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Review

# Practical experience of high throughput real time PCR in the routine diagnostic virology setting

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## Abstract

The advent of PCR has transformed the utility of the virus diagnostic laboratory. In comparison to traditional gel based PCR assays, real time PCR offers increased sensitivity and specificity in a rapid format. Over the past 4 years, we have introduced a number of qualitative and quantitative real time PCR assays into our routine testing service. During this period, we have gained substantial experience relating to the development and implementation of real-time assays. Furthermore, we have developed strategies that have allowed us to increase our sample throughput while maintaining or even reducing turn around times. The issues resulting from this experience (some of it bad) are discussed in detail with the aim of informing laboratories that are only just beginning to investigate the potential of this technology.

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*Keywords:* RT-PCR; Virus diagnosis; Sensitivity

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## 1. Introduction

The advent of polymerase chain reaction (PCR) has transformed the utility of the virus diagnostic laboratory. In comparison with traditional methods, PCR offers a highly sensitive and specific result within 24–48 h. The routine use of this test in diagnostic laboratories has led to many benefits including improved patient management, and increased ascertainment of previously under-diagnosed and undetectable viruses.

The advent of real time PCR technologies has further improved upon these already significant benefits (Arya et al., 2005; Aslanzadeh, 2004; Bustin and Nolan, 2004; Mackay, 2004; Tan et al., 2004). In comparison to traditional gel-based PCR assays, real time PCR offers increased sensitivity and specificity in a rapid format (turn around time from sample receipt to result <5 h). Unlike traditional systems, which rely upon endpoint analysis, real time PCR assays visualise the reaction as it is taking place allowing quantification and reaction analysis (e.g., PCR efficiency). Since real time PCR reactions are performed in a closed system (no gel analysis needed) the risk of contamination has been substantially reduced. This has also reduced the requirement for a stringent laboratory structure. The increasing number of chemistries and platforms available for real time PCR have reduced its overall cost significantly making this an increasingly attractive technique.

Over the past 4 years we have introduced a number of qualitative and quantitative real time PCR assays into our routine testing service. These include assays for the detection of influenza A, B and C, human metapneumovirus, respiratory syncytial viruses (RSV) A and B, rhinovirus, parainfluenza viruses 1–4, coronaviruses NL63, OC43 and 229E, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Pneumocystis jiroveci*, varicella zoster virus (VZV), herpes simplex virus (HSV) 1 and 2, cytomegalovirus (CMV), Epstein Barr virus (EBV), HHV-6, HHV-7, norovirus, adenovirus, rotavirus, astrovirus, sapovirus, erythrovirus B19, mumps, *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Nisseria gonorrhoeae* and enterovirus. Each year we carry out more than 84,000 PCR tests. During this period we have gained substantial experience relating to the development and implementation of real time assays. Furthermore we have developed strategies that have allowed us to increase our sample throughput while maintaining or even reducing turn around times.

The issues resulting from this experience (some of it bad) are discussed in detail below with the aim of informing labo-

ratories that are only just beginning to investigate the potential of this technology.

## 2. Developing and optimising a real time PCR assay

### 2.1. Which real time PCR chemistry is best for viral diagnosis?

There are numerous chemistries available to carry out real time PCR. These include dual labelled probes (often known as TaqMan<sup>TM</sup> probes), minor groove binding (MGB) probes, molecular beacons, fluorescence energy transfer (FRET) probes, intercalating dyes (such as SYBR green) and more recently developed fluorescent labelled primers such as Sunrise<sup>TM</sup>, Lux<sup>TM</sup> or Scorpion primers<sup>TM</sup>. The advantages and disadvantages of each chemistry are discussed (Arya et al., 2005; Aslanzadeh, 2004; Bustin and Nolan, 2004; Mackay, 2004; Tan et al., 2004) (Table 1).

Most of the published real time probe based PCR assays for viral diagnosis utilise either molecular beacons or dual labelled probes although more recent publications tend to favour the use of dual labelled probes. Currently all our real time PCR tests are dual labelled probe assays. However, we do have experience of both methods and have noted that there are several important differences between these two systems, which should be considered before developing or implementing a diagnostic virology test.

Molecular beacons are very specific (Tyagi and Kramer, 1996). The specificity is a direct result of their structure. In free solution a molecular beacon adopts a hairpin-loop conformation in which the reporter fluorescence is quenched by its proximity to the quencher molecule (Fig. 1). This is a very stable state and a molecular beacon will only bind to a target sequence, become linear and fluoresce if it is highly complementary. Any nucleotide differences between the beacon and the target sequence will greatly reduce the target binding efficiency of the probe. As a result molecular beacons have an increased propensity for false negative results. We encountered this problem during the implementation of a previously published molecular beacon based test for the detection of parainfluenza viruses. During the initial assessment, this detected all culture/direct immunofluorescence parainfluenza 3 positive samples detected between 2001 and 2002. However, all parainfluenza 3 positive samples detected by DIF or culture in 2003 were negative when tested with this assay. To assess whether the primers were amplifying the parainfluenza 3 viral RNA, SYBR green was

Table 1  
Comparisons of the various technologies available for real time PCR

Chemistry	Advantages	Disadvantages
Molecular beacons	Specific	Susceptible to probe mis-match, expensive, reduced fluorescence, less available
Dual labelled probes	Specific, many publications available, increased fluorescence, less susceptible to probe mismatch. Many manufacturers	Probe mismatch can lead to false negatives
Minor groove binders	Specific, increased fluorescence produced, can be used in small conserved areas, designed for SNP detection	Susceptible to mismatch, few suppliers
Labelled primers (e.g., sunrise, scorpion, and lux)	Cheap (no probe needed), sensitive as probe based technology, less homology needed (no probe region)	Primer-dimer formation, strict design criteria
FRET probes	Can readily detect single nucleotide differences, exact match to DNA for signal to be released, can be used in non-PCR amplification assays	Requires strict optimization of probe design, requires accurate thermal denaturation profiles to prevent interference with hybridisation
Intercalating dyes	Cheap, use on large regions	Primer-dimer, less specific than other methods, variable melting peaks

For full details of each system including FRET probes can be found in Mackey et al. (2002).

added to the PCR reaction in place of the molecular beacon. The formation of PCR product was observed. Using melt curve analysis, identical melting peaks were observed in all parainfluenza 3 samples and controls (Fig. 2). Running the PCR products on an agarose gel and observing a band of the expected size confirmed the successful amplification of parainfluenza 3 RNA by the primers. Based on these results we deduced that the molecular beacon was no longer complementary to the amplified target sequence. Consequently, following analysis of more recently available sequences in the database, a new molecular beacon was designed, which detected all 2003 parainfluenza 3 samples.

