. The run file is now open in **SEQ** mode.
4. On the **SEQ** screen, the top, middle, and bottom panels are plate Overview (**I**), Pyrogram (**II**) and Histogram (**III**) (Fig. 34.2).
5. To view a reaction, click it in the plate Overview (**I**). The run result will populate in the Pyrogram (**II**), Histogram (**III**), and the **Well Information** panel (**IV**). The info in **General Warnings** is useful for base calling analysis.
6. The called sequence of the selected sample is displayed on the top of the Pyrogram panel.
7. Sequencing quality is colour coded, Blue: Passed; Yellow: Check; Red: Failed.
8. Called sequences of all samples can be exported by clicking **Reports → SEQ Analysis Results**, then choose **All wells** and **Passed + Checked**. Save it into a folder.

Fig. 34.2. Analysis of PyroLN12 run

Panel I summarizes the run results; the quality of base-calling is colour coded (blue, passed; yellow, checked; red, failed). The algorithm-called sequences of selected wells are listed at the bottom of panel I. The raw sequencing signal is in panel II for a selected well and the histogram of the sequencing results is in panel III. Panel IV contains error messages.

Making a diagnosis and genetic typing using BLAST (Basic Local Similarity Searching Tool)

1. Go to the nucleotide Blast website at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
2. Copy the called sequences (only blue- and yellow-coloured sequence) and paste into the query sequence box.
3. Perform Blast search using the program optimized for "Somewhat similar sequences".
4. Among all the hit sequences, only top hits with the highest maximum scores are considered.
5. The sample is positive if the top hits contain RABV sequence(s).

Additional notes

1. Reconstituted enzyme mix and substrate mix of the PyroMark Advanced reagent can be stored at 4 ˚C and last for one week. Unused aliquots can be stored at –20 ˚C. Do not thaw or freeze the reconstituted enzyme and substrate mix more than three times.
2. High-quality base calling of pyrosequencing usually has an initial peak value above 20. The initial peak height of a positive control is usually around 45–55 relative light units. If the initial peak value is lower than 20, discard the constituted enzyme and substrate mix.
3. **Never freeze and thaw the nucleotides of the kit.** Freeze–thaw cycling raises background peaks, making base call more difficult.
4. Cartridges should not be used more than 30 times. Discard the cartridge if it is blocked. Keep a record of usage.
5. A Filter Probe can be used 100 times. It needs to be cleaned using sonication or replaced when usage reaches 100 times. Keep a time record of usage.
6. Streptavidin Sepharose beads (6%) should not exceed 1.0 µL per reaction. Overuse will cause baseline drifting.
7. Rinse troughs with high-purity water. Air dry and place them back into their position in the station.
8. The cartridge should be cleaned as soon as possible after a run has been completed to prevent a blockage in the cartridge.

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Part 6. Production of biologicals

Chapter 35
Regulatory perspectives on the design of human rabies biologicals

Introduction

The regulation of medicines demands the application of sound scientific, medical and technical knowledge, and operates within a legal framework. While medicines regulation is often associated with administrative aspects, far more relevant is the science that supports it. All medicines should meet three main criteria: to be of acceptable quality, to be safe, and to be effective. Any judgements about these criteria should be based on solid science.

The use of unsafe and low-quality medicines could lead to treatment failures, adverse effects, resistance to medicines and even death. In the case of rabies, the quality, safety and effectiveness of medicines are essential as the onset of clinical disease can only be prevented effectively by timely administration of rabies vaccine and rabies immunoglobulins (RIG) in the event of a severe exposure (WHO category III). Ineffective or poor-quality medicines could have detrimental effects on patients, and also undermine the community's trust in health systems, medical professionals, manufacturers and distributors. Moreover, financial resources spent on ineffective and poor-quality medicines are lost – whether by patients or governments. This is a concern for rabies, which occurs mostly in developing countries with limited resources.

Institutions such as WHO and national regulatory authorities ensure that the manufacture, use and distribution of medicines are adequately controlled. Medicines regulation is based on a number of documents including pharmacopoeial monographs, WHO guidance documents and regional guidelines (published e.g. by the United States Food and Drug Administration and the European Medicines Agency). These documents describe the requirements necessary to ensure that the safety and efficacy of medicines such as rabies biologicals are acceptable, assuring for instance that a tested vaccine does indeed induce neutralizing antibodies.

A fundamental of medicines regulation is the evaluation of their quality. Quality control of rabies biologicals should be ensured at two levels: by the manufacturer and by a national control authority, e.g. the national rabies laboratory or national veterinary service laboratory. Quality control will, for instance, ensure that the potency of RIG for rabies post-exposure prophylaxis (PEP) is correctly and accurately determined using virus neutralization assays in line with the regulatory requirements, e.g. according to pharmacopoeial monographs *(1)* as further described in this chapter.

Medicines regulation also addresses necessary inspections of manufacturers, ensuring that the medicines are compliant with good practice (GxP) regulations. The "x" in GxP is a variable that stands for manufacturing, clinical, laboratory, or clinical laboratory. For example, GMP (Good Manufacturing Practice) covers all

aspects of production from starting materials, premises and equipment to the training and personal hygiene of staff. Detailed, written procedures are essential for each process that could affect the quality of the finished product. Systems must be available to provide documented proof that correct procedures are consistently followed at each step in the manufacturing process, each time a product is made.

Regulatory requirements evolve over time following the course of scientific progress. Substantial scientific advances have been made to improve the methods of producing rabies vaccines and RIG and in developing new assays and tests. Major advances in molecular biology techniques have been extensively applied, for instance to express recombinant monoclonal antibodies directed against the rabies virus (RABV) glycoprotein for testing in clinical trials *(2, 3).* Thus, additional regulatory requirements need to be taken into consideration nowadays by rabies researchers and professionals, such as the existing guidelines and pharmacopoeial monographs for monoclonal antibodies *(4, 5).*

There has been a great deal of efforts to align regulatory requirements across the world. The establishment of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) in 1990, a collaborative initiative among the European Union, Japan and the United States with observers from WHO, the European Free Trade Association and Canada, was a milestone in facilitating the harmonization of medicines regulation *(6).* ICH guidelines focus primarily on technical requirements for new, innovative medicines.

Outside of the ICH regions and associated countries, many regulatory requirements have been addressed by WHO. In the field of quality assurance of medicines, WHO plays an important role, especially for those countries with few means to develop their own quality controls, and helps national competent authorities with various activities such as creating nomenclatures, guidelines, delivering training and awareness courses, and fostering discussions surrounding regulatory science to build and increase capacity.

A regulatory tool particularly relevant for developing countries is the “WHO prequalification of medicines”, a process which vets promising medicines around the world. Prequalification is often a condition under which international donors, such as the vaccine alliance GAVI, consider procuring them. The WHO Prequalification of Medicines Programme helps ensure that medicines supplied by procurement agencies meet acceptable standards of quality, safety and efficacy *(7)*.

The work of WHO is supported and complemented by other organizations and authorities, using specific regulatory pathways such as the European Union Article 58 of Regulation (EC) No 726/2004. This procedure enables the European Medicines Agency (EMA) to assess products and provide scientific advice for WHO. A procedure based on Article 58 includes an EMA assessment of the quality, safety and efficacy of a medicine intended for use outside the European Union, based on the same standards and procedures as those for medicines marketed in the European Union. Article 58 has been applied successfully in recent years, for example for assessment of the malaria vaccine Mosquirix and anti-HIV medicines *(8).*

Regulatory challenges for rabies biologicals

To facilitate the availability of safe, effective and good-quality medicines, products such as RIG and vaccines need to be tested in laboratories, as mandated by their pharmacopoeial monographs. One challenge in testing rabies biologicals is the need for high-containment biosecure laboratories, e.g. for measuring the neutralizing potency of RIG or neutralizing antibodies in serum following vaccination. Serological methods to analyse vaccine-induced humoral responses to RABV include the fluorescent antibody virus neutralization (FAVN) test (see Chapter 20), the rapid fluorescent focus inhibition test (RFFIT), discussed in Chapter 19, and the enzyme-linked immunosorbent assay, ELISA *(9)* as well as variations of the aforementioned assays *(10, 11)*.

The routinely used FAVN assay and RFFIT are the assays of choice with OIE/WHO reference laboratories and make use of live virus neutralization assays. The standard methods to test for neutralizing antibodies against RABV involve high containment, Biosafety Level 3 facilities; however, most rabies-endemic countries have resource-limited laboratories and performing the assays under high containment presents several financial and logistical challenges. To address these issues, several techniques have been developed, including the pseudotype neutralization assay (PNA) *(12, 13).* Pseudotypes are viruses that carry the genome and core of one virus and the envelope of another. RABV pseudotypes have been constructed using lentiviral backbones and an analytical method established that utilizes these replication-incompetent viruses to accurately measure neutralizing antibody titres. This method has the advantage of allowing experiments to be conducted in category 2 biosafety laboratories as the pseudotypes cannot replicate and are unable to cause a lasting infection *(12, 13)*. The assay also benefits from detecting virus neutralizing antibody alone, in contrast to ELISA, and gives a more detailed picture of the protective antibodies present (as not all virus-binding antibodies are necessarily neutralizing). Furthermore, the RABV pseudotypes were demonstrated to be stable during freeze–thaw cycles and storage at room temperature, confirming that the proposed analytical method could be a useful option for conducting neutralization studies in regions most affected by these infections *(12, 13)*. Although not currently registered in pharmacopoeias, such an assay would be ideally suited for use in resource-limited countries and should be incorporated in the relevant guidance and monographs in future.

Apart from the financial and logistical challenges associated with current neutralization assays, low accessibility to medicines for post-exposure prophylaxis (PEP) is problematic across many rabies-endemic countries. Access to the recommended complete PEP regimen components such as RIG remains insufficient. Human RIG (HRIG) is widely unavailable in rabies-endemic countries and its supply depends on a limited number of vaccinated individuals as donors *(14)*. Cheaper equine rabies immunoglobulins (ERIG) are available in limited quantities but several manufacturers are discontinuing ERIG production. The remaining ERIG manufacturers are mainly local ones with limited capacities for expansion. In the past, there were also some safety concerns regarding adverse events in recipients of ERIG, although purification techniques have advanced and the safety profile of ERIG has greatly improved. Adverse events with ERIG have been mitigated by generating antibody fragments lacking the equine antibody Fc region, i.e. $F(ab')_2$ fragments, but these are less potent than whole antibodies and usually have only

a few days of half-life. Thus, a widely available product that could be used alongside HRIG and ERIG would be highly desirable.

The use of monoclonal antibodies (MAbs) could address the low availability of RIG and overcome variability in specificity and potency. MAbs can be produced in cell lines using large bioreactors and can therefore easily be expanded to address the low availability of RIG. While MAbs have significant promise as rabies PEP agents, they are not without limitations, including expense, but some low-cost production platforms have been described *(15, 16)*. As MAbs come in the form of a concentrated product, they may also be more effective than polyclonal serum at wound infiltration, and thereby reduce the introduction of excess volume at the site of intramuscular injection, which in turn could lead to a better local tolerability *(2)*.

While MAbs are a step ahead in terms of scalability and lot-to-lot consistency, navigating the complicated clinical development path for rabies MAbs and associated ethical considerations remains complicated. Their effect may be difficult to measure in any feasible clinical trial, particularly for phase III efficacy studies. Most trials so far have been conducted in the non-RABV exposed population, which allow the study of different components and combined regimens of established and proposed PEP. Initial exploration of tolerability of a novel rabies MAb and information about adverse events can be understood from these types of trials.

While early stage clinical trials in non-RABV exposed healthy volunteers can provide some information about the safety and tolerability of a medicine, the relation to protection against disease when used after exposure may be complex. A range of factors could potentially contribute to the absence of RABV after receiving PEP following a suspected exposure; thus, the absence of clinical disease may not indicate the effect of the antibody component. Differences in bite sites, viral strains and animal vectors might influence any studies in a population potentially exposed to RABV. The risk of developing rabies after a suspected exposure depends on multiple factors, such as whether the biting animal was rabid, whether the animal was shedding RABV, how close the bite was to the central nervous system, whether the bite site was thoroughly cleaned, and whether an appropriate rabies vaccination series was initiated and completed.

While a placebo control would put patients at inappropriate risk and might not be ethically possible for rabies in a clinical trial setting, the use of HRIG for comparison presents some challenges including its low availability and the fact that the efficacy of the RIG/vaccine combination has not been rigorously tested under controlled conditions, as stated in the Imogam HRIG product label. Close attention must therefore be paid to the clinical trial design, to ethical considerations of such testing in adults and children, and to measurements that might aid our understanding about whether a new rabies MAb product provides early protection without increasing vaccine interference.

Worldwide, several rabies MAbs are in various stages of development *(17)*. One MAb has received a marketing authorization in a pioneering project completed by MassBiologics and the Serum Institute of India *(3, 18).* A phase I clinical trial and a phase II/III trial were undertaken for this Mab. The phase II/III randomized, single blind, non-inferiority study was conducted in 200 participants with WHO category III suspected rabies exposures. Study participants received proper

wound care followed by injections of either the investigational MAb or the standard HRIG treatment in combination with the vaccine *(18)*. On Day 0, participants received either the MAb or HRIG (1:1 ratio) into wounds and, if required, five doses of rabies vaccine intramuscularly on days 0, 3, 7, 14 and 28. The primary end-point was the ratio of day 14 geometric mean concentration of rabies virus neutralizing antibodies (RVNA) activity as measured by RFFIT for MAb recipients relative to HRIG recipients. Initially, only patients with category III exposures on the lower extremities were enrolled, followed by evaluation in patients with any type of category III exposure after interim analysis. No case of PEP failure or rabies was observed during the study period. The PEP regimen containing the MAb was safe and demonstrated non-inferiority to HRIG PEP in neutralizing antibody production. A marketing authorization was received in India in October 2016 and an event announcing the launch of this antibody (branded as Rabishield) was held in October 2017 in Mumbai. Two presentations of the MAb are available, including a 100 IU/2.5 mL (40 IU/mL) vial and a 250 IU/2.5mL (100/mL) vial *(19)*. According to the Serum Institute of India, Rabishield might be offered at a cost 25% cheaper than existing RIGs *(20)*.

It will be interesting to see how this innovative product is taken up by medical professionals. The availability of this MAb could fill critical public health gaps. As it is made by recombinant technology, it will be less prone to problems such as availability, safety and purity. It should be recommended for use in public health programmes, depending on the epidemiological and geographical setting, with monitoring of its safety and efficacy (clinical outcomes) during post-marketing use. The advent of this MAb presents an important step in making rabies PEP more accessible, and cost savings compared with RIG might be even more pronounced once additional MAbs are licensed.

Another project on rabies MAbs, initiated by the WHO Rabies Collaborating Centres *(21)*, provided several MAbs for inclusion in an antibody cocktail. Two MAbs were selected based on their strong potencies and different epitope specificities and were transferred to other parties and manufacturers. Preclinical studies of the WHO MAbs were undertaken by multiple groups *(22, 23)*; the Indian company Zydus Cadila has taken the MAbs into clinical trials.

Another project on a Mab cocktail comprising two MAbs was undertaken by Crucell *(2)*; however, the company was sold following phase II clinical trials and product development was discontinued. Clinical trials for a MAb combination have also been initiated by Synermore Biologics, China *(17, 24)*. Finally, several other companies including the Korean biologicals manufacturer Celltrion have generated strong preclinical data *(25)* which could pave the way for clinical studies of their MAbs.

The following sections will focus on general quality requirements for rabies biologicals, whether they are MAbs, RIGs or rabies vaccines.

Specifications and controls

A specification is a list of tests and methods with appropriate acceptance criteria, such as numerical ranges or other criteria for the tests described. The tests concern the active substance, finished product or, potentially, materials at other stages of their manufacture. “Conformance to specification” means that the drug substance and drug product, when tested according to the listed analytical procedures, will be compliant with their prespecified acceptance criteria.

Specifications are just one part of a total control strategy designed to ensure quality and consistency of products. Other parts of this strategy include extended characterization during development, compliance with Good Manufacturing Practice (GMP), validation of the manufacturing process, validation or qualification of the analytical methods, quality of raw materials, in-process testing and stability studies. Specifications are chosen to corroborate the quality of the active substance and finished product and should not focus on all quality attributes, but rather on those that are most relevant for the safety and efficacy of the medicine.

Minimum standards for specifications are listed in monographs and usually include at least identity, potency and impurities. Additional product-specific specifications are set by the manufacturers and must be assessed by regulatory authorities before approval of products. Specifications and limits can be set for both the active substance (often also referred to as drug substance) and the finished product (often also referred to as drug product).

Pharmacopoeial specifications apply to all products across a class (e.g. rabies cell culture vaccines), independent of their manufacturer. These limits are decided by pharmacopoeial committees, e.g. at the European Directorate for the Quality of Medicines (EDQM) in Strasbourg, France. Conversely, product-specific specifications are set for each individual product by their respective manufacturer. Since specifications are chosen to confirm the quality rather than to fully characterize each product batch, the manufacturer must provide the justification for including and/or excluding testing for specific quality attributes. The following points are usually taken into consideration by manufacturers and regulatory agencies when reviewing proposed specifications: specifications should be based on data obtained from lots used to demonstrate manufacturing consistency; they should account for the stability of drug substance and drug product; they are linked to qualified or validated analytical procedures; and they should be based on data obtained for lots used in preclinical and clinical studies.

Both pharmacopoeial specifications and product-specific specifications for representative rabies biologicals are further described in the following paragraphs. The setting of specifications and limits is often accompanied by appropriate control standards, such as the international standard for rabies immunoglobulins. Standards are important to ensure that repeatability and reproducibility are maintained. The first international standard for HRIG was established in 1985 and the second was established in 1993 *(26)*.

Pharmacopoeial specifications

Pharmacopoeias contain important requirements pertaining to certain analytical procedures and acceptance criteria, which, where relevant, are part of the evaluation of either the active substance or the finished product. Such monographs, applicable to biological products including rabies vaccines or immunoglobulins, generally include, but are not limited to, tests for sterility, endotoxins, microbial limits, volume in container, uniformity of dosage units and particulate matter. Compliance with available monographs is mandatory, but all tests listed in a monograph do not necessarily have to be performed at release. When agreed by the competent authority, alternative (validated) methods may be used for control purpose.

Several pharmacopoeias are in use around the world, e.g. the European Pharmacopoeia (Ph. Eur.), the British Pharmacopoeia (BP) and the United States Pharmacopoeia (USP). These monographs contain the basic requirements for medicines, and their content should theoretically also be largely applicable to other regions. As an example, the Ph. Eur. monograph with the specifications for HRIG is summarized below (for full details, refer to Ph. Eur. monograph 0723, *Immunoglobulinum humanum rabicum*). The Ph. Eur. specifications for RIG include requirements for definition/identity, potency limits and methods, culture medium, storage and labelling:

Definition

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from donors immunized against rabies. It contains specific antibodies neutralizing the rabies virus. *Human normal immunoglobulin for intramuscular administration (monograph 0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intramuscular administration (0338)*, except for the minimum number of donors and the minimum total protein content.

Potency

The potency is determined by comparing the dose of immunoglobulin required to neutralize the infectivity of a rabies virus suspension with the dose of a reference preparation, calibrated in international units (IU), required to produce the same degree of neutralization. The test is performed in sensitive cell cultures and the presence of unneutralized virus is revealed by immunofluorescence. The IU is the specific neutralizing activity for rabies virus in a stated amount of the International Standard for anti-rabies immunoglobulin. The equivalence in IU of the International Standard is stated by WHO.

Human rabies immunoglobulin BRP is calibrated in IU by comparison with the International Standard.

Methods

The method for the neutralization assay in suitable cells such as the BHK-21 cell line is described in detail in the monograph.

The stated potency is not less than 150 IU/mL. The estimated potency is not less than the stated potency and is not greater than twice the stated potency. The confidence limits (P = 0.95) are not less than 80% and not more than 125% of the estimated potency.

Culture medium

The culture medium for growth of BHK-21 cells is described in the monograph.

Storage and labelling

The monograph states requirements for storage and labelling (in IU).

As RIG is derived from blood donations, certain regulatory requirements for blood products apply. These products need to be treated to eliminate or reduce any risks of transmission of infectious agents. Briefly, plasma donors are initially screened for exposure to a range of viruses. After fractionation with cold ethanol of plasma from vaccinated donors, the HRIG products such as HyperRAB S/D and Imogam Rabies-HT are treated to eliminate potential pathogens. Use of HRIG in the USA has not resulted in any known cases of transmission of infectious agents *(14)*.

For blood products such as HRIG, only donations from qualified donors ("Regular donors") are accepted for fractionation. To qualify, applicant donors ("First time donors" and "Repeat donors") usually have to pass a history of two accepted donations given within 6 months of each other. For source plasma, the National Donor Deferral Registry (NDDR) allows donor deferral information to be shared on a confidential inter-company nationwide basis. This ensures that any donor who has been deferred at one centre under NDDR criteria may not donate at another *(27)*. Each manufacturer also operates an inter-centre deferral to ensure that "higher risk" donors are excluded. Donors are encouraged to donate regularly, resulting in frequent virus testing and review of post-donation information. A qualified donor who has not donated plasma for 6 months reverts to applicant donor status. All donations are tested at least for Hepatitis B surface antigen (HBsAg), anti-HCV and anti-HIV 1 and 2 antibodies. The lower incidence of positive results in qualified than in applicant donors confirms the effectiveness of donor selection, testing and exclusion in limiting the risk of transmitting infections in plasma.

A three-stage system is usually in place to ensure the safety of blood products such as RIG, namely:

1. Selection of healthy donors, with all donations tested and traceable to the donor;
2. Further safety tests of plasma minipools and pools in advance of the manufacturing process; and
3. Virus removal and inactivation steps during the manufacturing process (e.g. low pH virus inactivation steps, virus filtration, heat treatment).

Manufacturers' product specifications

In contrast to the pharmacopoeial specifications mentioned in the preceding paragraphs, product-specific specifications are set for each individual product by their respective manufacturer and are part of the registration process for each individual rabies vaccine and immunoglobulin. These specifications are critical quality standards that are proposed and justified by the manufacturer and reviewed by regulatory authorities as conditions of approval. They vary depending on the manufacturing process and are usually part of commercially confidential information provided in marketing authorization dossiers; hence they will not be described here. However, some general considerations apply: specifications set by the manufacturer should take into account the control of raw materials and excipients, in-process testing, process evaluation or validation, batch analysis data and stability. Finished product specifications should normally also be justified with reference to batch analysis data from clinical trial batches and the limits for potency/purity/impurities should be clinically qualified.

An in-depth characterization of a biological product by appropriate methods is necessary to allow suitable specifications to be set. Extensive characterization is performed in the development stages of a product and occasionally after licensing following substantial process changes.

Heterogeneity may be observed during manufacture and/or storage of the drug substance or drug product. The degree of this heterogeneity should be evaluated, to assure consistency between production lots. When these variants have properties closely related to those of the desired product with respect to activity, efficacy and safety, they are considered product-related substances. When process changes and degradation products result in heterogeneity patterns which are not clinically qualified, i.e. they differ from those observed in the material used during preclinical and clinical development, the significance of these alterations must be further investigated.

Purity

The absolute as well as relative purity should be analysed using suitable analytical methods. Traditionally, the relative purity of a biological product is expressed in terms of specific activity (that is, units of biological activity per mg of product) which could be highly method-dependent. Thus, the purity of the drug substance and drug product is usually assessed by a range of analytical methods. For the purpose of lot release, an appropriate set of methods is selected and justified for determination of purity.

Impurities

The manufacturer should assess impurities, either process or product-related. When adequate quantities of impurities can be enriched, they should be evaluated to the extent possible, including their impact on biological activity. Product-related impurities (e.g. precursors, certain degradation products) encompass molecular variants arising during manufacture and/or storage, which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety. Process-related impurities are those that are derived from the manufacturing process, i.e. cell substrates (e.g. host cell proteins, host cell DNA), cell

culture (e.g. inducers, antibiotics, or media components), or materials used in downstream processing. The acceptance criteria for impurities should be based on data obtained from lots used in preclinical and clinical studies and manufacturing consistency lots.

Contaminants

Contaminants include all adventitiously introduced materials not intended to be part of the manufacturing process, such as chemicals or microbial proteases. Contaminants should be strictly avoided and/or suitably controlled with appropriate in-process acceptance criteria or action limits for drug substance or drug product specifications.

In-process controls

In addition to specifications for the active substance and the finished product, so called in-process controls (IPC) and tests are performed at critical decision-making steps during manufacture. These data should be used to confirm consistency of the process during the production of either the active substance or the finished product. The results of in-process testing may be recorded as action limits or reported as acceptance criteria. Performing such testing may eliminate the need for testing of the active substance or finished product. In-process testing for adventitious agents at the end of cell culture is an example of testing for which acceptance criteria should be established. Data obtained during development and validation runs should provide the basis for provisional action limits to be set for the manufacturing process. These limits, which are the responsibility of the manufacturer, may be used to initiate investigation or further action. They should be further refined as additional experience and further data are obtained after product approval.

Raw materials and excipient specifications

The quality of the raw materials used in the production should meet standards. Moreover, the quality of the excipients should meet pharmacopoeial standards. Otherwise, suitable acceptance criteria should be established for any non-pharmacopoeial excipients.

Release limits vs shelf-life limits

The stability of the medicine should be established, and an appropriate shelf-life should be set. The limits might be different for release and during shelf-life, i.e. limits are usually tighter for the release than for the shelf-life of the drug substance or drug product, e.g. in the case of potency and degradation products.

Future perspectives

An interesting development that could help advance the regulation of medicines such as rabies biologicals is the launch of an African Medicines Agency (AMA) *(28)*. The AMA is intended to be an organ of the African Union legally mandated by Member States to provide a platform for coordination and strengthening of ongoing initiatives to harmonize the regulation of medicines. The remit

of the AMA will be to speed up the availability of affordable medicines that are needed on the continent and reduce dangerous, poor-quality and falsified medicines. It will protect public health across 54 Member states, serving 1.13 billion African people. Like the EMA, its European counterpart, the AMA will not replace national regulators, who will continue their work to register medicines that are safe and efficacious for their own populations. Instead, the AMA will provide regulatory guidance, oversee emerging issues such as pandemics, review adverse effects of medicines and vaccines, and conduct inspections of manufacturing facilities to check that medicines are being manufactured at good international manufacturing quality standards. The agency has been set up by African Heads of State and Government with help from the WHO Regional Committee for Africa, as the result of a longstanding strategy to improve regulatory capacity on the continent *(29)*.

The establishment of the AMA, together with the advent of rabies MAbs and innovations such as pseudotype neutralization assays, should contribute to the widespread availability of high-quality, safe and effective rabies biologicals in the future.

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Chapter 36
Regulatory issues in the development of animal biologicals for rabies

Introduction

Vaccinating domestic animals against rabies creates an effective barrier between the human population and rabies reservoirs. In multiple countries, mass canine vaccination yields a concomitant decrease in the incidence of human rabies cases *(1–4)*. For this reason, many countries require vaccination of dogs and cats, and have strict requirements for importation of these animals regarding rabies vaccination status. Under some conditions of herd health management, it may be advisable to vaccinate livestock as well, especially in areas endemic for rabies where exposure to lyssavirus virus vectors is likely, such as with vampire bat exposure in the New World *(5)*. In addition, vaccination of wildlife reservoirs can be a powerful tool for controlling endemic rabies in susceptible wildlife populations *(2–4, 6, 7)*. These efforts can further reduce human exposure by decreasing the likelihood of direct human contact with a rabid wild animal, and the likelihood of domestic animal contact and subsequent secondary human exposure. Hence, use of veterinary rabies vaccines can substantially reduce human exposures, resulting in fewer human deaths and reduced need for expensive post-exposure prophylaxis (PEP) in humans. Similarly, licensed diagnostic kits may be used for the detection of viral antigens, antibodies and amplicons from suspect animals.

