World Health Organization

Regional Office for South-East Asia

ESTABLISHMENT OF PCR LABORATORY IN DEVELOPING COUNTRIES

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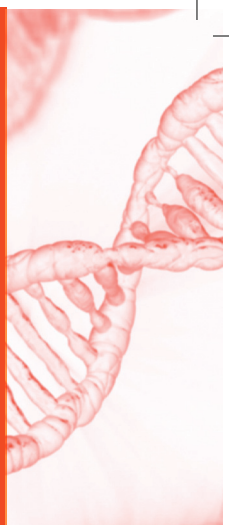
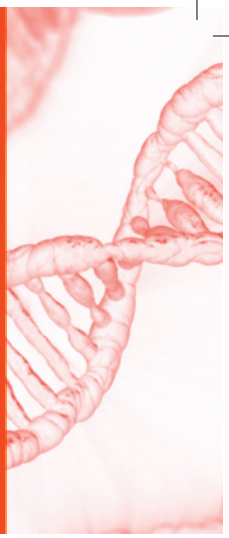


ABBREVIATIONS



BHQ	Black hole quencher
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
Cy5	Cyanine 5
DABCYL	4-dimethylaminobenzene-4'-sulphonyl (4- (4'-dimethylaminophenylazo) benzoic acid)
cDNA	Complementary DNA
Ct	Threshold cycle
Rn	Normalized reporter
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
EBV	Ebstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FAM 6	carboxy-fluorescein
FRET	Fluorescence resonance energy transfer
HHV 6	Human herpes virus 6
HIV	Human immunodeficiency virus
HSV	Human herpes virus
HTLV	Human T lymphotrophic virus
JOE	6-carboxy-4',5'-dichloro-2', 7'-dimethoxyfluorescein

NTC	Non-template control
PCR	Polymerase chain reaction
ROX	6-carboxy-X-rhodamine
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SARS	Severe acute respiratory syndrome
SOP	Standard operating procedures
TAMRA	6-carboxytetra-methylrhodamine
Tm	Melting temperature
VTM	Viral transport medium
VZV	Varicella zoster virus

FOREWORD

Molecular biology has revolutionized modern diagnostic technology in the past two decades. Polymerase chain reaction (PCR) has now become the method of choice in early and accurate diagnosis of most infectious diseases, and has become an indispensable research tool. With this revolutionary, yet relatively inexpensive, molecular genetics technology, it is possible to generate millions of DNA copies from a single strand of DNA.

PCR has significantly helped in early commencement of specific interventions for disease control, and also plays a critical role in understanding disease epidemiology, thereby unravelling the transmission dynamics of the disease.

The results of PCR are reproducible and comparable between different laboratories, and hence are globally accepted. Affordability has further increased its acceptance and utility in developing countries also.

The utility of this technology was amply demonstrated during the influenza pandemic and outbreaks of emerging viral infections such as SARS, Ebola, MERS CoV, ZIKA and many other communicable diseases.

Apart from rapid diagnosis of infectious diseases, PCR can be used in a variety of applications in forensic and medical areas. Likewise, it can be widely used in a variety of research purposes in the field of molecular genetics.

Good laboratory capacity is integral part of strong health system. Availability of PCR as point of care technology has significantly contributed in early detection of diseases which helps in timely prevention, treatment, and containment of diseases. It is now widely recognized that PCR technology has strengthened health systems by firming laboratory services. In days to come the utility and application of PCR is bound to increase manifold.

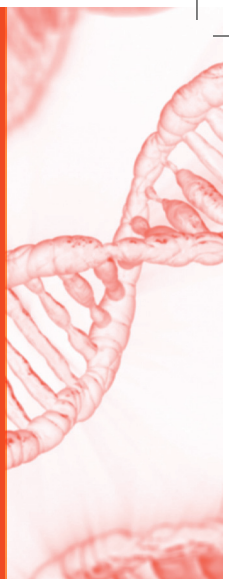
During the past few years, WHO has been providing technical assistance to all Member States in the South-East Asia Region to establish and maintain PCR facilities for public health actions. It is a matter of great pride that all 11 member states of the WHO South East Asia now have national capacity to efficiently apply PCR technology in response to public health emergencies. WHO is also assisting Member States of the Region with tools to assure the quality of the results of PCR.

This document has been developed to assist Member States in expanding their PCR facilities within the country and also in forging networks that can collaborate to further improve the utility of this application for the benefit of people.

We hope that laboratory professionals, medical technicians and technologists, and students and their supervisors will find this document simple, practical and useful.

Dr Poonam Khetrapal Singh
Regional Director
WHO South-East Asia Region
May 2016

1 INTRODUCTION



PCR is the abbreviation for “polymerase chain reaction”. PCR is a method used for amplifying DNA. In April 1983, Kary Mullis stumbled across a process that could make unlimited numbers of copies of genes now known as PCR. Since then, PCR has become popular as a diagnostic tool worldwide .

The name “ polymerase chain reaction” is derived from the deoxyribonucleic acid (DNA) polymerase used to amplify a piece of DNA by in vitro enzymatic replication. This process is known as a “chain reaction” because the original DNA template is exponentially amplified in every cycle of replication. The PCR has been extensively modified and is widely used in molecular biology, microbiology, genetics, diagnostics, and clinical, forensic and environmental laboratories, besides several other applications.

PCR methodologies have clearly evolved over the years from the engineering of thermostable polymerases and construction of automated thermocyclers to newer digital PCR (d-PCR) methodologies and associated microfluidic devices for handling high-throughput PCR applications. However, the fundamental procedure – denature, anneal, extend – has not changed much; countless PCR variations have been created and applied to answer unique biological questions. “Real-time” PCR is a modification that combines the objectivity of fluorescence detection with the simplicity of a basic PCR assay. “Real-time” PCR has earned wider acceptance due to the reduced risk of carryover contamination and improved sensitivity, rapidity and reproducibility. It is accepted as the “gold standard” for diagnosis of several viruses, and quantification of viral load in clinical samples – an indicator of active infection, disease progression and therapeutic response to antiviral drugs. The substantial monetary investment required for “real-time” PCR instrumentation and reagents is a major stumbling block for its routine use in most diagnostic laboratories. However, it is cost effective in high throughput laboratories and can become a feasible option for many other laboratories as more indigenous and less expensive kits/reagents are made available in the future.

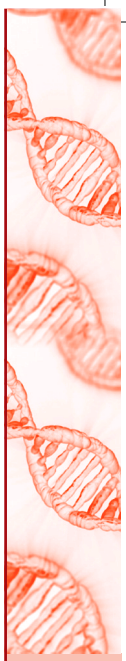
Diseases caused by viruses continue to pose a significant diagnostic and public health challenge worldwide. Since the turn of the century, several new viruses such as Nipah, SARS and H5N1 influenza virus have been identified, while few others, such as West Nile, Chikungunya, Influenza A (H1N1) pdm 09, MERS Corona, Ebola, Yellow Fever and Zika viruses have re-emerged to cause epidemics. Most laboratories, especially in developing countries, were ill-equipped to perform the PCR-based assay recommended for diagnosis of these infections. The positive aspect, however, is that several laboratories in developing countries were upgraded with infrastructure and technical training to perform PCR-based diagnosis of the pandemic influenza virus.

Any virus, including those that are fastidious or noncultivable, can potentially be detected using this technology; multiple pathogens can be identified in a single sample, using a single test (multiplex PCRs). Since only a small volume of the sample is required, PCR is especially valuable for clinical specimens such as CSF and ocular fluids that are usually available in limited quantity. An additional advantage of PCR is its ability to detect the viral nucleic acids even when the viability of virus is lost, most often due to storage or transport at inappropriate temperatures. Once established for viral diagnosis, the capacity of a PCR laboratory can be expanded to include diagnostic assays for bacterial, mycobacterial and fungal pathogens as well.

Despite the numerous advantages, the widespread acceptance and use of nucleic acid amplification techniques such as PCR have revealed several potential shortcomings. Standardized procedures for amplification methods are not yet widely available, and dramatic interlaboratory variability in test results using the same methods is not uncommon. Amplicon contamination in PCR laboratories continues to pose a significant problem. Due to the high sensitivity of these assays, low levels of clinically insignificant pathogens may be detected and mislead clinicians. These challenges underline the need for laboratories to recognize the benefits and limitations of each test and provide the appropriate interpretation of test results to clinicians. It is also essential for laboratories to participate actively in interlaboratory quality control programmes and communicate with each other to address problems such as standardization and optimization of PCR assays on a continual basis.

Scope

This manual is designed to offer a basic knowledge of the principles and utility of PCR-based assays for diagnosis of viral infections. It includes a few protocols of commercially available nucleic acid extraction kits and PCR assays, as well as the protocols of PCR assays developed, standardized and available for routine diagnosis at the Department



of Neurovirology, National Institute of Mental Health and Neuro Sciences (NIMHANS) in Bangalore, India.

Each laboratory must develop its own standard operating procedures (SOPs) depending on the diagnostic tests they can offer. The choice of molecular diagnostic tests such as PCR depends on the endemic diseases/objective of the laboratory and type of patients that the hospital/laboratory caters to (e.g. primary health care and specialized health-care facilities for particular disorders/diseases/priority areas of national laboratories structure, etc.), the purpose of testing (screening, diagnosis, therapeutic response/drug resistance, epidemiological surveillance, etc.), prevalence of the disease/virus sought, cost effectiveness, as well as the availability of infrastructure, clinicians and technically skilled laboratory staff/staff expertise in the laboratory.

This manual provides primary guidelines to assist clinical and laboratory personnel in developing countries to establish a PCR diagnostic facility, and thereby expand their diagnostic profile.

Development process

First edition of this book was published in 2011. Since then, there have been considerable developments that have occurred in the use of PCR technology and therefore it was thought appropriate to update the manual.

Guidelines development team

WHO wishes to acknowledge the support provided by several experts in drafting, reviewing and finalization of this document (Annex 1).

2 PRINCIPLES OF POLYMERASE CHAIN REACTION

Polymerase chain reaction or PCR is a simple laboratory technique to obtain multiple copies of specific DNA fragments from samples that may even contain only minute quantities of DNA or RNA.

Essential components of polymerase chain reaction

1. **Template DNA** – is the DNA that contains the target sequence of interest to be amplified during the PCR.
2. **Primers** – a pair of synthetic oligonucleotides (forward and reverse primers) that are complementary to the 3' ends of each of the two strands of target DNA.
3. **Thermostable DNA polymerase** – enzyme such as Taq polymerase (originally isolated from thermophilic bacterium Thermus aquaticus) is a vital ingredient of a PCR to catalyse the template-dependent synthesis of DNA.
4. **Divalent cations** – usually Mg^{2+} are required in optimum concentration for the activity of most thermostable DNA polymerases as well as for several other steps in PCR.
5. **Deoxynucleoside triphosphates (dNTPs)** – Equimolar amounts of each dNTP (dATP, dCTP, dGTP, dTTP), which are building blocks used by the DNA polymerase enzyme to synthesize a new strand of DNA.

6. **Buffer solution –** to maintain a suitable ionic environment for optimum activity and stability of the DNA polymerase.

Steps of PCR

The PCR typically consists of three basic steps:

1. **Denaturation:** The first step of a PCR where the sample is heated to separate or denature the two strands of the DNA (>90 ^{0}C).
2. **Annealing:** Following the denaturation step, the reaction temperature is lowered (usually 3-5 ^{0}C below the Tm of primer) to allow the oligonucleotide primers to bind to the single strands of the template DNA
3. **Extension:** The final step of the PCR where the temperature is raised, typically to 72 °C, allowing specific enzymes to synthesize a new DNA strand complementary to the DNA template.

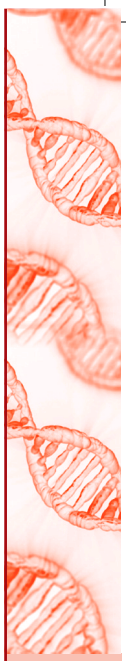
One thermal cycle of these three steps theoretically doubles the amount of DNA present in the reaction. Typically about 25 to 45 cycles of PCR are performed depending upon the type of PCR used, the amount of initial template DNA and the number of amplicon copies desired for post-PCR processing.

The PCR is commonly performed in a reaction volume of 10-200 µl in small reaction tubes (0.2-0.5 ml volumes) in a thermocycler that heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.

Post-PCR analysis/processing

Post PCR detection system must accurately and reproducibly reflect the nature and quantity of the starting DNA template. Specialized methods used in post-PCR analysis are usually tailored depending on specific applications.

The simplest method uses agarose gel electrophoresis. After the electrophoresis, PCR products can be visualized by staining the gel with fluorescent dye such as ethidium bromide, which binds to DNA and intercalates between the stacked bases. Confirmation of size of the DNA product is done by comparing the size with the DNA ladder. The appearance of the discrete band of the correct size may be indicative of a successful PCR amplification.



Other methods used for post-PCR analysis are

1. **Sequencing of the PCR product**
   - Is the gold standard but expensive and not widely available.
   - PCR product may be sequenced directly or cloned before sequencing.
   - However, it is the test of choice in outbreak situations where there are serious public health and/or medical-legal implications.
   - Sequencing can be used to confirm results of other molecular epidemiological assays. As a matter of fact, all other assays can be considered as simpler screening assays.
2. **Restriction Fragment Length Polymorphism (RFLP)** – very simple, rapid and economical technique but the result may be difficult to read.
3. **ELISA** – the PCR product obtained can be detected by performing an ELISA.
4. **Hybridization with a specific oligonucleotide probe** – a wide variety of formats are available e.g. dot-blot, Southern blot, reverse hybridization, DNA enzyme immunoassay etc.

Variations of the basic PCR technique

» **Reverse transcriptase PCR (RT-PCR)**

In reverse transcriptase or RT-PCR, a strand of RNA is initially reverse transcribed into its complementary DNA or cDNA using the reverse transcriptase enzyme. The resulting cDNA is further amplified by PCR. The reverse transcription step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) depending on the properties of the reverse transcriptase enzyme used. The RT-PCR is used for detection of RNA viruses in clinical samples and in gene expression studies.

» **Multiplex PCR**

Multiplex PCR refers to the simultaneous amplification of multiple selected target regions in a sample using different pairs of primers. In this version, multiple primer pairs are employed in the amplification mix so as to facilitate detection of multiple targets. Amplification products are finally differentiated by gel electrophoresis, sequence-specific oligoprobes or in a real-time format, by melting curve analysis. Since multiplex PCR can be used to detect multiple genes of interest in one

specimen, it can minimize the number of separate reactions and help conservation of time, reagents and samples that are of limited volume.

» **Nested PCR**

Nested PCR involves two successive PCRs, where the amplification product from the first PCR reaction is used as the template for the second PCR. Either one of the primers (semi-nested PCR) or both the primers (nested PCR) used in the second PCR may be different from the primers used in the first PCR.

It has been employed to detect organisms present in low copy numbers in specimens, and has the benefits of enhanced sensitivity and specificity, the latter resulting also from a cleaner template provided by the first amplification.

» **Real-time PCR**

Real-time PCR method is used for the detection and quantitation of an amplified PCR product as the reaction progresses in 'real time.' (9)

This new approach of PCR is based on the incorporation of a fluorescent dye where the increase in fluorescence signal, generated during PCR, is in direct proportion to the amount of the PCR product.

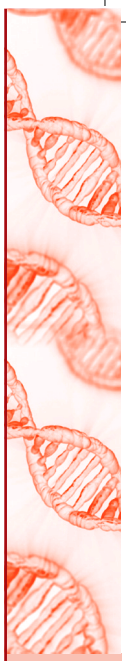
This modification avoids the requirement of a separate amplicon detection step, by employing fluorescent amplicon detection technology (using DNA-intercalating dyes such as SYBR Green or sequence-specific oligonucleotide chemistry such as TaqMan probes). Here, the fluorescent molecules added to the PCR mixture produce fluorescent signals which are detected simultaneously with the progress in amplification. Use of a closed system, reduced turnaround time, dynamic range of target detection, and feasibility for quantitation are a few of the advantages of this method.

» ***In-situ PCR***

The PCR amplification reaction takes place within the cell which is often fixed on a slide. It can be employed for the detection of nucleic acid in small tissue samples. The PCR master mix is directly applied onto the sample on a slide, and then both are covered using a coverslip, and the latter is subjected to amplification in a thermocycler with a slide adaptor or *in-situ* adaptor.