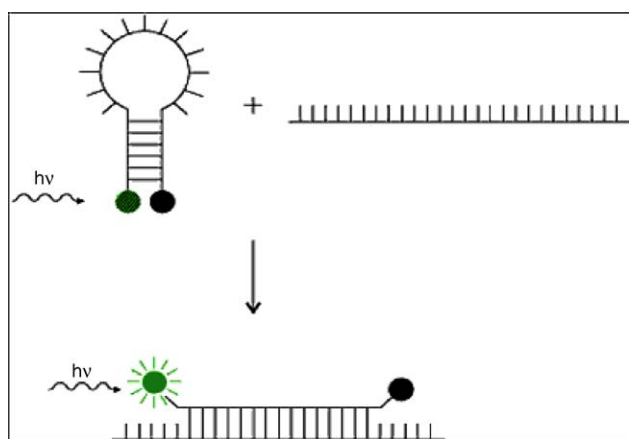


Fig. 1. Molecular beacons contain fluorescent and quenching dyes and are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher into close proximity. When a molecular beacon hybridizes to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation. Unlike dual labelled probes, molecular beacons are designed to remain intact during the amplification reaction (<http://probes.invitrogen.com/handbook/figures/0709.html>).

Unlike molecular beacons, dual labelled probes are in a permanent linear conformation (Lee et al., 1993) (Fig. 3). Like primers, they can tolerate a small number of mismatches between the probe and target and still bind to the target. Consequently, dual labelled probes are less likely to result in false negative reactions and may, in comparison with molecular beacons, be of greater use in viral diagnosis where occasional changes in even the most conserved target sequence may be expected to occur (although it should be noted that mismatches can also lead to false negative reactions with dual labelled probes). However, either method will be useful if targeting a highly conserved region.

The second difference between molecular beacons and dual labelled probe chemistries is related to the normalised

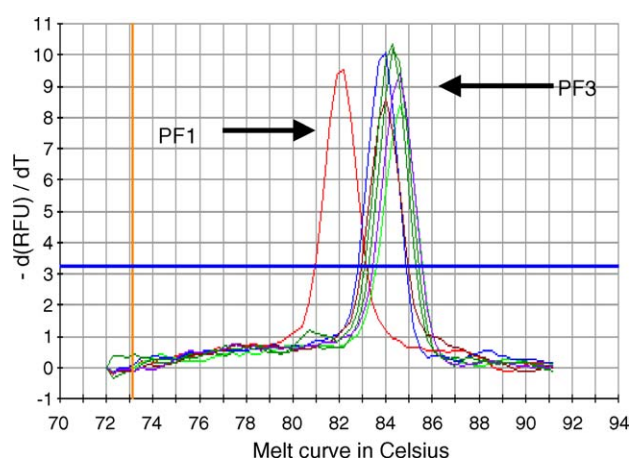


Fig. 2. Figure showing assessment of whether real time molecular beacon primers were amplifying the parainfluenza 3 viral RNA. In this reaction, SYBR green was added to the PCR reaction in place of the molecular beacon (all samples negative by molecular beacon assay). The formation of PCR product was observed. Using melt curve analysis, identical melting peaks were observed in all parainfluenza 3 samples and controls (<http://probes.invitrogen.com/handbook/figures/0710.html>).

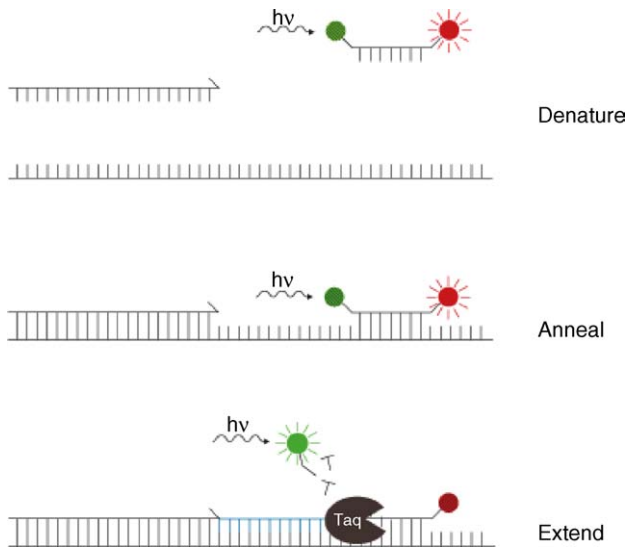


Fig. 3. Dual labelled probes (also known as Taqman™ probes) are oligonucleotides that contain a fluorescent dye on the 5' base, and a quenching dye located on the 3' base. When excited the fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a non-fluorescent probe. Dual labelled probes are designed to hybridize to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which the probe is bound, the 5'-exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs, allowing detection of the signal from the reporter dye. Fluorescence increases in each cycle, proportional to the rate of probe cleavage.

change in fluorescence ( $\Delta R_n$ ) produced during successful real time PCR. We have found in most, but not all cases, that dual labelled probes produce a greater fluorescent change than molecular beacons (Fig. 4). A larger  $\Delta R_n$  allows easier interpretation of results, as low positive results may be more easily differentiated from the variable background. Dual labelled probes provide a greater fluorescent change as the

reporter dye is irreversibly released from the quencher during the extension stage of each PCR cycle. Consequently there is a cumulative and permanent record of successful amplification, which is added to during subsequent PCR cycles. Molecular beacons are not destroyed at the end of each cycle, but return to free solution during the denaturation phase and revert back to their hairpin-loop structure. Consequently there is no accumulation of free reporter dye and the extra fluorescence produced is less after each cycle than when compared to dual labelled probes.

## 2.2. Development of new real time assays based on dual labelled probes

Since real time PCR is a relatively new technique, published assays may not be available for all viral pathogens. As a result many laboratories may wish to develop novel in-house real time PCR assays. The initial stages in developing a real time PCR assay are the same as those required for designing traditional gel based PCR tests. The first step is to identify a conserved region of the viral (or other pathogen) genome in which to design the assay. A literature review will often reveal which genes are conserved, and most often these will be genes encoding non-immunogenic proteins. Once a gene is identified, a BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>) is performed to locate the most conserved regions within this gene. As real time amplicons are short and contain a third oligonucleotide (i.e., the probe), the ideal region to design an assay would be 100–150 nucleotides long with 3 regions of 30 bases devoid of all base degeneracies. It is best to find a conserved region of 400–500 bases to allow the software to identify a number of potential assays. Several software programs are available to design real time assays, and often software is provided by the instrument supplier. Beacon Designer (Pre-

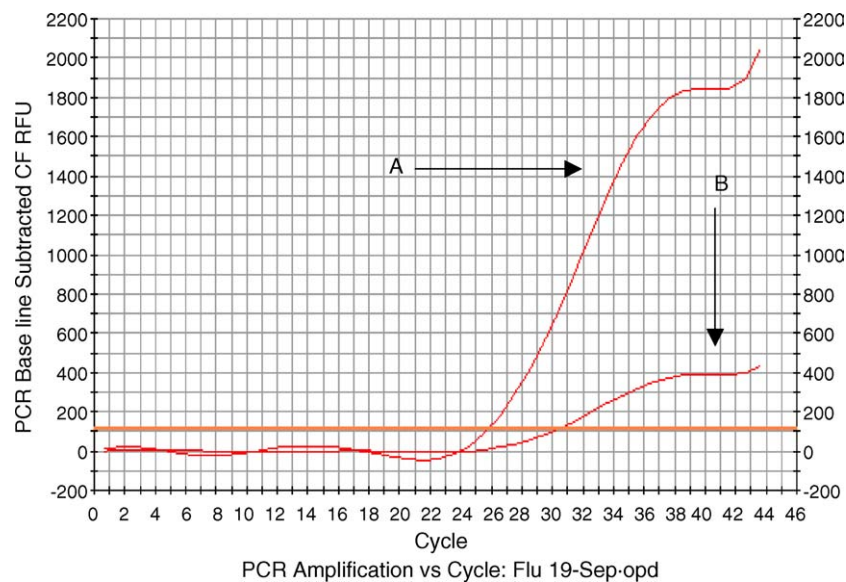


Fig. 4. Comparison of the  $\Delta R_n$  produced when using dual labelled probes (A) vs. molecular beacons (B).