Regulatory considerations concerning vaccines for use in domestic animals

Regulatory approval of rabies vaccines for use in domestic animals should be based upon solid evidence of their safety, purity, potency and efficacy. Early vaccines were based upon rabies virus (RABV) grown in adult or suckling animal brain tissue *(3)*. While these products provided a much-needed tool for control of rabies in domestic animals, they are now obsolete and, given the high rates of adverse events, should not be considered for use. Tissue culture origin, inactivated vaccines and recombinant vaccines are now the preferred candidates for use in domestic animals.

Worldwide, many vaccines are available for use in domestic animals. For example, products available from the United States, Canada, the European Union, Australia, New Zealand and Japan have met rigorous standards of safety, purity, potency and efficacy as required by these countries and regions. While the requirements for approval of rabies vaccines have not been harmonized worldwide, competent regulatory authorities use similar principles to assure that products are safe, pure, potent and effective.

Evaluation for safety should include laboratory studies in host and non-host animals, as well as large-scale field studies. Batch safety should be confirmed either by laboratory and/or host animal testing and through demonstration of a high level of consistency in production.

Evaluations for purity should begin with using a Master Seed and Master Cell concept. The cell cultures and virus seeds used should be characterized thoroughly and shown to be free of adventitious agents, before being approved for vaccine production. In addition, purity checks should be done at various stages of production. These might include testing of working seeds, production seeds and harvested bulks. Finally, each batch should be tested for mycoplasma, fungal, and bacterial contamination after filling of final containers. Assays should be well validated and should include proper controls to ensure assay integrity.

Potency testing will be dependent upon the nature of the product. Currently, the standard approach for inactivated rabies vaccines for most countries is the NIH test or a modification of this assay *(8)*. Briefly, mice are vaccinated with finished product, then challenged with a standard rabies challenge virus (see Chapter 42). This assay has been in place since the 1950s, and has several drawbacks. The assay takes at least one month to perform, and the outcome is highly variable. This results in frequent "no tests" because of the stringent validity requirements, requiring frequent retests. The assay is costly to run, represents a human health risk and results can vary dramatically from one operator to the next based on experience with the assay. In addition, the test relies on a standard reference vaccine, which must be replenished or replaced frequently *(9).* Efforts have been made to replace the mouse potency assay, but because most inactivated rabies vaccines for animals include an adjuvant, the development of an ELISA or other assay platform is complicated by the need to break the emulsion or dissociate antigen from the adjuvant *(8, 10)*. This has proven challenging and, to date, there is no well-characterized, well-validated assay to replace the National Institutes of Health test for veterinary vaccines. However, efforts are ongoing to define a replacement test or battery of tests to measure potency *(10, 11)*. Currently, most of the work being done is based on measurement of the trimeric form of the RABV glycoprotein (G).

Recombinant vaccines for use in domestic and feral animals have been developed using various viral vectors, including non-virulent viruses such as raccoonpox, canarypox and others *(4, 12)*. The RABV G gene is spliced into the vector, and the products are replication-competent in tissue cell culture, but most are replication-limited in vaccinates. Potency can be based on a simple virus titre accompanied with confirmation of protein expression. The titre for finished product should be based on the titre used in the pivotal efficacy trials, with some overage included to account for assay variability and titre loss over shelf-life.

Efficacy should be based on host animal vaccination or challenge studies. The challenge phase of the efficacy trial should take place at the end of the recommended revaccination period; that is, if the product is labelled for annual revaccination, the challenge event should occur at least one year after the vaccination event, and so forth for other duration of immunity claims. Products for domestic animals should result in a prevented fraction that approaches 85–90%.

Relevant regulatory authorities should require that manufacturers of RABV vaccines have a vaccinovigilance or pharmacovigilance programme that is regularly reviewed and monitored. While no vaccine can be expected to provide 100% efficacy, reports of lack of efficacy should be investigated thoroughly. At the very least, a complete history of the animal involved should be obtained, the conditions of product administration should be determined, and retention samples of

the product serial or batch should be tested for potency. If vaccine failure in an individual case is determined to be the result of a lack of sufficient potency, or if a cluster of reports occurs, a product recall should be conducted. End users should be notified, and animal revaccination should be strongly encouraged.

Regulatory considerations for use of vaccines in wildlife

Many countries have used wildlife vaccination campaigns to address epizootic outbreaks in wildlife reservoirs, such as raccoons, foxes, coyotes, and other meso-carnivores. Early work in this area involved trap/vaccinate/release campaigns using conventional parenteral vaccination. These efforts are resource intensive, and success is variable *(3)*.

Subsequent efforts that have shown success involve distribution of vaccine-laden baits. These products are either highly attenuated modified-live RABV or recombinant virus vectors containing the RABV G gene *(3)*.

Regulatory considerations for wildlife vaccines should include similar standards as those for domestic animals regarding safety, purity and potency. However, efficacy requirements may be adjusted species-by-species based on disease prevalence, animal distribution density, migratory patterns, species behaviour and other factors. For example, it might be possible to disrupt an epizootic with a slightly less efficacious vaccine if the baiting programme achieves adequate coverage, but this is dependent upon the nature of the target species and factors specific to the disease situation. Regulatory authorities should work closely with wildlife specialists and other experts to develop a rational approach for establishing efficacy requirements for wildlife vaccines.

Diagnostic test kits for use in animals

Diagnostic tests for suspected rabies cases in animals should be conducted by well-trained personnel, using well-validated assays. Currently, there are no testing protocols for use in living animals; the “gold standard” is based on the direct fluorescent antibody test or DFAT (see Chapter 11), using postmortem brain tissue *(12)*. Most developed countries have established testing protocols, and laboratories engaged in testing have appropriate quality systems in place and participate in regular proficiency testing.

Routine use of point-of-care diagnostic test kits is controversial. Although these types of tests can be useful, they should only be used as screening tools, or in situations where < 100% sensitivity and specificity is tolerable. An example situation would be attempts to conduct disease surveillance in wildlife, where the results are not being used to make management decisions for individual animals, especially in situations of human PEP.

Point-of-care kits should be well characterized and validated for sensitivity, specificity, ruggedness, repeatability and reproducibility. Pre-marketing evaluation should include a robust field trial to establish and confirm those parameters. End use should be limited to researchers and wildlife management officials.

Regulatory oversight of diagnostic test kits varies considerably from region to region. For example, in the United States, point-of-care kits for veterinary use

must be licensed by the United States Department of Agriculture's Center for Veterinary Biologics. Other countries and regions have requirements ranging from a similar model to limited or no regulatory oversight of such products. Authorities considering the approval and use of point-of-care kits should ensure that the products have adequate data supporting sensitivity and specificity claims.

Use of post-exposure prophylaxis for unvaccinated domestic animals

After exposure to a vaccinated animal, an immediate vaccine booster is recommended. Annually, in many countries, thousands of naive animals are euthanized after rabies exposure. Many are unvaccinated because they are too young (i.e. less than 3 months of age). There are currently no well-defined PEP protocols recommended for use with such naive domestic animals. However, objectively, there is no reason why such protocols could not be developed. Historically, supplies of anti-rabies immunoglobulin have been limited, costs are very high, and shortages have been commonplace. This situation raised ethical considerations around the use of a scarce commodity in animals when people in many parts of the world have no access to these life-saving materials.

Recently, monoclonal antibodies have been developed that are much less expensive to produce and can likely be supplied in large quantities *(13, 14)*. When these products have been fully evaluated, their availability may change the current paradigm related to using such products in exposed or potentially exposed naive animals. Evaluation of such products should be done using host animal exposure-prophylaxis studies as agreed upon by relevant regulatory authorities.

Future considerations

As technical advances continue, it can be expected that the tools available for rabies diagnosis, prevention and control in animals will expand. Next-generation products should be safer, more efficacious, more user-friendly and, hopefully, more economically sound. Given the status of rabies as a neglected tropical disease, future developments should allow for improved ability for enhanced, decentralized laboratory-based surveillance, to detect and reduce endemic rabies and respond effectively to rabies epizootics as they arise. Efforts should continue to educate at-risk regions as well as funding agencies as to the products and methods available. These efforts should include information related to product attributes and limitations to help ensure the most efficient allocation of limited resources, particularly as considered by regulatory authorities for all relevant species at risk.

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Chapter 37
Preparation of fluorescent antibody conjugate for the direct fluorescent antibody test

Introduction

During the past several decades, various live virus or purified antigens have been used to produce polyclonal or monoclonal antibodies for rabies diagnosis, as described in this manual and elsewhere *(1–8).* Monoclonal antibodies are characterized by their specificity of binding, their homogeneity and their ability to be produced in large quantities. For example, all the antibodies produced by descendants of one hybridoma are identical, making them powerful in testing for the presence of a desired epitope *(4)*. In 1975, Köhler and Milstein developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity *(5)*. In an animal, antibodies are synthesized primarily by plasma cells, a type of terminally differentiated B lymphocyte. Polyclonal antibodies are a mixture of antibodies that are secreted by different B cell lineages. These antibodies are a collection of immunoglobulin molecules that react against a specific antigen, and each identifies or recognizes a different epitope(s) on an antigen.

In 1973, Dean and Abelseth used inactivated rabies virus (RABV) infected mouse brain suspensions as a source of antigen to immunize animals and from the hyper-immune serum obtained concentrated specific antibodies *(3)*. Generally, antibodies are typically produced by inoculating a suitable mammal, such as mice, rabbits, goats, chickens, guinea pigs, hamsters, horses, rats and sheep. Larger mammals are often preferred, as greater volumes of serum can be harvested. The basic principle is that an antigen is injected into the mammal, and this induces the B-lymphocytes to produce immunoglobulins (e.g. IgG) specific for that antigen.

The primary goal of antibody production in animals is to obtain high titre, high affinity antisera for use in experimentation or diagnostic tests. The antigen may be administered with an adjuvant to improve or enhance the immune response to antigens. This chapter outlines the methods used to obtain polyclonal antibodies from goats and their labelling with fluorescein isothiocyanate (FITC). At least two animals per antigen should be used, as this reduces the failure resulting from non-responsiveness to antigens of individual animals. Purification of ribonucleoprotein (RNP) from baby hamster kidney (BHK) cells infected with a laboratory strain, such as Evelyn Rokitniki Abelseth (ERA) and/or Mokola virus 97/252 (MOKV), is described. The RNP is purified by employing ultracentrifugation in a cesium chloride gradient; the resulting RNP is checked for intactness and authenticity by immunoblotting with a specific lyssavirus antibody. The protein product(s) can be used for immunizing goats to raise hyperimmune serum, which is subsequently labelled with either biotin for use in the direct rapid immunohistochemistry test (DRIT), as described in Chapter 12, or the FITC for detection of lyssavirus antigens in the direct fluorescent antibody test (DFAT); see Chapter 11.

Ammonium sulfate precipitation is one of the most commonly used methods for removing proteins from solution. Water molecules are removed from the proteins, thereby decreasing their solubility. Although other salts, such as sodium sulfate, are sometimes used, precipitation of antibodies is commonly done with ammonium sulfate. Ideally, only the highest purity ammonium sulfate should be used. The concentration at which antibodies will precipitate varies from species to species. One disadvantage of using ammonium sulfate is that during the precipitation step, other high molecular weight proteins are trapped in the large flocculent precipitates, thereby improving the purity of the preparation.

Antigen production for antibody generation

The antigens are purified from BHK-21 cells [ATCC CCL-10] infected with ERA and/or MOKV (Fig. 37.1A). The cells are ruptured by five freeze–thaw cycles, homogenization and detergent action to disrupt the virus envelope. Lipids are removed using solvent and centrifugation. Proteins are separated by polyethylene glycol 8000 (PEG) precipitation followed by elution. Finally, the RNP is purified on a cesium chloride gradient and is dialysed against phosphate buffered saline (PBS) buffer for use in immunization and antibody induction.

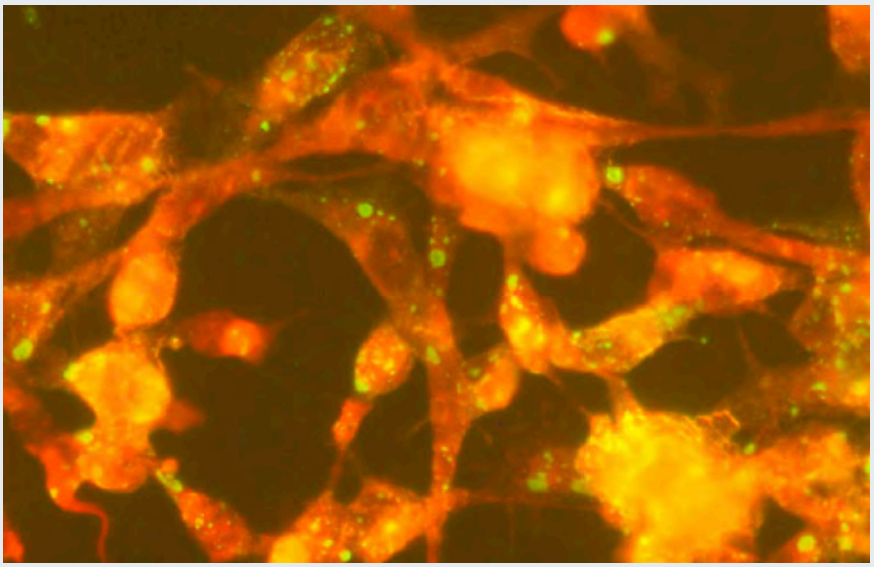

Fig. 37.1A. RABV-infected murine neuroblastoma cells stained with FITC-labelled conjugate

Protocol

1. Virus isolation and titration

1.1 Lyssavirus infection is confirmed by the DFAT as described *(3).* From a lyssavirus-infected sample selected for RNP and hyperimmune serum production, prepare a 10% (w/v) brain tissue homogenate in tissue culture (TC) medium consisting of Dulbecco's Modified Eagle Medium (DMEM F-12), supplemented with 10% fetal bovine serum (FBS) and 5% antibiotics/antimycotic. Note: It is crucial that cells are actively growing and healthy.

1.2 Centrifuge the homogenate at 1076 x *g* (2500 r/min in a Sorvall centrifuge) for 30 min to separate tissue debris, then transfer the supernatant into a sterile 2.0 mL or 5.0 mL polypropylene tube. Repeat this step if necessary to clarify the supernatant further. The supernatant is now ready for use as inoculum.

1.3 Trypsinize a confluent T25 cm^2 monolayer of BHK cells and resuspend the cell suspension in 20 mL of TC medium, then infect the cell suspension with a specific lyssavirus species at a multiplicity of 0.1. Mix gently by swirling and distribute 200 µL of the cell suspension into three adjacent wells in two separate 96-well plates as monitor plates. Incubate the two monitor plates and flask at 37 °C in a humidified incubator with 5% CO_2 for up to 72 h. After 48 h, fix the first monitor plate with 80% cold acetone for 15 min and air dry for 5 min at room temperature and then stain the monolayer (see Chapter 11). Dilute the current batch of the FITC-conjugated anti-lyssavirus polyclonal antibody to a working concentration with PBS (pH 7.2–7.4). Distribute 50 µL of the conjugate into each well and incubate the plate in a humidified chamber or container at 37 °C for at least 45–60 min. Remove the plate from the incubator, discard the conjugate and rinse three times with PBS (pH 7.2–7.4) to remove unbound conjugate and excess buffer. Blot the plates dry on a stack of paper towel, view under a fluorescent microscope, then record the observations.

1.4 Keep the flask in the incubator until the last monitor plate has been acetone-fixed and stained as in step 1.3. Harvest the supernatant when the infection is 80–100% and establish the virus titres using the Spearman–Kärber method (see Chapter 20). Briefly, remove the flask from the incubator and freeze–thaw three times at –20 °C and room temperature. Harvest the supernatant and clarify at 1076 x *g* (2500 r/min) for 30 min to remove cell debris and store at –70 °C until required. The virus titre should be at least 10^5 tissue culture infectious doses ($TCID_{50}$) for use in subsequent steps. Otherwise, repeat the process as in step 1.1.

2. Virus propagation and cell harvesting

2.1 The virus supernatant with a known titre is used to infect BHK cells re-suspended in TC medium. Briefly, determine the cell concentration of BHK cells from a confluent T75 cm^2 flask and re-suspend in 10.0 mL of TC medium using a haemocytometer. Detach the BHK cells from 16 T75 cm^2 [or T150 cm^2] from the surface of the flask using trypsin, pool aliquots together and re-suspend into a total volume of 1000 mL with TC medium. For the virus use the formula: volume of virus stock to be added = multiplicity of infection x number of cells / (virus titre) to calculate how much virus is required to infect the re-suspended cells.

2.2 Infect the re-suspended BHK cells with a lyssavirus at a multiplicity of infection (MOI) of 0.1 and mix by gentle swirling. Sub-culture about 25 mL of infected BHK cells into 40 x T175 cm^2 flasks or T150 cm^2 and 200 µL into a specific well (take note of the flask from which it originated) into two separate 96-well monitor plates (i.e. one can also use 60- or 72-well plates). Incubate both the monitor plates and the flasks at 37 ˚C in a humidified incubator with 5% CO_2 for 72 h. After 48 h, fix the first monitor plate with 80% cold acetone for 15 min and repeat **step 1.3** after 72 h.

2.3 Harvest the BHK cells from the flasks when the monolayer infection is between 80% and 100% on the monitor plate. Remove the flasks from the incubator and carefully pour off TC medium into virucidal solution.

2.4 Scrape the infected monolayer of cells from the surface of the flask using a clean, sterile cell scraper into a 50-mL polypropylene centrifuge tube and collect the pellet of BHK cells by centrifuging at 2988 x *g* (5000 r/min) for 5 min. Re-suspend the cell pellet in 8.0 mL of cold STE buffer (pH 7.8), and store at –70 °C until required.

3. Ribonucleoprotein (RNP) purification

3.1 Thaw the infected pellet of cells under running cold water and once thawed transfer the sample into a Dounce homogenizer placed in a beaker filled with crushed ice. Homogenize the cell pellet with 10–20 strokes of the loose pestle, followed by 10 to 20 strokes of the tight pestle.

Note: It is important that the suspension does not become warm. Sterilized glass beads may also be added to infected pellets; when thawing suspension, shake vigorously.

Add 0.2 mL of cold 10% IGEPAL [Sigma Aldrich, USA] for every 1.0 mL of the cell homogenate and stir gently for 30 min using a magnetic stirrer in a cold room (4 °C) or mix the cell homogenate with 10 strokes of the loose pestle without forming foam.

3.2 Add an equal volume of cold 1,1,1,2,3,4,4,5,5,5 decafluoropentane and mix well by inverting the tube several times. Stir the reaction mixture vigorously with a magnetic stirrer for 30 min in a cold room, then centrifuge the reaction mixture for 20 min at 2988 x *g* (5000 r/min) at 4 ˚C to separate the phases. Harvest the aqueous layer into a clean 50 ml polypropylene centrifuge tube using a sterile glass Pasteur pipette and store the aliquot at 4 ˚C. Add cold STE buffer (pH 7.8), equivalent to the starting volume of the cell homogenate to the inter-phase and bottom layer. Mix the solution by inverting the tube, then stir vigorously at 4 ˚C for 30 min using a magnetic stirrer. Centrifuge the reaction mixture at 2988 x *g* (5000 r/min) at 4 ˚C for 20 min and harvest the aqueous layer. Pool the aqueous layer supernatant with the previously harvested sample and estimate the total volume of the pooled supernatant.

3.3 Add a final concentration of 0.3 mol NaCl and continue stirring until completely dissolved. For every 1.0 mL of the solution, slowly (over 4–6 h), add 0.06 g of PEG (polyethylene glycol) 8000 while stirring at 4 ˚C overnight.

3.4 Centrifuge the mixture at 7649 x *g*(8000 r/min) for 30 min at 4 °C and discard the supernatant. Remove traces of moisture from the centrifuge tube using strips of Whatman No. 4 filter paper to absorb the moisture and allow to air dry for 5 min. Add about 500 µL of cold diluted STE buffer (1:4) and break the pellet using a Pasteur glass pipette, then stir at 4 °C overnight.

3.5 Centrifuge the reaction mixture at 7649 x *g* (8000 r/min) for 30 min at 4 °C and harvest the supernatant using a sterile Pasteur glass pipette into a sterile 50.0 mL polypropylene centrifuge tube. Add 500 µL of cold diluted STE buffer (1:4) to the pellet and solubilize by stirring at 4 °C for 4 h. Collect the supernatant by centrifugation at 7649 x *g* (8000 r/min) for 30 min and repeat the same process as previously. Harvest the supernatants and pool with the previous supernatant, then clarify by centrifugation at 11 952 x *g* (10 000 r/min) for 30 min to remove any cell debris.

3.6 Prepare the different cesium chloride (CsCl) solution densities or gradients of 1.2 g/mL, 1.3 g/mL and 1.4 g/mL with STE buffer (pH 7.8).

Note: The supernatant should not exceed 1.5 mL due to size limitation of the centrifuge tube to be used.

Overlay the supernatant with 3.0 mL of each CsCl solution starting with 1.2 g/mL, 1.3 g/mL and 1.4 g/mL. Balance the tubes by adding 1.3 g/mL of cold STE buffer. Centrifuge the tubes at 266 676 x *g* (38 000 r/min) using a high-speed rotor (e.g. SW 41 rotor) for 16 h or overnight. Visualize the bands by placing the tube underneath a light source and a black sheet behind the tube (see Fig. 37.1B).

3.7 Harvest each opaque band into a separate and sterile 2 mL Eppendorf tube. Dialyse the harvested bands using nitrocellulose membrane against 2000 mL of STE buffer (pH 7.8) for 24 h with an STE buffer (pH 7.8); change every 2 h. Finally, dialyse against 2000 mL of PBS (pH 7.2–7.4) overnight. Distribute the harvested and dialysed proteins into labelled cryotubes and store at –70 °C until required.

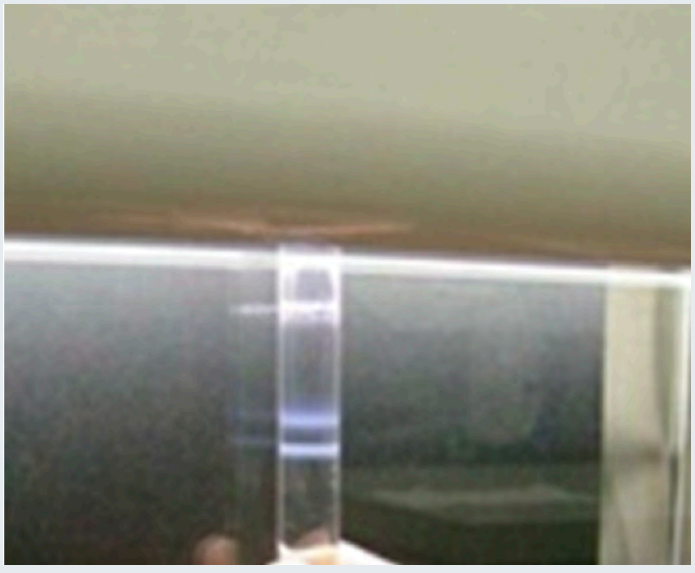

Fig. 37.1B. Ultracentrifuge tube demonstrating the two opaque bands observed after RNP purification from infected MNA cells

4. SDS-PAGE and western blotting

4.1 Assemble the plates on the casting stand with the short glass facing the front and securely tighten to avoid leaking of the gel. Cast two gels, one for the Western Blot analysis and the other for the Coomassie blue staining.

4.2 Prepare both resolving and stacking gels according to Table 37.1, starting with the resolving gel.

Table 37.1. Ratios of reagents used to prepare both resolving and stacking gels for SDS-PAGE and Western Blot analysis

Item	Resolving gel (10%)	Stacking gel (4%)
Distilled water	4.00 mL	2.80 mL
Acrylamide mix	3.30 mL	0.83 mL
1.5 mol Tris pH 8.8	2.50 mL	None
0.5 mol Tris pH 6.8	None	1.30 mL
10% sodium dodecyl sulfate (SDS)	0.10 mL	0.05 mL
10% ammonium persulfate (APS)	0.10 mL	0.05 mL
TEMED	0.004 mL	0.005 mL

4.3 Add the resolving gel between the plates, about 5 cm from the top, and overlay with 2% SDS to prevent bubbles from forming within the resolving gel. Allow the gel to polymerize for at least 30 min. Pour off the 2% SDS, then add 3 mL of the stacking gel on top of the resolving gel. Insert the comb gently between the plates and allow polymerizing for at least 5 min. Dilute samples and controls (10 µL) with an equal volume of sample loading buffer in a locking top Eppendorf tube and boil for 5 min. Mount the casting plates together with the gel onto a mini gel electrophoresis apparatus assembly with the short glass plate facing inwards and add about 20 µL of each sample into the wells. Fill the chamber with running buffer (see Annex) and electrophorese the samples at 100 V for 90 min or turn off when the dye is about a centimeter from the bottom. Remove the gels and cut-off the stacking gel. Clearly mark the gels by cutting off the bottom corner to indicate the sequence of the samples on the gel. Place the gel in a large Petri dish, add stain solution (see Annex) to cover the gel and shake for 10 min (at 150 r/min). De-stain the gel with de-staining solution until clear.

4.4 For the Western Blot analysis, add running buffer into the gel contained in a Petri dish and allow to equilibrate for 15 min at room temperature with shaking. Cut two pieces of filter paper and nitrocellulose membrane to match the size of the gel and place each in a separate Petri dish containing running buffer. Place on the base of blotting apparatus filter paper, nitrocellulose membrane, gel, filter paper then roll out the bubbles using a Pasteur pipette.

4.5 Attach to power supply and run at a constant 12 V for 40 min. Remove the membrane from the blotting apparatus and block with 5% skimmed milk in PBS (pH 7.2–7.4) buffer. Allow blocking for 60–120 min at room temperature with shaking. Air dry the membrane and mark the top of the membrane to indicate the sequence of the samples. Dilute a labelled anti-lyssavirus mono-

clonal antibody with horse radish peroxidase (HRP) in a 1:5 ratio known to react with specific lyssavirus species nucleoprotein or the target protein and add into the Petri dish containing the membrane. Incubate for 60–120 min with shaking at room temperature. Wash the membrane three times with PBS (pH 7.2–7.4) buffer and add chromogenic substrate buffer (TMB) to visualize the bands (Fig. 37.1C).

