

A beginner's guide to RT-PCR, qPCR and RT-qPCR

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The development of the polymerase chain reaction (PCR), for which Kary Mullis received the 1992 Nobel Prize in Chemistry, revolutionized molecular biology. At around the time that prize was awarded, research was being carried out by Russel Higuchi which led to the discovery that PCR can be monitored using fluorescent probes, facilitating quantitative real-time PCR (qPCR). In addition, the earlier discovery of reverse transcriptase (in 1970) laid the groundwork for the development of RT-PCR (used in molecular cloning). The latter can be coupled to qPCR, termed RT-qPCR, allowing analysis of gene expression through messenger RNA (mRNA) quantitation. These techniques and their applications have transformed life science research and clinical diagnosis.

Why are RT-PCR, qPCR and RT-qPCR not one and the same?

When discussing this topic, it is important to highlight the common misconception that RT-PCR, qPCR and RT-qPCR are synonymous. Indeed, the similarities between the closely related techniques often result in the incorrect use of the acronyms. In an attempt to prevent this, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, first published in 2009, proposed a standardization of abbreviations. They stated that 'RT-PCR' should only be used to describe reverse transcription PCR and not real-time PCR, as is often confused. Reverse transcription PCR allows the use of RNA as a template to generate complementary DNA (cDNA). Using the reverse transcriptase enzyme, a single-stranded copy of cDNA is generated. This can then be amplified by a DNA polymerase, generating double-stranded cDNA, feeding into a standard PCR-based amplification process (see Figure 1A). This technique can be used in molecular cloning of genes of interest (GOIs), but most commonly, it serves as the first step in RT-qPCR. According to MIQE, the acronym 'qPCR' describes quantitative real-time PCR, which is the PCR amplification of DNA in real time, measured by a fluorescent probe, most commonly an intercalating dye or a hydrolysis-based probe, enabling quantitation of the PCR product (see Figure 1B). This technique is used to detect the presence of pathogens and to determine the copy number of DNA sequences of interest. The final acronym 'RT-qPCR' is used for reverse transcription quantitative real-time PCR. This is a technique which combines RT-PCR with qPCR to enable the measurement of RNA levels through the use of cDNA in a qPCR reaction, thus allowing rapid detection of

gene expression changes (see Figure 1C). Despite these standardized abbreviations, it is important to note that this nomenclature guideline is not always adhered to, and qPCR is commonly used to describe RT-qPCR. Similarly, RT is used to denote real-time PCR rather than reverse transcription, thus causing confusion over which method is being described. For this Beginner's Guide, we will be using the MIQE abbreviations as described above.

Overview of qPCR and RT-qPCR

Quantitative PCR, whether involving a reverse transcription step or not, is routinely used in molecular biology labs and has revolutionized the way in which research is carried out due to its relatively simple pipeline (Figure 2). Its advantages over standard PCR include the ability to visualize which reactions have worked in real time and without the need for an agarose gel. It also allows truly quantitative analysis. One of the most common uses of qPCR is determining the copy number of a DNA sequence of interest. Using absolute quantitation, the user is able to determine the target copy numbers in reference to a standard curve of defined concentration in a far more accurate way than ever before. RT-qPCR, on the other hand, allows the investigation of gene expression changes upon treatment of model systems with inhibitors, stimulants, small interfering RNAs (siRNAs) or knockout models, etc. This technique is also routinely used to detect changes in expression both prior to (as quality control) and after (confirmation of change) RNA-Seq experiments.

Sample preparation

The most crucial step in the qPCR and RT-qPCR pipeline is arguably sample isolation. No matter how good your assay design is, if the starting material is contaminated