» **Digital PCR (dPCR)**

Digital PCR is a refinement of conventional polymerase chain reaction methods that can be used to directly quantify and clonally amplify nucleic acids including DNA, cDNA or RNA. The key difference between dPCR and traditional PCR lies in the



method of measuring nucleic acid amount, with the former being a more precise method than traditional PCR, though also more prone to error in the hands of inexperienced users. PCR carries out one reaction per single sample. dPCR also carries out a single reaction within a sample; however, the sample is separated into a large number of partitions and the reaction is carried out in each partition individually. This separation allows a more reliable collection and sensitive measurement of nucleic acid amounts. The method has been demonstrated as useful for studying variations in gene sequences – such as copy number variants and point mutations – and it is routinely used for clonal amplification of samples for "next-generation sequencing."

Real-time PCR

The procedure for Real-time PCR follows the general principle of the traditional polymerase chain reaction. However, unlike traditional PCR where endpoint detection of the amplification products is performed, in Real-time PCR the amplified DNA is detected as the reaction progresses. Real-time PCR technology is based on the detection and quantitation of a intercalating fluorescent dye such as SYBR Green, which only emits light upon excitation when bound to double stranded (ds) DNA. Another approach is using fluorescently labeled oligonucleotide probes e.g. TaqMan. These probes bind to their target sequence and fluoresce only when the target-probe interaction has been achieved. Probe-based systems provide highly sensitive and specific detection of DNA and RNA. The probes are attached with both reporter and quencher fluorescent molecules.

Three types of fluorescent molecules can be defined by their function as follows:

» **Reporter –** (donor dye)

The fluorescent signal from the reporter is the one that is monitored during the course of the experiment, e.g. FAM, JOE, SYBR GREEN, CY5

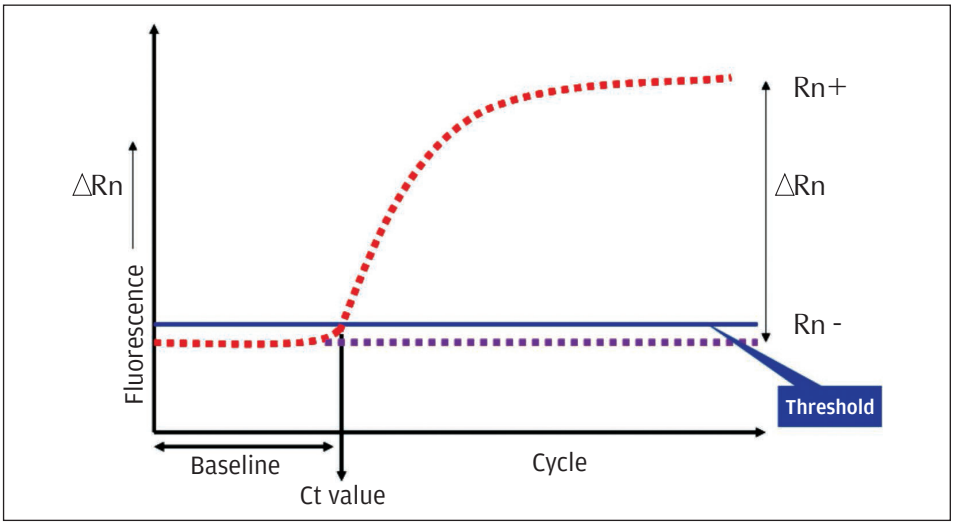
» **Quencher –** (acceptor dye)

It is responsible for the initial quenching of the reporter signal. E.g. TAMRA, DABCYL, BHQ

» **Reference –** (Passive reference dye)

It is common to all reactions, does not interact with the assay components and is used to normalize the signal generated from well to well. E.g. ROX, FLUORESCEIN

Figure 2.1: Real-time PCR Curve



Real-time PCR analysis terminology (Figure 2.1)

X axis: Number of PCR cycles

Y axis: Intensity of fluorescent signal

Threshold: It is the numerical value assigned for each run to calculate the Ct value for each sample. The threshold is generally set in the exponential part of the PCR curve. It is usually 10X the standard deviation of Rn for the early PCR cycles (baseline).

Baseline: The initial cycles of PCR during which there is little change in fluorescence signal (usually cycles 3 to 15).

Ct (threshold cycle): The cycle number at which the fluorescence generated within a reaction crosses the threshold is called the threshold cycle or Ct value. The Ct value is inversely proportional to the starting quantity of template DNA.

Rn (normalized reporter signal): Rn is the intensity of fluorescence emission of the reporter dye divided by the fluorescence emission of the passive reference dye. Rn+ is the Rn value of a reaction containing all components, including the template and Rn- is the Rn value of a reaction that does not contain any template.

ΔRn (delta Rn, dRn): The ΔRn value is determined by the following formula: (Rn+) – (Rn-) and is the magnitude of the fluorescent signal generated during the PCR at each time point.

Real-time PCR chemistry

All chemistries used in real-time PCR allow detection of PCR products by generation of a fluorescent signal. Two types of chemistries commonly in use are

Non-specific fluorescent compounds like SYBR Green that intercalate with any double stranded DNA to emit a strong fluorescent signal (Figure 2.2).

Sequence-specific DNA probes that are oligonucleotides labelled with a fluorescent reporter and quencher moiety, and generate fluorescence only after hybridization of the probe with its complementary DNA target. TaqMan probes, Molecular Beacons and Scorpions are sequence-specific probes that depend on Flourescent Resonance Energy Transfer (FRET) to generate the fluorescence signal (Figure 2.3).

Figure 2.2: SYBR Green chemistry

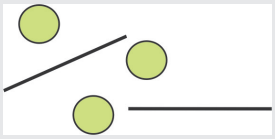
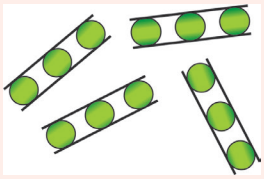
SYBR Green fluoresces when it binds to double- stranded DNA	
When DNA is denatured SYBR Green is released causing a decrease in fluorescence	
As more PCR products are generated SYBR Green binds to more double-stranded DNA causing a net increase in fluorescence detected by the machine	
Advantages • Simple and rapid screening of amplicon accumulation • Relatively inexpensive compared to probe- based assays	**Disadvantages** • Non-specific • Has to be coupled with melt curve analysis to increase specificity. Not ideal for multiplexing

Figure 2.3: The Taqman Probe

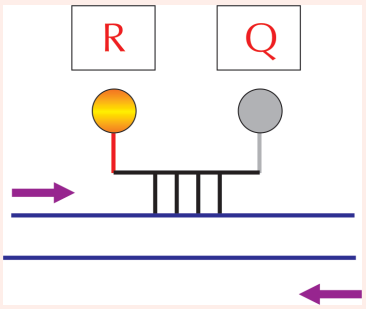
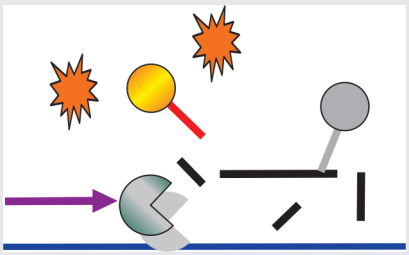
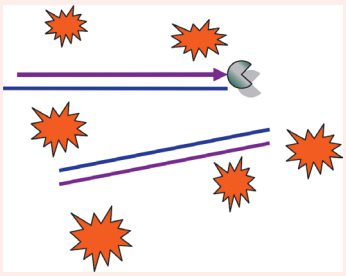
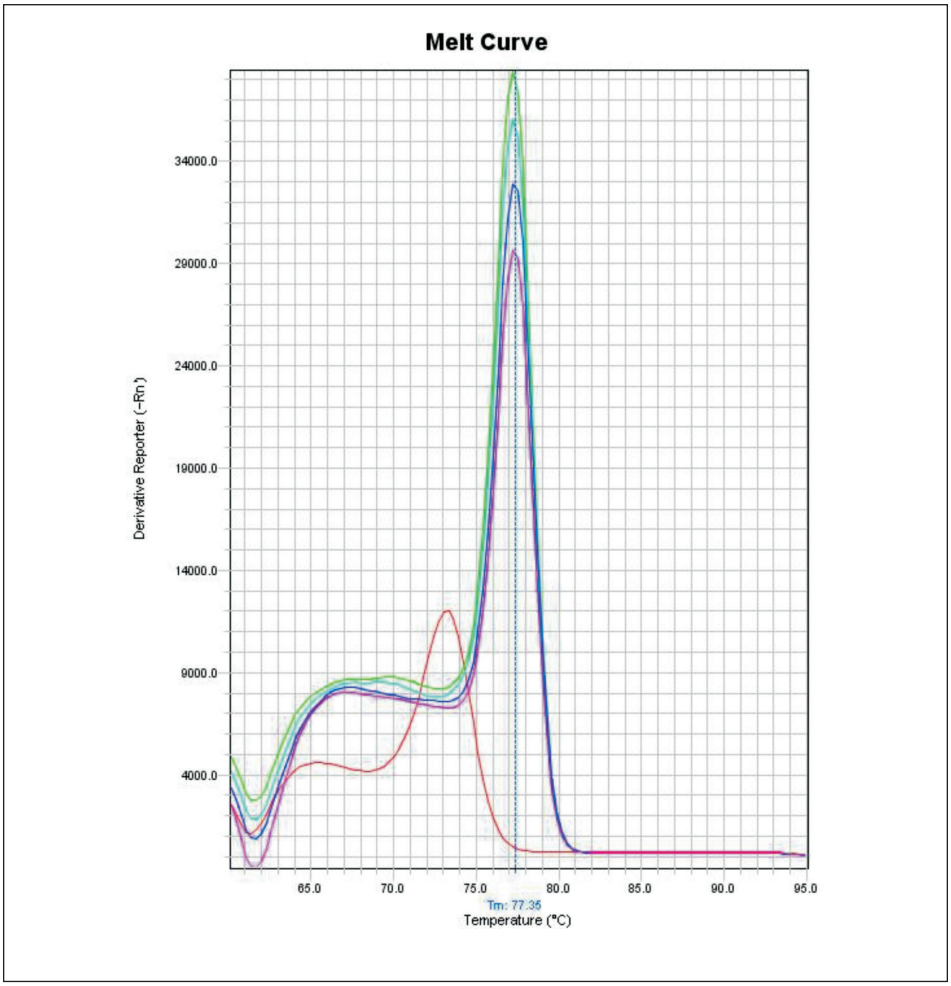
TaqMan reagent-based chemistry uses a fluorogenic probe for detection of specific PCR product as it accumulates during PCR cycles. When the probe is intact, reporter dye (R) emission is quenched due to its proximity to the Quencher (Q)	
During each extension cycle, as the primer extends, the probe is cleaved by the 5' nuclease activity of Taq DNA polymerse, releasing the reporter that generates a fluorescent signal.	
As more PCR products are generated there is a net increase in fluorescence detected by the machine	
Advantages • High specificity • No post-PCR processing required • Ideal for multiplexing	**Disadvantages** • Expensive • Unique probe has to be designed for each target

Figure 2.4: Melt Curve Analysis



Since SYBR Green binds to any double-stranded DNA, after real time PCR amplification, the machine is programmed to perform a melting profile of the products to ascertain the specificity. All PCR products for a particular primer pair should have the same melting temperature (Tm) - unless there is contamination, mispriming or primer-dimer artifact. In this melting curve (Fig 2.4), all samples are run with the same primer pair, but the sample that contained no DNA (the red line) shows a melting curve with a lower Tm compared with other samples; this is probably due to a primer-dimer artifact.

Quantification in real-time PCR

Relative quantification – Relative quantification is commonly used in gene expression studies. A gene of interest in a given sample is compared to the same gene in another reference sample (such as an untreated control sample) and the results are expressed as fold-up or down-regulation of treated versus untreated sample.

Absolute quantification – Absolute quantification is the process that determines the absolute quantity of a single nucleic acid target sequence within an unknown sample (e.g. viral load assays). Serial dilutions of samples with known quantity are amplified to generate a standard curve. An unknown sample can be quantified based on this curve (Figures 2.5 and 2.6).

Figure 2.5: Real-time PCR-absolute quantification amplification plot (linear)

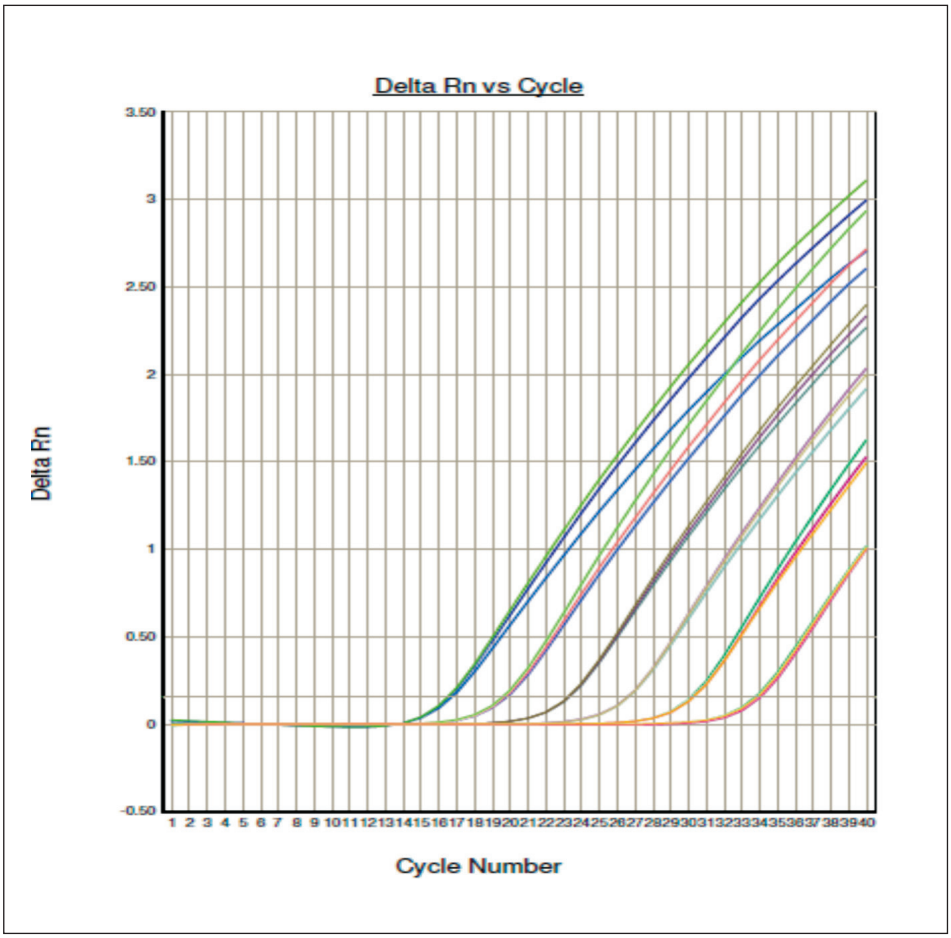
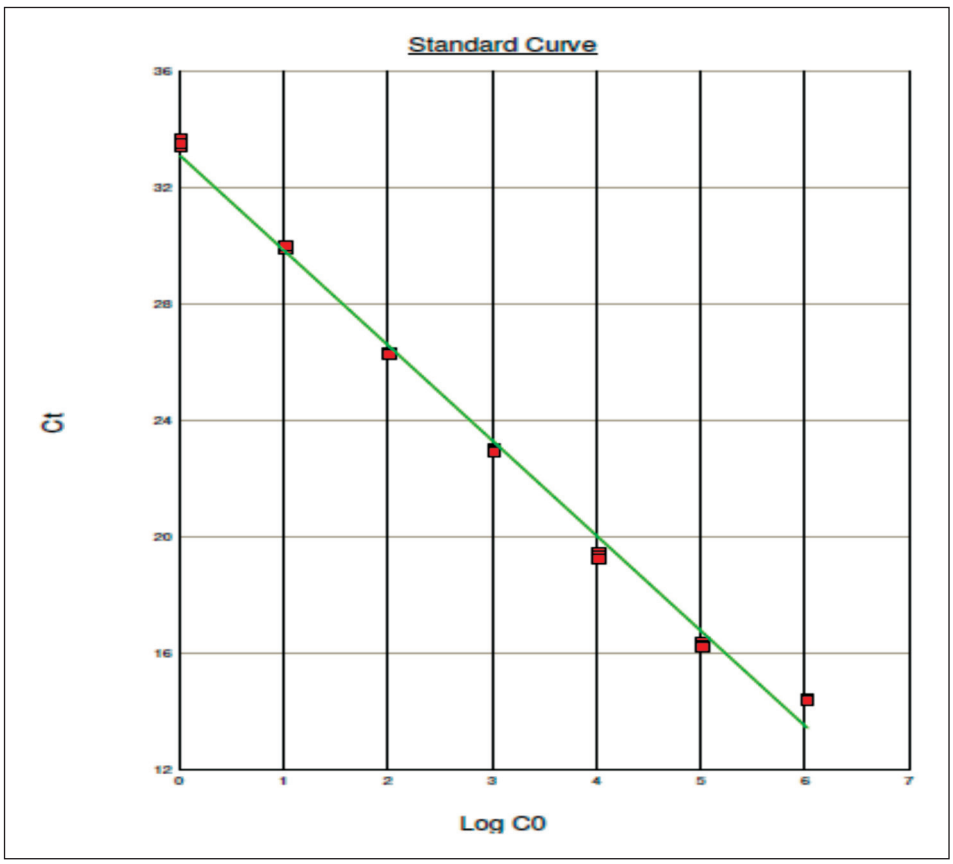


Figure 2.6: Absolute quantification-standard curve



Application, advantages and disadvantages of PCR types

Various applications, advantages and disadvantages are summarized in Table 2.1.