Table 2

Main factors to consider when developing a dual labelled probe PCR assay

Factors to consider when developing a dual labelled probe PCR assay

Identify a conserved region of the viral (or other pathogen) genome  
 Identify a region within this area of ~400–500 bases in length  
 Check that the probe sequence contains more C residues than G residues  
 Ensure that the probe does not begin with a G  
 The optimal primer  $T_m$  values are 58–60 °C  
 The optimal probe  $T_m$  should be ~10 °C higher  
 The amplicon should not exceed 150 bp in length  
 Primers should not contain more than 2/5 G or C residues at the 3'  
 Check the amplicon for secondary structure, and for specificity

mier Biosoft) can be used to design either molecular beacon assays or dual labelled probe based assays, and has additional functionality such as BLAST and secondary structure searching. Primer Express (Applied Biosystems) is another useful tool for designing Taqman based assays, and is the only software currently available for designing assays based on Applied Biosystems (ABI) MGB probes. Once the software has suggested a primer and probe set, it is important to ensure that they meet the criteria (Table 2).

Assuming the primers and probe meet these criteria, it is advisable to check the amplicon for secondary structure, and for specificity. Secondary structure prediction software

is available on the internet, for example, Michael Zukers' m-fold server (<http://www.bioinfo.rpi.edu/~zukerm/rna/>) is particularly useful. A highly structured amplicon (higher  $-\Delta G$ ) may reduce the efficiency of reverse transcription or primer annealing (Fig. 5). This may reduce the overall sensitivity of the assay.

The final stage of the design process is to check the amplicon for specificity using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>). The assay should be specific for the sequence/organism of interest, and should not detect other sequences. Non-specific matches may be picked up, but closer analysis of the primer and probe binding sites often confirms that these sequences will not be amplified or detected due to multiple base changes.

### 2.3. Optimising a new real time PCR test

When performing gel based PCR it is essential to fully optimise primer concentrations to achieve the best sensitivity of the assay and best end-point signal (brightness of band) (Gunson et al., 2003).

In real time PCR, the signal is detected early in the amplification process, and therefore the end-point variation seen in gel-based assays does not affect the result. Also, careful design of the assay can reduce primer–dimer formation

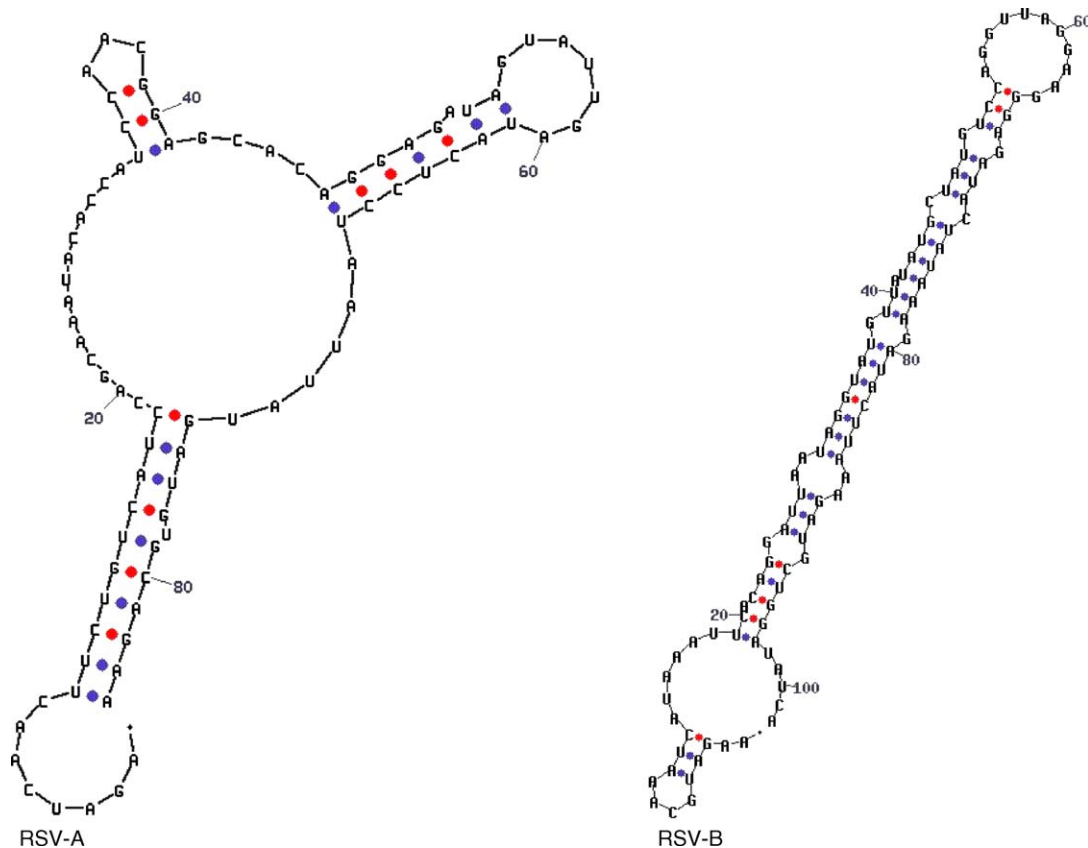


Fig. 5. Structure within PCR amplicons may affect the sensitivity of an assay. Respiratory syncytial virus (RSV)-A detection limit is  $10^2$  copies/reaction, while RSV-B which is more structured has a detection limit of  $10^4$ – $10^5$  copies per reaction.

and increase the efficiency of the specific amplification reaction. Consequently, many manufacturers of real time PCR equipment and oligonucleotide primers and probes no longer recommend optimising primer and probe concentrations for real time Taqman assays.

Despite this we still perform an initial optimisation of both primer and probe concentrations to ensure we are running our real time PCR assays at their most sensitive and efficient. Although for the majority of our assays the optimal concentrations are 1000:1000:300 nM (forward:reverse:probe), we have observed on several occasions that the optimal primer and probe concentrations were different to the values recommended. Our method for primer and probe optimisation is available online ([www.clinical-virology.org](http://www.clinical-virology.org)). However, other methods will also be available.

Optimisation of a real time PCR requires positive control material. Where positive control is not available (examples being a virus, which cannot be cultured or a highly pathogenic virus such as H5 influenza or SARS coronavirus), DNA or RNA oligonucleotide targets may be ordered. These are also useful as alternatives to plasmids as standards in quantitative assays. It should be noted that these oligonucleotide controls

must be ordered from a separate supplier to prevent contamination of the primer–probe set, and should be diluted in a separate laboratory prior to use as they may contain up to  $10^{17}$  target copies per ml, and are therefore a considerable source of potential contamination. We have observed contamination in erythrovirus B19 primers purchased several months after a full length oligonucleotide control was ordered from the same supplier (Fig. 6).