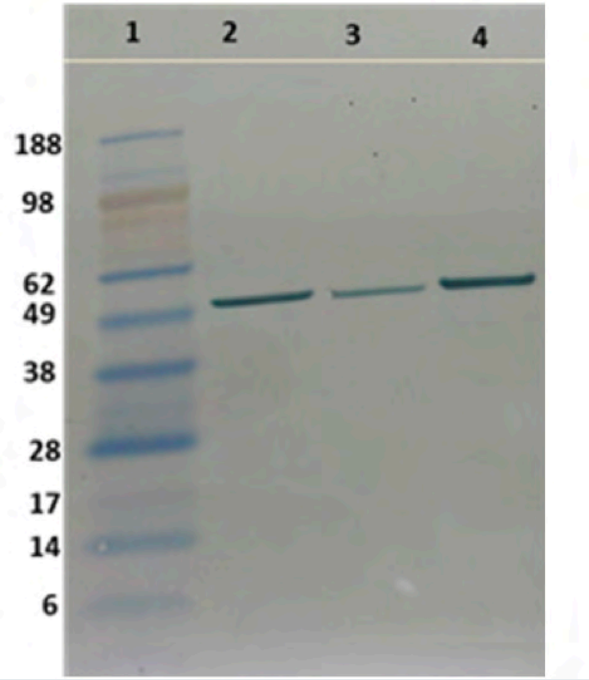

Fig. 37.1B. Ultracentrifuge tube demonstrating the two opaque bands observed after RNP purification from infected MNA cells

5. Immunization of animals

5.1 Obtain female goats aged 4–6 months. Provide all routine veterinary care, such as deworming (i.e. using anthelminthics [such as Valbazen Ultra and closantel, or similar]) and observe for 7 days. Collect 25 mL of blood from the jugular vein before the animals are immunized to obtain baseline antibody data.

5.2 Immunize the goats intramuscularly into the biceps femoris on day 0 with the RNP of interest (e.g. ERA RABV) together with complete Freund's adjuvant in a total volume of 1 mL (1:1 ratio). It is recommended that the adjuvant is well emulsified prior to immunization.

5.3 On day 21, collect 25 mL blood through the jugular vein and administer a booster with ERA RNP together with incomplete Freund's adjuvant in a total volume of 1 mL (1:1 ratio). Separate serum by centrifugation at 2988 x *g* (5000 r/min) and establish antibody titres by performing the immunofluorescence assay (IFA) as described in Chapter 21 of this manual.

5.4 On day 42, collect blood (25 mL) from the goats, separate serum by centrifugation and establish antibodies titres using IFA as described previously. Administer further booster doses on day 49 if a low antibody titre is observed (< 1:1000).

5.5 On day 63, collect a sufficient blood sample (at least 50 mL) into sterile 250 mL bottles. Allow the blood to clot and collect serum by centrifugation as done previously. Establish the antibody titre by IFA and store serum at –20 ˚C if > 1:10 000 until required. Administer a further booster with a different lyssavirus species (e.g. MOKV RNP) to generate broadly cross-reactive polyclonal antibodies.

5.6 On days 77 and 91, collect sufficient blood (at least 50 mL) from the jugular vein of animals into sterile 250 mL bottles. Allow the blood to clot, collect serum and clarify by centrifugation as done previously. Establish the antibody titre by IFA and store serum at –20 ˚C until required. Once the titre is adequate, sedate the animals, conduct a final bleeding and serum collection, euthanize the animals appropriately and incinerate the carcasses upon completion of the last collection.

6. Ammonium sulfate precipitation of immunoglobulins

A maximum of 25 mL of serum should be processed at any one time. All solutions are prepared in advance and stored at 4 ˚C until required. Each newly prepared batch of FITC-conjugated immunoglobulin is tested for sensitivity and specificity against known circulating lyssaviruses in the geographical area.

6.1 Thaw serum and centrifuge at 12 000 r/min for 10 min at 4 ˚C.

6.2 Remove serum and place in a 100-mL sterile Schott bottle.

6.3 Add a stir bar and place the Schott bottle on the magnetic stirrer in a cold room at 4 ˚C.

6.4 Add 1.0 mL cold saturated ammonium sulfate for every 1.5 mL of serum, dropwise, with constant stirring at low speed to minimize protein denaturation.

6.5 Stir the mixture overnight at 4 ˚C. A white precipitate should form.

6.6 Collect the precipitated proteins by centrifuging the mixture at 5000 r/min for 30 min at 4 ˚C.

6.7 Discard the supernatant and re-suspend the sediment in 0.01 mol PBS until the final volume equals that of the original serum (in step 6.1 above).

6.8 Add an equal amount of cold saturated ammonium sulfate dropwise with constant stirring for 30 min and repeat steps 6.5–6.6 twice.

6.9 Re-suspend the final sediment in PBS (in half the original volume of serum).

6.10 Place this volume in a dialysis bag and dialyse the bag in a 2 L-beaker containing PBS.

6.11 Change the buffer and leave stirring at 4 ˚C.

6.12 Check the PBS for the presence of sulfate ions. To 2–3 mL of PBS collected in a tube, add 1–2 drops of acidified saturated barium chloride, and watch for the formation of a visible white precipitate, barium sulfate. If sulfate ions are present, replace the PBS and let stir for another 2 h. Replace the 0.01 mol PBS at the end of the day and leave stirring at 4 ˚C overnight.

6.13 If sulfate ions are no longer present, replace 0.01 mol PBS, and let stir for a further 2 h.

6.14 Collect the immunoglobulins from the dialysis bag into a graduated cylinder and record the volume.

6.15 Determine the protein content on an aliquot using a protein assay kit or other protein concentration determining method. Calculate the total amount of protein. Continue on with labelling only if the amount of protein exceeds 2 mg/mL.

7. Labelling of the globulins

7.1 Add carbonate/bicarbonate buffer dropwise under constant stirring. The amount of buffer should not exceed 1/10th of the total volume of the collected globulins.

7.2 Monitor the pH of the globulins/carbonate buffer.

7.3 Add FITC (0.01 mg FITC per mg of protein) while stirring.

7.4 Let stir overnight or for a minimum of 8 h at 4 °C.

7.5 Centrifuge conjugate at 12 000 r/min for 10 min.

7.6 Remove untagged FITC from the conjugate [either with the Centriprep Centrifugal Filter Concentrator or Vivaspin tubes].

7.7 Centrifuge tubes at 2400 r/min for 20 min at 4 °C.

7.8 Repeat the process until all excess dye is removed and the filtrate is clear.

7.9 Centrifuge the final product at 6140 r/min for 1 h at 4 °C.

7.10 Withdraw 0.2 mL of conjugate into a vial and determine the working dilution for DFAT and the RABV tissue culture isolation test (RTCIT; see Chapter 9).

7.11 Aliquot 1 mL of conjugate concentrate into sterile labelled containers and store at –70 to –80 °C, or 0.6 mL into small vials and freeze-dry. Each vial should contain the following information: identity of animal, year serum was collected, bottle number of serum, vial number of conjugate and date the conjugate was bottled. The hyperimmune serum produced as described above can be labelled with FITC for use in lyssavirus diagnosis or biotinylated for the DRIT assay *(2).* As this is a polyclonal hyperimmune serum, it is capable of detecting a large spectrum of lyssaviruses. However, these preparations should be validated against commercial conjugates such as those manufactured by Centocor, Fujirebio or others *(7).*

8. Experimental tips

8.1 Viral isolation and titration

Select a highly infected brain tissue sample or further passage the virus in mice to increase virus titres for the initial preparation of 10% brain tissue homogenate.

8.2 Ribonucleoprotein purification

Avoid foam and heat formation at any step of the experiments. The foam and heat will decrease the yield and denature the protein of interest. Check the pH of diluted STE buffer, as it should not be below pH 7.8 or exceed pH 8.3. It will also affect the yield and denatures the protein.

8.3 SDS-PAGE and Western blot analysis

Always add TEMED last and quickly add to the glass plates as it will polymerize with the tube. Protein concentration can be established using the nanodrop or with any other appropriate protein determining method.

9. Critical parameters and trouble shooting

9.1 Infection and harvesting of BHK cells

It is recommended that the monolayer should be at least 80% infected before harvesting to ensure maximum yield of the target protein (RNP). Use clean and sterile cell scrapers to avoid contaminating particles within the end product. Avoid foam formation during homogenization of infected MNA cells and subsequent steps, since the target protein has been released from the cells. Slowly and carefully homogenize BHK cells as mentioned above. This step will determine if you will obtain a good yield.

10. Precautions

Wear protective clothing when handling virus and work in Biosafety Level 2 or 3 facilities, especially when growing up virus and infecting BHK cells.

11. Alternative materials and methods

For SDS-PAGE, pre-cast gels as well as buffers are available commercially. There are also nitrocellulose membranes and semi transfer blots commercially available, which shorten the time required to complete the analysis.

12. Time considerations

Adequate equipment, supplies, planning, budget and staff are needed for each of the steps in antigen preparation, immunization, antibody production and conjugation over the time frames suggested above.

13. Limitations

Facilities are needed to handle live virus, since lyssaviruses are classified as Biosafety Level 2 or 3 viruses, depending on the recommendations for each laboratory performing the procedure.

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Annex 1. Materials

Reagents

- sodium chloride, molecular grade
- Tris-base, molecular grade
- ethylene diamine tetra-acetic acid (EDTA), molecular grade
- IGEPAL CA-630 (Sigma 1-3021)
- polyethylene glycol PEG 8000
- cesium chloride, optical grade
- Premix 30% acrylamide/Bis 29:1
- mol Tris buffer pH 8.8
- 0.5 mol Tris buffer pH 6.8
- nitrocellulose membrane
- protein, molecular weight (MW) standards
- sodium dodecyl sulfate (SDS), molecular grade
- N,N,N',N'-Tetramethylenediamine (TEMED)
- tissue culture grade water or double distilled water
- DMEM F-12
- fetal bovine serum (FBS)
- penicillin streptomycin and amphotericin
- trypsin
- ammonium sulfate
- FITC-conjugated anti-lyssavirus polyclonal antibody
- 1,1,1,2,3,4,4,5,5,5 Decafluoropentane

Equipment

- centrifuges (Sorvall RC-3 and Beckman ultracentrifuge)
- centrifuge rotors (SL-50T and SW 41 T)
- Beckman ultracentrifuge tubes, ultra-clear
- hard polypropylene tubes 35 mL, round bottom, screw cap (e.g. Oakridge tubes)

  Note: Propylene tubes do not bind antigen as compared with polystyrene tubes. It is advisable therefore to use hard polypropylene tubes, as they are able to handle high centrifugal forces.

- electronic balance
- Pasteur pipettes
- dialysis tubing, 12–14000 MW cut-off, 10 mm flat-width
- magnetic stirrer
- cold room or refrigerator set at 4 °C
- semi-dry protein transfer apparatus
- gel casting trays, gel combs and plates

- running chamber
- mini gel electrophoresis tank and power supply
- Petri dishes (large)
- shaker (mini)

Biologicals

- BHK-21 cells (CL1300 [European Cell Culture Collection, Salisbury, UK])
- *Lyssavirus* species (MOKV, ERA, etc.)
- purified ribonucleoprotein (RNP)
- laboratory animals – for this SOP, young female goats aged 4–6 months

Labelling of the hyperimmune serum

- serum, thawed, prior to use
- magnetic stirrer with bar
- crushed ice in a bucket
- saturated ammonium sulfate (see Annex)
- 0.01 mol PBS (12 L and 100 mL sterile)
- acidified and saturated barium chloride (see Annex 2)
- carbonate/bicarbonate buffer (see Annex 2)
- 5 mmol EDTA/100 mmol sodium bicarbonate (see Annex 2)
- dialysis bags. Spectra pro 1 membranes (6000–8000 MW cut-off), 20.4 x 30.5 cm with closures
- FITC reagent
- Viva spin
- Sorvall RC-5C centrifuge with fixed angle rotor (SS-24)
- glassware 2 L beaker, and graduated cylinder

Annex 2. Reagents

RNP purification

STE buffer pH 7.8

- Sodium chloride (NaCl)
- Tris (hydroxymethyl) aminomethane
- EDTA
- Measure 1000 mL of distilled water
- Dissolve the salts in 90% of the total volume of water
- Adjust the pH to 7.8 with 10 N HCl

Phosphate buffered saline (PBS) pH 7.2–7.4

- Sodium chloride (NaCl)
- Sodium phosphate, dibasic (Na2HPO4)
- Potassium phosphate, monobasic (KH2PO4)
- Distilled water to prepare 20 L
- Adjust the pH to 7.2–7.4 by adding either 10 N HCl or NaOH pellets

SDS-PAGE

Sample loading buffer (4x)

- 50 mmol Tris-HCl, pH 6.8
- 2% SDS
- 100% glycerol
- 1% 14.7 mol ß-mercaptoethanol
- 12.5 mmol mol EDTA
- 0.02% bromophenol blue
- Distilled water

Staining solution

- Use at 1x for loading samples onto SDS-polyacrylamide gels
- 0.25 % Coomassie blue R-250
- 50% methanol
- 10% acetic acid
- 39.75% distilled water

Destaining solution

- 20% methanol
- 10% acetic acid
- 70% distilled water

Running buffer

- 10% SDS
- Glycine
- Tris base
- 1000 mL distilled water

Western blot analysis

- Running buffer (pH 8.3)
- 25 mmol Tris base
- 192 mmol glycine
- 20% methanol
- 1000 mL distilled water

Washing buffer (pH 7.4–7.6)

- 50 mmol Tris
- 200 mmol NaCl
- 0.5% Tween

Saturated ammonium sulfate

- Add sufficient amount of ammonium sulfate to 400 mL of double distilled water to produce a saturated solution (undissolved ammonium sulfate must be visible).
- Store at 4 ˚C.
- Before use, dispense the saturated solution into a beaker, being careful not to disturb the undissolved sediment, adjust to pH 7.0 with 4N HCl.

Acidified saturated barium chloride

- To 50 mL of water add enough barium chloride to prepare a saturated solution.
- To 20 mL of saturated barium chloride add 1–2 drops of 4N HCl.
- Mix well and store at room temperature until needed.

Carbonate-bicarbonate buffer

- Add 0.3 g of Na_2Co_3 to 25 mL of sterile double distilled water.
- Add 1.85 g of $NaHCO_3$ to 20 mL of sterile double distilled water. Mix well.
- Combine both and add sterile double distilled water to make a final volume of 50 mL.
- Verify pH and adjust if necessary with 10N NaOH.
- Store at 4 ˚C.

mmol EDTA/200 mmol sodium bicarbonate

- Add the following to 1000 mL of sterile double distilled water:
- EDTA 1.46 g
- $NaHCO_3$ 16.8 g
- Mix well and store at 4 ˚C until needed.

Preparation of dialysis bags

- Place 500 mL of the EDTA/sodium bicarbonate solution into a beaker and bring to boil.
- Place dialysis bags into the boiling solution and leave for 5 min.
- Bring the remaining 500 mL to boil and immerse the dialysis bags in this solution and leave for 5 min.
- Remove bags, place in sterile PBS, and store at 4 ˚C until needed.
- Rinse bags well in PBS before use.

Chapter 38
Anti-rabies monoclonal antibody production using mammalian expression systems

Introduction

The overall applications for monoclonal antibodies (MAbs) have expanded during the past several decades. In the field of rabies, MAbs have been used not only in research but also for diagnostic purposes, and evaluated as a replacement for rabies immunoglobulins (RIG). Moreover, MAbs have proven to be very useful tools in research to identify antigenic sites, including for typing of rabies virus (RABV). To aid identification of RABV in tissue in rabid animals or human rabies victims, MAbs have been employed over the past several decades. Finally, a number of groups have identified MAbs that could potentially replace RIG in a post-exposure prophylaxis (PEP) setting for severe exposures to RABV, or so-called category III exposures *(1)*. Today, RIG is obtained from rabies-immunized human or equine donors *(2,3)* and is limited in supply, partially due to the complexity and inconsistency of the manufacturing process. Using standard DNA recombinant technology, MAbs can be expressed in mammalian cell systems from which they can be produced in large quantities and, more importantly, produced consistently.

Clearly, the use of MAbs has played a large role in research, since the original concept of hybridoma technology was introduced in 1975 *(4)*. MAbs can be used as valuable tools in basic research to detect or purify a specific antigen in a variety of ways, but also for diagnostic purposes and as treatment and/or prevention of disease. In the context of rabies, all of the above options apply. For example, MAbs have long been of importance with the pioneering work described by Lafon and colleagues *(5)* to define the antigenic sites on the RABV glycoprotein. Furthermore, MAbs directed against RABV antigens, labelled with fluorescein isothiocyanate (FITC) or biotin, are used as tools for postmortem diagnostic testing, such as the direct fluorescent antibody test (DFAT) *(6)* or the direct rapid immunohistochemistry test (DRIT) *(7)* respectively, to determine whether a bite victim was truly exposed to RABV from a rabid biting animal. Finally, based on the recommendations of WHO in 2002 *(1)* the use of anti-RABV MAbs as alternatives for equine RIG (ERIG) and human RIG (HRIG) has been explored and begun in practice *(8–10).*

WHO recommends administration of PEP to all patients with category III exposure or to immunodeficient patients with category II exposure to RABV *(1)*. A major component of PEP is passive immunization from RIG. However, given a lack of supply, financial constraints and noncompliance with WHO rabies exposure guidance, many patients die because no PEP or RIG is administered *(11,12)*. To illustrate, in a recent study in the Philippines, several bite victims had inadequate knowledge of proper wound management, and some had resorted to “tandok”, a folk medicine practice where an animal horn is placed over the bite wound to suck out the virus (unpublished data). Access to RIG in the developing world is

particularly poor – a survey in India revealed that only 2.1% patients bitten by a rabid animal received RIG *(13)*.

Recombinant MAbs can be obtained through hybridoma technology, or phage display technology. The former depends on antibody responses in animals and subsequent generation of hybridoma cells expressing the antibody of interest, whereas the latter involves cloning of immunoglobulin gene segments to create antibody libraries with large numbers of specificities from which antibodies with desired specificities can be selected *(14)*.

The expression and purification of MAbs in mammalian expression systems are suitable for use in research settings or for prevention, treatment and diagnosis of rabies. This chapter describes techniques for expressing MAbs in mammalian cell lines, purifying MAbs via Protein A chromatography, and desalting with both manual and automated methods. Generic steps for production and purification are discussed, in addition to analysis of product quality.

Materials

Reagents

- RABV glycoprotein
- FITC-labelled anti-rabies and control reagents
- cell culture medium
- expression vectors
- fetal bovine serum (FBS)
- phosphate buffered saline (PBS)
- transfection reagent [e.g. Lipofectamine or FuGene].

Equipment

- CO_2 incubator
- ELISA reader
- Protein A sepharose column
- size exclusion high-performance liquid chromatography (HPLC) column
- fluorescence microscope
- gel electrophoresis equipment

Biological materials

- human embryonic kidney (HEK) 293T cells
- Chinese hamster ovary (CHO) cells
- mouse myeloma cells (NS0, SP2.0)
- challenge virus standard strain (CVS)-11 (or other laboratory RABV strain)
- non-expressing bacteria cells (e.g. DH1, DH5α™, XL1Blue)

Methods

Cells and cell culture

Successful MAb production can be achieved in commercially available cell lines such as HEK293T, CHO or mouse myeloma cells (NS0 or SP2.0) depending on the user requirements. If a specific hybridoma is available, these cells can be used as well for antibody production.

Preparation of expression vector

A variety of standard expression vectors can be used to express MAbs. Because MAb purification is mainly based on natural binding properties of the antibodies, the use of tags is not needed. Typically, the heavy and light chains are cloned into a single vector, each under control of its own promotor. If such plasmids are not available, separate vectors can be used to express both antibody chains. In either case, standard cloning techniques can be employed to obtain the expression plasmid(s).

Preparation of mammalian cell stocks

Quickly thaw a vial of cells and transfer them into pre-warmed media: for HEK 293T, CHO, grow cells in 10 mL DMEM [Dulbecco's minimum essential medium] (with 10% FBS) overnight in a 25 mL flask. For NS0, SP2.0, grow cells in 10 mL MEM at 37 °C with 0.5% CO_2 at 37 °C with 10% CO_2 overnight in a 25 mL flask. In all cases, after growth, re-suspend cells in PBS and harvest.

Transfection and clone selection

Plate out adherent cells at 3.5×10^6 cells into a 96 mm dish. Before transfection, incubate the plasmid (2 µg of each) with 10 µL transfection reagent in serum-free medium for 30 min. Add plasmid to cells for 5 h before adding DMEM with 10% FBS and incubating overnight. Add growth medium containing appropriate antibiotic. Wash cells twice a week for 3 weeks to remove dead cells. Pick and transfer clones to a 96-well plate. After several days of further growth under selective pressure, cell culture supernatants can be directly analysed for the presence of antibody using Protein A HPLC or Octet system [ForteBio]. Alternatively, antibody concentration can be quantified by ELISA specifically detecting mouse or human MAbs. With each approach, the assay results can be used to select the highest expressing clones to be expanded further using 24- and 6-well plates before transferring to T25 or T80 culture flasks and growing for several days before flash freezing cells (–80 °C). The process is repeated to select the highest expressing clones and to ensure the construct is stably expressing MAbs. At this stage, selective pressure is typically no longer needed.

Depending on the requirements, it could also be considered to produce small batches through transient transfection. The principles are the same as those described above but this approach has limitations with respect to the amount of antibody that can be produced in one run. Nowadays, new transient transfection systems or kits are commercially available that can produce at relatively high titres (e.g. 2–3 g/L), such that for a research setting it will yield more than sufficient material to perform a large set of experiments. For application such as diagnostic

purposes, where batch to batch consistency is preferred, a stable clone should be considered as most optimal.

Production of MAb using HEK 293T or CHO cells

Thaw a vial of frozen cell clone stocks or take cells from ongoing cultures. Inoculate clones to a target concentration of 1 × 10^6 viable cells/mL into DMEM containing 10% FBS at 37 °C in 10% CO_2. Expand the cells to the desired biomass in T flasks and shaker flasks or roller bottles before production is started. The total biomass will be highly dependent on user needs. Prior to the production phase, it is advised that serum containing medium should be washed away and replaced with DMEM, without serum, or lower concentrations of serum be used (e.g. up to 2%). The production phase is ideally performed in shake flasks, where again the selected working volume should be based on required yield. To monitor culture performance, daily samples can be taken to determine cell concentration and viability (using a cell culture analyser). In addition, MAb concentration in culture can be analysed by Protein A HPLC or Octet system (ForteBio). The culture should be harvested if the cell viability drops below 50%. Transfer medium to centrifuge bottles and centrifuge at 5000 x *g* for 20 min. Filter supernatant through 0.22 µm filter and collect for purification. For larger scale expression, roller bottles or bioreactors can be used instead of shake flasks. Ensure optimal inoculation cell densities are used according to the cell type.

Production of antibody using hybridoma cells

It is assumed that hydridoma cells are available that express the antibody of interest. It is up to the reader to assess whether the hybridoma is stable enough or if additional subcloning is required. To reach consistently high antibody titres, a relatively pure MAb cell population is recommended. Subcloning of hybridoma cell lines can be achieved by performing serial dilutions to eliminate non-antibody producing cells. Plate 1 to 0.5 cells per well in 96-well plates and culture them until visible colonies appear. Once the colonies appear large enough, they can be tested for antibody production by ELISA. Hybridoma cell lines can be grown in Roswell Park Memorial Institute medium (RPMI) plus 10% FBS during expansion, and grown at lower FBS (e.g. 2% but will be clone-dependent) or even in special serum-free hybridoma cell culture medium, such as EX-CELL Hybridoma medium (Sigma Aldrich) during antibody production. Depending on the antibody requirements, these cultures can be grown for 3–5 days to obtain small quantities or in bigger batches by increasing the number of flasks or expanded into larger production vessels. The yield is highly dependent on the hybridoma cell line itself. The choice of production medium with or without use of FBS is dependent on subsequent application of the purified MAb and the level of the purification procedure itself. To prevent potential background staining, binding or neutralization, production in the absence of serum is recommended. It must be noted that if a number of purification steps (as described below) are being executed, the serum impurities can be easily removed.

MAb purification

Depending on the end use of the MAb, different purification strategies can be executed. For research purposes, a straightforward Protein A chromatography will typically provide sufficient purity to perform a number of different experiments.

For diagnostic MAb tools, as well as MAb with the intention to use in humans, additional purification steps are recommended.

Protein A chromatography

Protein A has a high affinity for human IgG1/IgG2 and mouse IgG2a/IgG2b, moderate affinity for human IgM/IgA/IgE and mouse IgG3/IgG1, but no affinity for other human or murine immunoglobulins. Protein A chromatography can be performed via gravity columns or liquid chromatography systems. Use of the purest water available is recommended for use in all buffers (e.g. HPLC grade). Either prepacked columns can be used, or home-made columns can be generated using Protein A sepharose resin. Before purification of larger batches, it is advised to perform scouting experiments to ensure that optimal conditions are established for purification of antibody.

Performing Protein A separation on a gravity column

Each Protein A column will have manufacturer-specific protocols to follow. In short, columns must be equilibrated with binding buffer (e.g. PBS pH 7.4) before applying the sample with a syringe or peristaltic pump. Several wash steps to remove all unbound sample and impurities (protein presence should be determined by UV absorbance at 280 nm) can be implemented. This is somewhat dependent on the starting material and its purity. If highly enriched medium is used, and/or cell viability at the time of harvest was low, the column load can contain high levels of impurities. Using additional wash steps, in which a combination of high salt (up to 1 mol NaCl) and lower pH buffers (down to pH 5.5) are incorporated, such impurities can be removed prior to elution of the MAb. Bound MAb is displaced with elution buffer at low pH (range 3–3.6). Depending on the MAb in question, it is important to investigate which elution conditions are best for each antibody. It is known from experience that low pH can induce antibody aggregates at this stage of purification, which could be as high as 20%. Therefore, it may be helpful to achieve a higher recovery to explore slightly higher pH levels which are less harsh for the bound antibody. In addition, fraction collection into a high pH buffer (e.g. Tris buffer pH 8) to ensure a final pH in the more neutral range will also help to reduce aggregate formation. After elution, columns can be cleaned with 0.1 mol NaOH, flushed with PBS, and stored in 20% ethanol at 4–8 °C.

Performing a Protein A separation using liquid chromatography systems

By using liquid chromatography systems (e.g. FPLC, HPLC), a very similar yet much faster process is applied, but performed in an automated manner thereby obtaining more consistency between MAb batches.

Desalting columns

MAbs for research purposes can be processed further using desalting columns to exchange buffers and make them suitable for subsequent use. Desalting columns are very fast and efficient in doing so, with recoveries typically in the range of 90–100%. Similar to Protein A chromatography, this step can be performed either with bench top gravity columns or using liquid chromatography systems. In the case of gravity desalting columns, the manufacturer's protocol

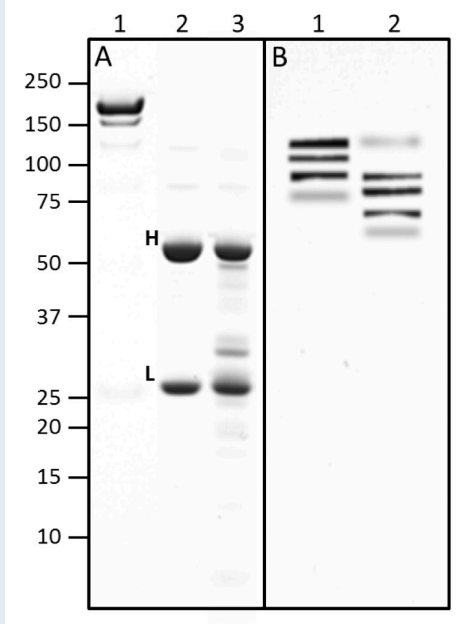
can be followed and typically works efficiently. The advantage of using liquid chromatography systems is that it provides a more consistent approach, but is not an immediate necessity for research MAb batches.

