Insight into PCR testing for surgeons

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Abstract

The most commonly used molecular diagnostic technique is the polymerase chain reaction (PCR). PCR detects a short section of genetic code of interest, a cancer gene, human mRNA or a pathogen's genome. It is used by every specialty in medicine and surgery, with increasing frequency and importance. In this article, the history, steps of the cycle, uses, forms, advantages and disadvantages of PCR are discussed. With the SARS coronavirus-2 pandemic having such an enormous impact on the delivery of elective surgery, decisions to proceed or defer are made by surgeons on a daily basis, based on PCR results. An understanding of these results is provided, what they tell us, what they do not and what other information is required to make these decisions. It is imperative to also look beyond PCR results, seeing the patient within the context of their symptoms, other pathology and imaging results, with the assistance of a medical virologist or microbiologist, in complex cases.

Keywords Polymerase chain reaction; Real time PCR; Quantitative and Qualitative PCR; Digital PCR; SARS CoV 2 PCR; Quality control; Interpretation; Ct (cycle threshold) value

Introduction

Molecular diagnostics are a range of techniques used to detect specific targets within a genome, or to decode the entire genome of interest (the complete set of genetic material within an organism or cell). The application of molecular biology has revolutionized infectious disease screening, diagnostics, treatment and the preventive medicine arenas, worldwide. It has also impacted on genetic medicine, oncology, haemato-oncology, transplant tissue typing, fertility services, and drug development and production, each to varying degrees. Beyond medicine, forensic science, paternity/ancestry testing, anthropologists and historians use these techniques to further their respective fields.

The most commonly used molecular diagnostic technique is the polymerase chain reaction (PCR). Initially described in 1971 by Kleppe and Khorana[1] and first practically demonstrated by Kary Mullis in 1983,[2] PCR is used for a multitude of applications including DNA fingerprinting, diagnosis of genetic disorders, detection of cancer associated genes, tissue typing and transplant donor selection, phylogenetic analysis, molecular epidemiological studies, and the diagnosis of infections due to pathogenic microorganisms, including variant species and drug resistance genotyping. As an example of the impact of PCR on health, by 21 June 2021, the cumulative number of laboratory PCR tests for SARS coronavirus-2 (SARS-CoV-2) exceeded one hundred million tests in the United Kingdom alone.[3] PCR results have provided the backbone of diagnosis, infection control and public health interventions during the pandemic thus far. These results are as vital to surgeons as the rest of hospital medicine. There is strong evidence of an increased mortality risk associated with surgery within 6 weeks of preoperative SARS-CoV-2 infection.[4,5] In one study the 30-day mortality rate in patients who did not have a preoperative SARS-CoV-2 infection was 1.4% (1973/137,104), for those with a diagnosis prior to surgery, the 30-day postoperative mortality rates were: 9.1% (104/1138) when diagnosed 0−2 weeks prior to surgery; 6.9% (32/461) at 3−4 weeks; 5.5% (18/326) at 5−6 weeks; and 2.0% (24/1202) at ≥ 7 weeks.[6] PCR results for patients will therefore be integral to decisions on the timing of elective operations for the foreseeable future (Table 1).

Polymerase chain reaction

PCR is a biochemical reaction almost identical to that which is found in most living cells. The procedure of nucleic acid replication is mirrored by PCR and used for the detection of an area of genetic code of interest. PCR involves the in-vitro exponential amplification of a small DNA or RNA fragment, from any living or dead structure, with a sufficiently intact genome present. For the replication of a DNA genome target, PCR uses an enzyme with DNA polymerase activity, this catalyses the synthesis of a complementary DNA strand from individual deoxynucleotide triphosphates (dNTPs), using a single-stranded DNA (ssDNA) template (Figure 1). The ssDNA is transformed during the process, in to a double-stranded DNA (dsDNA) form. For the detection of RNA, an additional initial step is required first, this is termed reverse transcription-polymerase chain reaction (RT-PCR). An enzyme with RNA-dependent DNA polymerase activity (also known as reverse transcriptase) uses RNA as the template, with the production of an initial complementary DNA (cDNA) strand (Figure 2). The cDNA strand, a single-stranded DNA structure (ssDNA), then acts as the template to produce a double-stranded DNA (dsDNA) copy of the original RNA. This uses the same process as HIV to transform its genome into one capable of integration into the genome of the human host. Further PCR cycles of replicating the dsDNA, within minutes, produces millions of copies of the target sequence. The amplified copies can then be detected using a variety of techniques to ascertain their presence and identify the DNA present.

Denaturation

PCR became a mainstream technology in the 1980s, after the discovery of *Thermus aquaticus*, a bacteria which thrives in hydrothermal vents and natural hot springs, in temperatures up to 100°C.[7] It contained a thermostable DNA polymerase, known as Taq DNA polymerase, forms of which are still in use today. The thermostable form was vital to improving the efficiency of the PCR reaction.[8] Prior to this, additional polymerase was required to be added after each of the cycle of the assay, of which there are

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SARS-CoV-2 preoperative results and important considerations