#### 2.4. Use of panels to assess newly developed PCR assays

Once the assay is optimised, it is essential to check the sensitivity and specificity of a new PCR assay by using a selection of sample ‘panels’. There is much debate about what is an acceptable validation process. These should minimally include clinical samples known to be positive by the current standard assay and should consist of the sample types commonly submitted to be examined for the virus in question. Clinical samples tested negative by the previous method should also be examined to determine if the new assay is more sensitive than the current test, and samples known to be positive for other agents should be tested to confirm assay

Label	Reaction components	Mean $C_T$ value
1	Supplier A Salt free negative control	21.08
2	Supplier A Salt free Positive control (-7)	21.10
3	Supplier A HPLC negative control	33.28
4	Supplier A HPLC Positive control (-7)	30.68
5	Supplier B negative control	40.00
6	Supplier B Positive control (-7)	29.93

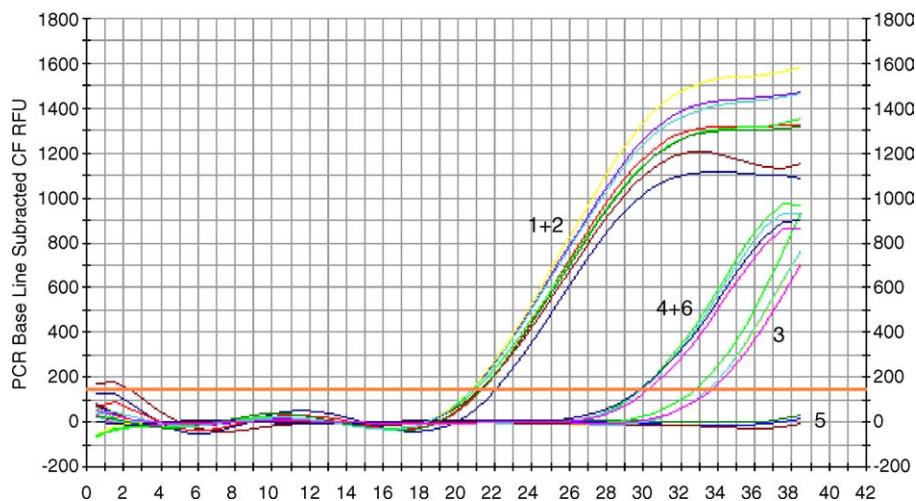


Fig. 6. Contamination of primer and probes with assay target produced at the same facility. Label 1, reaction component: supplier A salt free negative control, mean  $C_T$  value: 21.08; label 2, reaction component: supplier A salt free positive control (-7), mean  $C_T$  value: 21.10; label 3, reaction component: supplier A HPLC negative control, mean  $C_T$  value: 33.28; label 4, reaction component: supplier A HPLC positive control (-7), mean  $C_T$  value: 30.68; label 5, reaction component: supplier B negative control, mean  $C_T$  value: 40.00; label 6, reaction component: supplier B positive control (-7), mean  $C_T$  value: 29.93. A full length DNA oligonucleotide representing the amplicon of a B19 real time PCR assay was synthesised by supplier A. During a later investigation into assay contamination following a reagent change, primers and probes were again purchased from supplier A (salt free and HPLC purified), and from an alternative supplier B. The reagents purchased from supplier B (5 and 6) were clean, whilst those from supplier A (1 + 2) were contaminated with the previously synthesised positive control, even after HPLC purification (3 + 4).

specificity. Serial dilution series of known positive samples may also be prepared, and tested in parallel in the new and previous assay systems to determine which assay is more sensitive. Ideally these dilution panels should represent all subgroups of the target virus to ensure the test is sensitive for all types. A new test must be at least as sensitive as the assay in current use, and should ideally be able to detect a wider range of virus subtypes/variants.

An additional method to validate a new assay is to test the assay using samples from an external quality assurance program. Panels may be obtained from various sources, including National External Quality Assessment Service (NEQAS) and Quality Control for Molecular Diagnostics (QCMD), and the expected results may be compared with those obtained from the old and new assays in parallel. The use of such panels also allows the comparison of assays currently in use by different laboratories.

### 3. Factors aimed at increasing throughput and reducing turn around times

When implementing a newly designed or previously published assay a number of changes can be made in order to reduce the turn around time of the assay and increase laboratory throughput.

#### 3.1. Multiplex real time PCR

Multiplex real time PCR assays allow the detection of multiple pathogens within a single tube. The utilisation of such assays reduces overall testing costs and turn around times, enabling a high throughput. There are a number of multiplex real time PCR assays described in the literature (Draganov and Kulvachev, 2004; Gunson et al., 2005; Hindiyeh et al., 2001; Richards et al., 2004; Templeton et al., 2004). We recently described 4 triplex assays designed to detect 12 respiratory viral pathogens.

Designing a multiplex real time PCR is a complicated process often requiring a great deal of trial and error. Below are outlined some general criteria that may prove useful when designing such assays. In order to design appropriate primers and probes users should follow the development protocols outlined above. However, care should be taken to ensure that there is no primer or probe interaction that may reduce the sensitivity or efficiency of the PCR reaction. Most primer design software will allow primer–probe interactions to be examined. In order to optimise the multiplex assay each separate PCR should be optimised separately before being assessed when added together (see above section for details). Further experiments should include assessing the sensitivity of the multiplex assay for the simultaneous detection of mixed infections (real or spiked) and low copy targets in high copy backgrounds. Ideally, no loss in sensitivity should be observed when additional primers are added. However, if the multiplex assay is less sensitive, altering the ratio of

primers/probes concentrations may prove useful. Alternatively, changing the concentration of PCR reagents (enzyme,  $Mg^{2+}$ , dNTPs etc.) may also be beneficial. Some manufacturers are now producing real time reaction mixes specifically designed for use with multiplex assays, and provide guidelines on the optimal primer and probe concentrations to use. However, if all these factors fail to improve the sensitivity of the multiplex assay then some or all of the primer and probes may have to be redesigned.

The number of targets detected in one assay is limited by the number of detection channels available on the real time platform and the number of fluorescent-labelled dyes available. Newer machines tend to have five channels. Although there are many fluorescent dyes available, many of the excitation/emission spectra overlap and thus only certain combinations can be used. At present we are using FAM, VIC, and Cy5 detectors as these are optimal for the filter set utilised in the ABI 7500 (please note that this may differ when using other platforms).

#### 3.2. Syndrome based testing policies

Syndrome based testing policies are ideal for rapid, high throughput testing. In our laboratory we offer a number of such “menus”, which negate the need for clinical coding and allow samples to be tested immediately upon receipt (Table 3). For example, all CSF samples from patients with neurological illnesses such as encephalitis or meningitis are tested for enterovirus, HSV (1 and 2), VZV, EBV, CMV and HHV-6 regardless of patient or clinical details. Similar testing protocols are in place for urethritis, gastroenteritis, respiratory illness and eye infections. However, although such policies aid high throughput and reduce turn around times (sample receipt until when result is ready), it should be noted that they may be more expensive and will occasionally produce results that are difficult to interpret, e.g. herpes viruses in respiratory samples (see below).