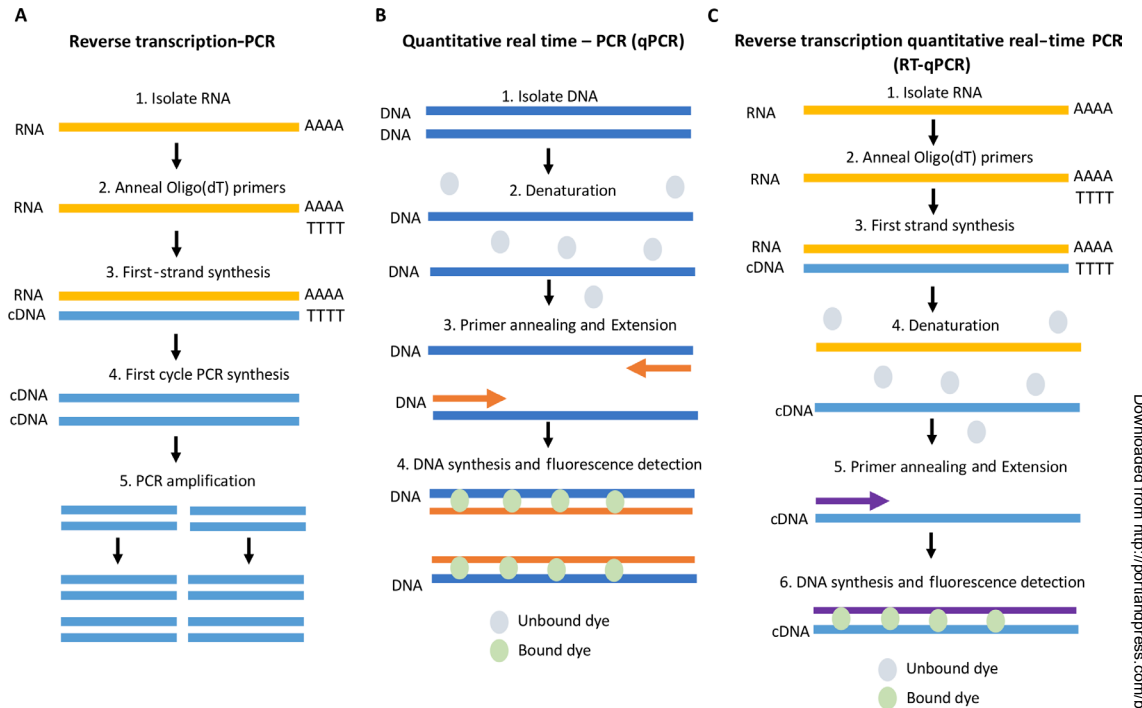


Figure 1. Schematic comparing RT-PCR, qPCR and RT-qPCR. (A) RT-PCR workflow. RNA is isolated and cDNA is generated via reverse transcription (RT); PCR is then carried out to amplify areas of interest. (B) qPCR schematic. DNA is isolated and amplified; amplification is quantitated using a probe which fluoresces upon intercalation with double-stranded DNA. (C) RT-qPCR procedure. RNA is isolated and cDNA generated before commencing a qPCR procedure.

or degraded, you will not get accurate results. A good-quality sample is the starting block of good-quality data. When isolating DNA for qPCR, it is essential that it is free from contaminants that may inhibit the reaction. Most often, extraction is carried out using commercially available kits, which have the advantage of being user-friendly, simple and quick, especially when integrated with a robotic system. The type of RNA extraction carried out depends on the type of RNA required. The most common extraction method used is with total RNA extraction kits. This isolates messenger RNA (mRNA;

the precursor of protein synthesis), transfer RNA (tRNA; decodes mRNA during translation with the ribosome) and ribosomal RNA (rRNA; reads the amino acid order during translation and links them with the ribosome), but often (not always) fails to isolate smaller RNAs such as non-coding RNA (ncRNA; functional RNA transcribed from DNA, but not translated into proteins) and micro RNAs (miRNAs; regulate gene expression by inhibiting mRNA translation). With the explosion of interest in enhancer RNAs (eRNAs; small RNAs transcribed from enhancers) which can vary in length

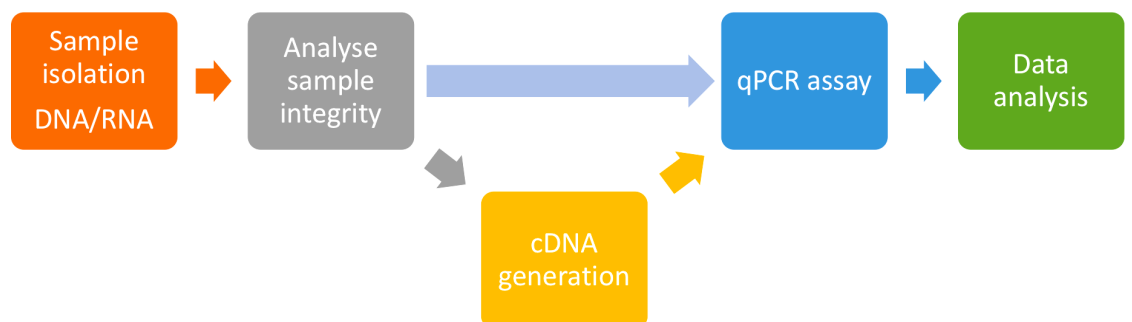


Figure 2. Workflow of a standard qPCR and RT-qPCR experiment. Following sample isolation, the integrity is analysed prior to cDNA generation and commencement of the qPCR assay using either intercalating dyes or hydrolysis probes. Fluorescence is detected throughout the PCR cycles and used to generate an amplification curve which is used to quantitate the target sample during data analysis.