1.	RNA detection does not always evidence current infectiousness	The RNA genome of SARS-CoV-2 can be detectable for up to four months after diagnosis in some patients. Semi-quantitation (C_t value) is vital to help differentiate current acute infection, along with symptoms, evidence of past infection (inc. SARS-CoV-2 antibody results). One or two repeat swabs (at 24 and 48 hours) are required to exclude early acute infection in the event of an asymptomatic low level RNA detection
2.	No detection of SARS-CoV-2 RNA in the upper respiratory tract, does not exclude acute infection in symptomatic patients	SARS-CoV-2 pneumonia can present with virus having been cleared from the upper respiratory tract. The virus is only then detectable on a bronchoalveolar lavage or endotracheal secretion sample. Induced sputum is not recommended due to the infection control risks.
3.	Length of infectiousness: criteria for releasing COVID-19 patients from isolation	**Symptomatic**: 10 days after symptom onset, plus at least 3 additional days without symptoms (including without fever, and without respiratory symptoms).[a,e] **Asymptomatic**: 10 days after positive test for SARS-CoV-2.[a] **Severe infection (ITU/HDU)**: 20 days then advice should be sought from a virologist/ microbiologist/infection specialist.[b,e] **Immunocompromised**: 20 days then advice should be sought from a medical virologist/ microbiologist/infection specialist.[b,e]
4.	Time since onset of SARS-CoV-2 infection and decision to proceed with emergency surgery	Essential urgent surgery should take place.[c]
5.	Time since onset of SARS-CoV-2 infection and decision to proceed with elective surgery	Surgery within 7 weeks of SARS-CoV-2 infection is associated with increased morbidity and mortality. Elective surgery should not be scheduled within 7 weeks of a diagnosis of SARS-CoV-2 infection, unless outweighed by the risk of deferring surgery such as disease progression or clinical priority[d]
6.	Immunization	**Patients**: Patients should be encouraged, where possible to receive two SARS-CoV-2 immunizations prior to surgery **Staff**: Surgical teams should protect themselves by receiving two SARS-CoV-2 immunizations and any follow up boosters offered. If your team has any concerns regarding immunization, advice should be sought from a medical virologist/ microbiologist/infection specialist
7.	Personal protective equipment	PHE COVID-19: Guidance for maintaining services within health and care settings

[a] https://www.who.int/news-room/commentaries/detail/criteria-for-releasing-covid-19-patients-from-isolation%20
[b] https://www.ecdc.europa.eu/sites/default/files/documents/Guidance-for-discharge-and-ending-of-isolation-of-people-with-COVID-19.pdf%20
[c] Emergency surgery during the COVID-19 pandemic: what you need to know for practice.
[d] SARS-CoV-2 infection, COVID-19 and timing of elective surgery.
[e] SARS-CoV-2 can be detected in upper respiratory samples approximately two days before the onset of symptoms.

Table 1

commonly up to forty. This was due to the denaturation of the polymerase caused by heat, integral to the process. This is due to the first step in a PCR cycle being to denature the dsDNA present (separate the dsDNA in to two separate ssDNA strands). The wells are heated up to 95°C, which disrupts the hydrogen bonds between the complementary bases of the dsDNA, producing two single DNA strands. The initial denaturation step lasts 30 seconds to 5 minutes (but can be up to 10 minutes, for templates with highly complex structures), ensuring that all complex dsDNA molecules are separated into single strands.

Annealing
The second step in a PCR cycle is to cool the reaction, usually to 45−70°C for the annealing (attachment) step, which takes between 20 seconds and 1 minute. Primers are a short complementary nucleotide sequence, specific to the required target DNA or RNA template. They are usually 18−30 bases in length, the shorter the primers are, the more efficiently they will anneal to the target. The annealing of primers is the production of hydrogen bonds between the complementary base pairs of the primer and the specific area of the target ssDNA. This results in a short section of dsDNA which is used as an attachment point for the DNA polymerase to adhere. Design of primers is fundamental to the quality of an assay, complementary areas between the primers can cause inhibition of effective amplification of the desired target (Figure 3). The primers (and probes, used in real-time PCR) provide the high specificity of PCR, when desired, which is dependent on both length and the annealing temperature used. The primers are chosen for attachment to the 5′ to 3′ (plus strand), this is the reverse primer and the 3′ to 5′ (negative strand) is complementary to the forward primer. The amplicon is the product of the PCR reaction, it is the length of base pairs (bp) from the 5′ end of the forward primer to the 5′ end of the reverse primer (Figure 4). Typical conventional PCR amplicons are designed to be 200−1000 bp and real-time amplicons 70−200 bp in length.
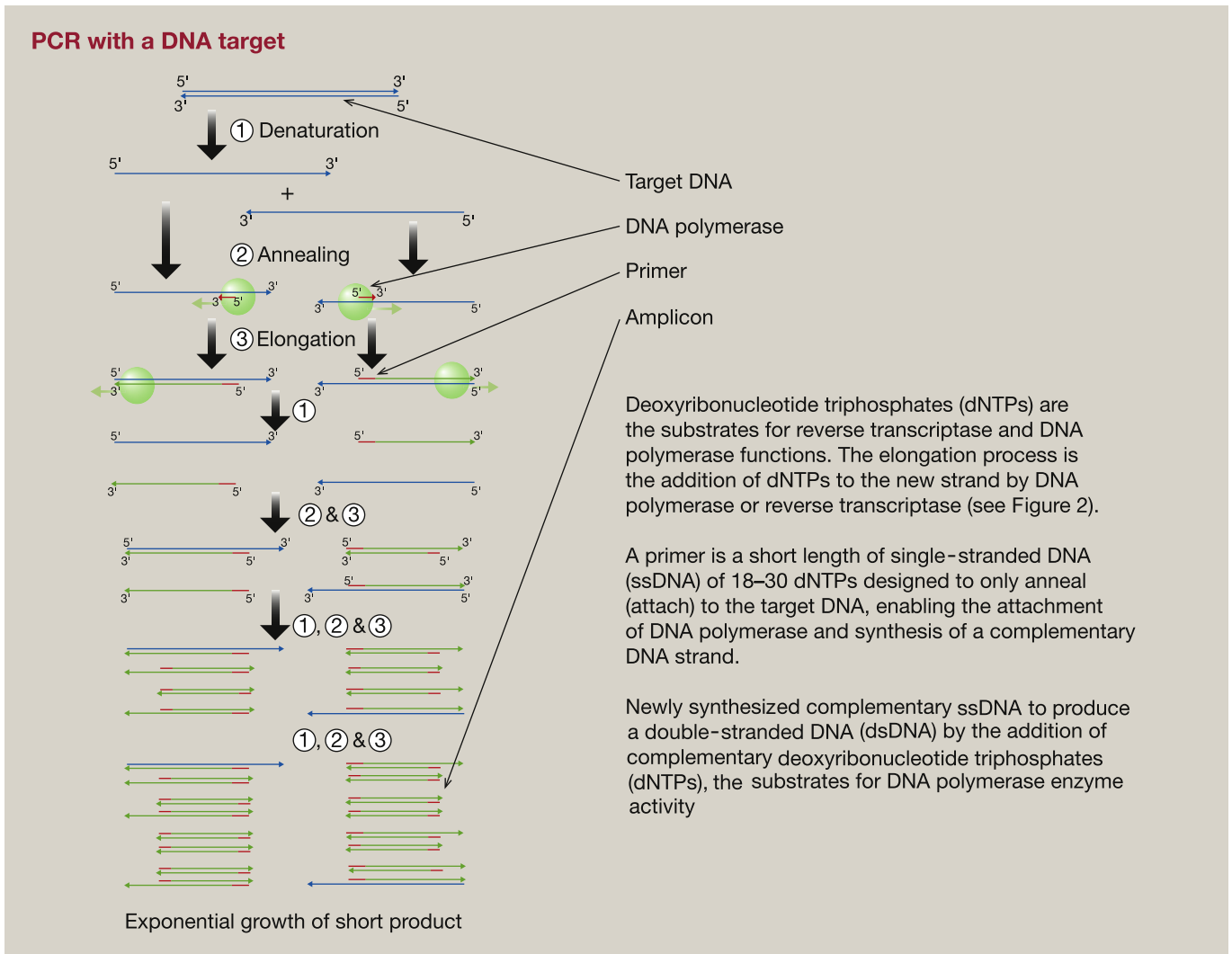
PCR with a DNA target