Table 3  
Examples of sample led testing “menus”

Syndrome	Tests carried out
Gastroenteritis	Norovirus, astrovirus, rotavirus, adenovirus and sapovirus
Respiratory illness (immunocompetent)	Influenza A, B and C RSV A and B, rhinovirus, human metapneumovirus, coronaviruses (OC43, NL63, 229E), parainfluenza viruses 1–4, adenovirus
Respiratory illness (immunocompromised)	Influenza A, B and C RSV A and B, rhinovirus, human metapneumovirus, coronaviruses (OC43, NL63, 229E), parainfluenza viruses 1–4, adenovirus, HSV, CMV, VZV, PCP
Neurological illness	CMV, EBV, enterovirus, HHV-6, VZV, HSV (1 and 2)
Infections of the eye Urethritis screen	Adenovirus, VZV, HSV and <i>C. trachomatis</i> <i>C. trachomatis</i> , <i>N. gonorrhoeae</i> , <i>M. genitalium</i>



Table 4  
Manufacturers' websites

Manufacturer	Website address
ABI	<a href="http://www.appliedbiosystems.com">www.appliedbiosystems.com</a>
Autogen	<a href="http://www.autogen.com/">http://www.autogen.com/</a>
Beckman	<a href="http://www.beckman.com/products/pr_lab_auto.asp">www.beckman.com/products/pr_lab_auto.asp</a>
Biomerieux	<a href="http://www.biomerieux.com/">www.biomerieux.com/</a>
Corbett	<a href="http://www.corbettresearch.com/index2.html">www.corbettresearch.com/index2.html</a>
Hamilton	<a href="http://www.hamiltoncompany.com/newdev/robotics/roboticsindex.asp">www.hamiltoncompany.com/newdev/robotics/roboticsindex.asp</a>
Kingfisher	<a href="http://www.thermo.com/com/cda/product/detail/1,1055,19598,00.html">www.thermo.com/com/cda/product/detail/1,1055,19598,00.html</a>
Qiagen	<a href="http://www1.qiagen.com/Products/Automation/">www.1.qiagen.com/Products/Automation/</a>
Roche	<a href="http://www.roche.com/prod_diag_lab">www.roche.com/prod_diag_lab</a>
Tecan	<a href="http://www.tecan.com">http://www.tecan.com</a>

### 3.3. Automated extraction and liquid handling equipment

Automation of the extraction and liquid handling process has led to significant improvements in turn around times and allows high throughput with a reduced risk of user error. Many manufacturers now supply automated equipment for the extraction of nucleic acid from diagnostic samples (Table 4). Some manufacturers provide open platforms, which can be used with other suppliers' kits and reagents, while others provide complete extraction solutions. Although universal extraction kits (DNA and RNA pathogens and most specimen types) are available, it should also be noted that different kits can be used for particular samples types and pathogens (e.g., RNA or DNA) and may be more sensitive for a particular application. Although automated extraction has many advantages, laboratories should also consider complementing this service with a manual extraction system. This can be used for testing emergency samples that have arrived in the laboratory after an automated extraction has begun or for samples requiring special processing, not suited for automation, e.g. tissue. Many suppliers also supply automated liquid handling equipment, which can facilitate the set up of large numbers of PCR reactions.

### 3.4. Reduced PCR cycling times

Traditionally most published and in-house developed real time PCR methods consist of the following standard parame-

ters: a Taq DNA polymerase activation step (usually 95 °C for 2–15 min depending upon PCR kit manufacturer) followed by 40–50 cycles of 95 °C denaturation for 15–30 s and an annealing/extension cycle of 60 °C for 60 s. If an RNA virus is to be detected, an additional 30 min reverse transcription step is required before the Taq DNA polymerase activation. Overall the reaction run time for a real time PCR is between 80 and 100 min. We have repeatedly shown using dilution series of a number of DNA and RNA viral pathogens that reducing the duration of the reverse transcription step, the denaturing and annealing/extension step by 50% can reduce the reaction run time of the assay significantly without any concurrent loss in sensitivity (Table 5). Overall our reaction run time has reduced to approximately 60 min (70 min for RT-PCR), freeing up PCR machines for further tests and allowing more testing within the working day.

### 3.5. Standardised real time PCR conditions

Most dual labelled probe real time PCR assays are designed to utilise the same PCR parameters (i.e., denaturation step of 95 °C for 30 s and an annealing and extension step of 60 °C for 60 s). Theoretically, multiple different real time PCR assays can be carried out at the same time on the sample plate. We have also shown that, where DNA and RNA reagents are purchased from the same supplier, and therefore have identical Taq activation requirements, DNA assays do not suffer any loss in performance when run through RT-PCR cycling conditions. This will allow laboratories greater flexibility and provide a rapid service.

### 3.6. Pre-prepared real time PCR reagents

Pre-prepared, frozen real time PCR reagents are user friendly and lead to reduced TRT and improved quality control (QC) when compared to the preparation of PCR mixes from separate reagents. We have assessed two different methods of pre-prepared real time PCR reagents: frozen aliquots of pooled primers and probes, and frozen aliquots containing all real time PCR reagents. Both systems have been assessed over relatively short time period (up to a maximum of 10 weeks, which corresponds to the maximum period of time a pool would last before running out). Ideally these would

Table 5  
Comparison of reduced cycling times and normal cycling times on numerous pathogens

	EBV		Adenovirus		Norovirus		Rotavirus	
	Ct (normal)	Ct (reduced)	Ct (normal)	Ct (reduced)	Ct (normal)	Ct (reduced)	Ct (normal)	Ct (reduced)
Dilution								
10-1	19.5	19.1	25.1	25.7	22.88	21.92	26.1	25.57
10-2	23.1	22.8	30.6	29	26.36	25.95	28.83	27.54
10-3	27	26.8	33.4	33.6	29.81	30.03	32.71	30.41
10-4	30.8	30.2	–	–	34.88	33.76	–	–
10-5	35	33.8	–	–	–	–	–	–
Total time taken (min)	100	60	100	60	120	70	120	70

Table 6  
Advantages and disadvantages of pooled primer/probes and frozen aliquots of total mastermix (including enzyme)

Method	Advantages	Disadvantages
Pooled/primer probes	Flexible  Less waste than frozen mastermix Good QC Easy and rapid to use	User must combine two or three tubes
Frozen mastermix	Easier than pooled primer/probe method Can be stored in plate format Excellent QC	Expensive waste  Less flexible than primer/probe method

have been assessed over a longer period. We find that the pooled primer and probe approach best suits seasonal assays such as those for respiratory pathogens, whereas the latter approach is more suited to assays which are performed regularly throughout the year on a standard number of samples. The advantages and disadvantages are listed in Table 6.