considerably, it is essential that the extraction methods are carefully considered to ensure isolation of the RNA of interest. In addition to extraction considerations, it is essential that RNA is not contaminated with DNA, since this cannot be distinguished from cDNA in the qPCR reaction. To overcome this, most protocols rely on the use of a DNase I treatment which digests any DNA.

During isolation, sample degradation is always a possibility. Accordingly, any good pipeline will involve a quality control step to assess the integrity of the sample. This can be done quickly by evaluating the A260/280 ratio (comparing the absorbance at 260 vs 280 nm, a measure of contamination by proteins) and the A260/230 ratio (260 vs 230 nm, an indication of the presence of organic contaminants) of the sample; however, this is not very accurate and is subject to interference from several factors. A more accurate measure is the use of a virtual gel electrophoresis system such as the Aligent Bioanalyser. This system works using a chip that separates RNA based on size and detects RNA by fluorescent dyes. This is then translated to a computer which, using an algorithm, produces an RNA integrity number (RIN) which represents the quality of the sample, with 10 being the highest.

The final step in sample preparation for RT-qPCR is the generation of cDNA. cDNA utilizes RT-PCR (Figure 1) to generate cDNA from the RNA template using a reverse transcriptase. This can be done employing oligo(dT) primers, which anneal to the polyA tail of RNA, or using random hexamers (primers of six to nine bases long, which anneal at multiple points along the RNA transcript). Generally, a mix of the two primers is thought to be best as it enables amplification of polyA tail RNA (mainly mRNA) and non-polyA-containing RNA (tRNA, rRNA, etc). In addition to the primer consideration, cDNA generation can be part of the qPCR

experiment (termed one-step RT-qPCR) or is generated separately from the qPCR (two-step RT-qPCR), as shown in Figure 3. The advantage of one-step RT-qPCR is that there is less experimental variation and fewer risks of contamination, as well as enabling high-throughput screening; hence, this option is usually used for clinical screening. However, it does mean that the sample can only be used a limited number of times, whereas two-step RT-qPCR enables more reactions per sample and flexible priming options and is usually the preferred option for wide-scale gene expression analysis, but does require more optimization.

Detection methods

The next most important decision when designing your experimental pipeline is choosing the method of detection. All are based on the emission of fluorescence, but the chemistry behind them differs. One method is the use of a fluorescent dye which binds non-specifically to double-stranded DNA as it is generated. SYBR[®] Green is the most common intercalating dye and emits a fluorescent signal upon intercalating with newly synthesized DNA. The more the DNA generated in the qPCR reaction, the more fluorescence is detected (Figure 4A). The second method of detection uses hydrolysis probes such as TaqMan[®] probes, which depend on Förster Resonance Energy Transfer (FRET) preventing the dye moiety from emitting a signal via the quencher when the probe is intact (Figure 4B). These probes are specific sequences which are designed to bind downstream of the qPCR primers. The 5' end of the probe is labelled with a fluorescent reporter such as the carboxyfluorescein (FAM) moiety; on the 3' end is a quencher molecule which prevents fluorescent emission when in close proximity to the reporter. As