Additional purification steps

To achieve highly purified MAbs with high-quality standards, a combination of anion and cation exchange chromatography steps should be implemented. This is most likely more applicable to MAbs used for diagnostic purposes or for human use, for which the latter obviously would have to be produced under GMP (good manufacturing practice) conditions (which are beyond the scope of this chapter). However, in a research setting, there may also be reasons to include more purification steps (e.g. if high levels of aggregates have been observed during the Protein A purification step). Antibody aggregates can cause nonspecific binding and false–positive results during experimental evaluation, which can be prevented by additional purification steps to remove the aggregates. Most suitable in that case would be a cation exchange chromatography step, in which the MAb is bound to the column at low salt conditions and eluted with a high salt gradient. The monomer MAb species will elute before the aggregate peak and hence separation can be achieved. The detailed conditions are highly dependent on the MAb, type of resin, pH and buffer conditions, such that this will have to be explored further by the reader. Generally, a binding buffer at 25–50 mmol NaCl and a pH range of 5–6.5 dependent on the isoelectric point (pI) of the MAb can be used as starting point. Elution up to 1 mol NaCl can be achieved using a linear gradient. If additional impurities are still present, anion exchange chromatography could be explored to further purify the antibody.

MAb product testing

To assess the quality and purity of antibody, various analytical techniques can be used. The MAb concentration can be determined by a standard UV280 method using a nominal value of 1.5 as the theoretical extinction coefficient. Purity is most often assessed using SDS-PAGE to separate proteins by molecular weight. If the purified MAb has incurred significant insertions or deletions, this should be detected by SDS-PAGE. Non-reducing SDS-PAGE and reducing SDS-PAGE will indicate intact antibody and heavy and light chains, respectively. Precast gels (4–12%) will yield suitable results. Fig. 38.1A shows a composite SDS-PAGE of a purified intact human IgG MAb under reducing and nonreducing conditions, as well as a stressed MAb sample to illustrate the presence of antibody fragments. If the purified MAb shows such fragments, the batch needs to be discarded and a novel MAb production should be initiated. In addition, isoelectric focusing (IEF) can be employed to assess charged isoforms of the MAb and their respective pI. Furthermore, IEF will detect if there have been events that altered the overall charge of the purified MAb, protein degradation or protein deamidation during purification and/or storage. Therefore, this method is also suitable to determine MAb consistency between antibody productions and to detect potential variations. Fig. 38.1B shows an intact MAb sample in comparison with a stressed sample in which the antibody is purposely degraded. Such observations in the purified sample will indicate that the intact MAb has been altered and should be replaced with novel MAb batches. The theoretical molecular weight and pI for the MAb that is being purified can be determined using an online tool such as ExPASy (http://web.expasy.org/compute_pi/) to assess pI of the purified MAb.



By courtesy of Wilfred E. Marissen, Crucell Holland, The Netherlands

Fig. 38.1. Monoconal antibody (MAb) analysis by SDS-PAGE and IEF

A: Purified MAb (10 µg) was loaded onto NuPAGE Novex 4–12% Bis-Tris gel under nonreducing (lane 1) or reducing (lane 2) conditions. Intact IgG and heavy (H) and light (L) chains are visualized with Coomassie Blue staining solution. A MAb sample exposed to low pH (2.7) was loaded under reducing conditions (lane 3) to illustrate occurrence of potential fragments in purified MAb batches.

B: Purified MAb (20 µg) was loaded onto a FocusGel 3–10 24S IEF gel to separate the charged isoforms (lane 1). Additionally, a purified MAb sample was pretreated at high pH (9.5) before loading onto isoelectric focusing gel to illustrate the effect of protein degradation or deamidation (lane 2). Note that several bands will be observed, which will be specific for each sample due to multiple protein states with different isoelectric points.

Size exclusion HPLC (SE-HPLC) can be used to detect aggregates as well as fragments in the purified MAb product. A small amount (10–20 µg) of antibody can be applied to a SE-HPLC with an appropriate cut-off and equilibrated using a suitable buffer (e.g. 50 mmol sodium phosphate, 250 mmol NaCl [pH 7.0]) at a flow rate of 0.15 mL/min. Absorbance at A_{280} or A_{214} can then be used to determine the presence of aggregates and/or fragments. Aggregates (i.e. dimers, trimers, tetramers and oligomers) can form during the purification steps (e.g. protein A elution at low pH), during longer term storage or during multiple freeze–thaw cycles. The monomer intact MAb will be preceded by aggregates and elute prior to potential fragments that may be present in the sample (Fig. 38.2). A monomer concentration of > 95% should be achieved to have a MAb batch that yields reliable results when used in experiments. High aggregate content could result in background staining, or generate other false–positive results.

To complement the analytical quality testing, additional assays that test for functionality can be included. For instance, the specific binding of the MAb to its target can be tested using an ELISA method. Depending on the MAb target, purified RABV glycoprotein or nucleoprotein can be coated onto 96-well plates. Coating conditions for the purified antigen would have to be determined, but typically a target of 0.5 µg/mL as coating concentration will be a good starting point. After overnight incubation, each well is blocked with 0.3 mL of a 3% BSA (bovine serum albumin) solution for at least 1 h at room temperature. Then, the wells are washed and each well is incubated with MAb samples (a range of concentration can be used) or control samples for another 1 h. The plate is washed again and incubated with conjugate antibody for 1 h at room temperature, washed once and incubated with TMB substrate for 5–10 min. The reaction is stopped by the addition of 1 mol sulfuric acid and the absorbance is read by an ELISA plate spectrophotometer. Alternatively, inactivated RABV, either prepared in-house or obtained as a rabies vaccine, could be used although typically the signal is less strong compared with purified antigens. If purified RABV glycoprotein is not available, commercially available RABV glycoprotein pre-coated ELISA plates [e.g. Platelia

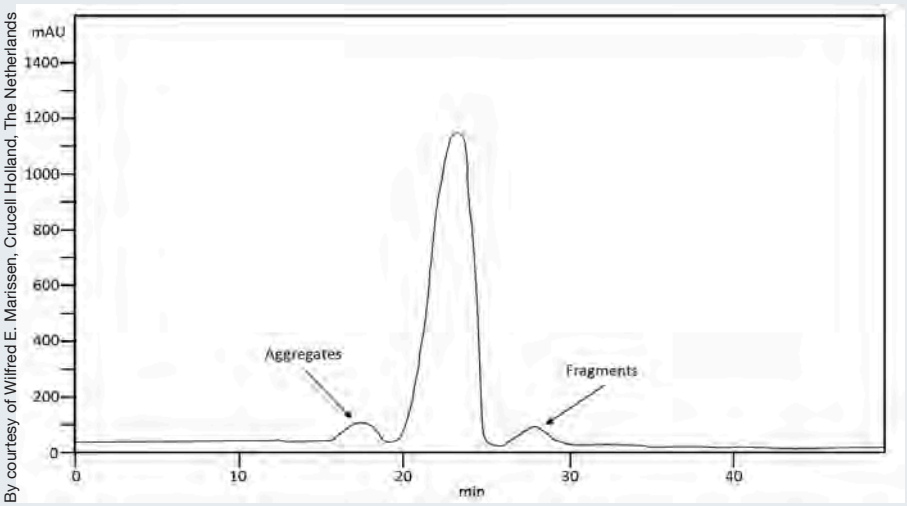


Fig. 38.2. Determination of aggregate levels using SE-HPLC

Purified MAb (20 µg) was injected onto a TSKgel SuperSW3000 column equilibrated in 50 mmol sodium phosphate, 250 mmol NaCl (pH 7.0) at a flow rate of 0.15 mL/min. Absorbance was measured at 214 nm. Elution of aggregates, monomer IgG, and fragments, respectively can be observed.

kit, BioRad] can be used. Demonstration of strong binding indicates correct MAb identity and functionality. Weak or lack of binding would suggest a loss of tertiary structure, mutations in the complementarity determining regions, or instability of the MAb, among others. Specific binding could also be assessed by flow cytometry using cell lines expressing RABV glycoprotein on the cell surface *(15)*.

Additional functionality can be assessed by analysis of in vitro potency testing such as RFFIT (see Chapter 19) if the MAb is directed against RABV glycoprotein. The capability of a MAb to neutralize RABV will be the ultimate test of its functionality and proof of appropriate quality. For each MAb, the 50% neutralizing titre against RABV (e.g. CVS-11 laboratory strain) can be determined and used to calculate an effective (EC_{50}) or inhibitory (IC_{50}) concentration. Most often, the MAbs are benchmarked against the 50% neutralizing titre (2 IU/mL) of a standard reference serum (e.g. standard RIG, lot R3); however, caution should be taken in doing so as neutralizing responses of a polyclonal antibody mixture versus a highly purified MAb may not necessarily be parallel. Lack of parallelism may result in an under- or over interpretation of MAb potency. In such a case, it is better to establish one's own MAb reference standard that can be used to assess the potency of MAb batches and report MAb potency in EC_{50} in ng/mL. If required, several purified MAbs can be ranked according to potency. Alternatively, if the appropriate biosafety level laboratory for working with virus is unavailable, in vivo neutralization testing using rabies pseudoviruses can be used, as described previously *(10)*.

Interpretation of results

The methods presented here allow quick expression of RABV MAbs at small scale but also at larger scale to obtain larger batches of MAb. Purification of antibodies can be performed using standard purification techniques as described that can be supplemented with additional purification steps depending on the end-user requirements. The quality of the purified MAb is demonstrated by SDS-PAGE, IEF, SE-HPLC, ELISA, flow cytometry or RFFIT. The MAbs are suitable for both in vivo and in vitro research purposes. If the MAb is used for in vivo testing, analysis of bioburden and endotoxin levels is highly recommended to confirm that the MAb batches are suitable for animal testing.

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Chapter 39
Generation of anti-rabies single domain antibodies by display technologies

Introduction

Rabies virus (RABV)-specific, cross-neutralizing llama-derived heavy-chain antibody fragments (VHH, or nanobodies) can rapidly be generated from immunized llamas using phage display technology. Phage display is a well-established technique that uses bacteriophage to connect genotype with phenotype by expression and display of proteins that can be selected from large protein-encoding libraries. The technique was first described in 1985 *(1)*. Proteins displayed may be peptides, antibody fragments such as single-chain antibody fragment (scFv), antigen-binding fragments (Fabs) or, as described here, variable heavy-chain fragments (VHH). The VHH are the antigen-binding, variable part of "heavy-chain only" antibodies present in Camelidae family members, such as llamas *(2)*. The VHHs are small (12–15 kDa), biophysically stable molecules with good solubility characteristics and similar affinities to conventional antibodies *(3).* Llama-derived VHHs have proven to be powerful virus-neutralizers, including for RABV *(4–6)*. Their single-chain nature allows construction and production of multimeric molecules using the same or different VHH building blocks joined by flexible Glycine$_4$Serine (G_4S) linkers, thereby targeting either one or two different epitopes on the same molecule *(5,7)*. By linking two VHH into bivalent (two identical VHH) or biparatopic (two different VHH) constructs, the neutralizing potency can be increased to the picomolar range. In mouse challenge models, the protective effect further improves significantly by increasing the half-life through linkage with a third VHH targeted against serum albumin. Although some interference with the antigenicity of rabies vaccine is observed, combined use of anti-rabies VHH and vaccine can act synergistically to protect mice after RABV exposure *(8, 9)*. These properties make them promising molecules for prophylactic and therapeutic purposes, as well as for diagnostics and research. Ablynx (Sanofi) is developing VHH-based therapeutical proteins (trademarked as Nanobodies®).

The principles of phage display and panning are illustrated in Fig. 39.1.

Methods

Immunizations

Inactivated rabies vaccine Mérieux HDCV for use in humans contains the Wistar strain of the Pitman Moore RABV grown on human diploid WI38 lung cells (PM/WI38 1503 3M). It contains human albumin, but no adjuvant. Intramuscular injection of the vaccine suspension, divided over two spots (0.5 mL/spot, corresponding to 2.5 IU), was performed in the neck of llamas on days 0, 7, 28, 35 and 57. Blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA)

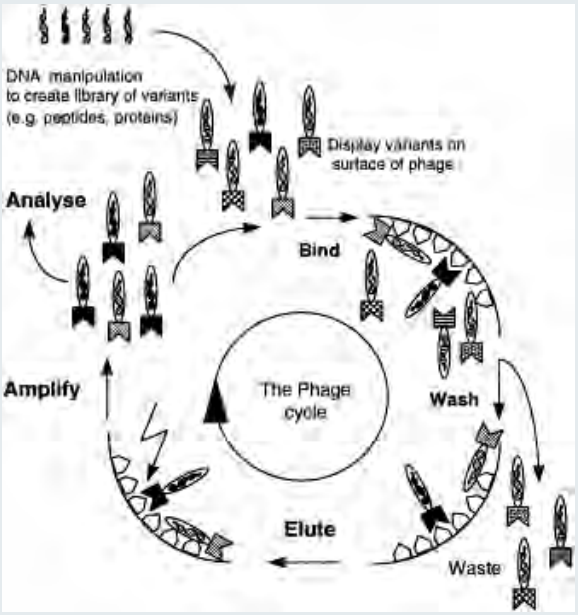

Fig. 39.1. Principles of phage display and panning

Source: reference *(12)*

at time points as indicated in Table 39.1.

The neutralizing potency of the immune serum from immunized llamas can be determined with the rapid fluorescent focus inhibition test (RFFIT) as described *(10)*. A serum titre of 0.5 IU/mL indicates a seroconversion. The serum titre of neutralizing antibodies in two immune llamas is shown in Table 39.1. They both had a titre in the range of 15–35 IU/mL after repeated immunization with HDCV (Table 39.1).

Immune variable heavy-chain fragment library construction for phage display

Immune VHH library construction is divided into five parts: (i) isolation of total RNA, (ii) cDNA synthesis, (iii) polymerase chain reaction (PCR) amplification of VHH (variable domain fragments, derived from llama heavy-chain immunoglobulins *(2)*), (iv) ligation into phagemid vectors and (v) expression of VHH containing phage for selections (caution: work on ice as much as possible).

Isolation of total RNA

Isolate peripheral blood lymphocytes (PBL) from total blood using routine ficoll gradients (PBL1-3 can be combined or used alone depending on the immune response). Total RNA from PBL can also be extracted using the RNeasy Midi Kit [Qiagen] following the manufacturer's protocol. Lymph nodes may be indistinguishably enlarged, which makes them difficult to find and therefore unsuitable for use as a source of lymphocytes.

Determine the OD_{260}/OD_{280} ratio as an indication of the quality of the purified RNA (should be around 2). Estimated total RNA amounts from 150 mL blood are ~ 200–1000 µg. Determine the integrity of the sample on a 1% agarose gel in a tank (combs and tray) cleaned with 0.1 % SDS (at least for 1 h and rinse with ddH_2O) to avoid degradation. Three bands of RNA should be observed: ~1500 bp, 800 bp and a band below 200 that corresponds to 28S,

Table 39.1. Immunizations, blood collection and sero-conversion

Day	Immunization (vaccine potency IU)	Blood collection	RFFIT titre* (50% dilution)	
			Llama 1	Llama 2
0	2.5	10 mL pre-immune blood	< 0.5 IU/mL (1/9)	< 0.5 IU/mL (1/9)
7	2.5	–		
27		10 mL immune blood	2 IU/mL (1/66)	6 IU/mL (1/179)
28	2.5	–		
35	2.5	–		
37		10 mL immune blood	22 IU/mL (1/674)	27 IU/mL (1/789)
42		150 mL immune blood (PBL1)	37 IU/mL (1/989)	33 IU/mL (1/896)
49		150 mL immune blood (PBL2)	23 IU/mL (1/674)	15 IU/mL (1/441)
57	2.5	–		
62		150 mL immune blood (PBL3)	22 IU/mL (1/673)	35 IU/mL (1/1071)

IU, international unit; RFFIT, rapid fluorescent focus inhibition test, virus-neutralization test

18S and 6S rRNA, respectively. Occasionally, a band of 70–100 bp can be visible representing tRNA. Possible DNA contamination will be above 10 000 bp.

cDNA synthesis

Use random primers and the SuperScript III First-Strand Synthesis System for RT-PCR according to the manufacturers' recommendations to amplify VHH encoding gene fragments. [The QIAquick PCR Purification Kit can be used for clean up, but is not a necessary step.]

PCR amplification

This is divided into two amplifications: the first step amplifies conventional and heavy-chain antibody genes from cDNA, resulting in antibody fragments from FR1 to CH2 of VHH using two framework 1 (FR1) specific primers (051 and 052) and a CH2-specific primer (003). DNA fragments corresponding to 600 bp and separated from the conventional antibody-derived VH of around 900 bp are extracted from a 1% agarose gel and used as a template in the second nested PCR reaction using primers flanked with *Sfi*I and *BstE*II restriction sites in the 5' and 3' end, respectively (primers 050 and 003). These fragments are cloned into a phagemid vector upstream of a *c-myc* tag and a His_6-tag as well as gene3 for display on filamentous bacteriophage, as previously described *(11)*. Electrocompetent *Escherichia coli* TG1 are transformed, generating library sizes of around 10^8 and phage expressing VHH are prepared as described in Annex 2.

Phage display for selection of RABV-specific llama variable heavy-chain fragments

Selections for RABV glycoprotein (G protein)-specific VHH are performed on 8-well strips pre-coated with native G protein from the PV (Pasteur virus) strain (Platelia II Rabies plates). An overnight culture of *E. coli* TG1 is prepared from a single colony taken from a minimal M9 agar plate. Both the strips and the input phage, prepared using standard protocol (Annex 1), are blocked in superblock (Pierce) or 1% skimmed milk (Marvel) in PBS for 1 h at room temperature. Add 10 µL phage to 90 µL superblock per well and incubate with shaking for 2 h at room temperature. Remove the phage-containing solution in each well with clean filter tips and wash carefully 20 times with PBS + 0.05% Tween 80, followed by five times PBS. Use new tips for every well. Elute with 100 µL trypsin/well (1 mg/mL) for 15 min at room temperature with shaking. Stop the trypsin reaction by addition of 5 µL 4 mg/mL ABSF (adult bovine serum). Infect exponentially growing TG1 for 30 min at 37 °C and titrate for determination of output enrichment compared with control as described below. Infect 50 µL of eluted phage into 333 µL TG1 (OD_{600}=0.5) + 666 µL 2×TY. Infect for 30 min at 37 °C without shaking. Add 10 mL 2×TY amp100 Glu2 % and grow overnight at 37 °C, 250 r/min. Store grown culture as glycerol stock at –80 °C.

Output phage titration

Prepare serial dilutions (10^{-1}–10^{-5}) in a 96-well culture plate (10 µL output phage in 90 µL PBS); add 5 µL of phage dilutions to 95 µL of exponentially growing TG1 (OD_{600}=0.5). Infect for 30 min at 37 °C without shaking. Plate 5 µL drops in duplicate on LB/amp100/gluc2% plates and incubate at 37 °C overnight. Calculate the number of input phage when colonies have grown. Store remainder of eluted output phages as glycerol stock at –80 °C.

For calculation of input phage

Prepare serial dilutions of input phages in 96-well culture plate (10 µL output phage in 990 µL PBS); typically, 1E2, 1E4, 1E6, 1E8 and 1E10. Infect as described for output phage. Repeat the selections if needed to enrich for RABV-binding VHH expressed on phage. Use 1 µL of input phage in selection round 2. When enrichment is observed compared to the control (PBS or irrelevant coated protein), the overnight cultures of infected TG1 are diluted and plated on LB/amp100/gluc2% plates. Dilute enough to have individual colonies that are transferred to a 96-well plate (v-shaped) with 85 µL of 2×TY amp100/Glu2% and let them grow overnight at 37 °C. This is a master plate and is used for production of VHH and sequencing. The plate is stored at –80 °C in glycerol to be able to trace back the individual clones.

Periplasmic expression of variable heavy-chain fragments

From the overnight cultures in the 96-well plate, periplasmic extracts containing VHH are prepared by induction of exponentially growing *E. coli* TG1 with 1 mmol isopropyl-ß-D-1-thiogalactopyranoside (IPTG) and continued cultivation overnight at 37 °C (can be performed in 1–400 mL scale) at 250 r/min for production of VHH. After centrifugation, the bacterial pellet is freeze-thawed and re-suspended in PBS. The His_6-tag is used for purifications with Talon Metal affinity

resin [Clontech] according to the manufacturer's instructions. The concentration of the purified VHH is determined by OD_{280} and the purity (1 µg) by SDS-PAGE under reducing (with DTT) and non-reducing conditions. A band of around 15 kDa corresponds to a VHH.

Purified VHH can be characterized by enzyme-linked immunosorbent assay (ELISA), competition assays and virus-neutralization assays (RFFIT).

VHH encoding genes can be fused into multimeric constructs with flexible G_4S-linkers of different lengths to form bivalent or biparatopic molecules targeting one or more different epitopes.

Enzyme-linked immunosorbent assay

The RABV G pre-coated on 8-well strips (BioRad) can also be used to identify the G-specific VHH by addition of periplasmic extracts containing VHH, followed by incubation for 2 h at room temperature. After washing, add rabbit anti-VHH antibody (MCA, The Netherlands) (1/5000 dilution) and incubate for 1 h at room temperature. Add horseradish peroxidase-conjugated goat anti-rabbit IgG (1/10 000, Jackson) and incubate for 1 h at room temperature. Add 3,3',5,5'-Tetramethylbenzidine (TMB) substrate and read at 620 nm.

Competition assays can be set up using ELISA to home in on specific epitopes or to evaluate if the selected VHH bind to the same or different epitopes. Label the first VHH (VHH1) with biotin as described by the manufacturer [Thermo Scientific], and determine the concentration at 50% binding. Incubate this fixed amount of VHH1-biotin with a dilution series of a second VHH (VHH2) and evaluate competition by reduction in signal. Use cold unlabelled VHH1 as positive control for the competition assay.

Virus-neutralization assay (rapid fluorescent focus inhibition test)

The RFFIT is a virus-neutralization assay using baby hamster kidney (BHK)-21 cells as susceptible targets. It is performed according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals and as described in Chapter 19 of this manual. Serial three-fold dilutions of heat-inactivated serum samples are incubated with the CVS-11 strain of RABV in 8-well tissue culture chamber slides for 90 min at 37 °C. BHK-21 cells are then added to the serum–virus mixture and incubated for an additional 20–24 h at 37 °C and 5 % CO_2. Slides are acetone-fixed and stained with an FITC-coupled anti-nucleocapsid conjugate and evaluated using a fluorescence microscope to score the number of virus-infected cells (foci) under 200× magnification. The number of positive fields (of 20) with RABV-infected cells per well is recorded. The neutralization end-point titre is defined as the highest sample dilution at which 50% of the observed microscopic fields contain no infected cells. The in vitro neutralizing potency is expressed in International Units (IU)/mL in reference to "The 2nd International Standard for Anti-rabies Immunoglobulin, Human" from the United Kingdom National Institute for Biological Standards and Control (NIBSC, Potters Bar, Herts, UK).

Discussion

VHHs have been shown to be excellent virus neutralizers, including of RABV. Part of this success is due to what is termed "targeting precision". Antibodies should recognize their cognate epitope but not the adjacent amino acids of the epitope. Conventional antibodies often interact with these amino acids because they are large, whereas VHH often do not interact at all with adjacent amino acids. Moreover, due to their small size VHH often can interact with cavities of the paratope.

The monovalent nature of the molecules allows for multimerization to increase both potency and virus cross-neutralization *(5, 8)*. The length of the flexible linker can be varied to optimize intramolecular binding within a trimeric protein such as the RABV G, or between two different trimeric proteins, potentially preventing viral escape.

Experimental tips

Critical parameters and troubleshooting

All steps should be properly quality controlled before proceeding with the next steps. High-quality RNA is important. Always work RNase-free and on ice. TG1 must be kept on minimal M9 agar plates to maintain the sex pilus on TG1 for infection by the phage when grown to exponential phase. As a contamination control, always include TG1 both in culture medium and on plates when spotting.

Alternatives

Elutions can be performed with pH shock using 100 mmol triethylamine (TEA, pH 12) or 0.2 mol Glycine-HCl, pH 2.2 for 15 min at room temperature with shaking. Transfer eluted phage to a new well or tube and neutralize immediately.

Blocking solution can be exchanged with 1% skimmed milk (Marvel), 2% casein or 2% BSA (all dissolved in PBS). Even if biotinylated antigens are used for selections, milk-derived blocking agents can still be used, but superblock is preferred.

Precautions

- Use gloves when working with RNA.
- Clean bench and pipettes with RNase away [Molecular BioProducts].
- Use a separate set of pipettes for library constructions to avoid contaminations.
- Use QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit dedicated only for library construction.
- Clean gel chambers with 0.2 mol NaOH and rinse well with deionized or sterile water.
- Clean gels chambers with 0.2 mol HCl O/N and rinse well with deionized or sterile water before use.
- Work on ice.

- Make sure that there is no excess chromosomal DNA after RNA isolation because it will bind the RNA upon precipitation.
- To avoid contamination of phage, use filter tips for every step and clean carefully the bench with bleach before and after use. Preferably work in dedicated laminar flows.
- Never use pipettes for inoculation of TG1, but sterile stripettes to avoid contamination.

Time considerations

Immunizations take at least 6 weeks, RNA extraction and library preparation 1–2 weeks. Selections take 2–4 weeks dependent on complexity and rounds of selection.

Limitations

Displaying VHH by bacteriophage does not allow for affinity selections since more than one copy of VHH is displayed on each phage, with the exception of monomeric target proteins (should be kept in solution as a biotinylated protein).

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Annex 1. Materials

Reagents

For total RNA isolation

- RNAeasy Midi kit [Qiagen]
- 96% ethanol (RNase-free)
- 2 M NaAc pH 4 (RNase-free)
- RNase-free 1.5 mL microcentrifuge tubes
- double autoclaved distilled water
- RNase away solution [Molecular BioProducts, catalogue number 7002]

cDNA synthesis: SuperScript III First-Strand Synthesis System [Invitrogen]

Primers for amplification of VHH

- 051 (homology to FR1) 21 bp: GGCTGAGCTGGGTGGTCCTGG
- 052 (homology to FR1) 21 bp: GGCTGAGTTTGGTGGTCCTGG
- 003 (homology to CH2) 23 bp: GGTACGTGCTGTTGAACTGTTCC
- 050 (*Sfi*I containing FR1) 55 bp: CATTTGAGTTGGCCTAGCCGGCCATGGCAGAGGTGCAGCTGGTGGAGTC-TGGGGG

PCR reagents

- 20 mmol dNTP
- 10× buffer (+Mg) and Expand High Fidelity enzyme (3.5 U/µL) [Roche Diagnostics GmbH]
- TAE buffer and agarose
- DNA QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit [Qiagen]

Restriction enzymes and ligase

- *BstE*II (10 U/µL) and *Sfi*I (20 U/µL [Biolabs])
- T4 ligase (3 U/µL [Promega])

Antibodies

coupled anti-RABV nucleocapsid IgG – FITC conjugate [Bio-Rad Laboratories, France]

Platelia II Rabies kit [Bio-Rad Laboratories, catalogue number 355-1180], 8-well strips pre-coated with native RABV G

Phagemid vector for fusion of VHH with *c-myc*-His_6 and gene3 at sites *BstE*II 5' and *Sfi*I 3' *(11)*, which is identical to pHEN-1 *(13)*, but contains a hexahistidine tail for immobilized metal affinity chromatography (IMAC).