Table 2.1: Applications, advantages, and disadvantages of PCR types

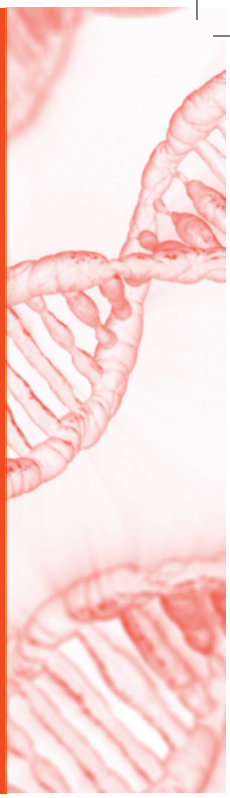
PCR Type	Target	Application	Advantages	Disadvantages
Conventional	DNA	• Amplification and detection of DNA sequences	• Easiest of the PCR types to perform • Low cost of equipment and supplies	• Normally produces only qualitative results • Requirement for post amplification analyses increases time and labour as well as risk of cross contamination and human error • Products should be confirmed by probe hybridization or sequencing
Real-time	DNA	• Amplification, detection and quantification of initial copy number of nucleic acid target	• Rapid potential for relative or absolute target sequence quantification • Usually eliminates requirement for post amplification analyses • Increased specificity because probes or melting curves are used • Totally closed tube analyses creates less potential for cross-contamination	• Requires more expensive equipment and reagents • Less flexibility in primer and probe selection • Less amenable to other downstream product confirmation analyses, such as sequencing due to small amplicon size

PCR Type	Target	Application	Advantages	Disadvantages
Multiplex	DNA	• Simultaneous amplification and detection of two or more different DNA sequences (can be performed as a conventional or real time procedure)	• Amplification of multiple target sequences in a single reaction reduces time and labour requirements	• Less flexibility in primer selection • Requires significant optimization • Generally has lower sensitivity and specificity
Nested	DNA	• Amplification and detection of DNA using external and internal primer sets in sequential steps	• Potentially more sensitive • Decreases the potential for nonspecific amplification	• More likely to produce false positives due to carryover of products from first amplification step • An additional room for sample preparation after the first amplification step is needed
Reverse Transcription (RT)	mRNA, rRNA, viral RNA	• Amplification and detection of RNA	• Amplification of all RNA types	• RNA is sensitive to degradation • Added RT step may increase time and costs as well as potential for contamination

Source: The United States Environmental Protection Agency (EPA), Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples, October 2004

3

STRUCTURE AND FUNCTIONS OF PCR MACHINES



A PCR machine is also called a thermal cycler. It rapidly changes temperatures (heating and cooling) for PCR reactions, thereby allowing the reaction to cycle between primer annealing (50-60 ^{0}C), DNA amplification (72 ^{0}C), and strand melting cycles (94 ^{0}C). The device has a thermal block with holes where tubes holding the PCR reaction mixtures can be inserted. The cycler then raises and lowers the temperature of the block in discrete, pre-programmed steps.

The earliest thermal cyclers were designed for use with the Klenow fragment of DNA Polymerase I. Since this enzyme is destroyed during each heating step of the amplification process, a new enzyme had to be added every cycle. This led to a cumbersome machine based on an automated pipettor, with open reaction tubes. Later, the PCR process was adapted to the use of thermostable DNA polymerase from *Thermus aquaticus,* which greatly simplified the design of the thermal cycler.

While in some old machines the block is submerged in an oil bath to control temperature, in modern PCR machines a Peltier element is commonly used. The Peltier effect bears the name of Jean-Charles Peltier, a French physicist who in 1834 discovered the calorific effect of an electrical current at the junction of two different metals. Another way to understand how this effect could cool a junction is to note that when electrons flow from a region of high density to a region of low density, this “expansion” causes cooling (as with an ideal gas). An interesting consequence of this effect is that the direction of heat transfer is controlled by the polarity of the current; reversing the polarity will change the direction of transfer and thus the sign of the heat absorbed/evolved. A Peltier cooler/heater or thermoelectric heat pump is a solid-state

active heat pump that transfers heat from one side of the device to the other. Peltier cooling is also called *thermo-electric cooling (TEC)*.

Present-day thermal cyclers contain a thermoelectric heat pump, which allows for a solid state solution for temperature control without the need for a constant temperature bath. It is fitted with a heated lid that serves to press against the top of the sample tubes. This feature, in turn, hinders water condensation on the lid and removes the need to top off samples in the tubes with PCR oil. Higher-end thermal cycler models have multiple thermal blocks that make multiple synchronous PCR reactions possible. The gradient thermal cycler allows the user to set different temperatures in specific sections of the thermal block, which is an extremely useful feature when testing appropriate temperatures for the annealing of primers. Quality thermal blocks are constructed from silver to obtain the most uniform temperature throughout the block and the quickest temperature changes.

While choosing a PCR machine, much care has to be exercised to choose the appropriate specifications for the machine being purchased. In particular, the following points should be considered before purchasing a PCR machine. A typical specifications list for a PCR machine may include the following features: (i) temperature range (4 °C – 99 °C), (ii) block heating rate (up to 3 °C/sec, (iii) block cooling rate (up to 2 °C/sec), (iv) block uniformity across wells (ffl 0.5 °C within 15 sec), (v) display resolution (0.1 °C), (vi) maximum programmed dwell time (9 hours 59 minutes 59 seconds), (vii) heated lid temperature range (95 °C – 120 °C), (viii) block modules (preferably silver, with provision for holding 0.2 ml or 0.5 ml tubes/strips), (ix) number of programmes (>50), (x) maximumnumber of programme stages (5), (xi) maximum number of programme steps (5), (xii) auto-restart facility (Yes), (xiii) temperature ramping (Yes), (xiv) pause facility (Yes), (xv) programme naming (Yes), and (xvi) run “end time” calculation (Yes).

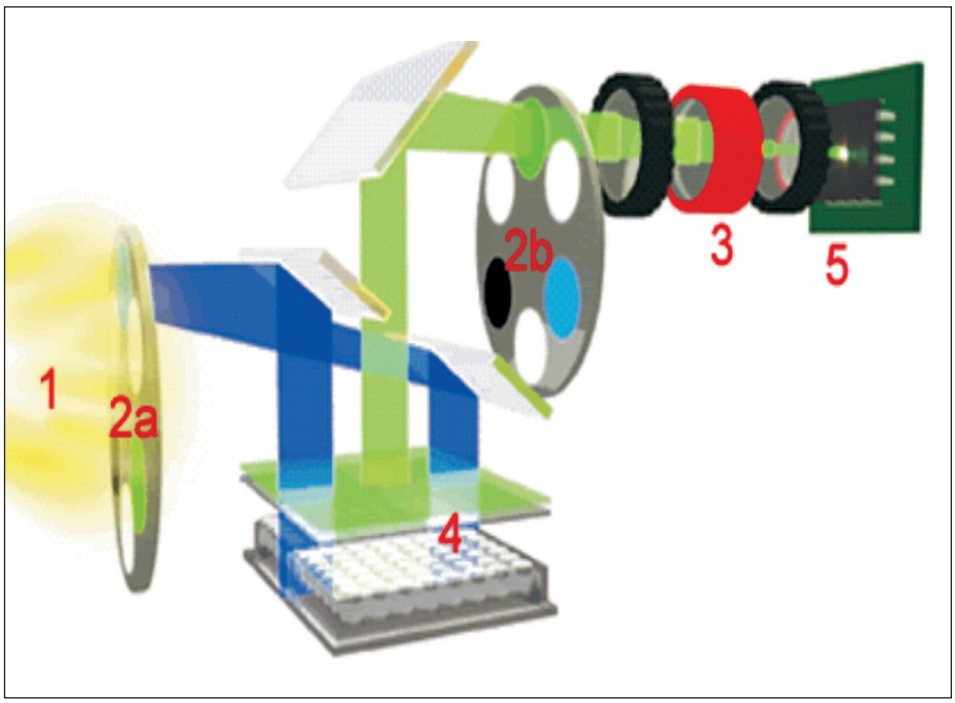
Real-time PCR thermal cycler

The real-time PCR thermal cyclers combine PCR product generation and recognition of the product into one integrated format that allows for the subsequent analysis of the captured data. One of the fundamental differences between a conventional PCR machine and a real-time PCR machine is that no post-PCR processing is involved in real-time PCR technology as the results of the reaction are available on the machine as soon as the run is over. Procedures such as gel electrophoresis and/or ELISA are not required to visualize a PCR result.

To accomplish these two tasks of amplification of DNA and detection of the product, a real-time PCR machine incorporates the traditional PCR thermal cycling technology

along with integrated fluorimeters and detectors that provide the ability to both excite a fluorochrome and detect the emitted light. The second difference is that the real-time PCR machine through a kinetic approach looks at the reaction in the early stages while it is still linear rather than the end-point. Consequently, the design of a real-time PCR machine incorporates a heating block much similar to the one found in conventional PCR machines. In addition, it has a sensitive camera that monitors the fluorescence in each well of the 96-well plates at frequent intervals during the PCR reaction. Figure 3.1 depicts a cross-sectional view of a real-time PCR machine. As DNA is synthesized, more fluorescent dye will bind and as a result the fluorescence signal will increase. This is captured by the camera and converted to analog signals that are plotted by the software as a typical graph wherein the X-axis depicts the number of cycles while the Y-axis depicts the amount of fluorescent signal accumulating with every cycle (Figure 2.5).

Figure 3.1: Representation of optical detection system layout



The real-time machine not only monitors DNA synthesis during the PCR, it also determines the melting point of the product at the end of the amplification reactions. The melting temperature of a DNA double helix depends on its base composition (and its length if it is very short). All PCR products for a particular primer pair should have

the same melting temperature, unless there is contamination, mispriming, primer-dimer artifacts, or some other problem. Since SYBR Green does not distinguish between one DNA and another, an important means of quality control is to check that all samples have a similar melting temperature.

After real-time PCR amplification, the machine can be programmed to do a melt curve (Figure 2.4), in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, and this will peak at the melting temperature (Tm). There are several suppliers of such equipment and these can range in cost depending on the features. For many laboratories, this represents a substantial investment but can result in savings, as the analysis of PCR products can be conducted without running agarose gels, representing savings in both time and money when processing a large number of samples.

There are several platforms available commercially on which real-time PCR machines have been designed. The fundamental difference between platforms is the ability to detect more than one fluorochrome with the addition of multiple excitation and detection channels. Many suppliers provide different models ranging from a relatively low-cost single-channel machine suitable for SYBR Green detection (e.g. Bio-Rad's DNA Engine Opticon) to a multichannel machine (e.g. the 7500 real-time PCR system from Applied Biosystems) that can be used for the detection of multiple fluorochromes at once (multiplexing). Many work best with specific chemistries and fluorochromes, so it is important to consider the cost and chemistry options available for each platform in addition to the cost and capabilities of the various thermal cyclers themselves.

Because real-time PCR reactions are quantified at each cycle by measuring fluorescence, most real-time thermal cyclers need to be calibrated for the particular tube or microtiter plate and microtiter plate seal used, and for the PCR reaction volume. Therefore, it is important to know which real-time thermal cycler will be used for the experiment before setting up the reactions.

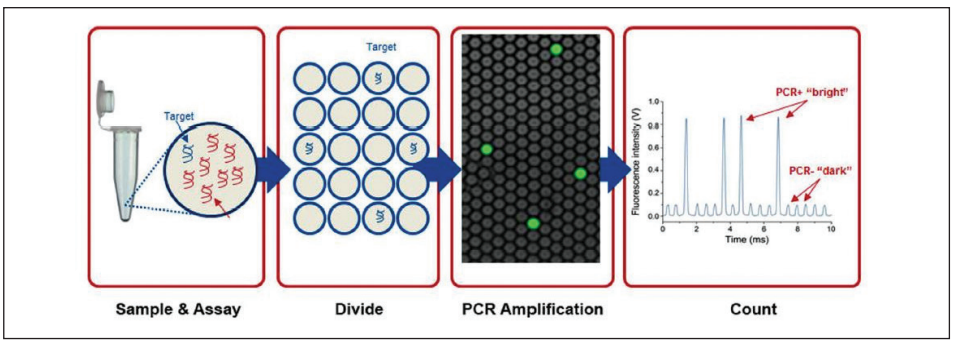
Detecting the PCR product in real-time involves the use of a fluorescent dye. These can be either nonspecific dyes, such as fluorescent DNA-binding dyes (e.g. SYBR Green I) or strand-specific probes (such as Taqman or molecular beacons), many of which use a phenomenon known as fluorescent resonance energy transfer (FRET) to distinguish between various products. For the initial optimization of the real-time PCR amplification, nonspecific fluorescent dyes from the SYBR Green family are most

commonly used, because they are more economical compared with strand-specific probes, and easier to optimize. When using this method of detection, a single, specific DNA fragment has to be obtained during PCR amplification, because any additional nonspecific DNA fragment accumulation will contribute to the fluorescence measured. Strandspecific probes need to be designed if nonspecific bands are amplified in the PCR reaction or if the amplification of more than one target sequence will be monitored in a single PCR reaction.

Digital PCR technology

Digital PCR measurements are performed by dividing the sample and qPCR assay mixture into a very large number of separate small volume reactions, such that there is either zero or one target molecule present in any individual reaction (Fig. 3.2). This is the fundamental concept for making ”digital” measurements. Thermal cycling is performed to end-point. Any target-containing compartments will become brightly fluorescent while compartments without targets will have only background fluorescence. A reaction with no target molecule is counted as a 0 (PCR-negative), and a reaction that has one target molecule is counted as a 1 (PCR-positive). When the entire set of divided reactions is counted, the total number of ‘positive’ reactions is equal to the number of original target molecules in the entire volume, and the total number of reactions multiplied by the individual reaction volume equals the total volume assayed. Thus, the absolute concentration of the target is easily calculated as being equal to the total number of target molecules divided by the total measured volume. Uncertainty in this “absolute” measurement comes only from error in the measured

Figure 3.2: Separation and digital counting provide sensitive, absolute quantification.



Digital PCR is performed by dividing the sample and the assay (e.g. qPCR hydrolysis probe and primers) into enough separate reaction chambers such that any reaction will contain either only 0 or 1 target molecule. Standard end-point PCR is performed and the number of fluorescent reactions counted. PCR-positive, “bright” reactions each contained 1 target molecule, and PCRnegative, “dark” reactions have no target .

volume or the presence of more than a single target molecule in a compartment, so dPCR methods that control for these two factors provide the highest accuracy.

When target molecules are divided into separate reaction compartments, the chances for more than one target molecule to be co-located in the same compartment can be calculated using Poisson statistics. When the number of target molecules is significantly smaller than the number of compartments (low occupancy), the chance of co-compartmentalization is small. Poisson statistics can be used either as a small correction factor (at low occupancy) or to calculate an estimated concentration (at high occupancy). dPCR platforms, which divide the sample into a larger number of compartments, will have the highest accuracy by directly counting single molecules (low occupancy). Similarly, dPCR performed using higher numbers of compartments provides the highest sensitivity—with limits of detection approaching 1 in 1 million, and the widest dynamic range of inputs—over 6 logs.

4

APPLICATIONS OF PCR TECHNOLOGY IN MEDICINE AND ALLIED SCIENCES

Introduction

Polymerase chain reaction (PCR), a revolutionary nucleic acid amplification technology, has brought in a quantum leap in all walks of modern biology. The technique involves amplification of genetic material to billions of copies from minute amounts of starting material in a matter of hours, and at times, even lesser duration. Perhaps no other technique has shown as much promise in as shorter a time-frame as PCR.

The use of PCR in molecular diagnostics has increased to the point where it is now accepted as the gold standard for detecting nucleic acids from a number of origins and it has become an essential tool in the research laboratory. Real-time PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination.

Point-of-care (POC) testing is defined as analytical testing performed outside the central laboratory using devices that can be easily transported to the vicinity of the patient. Microfluidic technologies have enabled miniaturization of PCR processes onto a chip device with potential benefits including speed, cost, portability, throughput and automation, thereby rendering PCR as a POC test. In the developing countries where high infectious disease burden is compounded by diagnostic challenges due to poor clinical laboratory infrastructure and cost constraints, the potential utility of PCR for POC testing is even greater.