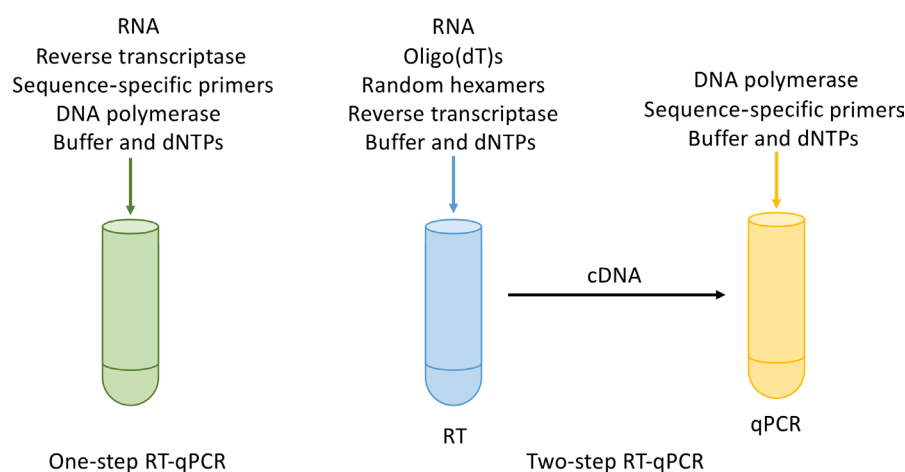


Figure 3. One-step vs two-step RT-qPCR. One-step RT-qPCR involves the generation of cDNA via reverse transcription and qPCR amplification of the target sequence in one reaction. Two-step RT-qPCR separates out the two steps (RT-PCR and qPCR), thus enabling more target sequences to be analysed in the qPCR reaction.

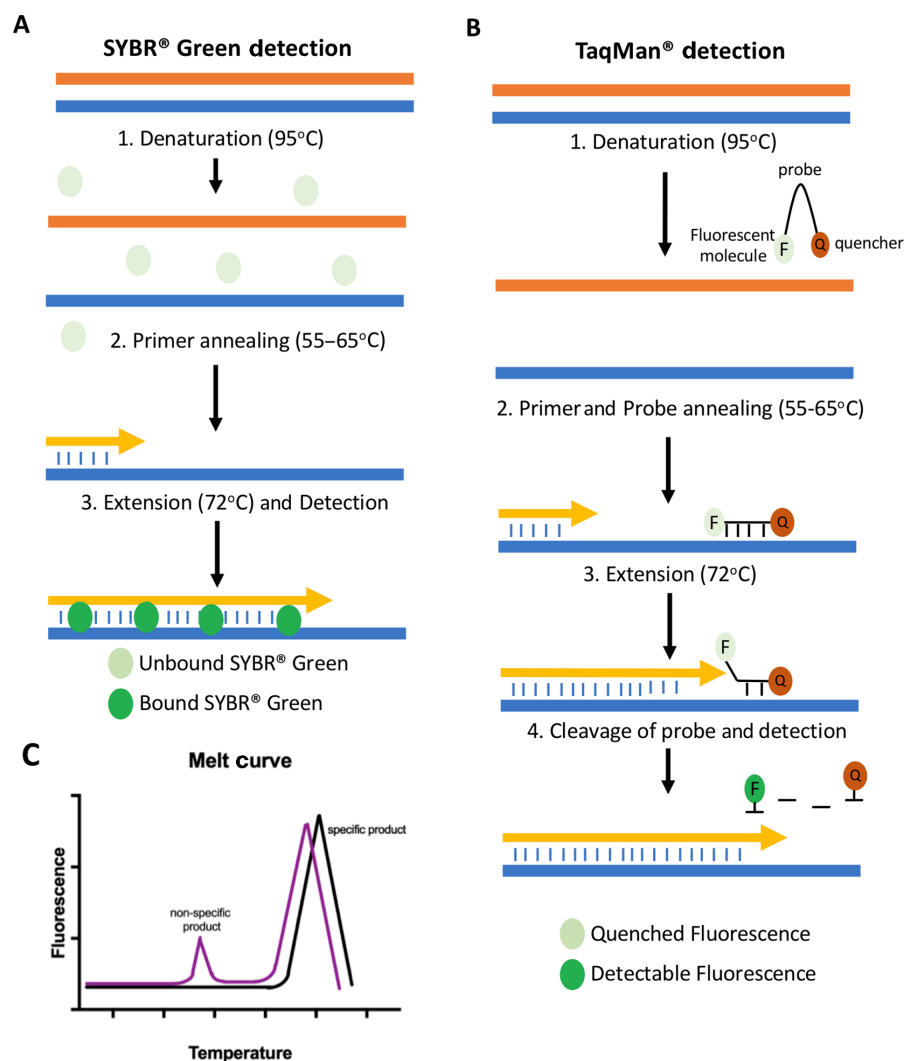


Figure 4. Comparison of intercalating dye and hydrolysis-based probe detection. (A) **SYBR® Green detection:** Following denaturation of cDNA, primers anneal and are extended. During extension, SYBR Green binds to the double-stranded DNA (dsDNA), emitting a fluorescent signal detected by the qPCR instrument. (B) **TaqMan® probe detection:** TaqMan probes bind downstream of the primers to single-stranded cDNA. During extension, the polymerase breaks up the probe, allowing the fluorescent signal to be detected due to the loss of proximity to the quencher moiety. (C) **Melt curve graph for primer specificity:** A melt curve measures the dissociation of dsDNA at high temperatures. A single DNA species (produced from a specific primer pair) will result in a single peak (black line), and multiple DNA species or primer dimers will result in two or more peaks (purple) and indicate non-specific primers.