### 3.6.1. Pooled primers and probes

We have introduced pooled primer and probes for the majority of our routine DNA and RNA tests. This has proved especially useful for our high throughput assays such as the 'Respiratory Screen', which consists of five triplex real time RT-PCR assays. For each multiplex assay, the operator needs only to mix three tubes containing pre-aliquotted reagents: an aliquot of mastermix containing ROX reference dye, one containing enzyme mix (RT + Taq), and an aliquot of primer probe pool (containing three sets of primers and probes, and sufficient water). In this way, mastermix can be prepared rapidly. The reagents have been carefully quality controlled and the possibility of pipetting or calculation errors at the time of preparation is reduced. The production of a large number of aliquots at the same time (sufficient for approximately 3000 tests) also facilitates inter-run reproducibility and assists in maintaining the quality of the results. While some mix is unavoidably wasted, the time saved and the reduced number of failed runs compensates for this cost, and during the summer months when sample numbers are much reduced, smaller aliquots can be prepared. Table 7 shows the CT values obtained from the coronavirus triplex assay using pooled primers and probed stored for up to 6 weeks, demonstrating

the stability of the reagents when stored at  $-20^{\circ}\text{C}$ . We have now been using the same lot number of pooled primer–probe for the coronavirus assay for in excess of 10 weeks without loss of performance. With this system and pooled controls (see below) in use, we are now able to provide an efficient and reliable.

### 3.6.2. Frozen pools of primers, probes, mastermix and enzyme

The use of aliquots of frozen mastermix (containing all PCR reagents except template) is an alternative to frozen primer and probe aliquots described above. The laboratory user need only remove the desired number of aliquots (or plates if frozen in this format), defrost and then add the template to be tested. Frozen aliquots are easier to use than the pooled primer and probe method, facilitate rigorous quality control and reduce the overall turn around times. However, they are less flexible than the primer probe aliquot system and wastage will be more expensive as it includes enzyme. Furthermore, any mistakes in the making up of the aliquots will result in the loss of primers and probe and expensive mastermix. We have shown, using positive controls, that both RNA and DNA mastermix from a number of companies (Applied Biosystems, Invitrogen, and Qiagen) can be frozen for at least 1 month with no loss of sensitivity.

### 3.7. Pooled controls

Positive and negative controls are an essential part of any diagnostic PCR service. Until recently, we, and many other laboratories, utilised two dilutions of a positive control for each virus to be tested (the end-point of a dilution series of cultured virus tested in the relevant assay (acting as a sensitivity control) and the dilution 1 log less dilute). As a result, for each robot extraction run of 96 wells, a substantial number of wells were required for the positive controls alone. The inclusion of negative extraction controls further reduces the possible number of extractions available for samples. The use of numerous controls increases the cost per sample and the turnaround times of the service.

Pooled controls are a significant improvement on the previous method and we now use separate pools containing 12 respiratory viruses, and 6 gastrointestinal pathogens. In order to develop a pooled respiratory or gastrointestinal control, each virus culture or stool extract was serially diluted and

Table 7  
Ct of positive control on frozen primer/probe pools at 4, 6 and 8 weeks

	Coronavirus OC43		Coronavirus 229E	
	Ct of control <sup>a</sup> in non frozen primer/probe pool	Ct of control <sup>a</sup> in frozen primer/probe pool	Ct of control <sup>a</sup> in non frozen primer/probe pool	Ct of control <sup>a</sup> in frozen primer/probe pool
4 <sup>b</sup>	23.51	23.6	27.06	27.45
6 <sup>b</sup>	15.75	15.9	19.29	19.26
8 <sup>b</sup>	19.65	18.3	22.6	22.6

<sup>a</sup> Controls were tested in triplicate.

<sup>b</sup> Please note that different positive controls were used each week.

an end point established. For the respiratory virus control a 'High' positive control pool was prepared by adding an equal volume of the dilution 2 logs above the end point for each of the 12 culture fluids. A further 1 in 10 dilution of this 'High' positive control was prepared to produce the 'Low' positive control. We now include just two respiratory controls on our robot extractions, freeing up an additional 22 wells for other samples. The preparation of a large volume of control at the one time allowed better QC and reproducibility to be achieved. Aliquots of control are stored at  $-70^{\circ}\text{C}$  and have been found to be stable for up to 3 months so far.

#### 4. Quality control issues

##### 4.1. The optimisation of new batches of primers

We have previously experienced lot-to-lot variation of both primers and probes resulting in reductions in test sensitivity. When a new batch of reagents is purchased we now run a performance test (using the new reagents at the same concentrations previously determined as optimal) by testing the 'old' and 'new' primer probe sets in parallel with the same positive control on the same PCR run. If the  $C_T$  and  $\Delta R_n$  values observed are comparable (newly prepared reagents must produce  $C_T$  values falling within two standard deviations of the mean value determined for the reagents previously in use (when testing identical positive controls), the new reagents are released for routine use. If the assay is less sensitive than the previous assay then primer and/or probe optimisation should take place. Ideally this should be done several weeks before the next batch is required for routine use as new probes or primers may need to be re-ordered. Our experience over the winter of 2004–2005 is that re-optimisation has not been required for any of the respiratory assays.

##### 4.2. Routine validation of each real time PCR run

For validation of each real time PCR run we recommend the following. The  $C_T$  of the positive control should be documented with each run and compared to the value derived from previous runs. This should help identify any loss in sensitivity that can be seen due to user error or degradation of PCR reagents. If the  $C_T$  falls significantly below the expected value the run should be repeated (outwith two standard deviations of the mean value determined by previous runs (when testing identical positive controls). If the  $C_T$  remains low or reduces further, new controls and PCR reagents may be required. In addition to this the overall fluorescence change should also be monitored with each run. Reductions in fluorescence may cause interpretation difficulties and may also highlight a problem in the PCR reaction. As with changes in  $C_T$ , large reductions in fluorescence may result in the need to repeat the PCR or introduce a new batch of controls and PCR reagents. Ideally real time PCR tests should include an internal control in order to ensure confidence in negative results.

There are many internal control PCR tests available targeting animal viruses (added to the sample before extraction) and synthetic controls (which are added to the mastremix), and human genes. However, the inclusion of such controls can be expensive as they may have to be carried out separately from the diagnostic assay. As a result many laboratories (including ourselves) do not use such controls on all tests.

##### 4.3. Quality control of quantitative assays

Any laboratories performing real time PCR assays can perform quantitative assays with the addition of suitable standard quantitative controls to the assay, although a uniform sample type is required to obtain meaningful results (e.g., blood, urine). Attempting to quantify virus in non-uniform sample types (such as respiratory samples or stool) is not recommended without thorough assessment of sampling reproducibility.

In common with many laboratories, we prepare our quantitative standards (oligos or plasmids) in bulk, test these for acceptable linearity and slope ( $-3.33$ ) for a good 10-fold dilution series (we allow a range of 3.18–3.45 which equates to a variation of  $\pm 5\%$  in the efficiency of the reaction), and then aliquot this into volumes sufficient to last 1 week at  $4^{\circ}\text{C}$ . Aliquots are stored frozen at  $-20^{\circ}\text{C}$  until required.

It is essential to track the  $C_T$  values of the controls to check that the assay is performing satisfactorily, and to enable a smooth transition to a new control set when required (Fig. 7). We record our  $C_T$  values in the form of a Shewart Control Chart (Davies, 2003). Newly prepared standards (produced annually) must have  $C_T$  values falling within two standard deviation of the mean value determined for the standards previously in use.