Applications of PCR in clinical microbiology

The applications of PCR in infectious disease diagnostics include specific or broad-spectrum pathogen detection, evaluation of emerging infections, surveillance, detection of agents of bioterrorism and antimicrobial resistance profiling. In the field of infectious diseases, the scope of PCR can be recognized under three areas, viz., diagnosis, epidemiology and prognostic monitoring. In this field, PCR can be an attractive strategy as it yields rapid results, has highthroughput along with high sensitivity and specificity. Practically, each type of PCR has applications in all branches of clinical medicine , biotechnology and forensic medicine.

Applications in virology

The PCR is currently being employed in the detection and quantitation of a number of DNA and RNA viruses, including Hepatitis C virus, HIV, Japanese encephalitis virus, human papillomaviruses, chikungunya virus, influenza viruses, rabies virus, cytomegalovirus and JC virus and Ebola. It has become a valuable tool in the diagnosis, clinical management and prognostic monitoring of HIV infection, in particular, and a number of other infections such as Hepatitis B virus. Quantitation of viral load assay to monitor the progress of infection is an important application in this regard.

Another very useful application of PCR in the field of HIV has been in early infant diagnosis, which has been a problem area to date. The PCR also remains the only reliable method of diagnosis for a few infections, as in the case of the most recent H5N1 and pandemic influenza A H1N1 (2009). Amenability of PCR to high-throughput platforms becomes a boon in rapid investigation of such outbreaks.

The PCR-based studies targeting conserved genetic regions of viral pathogens also provide valuable insights into the epidemiological patterns of infections in the areas affected. Conventional PCR methods are now increasingly replaced by real-time PCR techniques for rapid detection of many viruses andalso their advances in the development of fluorophores, nucleotide labelling chemistries, and the novel applications of oligoprobe hybridization have provided real-time PCR technology with a broad enough base to ensure its acceptance.

Recently, instrumentation has appeared that is capable of incredibly short cycling times combined with the ability to detect and differentiate multiple amplicons. New instruments are also flexible enough to allow the use of any of the chemistries making real-time nucleic acid amplification an increasingly attractive and viable proposition for the routine diagnostic laboratory. In many cases these laboratories perform tissue

culture to isolate virus and serological methods to confirm the identity of the isolate, which may take a considerable, and clinically relevant, amount of time.

Recent developments in multiplex real-time PCR have suggested a future in which easy identification, genotyping and quantitation of viral targets in single, rapid reactions will be commonplace. Of course, this technology is by no means restricted to virology, as significant achievements have appeared in the area of mutation detection, applying all the benefits described above to enhance the detection of genetic disease and, where applicable, allow quantification of the extent of such genetic changes.

In diagnostic laboratories, the use of PCR is limited by cost and sometimes the availability of adequate test sample volume. To overcome these shortcomings and also to increase the diagnostic capacity of the PCR, a variant termed multiplex PCR has been described. In multiplex PCR more than one target sequence can be amplified by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising the test utility. Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics, including gene deletion analysis, mutation and polymorphism analysis, quantitative analysis, and RNA detection. In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi and/or parasites. A representative list of such agents utility of multiplex PCR for diagnosis of viral infections is shown in Table 4.1.

Table 4.1: Representative list of applications of multiplex PCR to the diagnosis of infectious diseases

Infectious agent	Pathogen targeted	Clinical manifestation and/ or specimen
Virus	HIV-1, HIV-2, HTLV-1, and HTLV-2	Blood
	HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, and EV	Meningitis, encephalitis, or meningoencephalitis; CSF
Bacterium	*Haemophilus influenzae, Streptococcus pneumonia, Mycoplasma catarrhalis, and Alloiococcus otitidis*	Upper respiratory tract
	Campylobacter jejuni and Campylobacter coli	Human campylobacteriosis
	Actinomyces actinomycetemcomitans, Porphyromonas intermedia, and Porphyromonas gingivalis	Periodontal infection

Table 4.1: Representative list of applications of multiplex PCR to the diagnosis of infectious diseases

Infectious agent	Pathogen targeted	Clinical manifestation and/ or specimen
Bacterium	*N. gonorrhoeae and C. trachomatis*	Genital infections
	C. trachomatis, N. gonorrhoeae, Ureaplasma urealyticum, and M. genitalium	Genital infections
Parasite	*Giardia lamblia and Cryptosporidium parvum*	Diarrheal disease; water
	Leishmania spp.	Leishmaniasis
Combination	HSV, H. ducreyi, and T. pallidum	Genital ulcer disease
	HPVs, HSV, and C. trachomatis	Genital swabs
	Adenovirus, HSV, and C. trachomatis	Keratoconjunctivitis
	EV, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, M. pneumoniae, and C. pneumoniae	Acute respiratory tract infections

Applications in bacteriology

PCR, though not widely employed as a routine diagnostic method in bacteriology, has been helpful in the detection of sexually transmitted bacterial pathogens such as *Chlamydia trachomatis, Neisseria gonorrheae, etc.* Extensive protocols have also been developed for PCR-based detection of Mycobacterium tuberculosis and related species, and also for bacterial pathogens causing meningoencephalitis. Variations of the technique have also been employed in identification and characterization of antimicrobial resistance patterns in several general forms of bacteria (e.g. MRSA, MDR and XDR-TB, “Superbugs” with metallobetalactamase-1 gene).

Applications in mycology

The PCR techniques have also been employed for the detection of a number of localized (e.g. endophthalmitis) and invasive fungal diseases (e.g. aspergillosis). Lack of information regarding the ideal type of specimen to be tested, kinetics of fungal DNA during infections, and issues with standardization of protocols and interpretation of results have been hampering this area of PCR-based diagnostics.

Applications in parasitology

Protocols have been developed for PCR diagnosis of protozoal pathogens of humans and animals, including pathogenic Plasmodium spp., pathogenic amoebae, Giardia spp., Cryptosporidium spp., Microsporidia filarial parasites, etc.

Applications of PCR in noninfectious diseases

The PCR has been applied in the identification of gene mutations associated with malignancies (mutations in oncogenes/protooncogenes, e.g. BRCA1 mutations in carcinoma breast, p53 mutations in a number of tumours, etc.), metabolic errors and psychiatric disorders, etc. Prenatal screening and diagnosis can be attempted with the help of PCR on tissue samples obtained by amniocentesis, chorionic villus sampling or even from rare fetal cells in maternal circulation. Pre-implantation genetic diagnosis is also facilitated by PCR, wherein individual embryonic cells are tested for mutations.

Another major application of PCR has been in the study of gene expressions associated with specific diseases. Qualitative and quantitative data on gene expression profiles can be obtained by PCR-based methods. Both spatial and temporal patterns of gene expression can be studied in tissue samples by PCR-based methods. The PCR has greatly facilitated attempts towards personalized drug therapy. Specific polymorphisms/mutations of genes involved in drug metabolism can have important consequences in treatment outcome. The PCR-based pharmacogenomic methods have been employed in such attempts.

Applications in forensic medicine

Specific examples of PCR applications in this area include resolution of disputed paternity, and personal identification from specimens obtained at crime scenes (e.g. blood stains, hair strands, semen etc.) Variable Nuleotide Tandem Repeats (VNTR), Short Tandem Repeat Polymorphisms (STRPs), and Single Nucleotide Polymorphisms (SNP) are a few markers that are employed for such purposes.

Applications in biotechnology and allied fields

The PCR-based methods have been employed extensively in the field of biotechnology for the production of hybridization probes, development of disease-resistant and high-yield varieties of plants and animals and production of therapeutic proteins, etc. Another interesting application is the production of human vaccines using transgenic plants. Considerable success has been obtained with vaccines for diseases such as rabies and hepatitis B.

5 ESTABLISHMENT OF A PCR LABORATORY

Over the past two decades, the development of the PCR as the basic component of a molecular biology laboratory has occurred very rapidly. The PCR technique became indispensable in laboratories to amplify small amounts of template nucleic acid. Laboratory personnel at the same time, learned that PCR had a strong susceptibility to contamination from its own product. A number of precautions are required to be adopted when designing a PCR laboratory such that the laboratory is operated in a way that prevents contamination of reactions with amplified products from previous assays and cross-contamination between samples, both of which can lead to false-positive results.

Laboratory space arrangement

A PCR laboratory should contain two functional work areas: a pre-amplification area and a post-amplification area. These two areas should ideally be in separate rooms, or when space constraints exist, separate work stations/biosafety cabinets in a single room. Supplies and equipment should be dedicated to each work area and should not be interchanged between areas.

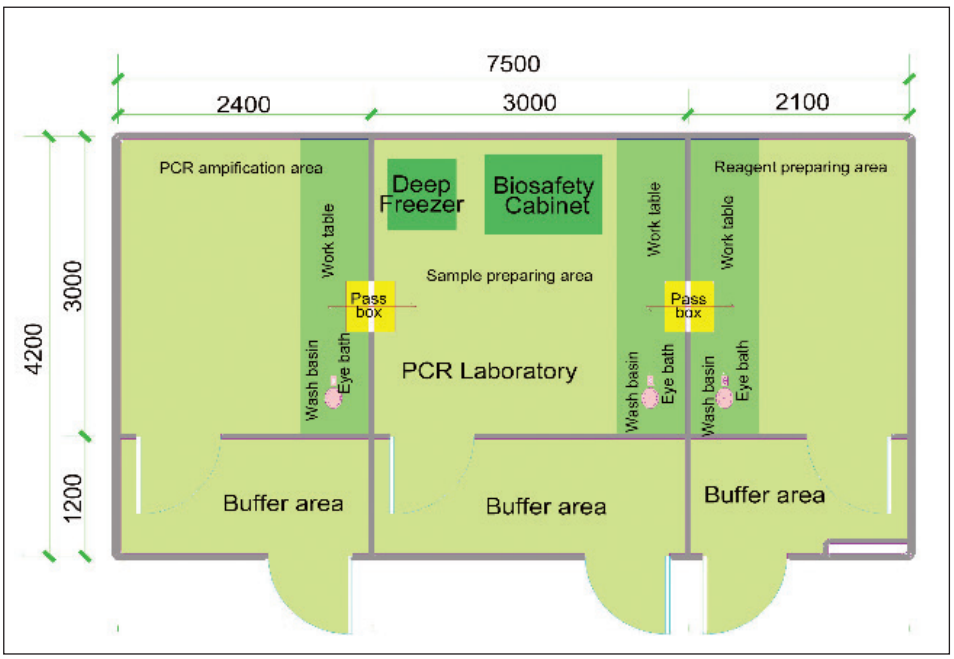
A laboratory performing PCR analyses on diagnostic samples should be divided into at least three physically separate rooms (Figure 5.1):

- Reagent preparation (using positive pressure to prevent the introduction of contamination)
- Sample preparation (using negative pressure to keep template nucleic acids in the room)

- Amplification and product detection (using negative pressure to keep amplified nucleic acids in the room)

A unidirectional workflow should be observed to reduce the chances for contamination to occur. No materials, supplies or equipment from the sample preparation room should be taken into the reagent preparation room. Similarly, nothing from the amplification and product detection room should be taken into the sample preparation room or the reagent preparation room.

Figure 5.1: A sketch depicting the model layout for a PCR laboratory.



Reagent preparation room

The reagent preparation room should be designated for the preparation and storage of PCR reagents. Preparation of master mixes and aliquoting of master mixes to PCR tubes should be performed in this room. To prevent cross contamination and to avoid repeated freezing and thawing, reagent-stock solutions should be aliquoted into smaller volumes. To deter contamination, the room should be under positive pressure. The reagent preparation room should have a dedicated set of adjustable pipettes with plugged, aerosol-barrier tips, laboratory coats and disposable gloves. Personnel should complete tasks in this room before working in the sample processing or amplification/detection rooms and should not return from these rooms to the reagent preparation room.

Sample preparation room

The sample preparation room should be designated for sample processing. This facility should be used for aliquoting of sample and preparation of positive and negative controls. As per the protocol used for extraction of nucleic acid the required quantity of clinical sample should be added to lysis buffer and then transferred to RNA /nucleic acid extraction area. The processed samples and controls are then added to tubes containing the PCR master mix in this room. PCR tubes should be capped as soon as the sample is added.

Workflow in a PCR laboratory

A unidirectional workflow should be used to reduce the potential for contamination.

Reagent Preparation Room
PCR master mix preparation and aliquoting room

↓

Sample Preparation Room
(a) Processing of sample (b) Isolation of nucleic acids
(c) Addition of sample to master mix aliquot

↓

Amplification and Product Visualization Room
(a) PCR thermal cycling (b) Confirmation
i. gel electrophoresis (conventional PCR)
ii. visualization (real-time PCR)

Amplification and product room

This room should be designated for PCR amplification and post-PCR analyses. The thermocycler /real-time PCR machine should be kept in this room. Gloves and laboratory coats should be worn at all times and removed before leaving the room to control amplicon contamination of other locations. All equipment used for amplification and product detection should be dedicated to this room, including adjustable pipettes with plugged, aerosol-barrier tips. This room should be kept under negative pressure. Although PCR amplification and post-PCR analyses may be performed in the same room, these activities can be conducted in different areas or different rooms to reduce the risk of contamination from amplified products.

Equipment in PCR laboratories

To ensure that pre-PCR and post-PCR events remain separated, each room must have its own separate set of equipment, reagents, pipette tips and racks, etc. used in that location only.

Thermocyclers and real-time PCR machines

Thermocyclers are essential to all PCR methods, and great care should be taken to ensure that they are well maintained and reliable. The block temperature of a thermocycler should be tested at least twice a year by the laboratory or under a maintenance agreement to ensure uniform heating throughout the block. Block temperature should be tested with an external probe that has been calibrated against a temperature standard. For testing, the probe is placed in several of the wells in the periphery and centre of the instrument. All temperatures should be within the manufacturers' specifications. The amplification programme used in each run should be printed to further verify the conditions of the PCR.

Real-time PCR instruments are equipped to perform fluorescence excitation and detection to monitor amplification throughout the PCR cycles. The design is usually different from the standard thermocycler, and calibration may be specific to the instrument design. Temperature, laser performance, alignment, and safety devices should be checked and optical systems calibrated. The machine should be serviced annually.

Real-time machines should be used with uninterrupted power supply (UPS) as this equipment is very delicate and, sensitive and also the laser needs to be protected from damage.

Centrifuges

Separate centrifuges, including microfuges, are required for pre- and post-PCR procedures. The manufacturers' instructions for calibration should be followed. The centrifuge should be balanced before use to increase bearing life and minimize vibrations.

Vortex mixer

The vortex is an important equipment required for reagent preparation in the PCR clean room and for nucleic acid extraction.

Gel electrophoresis chambers

This equipment is used to detect the PCR products after amplification.

Chambers should be inspected before each use to ensure that electrodes and buffer tanks are intact, and that power supply electrodes fit snugly. Gel electrophoresis chambers should be rinsed several times with water after each use in the designated product room.

Pipettes

Automatic, fixed-volume, adjustable, positive-displacement pipettes, and/ or micropipettes are used in the PCR laboratory. These should be calibrated quarterly by the manufacturer or a technician. Each pipette should be sterilized according to manufacturers' recommendation on a regular basis or whenever contamination is suspected.

Laminar-flow hoods/Biological safety cabinets (BSCs)

Laboratory users should pay careful attention to the specifications of the hood or cabinet to ensure that it is appropriate for its designated use by the laboratory.

Unit type	UV light	Airflow system	Use
PCR cabinet (Type A)	Yes	None	Reagent preparation only
PCR cabinet (Type B)	No	Intake filtered	Not recommended for any aspect of PCR preparation
PCR cabinet (Type C) / Laminar-flow hood	Yes	Intake filtered	Reagent preparation only
Class I biological safety cabinet	Yes	Exhaust filtered	Not recommended for any aspect of PCR preparation
Class II or III biological safety cabinet	Yes	Intake and exhaust filtered	All aspects of PCR

Class I cabinets have inward air flow and HEPA-filtered exhaust that provides personal and environmental protection, but no product protection. Class II and Class III BSCs

filter both air intake and exhaust, and prevent contaminants from entering and leaving the hood (reducing the likelihood of sample and work area contamination). Before use, hoods should be decontaminated using UV light for at least half an hour and cleaned with bleach or other effective nucleic acid inactivating agent. The airflow and HEPA filtration in all hoods should be monitored and certified as per manufacturers' recommendations at least annually.

Refrigerator

Separate refrigerators for temporary storage of sample, extracted RNA/nucleic acid and final amplification products should be maintained in the respective laboratory. Usually long-term storage is not recommended but if needed separate deep freezers (-80 °C) can be maintained.