DNA polymerase extends the primer, the probe is cleaved, enabling the reporter molecule to emit a fluorescent signal.

Since such probes are target specific, they inherently have greater specificity than intercalating dyes. Consequently, when you detect a signal using a probe, you can be confident that the signal is genuinely from your GOI, since it requires the primers and the probe to bind at the target sequence for signal detection. Intercalating dyes, however, are non-specific, and therefore, further downstream analysis in the form of a melt curve is required to ensure that the signal being detected is genuinely the target of interest (Figure 4C).

This can also be aided by the use of carefully designed primers and by validating their specificity, for which there are many examples online including the Harvard primer bank. More recently, new-generation intercalating dyes such as EvaGreen® have been developed, which have lower background noise and a stronger signal, enabling improved melt curve analysis and amplification detection. Despite their disadvantages, intercalating dyes are significantly cheaper to use than probes, as you can use the same dye for multiple different primer pairs (as long as the reactions are run separately). Since hydrolysis probes are sequence specific, every GOI

requires an individual set of primer pairs and probe. In consequence, this method is usually only chosen if the user wants to measure just a few targets of interest, such as in diagnostic testing. Since the development of the first commercial qPCR machines, instrumentation has come a long way in terms of both reliability and sensitivity. From the first machines, which could measure a small number of samples, we are now able to carry out high-throughput screening using 96- and 384-well plates. This advance is further enhanced through the development of detection systems. The detection of multiple emission spectra in many newer machines enables multiplexing of up to five or six colours at one time, facilitating high-throughput analysis in shorter periods of time.

Quantitation and data analysis

Real-time detection of the qPCR cycle results in an amplification curve with initiation, exponential and plateau phases (Figure 5A). This curve forms the basis of quantitation. When amplification starts, the level of fluorescence is low and is used to set the baseline level

of fluorescence. As the reaction progresses into the exponential growth, fluorescence reaches a level which is significantly higher than the baseline; this is referred to as the threshold level. The threshold level is the heart of quantitation, as the point at which your sample crosses this threshold is recorded as the Ct or Cq value. The threshold is set in the exponential phase, so the reading is not affected by reagent shortages, etc. in the plateau phase. The second crucial factor in quantitation is the use of a reference gene (RG), an endogenous control present in all samples at a consistent concentration which does not change in response to biological conditions. Often, genes such as GAPDH and β -actin are used; however, the levels of these transcripts can change in certain conditions, thus it is essential that the RGs are matched to the experiment.

To analyse the data, there are two types of quantitation methods to choose from, absolute and relative. Absolute quantitation is the most rigorous in terms of controls. Each reaction requires a standard of known concentration for the RG and GOI, for which a standard curve is generated using the log concentrations