A second issue with quantitative assays that do not use extracted material as quantitative controls is that these assays are sensitive to changes in extraction methodology or efficiency. We have recently moved to a more efficient extraction kit (QIAamp Virus Robot kit), but as our standards are plasmid or cellular DNA based and are not extracted alongside the specimens, we are now reporting higher viral loads for the same sample than with previous extraction procedures. This observed change in viral loads is only a problem during the crossover period from one extraction procedure to another, as subsequent samples will be analysed in the light of the new baseline level. To ensure intra-run extraction consistency, a positive or an internal control (of known quantity) should be extracted and run at the same time as the samples to be tested. This control should be monitored in the same way as outlined above (see routine validation of each real time PCR run).

##### 4.4. Continual assessment of the sensitivity of real time PCR primers and probe

Once an assay (or a number of assays) has been introduced into routine service it is important to re-assess the sensitivity of the assay in relation to current circulating viruses. This can

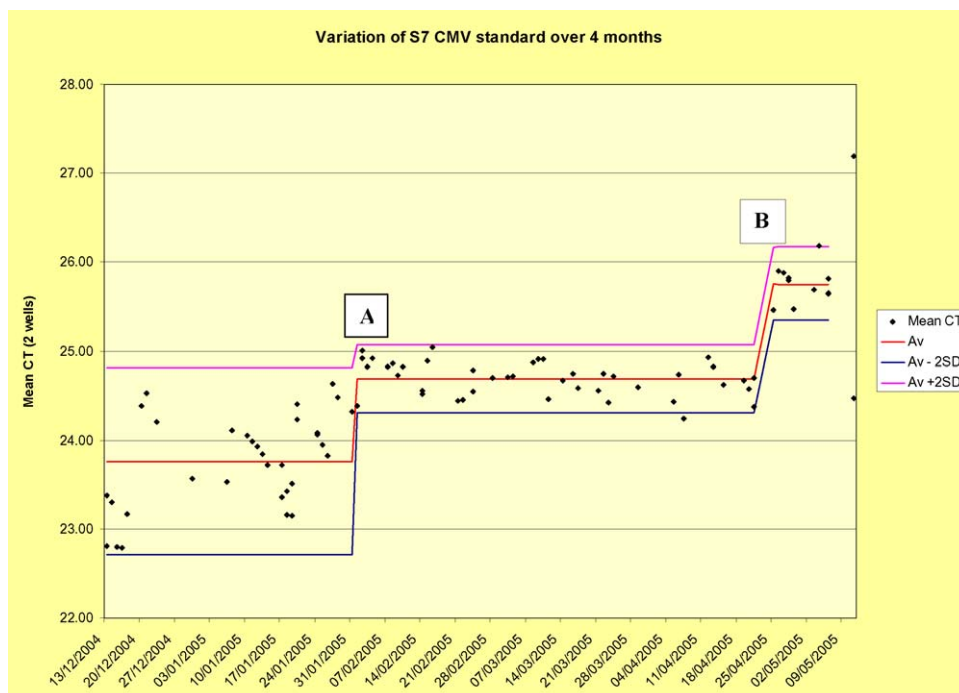


Fig. 7. Application of Shewart Control Chart to track potential changes in assay performance. The mean CT values obtained for the  $1 \times 10^7$  copies per ml standard were plotted over time. The average value of these CT values was calculated and plotted (red line) for each data set (along with two standard deviations above (pink line) and below (blue line) the average value). Two standard deviations are generally accepted as the warning level in such analyses. The first 'jump' (A) represents a change in the set of standards used, and while this is not ideal, results in a much more reproducible assay. The second jump (B) is caused by a change in the primer–probe pool in use, and shows a significant change in the sensitivity of the assay. As a result of this analysis another batch of primer–probe pool was prepared and the results obtained returned to the acceptable range. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.

be carried out using positive samples detected by an alternative test or by comparing primers and probe to new sequences stored in surveillance databases. Although most assays target a conserved region of the viral genome, small changes in the target can result in false negative reactions due to primer and/or probe mismatches. If a loss in sensitivity occurs primer or probe sequences may need to be updated or a new assay may have to be developed.

#### 4.5. Interpretation of results: potential pitfalls

Interpreting real time PCR results is a relatively straightforward process. In a fully optimised assay all positive results should show increases in fluorescence in a characteristic exponential curve. However there are still pitfalls that we feel users should be made aware of when interpreting data.

Occasionally samples may show "signal drift" (traces that increase in fluorescence as the PCR progresses but are not exponential) (Fig. 8). Signal drift can be produced for a number of reasons. True positive samples may show signal drift because of sub optimal PCR conditions, inhibition and primer mismatches. Occasionally negative samples may also show signal drift. This may be due to probe breakdown resulting in a fluorescence increase. Signal drift often occurs towards the end of the PCR reaction. Some platforms allow multicomponent analysis of weak positive traces. This allows users to

assess the changes of each fluorescent label in the reaction. Genuine positive traces will show an exponential increase in the fluorescent signal whereas signal drift is often due to a change in the normalisation dye (e.g., ROX). We currently repeat all positive samples with Ct's greater than 35 cycles as we feel these may be either low copy number positive samples or non-specific reactions.

Some 96 well real time plates require sealing with optically clear plate seals before PCR can take place. On occasion these may not seal properly and PCR reagents evaporate during cycling. As a result of this a curve may be produced mimicking a positive PCR reaction.

The correct placing of the threshold line is essential to allow accurate  $C_T$  measurement. Some of the computer software available with current real time PCR formats can automatically place the threshold line during result analysis. Any sample with fluorescence above this line will be regarded as positive by the computer. Always check the automatic placement of the threshold line as we have found that sometimes the computer will place it wrongly resulting in both false positive and negative results (Fig. 9). An alternative is to use a fixed threshold line. The use of such a system will ensure the real time assay is directly comparable to previous runs This should not preclude careful analysis of the data.

The increased sensitivity of real time PCR means that, like nested PCR, occasionally positive results will be obtained that