Freezer (-20 °C)

PCR clean reagents, enzymes, buffer, dNTPS and primers are required to be stored at -20 °C.The primers, dNTPS and water should be stored in small aliquots to avoid freezing and thawing effects and also to rule out contamination issues.

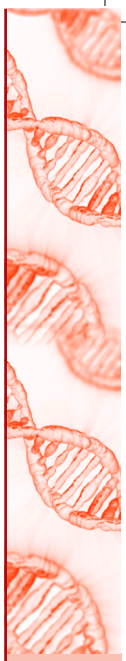
To verify that equipment is functioning properly, the laboratory should have a schedule for maintaining equipment. The schedule should include the set-up, calibration, repair, record-keeping, and normal operation of all equipment used in sample analysis. The results of all tests should be documented in an equipment logbook and/or electronic database. The logbook or database should be checked monthly by quality control (QC) personnel or the laboratory supervisor, and any problems and corrective actions managed. Equipment should be dedicated to a specific laboratory room, and instrument manuals from the manufacturer should be available.

Consumables

Disposable materials used in a PCR laboratory include pipette tips, sample tubes, PCR tubes and gloves. To reduce the contamination and degradation of the target nucleic acids, disposable materials should be of good quality.

Pipette tips

Special tips for PCR analysis include barrier tips and aerosol-resistant tips, both of which minimize cross-contamination of samples during pipetting. These tips can be purchased pre-sterilized and pre-loaded in hinged racks to provide tip protection and easy access. Pipette tips for PCR analyses should be RNase-free, DNase-free, and pyrogen-free.



Sample and PCR tubes

Polypropylene tubes that are certified DNase-, RNase-, and pyrogen-free are best recommended for PCR laboratories. The size and style of PCR tubes or reaction plates should be compatible with the block and lid height of the thermocycler/real-time machine. Thin-walled tubes provide the best heat transfer, ensuring that the reaction volume reaches its specified temperature in the shortest amount of time, thereby improving specificity and reproducibility. Tubes containing stored samples and reagents should be centrifuged briefly before opening to ensure that all liquids are at the bottom of the tubes.

Laboratory wear

Disposable gloves should be available in each section of laboratories used for PCR analysis. Gloves should be changed before leaving and entering each section of the laboratory and each time that contaminating DNA is potentially encountered. In addition to reducing potential contamination from samples, wearing gloves may protect the technician from potential chemical exposure and prevent sample contamination due to human DNases and RNase.

Dedicated laboratory coats should be available in each laboratory room. Laboratory coats should be removed and gloves discarded appropriately before leaving each room. Changing laboratory coats and gloves reduces the possibility of contamination with template or amplified nucleic acid. Laboratory coats should be cleaned regularly to reduce the possibility of contamination of the designated workspace and the PCR reaction. To eliminate the need for cleaning laboratory coats, disposable (single-use) laboratory coats may also be used.

Reagents

The reagents used in PCR amplification can be purchased or prepared inhouse. All reagents should be clearly labelled with name, expiration date, and relevant safety information. Reagents from different lot numbers should not be interchanged or combined together. Precautions should be taken to ensure that reagents are contamination free and storage conditions are well maintained.

Molecular-grade water or its equivalent from commercial sources should be used for all assays. Water purification systems that produce high-quality pyrogen and DNase/RNase-free water may be used. Diethylpyrocarbonate (DEPC) treatment can be used to eliminate RNase from plastic ware and from water which is required in RNA analysis. Reagent water is treated with a solution of 0.1% DEPC for several hours and

then autoclaved to degrade the DEPC completely. Proper autoclaving is necessary, because trace amounts of DEPC in a solution will lead to the modification of the purine residues in RNA by carboxymethylation. This leads to downstream effects in RNA experimentation (e.g. removing the ability of reverse transcriptase to bind RNA and synthesize DNA from an RNA template).

Commercially prepared reagents should be of molecular grade and should be stored according to manufacturers' recommendations. All reagents from new lots should be tested to ensure that they work properly by running a PCR positive control using the new reagents.

For reagents prepared in-house, criteria should be developed for expiration dates, functional acceptability, and storage conditions using product sheets from similar commercial products as guidance. The criteria should be documented in the laboratory standard operating procedures (SOP). Buffers should be inspected for precipitates or microbial contamination before each use.

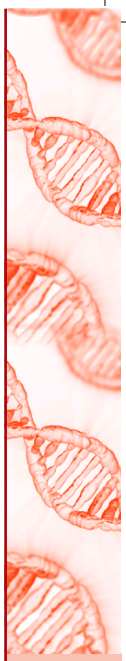
Commercially available kits

Many types of commercial kits are available for PCR applications. These products expedite and simplify procedures, such as the isolation of DNA and RNA and the purification of nucleic acids to remove contaminants.

Primers and Probes (oligonucleotides)

The PCR analyses require the use of short segments of chemically synthesized DNA (oligonucleotides or oligos). Primer sets are oligos that are designed specifically to prime the amplification of a portion of a target nucleic acid of interest. Primers and probes, containing a specific sequence of nucleotides, can be obtained commercially. Certification of the quality of the oligos, including method of purification, purity, and concentration, should be required from all commercial manufacturers.

Storage: Most oligos and DNA templates should be stored at -20 °C or -70 °C in either TE buffer (10 mM Tris-HCl and 0.1mM EDTA, pH 8.0) or molecular grade water. The TE buffer generally is the preferred storage buffer for oligos and DNA templates, because it may prevent DNA degradation. The pure, concentrated oligos should be stored in the original tube from the manufacturer and labelled with the primer name and concentration. To minimize the chance of contamination and degradation, these concentrated stocks should not be used on a regular basis. Diluted working stocks should be made for each oligo, and these working stocks should be used for all experiments. Before use, oligos should be thawed and mixed completely.



Enzymes

Enzymes are the critical requirement of a PCR and should be purchased from a commercial source to ensure purity. Quality assurance (QA) information with the enzymes should be obtained from the source. Each new lot of enzyme should be compared with old lots using known controls and samples.

Storage: The manufacturer's instructions on enzyme storage and use should be followed carefully. Enzymes are usually stored at -20 °C, and should never be left at room temperature. Insulated bench-top coolers or ice can be used to keep the enzyme cold in the laboratory, when being used on the bench top. PCR products may also be stored at -20 °C or -70 °C. RNA templates should be aliquoted and stored at -70 °C.

Personnel

Personnel working in a PCR laboratory should undergo training in the methodology that covers PCR and recombinant DNA theory and practice. The course work should also include biosafety in a PCR laboratory as well as quality issues and troubleshooting PCR-related problems. Hands-on training should be completed for each technique under the supervision of experienced personnel. The time required for training will vary depending on the trainee and technique. Personnel should demonstrate that they can successfully perform the method by testing confirmed positive and negative control samples before being allowed to analyse diagnostic samples.

6

SAMPLE COLLECTION, STORAGE AND SHIPMENT OF CLINICAL MATERIAL FOR DIAGNOSIS OF VIRAL INFECTIONS BY PCR

Laboratory diagnosis of viral infections requires a good understanding of the pathogenesis of the suspected virus, the type and duration of illness, stage of infection, age and immunization status of patient and the occurrence of outbreaks of a similar illness in the community to determine the most appropriate specimen and diagnostic test. Even a well performing molecular assay cannot restore the loss or degradation of viral nucleic acids due to inappropriate collection, handling or transport of the sample. Hence every laboratory should devise protocols to describe sample collection,

transportation to the laboratory, sample acceptance/rejection criteria and storage of sample and extracted nucleic acid prior to testing by molecular methods such as PCR.

Selection of appropriate specimens

Clinical symptoms can identify the target organ, where the highest level of viral replication usually occurs in an active viral illness, and help determine the appropriate specimens to be collected. The type of specimens to be collected for PCR diagnosis in common viral infections is summarized in Table 6.1.

Table 6.1: Specimens to be collected for diagnosis of viral infections by PCR

No.	System Involved	Disease/ Syndrome	Organism	Specimen(s)
1.	**Central Nervous System**	Encephalitis	HSV 1&2, CMV, VZV, EBV, HIV, Measles virus, Mumps virus, Rabies virus, Arboviruses, JC Virus	CSF Brain tissue (biopsy/ autopsy)
		Meningitis	Enteroviruses, HSV, Arboviruses, Mumps virus	CSF
2.	**Respiratory System**	Upper and lower respiratory tract infections	Influenza Viruses (A,B,C) Parainfluenza viruses, RSV, Adenoviruses, Rhinoviruses, Human coronaviruses, Human metapneumoviruses, Human bocavirus	Nasopharyngeal swab/aspirate Throat swab Nasal wash Broncheo-alveolar lavage Tracheal aspirate Sputum Pleural fluid Lung biopsy
3.	**Skin**	Vesicular/ Petechial rash	Adenoviruses Enteroviruses HSV HHV-6 VZV Measles virus Rubella virus Parvovirus B19	Vesicular swab/ aspirate Skin scraping/ biopsy Nasopharyngeal aspirates Throat swab

No.	System Involved	Disease/ Syndrome	Organism	Specimen(s)
4.	**Gastrointestinal System**	Gastroenteritis	Rotavirus Astrovirus Calicivirus Enteroviruses Adenovirus CMV	Stool Rectal swab
		Hepatitis	Hepatitis virus A,B,C,D,E, G Transfusion Transmitted virus (TTV) CMV EBV Parvovirus B19	Blood Serum Tissue specimen (biopsy/autopsy)
5.	**Urogenital System**	Haemorrhagic cystitis, Urethritis Cervicitis Genital warts Cervical carcinoma	Adenovirus BK virus HSV VZV HPV	Urine Urethral discharge/ swab Cervical swab Tissue biopsy
6.	**Ocular Tract**	Conjunctivitis Keratoconjunc-tivitis Chorioretinitis	Adenovirus Enterovirus HSV CMV VZV	Corneal scrapings Conjunctival swabs/ scrapings Aqueous/Vitreous fluids
7.	**Haematopoietic System**	Lymphoid disorders Disorders of red blood cells	EBV HIV HTLV-1 Parvovirus B19	Blood Bone marrow aspirate
8.	**Congenital/ Perinatal infections**	Multi-system involvement	CMV HBV HIV Parvovirus B19 Rubella virus	Blood Serum/Plasma Amniotic fluid Tissue biopsy

Specimen collection and storage

For many viral infections viral shedding begins before the onset of symptoms and decreases after the acute phase of illness. Since the possibility of detection of virus/viral nucleic acid is high during the early stages of the infection, specimens for diagnosis of viral infections by PCR must be obtained within one to three days of onset of symptoms.

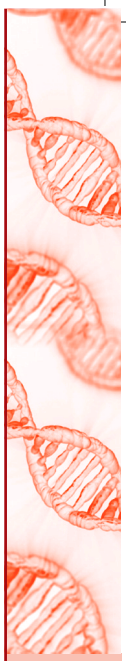
The success of molecular diagnostic methods such as PCR is essentially dependent on the quality of nucleic acid purified from a clinical specimen, which in turn is directly related to how the sample has been stored and/or transported after collection.

All samples to be processed for molecular diagnosis of any viral infection by PCR should be obtained under strict aseptic precautions. Each specimen should be placed in a separate sterile, nuclease free container labelled with the patient's name and identification number, the collection site, and date and time of collection. Swabs, scrapings and small pieces of tissue are placed in a container with 2-3 ml of viral transport medium (VTM). The VTM consists typically of a buffered salt solution to maintain appropriate pH and osmotic environment, protein such as bovine serum albumin to stabilize the virus, and antibiotics to prevent microbial contamination. It prevents the specimen from drying and helps maintain viral viability. Specimens like CSF, blood, urine, broncheoalveolar lavage fluid, vesicle fluid and stool specimens can be sent without transport medium.

The best yield and quality of DNA/RNA is obtained from a fresh specimen; however, if samples cannot be processed immediately, they must preferably be snap-frozen in liquid nitrogen and stored at -70 ^{0}C. Repeated freezing and thawing can lead to reduced yield of viral nucleic acid and should be avoided.

Compared with DNA that is very stable, RNA is extremely labile and susceptible to degradation by RNases, which are ubiquitous. Since the human skin is a common source of RNases, use of gloves while handling and processing samples and strict adherence to specimen storage temperatures can ensure a yield of good quality RNA from the sample.

Preservation of the integrity of viral nucleic acids in specimens during shipping and handling is crucial for PCR. An economical and convenient method is described for nucleic acid stabilization by using an RNA stabilizing solution (e.g. RNAlater) in plasma that is designed for the shipment of samples in tropical countries.



Special considerations for some common clinical specimens

Swabs

Sterile dacron or rayon swabs with plastic shafts or if available, flocked swabs should be used. Calcium alginate swabs or cotton swabs with wooden sticks should not be used, as they may contain substances that inactivate some viruses and inhibit some molecular assays. Swab specimens in VTM can be refrigerated for up to four days before processing. For long-term storage prior to nucleic acid extraction, the swab is swirled in the VTM fluid, squeezed off the fluid inside the tube and discarded. The VTM is then stored in aliquots at -70 ^{0}C.

Blood and bone marrow aspirate

Whole blood samples/bone marrow should be collected in EDTA tubes. Heparin should not be used as an anticoagulant since it is known to interfere with Taq polymerase enzyme used in PCR. Whole blood samples can be stored in the refrigerator at 2-8 ^{0}C for up to 48 hours before they are processed for nucleic acid extraction. Freezing of whole blood is not recommended since disruption of the RBCs results in release of large amounts of PCR inhibiting haem. For long-term storage of whole blood samples, preliminary purification steps such as lysis of RBCs using ammonium chloride followed by purification of leucocytes or separation of leucocytes by gradient sedimentation methods can be done. If serum/plasma is required for PCR, centrifugation of blood sample should be ideally done within six hours of collection. After centrifugation, serum/plasma samples can be stored at 2-8 ^{0}C for up to six hours and for long-term storage, in aliquots at -70 ^{0}C until they are processed for nucleic acid extraction.

An alternative specimen type to whole blood/ plasma whose use is more practical in the field in resource-limited settings is dried blood spots (DBS). DBS require a smaller volume of blood than plasma or serum, and their usage minimizes the need for cold-chain storage and transportation requirements. In addition, DBS specimens, once completely dried, are considered noninfectious and nonhazardous, allowing them to be shipped at a reduced cost since they can be transported at ambient temperature using a standard courier service. The use of DBS has been well established for Early Infant Diagnosis of HIV for the detection of HIV nucleic acid by PCR.

CSF and other sterile body fluids

CSF and other body fluids are collected in sterile, nuclease free, leak-proof containers. VTM is not recommended since dilution of sterile body fluids such as CSF, pericardial fluid, pleural fluid, aqueous and vitreous fluids, etc. is contraindicated. However since

these fluids, especially CSF, aqueous and vitreous fluids are known to contain proteins and other uncharacterized substances that can inhibit a PCR, extraction of nucleic acids is recommended before PCR testing.

Tissue specimens

Solid tissue samples obtained by biopsy/autopsy that have to be transported to another location should be cut into small pieces and placed in RNAlater (Ambion) solution to stabilize the cellular RNA in the tissue. Tissue specimens have to be minced and treated with proteolytic enzymes before extraction of nucleic acids. Nucleic acids can also be obtained from formalinized, paraffin embedded tissues after deparaffinization and extraction.