Deoxyribonucleotide triphosphates (dNTPs) are the substrates for reverse transcriptase and DNA polymerase functions. The elongation process is the addition of dNTPs to the new strand by DNA polymerase or reverse transcriptase (see Figure 2).

A primer is a short length of single-stranded DNA (ssDNA) of 18–30 dNTPs designed to only anneal (attach) to the target DNA, enabling the attachment of DNA polymerase and synthesis of a complementary DNA strand.

Newly synthesized complementary ssDNA to produce a double-stranded DNA (dsDNA) by the addition of complementary deoxyribonucleotide triphosphates (dNTPs), the substrates for DNA polymerase enzyme activity

Figure 1

Primers are chosen based upon the various needs of the assays.[8] Assays for the diagnosis of infection must be checked that the primers do not have complementarity to the human genome or any of the human microbiome or other pathogens.[9] The site should be a highly stable region to prevent false negative results due to mutations at the primer binding site. This is an on-going task to check that primer sites remain appropriate, without evidence of target sequences missed by the primer set, on at least an annual basis.

Elongation

Once the primers have annealed to the complementary target, the third extension or elongation step of a PCR cycle begins. The elongation step requires a rise in temperature from the annealing step to approximately 72–80°C. The DNA polymerase attaches to the 3' end of both forward and reverse primers. The subsequent nucleotide in the template strand on the 3' end of the primer is identified by the enzyme. It attaches the complementary deoxynucleotide (dNTPs, the building blocks of DNA molecules) synthesizing a new complementary DNA strand from the 3' to the 5' end of the template. At the end of the first PCR cycle of a dsDNA target (denaturation, annealing, elongation), two copies dsDNA template are present, each with one of the original strands. This is due to the use of both ssDNA strands as a template, with the forward primer attaching to the minus ssDNA strand and the reverse primer attaching to the positive ssDNA strand. In reality, tens of thousands of target sequence may be present in a reaction, so the first cycle will double the number of target sequences in the reaction. This is due to the excess of primers (and probes, if a real-time PCR) and DNA polymerase added to the well, enabling a duplication after each cycle until the dNTPs, primers, probes or functional DNA polymerase supply is depleted by the production of dsDNA.