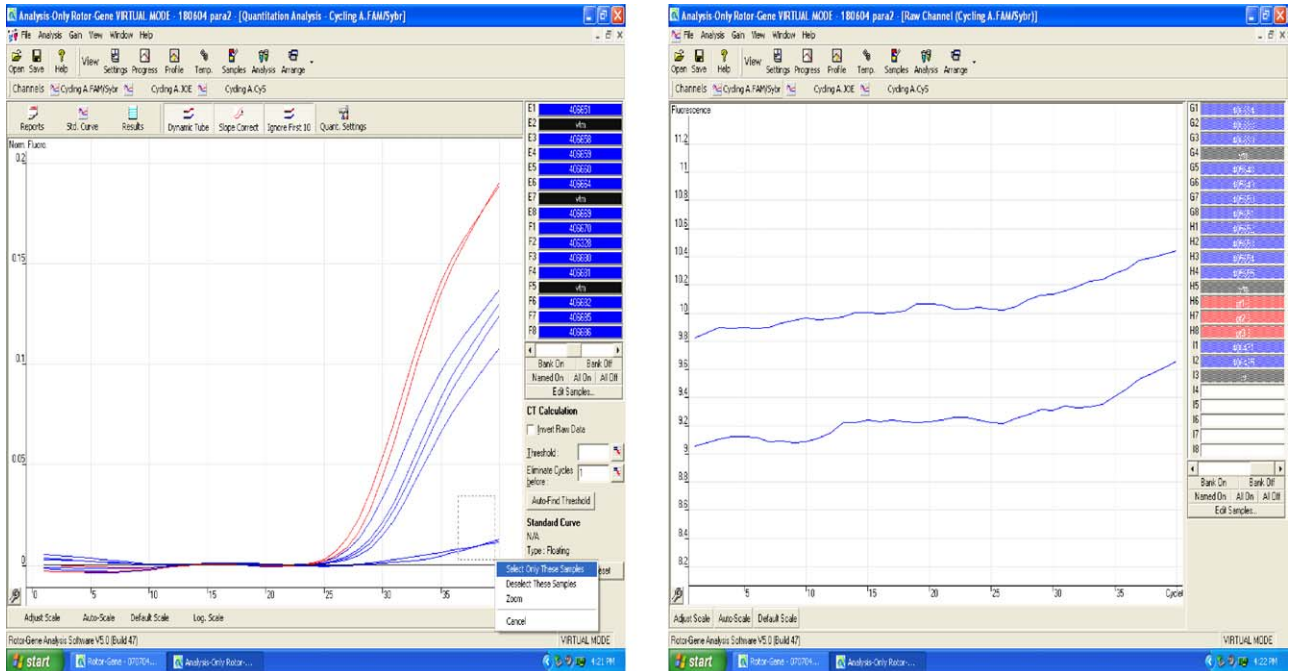


Fig. 8. Example of negative samples showing signal drift. The two sample shown in blue are showing an increases in fluorescence when examined using the quantification option (shown above left). Analysis of the raw cycling data (shown above right) shows that there is no increase in fluorescence usually associated with a positive sample.

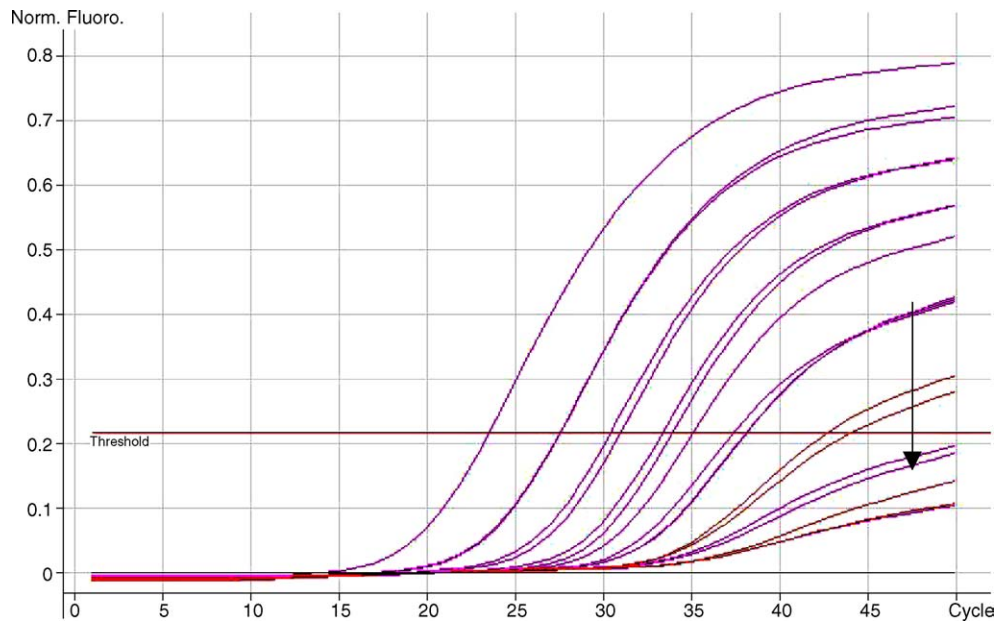


Fig. 9. An example of a computer placed threshold resulting in false negative readings (arrowed).

are not in keeping with accepted knowledge. For example, herpes viruses in throat swabs should be interpreted with care. We often detect low positive EBV, HHV-6, HSV or CMV in throat swabs in cases of respiratory infection. Whether these are the cause of disease or unrelated re-activations are unclear. Although these findings may be irrelevant to the clinical illness it is important that these results are not ignored. For as we gain more experience with these sensitive assays we may

identify new, previously unrecognised syndromes attributed to particular viral pathogens.

## 5. Final comment

There is no doubt that in the coming years an increasing number of virology laboratories will utilise real time

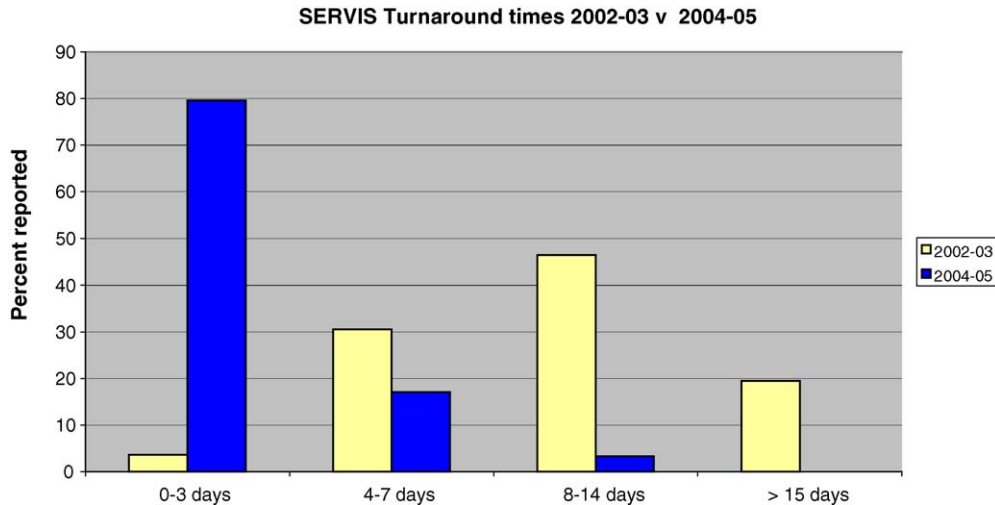


Fig. 10. The turn around time of respiratory samples in 2002–2003 to 2004–2005.

PCR assays. As a result virology laboratories will be able to offer more tests and process more samples while reducing turn around times. This can be highlighted by our winter respiratory surveillance service (SERVIS), which currently tests for 12 pathogens. During the 2004–2005 season 509 samples were tested with 80% of results reported within 3 days of the samples arriving in the laboratory (Fig. 10). For 2002–2003 when gel based PCR was used to detect Influenza A and B, RSV and picornavirus, 554 samples were tested in total. Only 3.6% of results were available in 3 days with most results returned to users within 14 days. With a slightly extended working day, real time PCR results ought to be reported within 36 h of receipt.

The routine use of real time PCR will have several benefits. First it will aid patient management (prognosis, treatment guidance and infection control) and may assist in the development of new antiviral therapies. Real time PCR will also improve the sensitivity of the surveillance of viral pathogens, increasing our understanding of these important infections, providing accurate assessments of the morbidity and economic cost of disease and facilitating the implementation of public health prevention measures.

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