Table 6.2: Long-term storage of extracted nucleic acids

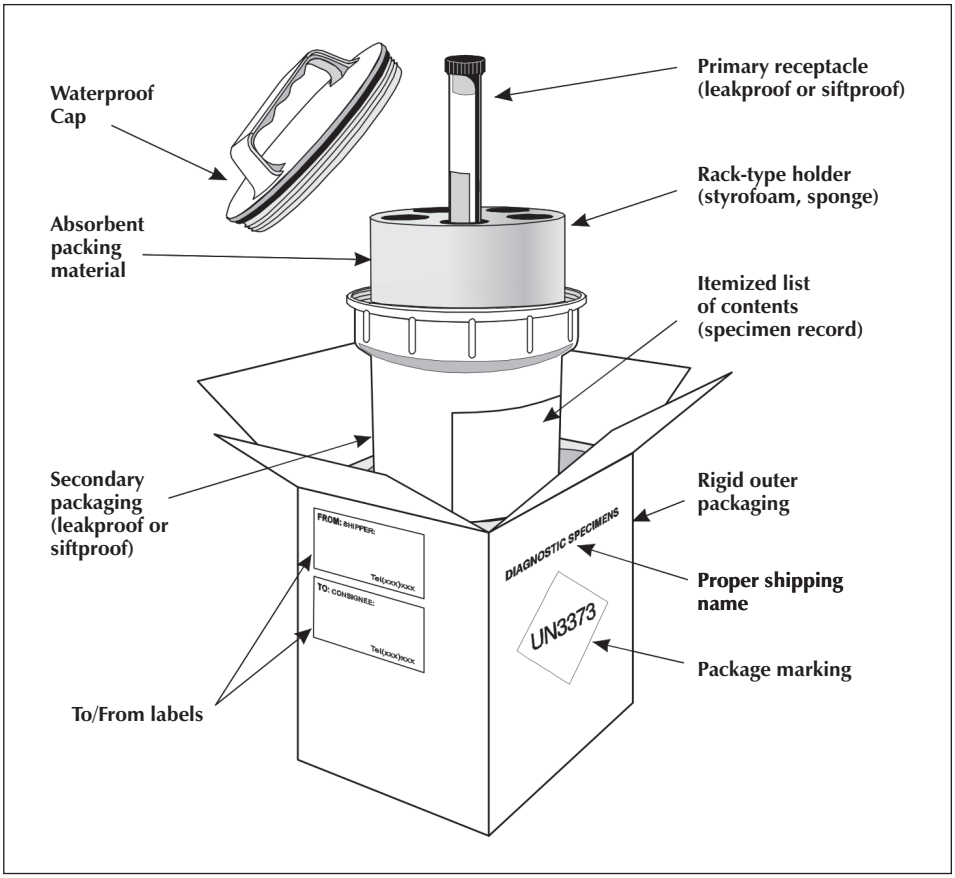
Nucleic Acid	DNA	RNA
Storage medium	1. TE Buffer-(Tris-EDTA Buffer) (10 mmol/L Tris, 1mmol/L EDTA, pH 7.4-8.0) 2. Sterile nuclease free water	1. Store RNA as precipitate in ethanol 2. TE Buffer 3. Sterile nuclease free water
Storage method	Divide DNA into small aliquots and store in sterile, nuclease free tubes.	Divide RNA into small aliquots and store in sterile, nuclease free tubes or diethylpyrocarbonate (DEPC) treated tubes. RNA can be reverse transcribed to cDNA and stored for better long term stability.
Storage Temperature	Recommended -70^{0}C Store at -200C if -70^{0}C not available Avoid repeated freeze-thaw of samples.	

Transport of specimens

Place the tightly capped and labelled specimen container and the appropriate laboratory requisition form into separate compartments of a plastic specimen 46

Establishment of PCR laboratory in developing countries transport bag. Appropriate packaging ensures safety from biohazardous specimens and prevents introduction of microbial contaminants during transit. (Figure 6.1) All specimens should be delivered to the laboratory as soon as possible after collection, since loss of infectivity occurs over time; though viability of the virus is not essential for molecular assays like PCR, the same sample may be required for viral culture or other assays.

Figure 6.1: Packing of specimens for transport to laboratories



If immediate delivery is not possible, refrigerate specimens (2-8 ^{0}C), or place them on wet ice or a cold pack. Loss of viability is slower at refrigeration temperature. As far as possible, do not freeze samples. If samples must be frozen, freeze them rapidly at -20 ^{0}C or -70 ^{0}C.

7 CONVENTIONAL PCR PROTOCOLS

Protocol 7.1 – RNA extraction using QIAamp Viral RNA Mini Kit (Qiagen)

Introduction

The first step to any PCR is the nucleic acid extraction procedure. A high yield of purified RNA is vital for increasing the sensitivity of a PCR.

Principle

The QIAamp Viral RNA Mini Kit (Qiagen) is designed for fast and easy extraction, and purification of viral RNA from clinical samples. After optimal lysis, the sample is loaded onto the QIAamp mini spin column and the RNA that is absorbed onto the QIAamp silica membrane is washed for removal of any residual contaminants. Purified RNA is eluted from the QIAamp mini spin column in a concentrated form in a RNase-free buffer for direct use in PCR.

Requirements

Equipment	Consumables	Reagents
• Refrigerator • Freezer (-700C) • Micro pipettes • Microcentrifuge • Timer • Vortex mixer	• Tissue paper • Disposable gloves • 70% Ethanol • 1.5ml microcentrifuge tubes • Sterile, nuclease-free filter tips • Ethanol (96-100%) • Marker pens	**QIAamp Viral RNA Minikit (Qiagen)** • QIAamp Mini Spin columns • Collection tubes (2ml) • Buffer AVL • Buffer AW1 (Concentrate) • Buffer AW2 (Concentrate) • Buffer AVE • Carrier RNA

Storage of reagents

All QIAamp Viral RNA Mini Kit reagents should be stored at room temperature, ethanol (96-100%) at 4 ^{0}C, reconstituted aliquots of carrier RNA at -20 ^{0}C.

Test procedure[1]

» When using a new QIAamp Viral RNA Mini kit add alcohol to buffers AW1 and AW2 as indicated on the bottles.

» Reconstitute carrier RNA (310 µl of AVE into tube containing 310 µg Carrier RNA). Aliquot and store at -20 ^{o}C.

» Determine the number of samples and prepare working AVL buffer by mixing 560 µl of AVL and 5.6 µl of reconstituted carrier RNA for each sample.

» All centrifugation steps should be carried out at room temperature.

» Pipette 560 µl of working AVL buffer into a 1.5 ml microcentrifuge tube.

» Add 140 µl of the sample and vortex the tube briefly.

» Incubate at room temperature (15–25 ^{o}C) for 10 minutes.

» Centrifuge briefly to remove drops from inside the lid.

» Add 560 µl of ethanol (96–100%) to the tubes. Vortex tube briefly.

» Centrifuge to remove drops from inside the lid.

» Pipette 630 µl of the mixture into the QIAamp Spin column placed in the 2 ml collection tube without wetting the rim, close the cap.

» Centrifuge at 8000 rpm for 1 minute. Discard the collection tube containing the filtrate.

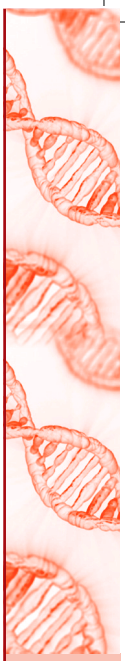
» Place the spin column into fresh collection tube.

» Add the remaining 630 µl of the mixture into the spin column.

» Centrifuge at 8000 rpm for 1 minute. Discard the collection tube containing the filtrate.

» Place the spin column into fresh collection tube.

[1] Handbook – QIAamp Viral RNA Mini and Blood Mini Kit. (Qiagen)



» Add 500 µl AW1 buffer into the spin column and centrifuge for 1 minute at 8000 rpm. Discard collection tube containing the filtrate.

» Place mini spin column into a fresh collection tube and add 500 µl of AW2 buffer.

» Centrifuge for 3 minutes at 14 000 rpm to completely remove the residual AW2. Discard the collection tube containing the filtrate.

» Place the spin column into appropriately labelled 1.5 ml microcentrifuge tube.

» Add 60 µl of Buffer AVE into the spin column. Incubate for 1 minute at room temperature.

» Centrifuge for 1 minute at 8000 rpm.

» Discard the spin column and store the eluted RNA at -70 ^{0}C to be used for PCR

» An extraction should be performed on distilled, deionized, sterile water as an extraction control for each set of extractions.

Protocol 7.2 – Reverse Transcription: synthesis of complementary DNA (cDNA) from RNA using high-capacity cDNA reverse transcription kit Applied Biosytems

Introduction

In a two-step PCR for amplification of viral RNA, the first step involves conversion of viral RNA into complementary DNA (cDNA.)

Principle

The reaction involves the use of random primers for the synthesis of cDNA from RNA. The enzyme required for the conversion is reverse transcriptase.

Requirements

Equipment	Consumables	Reagents
• Refrigerator • Freezer (-70 ^{0}C) • Micro pipettes • Microcentrifuge • Timer	• Tissue paper • Disposable gloves • 70% Ethanol • 0.2 ml microcentrifuge tubes • Sterile, nuclease-free filter tips • Marker pens	• High – Capacity cDNA Reverse Transcription Kit (Applied Biosystems) • Nuclease-free water

Storage of reagents

Reagents should be stored at -30 ^{0}C

Test procedure

» Take 50 µl of extracted RNA in a microcentrifuge tube and incubate at 65 0C for 10 minutes in a thermocycler.

» Transfer the tubes to a beaker containing ice for 2 minutes.(Snap chilling)

» Thaw all the reagents from the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems) completely before use, and keep on cold rack during the assay set-up.

» Calculate the number of reactions to be set up and prepare the reaction mix using the reagents as follows.

Reagents	Volume of reagent added per reaction (in µl)
10x RT buffer	5
25x dNTP's	2
10x Random primers	5
Reverse transcriptase enzyme	2.5
Nuclease-free water	9.5
RNAse inhibitor	1
TOTAL	25

» Aliquot 25 µl of the reaction mix into 0.2 µl microcentrifuge tubes labelled appropriately.

» Carefully carry the tubes to the Template Addition Hood.

» Add 25 µl of template RNA into the respective tubes containing reaction mix.

» Mix well taking care not to generate air bubbles.

» Transfer the tubes to the thermocycler.

» The thermocycling profile is as follows.

Synthesis	37 ^{0}C for 120 min
RT Inactivation	95 ^{0}C for 5 min
Hold	4 ^{0}C

» The tubes are stored at 4 ^{0}C until testing or at -70 ^{0}C, if the PCR is not performed the same day.

Protocol 7.3 – Conventional reverse transcriptase (RT) PCR assay for Chikungunya virus

Introduction

Chikungunya, a mosquito-borne RNA virus has assumed public health importance due to the large-scale outbreaks it has caused since 2005. Incapacitating arthralgia is the hallmark of the chikungunya virus infection. Molecular methods such as PCR have been found to be rapid and sensitive for detection of Chikungunya viral nucleic acids from serum, plasma or CSF samples.

Principle

It involves the conversion of viral RNA into complementary DNA followed by the amplification of the target gene by PCR that uses forward and reverse primers and a heat-stable DNA polymerase. The reaction mix also contains a buffer with various ions such as Mg2+ and dNTPs.

Primers: The target region that is amplified is the envelope glycoprotein, E1, of Chikungunya virus and the expected size of the amplicon is 646 base pairs.

Forward primer	5'CGT GGT GTA CAA AGG TGA CG 3'
Reverse primer	5' ACG CCG GGT AGT TGA CTA TG 3'

Definitions

- **NTC -** Non Template Control
- **PC -** cDNA from known positive clinical specimen or culture isolate.
- **EC -** Extracted-water negative control (RNA extraction performed on distilled, deionized, sterile water followed by cDNA synthesis).

Requirements

Equipment	Consumables	Reagents
• Refrigerator • Freezer (-30 ^{0}C) • Freezer (-70 ^{0}C) • Micro pipettes • ABI Veriti thermocycler • Vortex mixer • Microcentrifuge	• Tissue paper • Disposable gloves • 1.5 ml and 0.2 ml microcentrifuge tubes • Sterile, RNase/DNase free 10 µl, 200 µl, and 1000 µl filter tips.	• 10 X PCR buffer • Forward and Reverse Primers • Taq DNA polymerase • 10mM dNTP mix • Nuclease free water

Storage of reagents

All reagents should be stored at -30 ^{0}C

Test Procedure

RNA Extraction from clinical sample – refer to Protocol 7.1

Reverse Transcription (cDNA synthesis) – refer to Protocol 7.2

PCR

» Prepare template worksheet.

» Switch on the PCR thermocycler and programme the thermal cycling profile for the run.

» Remove stored cDNA from -20 ^{0}C and allow it to thaw completely.

» In PCR clean room hood, gather reagents for the PCR and allow them to thaw completely before use. Mix the contents well before use.

» The composition of the master mix for a single reaction is as follows:

Reagents	Volume per reaction (µl)
10 X PCR buffer	2.5
Forward primer (10pm)	1.0
Reverse primer(10pm)	1.0
$MgCl_2$	1.0
dNTPs(10mM)	0.5
Taq DNA Polymerase	0.5
Nuclease free water	17.5
Total	24.0

» Calculate the number of reactions required per assay including NTC, PC and EC.

» Prepare the master mix accordingly.

» Aliquot 24 µl of the prepared master mix into appropriately labelled 0.2 µl microcentrifuge tubes.

» Add 1 µl of nuclease-free water to NTC tube in the PCR clean room.

» Close the tubes. Carefully transport the tubes to template addition room.

» Add 1 µl of cDNA template (sample/PC/EC) to corresponding tubes and mix well, taking care not to generate air bubbles

» After addition of the template to the tubes, close the tubes and transfer them to the thermocycler.

» Cycling conditions for the PCR are as follows:

First holding stage	94 ^{0}C/10 minutes
PCR amplification (35 cycles)	95 ^{0}C/ 45 seconds
	58 ^{0}C/45 seconds
	72 ^{0}C/45 seconds
Final Extension	72 ^{0}C/7 minutes

Detection of PCR end products by agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA molecules based on their size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field. Shorter molecules move faster and migrate further than larger ones. The DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and fluoresces under UV light.

Requirements

Equipment	Consumables	Reagents
• Gel casting trays • Gel electrophoresis unit • UV-transilluminator • Micro pipettes	• Tissue paper • Disposable gloves • Sterile, nuclease-free filter tips	• Agarose • 1X TAE (Tris-Acetate-EDTA) buffer • Molecular weight DNA ladder • 1% ethidium bromide solution • Sample loading dye

» Prepare the gel tray by sealing the edges with the adhesive tape and place the gel comb in position.

» Prepare 2% agarose gel by adding 0.8g of agarose to 40 ml of 1X TAE buffer in a conical flask.

» Dissolve the agarose by heating in a microwave oven.

» The temperature of the solution is brought down to ~60 ^{0}C

- 1 µl of ethidium bromide is added into the solution and mixed well.
- The gel is slowly poured onto a gel casting tray and allowed to solidify.
- After solidification, the comb and the adhesive tape are carefully removed
- The gel tray is kept inside the electrophoresis tank and 1X TAE buffer is added to a level that immerses the gel.
- 10 µl of each sample is mixed with 3 µl of sample loading dye and loaded into well.
- The molecular weight ladder is loaded into to the first well, followed by NC, PC, EC and samples.
- The power pack is connected to gel tank and electrophoresis is carried out at 100V for 30 min.
- At the end of the run, the power supply is disconnected.
- The gel is removed from the electrophoresis tank, placed inside a gel documentation system with UV transilluminator and observed.

Interpretation of results

- A positive result is indicated by the presence of the DNA band corresponding to 646 base pairs in relation to the molecular weight ladder.
- There should be no spurious bands observed in the lane in which NC and EC are loaded.

8 REAL-TIME PCR PROTOCOLS

Protocol 8.1 – One-step real-time RT PCR for 2009 H1N1 Influenza A (CDC Protocol)[2]

Introduction

A novel strain of Influenza virus 2009 A/H1N1, identified in April 2009 has emerged as a global pandemic agent. This novel influenza virus contains a combination of swine, avian and human influenza virus genes and can cause a febrile respiratory infection ranging from self-limited to severe illness. A one-step Real-time RT PCR protocol developed by CDC is used for laboratory diagnosis of 2009 H1N1 virus. Nasal/throat swabs, broncheoalveolar lavage, tracheal aspirates, nasopharyngeal /oropharyngeal aspirates or washes are the recommended specimens for diagnosis of 2009 H1N1 infection.

Principle

This PCR includes a panel of four sets of oligonucleotide primers and dual labelled hydrolysis (Taqman) probes for the qualitative detection of 2009 H1N1 Influenza A virus in respiratory specimens. Viral RNA is reversely transcribed with primers followed by TaqMan real-time PCR. Each sample RNA is tested for 4 targets by different primer/probe sets – Influenza A (InfA), Universal swine influenza A (swA), Swine H1 (SwH1) and RNase P (RP) which serves as an internal positive control.

[2] CDC Protocol of real time RT PCR for Swine Influenza H1N1 http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf

Requirements

Equipment	Consumables	Reagents
• Refrigerator • Freezer (-30 ^{0}C) • Freezer (-70 ^{0}C) • Micro pipettes • ABI Realtime PCR machine • Vortex mixer • Microcentrifuge • Plate Centrifuge	• Tissue paper • Disposable gloves • 70% ethanol • 1.5 ml sterile microcentrifuge tubes • Sterile, RNase/DNase free 10 µl, 200 µl, and 1000 µl filter tips • 48/96 well polypropylene plate • MicroAmp™ Optical Adhesive Film • Adhesive film Applicator	• Nuclease-free Water • AgPath-ID One step RT PCR kit (Applied Biosystems, USA). – Assay Mix – Mix 1- Inf A primers and Probes – Mix 2- sw A Primersand Probes – Mix 3- sw H1 Primers and Probes – Mix 4- RNase P Primers and Probes – Enzyme Mix – 2X PCR Buffer

Storage of reagents

Reagents should be stored at -30 ^{0}C

Test procedure

RNA Extraction – Refer to Protocol 7.1

One-step real-time PCR

» Prepare plate template work sheet.