The choice of DNA polymerase is important when designing a PCR assay, the available enzymes have different characteristics, including specificity, thermostability, fidelity and processivity. Hot start enzymes, for example, have specific antibodies bound to the polymerase. During the initial denaturation step, the bound antibodies are degraded by the 95°C heat, which activates the hot start polymerase. This inhibits the potential for non-specific amplification during the reaction setup. This can also be accomplished by the PCR set up being completed with the plate on ice, which significantly reduces the activity of
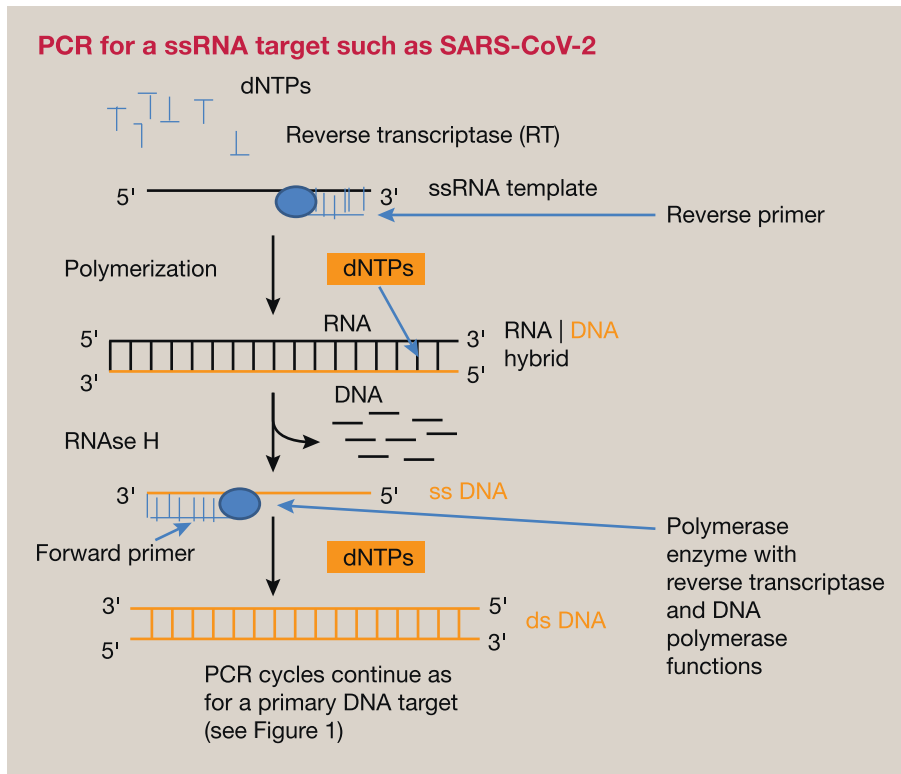
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Figure 2

polymerases, inhibiting non-specific amplification at room temperature. Weighing the advantageous and disadvantageous characteristics of the available enzymes (including the considerable variation in cost), inform the choice for the specific assay and result requirements.

The steps in a PCR cycle initially required the manual movement of tubes back and forth between three water baths of different temperatures. The automation of PCR was started by the invention of the thermocycler, a machine capable of rapidly heating and cooling individual tubes and then plates of multiple wells. This enabled PCR to move from a small scale, labour intensive procedure, to one able to be adapted to use on a large scale.

Conventional PCR

Prior to the PCR reaction taking place, a sample of interest may need to be rendered uninfectious, most commonly using a lysis buffer containing guanidinium thiocyanate and a detergent.[10] This disrupts the cell membranes, viral envelopes, bacterial
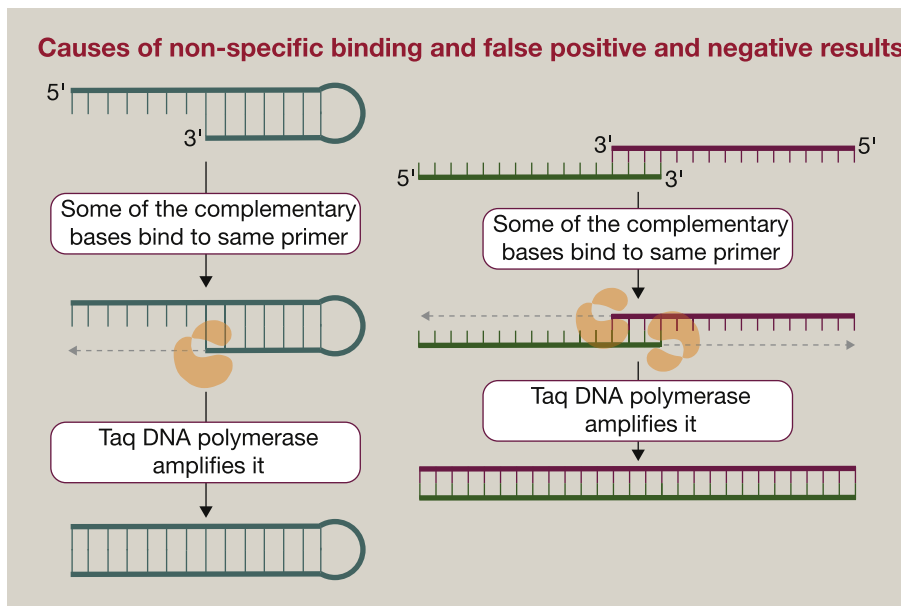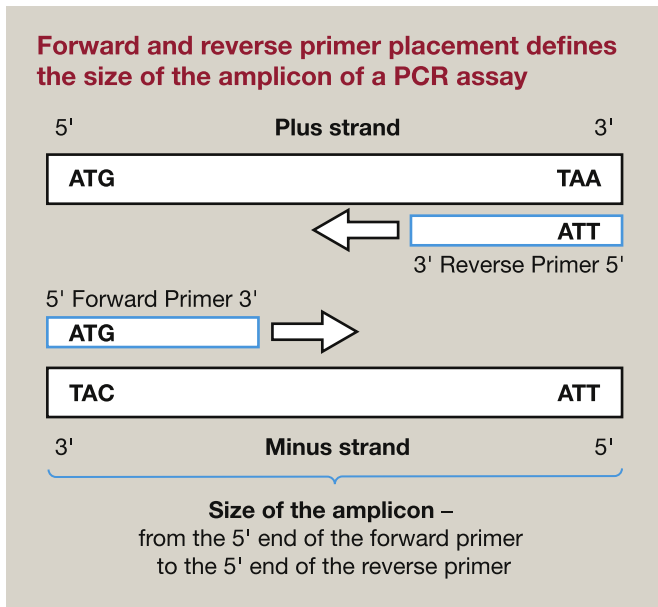