Equipment

- spectrophotometer (UV: preferably 230–310 nm and 600 nm) and cuvettes (if necessary)
- table centrifuge
- PCR thermocycler
- DNA electrophoresis systems cleaned with NaOH and HCl
- UV/VIS camera for DNA gel and agar plate imaging
- UV camera for slicing out DNA fragments
- incubator at 50 °C and 60 °C and 16 °C (without shaking)
- incubator at 37 °C with shaker
- electroporator
- fluorescence microscope [Olympus IX73]

Biological materials

- rabies vaccine Mérieux HDCV [Sanofi Pasteur MSD]
- BHK-21 cells (ATCC CCL-10)
- CVS-11, a reference laboratory strain of RABV, genus *Lyssavirus*, family Rhabdoviridae, ATCC VR959)
- *Escherichia coli* TG1, electrocompetent *Escherichia coli* TG1 [Stratagene]
- M13KO7 Helper phage

Laboratory animals

Two llamas (*Lama glama*) [purchased from N.V. Neerhofdieren Bocholt] were located at the animal facilities of the Belgian Scientific Institute of Public Health (WIV-ISP, authorization no. LA1230177). All experimental procedures were approved by the Ethical Committee of the WIV-ISP and the Veterinary and Agrochemical Research Centre (CODA-CERVA).

Annex 2. Phage preparation, composition of solutions and preparation of helper phage

Phage preparation

Precipitate phage with $^1/_4$ volume 20% PEG6000/2.5 mol NaCl, for 10–15 min on ice.

Centrifuge 5 min, max speed, remove supernatant. The pellet contains your precipitated phage.

Repeat the centrifugation, remove remaining supernatant and leave the tubes head down on a tissue to remove all PEG.

Re-suspend the pellet in $^1/_2$ volume PBS. Centrifuge 5 min, max speed, and take the supernatant into a new microcentrifuge tube. If there is still a pellet (cellular debris), repeat the centrifugation step and transfer the supernatant to a new microcentrifuge tube.

Composition of media, buffers, solutions

2×TY media (1 L)

1. Dissolve 16 g tryptone, 10 g yeast extract, 5 g NaCl, deionized water to 1 L.
2. Autoclave.
3. Add antibiotics (100 µg/mL ampicillin or 50 µg/mL kanamycin) when the media has cooled down to 55°C.

LB (Luria broth) media (1 L)

1. Dissolve 10 g tryptone, 5 g yeast extract, 5 g NaCl, deionized water to 1 L.
2. Autoclave.
3. Add antibiotics (100 µg/mL ampicillin or 50 µg/mL kanamycin) when the media have cooled down to 55 °C.

LB agar (1 L)

1. Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
2. Adjust the pH of the medium to 7.0 using 1N NaOH.
3. Autoclave.
4. Add antibiotics (100 µg/mL ampicillin or 50 µg/mL kanamycin) when the media have cooled down to 55 °C.

Minimal (M9) agar plates (1 L)

1. Dissolve 15 g Difco Bacto agar in 888 mL H_2O and autoclave.

2. Prepare 100 mL 10×M9 salts (60 g Na_2HPO_4, 30 g KH_2PO_4, 10 g NH_4Cl, 5 g NaCl, final volume 1 L autoclaved and stored at 4 °C), 1 mL 1 mol $MgSO_4$, 0.1 mL 1 M $CaCl_2$, 1 mL 1% thiamine HCl (filter sterilized), 10 mL 20 % glucose and add to the autoclaved agar solution when the temperature reaches 60 °C.
3. Fill to final volume of 1 L with deionized water when the temperature reaches 60 °C, pour plates.

20 glucose (w/v) (100 mL)

1. 20 g glucose, add to 100 mL with deionized water.
2. Autoclave.

20% glycerol in 2×TY (90 mL)

Add 30 mL 60% glycerol (autoclaved; do not autoclave 100% glycerol) to 60 mL sterile 2×TY.

1×TAE (1 L)

20 mL 50×TAE and fill to 1 L with deionized water.

TE buffer (100 mL)

1 mL 1 mol Tris-HCl, 0.2 mL 0.5 mol EDTA pH 8.0, 98.8 mL deionized water.

Preparation of M13KO helper phage

1. Grow an overnight culture of TG1 in LB media at 37 °C starting from a single colony grown on a minimal M9 plate.
2. Inoculate 1:100 in LB and grow to log phase (OD_{600}: 0.6–0.9).
3. Make top agar (2×TY-agar 0.75 %) and let it cool to 50 °C in a water bath.
4. Streak 1 µL of a helper phage stock (e.g. ~3×10^{12} pfu/mL) and 1 µL of a 100× diluted stock onto the surface of a pre-warmed 2×TY plate.
5. Add 5 mL of the TG1 culture to 30 mL of top agar (at 50 °C).
6. Immediately pour 3 mL of TY top agar containing the TG1 culture across the plate from the end towards the start of the streak. Allow the top agar to solidify for a few minutes and incubate at 37 °C overnight. Do not forget to inoculate new TG1.
7. Pick four well-separated single plaques (small ones) with a glass pipette and drop into 4×4 mL of 2×TY with a 1:100 dilution of an overnight TG1 culture.
8. Grow for 2 h at 37 °C.
9. Dilute the 4 mL cultures into 100 mL of 2×TY in a 2 L flask, preferably baffled for good aeration.
10. Grow for 1 h at 37 °C. If baffled flasks are available, add 300 mL of 2×TY with kanamycin (final concentration: 25 µg/mL) and grow overnight. If baffled flasks are not available just add kanamycin (final concentration: 25 µg/mL).
11. Spin down the bacteria in 50 mL tubes for 15 min.

12. Pour 40 mL of the supernatant in tubes with 10 mL 20 % PEG6 000/2.5 M NaCl and incubate on ice for at least 30 min. Spin down for 15 min and discard the supernatant, leave the tubes head down on tissue paper to remove all PEG.
13. Re-suspend the pellet (for 4×400 mL: 4 times 8 pellets) in 1 mL of filter sterile TE buffer. Centrifuge the bacteria in microcentrifuge tubes to remove remaining cell debris for 5 min at 4 °C.
14. Combine all supernatants (approximately 32 mL) and add an equal volume of 100% sterile glycerol.
15. Determine the titre of the phages by making dilutions of 10^4 to 10^{14} in PBS and plate 100 µL of these dilutions on 2×TY plates and add top agar with TG1 (see above). Make aliquots and store at –80 °C.

Chapter 40
Production of monospecific polyclonal rabies virus antibodies in birds

Introduction

An antigen-specific IgY was purified from egg yolk laid by intramuscularly immunized hens independently by Jensenius *(1)* and Polson *(2)* during the 1980s. Since then, numerous reports have demonstrated that IgY is the functional equivalent of mammalian IgG to be used as an immunological tool in diagnosis as well as in passive immunization, administered either systemically or orally to individuals for prevention of infectious diseases.

Recent advances in molecular biology, together with a newly invented method of producing antigen-specific IgY, have created opportunities to develop a safe, convenient and inexpensive way of manufacturing various immunodiagnostics *(3).* These methods have already led to the development of orally administered agents for the prevention of enteric colibacillosis, dental caries and human rotavirus infection *(4–6)*. The method of producing IgY antibodies has certain advantages over their production from mammals, in that: there is no need to bleed animals; it is easy to purify a large amount of antibody; and it is feasible to produce a specific antibody to a small amount of antigen that is poorly immunogenic in mammalian hosts *(3, 7, 8)*.

The technique of producing polyclonal antibodies in birds has great potential to advance rabies virus (RABV) diagnosis, research and use of biologicals, with the possibility of in-house production in developing countries *(9, 10).*

Advantages of IgY compared with IgG

Comparison of the methods for preparation of IgY and serum IgG and advantages in preparation of antibody using hens instead of animals are summarized in Table 40.1. Of note is that preparation of IgY requires only collection of eggs whereas preparation of serum IgG involves drawing blood or euthanasia of animals (Fig. 40.1).

Egg yolk contains a considerable amount of IgY (around 100–150 mg/egg), and a laying hen produces an average of 240 eggs per year *(11, 12).* Therefore, one immunized hen could produce > 30 g of IgY a year, and > 60% of IgY in the egg yolk could be isolated with > 95% purity by a simple purification method. Conversely, exsanguination produces an antiserum of only 50 mL per rabbit wherein only 1400 mg of purified IgG could be isolated *(6,13)*.

Immunochemical differences between IgY and serum IgG are listed in Table 40.2. Of note is that IgY can be used in diagnosis because it is not associated with mammalian complement or rheumatoid factors (RF), and its binding

with human and bacterial Fc-receptors on cell surfaces is less than that of IgG. Also, IgY does not bind to protein A or G, as does IgG. These immunological properties are superior for IgY to avoid false–positive results due to human serum characteristics in diagnosis.

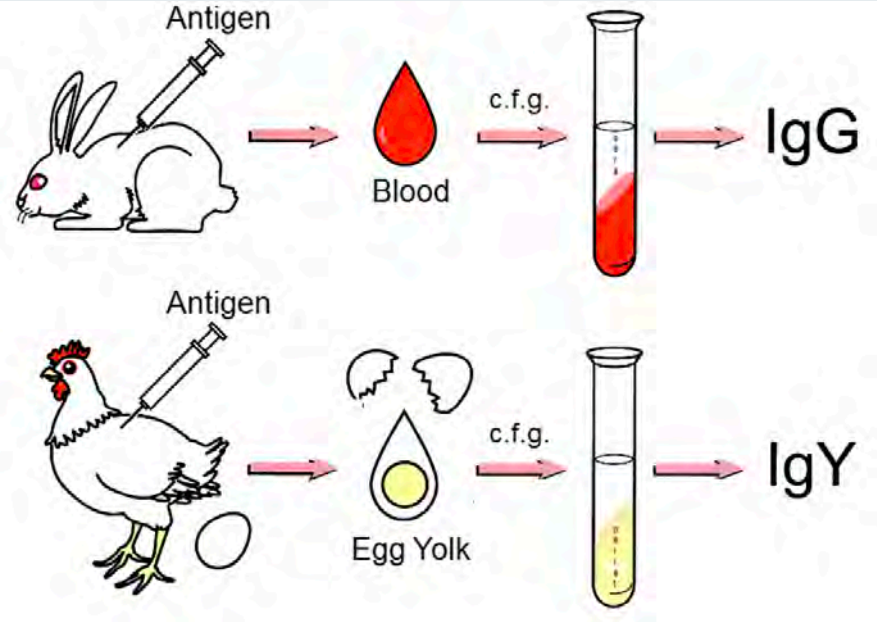

Fig. 40.1. Preparation of specific polyclonal antibodies

Table 40.1. Comparison of a specific antibody preparation

		Rabbit immunization	Immunity egg way
1.	Extraction source of an antibody	Blood of rabbit	Hen egg yolk
2.	A specific antibody preparation	(1) Immunity to rabbit (2) Exsanguination (3) Serum separation (4) Purification of the IgG	(1) Immunity to chicken (2) Egg collection and yolk separation (3) Water solubility, protein separation (4) Refinement of the IgY
3.	Class of the antibody	An IgG in addition to a IgA and a IgM are included in the serum	Yolk includes only IgY and purification is easy.
4.	Animal culture way	Mass rearing is difficult	It's possible to raise in quantities (large-scale poultry farming).
5.	Immunization	Rabbit is fixed and performed	An immunization way is systematized for the purpose of chicken disease prevention.
6.	Antibody manufacturing scale	The laboratory level	Industrial scale mass production is possible.

Table 40.2. Comparison of yolk (IgY) and serum (IgG) in mammalian antibodies

a.	The molecular weight of IgY is about 180 000 and of IgG is about 15 000. The H chain is large, has a constant region and consists of four domains. The IgG has three domains.
b.	The isoelectric point of IgY is about 6.0, which is almost 1 pH unit lower than IgG.
c.	The thermal denaturation temperature of IgY is 73.9 °C; the IgG of rabbits is 77 °C.
d.	Something has radical glucose at the end in a sugar chain of IgY.
e.	IgY does not activate a complement of the mammals.
f.	IgY does not combine with proteins A and G (IgG binding protein).
g.	IgY does not combine with a rheumatic factor (autoantibody to an Fc radical of an IgG).
h.	IgY does not combine with the Fc receptor of a mammalian cell.

Materials and methods

Immunization

Rhode Island Red hens were used in this experiment as the donor host of egg yolk. The anti-rabies IgY was purified from the egg yolk of hens immunized a part of the G gene encoding truncated protein (rG-F2), the recombinant nucleoprotein (rN) and the P protein (rP) of RABV, CVS-11 strain *(9,10).*

Preparation of RABV antigens

Preparation of rG-F2, rN and rP of RABV was carried out according to a protocol reported elsewhere *(14,15).* The rG-F2, rN and rP expressed in *Escherichia coli* DH5α after transformation by plasmid DNAs were purified using a nickel-nitrilotriacetic acid column [Qiagen] and the expected sizes of purified proteins were confirmed by SDS-PAGE *(9,10,15)*.

Preparation of water in oil emulsion of antigens

Counter-Lock type glass syringes (5 or 10 mL volume) were connected to each other by specially ordered 0.5 cm length jointing stainless tube (0.5 mm inner diameter) and used to prepare a stable water-in-oil emulsion of antigens. For one immunization to a hen, an antigen solution (1 mL) containing about 0.3 mg of recombinant protein was emulsified with 1 mL of Freund's Complete Adjuvant by passing the mixture in the syringes back and forth through the joint tube. One drop of the emulsion was dropped through a 23-gauge needle attached to the syringe on water to confirm its intactness. For booster shots, an antigen solution (0.5 mL) was emulsified with Freund's Incomplete Adjuvant (0.5 mL) as described above. The emulsion prepared can be stored for several days in a refrigerator before use.

Immunization of hens and collection of samples

Two hens were used for each recombinant antigen (rGF-2, rN, and rP). The emulsion (1–2 mL) was injected by a 23-gauge needle intramuscularly into several sites evenly under the hen's wings. Booster shots were injected in a manner similar to the first shot twice, with an interval of 2 weeks. Blood samples (about 1 mL) were

drawn from the wing vein on every immunization day, and serum was separated by conventional methods and stored frozen until use for determination of antibody titres by an enzyme-linked immunosorbent assay (ELISA) using the recombinant antigens (rGF-2, rN, and rP) during the solid phase *(16)*.

Egg production after immunization

The hens' weight changed little from the first immunization for 18 weeks and maintained at around 2.0 kg. The egg-laying rates of the immunized hens dropped drastically from 80% to 20% during the 2 weeks after the first immunization; however, these rates recovered individually to 70–80 % after the third immunization. The egg-laying rates of immunized hens were almost identical to those of unimmunized hens, starting at around 80% and maintaining at around 60% at 18 weeks after the first immunization. Total egg production per hen averaged 98 eggs in 18 weeks, resulting in 78% of the average egg-laying rate.

Eggs were collected daily and stored in a cold room up to one month to separate egg yolk from egg white by using a yolk separator instrument, followed by breaking the eggs. The egg yolk was then rotated carefully on paper towels to remove excess egg white from the yolk membrane. An aliquot of yolk from an egg laid every 2 weeks after the first immunization was diluted with an equal volume of water containing 0.05% NaN3 as a preservative and stored in the cold room until titrated. The egg yolk samples were pooled in alternate weeks and frozen until IgY separation.

The ELISA values of egg yolks obtained from individual hens against antigens (rGF-2, rN, and rP) generally increased after the second immunizations and reached a maximum at 6 weeks after the third immunization. The ELISA values decreased gradually after a maximum during the immunization period *(16)*. Control egg yolk from unimmunized hens did not bind any antigens at all. Pooled egg yolks between 6 to 8 weeks of the individual hens were selected for further IgY purification.

Purification of IgY

The modified λ-carrageenan method was used, as summarized in Fig. 40.2. Briefly, the stored egg yolk (100 g) was mixed and homogenized with 700 mL of 0.36% NaCl. The homogenate was mixed slowly with 400 mL of 0.4% λ-carrageenan solution to confirm generation of floating lipoprotein coagulum, while being gently stirred with a spatula. The mixture was then left for 1 h at 20 °C followed by centrifugation at 7000 × *g* for 30 min. The supernatant was filtered with filter-paper, precipitated with 15% (w/v) sodium sulfate three times, then dialysed against 10 mmol disodium hydrogen phosphate. Purified IgY fractions were stored at –80 °C until use after the dialysates were centrifuged and filtrated through 0.45 µm syringe filters.

Egg yolk generally contains IgY of about 1% (w/w). λ-carrageenan precipitate most lipoproteins in egg yolk by the mode of the ionic binding in slightly acidic pH of around 6. Since the IgY is a water-soluble protein in egg yolk, it is recovered

in the supernatant after precipitating the λ-carrageenan-lipoproteins complex. The IgY recovery is generally 68% with 29% purity in the water-soluble fraction. A salting out procedure using sodium sulfate performed quite well to precipitate IgY. This procedure was repeated several times by monitoring IgY purity using analytical SEC-HPLC. The final dialysate contained quite pure IgY with 61% of recovery and 98% of purity *(16)*.

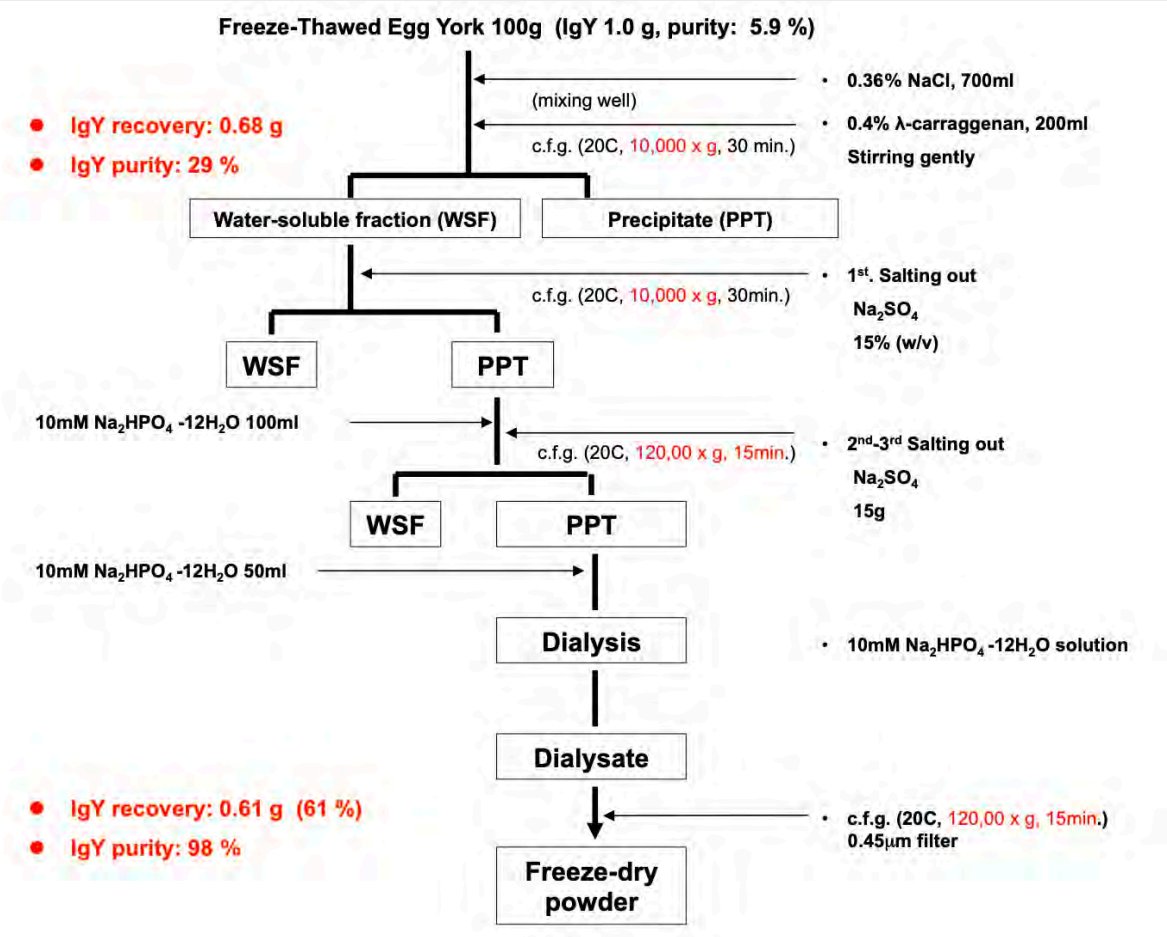

Fig. 40.2. Modified λ-carrageenan method

Antibody titres of egg yolks

The ELISA values of IgY obtained from individual hens against antigens (rGF-2, rN and rP) are shown in Fig. 40.3. The values generally increased after the second immunizations and reached a maximum at 6 weeks after the third immunization individually. The ELISA values decreased gradually after a maximum during the immunization period. Thus, an appropriate booster shot is needed to maintain high antibody titres. Control egg yolk from unimmunized hens did not bind any antigens. Pooled egg yolks between 6 to 8 weeks of the individual hens were selected for further IgY purification.

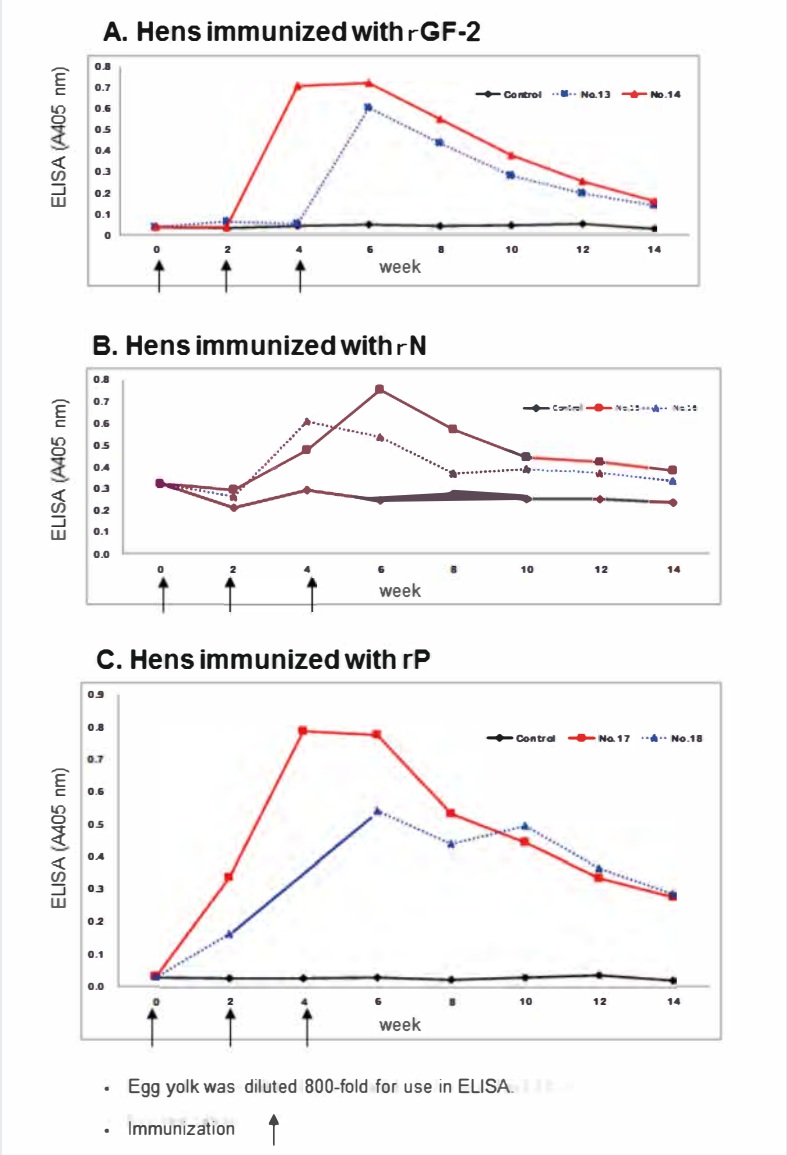

Fig. 40.3. Changes in antibody activity in egg yolk

Detection of RABV antigens by IgY

Immunological staining of RABV antigens has been reported with rabbit IgY developed by the immunization of RABV proteins (rGF-2, rN and RP), as described previously, using brain tissues of mice infected with RABV and rabid dogs, which had been submitted and diagnosed by using fluorescence isothiocyanate (FITC)-conjugated anti-rabies monoclonal antibody at the Research Institute of Tropical Medicine in the Philippines *(9, 15, 17–19)*. The specific binding to the N and P proteins of RABV was also detected by anti-rN IgY and anti-rP IgY, respectively *(9, 10)*. The viral P antigens in the Ammon's horn of two rabid dogs (No. 64 and No. 86) are shown in Fig. 40.4. Small transverse sections (2–3 mm in thickness) of Ammon's horn, one a slide, were fixed with cold acetone overnight and incubated with anti-rP Ig Y for 30 min at 37 °C in a high-humidity chamber. After washing, the slides were further incubated with FITC-conjugated anti- chicken IgY rabbit IgG fraction for 30 min. The slides were examined using a fluorescence microscope.

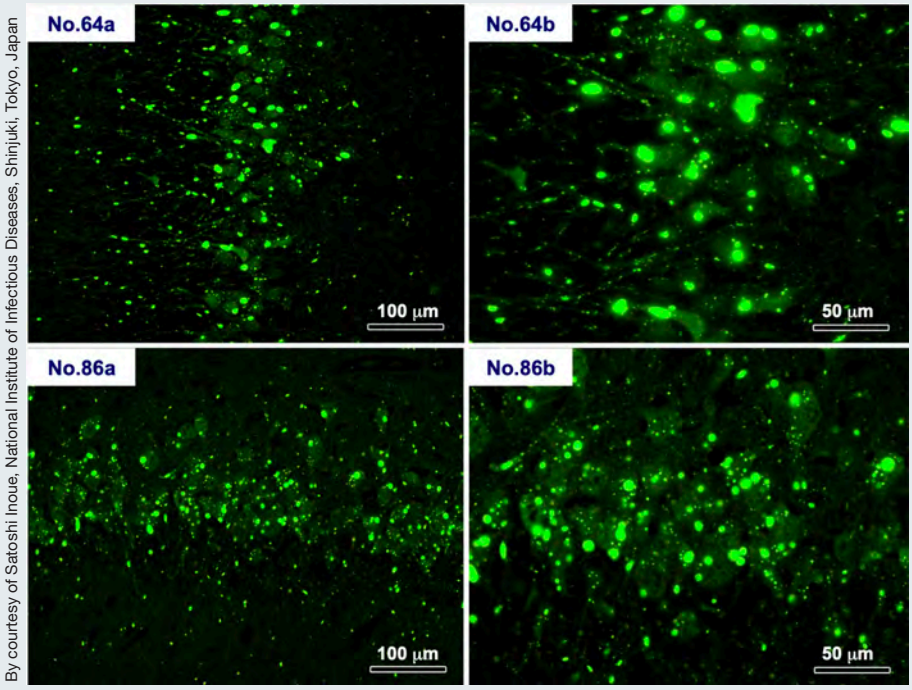

Fig. 40.4. IFA staining of rabies virus P proteins in Ammon's horn of rabid-dog brain

Discussion

The anti-rN and rP IgY were shown to bind specifically to the respective proteins of the CVS-11 strain of RABV by western blotting, indirect fluorescent antibody test and immunohistochemistry, indicating that IgY to RABV recombinant proteins could serve as a reagent for diagnosis of RABV infection *(9, 10)*. The progression of the signs in RABV-infected mice was also substantially delayed by the inoculation of anti-rG-F2 IgY as PEP at the proximal site of RABV inoculation *(10)*. Therefore, anti-RABV IgY prepared following this method is suitable not only for diagnosis but also for potential therapeutic use instead of antibodies originating from mammals.