» Determine the number of reactions (N) to set up per assay. For each reaction mix (i.e. mix for each target gene), it is necessary to make extra reaction mix to allow for NTC (No Template Control) PC (Positive Control), EC (Extraction Control-water) reactions and pipetting errors.

E.g.: If, No. of Samples (S) = 10,
No. of reactions (N) to set up will be S (10) + NTC (2) + PTC (1) +
EC (1) + Pippetting error (2) = 16 reactions

» Calculate the amount of each reagent to be added for each reaction master mix as shown below

Reagents	Volume of Reagent Added per Reaction (in µl)
2X PCR Reaction Buffer	12.5
Nuclease free water	6
Enzyme Mix	1
Assay Mix	0.5
Total	20

» Thaw all the reagents completely before use, and keep on cold rack during the assay set up.

» Prepare reaction master mix for each target gene as calculated previously. Mix well before addition.

» Pipette 20 µl of each reaction master mix into appropriate well of the PCR plate, according to the plate template.

» Add 5 µl of nuclease free water to the NTC wells in the PCR clean room.

» Cover the plate and carefully transport it to the template addition hood.

» Inside the template addition hood, add 5 µl of the extracted RNA (samples/PC/EC) into the respective wells according to the PCR template.

» NOTE: PC should be always added last.

» Seal the plate with adhesive film.

» Centrifuge the plate briefly to spin down the contents and to remove trapped air bubbles, if any.

» Place the reaction plate in the PCR machine

» Program the thermocycler as follows:

Reverse transcription	50 ^{0}C for 30 min
Taq activation	95 ^{0}C for 10 min
PCR amplification (45 cycles)	95 ^{0}C for 15 sec
	55 ^{0}C for 30 sec*

* Fluorescence data are collected during the 55 OC incubation step

Interpretation of results

Validity criteria

» The NTC should not exhibit fluorescence that crosses the threshold line.

» All clinical samples should be positive for RP (Ct < 37), indicating that the specimen is of acceptable quality.

» The extraction water control should NOT exhibit fluorescence that crosses the threshold line.

» PC should give a positive result with the InfA, swInfA, swH1, and RP reactions (Ct < 40).

Positive result

» 2009 H1N1 influenza A positive- Sample positive for all 4 targets (Ct value of 40 or less).

» Positive for other Influenza A viruses- Sample positive for InfA and RP (Ct value of 40 or less).

» Negative result.

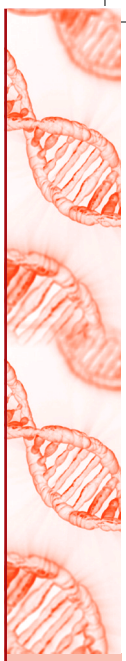
» Negative for Influenza A- Sample should be negative for Inf A, sw A, sw H1 and positive for RP.

Protocol 8.2 – Quantitative Taqman real-time PCR assay for human immunodeficiency virus (HIV)[3]

Introduction

The nucleic acid-based diagnostic assays for the quantitation of plasma human immunodeficiency virus (HIV) RNA levels are used to monitor disease progression and the response of patients to antiretroviral drug therapy. Baseline CD4 cell counts and HIV-1 RNA levels have been shown to predict immunologic and virologic responses in HIV infected patients on anti-retroviral therapy.

[3] Kamat A, Ravi V, Desai A, Satishchandra P, Satish KS, Borodowsky I, Subbakrishna DK, Kumar M. 2007. Quantitation of HIV-1 RNA levels in plasma and CSF of asymptomatic HIV-1 infected patients from South India using a TaqMan real time PCR assay. Journal of Clinical Virology; 39: 9 -15



Principle

This quantitative real-time assay is based on the Taqman principle. During PCR, forward and reverse primers hybridize to a specific sequence product. An oligonucleotide probe, labelled with a 5'reporter dye (FAM) and a downstream, 3'non-fluorescent quencher dye, hybridizes to one of the strands of the target DNA of the amplicon. The Taq polymerase which possesses 5'–3' exonuclease activity cleaves the probe as the primers are extended. The reporter dye and quencher dye are separated on cleavage, resulting in an increase in fluorescence which is directly proportional to the quantity of the amplicon.

Primers and probe: The primers and probe were selected in the gag region of an Indian isolate of HIV-1 subtype C.

Forward primer	5' ACC CAT GTT TAC AGC ATT ATC AGA AG 3'
Reverse primer	5' GCT TGA TGT CCC CCT ACT GTA TTT 3'
Probe	5'AGC CAC CCC ACA AGA TTT AAA CAC CAT GT 3'

Definitions

- **NTC:** Non template control
- **Standards:** A reference sample with known copy numbers of the viral RNA is diluted five fold from 1:5 to 1:3125. These 5 standard dilutions are run in triplicates to generate a standard curve.
- **EC:** Extracted-water negative control (RNA extraction performed on distilled, deionized, sterile water followed by cDNA synthesis).

Requirements

Equipment	Consumables	Reagents
• Refrigerator • Freezer (-30 ^{0}C) • Freezer (-70 ^{0}C) • Micro pipettes • ABI Realtime PCR machine • Vortex mixer • Microcentrifuge • Plate Centrifuge	• Tissue paper • Disposable gloves • 1.5 ml sterile microcentrifuge tubes • Sterile, Rnase/Dnase free 10 µl, 200 µl and 1000 µl filter tips • 48/96 well Polypropylene PCR plate • MicroAmp™ Optical Adhesive Film • Adhesive film • Applicator	• TaqMan® Universal PCR Master Mix • Primers and probes • Nuclease free water

Storage of reagents

All reagents should be stored at -30 ^{0}C

Test procedure:

RNA Extraction – Refer to Protocol 7.1

cDNA synthesis – Refer to Protocol 7.2

Real-time quantitative PCR

» Prepare the PCR template worksheet.

» Switch on the PCR machine computer, feed in the data according to your PCR template worksheet and programme the thermal cycling profile for the run.

» Remove stored cDNA of samples and standards from -20 ^{0}C and allow them to thaw completely.

» Allow the PCR reagents to thaw completely and mix the contents well before use.

» The composition of the master mix for a single reaction is as follows:

Reagents	Volume per reaction(µl)
TaqMan® Universal PCR Master Mix	12.5
20X primer probe mix	1.25
Nuclease free water	6.25
Total volume	20

Note

- For each run, include 5 standards, 1 NTC and 1 EC each in triplicate (unknown samples are added in duplicate if included in the PCR assay.)
- Number of reactions to be prepared for each standard/NTC/EC=5 (to accommodate pipetting error)
- Total number of reactions = (5+1+1) X 5 = 35 reactions
- The total volume of the master mix is 20x35 =700 µl
- Aliquot 80 µl (20 x 4) of the prepared master mix into appropriately labelled 0.5 µl microcentrifuge tubes for each of the standard, NTC and EC.
- Add 20 µl of nuclease free water to NTC tube in the PCR clean room.

- Close the tubes. Carefully transport the tubes to template addition room.
- 20 µl of cDNA template/EC is added to corresponding tubes and mixed well, taking care not to generate air bubbles
- After addition of the template to the tubes, 25 µl of the mix is added to the 96 well reaction plate according to the template worksheet prepared.
- The plate is sealed using the optical adhesive film.
- Spin the plate for five minutes to remove any air bubbles present in the wells.
- Place the plate in the machine and run it as per the following cycling conditions

1st holding stage	50 °C/2 minutes
2nd holding stage	95 °C/10 minutes
PCR amplification (40 cycles)	94 °C/15 seconds
	60 °C/1 minute

Interpretation of results

- Amplification data are analysed by instrument software.
- Validity of the test: The NTC should not show any amplification and all standards should show appropriate Ct values.
- The software generates a standard curve using the copy number (CO) of the known standards and their respective Ct values. There should be a linear relation between the log CO and the Ct values generated.
- The Ct values of the samples are extrapolated on the standard curve and the copy number in the unknown sample is generated by the instrument software.

9

QUALITY IN PCR

Quality control can be defined as a system of routine and consistent steps taken to ensure integrity, accuracy and reproducibility results. It helps identify and correct errors/omissions that might occur during processing as well as recording and archiving results and data. On the other hand, quality assurance is a planned system of review of procedures, preferably performed by an independent third party laboratory, which assesses procedures and verifies that quality objectives have been, and are being met.

Like any other laboratory procedure, accuracy and consistency in PCR procedures is highly dependent on instituting and maintaining highest level of quality in all stages.

Factors influencing quality of PCR in the laboratory

(1) Sample integrity

(2) Extraction of nucleic acid

(3) Quality of reagents

(4) Thermocycler

(5) Use of appropriate controls

(6) Contamination

(7) Design of assay

Sample Integrity: Sample collection and transport conditions impact the results of PCR analysis. Laboratories should develop a detailed sampling and processing standard operating procedures (SOPs) for each new method. The SOPs should define the range of acceptable sample volumes, sample handling protocols and the time samples can

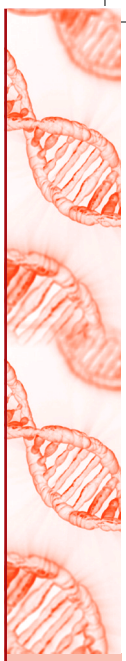
be held before beginning sample processing. It also should describe the use, cleaning and sterilization of any sampling apparatus.

The procedures described in the SOPs should be designed to preserve the integrity of the target nucleic acid sequence in the sample.

Extraction of nucleic acid: Some nucleic acid extraction methods require the lysis of the target organism and the isolation of its nucleic acid before proceeding to PCR. If the nucleic acids of the organism are isolated before addition to the PCR, the performance of this part of the method also should be assessed independently and as part of the entire analytical process. The efficiency of nucleic acid isolation varies with sample type and extraction procedure. Ideally, an isolation technique should meet the following goals:

- Demonstrate a high efficiency of target nucleic acid recovery
- Maintain nucleic acid integrity and minimize fragmentation
- Provide sufficiently pure nucleic acid, free from PCR inhibitors
- Minimize the use of dangerous chemicals
- Reproducible

Reagents: The reagents used in PCR amplification can be purchased or prepared in-house. Care should be taken to ensure that reagents are maintained contamination-free. All reagents should be clearly labelled with name, expiration date and relevant safety information. Reagents from different lot numbers should not be interchanged without prior functional validation. Moleculargrade water or its equivalent from commercial sources should be used for all assays. Laboratories also may use water purification systems that produce highquality pyrogen and DNase/RNase-free water. Diethylpyrocarbonate (DEPC) treatment can be used to eliminate RNase from water used in RNA analysis. Addition of DEPC results in the covalent modification of nucleases (such as RNase), causing them to lose their function. Proper autoclaving is necessary, because trace amounts of DEPC in a solution will lead to the modification of the purine residues in RNA by carboxymethylation. All reagents should be diluted and working stocks should be made for all critical reagents such as primers, probes and enzymes. These working stocks should be stored at appropriate temperatures and used for all experiments. Primers and probes should be free from other contaminating sequences and enzymes. Impure oligos will decrease the specificity of the procedure. Certification of the quality of the oligos, including method of purification, purity and concentration, should be required from all commercial manufacturers. Functional validation should



be performed on every new lot of primers and probes by comparing their performance against older sets of known quality.

Thermocycler: Thermocyclers are essential to all PCR methods, and care should be taken to ensure that they are well maintained and reliable. The manufacturers of the instruments recommend procedures to maintain the instrument. The block temperature of a thermocycler should be tested at least twice a year (or at the frequency specified by the manufacturer) by the laboratory or under a maintenance agreement to ensure uniform heating throughout the block.

Real-time PCR instruments are equipped to perform fluorescence excitation and detection to monitor amplification throughout the PCR cycles. The design is often different from the standard thermocycler, and calibration may be specific to the instrument design and should be carried out according to manufacturers' specifications. For example, several real-time platforms do not have heating blocks but rotor designs, with samples spinning in an air chamber that is heated and cooled. Temperature can be calibrated for this type of instrument using a calibration rotor provided by the manufacturer.

Use of appropriate controls: Laboratories using PCR should analyse positive and negative QC samples on a routine basis to demonstrate adequate performance of PCR-based methods. The actual number of controls will depend on the experimental design and expected variability. The method for preparation of controls should be documented in the laboratory's SOPs. This helps promote consistency between analysts. SOPs for a particular method should also document QA procedures, such as the types and frequency of QC controls, and corrective actions for positive and negative control failures.

PCR positive controls (PC) are used to verify that the PCR master mix and reagents were prepared correctly to produce amplification of the target nucleic acid.

Negative control (NC) samples are used to verify that no contaminating nucleic acid has been introduced into the master mix or into samples during sample processing. These negative controls are clinical samples earlier confirmed not to contain target nucleic acid.

No template control (NTC) is used to verify that no contaminating nucleic acid has been introduced into the master mix. These controls are prepared when no template is added to the master mix. They are prepared as separate samples to which aliquots of molecular-grade water or buffer are added to the master mix in place of clinical sample. A negative result with this control indicates that the master mix and final processing reagents are not contaminated.

Contamination: Contamination remains an important issue for laboratories performing PCR procedures for the detection of infectious agents. There are a number of approaches to control PCR contamination, and the degree of stringency that is required in a laboratory is often determined by the assay being performed.

Contamination in PCR occurs primarily because of:

(1) Generation and spread of aerosols;

(2) Contaminating materials present on hands, clothing or hair, introduced into PCR mixes;

(3) Fomites – Laboratory coats, gloves, vortexers, pipettes etc;

(4) Circulating PCR amplicons (aerosols/fomites);

(5) Positive control specimens; and

(6) DNA clones used for synthesis of positive control material

Contamination can be prevented to a great extent by :

(1) Compulsory wearing of a laboratory coat and disposable latex/ neoprene gloves and changing the laboratory coat and disposing the gloves upon moving from one area to the next in the PCR annexe.

(2) Maintaining strict unidirectional flow of material and personnel in the PCR annexe, from the sample processing room to the pre-PCR room to the PCR amplification room to the post-PCR analysis room.

(3) Keeping all bottles and reaction tubes sealed for as long as possible.

(4) Using as few manipulations as possible.

(5) Avoiding vigorous and excessive vortexing, pipetting and spillages in the PCR areas.

(6) Centrifuging vials containing specimens, nucleic acids, PCR mix ingredients and PCR amplicons, before opening them.

   (a) Aliquoting reagents into single-use volumes

   (b) Autoclaving /incinerating contaminated utensils (glassware, reaction tubes, pipette tips, buffers etc.)

   (c) Cleaning the lab benches periodically with 1N HCl (causes depurination and hydrolysis of contaminant nucleic acid molecules), or expose the workstations overnight to UV light.

(d) Working within an easily-cleaned Biological Safety Cabinet or PCR workstation, preferably one with a built-in UV source.

(e) Treatment of surfaces with psoralene compound may be considered in cases of recalcitrant contamination.

(f) Substitution of dTTP in PCR mix by dUPT

(g) Using "anti-contamination primers" (which amplify sequences from within the contaminating vector itself)

Design of the assay – quality considerations: while designing a PCR assay for detection of infectious agents, the following paratmeters need to be considered:

(1) The quality of enzymes used i.e. exonuclease activity, reverse transcription activity, half-life, storage, buffer requirements

(2) Primer design and synthesis: Important parameters to consider are sequence specificity, GC content and secondary structure. Also of critical importance is the purity and integrity of template (purified DNA) and primer design. There are several software programs available online for designing primers. The best primers have 18–24 bases, no secondary structure (i.e. hairpin loops), balanced distribution of G/C and A/T pairs, are not complementary to each other at the 3' ends and have melting temperatures (Tm) about 5–10 OC below the annealing temperature, which is usually between 55 and 65 OC. The Tm for both primers should be similar for best results. Purification of primers is also of importance and primers purified by HPLC should be preferred.

(3) Reaction mix: The proportions of the reaction mixture have a huge influence of the quality of PCR results. There is a general formula for concentrations of template, enzyme, primers and nucleotides to use, but this can be tweaked a little bit. Optimal primer concentrations are between 0.1 and 0.6 micromoles/L. The amount of template varies depending on the type of DNA (human, bacterial, plasmid). With very low amounts of template there are other strategies to improve results, like increased cycle numbers or use of "hot start". Always test new primers with a positive control reaction, to be sure they work under your specific experimental conditions.

(4) Number of cycles, reaction cycle lengths, temperatures, and number of cycles, all have a critial role in determining how well a PCR will work. The initial heating step must be long enough to completely denature the template and cycles must be long enough to prevent melted DNA from reannealing to itself. Increased yield can be achieved by increasing the extension time about every 20 cycles,

to compensate for less enzyme to amplify more template. Usually less than 40 cycles is enough to amplify less than 10 template molecules to a concentration large enough to view on an ethidium bromidestained agarose gel.