Figure 3

Forward and reverse primer placement defines the size of the amplicon of a PCR assay
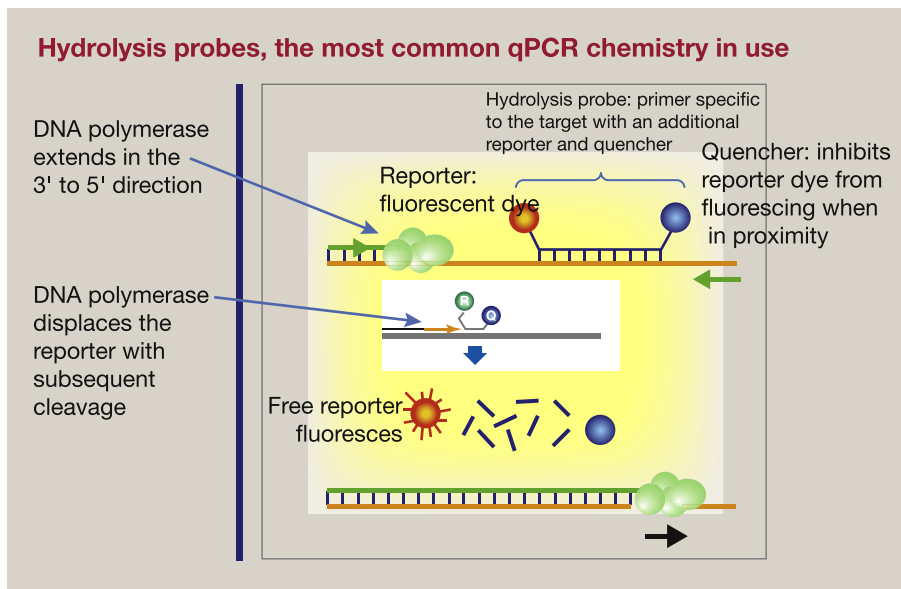

Figure 4

and fungal cell walls, releasing their contents into the solution. Proteins are denatured; this inactivates the nucleases (DNase and RNase) which would otherwise start to degrade the nucleic acids present. The stabilized DNA and RNA then undergo nucleic acid extraction,[11] they are lifted from the sample mixture, leaving behind all the other sample components. The purified nucleic acid is then added to PCR grade water, the extracted nucleic acid has removed multiple potential causes of PCR inhibition, leaving the concentrated purified nucleic acids in a stable form which can be stored long term. The quality of the nucleic acid extraction determines the stability, presence of residual inhibitors and concentration of RNA/DNA for use in PCR. Most commonly the nucleic acid extract is used directly by adding to the primers, dNTPs, DNA polymerase (with or without additional reverse transcriptase activity) and PCR buffer. The PCR buffer provides a

suitable chemical environment for the activity of DNA polymerase and includes Tris—HCl and potassium chloride which stablize the pH of the reaction. Magnesium chloride is included as magnesium ions act as cofactors for DNA polymerase.

After completion of the PCR cycles, the detection of the amplicons produced, was historically carried out using the gel electrophoresis technique.[12] The products of PCR were pipetted into wells of the gel block, and when present they migrated electrophoretically across the block from the negative electrode towards the positive. They are separated according to their molecular size, because the distance travelled by each DNA fragment, during a specific period of time is unique to its molecular size. The position on the gel block is then compared against a DNA ladder, which is made up of a mixture of DNA fragments of pre-determined sizes and the presence of a specific DNA fragment the size of the expected amplicon. DNA is not visible by eye, most commonly it was visualized by staining with ethidium bromide, a fluorescent dye that intercalates into DNA. This is a highly carcinogenic, cytotoxic and mutagenic compound, which is hazardous to human health and the environment. Visualization occurred using a UV transilluminator, at the terminal stage, when the result became identifiable. Now termed conventional PCR, it is no longer used commonly for diagnostic purposes. Despite alternatives to ethidium bromide now being available, the two-step process of conventional PCR, with the subsequent detection of the amplicon, limited the number of samples which could be tested, increased the risk of amplified product contaminating other results and was labour intensive.

Real-time PCR/qPCR

The most commonly used form of PCR in the developed world today is real-time PCR (qPCR or qRT-PCR).[13] The difference from conventional PCR was the introduction of the detection of the target simultaneously with the amplification occurring in the well. This had the advantage of dramatically reducing the turnaround time and the risk associated with post-amplification handling, causing contamination (false positive results). The