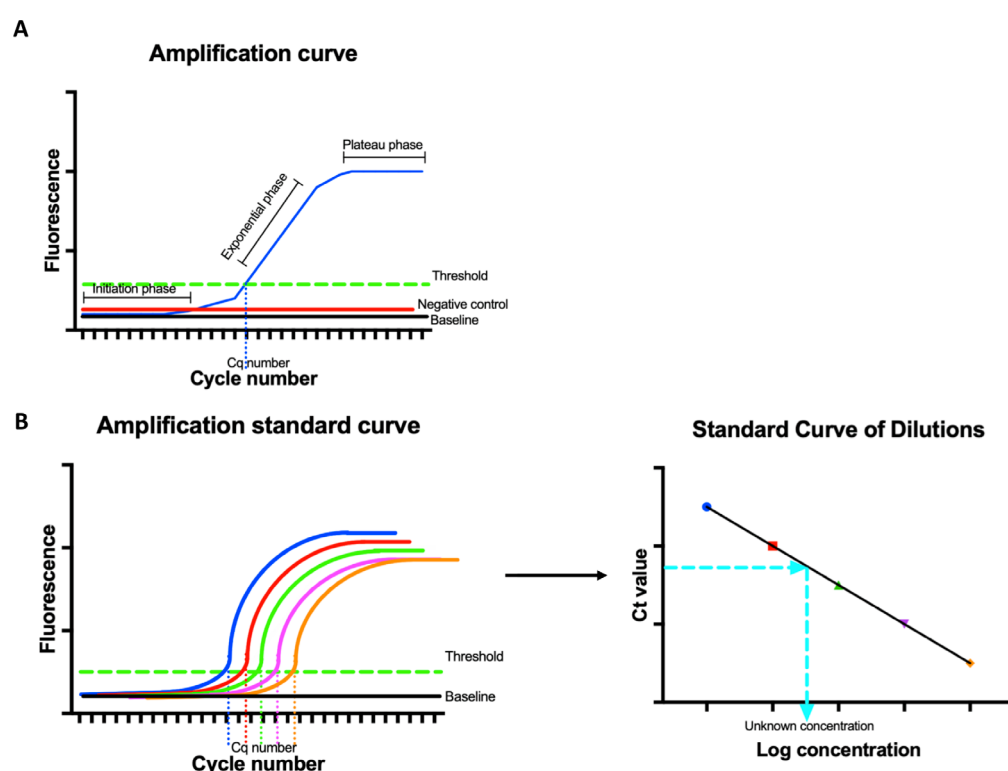


Figure 5. Quantitation of RT-qPCR and qPCR. (A) Amplification curve generated during the run as the reaction is measured in real time. Due to fluorescence detection, an amplification curve is generated (blue curve) which involves an initiation phase [low level of fluorescence, often termed the baseline (black line)]. A threshold is determined (green line) once the amplification curve is in the exponential phase, and where the amplification crosses this line determines the Cq/Ct value used to quantify data. Negative controls, i.e., water controls, should be around the baseline value. (B) A serial dilution of standards of known concentration are used to generate a amplification curve, which when Cq values are plotted against their log concentrations produce a standard curve. Target sequences of unknown concentrations can then be accurately quantified using their Cq as shown by the hashed cyan line.

and the Ct value (Figure 5B). This standard curve can then be used to quantitate the concentration of the unknown experimental samples and is often used for identifying DNA copy numbers. The second approach is relative quantitation, which enables you to calculate the ratio between the RG and the GOI. The accuracy of this quantitation depends on the RG; therefore, it is crucial that this remains unchanged, so as to prevent erroneous results. The method used to express the ratio between the RG and GOI is called the delta delta Ct method ($2^{-\Delta\Delta C_t}$). The Ct (Figure 5A) of the RG is removed from the GOI Ct, so as to remove any errors in sample loading. This generates a ΔC_t value for all samples, which is then compared back to a control sample to generate the $\Delta\Delta C_t$. This method is generally used for comparing healthy vs disease samples, etc.

Covid-19: the new frontier for real-time PCR assays

RT-PCR has been used to detect the viruses responsible for respiratory infections in public health for many years. With the recent outbreak of SARS-CoV-2, the virus causing Covid-19, the use of real-time RT-PCR has come to the forefront of research. The conversion of RT-PCR testing

to real-time RT-PCR or RT-qPCR allows high-throughput screening of patients, which is critical during a public health emergency. These tests have been rapidly designed following the deposition of the SARS-CoV-2 genome allowing prompt design of primers and probes specific for Covid-19. The most common test for SARS-CoV-2, which has been implemented by the World Health Organization (WHO), Public Health England (PHE) and National Health Service (NHS) laboratories, is real-time RT-PCR (RT-qPCR) using a system similar to TaqMan probes. The Drosten group, based in Berlin, has designed a real-time RT-PCR assay which detects the *RdRp* gene of SARS-CoV-2 and involves isolation of RNA and subsequent one-step real-time RT-PCR using fluorescent probes designed for the *RdRp* cDNA. A second collaborative group based in Hong Kong has designed a similar test employing two one-step RT-qPCR assays using fluorescent probes for alternative SARS-CoV-2 genes, called ORF1b and the N gene. These two real-time assays can be scaled up onto large automated qPCR machines, thus enabling rapid detection with high sensitivity and selectivity over similar coronaviruses such as the virus causing SARS. Consequently, it is clear that as well as being a powerful investigative technique in life sciences research labs, this technique is a strong contender for rapid diagnostics in current and future public health emergencies. ■

Further reading

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