(5) Micropipettes: As the results of PCR are depended on accurate quantities of the enzymes and primers used, it is very essential that micropipettes are regularly calibrated and documented.

(6) Reaction parameters

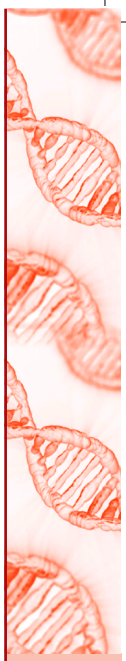
- Mg ion concentration should be between 0.5 mM-5.0 mM
- Primer concentration should be between- 0.1 µM-1.0 µM
- dNTP concentration should be between 20 µM-200 µM
- Enzyme concentration can range from 0.2-2.0 units/reaction.
- pH: 8.5-10
- Type of buffer used: Sodium, potassium or ammonium ions.
- Additives added if any - DMSO, Betain.
- Templates - RNA/DNA, (un)diluted

Quality considerations specific to RT-PCR

Reverse transcription PCR (RT-PCR) is done for detection of RNA viruses by first converting the RNA to its complementary DNA (cDNA). As this involves many steps and additional reagents, RT PCR is sometimes associated with problems related to false positive and false negative PCR results. Problems likely to cause false-positive results in RTPCR are:

(1) Contamination with genomic DNA. This can be prevented by removing contaminating genomic DNA by adding RNase-free DNase to extracted RNA and incubating it for 1 hour at 37 °C, and heat-inactivating the DNase prior to RT steps.

(2) Amplification of pseudogene sequences. Pseudogenes are nonfunctional genes that lost their functional significance over the course of evolution .They may still show sequence homology to the original functional gene intron-free pseudogenes may lead to falsepositive results in RTPCR if residual DNA is present in the reaction mix and if RT primer-annealing site is not affected by mutations. It is highly recommended to include a DNA digestion step in the RTPCR protocols to prevent amplification of pseudo genes.



In the worst case scenario of PCR contamination it is advisable to:

- Shut down the offending PCR laboratory
- Discard all the working solutions
- Thoroughly decontaminate all work surfaces, laboratory coats, pipettes etc.
- Order new supplies of PCR reagents.

Biosafety in PCR laboratories

The biosafety levels followed in PCR laboratories are similar to any other microbiology laboratory depending on the type of the microbe handled. Adherence to the required bio-safety level is critical while handling the clinical samples and extracting the nucleic acid from the sample. For some viruses likely to spread by aerosols like H1N1, working in a bio-safety level III cabinet is mandatory. While working, it is necessary to wear personal protective equipment (PPE) and take a shower before exiting. For viruses such as rabies and Japanese encephalitis either BSL II or BSL III cabinet can be used.

Safety aspects related to the use of ethidium bromide staining of agarose gels

Ethidium bromide is a DNA-intercalating dye and helps in visualization of DNA band; usually used at a concentration of 0.5 µg/ml. It is a mutagenic agent. People who perform conventional PCR often have repeated exposure to ethidium bromide. They should take the following precautions:

- Use a chemical safety hood to prepare ethidium bromide containing gels.
- Avoid direct contact and inhalation of fumes.
- Use filter tips to dispense the agent into molten agarose.
- Use vinyl gloves if possible, while handling ethidum bromide containing gels.
- After detection of DNA bands, dispose of the gels in the hazardous waste, close to their site of preparation.
- Decontamination of solutions with activated charcoal followed by incineration
  - Add 100mg of powdered activated charcoal for each 100ml of solution; keep at RT x 1 hr with intermittent mixing; filter through a Whatman No.1 filter and discard the filtrate; seal the filter and charcoal in a plastic bag and dispose in hazardous waste.
  - Commercial decontamination resins available (e.g., Amberlite XAD-16 from Sigma-Aldrich, EtBr Green Bag (Q.BIOgene)) Gels to be discarded alongwith hazardous waste, close to their point of preparation

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CARE AND MAINTENANCE OF PCR MACHINES

Great care should be taken to ensure that PCR machines are well maintained and reliable. Care and maintenance of a PCR machines is essential for the following reasons: (i) it is a sophisticated equipment and not routinely used in most laboratories, (ii) it is expensive to buy and repair, (iii) a preventive action avoids breakdowns/repairs and (iv) it requires specialized, trained engineers to repair it. It is a good laboratory practice to ensure that PCR machines are maintained by the manufacturer and/or an authorized service engineer. This would avoid break down of equipment, prevent disruption of clinical laboratory services and ensure credibility of laboratory results.

General tips for care and maintanence of a PCR machine

- Keep the machine in a cool, dry place.
- Always use an uninterrupted power supply (battery back up) – Do not connect the machine directly to the power source.
- Keep the room and the area around the machine “dust free”.
- Switch off the machine when not in use. Disconnect the power supply from the electrical source at the end of the day.
- Fix responsibility among users for each machine.

- Maintain a log book – Who used it?; When was it used?; What was the condition of the machine?
- Keep the manual of the machine near the machine.
- Paste the business card of the service engineer on the side of the machine.
- Paste a note near the machine indicating the due date for the next service.

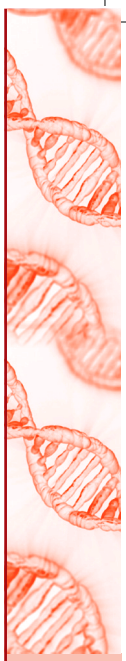
Typically, the manufactures of the instruments have developed recommended procedures to test and maintain the instrument. However, it is essential that a check list for the care and maintenance of PCR machine be prepared in every PCR laboratory and that would include the following essential points:

- Safety of the machine and user –electrical safety, chemical safety
- Cleanliness of the machine and its surroundings
- Temperature verification
- Checking and calibration of fluorescence in real time PCR machines
- Use of calibration kits
- Schedule of visit by service engineer
- Basic training for staff in care and maintenance of PCR machine

Safety: PCR machines are essentially heating blocks and therefore they use electricity. Electrical safety should be given prime importance and all safety practices used for any electrical appliance in a laboratory should also be adopted while using a PCR machine. The lid/door of an instrument should never be opened when the machine is in operation. The plugs and power points should be checked periodically by the laboratory for their physical integrity and quality of power supply (voltage, earthing, etc.). Fuses should be checked periodically and replaced when blown up. It is a god practice to disconnect the PCR machine from the power source at the end of every day so that the equipment is protected from any surge in power that might occur when laboratory personnel are away.

Chemical safety is also to be given top priority in a PCR laboratory. Staff should use appropriate precautions in handling hazardous chemicals and clean the machine immediately in the event of a spill.

Cleanliness of machine: The PCR machine uses sophisticated electronics to run its applications and hence requires to be kept in a dust free environment. The real time



PCR machines also have sophisticated lasers, lenses and optical pathways which require that the environment in the laboratory be dust free. Cleaning of PCR machines has to be carried out on a daily basis using practices recommended by the manufacturer. In addition periodic checking of individual wells in a PCR heating block must be carried out to ensure that there is uniformity of thermal cycling in all the wells. Spillage reagents into the well of a PCR block is a very common incident in a laboratory and therefore checking of wells should become part of daily routine in the laboratory. Cleaning of wells in a PCR block may be carried out using solutions recommended by the manufacturer. Use of individual ear cleaning buds for each of the wells is highly recommended.

Temperature verification: The block temperature of a thermocycler should be tested at least twice a year (or at the frequency specified by the manufacturer) by the laboratory or under a maintenance agreement to ensure uniform heating throughout the block. Monthly testing should be performed by laboratories performing a large number of reactions. Block temperature should be tested with an external probe that has been calibrated against a temperature standard. For testing, the probe is placed in several of the wells in the periphery and centre of the instrument. All temperatures should be within the manufacturers' specifications. The amplification program used in each run should be printed to further verify the conditions of the PCR. This also allows the analyst to determine if there were any instrument malfunctions during the reaction. If the thermocycler does not have software with this capability, then the cycling program used should be documented in the laboratory's SOP with results of monthly calibration checks.

Temperature verification in real-time PCR machines requires proper understanding of how the machine functions. Indeed the design is often different from the standard thermal cycler, and calibration may be specific to the instrument design and should be carried out according to the manufacturer's specification. For example, several real-time platforms do not have heating blocks but rotor designs, with samples spinning in an air chamber that is heated and cooled. Temperature can be calibrated for this type of instrument using a calibration rotor provided by the manufacturer.

Recently, there are mobile, personal measurement devices that are available commercially to monitor temperature of a PCR machine. These can be used daily, weekly or monthly to monitor the performance and temperature drift of thermal cyclers. They measure temperature accuracy – deviation of the actual block temperature from the set temperature. They also monitor uniformity – the spread of the temperature between wells on the same block and also provide data on the speed, or ramp rate,

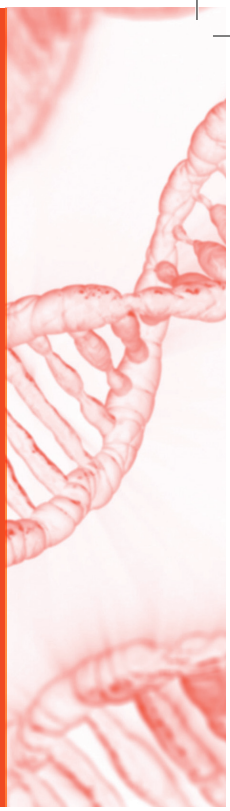
at which the cycler changes temperature and report on any under or overshoots – all significant factors affecting the success of your PCR reactions.

Checking and calibration of fluorescence: Real-time PCR instruments are equipped to perform fluorescence excitation and detection to monitor amplification throughout the PCR cycles. Laser performance, alignment, and safety devices should also be checked and optical systems calibrated. This may involve analysing fluorescent test trays to confirm consistent detection from each well. For rotor designs, each tube is passed by the same detector, providing greater sample-to-sample consistency. Spectral calibration files may also need to be recalibrated to confirm that the excitation signals from the range of fluorophores used can be separated, and gain settings adjusted. Analysts can perform part of this calibration, but service technicians trained by the equipments supplier will be required to carry out a full maintenance and calibration service. A full service should be performed at least annually.

Use of calibration kits: Most manufacturers of real-time PCR machines recommend the use of calibration kits to check the performance of the machine. These kits not only help in verifying the cycling efficiency of the thermal cycler in each well but also help monitor the spectral analysis as well the alignment and integrity of the optical pathways used for fluorescence detection. It is therefore good laboratory practice to procure these calibration kits and use them periodically to ensure that the equipment is performing optimally.

11

TROUBLE SHOOTING PCR



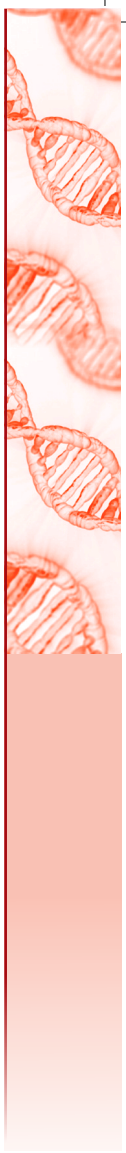
A laboratory needs to implement a number of precautions when using PCR to generate large amounts of a desired product. At one end, multiple nonspecific and unwanted products may get amplified, at times leading to the masking of the desired product. On the other extreme, no product may be amplified. The general corrective action at this juncture is to vary one or more of the many optimization variables such as Mg2+ concentrations, buffer pH and cycling conditions that are known to contribute to primer-template fidelity and primer extension. However, most often these variables are interdependent. For example, because dNTPs directly chelate a proportional number of Mg2+ ions, an increase in the concentration of dNTPs decreases the concentration of free Mg2+ available to influence polymerase function.

Guide for PCR troubleshooting

Problem	Causes	Actions
No PCR product	Inhibitors present	Reduce volume of sample in the mix. Check ethanol was evaporated from DNA extractions.
	No of cycles not sufficient	Check temperature and cycle time. Repeat the run with an additional five cycles
	Error in gel analysis	Check wells on gel loaded correctly, correct loading buffer was added to samples, EtBr is added to gel and UV settings are correct

Guide for PCR troubleshooting

Problem	Causes	Actions
No PCR product	Incorrect annealing temperature	Run a temperature gradient. Decrease annealing temperature and /or increase elongation time depending on product size.
	Incorrect $MgCl_2$ concentration	Run a $MgCl_2$ gradient of 0.5 mM increments between 1.5 mM and 4.0 mM
	Nucleotides degenerated	Store nucleotides in small aliquots at -20 °C. Thaw on ice.
	Primer dimers	Increase temperature and/or decrease MgCl2. check self complementarity of primers on primer design software, Redesign primers.
	Primer design error	Check primer specificity. Recheck primer parameters on primer design software. Redesign primers if required.
	Set-up	Check that all components are added correctly.
Low yield	Annealing temperature not optimal	Run a temperature gradient in 2 °C increments
	MgCl2 concentration not optimal	Run a MgCl2 gradient of 0.5 mM increments between 1.5 mM and 4.0mM
	Buffer not optimal	Use a NH4 based buffer instead of KCI based buffer for greater yield
	Insufficient template	Increase template concentration
	Insufficient primers	Increase primer concentration
	Insufficient cycles	Increase number of cycles
	Secondary structure in template	Use touchdown PCR, add adjuvant such as DMSO, BSA or Betaine, or use a hot-start Taq DNA polymerase
	GC-rich template	Add adjuvant such as DMSO 5% (v/v, final concentration) or BSA (0.1 to 0.8 :g/l final concentration).



Guide for PCR troubleshooting

Problem	Causes	Actions
Low yield	Extension time too short	For long products (>2kb), extension time (in mins) should be approximately equal to the number of kb in the amplicon.
	Long denaturation inactivating enzyme	Only use a 2 minute denaturation time for polymerases which do not require a hot-start.
	DNA not clean or contains inhibitors	Check template is clean. Check all ethanol was evaporated from DNA extractions. If inhibitors are present diluting DNA can improve the reaction.
	Sample evaporating during thermal cycling	Check levels in wells after cycling. Ensure screw-down lid is pressing firmly on plate. Use high quality adhesive seals and rigid PCR plates.
Multiple non- specific amplification products	Reaction conditions not optimum	Optimize annealing / elongation temperature, $MgCl_2$ concentration, keep reactions on ice while mixing, use a hot-start Taq DNA polymerase
	Annealing temperature not optimal	Decrease annealing time. Run a temperature gradient in 2 ^{o}C increments
	Several targets present in the template	Redesign primers with higher specificity
	Buffer not optimal	Use a KCI based buffer instead of a NH4 based buffer for greater specificity
	Primers not specific	Primers should not be self complementary. Prevent 3 successive Gs or Cs at 3' end.
	Excess primer	Decrease primer concentration
	Excess template	Decrease template concentration
	Contamination	Check no template control (NTC) for bands
Smeared product	Set up	Use a hot-start Taq DNA polymerase
	Annealing temperature not optimal	Run a temperature gradient in 2 ^{o}C increments

Guide for PCR troubleshooting

Problem	Causes	Actions
Smeared product	MgCl2 concentration not optima	Run a MgCl2 gradient of 0.5mM increments between 1.5 and 4.0mM
	Buffer not optimal	Use a KCI based buffer instead of a NH4 based buffer for greater specificity
	Primers not specific	Blast primers to check specificity. Redesign primers
	Overabundance of primer	Decrease primer concentration
	Overabundance of template	Decrease template concentration
	Annealing time too long	Decrease time of annealing step
	Template degraded	Minimize freeze thawing of DNA.
	Extension time too short	For long products (>2kb), extension time (in mins) should be approximately equal to the number of kb in the amplicon.
Band in No template control (NTC) - contamination	Reagents contaminated	Use fresh aliquot of reagents
	Pipettes contaminated	Clean and sterilize pipettes. Use filter tips. Always use different pipettes for pre- and post-PCR.
	Work area contaminated	Clean work bench as well as the room.
	Aerosol contamination	Use a master mix to minimize pipetting steps, use filter tips, close lids on all tubes and expel reagents carefully. Change gloves regularly.
Wrong size band amplified	Contamination	Check no template control for bands
	Incorrect primers or template	Check labels on primer and template. Check set up.

Adapted from
1. www.abgene.com/downloads/PCR_troubleshooting_guide.pdf
2. http://www.scedosporium-ecmm.com/Protocols/PCRtroubleshooting.pdf

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Polymerase Chain Reaction (PCR) has significantly helped in early diagnosis and commencement of specific interventions for diseases control. It also plays a critical role in understanding the disease epidemiology and unraveling the transmission dynamics of the disease. This manual intends to provide primary guidelines to assist health lab personnel in developing countries to establish a PCR diagnostic facility for efficient support to patient care as well as public health actions.

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