Hydrolysis probes, the most common qPCR chemistry in use
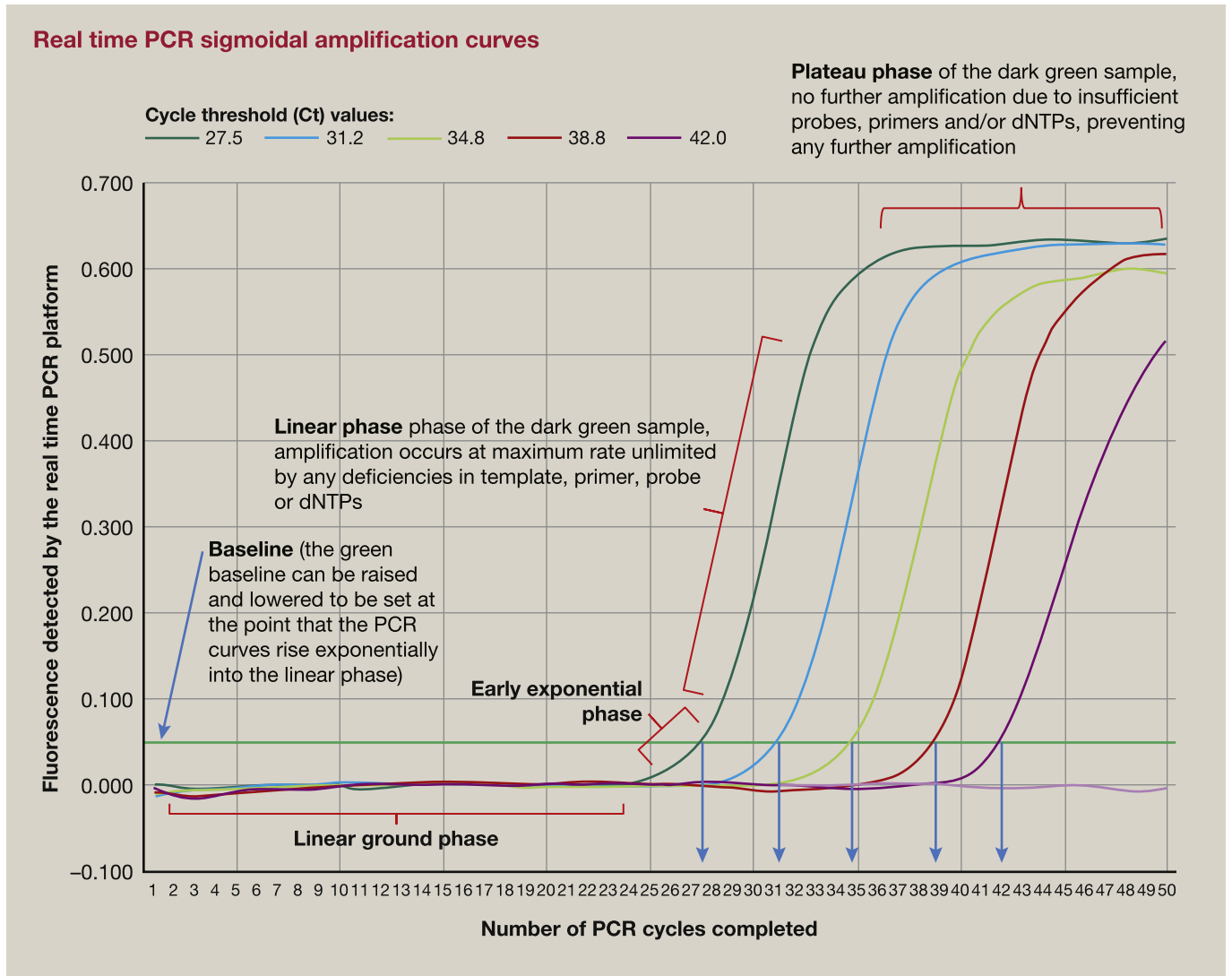

Figure 5

Figure 6

technique also has the ability to semi-quantify the concentration of the target sequence, hence the use of the term qPCR (quantitative PCR) for DNA targets and qRT-PCR for RNA targets, which are synonymous with qPCR. Real-time PCR has a significantly increased specificity, sensitivity, safety and turnaround time, compared to the conventional PCR method.

One of the most popular detection chemistries[14] for qPCR uses a florescent labelled probe, a hydrolysis probe (Figure 5). A probe is a short oligonucleotide sequence (a primer) with a fluorescent dye, the reporter, and quencher attached. The quencher prevents the dye from fluorescing, when the probe is intact, due to their close proximity. The use of the probe (in addition to the primers) increases the specificity, by a further complementary annealing step being required. The detection occurs after the cleavage of the reporter dye, removing it from the influence of the quencher. The fluorescence emitted from the free dye is detected using a qPCR instrument, a thermal cycler with additional optics for fluorescence excitation and emission collection with associated analysis software. A graphical representation of the fluorescence intensity against the amplification

cycle illustrates the results of a qPCR run (Figure 6). The linear ground phase represents the PCR cycles where the fluorescence emission has not risen above the background, providing no evidence of amplification of the target. The early exponential phase starts where the fluorescence emission is starting to rise above the background. The baseline is set in the early exponential phase, at the point that the PCR curve starts to rise exponentially into the linear phase. The cycle threshold (C_t) value is read at the crossing point of the curve and the baseline. C_t is the number of PCR cycles completed at that crossing point. The terms C_t, quantification cycle (Cq) crossing point (Cp) and take-off point (TOP) are all found in the literature and are identical in meaning, each commercial company producing the real-time instruments using their own name. The log linear phase occurs when the amplicons are produced exponentially with each PCR cycle, when all required components are available, to supplying optimal replication with no restrictions. The plateau phase occurs when some or all are limited. The primers, probes, dNTPs and functional enzyme are initially limited in the plateau phase and then absent to prevent any further release of fluorescence.