Trott and colleagues reported that older hens generally had higher IgY titres than younger hens *(20)*. In our experience, older hens also tend to tolerate severe immunization. We used Rhode Island Red hens aged around 300 days for immunization. To avoid a reduction in egg production due to the inflammation in the hens, Freund's Complete Adjuvant with recombinant antigens was applied for primary immunization and Freund's Incomplete Adjuvant was applied for booster immunizations. Higher antibody activity and purity of the IgY would enable the application of IgY in diagnosis as well as a passive immunization therapy.

The most common injection route is the intramuscular route. Chang and colleagues demonstrated that intramuscular immunization results in higher levels of specific IgY when compared with the subcutaneous route *(21)*. However, intramuscular injections into the hen's legs sometime cause limping and improper feed intake and therefore we chose to inject the muscle under the hen's wing. Although egg-laying rates drastically dropped after the first injection for several weeks, immunized hens recovered well to achieve comparable egg-laying rates (around 80%) to those of the unimmunized hens, which lasted for 4 months.

IgY is one of the water-soluble proteins in egg yolk. Therefore, separation of the water-soluble IgY from abundant yolk lipoproteins is the first step of IgY purification. Many purification methods for IgY have been developed that involve precipitation of lipoproteins with polyethylene glycol or poly-anions, such as dextran sulfate, sodium alginate, xanthan gum, λ-carrageenan and pectin *(1, 2, 20, 22–24)*. These lipoprotein-coagulating agents work effectively to retain the water-soluble proteins in the supernatant after centrifugation. Thereafter, IgY is generally isolated in pure form by salting out using either ammonium sulfate or sodium sulfate. The λ-carrageenan method was further modified to improve IgY recovery (61%) and its purity (98%).

Tan and colleagues developed a cost–effective and efficient IgY purification method to obtain IgY with high recovery and purity by employing the existing commercial IgY isolation kits *(25).* They obtained chicken IgY of high yields (60 mg) and high purity (about 80%) using pectin and λ-carrageenan in the presence of calcium chloride to precipitate egg yolk lipoproteins while retaining IgY in solution followed by salting out IgY with higher purity. The modified λ-carrageenan method shown in this protocol might be superior to any other reported methods for obtaining the highest purity of IgY of > 90% in protein basis, and suitability for large-scale purification of chicken IgY.

The method of producing antigen-specific antibodies in egg yolk (IgY) can provide new opportunities to develop a safe, convenient and inexpensive way of manufacturing various immunodiagnostics.

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Chapter 41
Plant production of monoclonal antibodies for rabies

Introduction

Virus neutralizing antibodies (VNA) are a key component of immunity against viral infections, and act by preventing or modulating viral disease progression. Polyclonal antisera are still widely used for the prevention and/or post-exposure prophylaxis (PEP) of many infections and are important products because they can provide immediate protection even in immunocompromised individuals. They can also be reasonably cheap to manufacture, allowing greater accessibility in low and middle-income countries (LMICs). In rabies, passive immunization with rabies immunoglobulin (RIG) is well established for PEP. RIG is a WHO essential medicine and the preventive method of choice in most countries where rabies is endemic *(1)*.

Polyclonal antisera have drawbacks however, being derived either from human donors or large animals with consequent risks related to the use of blood products. They are often costly and/or in scarce supply and are, by their nature, mixtures of active compounds that exhibit significant batch-to-batch variability. In addition, the active pharmaceutical ingredient, i.e. the neutralizing antibodies, is poorly defined. For these reasons, monoclonal antibodies (MAbs) have been explored as alternatives to RIG by many groups *(1–4)*.

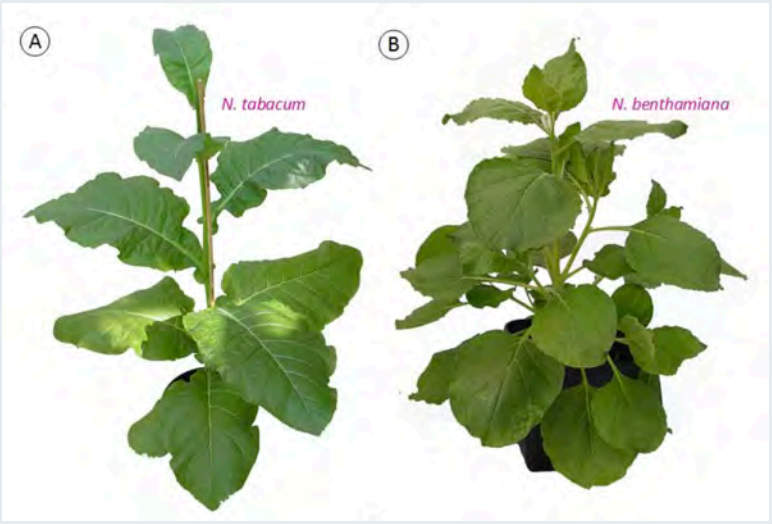
Clinical MAbs are manufactured by mammalian cell fermentation *(5)*. This is an established industry with a number of successful blockbuster antibody drugs. However, a monoclonal antibody RIG replacement product would impose specific constraints, which are not addressed by the commercial MAb sector. Firstly, to ensure adequate viral coverage and prevent viral escape a combination of at least two MAbs would likely be required *(6)*. At present there are no commercial MAb products comprising more than one antibody. Secondly, antibody production would be needed at an abundant scale. Every year, more than 15 million people worldwide receive a post-bite vaccination and require PEP (WHO factsheet 2017), suggesting that production of hundreds of kilograms annually of rabies MAbs would be required. Thirdly, rabies is predominantly a disease of LMICs, so to ensure accessibility and availability of any RIG replacement product, the investment cost for manufacturing and cost of goods needs to be low. Specifically, the disincentive of upfront costs for product and clinical development for a product primarily for LMICs has been an important reason why there has been virtually no interest from the pharmaceutical industry to address this unmet medical need.

Molecular pharming – the use of plant biotechnology to manufacture pharmaceuticals – offers some potential solutions, particularly in relation to cost, scalability and technology transfer to under-developed regions *(7, 8)*. The application of molecular pharming to rabies MAb production has been explored by different groups *(9–11)* and the antibodies produced in plants have demonstrated

viral neutralization equivalent to their counterparts produced in mammalian cells. For example, two MAbs identified by WHO rabies collaborating centres as potential clinical candidates *(6)* have been successfully produced in plants *(3, 12)*, one of which (E559) was also demonstrated to protect against rabies virus (RABV) in a hamster challenge model *(12)*.

There are two mainstream approaches to producing MAbs in plants. The pioneering work on expression of antibodies in plants was developed in transgenic *Nicotiana tabacum (13)*. The rabies MAbs from plants that have been described in the literature to date have also been produced by generating stable transgenic *N. tabacum* plants (Fig. 41.1A), in which the heavy and light chain MAb genes were incorporated into the plant nuclear genome, transgenic plant lines were regenerated and the plant lines underwent standard plant breeding to achieve homozygosity and genetic and phenotypic stability *(14)*. This transgenic approach is relatively slow but has the advantage of resulting in transgenic seed that are easy to store, distribute and grow at massive scale at low cost.

The second approach is to express recombinant MAbs transiently in plants. Although this rapid gene expression system was also initially developed in *N. tabacum (15, 16)*, *N. benthamiana* (Fig. 41.1B) became more widely used because it allows the use of inhibitors of post-transcriptional gene silencing, such as p19 from tomato bushy stunt virus *(17)*. In transient expression systems, vectors based on plant viral elements or the Ti plasmid of *Agrobacterium tumefaciens (18)* containing the MAb genes are introduced into plants by a process called agro-infiltration *(19, 20)*. This results in transfection of all the cells in the agro-infiltrated plant tissue and short-term, or transient, expression of the MAb genes, within a few days. The ZMapp antibodies used in the 2014 West Africa Ebola outbreak were manufactured using this approach *(21)*. The key advantages of transient expression lie in the simplicity of the technique, the speed of expression and the high antibody yield achievable at laboratory scale. This transient expression technique for antibodies in plants could be extremely valuable to researchers in the rabies field, even at laboratory scale where only a few milligrams of antibodies are required for use as a reagent. This chapter describes the detailed methodology and how to get started.



By courtesy of Julian K.C. Ma, St George's Hospital Medical School, London, UK.

Fig. 41.1. *Nicotiana tabacum* cv. Petit Havana SR1 (A) and *Nicotiana benthamiana* (B)

The plants were grown in the greenhouse and are shown at about 6–8 weeks old. Whereas *N. benthamiana* potentially reaches 0.5 m in height at maturity, *N. tabacum* may grow to 2 m at maturity in the greenhouse, and more in the field.

Key features of the stable transgenic (GM plant) and the transient gene expression via agro-infiltration approaches are summarized in Table 41.1. Of note, the initial cloning work is virtually the same for both approaches and some vectors (e.g. pTRA) are well suited for both tasks.

Table 41.1. Comparison of key features of stable transgenic (GM plant) and transient gene expression (agro-infiltration)

Feature	GM plant	Agro-infiltration
Level of integration	High	Mid
Setup time	6 months to generation of first transformants	4–6 weeks from seed to plant ready for infiltration
Scalability	Agricultural	Good to several kg biomass, then requires sophisticated facility
Flexibility	Limited	High, particularly useful for product development
Skills required	Plant tissue culture	Basic microbiology
Protein accumulation	Generally lower	Generally higher
Stress level	Lower	Higher, both biotic and abiotic stress factors
Containment	As for all GM plants. Deregulation may be approved.	Plants can be grown anywhere; agrobacteria and infiltrated plants require containment;
Robustness	Very robust	More input parameters, more variable

Rabies reagents produced in plant expression systems

Several rabies antibodies and antigens have been expressed in plants using both stable transgenic approaches as well as transient gene expression via agro-infiltration. While the production of rabies antibodies has generally been very successful (Table 41.2), rabies antigens so far have been more challenging (Table 41.3). Specifically, high-level expression of the full-length ectodomain of the envelope glycoprotein has not been achieved. However, several small fragments such as the RVG peptide that binds to nAChR have been produced successfully.

Expression vectors for *Agrobacterium*-mediated transformation

For plant molecular pharming applications, the systems mostly used nowadays are based on (i) a binary vector derived from *A. tumefaciens* (*Rhizobium radiobacter, A. radiobacter*) and (ii) various genetic elements from plant viruses. Genetic elements from plant viruses are widely used because many plant viruses accumulate to very high levels in plant cells. To achieve this, plant viruses evolved different mechanisms, including highly efficient 5' and 3' untranslated regions (UTR) as translational enhancers, inhibitors of post-transcriptional gene silencing and amplification of genomic and subgenomic RNAs. Learning from nature, these genetic elements have successfully been harnessed in various ways to create the powerful plant expression strategies that are now available.

Table 41.2. Rabies virus antibodies produced in plants

Antibody ID	Type	Expression strategy / Host plant	Reference
SO57	Human IgG1	Transgenic *N. tabacum* cv. Xanthi	Ko et al., 2003 (10)
		Suspension cells of transgenic *N. tabacum* cv. Xanthi	Girard et al., 2006 (9)
62-71-3	Mouse–human chimeric IgG1	Transient *N. benthamiana*	Both et al., 2013 (3)
	scFv-RVG fusion	Transient *N. benthamiana*	Phoolcharoen et al., 2017 (22)
E559	Mouse–human chimeric IgG1	Transgenic *N. tabacum* cv. Petit Havana SR1	van Dolleweerd et al., 2014 (12)
8C5 10H5 4H3 7A2	Human IgG1	Transient *N. benthamiana*	Unpublished

Table 41.3. Rabies virus proteins produced in plants

Protein	Type	Host Plant / Expression strategy	Reference
N	Full-length	Transgenic tomato, transient *N. benthamiana*	Perea Arango et al., 2008 (23)
G, N	G-N chimeric peptide fused to AIMV coat protein	Tobacco and spinach / recombinant plant virus	Yusibov et al., 2002 (24)
G	Full length	Transgenic tomato	Mc Garvey et al., 1995 (25)
	Full length chimeric	Transgenic *N. tabacum*	Ashraf, 2005 (26)
	Full-length G fused to B sub-unit of cholera toxin	Transgenic *N. tabacum*	Roy, 2010
	Full-length	Transgenic *N. tabacum*	Yadav, 2012
	Full length G fused to B sub-unit of ricin toxin	Hairy root cell culture derived from transgenic tomato	Singh, 2015
	VLP, co-expression with M	Transient *N. benthamiana*	D'Aoust, Medicago, patent application

A. tumefaciens is a natural genetic engineer with a type IV secretion system specialised to deliver genetic information to plant cells to cause crown gall disease *(27).* A particularly important feature of this system is that it has evolved to transfer single-strand DNA efficiently across the plant cell wall and the plasma membrane to shuttle it into the plant nucleus *(28, 29).* Fortunately, the only *cis* elements required are the left and right border 25 base pair (bp) repeat sequences, and all other necessary virulence genes can be provided in *trans*. This led to the development of T-DNA binary systems, where the natural tumour inducing Ti plasmid was disarmed by eliminating the transfer DNA region carrying the plant oncogenes and an artificial T-DNA was introduced into a shuttle vector that can replicate in both *E. coli* and *A. tumefaciens.* Such a pair of plasmids is called a T-DNA binary system. The disarmed Ti-plasmid is also referred to as helper plasmid and the shuttle vector as T-DNA plasmid. There are many different variants of the latter, inclu-

ding pTRA *(30)*, pEAQ *(20)*, magnICON *(31)*, pRIC, pORE, pGREEN, pCAMBIA and pBIN. Importantly, not every helper plasmid can be combined with every T-DNA plasmid and it is vital to ensure compatibility for the origin-of-replication and antibiotic resistance genes.

In the pTRA vector, gene expression is controlled by a duplicated CaMV-35S promoter, the 5'UTR from tobacco etch virus, a CaMV-35S 3'UTR and transcriptional terminator and scaffold attachment regions of the tobacco RB7 gene. An essential element is the inclusion of a leader sequence to target transgene expression products to the plant secretory pathway. Leader sequences can be derived from either mammalian or plant sequences *(34)*. Genes of interest are inserted using convenient restriction sites. For therapeutic antibodies, the accuracy of signal peptide cleavage is critical, whereas for research and diagnostic purposes, heterogeneity at the N-terminal end is not an issue. Details for the pTRA vectors currently used for producing human antibodies at high levels *(32)* are shown in Table 41.4 and Fig. 41.2.

Table 41.4. Genetic elements used in pTRA plasmids

Group	Element	Description
Backbone	ColE1 ori	High copy origin of replication for *Escherichia coli*
	RK2 ori	Low copy origin of replication for *Agrobacterium tumefaciens*
	bla	β-lactamase, resistance to ampicillin/carbenicillin
T-DNA	LB	Left border, start of transfer-DNA (clockwise)
	RB	Right border, end of transfer-DNA
DsRed scorable marker gene expression cassette	Pnos	Nopaline synthase promotor
	CHS	5'UTR of the chalcone synthase gene from parsley
	DsRed-H_6KDEL	Red-fluorescent protein from *Discosoma* spp. with a His_6 tag for IMAC purification and a KDEL tag for ER-retrieval
	pAnos	3'UTR and termination of transcription from the nopaline synthase gene
Recombinant Antibody gene expression cassette	2x P35S	Duplicated promotor from the 35S RNA of Cauliflower Mosaic Virus
	TL	5'UTR from tobacco etch virus
	SP	Signal peptide
	HC	Mature antibody heavy chain
	pA35S	3'UTR and termination of transcription from the 35S RNA of Cauliflower Mosaic Virus
SAR	SAR	Scaffold attachment regions from the rb7

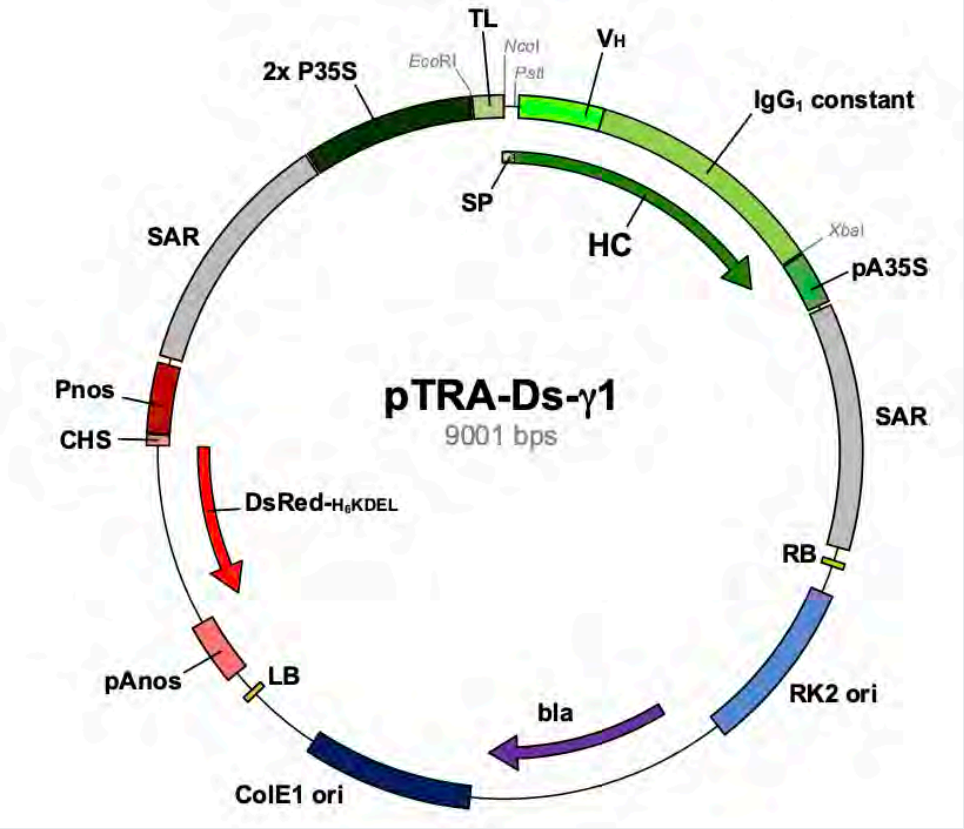


Fig. 41.2. Plasmid map for pTRA-Ds-g1 used for expression of human IgG1 antibodies

The same plasmid is also used to express the human light chain by replacing the PstI–XbaI fragment. The elements comprising the expression cassette for the antibody are depicted in grades of green colours. The cassette for the DsRed marker gene is shown in grades of red. The region (clockwise) between the left border (LB) and the right border (RB) comprises the T-DNA that is transferred to the plant cell. Details of the various elements are given in Table 41.4.

Setting up a plant expression system yourself

The practical aspects of getting started with a plant-based transient expression system for MAbs assumes that a standard molecular biology laboratory is available for conducting the basic recombinant DNA and protein analysis work. Consideration is given to a minimal setup scenario and estimate of the costs required to become operational.

N. benthamiana seeds typically cost a few dollars only but shipping costs may apply and not every vendor will ship seeds to another country. It is often easiest therefore to identify a national collaborator who already has *N. benthamiana* seeds and can spare a few. As seed amplification has a lead-time of about 80 days, it is advisable to use the first seeds to grow plants for making more seeds. A single plant can produce seeds sufficient for a year or longer, and seeds are viable for many years if stored properly in dry, dark and temperate conditions.

The next steps are to obtain the *Agrobacteria* with the helper plasmid and the corresponding T-DNA plasmid. The Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures – charges US$ 120–180 all-inclusive for freeze-dried *R. radiobacter* GV3101 (*A. tumefaciens*) carrying the helper plasmid pMP90RK.

Handling fees for the T-DNA plasmids can range from €50 to €500 largely depending on the legal entities and collaborators often can receive the material free of charge from universities.

Plants can be grown on a metal rack that can be obtained for less than US$ 100 from a do-it-yourself store and standard fluorescent lamps. This means that a growth rack with three levels can be setup for less than US$ 500. Plastic plant pots and good-quality compost can be purchased from a local garden centre. The cost for an entire basic setup would be less than US$ 800. Many other items that may be helpful are also inexpensive as many are sold as household goods.

The running costs are also very favourable and include standard laboratory plasticware such as tips, gloves, Petri dishes, paper towels, plastic foil, cellophane, transparent tape, 1 mL syringes, and Eppendorf and Falcon tubes. The infiltration medium costs less than US$ 2 per litre at small scale, and is cheaper than the media used for growing *E. coli* and agrobacteria. The main cost drivers are the reagents and materials needed for analysis of expression, purification of the target protein and its characterization. This is highly specific to the target protein and therefore we do not consider this here any further.

It is helpful – albeit optional – to co-express the MAb genes with a reporter gene that can easily be detected at low cost. We favour the red-fluorescent protein from *Discosoma* spp. for several reasons, including its high level of expression in different cellular compartments and plant species, high stability and ease of non-invasive macroscopic and microscopic detection.

Antibody purification by protein A affinity chromatography is a standard procedure that is used once the plant extract has been clarified and filtered. The overall setup and running costs for a minimal setup scenario that has a capacity for producing > 20 antibodies at the 2–5 mg scale per year is summarized in Table 41.5. The apparatus for SDS-PAGE if not already available is the highest cost factor.

Table 41.5. Summary of set-up and running costs for making more than 20 x 2 mg antibodies per year with the agro-infiltration technique

Category	Approximate cost (US$)	
	Set-up	Running
Agro-infiltration	800	500
DsRed detection	100	–
SDS-PAGE	1200	150
Protein-A matrix	–	250
General consumables	–	200
Total	2100	1000

Methods

Protocols for cloning antibody genes are extensively described in the literature *(33)*. The cloning of full length light and heavy chain genes is not difficult *(12)*. Nowadays, if the gene sequences have been determined, we routinely purchase synthetic heavy chain variable regions (V_H) and V_L chain genes, link them to the heavy and light chain constant regions of choice, and clone the complete heavy and light chain genes into binary T-DNA vectors. Using synthetic genes is advantageous because it allows codon optimization, which may improve expression levels in some cases. Codon optimization is still somewhat of a dark art, and DNA synthesis companies are not usually transparent in their algorithm design *(34)*. The Invitrogen GeneArt Gene Synthesis plant codon optimization service has been used with success by many plant groups.

The recombinant plasmid is amplified in *E. coli*, isolated and used to transform *A. tumefaciens*. Commonly used laboratory strains of *A. tumefaciens* include LBA4404 [available from Clontech and ThermoFisher], EHA105 [available from LifeScience Market], GV3101 and GV3101:pMP90RK [(available from Leibnitz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, DSM-12364 and DSM-12365]. Recombinant agrobacteria carrying the expression constructs are selected on agar plates containing appropriate antibiotics – for pTRA, this is carbenicillin.

The host range of *A. tumefaciens* depends both on the Ti plasmid and the genetic background of the isolate. A description of the chromosomal backgrounds and Ti plasmid derivations for the strains LBA4404 and GV3101 can be found in *(35)*. These commonly used strains have a broad host range, meaning that many important crops such as beans, lettuce, pumpkin, carrot, rapeseed and members of the Solanaceae family, as e.g. tomato, potato and tobacco, can be used. Here we focus on two related species from the genus *Nicotiana*, i.e. *N. tabacum* and *N. benthamiana*, which are the workhorses of Plant Molecular Pharming due to their ease of cultivation, rapid growth, prolific seed production, efficient transformation and high accumulation of foreign proteins.

Antibodies are expressed transiently in *N. benthamiana* by introducing the recombinant agrobacterium into leaf tissue by a process called agro-infiltration. At laboratory scale, this method is very simple and requires only basic skills and standard laboratory equipment *(36)*. The only additional requirement to a standard molecular biology or protein biochemistry laboratory is a space to grow a few plants. As expression levels are usually high, 10 plants would likely be sufficient and, in good cases, even a single plant leaf can provide a milligram of reagent. The plants are not genetically modified, and the only laboratory safety requirements relate to the use of recombinant bacteria (*E. coli* and *A. tumefaciens*). Even when the plants have been agro-infiltrated and are expressing recombinant protein, they are not regarded as genetically modified. However, as recombinant *A. tumefaciens* is still present, normal containment regulations for genetically modified bacteria apply. The plants and compost are usually disposed of by the same process as for any biological waste.

The antibody expression steps (Fig. 41.3) are:

1. Grow *N. benthamiana* plants.
2. Produce a liquid culture of recombinant agrobacteria.
3. *Agro*-infiltrate by syringe or vacuum infiltration.
4. Allow transient expression to occur and protein to accumulate.
5. Extract and purify protein.

An indicative timeline for antibody expression is shown also in Fig. 41.3.

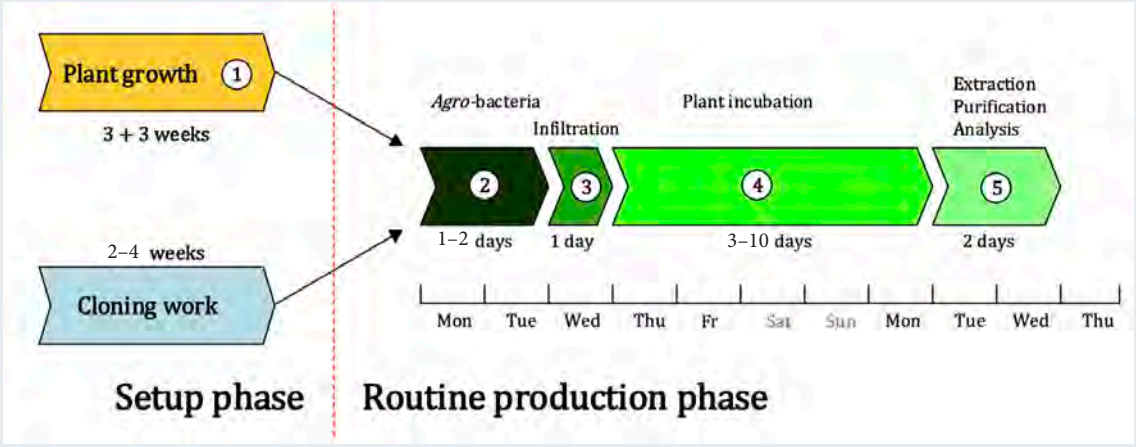


Fig. 41.3. Antibody expression by the agro-infiltration method, with indicative timelines

1. Grow N. benthamiana plants

N. benthamiana seeds can be obtained from commercial sources or through academic collaboration. Note that there are different ecotypes that differ in their susceptibility to plant viruses and in their performance in agro-infiltration *(37)*, which depends on a natural loss-of-function in the RNA-dependent-RNA-polymerase 1 gene *(38)*. Once obtained, seeds can be amplified easily and set aside in storage by keeping a few plants for flowering, self-pollination and seed-setting.

Nicotiana seeds are grown in standard commercial compost for about 6 weeks. It is beneficial to have two growth phases. Germination and seedling growth until a size of about 1–2 cm is typically done in pots or trays at a high plant density. Strong seedlings are then replanted into individual pots or plant trays. We typically grow three times as many plantlets as we intend to use. The growth conditions for the wild type plants are flexible. *Nicotiana* species prefer warm climates and can be grown at temperatures of 18–30 °C. They also tolerate high humidity, but this may cause moulds to grow excessively on top of the soil. The plants are usually grown under long-day conditions, e.g. with lighting on an 18 h (day) and 6 h (night) cycle. Many laboratories use standard fluorescent tubes attached to a timer switch. By 6 weeks, the plants are generally 10–15 cm high, and each plant will occupy a circular space of approximately 10–15 cm diameter.