Advantages and disadvantages of qPCR

Advantages of qPCR	High sensitivity (but highly variable dependent on the assay, ask for the lower limit of detection in IU/mL for SARS-CoV-2)
	Wide dynamic range for quantification (7–8 Log_{10})
	High specificity
	Can be used to detect and subtype every species of microorganism
	Cost effective (e.g. in-house PCR, £14 for a panel of 12 pathogens)
	Can be scaled up to 10,000s results per day in a single laboratory
	Automated options such as Abbott Alinity m with lower turnaround time (2.5 hours for Resp-4-Plex assay: SARS-CoV-2, Flu A, Flu B, & RSV, ∼300 results in 8 hours)
Disadvantages of qPCR	The genomes of inactivated pathogens are detected and give the same results as replicating, infectious viruses, bacteria, fungi and protozoa. Patients on effective bactericidal antibiotics initially provide the same results as those with high level resistant untreated bacteria. Culture is the only option which definitively identifies live pathogen
	Highly skilled workforce required to avoid contamination (false positive results) using open platforms with manual set up (drastically reduced by automation)
	Turnaround time varies but is significantly longer than cartridge based qPCR (∼2.5 hours for automated commercial, 4–6 hours in-house on platform turnaround time, commonly batched in 96 or 384 well plates)
Advantages of cartridge-based qPCR: GeneXpert	High sensitivity
	High specificity
	Short turnaround time (SARS-CoV-2, Flu A, Flu B and RSV in 40 mins on platform turnaround time)
	Can be used as Point of Care Test (POCT) on the ward/in theatre
Disadvantage of cartridge-based qPCR: GeneXpert	Limited number of targets offered by the companies supplying the platforms.
	Limitation to platform and cartridge availability
	High cost per test (∼£35 SARS-CoV-2 only, ∼£42 SARS-CoV-2, influenza A/B and RSV)
Advantages of cartridge-based film array: Biofire	High sensitivity
	Broad syndromic based targets
	Short turnaround time (40 mins–1 hour on platform turnaround time)
	Can be used as point-of-care test (POCT) on the ward/in theatre
Disadvantage of cartridge-based film array: Biofire	Lower specificity (endpoint melting curve data, not amplification curve analysis as for qPCR)
	Extremely high cost per test (∼£100)
	Limitation to platform and cartridge availability

Table 2

Opposite to conventional wisdom, low C_t values indicate samples with a high amount of starting target sequence, because they take a smaller number of cycles of qPCR to produce detectable fluorescence which crosses the baseline. These samples have a high 'viral load', which cannot be compared to other assays (unless a standard is used) but can provide a useful guide to indicate the approximate concentration of target present. Sample type is important here, defined sample volumes such as whole blood, plasma, serum and cerebrospinal fluid results can be compared to another sample of the same type on the same qPCR run. C_t values of different sample types are not comparable in the same patient. For example, nose and throat swabs will be dependent on sampling technique and the highly variable nature of the respiratory secretions collected. Two swabs taken at the same time, from the same patient will be expected to have significantly different C_t values. A nose and throat swab C_t value is not comparable to a bronchoaveolar lavage (BAL) for example, the result only indicates what is present in the location sampled and the results can be expected to be different in both locations. For example, patients with

COVID-19 can have negative qPCR results in the nose and throat and a Ct of 15 in the BAL, taken on the same day. High C_t values conversely indicate samples in which the target concentration is low. Therefore the C_t value is important for semi-quantitative detection of a specific target sequence, which was not feasible with conventional PCR detection methods. For every patient it is imperative to also look beyond PCR results, seeing the patient within the context of their symptoms, other pathology and imaging results, with the assistance of a medical virologist or microbiologist, in complex cases.

All real-time PCR assays, however, are not equal with regards to their sensitivity. Throughout the literature C_t values are compared between different assays for the same pathogen; however, C_t values vary widely even for identical samples on different assays, in different laboratories using different protocols. In one international external quality assessment (EQA) scheme for SARS-CoV-2 qPCR, one identical sample tested across 66 laboratories, each using the N gene target, had a variation of 18 C_t from the highest to lowest reported for the same specimen.[15]

An important consideration is that detection of DNA by PCR alone, does not imply pathogenesis. This is a vital point often missed in the literature, leading to erroneous conclusions. A causal association between cardiac disease and parvovirus B19 continues to be frequently postulated following the detection of DNA by PCR in endomyocardial biopsy specimens. This was elegantly dismissed for the majority of cases by Virologists in Freiburg, Germany.[16] Parvovirus B19 DNA was tested for in myocardial samples from cadavers who died from a non-cardiac aetiology; 46 of 48 seropositive (those with detectable IgG, evidence of past infection) patients myocardial samples had parvovirus B19 DNA detected. Conversely, none of the 21 seronegative individuals (no IgG detectable, no evidence of past infection) had parvovirus B19 detectable. So presence of parvovirus B19 DNA alone does not provide evidence of primary acute infection, it merely represents past infection. A significant number of papers propose parvovirus B19 as a cause of cardiac disease in adults, evidenced by detection of DNA alone in cardiac biopsies. If an acute pathogenic process is being proposed, evidence of acute infection is vital in the form of IgM or high level viraemia (10^6 IU/mL). Asymptomatic lifelong persistence is known to occur in immunocompetent individuals infected with parvovirus B19, JC virus, BK virus, polyomavirus simian virus 40 (SV40), hepatitis B in hepatocytes and all herpesviruses.

Multiplex PCR is a variation of qPCR which dramatically improved its efficiency. Multiplexing involves the addition of primers and probes sets, for more than one target sequence into a single PCR well. The probes are labelled with different fluorescent dyes (detected at different wavelengths) and each target sequence produces their own individual amplification curve, if present, during the qPCR run. This technique has been used to produce tests with two (duplex), three (triplex) and even four (quadriplex) targets per well. This has reduced the costs and turnaround times associated with qPCR and have made it possible to use a syndromic approach for disease diagnosis. Respiratory panels consisting of viral and bacterial pathogens are currently multiplex qPCR results or variations on this technology. Meningitis/encephalitis pathogen panels similarly have transformed diagnostic efficiency, increased the number of diagnoses made and improving the management of these infectious diseases dramatically.

Quantitative PCR

The only quantitative information directly available via qPCR is the comparison of samples with a standard volume, which are tested on the same run. Under these circumstances a 3.3 C_t change represents a 1 Log_{10} (or 10 fold) change in the quantity of the genetic template between the samples. 6.6 C_t change indicates a 2 Log_{10} (or 100-fold) and 9.9 C_t a 3 Log_{10} (or 1000-fold) increase or decrease and so forth.