It should be emphasized that growing *Nicotiana* plants is entirely straightforward, and within the grasp of anyone who has ever grown any kind of plant before. Although we recommend conditions for growth, in general it is difficult to get this stage wrong. For those without horticultural experience, the only requirement is to remember to water the plants!

2. Produce a liquid culture of recombinant agrobacteria

A. tumefaciens transformed with pTRA harbouring antibody genes is grown overnight on an orbital shaker (220 r/min) in complex media such as Luria-Bertani (LB) broth, YEB or PAM *(39)*, 100 mg/mL rifampicin and 50 mg/mL kanamycin and 50 mg/mL carbenicillin at 26–28 °C. It is important to ensure that the temperature does not exceed 30 °C because the plasmids can be lost when the cells grow too quickly.

Agrobacteria grows more slowly than *E. coli.* If starting from a single agrobacterium colony from an agar plate, this should be streaked onto an LB (Luria–Bertani broth) or YM (yeast minimal) agar plate and incubated at 28 °C for 2 days. Then 3 to 5 loopfuls of the culture are inoculated in liquid media to grow at 28 °C overnight. It is advisable to prepare a cell bank, i.e. aliquoted glycerol stocks of 50–500 µL stored at –80 °C.

After centrifugation, the bacterial pellet is resuspended in infiltration solution containing 0.1 mmol acetosyringone (for low yield constructs), 10 mmol MES and 10 mmol $MgCl_2$. The final OD_{600} of the bacterial suspensions should be adjusted to 0.5–1.0 for syringe infiltration, or 0.1–0.25 for vacuum infiltration (see below). Before scaling up, it can be worthwhile to run a titration experiment to determine the optimal OD_{600} for a particular gene of interest.

Full length IgG antibodies derive from two gene products. While both transgenes can be introduced on the same vector, this is not necessary. Individual heavy chain and light chain vectors can be prepared and simply mixed in a 1:1 ratio prior to *Agro*-infiltration, resulting in similar or sometimes even higher yields *(32)*. The transfection process is highly efficient, so both genes will be co-transferred into a large proportion of the transfected plant cells.

The same applies to the use of inhibitors of post-transcriptional gene silencing. A vector encoding, for example, the p19 silencing suppressor can also be co-infiltrated; however, this is typically done at lower levels, i.e. at a 1:5–1:10 ratio.

3. Agro-infiltrate by syringe or vacuum infiltration

This is the process by which the agrobacterial suspension is introduced into the intracellular spaces of the plant leaves. The anatomy of a plant leaf is indicated in cross section in Fig. 41.4, illustrating the intercellular spaces that are present primarily within the spongy mesophyll cell layer. Agrobacteria are introduced through the stomata, which are normal structures in the lower surfaces

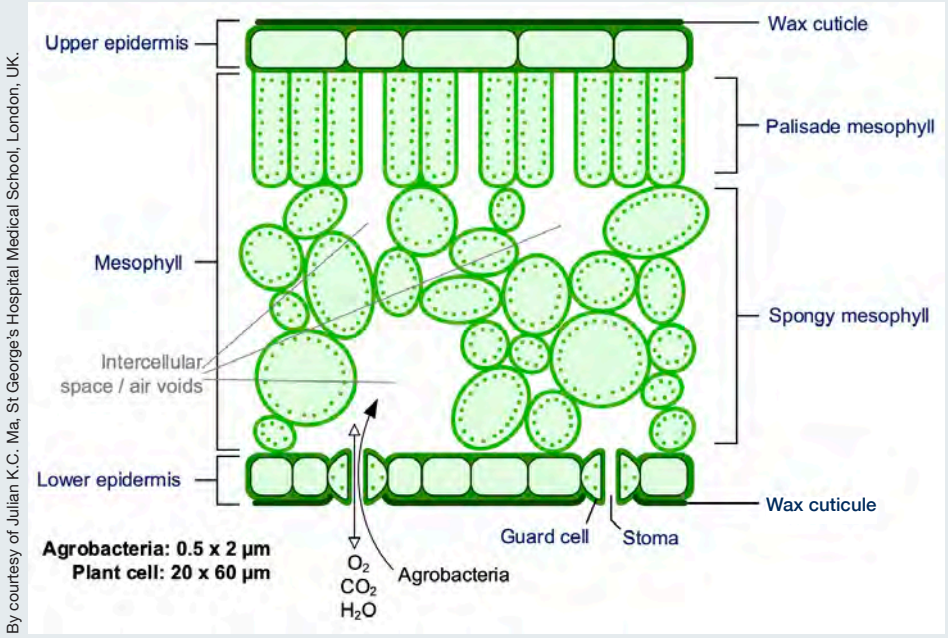


Fig. 41.4. Schematic representation of a leaf cross-section showing the general organization of the different cell types and the intercellular space targeted by agroinfiltration

of leaves that allow gaseous exchange and transpiration. It is helpful to ensure that the plants are well watered before agroinfiltration. When the stomata are closed, it is almost impossible to infiltrate the leaves. In this case it can help to punch little holes into the leaf or make small scratches into the lower epidermis to facilitate entry of the bacterial suspension. These injuries should, however, be as small as possible as the affected tissue will ultimately die. The aim is to fill the air spaces with agrobacterium solution, and this is most simply done using a needle-less syringe on individual plant leaves. Alternatively, a whole plant can be agro-infiltrated at once using a simple vacuum device. There are numerous videos demonstrating these techniques online (search for "agroinfiltration") or see *(40)*.

Using a 1 mL syringe pressed gently against the underside of the leaf (the abaxial surface), the bacterial suspension can be introduced directly into the leaf intercellular space. It is helpful to press a finger against the syringe nozzle from the other side of the leaf. The infiltrated area is evident by the growing "wet" patch that develops. Within a single leaf, the major veins often restrict the spread of the infiltration patch, so the procedure is repeated in different areas until most of the leaf is infiltrated. Expression of the gene of interest will be restricted to the area of infiltration, so it is possible to express multiple constructs on the same leaf.

To infiltrate entire plants, a larger volume of recombinant agrobacteria is required, typically 1–2 L. The plant is immersed (upside down) in the bacterial suspension, within a dessicator vessel or pressure cooker attached to a vacuum pump. Precautions must be taken to prevent the compost from falling out of the plant pot, e.g. by wrapping the pot in plastic film. A vacuum is applied for 1–2 min at 100 mbar to draw out the air in the intracellular leaf spaces. On release of the vacuum, the agrobacterium solution is sucked back into the leaf. The plants are taken out of the suspension and excess liquid is allowed to drip off or be carefully removed with paper towels.

4. Allow transient expression to occur and protein to accumulate

Following infiltration, the air space needs to be restored within the plant leaves to facilitate gas exchange *(41)*. This also concentrates the Agrobacteria to the cell walls where they can initiate the gene transfer process. Usually no active intervention is necessary, but if problems arise, such as plant tissue death or rotting, it is usually helpful to incubate the plants for a couple of hours under low humidity or expose them to an airstream after agro-infiltration.

The plants are then grown for 4–10 days at 22 °C. It has been shown that the transformation efficiency is temperature-dependent and drops sharply < 19 °C and > 23 °C *(42)*. As before, lighting is typically maintained on a 16:8 h day:night cycle. Even though the infiltration medium contains a large amount of sugar and the leaf tissue does not depend on photosynthesis, incubation in the dark generally results in poor expression because the leaves cannot get rid of the excess liquid as easily.

Maximal recombinant antibody expression is usually seen at around 5 days, but this varies from target protein to target protein, so it is advisable to assay different plants daily starting from day 3. Detection of recombinant antibody can be performed either by ELISA or western blot or dot blot. A small sample of leaf (for example taken using a hole punch) is sufficient. The leaf tissue is homogenized in three volumes (volume/weight) of sodium phosphate (pH 7) buffer in a microcentrifuge tube, using a plastic or electric-pestle. After centrifugation, the supernatant can be applied to an ELISA plate pre-coated with a capture antiserum (and further detected using a second appropriate enzyme labelled antiserum), or 10 mL can be applied to SDS-PAGE and western blot using appropriate detection antisera.

For ease of detection, we often co-express a fluorescent marker protein, such as DsRed, which provides a simple, non-invasive and cost–effective way to quickly assess the experiment.

5. Extract and purify protein

Protein extraction involves homogenization of transfected leaf tissue in an extraction buffer. A simple buffer (e.g. sodium phosphate pH7) is commonly used *(14)*, and the addition of antioxidants and/or protease inhibitors is at the discretion of the individual *(43)*. Usually, provided the extraction is performed quickly and on ice, these are not required. Typically, 2–3 volumes of buffer are used to prepare the plant extract (volume/weight), using a micro pestle (manually), pestle and mortar or a standard kitchen blender. The resulting smooth homogenate is optionally passed through Miracloth before centrifugation at 18 000 r/min for supernatant clarification. The duration of centrifugation depends on the volume. At small scale (< 2 mL), 2 mins is sufficient, whereas at larger scale > 100 mL, up to 30 min may be necessary. The pH of the supernatant often needs to be re-adjusted, as the extract itself is slightly acidic and the buffer capacity may be insufficient, typically followed by another centrifugation step to remove further precipitates. The clarified extract can then either be applied directly to e.g. a self-packed disposable

column or the supernatant is then passed through a 0.45 µm filter followed by a 0.22 µm filter before being purified by affinity chromatography. Protein-G or Protein-A-sepharose is commonly used. Once the plant extract is loaded, the column is washed with three volumes of sodium phosphate buffer (pH 7.4), then the antibody is eluted with 100 mmol glycine (pH 2.5), followed by pH neutralization with 0.1 vol. 1 mol Tris base or 1 mol sodium acetate.

Scale-up options

The method described is adequate for most laboratory-based requirements for monoclonal antibodies and readily delivers high milligram quantities. Importantly, it is quick, economic and technically simple.

Also important are the options for scaling up production, particularly for the development of potential products. This will, of course, require more specialist facilities, but it is useful to know that such facilities exist. For scaling up transient expression, there are commercial facilities established around the world, for example in the UK (Leaf Expression Systems Limited), USA (e.g. Kentucky Bioprocessing, Inc. and Medicago) and Germany (Fraunhofer IME and Nomad Bioscience).

One of the advantages of the pTRA vector system is that the recombinant agrobacteria can be used both for transient expression as well as for transformation of tobacco tissue to produce stable transgenic plants. Although the process of generating and screening transgenic plants is relatively lengthy, this is the most appropriate solution for massive scale production of antibodies (as would be needed for MAbs used in rabies PEP for example). The path to the clinic for MAbs produced in transgenic plants has been made much clearer by the issuing of a Good Manufacturing Practice (GMP) licence to the Fraunhofer IME Institute (Aachen, Germany) for this process *(44)*, and the approval of a first-in-human Phase I clinical trial for a plant derived antibody *(14)*.

While a GMP licence has yet to be issued for transient expression of MAbs in *N. benthamiana*, plant antibodies produced by agro-infiltration have entered clinical trials under FDA oversight. These include the patient-specific antibodies produced by non-Hodgkin's lymphoma as an idiotype vaccine *(45,46)*, and ZMapp, a cocktail of three MAbs against Ebola Virus *(47)*. With several more antibodies in the pipelines of academia and start-up companies and with the first big pharmaceutical company entering the scene, it is foreseeable that research manufacturing capacity, regulatory guidelines and large-scale industrial plants will continue to grow.

Production costs

For those with a longer term interest in manufacturing antibodies in plants, cost aspects are considered in more detail below, starting with some general top-down considerations and then providing a bottom-up view of actual cost to establish plant antibody manufacturing.

Several authors have analysed the costs of production for recombinant proteins from plants. Across all the reported studies and in agreement with the experiences

of many researchers in the fields, some generally accepted statements, if not dogmas, have arisen. The first and most important is that recombinant protein yield has the biggest impact on cost. In particular, low yields present additional problems such as degradation, low solubility and high losses during downstream processing. The second dogma is that upstream processing, i.e. growing the plant biomass, is generally inexpensive, immediately followed by the third dogma, which states that downstream processing represents the major bottleneck and up to 90% of the overall costs.

Some 20 years ago, Kusnadi and colleagues reported the cost of producing a recombinant protein in plants, of US$ 5–60 per kilogram, assuming accumulation of 10% (w/w) of the total crop protein *(48)*. As an example, soybean has a total protein content of 38% and the price per metric tonne was US$ 350 in May 2017, i.e. the cost of total soybean protein currently is about US$ 1/kg. Thus, at an accumulation rate of 10%, the recombinant protein would cost ~ US$ 10/kg. If only we could grow transgenic plants expressing valuable and lifesaving antibodies in open fields!

An early techno-economic analysis was reported for MAb production in 2012, based on a production scale of 100 kg purified MAb annually in stable transgenic tobacco-based systems, assuming an expression level of 1 g/kg FW. Here, the cost of goods sold for production in greenhouses or in bioreactors was US$ 98/g and US$ 138/g respectively *(49)*.

In 2014, a similar analysis was performed for the agro-infiltration system including the investment and operating costs for the manufacturing facility *(50)*. The authors concluded that a 400 mg dose of a therapeutic enzyme would cost US$ 474, equivalent to US$ 1185 per gram. Adapting these numbers to the expression levels and purification yield of human MAbs suggests production costs of US$ 237 per gram.

Most recently, a detailed techno-economic analysis for MAb production in a transient plant-based platform was published *(51)*. The model analysis included evaluation of total capital investment, annual operating cost and cost of goods, and was based on published designs for a commercial-scale facility. At a production scale of 300 kg/year, the model predicted a total capital investment of US$ 122 M and cost of goods of 121 US$ per gram. Compared with the most recent data from the CHO manufacturing industry, this represents a significant reduction in capital investment and a > 50% reduction in the cost of goods *(52, 53)*.

The costs of a biological drug are also largely dependent on the required dose, i.e. the potency of the pharmaceutical protein. This is particularly true for virus-neutralizing antibodies and has been demonstrated impressively and documented for highly potent and broadly neutralizing HIV antibodies *(54, 55)*. Compared with the early HIV-neutralizing MAbs, the required dose for the latest bnAbs (broadly neutralizing antibodies) is not only 100–1000 times lower, but their increased breadth of neutralization greatly facilitates the development of a cocktail antibody product. The situation is similar for rabies MAbs, where some of the more recently discovered human monoclonal antibodies have higher potencies than the murine MAbs that were the first available antibodies.

A RIG product comprising a cocktail of at least three MAbs requires excellent and matching yields and potencies. The rapid and scalable transient gene expres-

sion by agro-infiltration of *N. benthamiana* provides unique opportunities for identifying and developing such a challenging product. Again, the low entry barriers and costs are key enabling features.

This chapter has described how recombinant proteins including antibodies can be expressed in tobacco plants and has summarized the state of the art in relation to RABV-related reagents. Its main aim is to convey the message that plant expression of MAbs is technically and practically simplistic and within the grasp of any biologist. This can become a powerful tool for research laboratories to manufacture useful amounts of important reagents and candidate drugs for early in vivo studies.

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Part 7. Potency determinations

Chapter 42
The NIH test for potency testing of vaccines

Introduction

The NIH test for potency was originally developed at the National Institutes of Health (Bethesda, MD, USA). The test measures the degree of protection conferred by inactivated rabies vaccines in immunized mice challenged with rabies virus (RABV). It is undertaken by vaccinating two groups of mice twice, 7 days apart, with dilutions of a reference vaccine and the vaccine being tested. Seven days after the last vaccination, the immunized mice and a control group of mice are challenged with the challenge virus standard (CVS) mouse-brain strain of fixed RABV. The mice are observed daily and the median effective dose (ED_{50}) of the reference and test vaccines is determined based on the number of survivors. The relative potency of the test vaccine is then calculated by comparing the ED_{50} of the test vaccine with that of the reference vaccine.

Methods

Reagents

- CVS stored frozen (at –80 °C or in liquid nitrogen) as a 20% mouse-brain suspension in a diluent containing a low percentage (2–5%) of heat-inactivated fetal calf serum (FCS), and distributed in aliquots of about 800 µL.
- Reference vaccine

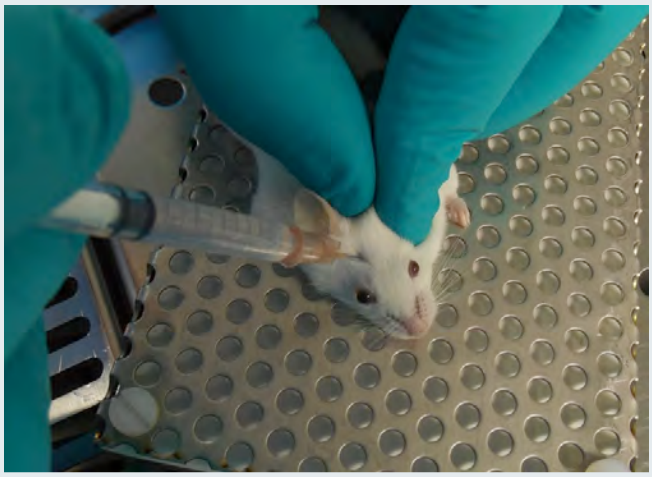
  Several reference vaccines are commercially available. For example, the Biological Reference Preparation (BRP) batch N°5, currently distributed by the European Directorate for the Quality of Medicines (EDQM), is a freeze-dried vaccine derived from the Pitman Moore strain of RABV produced in Nil-2 cell line and inactivated with ß-propiolactone. This reference vaccine has an assigned titre of 10 International Units (IU) per vial *(1)*. The sixth International Standard for Rabies Vaccine (07/162) is distributed by the National Institute for Biological Standards and Control (NIBSC, UK). This material was prepared from a bulk of Vero cell derived, Pitman Moore strain, produced by the same manufacturing process as that for the fifth International Standard for Rabies Vaccine. It has an assigned titre of 8 IU/vial *(2)*.
- Laboratories can prepare an internal reference vaccine provided it is calibrated against an International Standard (see above).
- 3- and 5-week-old Swiss or NMRI (Naval Medical Research Institute) female mice, or equivalent
- heat-inactivated FCS
- phosphate buffered saline (PBS), pH 7.4

- injectable anaesthetics (tiletamine in combination with zolazepam as an example) or anesthetics for inhalation (isoflurane).

Protocol

Preparation of the working CVS

1. Thaw rapidly the content of an ampoule of frozen virus under cold running water and dilute in PBS supplemented with 2% heat-inactivated fetal calf serum (FCS) so as to obtain a suspension containing approximately 10^3 LD_{50}/mL. The dilution factor will be calculated according to the titre of the stock solution.
2. Anaesthetize 3-week-old female Swiss/NMRI mice (the number of mice will be adapted according to the amount of required CVS vials).
3. Inoculate the mice intracerebrally (Fig. 42.1) with 0.03 mL of the suspension containing 10^3 LD_{50}/mL.



By courtesy of Alexandre Servat, ANSES, Malzéville, France

Fig. 42.1. Intracerebral injection into an anesthetized mouse

4. Observe the mice at least once daily to detect rabies clinical signs. Animal's deaths occurring during the 4 days after the intracerebral inoculation are considered nonspecific and cannot be attributed specifically to rabies.
5. Once paralyzed, euthanize the mice by acceptable standards (e.g. by cervical dislocation or CO_2 asphyxia).
6. Collect the brains, freeze them immediately and store at –80 °C.
7. Once the collection is complete, thaw, weigh and reduce the harvested brains to pulp using a sterile pestle and mortar, a tissue grinder, a mixer or another appropriate device. This procedure should be carried out in a biosafety cabinet to prevent the release of the virus in an aerosol. Add a sufficient volume of PBS supplemented with 2% heat-inactivated FCS (plus antibiotics) to obtain a 20% suspension by weight.
8. Assign a batch number to the suspension, centrifuge at 3000 x *g* / +4 °C for 30 min and immediately distribute the supernatant into sterile ampoules. Store at –80 °C or in liquid nitrogen.

   Note: Each step in preparing the working CVS must be carried out promptly, and in an ice-water batch or equivalent, to ensure the survival of the maximum possible amount of virus.

Determination of the LD_{50} of the working CVS

Before use as a challenge virus, the median lethal dose (LD_{50}) of each lot of the working CVS should be determined in 5-week-old mice as follows:

Remove one ampoule of the pooled working CVS from storage at –80 °C and thaw rapidly under cold running water.

Prepare serial 10-fold dilutions of the suspension in CVS diluent.

Anaesthetize groups of 10 mice and inoculate them intracerebrally (Fig. 42.1) with each dilution of the working CVS, each mouse receiving 0.03 mL.

Observe the mice for 14 days and record the number that die from rabies after the first 5 days and animals that are euthanized after evidence of stage 3 clinical signs (see Annex). Include any mice showing signs of rabies (e.g. paralysis, convulsions) on the 14th day.

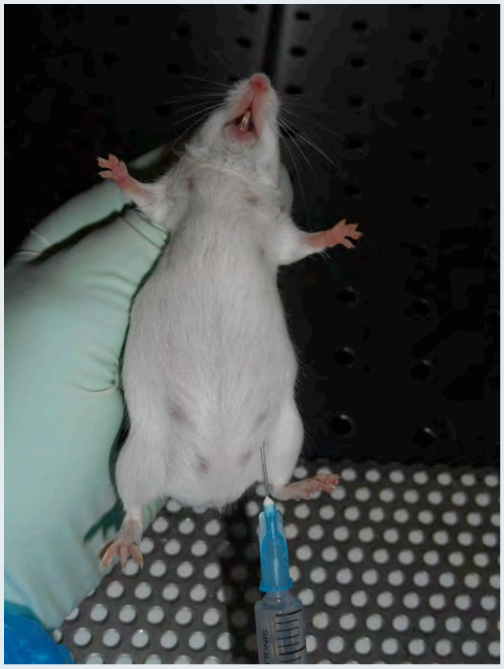
Calculate the LD_{50} of the working CVS using the Spearman–Kärber method or using an appropriate statistics software. A lot is generally considered satisfactory if the LD_{50} is between 10^{-6} and 10^{-8} dilutions inclusive. The maximum variation from test to test in the titre obtained should not exceed one 10-fold dilution when the same lot of challenge virus is used. The lot of working CVS may be used for as long as full potency is maintained as shown by mouse titration.

Immunization of mice

A four serial 5-fold dilution range of test vaccines and reference vaccine are performed in PBS. The initial dilution generally corresponds to 1/5, but may be adapted depending on the potency of the test vaccine and the reference vaccine. Dilutions may be performed in 15 mL conical centrifuge tubes as follows:

Dilution	Volume of vaccine	Volume of PBS
$10^{-0.7}$	2 mL of neat vaccine	8 mL
$10^{-1.4}$	2 mL of dilution $10^{-0.7}$	8 mL
$10^{-2.1}$	2 mL of dilution $10^{-1.4}$	8 mL
$10^{-2.8}$	2 mL of dilution $10^{-2.1}$	8 mL

1. Inject groups of sixteen 3-week-old female mice intraperitoneally (Fig. 42.2) with 0.5 mL of each dilution of the test vaccine and the reference vaccine. The mouse is manually restrained and is held in a supine position. The needle (23-gauge x 1”) and syringe (2.5 mL) are kept parallel to the vertebral column of the animal; the injection is made in the lower quadrant of the abdomen with an angle of about 10°.
2. Administer two doses of vaccine to each mouse one week apart.
3. Set aside enough mice for an adequate titration of the challenge virus to be made with at least 10 mice for each dilution of virus (a total of four dilutions, i.e. 40 mice).



By courtesy of Alexandre Servat, ANSES, Malzéville, France

Fig. 42.2. Intraperitoneal injection to lower quadrant of a mouse

4. Use a different needle and syringe to inoculate each group of mice. Where supplies are limited, use a single needle and syringe to inoculate the test vaccine and/or reference vaccine. In that case, mice receiving the most diluted vaccine should be inoculated first, followed by those receiving successively more concentrated vaccines. Mice receiving different vaccine concentrations should be housed separately.

Challenge of control and test mice

All mice are challenged intracerebrally (Fig. 42.1) 14 days after the first dose of vaccine as follows:

1. Take one ampoule of the pooled working CVS, put it on a bed of ice and thaw rapidly under cold running water.

2. Based on the previous titrations, dilute the CVS in PBS supplemented with 2% of heat-inactivated FCS to obtain the challenge dilution providing about 50 LD_{50} in 30 µL. From this challenge dilution, a three serial 10-fold dilution range is prepared to perform a titration of the virus as follows:

Dilution	Volume of vaccine	Volume of PBS
10^{-0}	Challenge dilution providing $50LD_{50}$/0.03 mL	–
10^{-1}	0.5 mL of dilution 10^{-0}	4.5 mL
10^{-2}	0.5 mL of dilution 10^{-1}	4.5 mL
10^{-3}	0.5 mL of dilution 10^{-2}	4.5 mL

All dilutions of CVS are held in an ice bath throughout the experiment.

3. Anaesthetize the mice to minimize or avoid the pain and distress associated with the intracranial inoculation procedure (following international regulations on animal experimentation). This anaesthesia has no adverse effect on the test results.

4. Challenge the immunized mice intracerebrally with 0.03 mL of the dilution containing 50 LD_{50} per 0.03 mL. Inoculate the control mice intracerebrally (syringe 1 mL, needle 26G / ½") with 0.03 mL of each dilution of the challenge virus. It is preferable to use a different syringe for each dilution of the challenge virus; however, if only one syringe is used, the 10^{-3} dilution must be injected first, followed by the 10^{-2} dilution, the 10^{-1} dilution and then the 10^{-0} dilution.

5. Observe the mice daily for 14 days to detect the appearance of typical rabies clinical signs. Generally shaky movements, trembling and convulsions (stage 3 of rabies clinical signs) are suitable humane end-points instead of lethality, to reduce the duration of animal suffering *(3)*. Record animals that die from rabies and animals that are euthanized after evidence of stage 3 clinical signs (see Annex). Include mice showing rabies stage 3 clinical signs on the 14th day.

Calculation of potency

The NIH potency test is a titration method based on quantal or "all or none" responses. In such dilution assays, a comparison between the dose–response relationships of the reference vaccine and the test vaccine is necessary. Linearization of these dose–response curves may be obtained by different transformations, such as probit, angular or logit. Statistics software may be helpful to simplify the calculations. Potency and confidence limits can be calculated by comparing the ED_{50} of the reference vaccine with the ED_{50} of the test vaccine.

When an in-depth statistical analysis is not possible (as described above), a volumetric method of calculation of potency should be used. This compares the 50% end-point dilution (vaccine dilution protecting 50% of mice) of the vaccine under test with that of the international standard (or equivalent national reference vaccine). The relative potency (RP) of the vaccine under test is determined by the formula:

$$RP = \frac{\text{reciprocal of ED50 of TV}}{\text{reciprocal of ED50 of RV}} \times \frac{\text{dose of TV}}{\text{dose of RV}}$$

Where

TV = test vaccine

RV = reference vaccine

Dose = volume of a single vaccinal dose, as stated by the producer.

For example, if the ED_{50} of the test vaccine is 1:90 and that of the reference vaccine is 1:70, the reciprocal values will be 90 and 70, respectively. If it is assumed that a single human dose of the test vaccine is 2 mL and that 1 mL of the reference vaccine represents a single dose for humans, then:

$$RP = \frac{90}{70} \times \frac{2\ \text{mL}}{1\ \text{mL}} = 2.6\ \text{IU/mL}$$

Minimum potency requirements

The relative potency of rabies vaccines for veterinary use should be determined using a recognized rabies reference vaccine and the batch of rabies vaccine used in a valid vaccination challenge test in the target species. The test should be carried out at the end of the period of immunity claimed by the vaccine producer. The relative potency value obtained in the NIH test should become the minimum value for all subsequent batches of the vaccine.

At its eighth meeting, the WHO Expert Committee on Rabies *(4)* suggested that inactivated veterinary vaccines with a potency of < 1.0 IU per dose, as measured by the NIH test, should not be licensed or released unless an adequately designed experiment has demonstrated a duration of immunity of at least 1 year in the species for which the vaccine is to be used. The Committee recommended that highly purified, modern rabies vaccines for human use should have a minimum potency of 2.5 IU per dose *(5,6)*.

Modified NIH test

To comply with the 3Rs principle, which aims to replace or reduce animal use and refine experimental procedures, a modified NIH test, based on a single immunization instead of two, may be used as described in the *European Pharmacopeia for rabies inactivated vaccines for veterinary use (7)*. For laboratories testing numerous batches of vaccine every year, and having a strong expertise and testing history, groups of 10 mice (instead of 16) may be used as well for the test vaccine and the reference vaccine *(8)*.

Single dilution test

The single dilution test is a simplification of the NIH test. The aim of this test is to determine whether a rabies vaccine satisfies the minimum potency requirement without assigning a precise value to it. The test provides qualitative results. It requires a homogeneous stock of challenge virus, well standardized methods of titration and laboratory animals of constant quality (consistent response to the vaccine and the challenge virus).

This test is particularly useful for testing multiple batches of vaccine within a short time and for reducing the number of mice used in the NIH test. However, given its own lack of precision, the minimum requirement for vaccines tested by the single dilution test is higher than that for vaccines tested by the standard NIH test.

Method

Before performing the single dilution test, a laboratory must have determined the titre of the reference vaccine (sixth International Standard for Rabies Vaccine, Biological Reference Preparation batch N°5, or any national reference vaccine calibrated against an international standard) several times in order to determine its ED_{50}. When the weighted mean of the ED_{50} of the reference vaccine has been calculated, the theoretical ED_{50} for a vaccine of the required potency can be determined using the formula:

$$D = D_m + \log_{10}(n) - \log_{10}(N) - \log_{10}(v)$$

Where:

D = the minimum ED_{50} required for the vaccine under test (decimal logarithm of the inverse of the arithmetical dilution);

D_m = the weighted mean of the ED50 obtained with the reference vaccine;

N = the required potency of the vaccine under test (IU/mL);

N = the required potency of the reference vaccine (IU/mL) – this information is provided in the insert supplied with the vaccine; and

V = the volume (mL) of a single dose of the vaccine under test, as stated by the manufacturer.

This dilution is then used for the vaccine under test. The standard NIH test protocol is followed using 10 mice vaccinated with the theoretical ED_{50} of the vaccine under test. To satisfy the minimum requirement, at least eight of the 10 vaccinated mice should survive after challenge.

Discussion

Potency tests of vaccines are quality control tests that provide manufacturers with information on the potency of their products before licensing. These tests are also widely used by regulatory authorities to ensure that marketed vaccines are sufficiently potent and effective. Many quality control tests for vaccines, notably the rabies vaccine potency test, are based on techniques described several decades ago and still rely on the use of laboratory animals. Nevertheless, the introduction of the 3Rs concept *(9)*, and the growing concern about ethics and animal welfare, have convinced regulatory authorities to promote animal reduction and refinement of the NIH test: anaesthesia before injection of intracerebral virus, definition of humane end-points *(10)*. Reduction of animal use in each dilution is now widely incorporated in guidelines and monographs and should be considered and applied by all laboratories carrying out the NIH test. Methods such as the single dilution test or the serological potency assay could also provide advances to go even further in the 3Rs approach.

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Annex

Progress of rabies virus infection in mice associated with stages of clinical signs

Stage 1: ruffled fur and hunched back

Stage 2: loss of alertness, slow and/or circular movements

Stage 3: trembling and shaking movements, weight loss, convulsions

Stage 4: paresis followed by signs of paralysis

Stage 5: moribund animals, prostration

Chapter 43
The serological potency assay for batch potency testing of inactivated rabies

Introduction

The serological potency assay (SPA) uses groups of mice immunized with a prediluted test vaccine or the reference standard vaccine adjusted to the minimum potency of 1 International Unit (IU) per dose. Blood samples from all mice are taken 14 days after immunization and the amount of rabies virus (RABV)-neutralizing antibodies induced after vaccination is determined using a serum neutralization test. The vaccine complies if the antibody titres obtained with the test vaccine are greater than or equal to the antibody titres obtained for the reference vaccine.

Laboratories willing to implement this alternative test are strongly encouraged to have a background in the mouse potency test and to conduct product-specific validations on rabies inactivated vaccines that they routinely control. This alternative method offers significant progress for the batch potency testing of rabies vaccines by significantly decreasing the number of animals (20 vs 148 for the NIH test) for one test vaccine and by avoiding the pain and distress of the intracranial challenge along with the signs associated with a lethal RABV infection.

Methods

Reagents

Reference vaccine

Several reference vaccines are commercially available. The Biological Reference Preparation (BRP) batch No. 5 is distributed by the European Directorate for the Quality of Medicines. It is a freeze-dried vaccine derived from the Pitman Moore strain of RABV produced in Nil-2 cell line and inactivated with ß-propiolactone. This reference vaccine has an assigned titre of 10 IU/vial *(1).* The sixth International Standard for Rabies Vaccine (07/162) is distributed by the National Institute for Biological Standards and Control (NIBSC), UK. This material was prepared from a bulk of Vero cell-derived, RABV Pitman Moore strain, produced by the same manufacturing process as the fifth International Standard, RAV. It has an assigned titre of 8 IU/vial *(2).*

Laboratories can prepare an internal reference vaccine provided it is calibrated against an International Standard (see above).

- Swiss/NMRI female mice, or equivalent, weighing 18–20 g
- phosphate buffered saline (PBS), pH 7.4
- xylazine and ketamine-based anaesthetics

Protocol

Immunization of mice

For immunization of mice, the reference vaccine and the test vaccine are diluted as follows:

1. Prepare a 1 IU/mL suspension of the reference vaccine in PBS.
2. Test vaccines are diluted according to their minimum approved specification. Rabies vaccines with a minimum specification at 1 IU/mL are used neat. Vaccines with a minimum specification at 2 IU/mL or 3 IU/mL are diluted 1:2 and 1:3 respectively in PBS.
3. Inject groups of 8–10 female Swiss/NMRI mice, weighing 18–20 g, intraperitoneally with 0.2 mL of each dilution of the test vaccines and the reference vaccine. Mice are manually restrained and are held in a supine position. The needle (23G x 1") and syringe (2.5 mL) are kept parallel to the vertebral column of the animal and the injection is made in the lower left quadrant of the abdomen with an angle of about 10°.

Blood sampling

After 14 days, mice are anaesthetized using a combination of xylazine and ketamine. Blood is collected by heart puncture under thoracotomy to obtain a large amount a venous blood with certainty.

1. Restrain the mouse on its back on an operating board.
2. Cut the skin around the xiphoid cartilage to expose the muscular wall of the thorax and abdomen.
3. Incise the abdominal wall just below the xiphoid cartilage and cut the diaphragm and thoracic wall at both sides of the sternum. The thoracic wall is pulled upwards and gripped with forceps.
4. Prepare a 1 mL syringe with a needle (21–23 gauge), insert the needle into the right ventricle of the beating heart, and collect the blood slowly and continuously by withdrawing the plunger.
5. Once bloods are collected from all mice, sera are extracted after centrifugation (2000 *g*, 15 min) and kept at –20 °C until determination of RABV neutralizing antibody.

Determination of RABV neutralizing antibodies

All sera from mice immunized with the reference vaccine and from mice immunized with test vaccine are tested individually for RABV neutralizing antibodies using a sero-neutralization assay such as the FAVN test *(3, 4)* or the RFFIT *(5)*.

Statistical analysis

The SPA uses the one-sided limit test (the Wilcoxon–Mann–Whitney exact test) lying on the ranking of all titres obtained with mice immunized with the test vaccine and the reference vaccine. Calculations must be performed using appropriate statistics software.

Validation of the SPA

The assay is invalid if more than two non-responders (RABV neutralizing antibody titre below the quantification limit) are observed in a group of 10 mice vaccinated with the reference vaccine.

A test vaccine complies with the SPA if the RABV neutralizing antibody titre is significantly higher ($p \leq 0.05$) than the RABV neutralizing antibody titre obtained with the reference vaccine.

Discussion

The SPA was first included in the 8th edition of the European Pharmacopeia as an official alternative to the NIH test for the batch potency test of rabies inactivated vaccines for veterinary use *(6)*. This test offers a real improvement in animal welfare by reducing significantly the number of animals used and decreasing the suffering entailed by the intracranial injection and the development of rabies clinical signs. The SPA for rabies vaccine batch potency testing, while not completely eliminating the use of experimental animals, contributes to the efforts to be made in the context of the 3Rs. Laboratories are strongly encouraged to switch from the NIH mouse challenge test to the SPA and to validate this test on each rabies vaccine that they routinely control for potency.

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Chapter 44
In vitro tests for rabies vaccine potency testing

Introduction

For more than 50 years, the NIH test *(1)* has been used to evaluate rabies vaccine potency before batch release. The test involves immunizing groups of mice intraperitoneally with the vaccine to be tested and administering an intracerebral challenge 14 days later with the challenge virus standard (CVS) strain (see Chapter 42). Although still required to assess vaccine potency by WHO *(2)* and the European Pharmacopoeia *(3)*, the NIH test has several drawbacks: results are highly variable *(4)*; live rabies virus (RABV) is used and requires strict biosafety measures; and large numbers of animals are employed and the severity of the challenge raises ethical concerns *(5)*. A less severe variation of this test has been developed: 2 weeks after intraperitoneal vaccination as above, mice are bled and RABV neutralizing antibodies (VNAs) are tested in an in vitro neutralization test (see Chapter 43). This test is already in use for veterinary vaccines *(6, 7)* and has been considered for human vaccines *(8)*. However, it sacrifices a large number of laboratory mice.

Today, both International *(9)* and European *(10)* recommendations encourage manufacturers and national control laboratories (Official Medicine Control Laboratories; OMCLs) to implement the "3Rs strategy" for the "Replacement, Reduction and Refinement" of laboratory animal testing. European Directive 2010/63/EU (in force since 1 January 2013) related to the protection and welfare of animals has reinforced the rules for vaccine manufacturers and laboratories involved in quality control of rabies vaccines as well as in rabies research *(11)*. Development, validation and use of in vitro alternative approaches have now become a priority; they are not only ethically sound but can also reduce batch testing costs and shorten the time for results to hours instead of weeks *(3)*.

Several immunochemical methods, such as the antibody-binding-test *(12, 13)*, the single radial immunodiffusion test *(14)* and the ELISA test *(15–19)* are recommended by the WHO Technical Report Series *(2)* and the European monograph *(3)* to quantify the antigen content in rabies vaccines. They are used by manufacturers to monitor the consistency of vaccine production and by the OMCL to assess the consistent formulation of batches of human vaccine *(20)*, even if the NIH test is still retained for potency.

At the surface of the RABV particle, the glycoprotein adopts a trimeric form *(21–25)*. In rabies vaccine, this native trimeric form constitutes the major immunogen to induce VNAs *(26)*, while the soluble or denatured glycoproteins are poorly immunogenic *(27, 28)*. The single radial immunodiffusion test requires a pre-treatment which may alter the membrane-anchored trimers of the glycoprotein into soluble or denatured forms *(14, 29)*. Hence, this test is less able to discriminate between immunogenic and non-immunogenic glycoproteins and thus less rele-

vant to appraise the immunogenicity of a vaccine lot. Conversely, the ELISA test is more sensitive *(14)*, preserves the native structure of the glycoprotein, and is thus more appropriate to determine the content of the natively folded trimeric glycoprotein. Studies have demonstrated good concordance between the NIH test and the antigen content evaluated by ELISA in vaccines, concluding that ELISA methods were suitable for the in vitro potency test and advocating that such tests might partly replace or even supplement the NIH test *(4, 18, 19, 30–33).* The complete avoidance of animal use is an achievable objective, and the European Pharmacopoeia now recommends the use of validated serological or immunochemical assays as alternatives to the NIH test *(3)*.

Method

The vaccine to be tested is incubated in a plate previously sensitized with anti-glycoprotein VNA, either polyclonal or monoclonal antibody. Bound antigens are subsequently identified by adding the same (or another) anti-glycoprotein antibody labelled with peroxidase, which is revealed in the presence of substrate and chromogen. Comparison of absorbance measured for the tested vaccine and the reference vaccine allows the determination of the glycoprotein content. The assay is functional for both purified anti-glycoprotein polyclonal antibodies and monoclonal antibodies concentrated with ammonium sulfate. The method to obtain and purify anti-glycoprotein polyclonal rabbit immunoglobulins G (IgG) or monoclonal mouse globulins has been extensively described in the previous edition of this manual *(34)*, as has the method to conjugate antibodies with peroxidase *(35)*. For the use of new reagents, such a defined and updated SOP may be available from commercial partners.

The following protocol is based on an indirect ELISA sandwich immunocapture using a monoclonal antibody D1 clone (mAb-D1) which recognizes the antigenic sites III (aa 330 to 338) of the trimeric RABV glycoprotein *(24, 36)*. This method was developed initially at the Institut Pasteur *(18, 30)* then optimized and validated by the Agence Nationale de Sécurité du Médicament et des produits de santé (ANSM) laboratory, i.e. the French OMCL *(4, 33)*. The monoclonal antibody (MAb) D1 is used for both coating and detection, which allows only trimers of the glycoprotein to be to recognized, i.e. the immunogenic RABV antigen. However, the same method may be applied using different MAbs (e.g. Wistar Institute MAb 1112) recognizing different antigenic sites of the RABV glycoprotein *(37)*.

Protocol

Microplate sensitization

The microplate is sensitized by adding to each well 200 µL of an appropriate dilution of anti-glycoprotein purified polyclonal or semi-purified MAbs in carbonate buffer 50 mmol, pH=9.6. Different dilutions of antibody must be previously tested to determine the optimal concentration, as about 1 µg/well is generally required.

Incubate the microplate for 3 h at 37 °C in a humidified atmosphere (or covered with a sealer sheet).

1. Aspirate carefully the well content, invert the microplate which is left drying on an adsorbant paper at laboratory temperature for 5 min.
2. Fill each well with 300 µL of test buffer: 0.3% bovine serum albumin (BSA), 5% sucrose dissolved in carbonate buffer 50 mmol, pH=9.6.
3. Incubate for 30 min at 37 °C.
4. Aspirate carefully the well content again, invert the plate which is left to dry on an adsorbant paper at laboratory temperature for 1 min.
5. The microplate can be immediately used or stored sealed at –20 °C until use. When the microplate is kept > 3 months, it must be tested before use.

The assay

1. The sensitized plate is washed 5 times with PBS-Tween, pH7 (washing buffer). Between each washing, the microplate is inverted and dried for 1 min on adsorbant paper.
2. The first well 1A (or all wells of the line 1) receive(s) 200 µL of PBS-Tween-BSA, pH7 and serves as a blank control.
3. Distribute 200 µL of eight serial 2-fold dilutions in PBS-Tween-BSA, pH7 of the reference vaccine in duplicate in wells of the lines 2 and 3 of the microplate. The lowest dilution must have a content about 1 µg/mL of rabies virus glycoprotein.
4. Distribute 200 µL of serial 2-fold dilutions in PBS-Tween-BSA, pH7 of each vaccine sample to be tested in the remaining wells, each dilution in duplicate.
5. Cover the microplate with an adhesive film and incubate for 1 h at 37 °C.
6. Remove the film and aspirate carefully the content of each well.
7. Wash five times with PBS-Tween, pH7.
8. Distribute 200 µL of an appropriate dilution in PBS-Tween-BSA, pH7 of peroxidase-labelled antibodies in all wells.
9. Seal the microplate and incubate for 1 h at 37 °C.
10. Aspirate carefully the labelled antibodies, wash the microplate six times with PBS-Tween, pH7, invert and dry it for 1 min on adsorbant paper.
11. Distribute to each well 200 µL of substrate-chromogen solution. Seal the microplate and incubate in a dark at room temperature for 30 min.
12. A yellow–orange colour develops, and the reaction is stopped by adding in each well 50 µL of stopping solution (4 mol sulfuric acid).
13. Carefully wipe the bottom of the microplate and place it in a spectrophotometer to determine the optical density at 492 nm of the negative control (blank), the reference vaccine and the tested vaccine.

Interpretation of the results

The reference vaccine must have a well-known glycoprotein content (µg/mL); this can be determined either directly when using purified viral particles (determination of total viral proteins then evaluation of the percentage of glycoprotein by SDS-polyacrylamide gel electrophoresis) or indirectly by ELISA when using a calibrated reference vaccine. This allows to design a reference curve showing the glycoprotein content in function of the optical density (OD), as seen in Fig. 44.1.

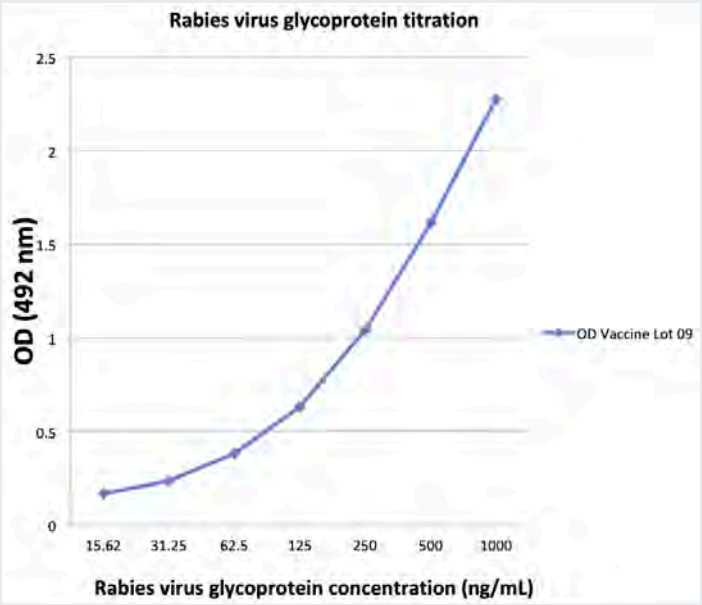


Fig. 44.1. Reference curve showing the glycoprotein content in function of the optical density (492 nm)

The evaluation of glycoprotein content in the tested vaccine is expressed in µg/mL by comparison to this reference curve. In the above example a vaccine diluted 1/32 which exhibits a mean OD for duplicate samples of 1.6 using MAb-D1 will content 32 x 500 ng/mL = 16 µg/mL of glycoprotein.

As the reference vaccine has been previously tested for its activity expressed in international units (IU/mL), the comparison of the mean ODs allows the in vitro potency of the tested vaccine to be evaluated. The tested vaccine potency is expressed as glycoprotein content in equivalent international units (EIU/mL).

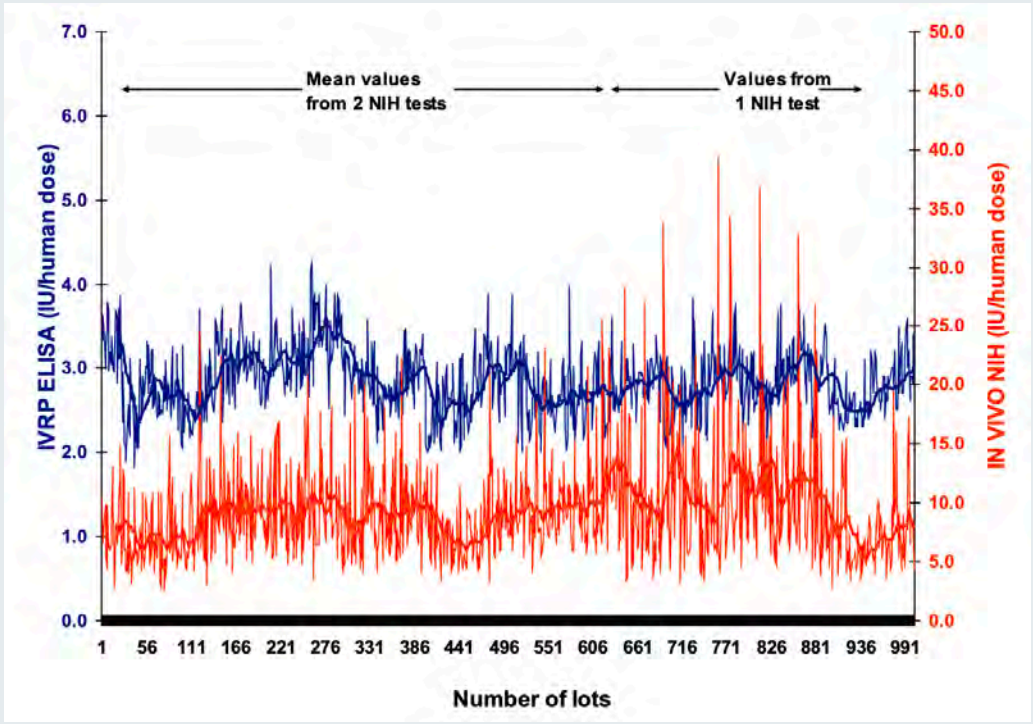
Precautions

- Vaccines or infected cell supernatants to be tested are often inactivated. Nevertheless, samples are considered potentially infectious, and health and safety precautions must be observed as described in Chapter 3 on Biosafety.
- All reagents must be adjusted to the laboratory temperature by waiting 10 min before use.
- Before working, a plan for distribution and identification of samples must be established.

- Reference antigen or vaccine and samples are diluted in tubes and not in the sensitized plate.
- If crude infected cell supernatant is tested, a non-infected cell supernatant should be used to eliminate possible nonspecific reactions.
- The quality of the results depends upon compliance with good laboratory practices. The washings can be carefully carried out with an automatic washer or by distributing manually washing buffer and drying the plate after inverting it on an adsorbant paper after each washing.

Discussion

For more than 1003 batches of human rabies vaccine to be released in the market, the French OMCL (ANSM) has monitored the glycoprotein content using the ELISA method described above and the NIH test performed at the manufacturer's site (Fig. 44.2). Although no correlation has been demonstrated statistically between the two tests, mainly because of the high variability of the NIH test (heterogeneity in mice and challenge procedure *(38)*, a concordance in the profile of results and the same pass or fail conclusions were obtained using in vitro and in vivo assays *(4)*. This concordance is logical since the NIH test evaluates protection of mice from an intracerebral challenge, i.e. the quantity of VNAs induced by vaccination, and the MAb D1 clone recognizes the native trimers of the glycoprotein that constitute the main RAVB immunogen *(36, 39)*. The recognized epitope is located at the level of the antigenic site III which is not only immunodominant for the induction of VNAs but also involved in neurovirulence, pathogenicity *(40–41)* and receptor recognition *(42)*.



Source: reproduced with permission from reference (4)

IU, international units; IVRP, in vitro relative potency; NIH, National Institutes of Health

Fig. 44.2. Comparison of the quantification of glycoprotein content by the ELISA method (blue line) and potency results by the NIH test (red line) for 1003 batches of human rabies vaccines

In summary, the in vitro ELISA method, which specifically quantifies a highly immunogenic epitope of correctly folded glycoprotein trimers, appears as efficient as the NIH test for measuring the capacity of a vaccine batch to induce VNAs that protect against a productive RABV infection. The glycoprotein quantification by ELISA thus mimics in vitro the capacity of rabies vaccine to induce humoral immunity. Additionally, it is able to discriminate sub-potent lots, in quality or in quantity, from potent ones *(4)*. Before proposing that an in vitro ELISA assay measuring the immunogenic glycoprotein could replace the NIH test, it is desirable to organize an international collaborative study for its improvement and standardization.

A workshop of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) entitled "International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing" (Ames, September 2010) *(43)*, concluded that the NIH test should be replaced by an alternative test showing agreement with the immune response and be able to discriminate between potent and sub-potent batches *(4)*. During the following workshop of the European Partnership for Alternatives to Animal Testing (EPAA) in 2012 *(44)*, it was decided that a standardized sandwich ELISA calibrated against the current international rabies reference standard would be an ideal alternative for rabies vaccine potency testing. An international collaborative pre-validation study including both manufacturers and regulatory bodies further compared various ELISA designs used by manufacturers and their national control laboratories for batch release for their ability to discriminate sub-potent from potent batches from different vaccine brands *(37)*. The most appropriate ELISA test remains to be formally validated under the umbrella of the European Directorate for the Quality of Medicines' (EDQM) Biological Standardisation Programme.

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Annex

Buffers and reagents

Coating (carbonate) buffer

Sodium bicarbonate 50 mmol

- $NaHCO_3$ 4.20 g
- Distilled water up to 1000 mL

Sodium carbonate 50 mmol

- $Na_2CO_3,10H_2O$ 14.30 g
- Distilled water up to 1000 mL

Carbonate buffer 50 mmol pH=9.6

Add to the sodium bicarbonate 50 mmol, the sodium carbonate 50 mmol until the desired pH is reached. Prepare fresh as required.

Other buffer

- Bovine serum albumin (BSA) 0.3 g
- Sucrose 5 g

dissolved in 100 mL of carbonate buffer, 50 mmol pH 9.6

Phosphate buffered saline (PBS) pH=7 concentrated 10 times (PBS 10X)

- NaCl 80.00 g
- KCl 2.00 g
- $Na_2PO_4,12H_2O$ 11.33 g
- KH_2PO_4 2.00 g
- Distilled water up to 1000 mL

Adjust pH=7 with 4N NaOH

Washing buffer: PBS-Tween pH=7

- PBS 10X 100.0 mL
- Tween 20 0.5 mL
- Distilled water up to 1000 mL

PBS-Tween-BSA pH=7

- PBS 10X 10.00 mL
- Tween 20 0.05 mL
- BSA (Fraction V) 0.50 g
- Distilled water up to 100 mL

Citrate buffer pH 5.6 for peroxidase substrate

- Tri-sodium citrate, $2H_2O$ ($Na_3C_6H_5O_7$, $2H_20$) 11.67 g
- Citric acid, $1H_20$ 2.17 g
- Hydrogen peroxide 30% (110 vol) 1.00 mL
- Distilled water up to 1000 mL

Substrate–chromogen solution

Ortho-phenylene diamine 50 mg

Citrate buffer pH 5.6 25 mL

Stopping solution: 4 N sulfuric acid

Dilution must be carried out in an ice bath

- Cooled distilled water 80.00 mL
- H_2SO_4, 36N 10.00 mL

Equipment

- Class II Biosafety Safety Cabinet when use of non-inactivated infected supernatant is used
- Laboratory fume hood for preparation of sulfuric acid solution
- Appropriate virucidal solution
- Classical laboratory equipment: refrigerated centrifuge, (multichannel) micropipettes
- 96-well MaxiSorp flat bottom plates in clear polystyrene for immunological assays (ELISA)
- Shaker/incubator for plates used at 37 °C

Microplate washer

- Microplate reader with multichannel absorbance reading (wavelength 492 nm)

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