When absolute quantification is required, a standard is tested simultaneously with the samples in question. The standard is provided at a known quantitation, in international units per millilitre (IU/mL) or rarely in the case of HIV, copies per millilitre (copies/mL). In most cases the use of copies/mL in the literature, with the exception of HIV, use in-house standards and therefore results are not comparable to any other assays results reported as copies/mL. Check the standard used is the same quantitation

standard, before comparing results across assays. A high, moderate and low level standard are usually used to produce a standard curve. The C_t levels of the standards are graphed with comparison of the C_t against the IU/mL standard curve. This standardization means that a hepatitis B quantitation or 'viral load' will be within 0.5 Log_{10} of the result produced using the same sample, anywhere in the world when using the same international standard. If the same sample is re-tested, results are expected to vary by up to 0.5 Log_{10}, so even when standards are used to provide quantitative results (IU/mL or copies/mL), only changes of >0.5 Log_{10} represent evidence of a genuine change. This is the reason that quantitative results should be considered in the Log_{10} value form, it enables the expected variation when repeating the same sample multiple times to be accounted for.

Interpretation of the quantitation of viruses which undergo latency requires careful attention. These are persistent infections where the complete viral genome is retained with in the host cell. A restricted expression of viral antigens occurs, but there is no production of daughter virions. Latency also requires that the latent state can be reversed to produce a lytic infection. Epstein−Barr virus (EBV), for example, undergoes latency in B-lymphocytes and asymptomatic reactivation may occur in immunocompetent patients with an acute illness. This represents non-pathogenic replication with the exception of haemophagocytic lymphohistiocytosis (HLH) which is highly clinically distinct. Detection of EBV DNA in the blood or any inflamed tissue is non-pathogenic in the immunocompetent until proven otherwise. On the contrary in severely immunosuppressed individuals, detection of high levels of EBV DNA require urgent assessment to investigate or treat post-transplant lymphoproliferative disorders (PTLD). EBV and CMV IgM are required to provide evidence of acute primary infection in the immunocompetent. Requests for quantitative EBV and CMV PCR should be restricted to use in immunocompromised patients (Table 2).

Digital PCR

Digital PCR offers an alternative quantification method which does not require standards or the production of a standard curve based.[17] The same reagents are used as for qPCR, the addition step involves the partitioning of the sample into separate nano-litre sized droplets, each droplet contains an individual opportunity for a PCR reaction to occur in parallel. Unlike qPCR, amplicon detection occurs at the end of the PCR cycles. The result is provided as an absolute amount of the target present in the sample by using the proportions of negative and positive signalled droplets without the need for standards to provide a reference concentration to compare against. Digital PCR has not yet become a standard tool within specialist virology centres due to technical limitations including the numbers of droplets produced, variation in volume of droplets, cross-contamination, and cost.

Quality control

The quality control of all nucleic amplification techniques is imperative. The nature of multiplying millions or billions of facsimiles of the target sequence results in a high risk of contamination of negative samples. Preventative measures against contamination to avoid false positive results include a

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strict one-way system, where amplified plates or any other object from the amplification location do not go back to the area where nucleic acid extraction and PCR plate set up occur. This is detail not usually available in the literature to assess; however, the use of negative controls should always be provided in papers. Negative controls enable the identification of false positive results, ideally they are known negative samples. It is vital that negative controls are processed in the identical way to the other samples for each run of PCR tested together. This includes any inactivation process, nucleic acid extraction, PCR plate set up and amplification step. These no template controls (NTC) serve as a control for extraneous nucleic acid contamination and when positive may indicate the need to discard all positive results on the run. In order to identify that all the required components have been added to the reaction, a positive control is required. This provides evidence that the negative results on the run are genuine and not due to failure to add a reagent to the wells. The internal control (IC) is an unrelated genetic sequence, which is added to the nucleic acid extraction step of all samples. The IC provides evidence of successful nucleic acid extraction, lack of inhibition of the PCR reaction and addition of all reagents. Ideally an IC is included in an assay as a duplex, with the primers and probes of both the primary target and that of the IC. The duplex option has the advantage that there are identical conditions and content in the single well, providing the best evidence that when the IC is detected at the expected level, there has been no inhibition of the PCR reaction and a undetected primary target is due to lack of presence in the sample and not a false negative result.

Beyond SARS-CoV-2 and infectious pathogens, PCR has been used for a wide variety of indications in surgical patients, for example, for TERTp mutation detection in spinal myxopapillary ependymoma[18] and to assess cancer cell dissemination to identify patients at high risk for peritoneal recurrence in preoperative pancreatic cancer.[19] Surgeons of every specialty will be using PCR results to inform their decisions about patients in the future, related to human and cancer gene expression informing their patient prognosis and the diagnosis of pathogens with implications for infection control procedures in theatre and for preoperative and postoperative treatment.

Production of reliable, repeatable, timely qPCR results is a continuous struggle with the current unprecedented volumes of work. If an urgent result will alter patient management the most effective intervention is to telephone the virology or microbiology laboratory before the sample is taken, to get advice on current turnaround and how to fast track a result. The fastest results are available after 40 minutes to 1 hour, but these cartridge based assays are severely limited in number, are expensive and may lack the capacity to provide a C_t value and therefore to differentiate if there is evidence of an acute infection, or residual nucleic acid from an infection weeks or even months before, such as the Biofire. Large-scale commercial platforms frequently have results available 2.5 to 4 hours after addition to the platform (but usually have a limited repertoire and some, such as the Panther Hologic, use alternative technology which lacks the capacity to provide a C_t value and has a lower specificity, requiring confirmation and quantitation) and in-house assays vary widely, due to the variation in technologies used. Contact your laboratory for local specific information on availability, limit of detection and turnaround time. ◆

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