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Using multiplex real time PCR in order to streamline a routine diagnostic service

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ABSTRACT

An increasing number of virology laboratories are now utilising in house real time PCR assays as the frontline diagnostic tests. As the number of tests on offer increases the natural progression from this will be to rationalise their service via multiplexing. Since 2003 we have introduced a large number of qualitative and quantitative multiplex real time PCR assays into our routine testing service. This paper describes the development of the multiplex assays, the problems encountered and the resultant benefits to the routine service.

1. Introduction

1.1. Overview of service development at the West of Scotland Specialist Virology Centre

The advent of PCR has transformed the utility of the virus diagnostic laboratory and, compared to traditional methods, has led to many benefits including improved patient management and increased ascertainment of previously under-diagnosed and undetectable viruses.[1–3]

The advent of real time PCR technologies has further improved upon these already significant benefits.[4–9] In comparison to traditional gel-based PCR assays, real time PCR offers similar or improved sensitivity and specificity in a rapid format (turn around time from sample receipt to result <5 h). 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 commercially available real time PCR chemistries and platforms has led to significant reductions in its overall cost.

Since 2003 we have introduced a number of qualitative and quantitative real time PCR assays into our routine testing service.[10–13] 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, *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, measles, rubella, *Chlamydia trachomatis*, *Nesseria gonnorhoeae*, *Treponema pallidum*, HBV, HCV, HCV genotyping, HEV, enterovirus and parechovirus.

We have developed strategies to enable an increase of sample throughput while maintaining or even reducing turn around times.

Of these developments, multiplexing a number of separate real time PCR assays into one test remains the most effective way of improving the rapidity, cost, ease of use and throughput of a PCR-based diagnostic service.[14–16] For example, in 2003 our frontline respiratory service consisted of five real time PCR assays that together allowed the detection of eight commonly encountered pathogens (Table 1). After further multiplexing our respiratory service still consists of five separate assays but can now detect 17 different viral and bacterial pathogens. In 2007, we applied the same principles to our non-respiratory diagnostic service. In this case all the multiplex assays were designed to test certain sample types that represent different disease syndromes. This work has reduced the number of tests carried out from 21 to 9 (Table 2).

Here, we describe the development of the multiplex assays, the problems encountered and the resultant benefits.

2. Issues relating to the development of the multiplex tests

2.1. Initial assessment of the multiplex assay

Since most of the components of the proposed multiplexes were initially available as singleplex assays, initial experiments were carried out to ensure that the addition of the extra primers and probes

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Table 1
The west of Scotland specialist virology centre respiratory service from 2003 to 2008.

Year	Format	Number of separate PCR tests	Targets detected (cumulative)
2003–2004	Real time PCR	5	Influenza A, B; RSV; adenovirus; *Rhinovirus*; *PF1, 2, 3*
2004–2005		5	Influenza A, B; RSV A + B; adenovirus; rhinovirus; PF1, 2, 3; *coronavirus NL63*, *229e*, *OC43*; *HuMPV*
2005–2006		6	Influenza A, B, C; RSV A + B; adenovirus; rhinovirus; PF1, 2, 3, 4; coronavirus *NL63*, *229e*, *OC43*; *HuMPV A + B*
2007–2008		5	Influenza A, B, C; RSV A + B; adenovirus; rhinovirus; PF1, 2, 3, 4; coronavirus *NL63*, *229e*, *OC43*; HuMPV A + B; *M. pneumoniae*

Table 2
The multiplexing of non-respiratory real time PCR tests.

Sample	Ancient testing	Number of wells pre-2007	Modern testing	Number of wells post-2007
Vesicle fluid	hsv-1/2, vzv	2	hsv1/2/vzv	1
Genital	hsv-1/2	1	hsv-1/2/syphilis[a]	1
Stool (non-outbreak)	Adenovirus, astrovirus, rotavirus, sapovirus	4	Adenovirus/astrovirus/rotavirus/sapovirus	1
Eye swab	Adenovirus, hsv-1/2, C trachomatis, vzv	4	Adenovirus/hsv-1/2/C trachomatis/vzv	1
Blood (transplant)	cmv, ebv, adenovirus	3	Adenovirus/cmv/ebv	1
CSF	Enterovirus, mumps, hsv-1/2, vzv, cmv, ebv, hhv6/	7	Enterovirus/parechovirus,[a] mumps, hsv-1/2/vzv, cmv/ebv/hhv-7	4
Total		21		9

Each line represents a well.
[a] Additional targets previously not included in the laboratory repertoire.

did not result in a loss of test performance. Additional primers or probe can result in unpredictable reduced sensitivity or the formation of non-specific products. The proposed multiplex assay was compared to the singleplex using end-point detection limit experiments, specificity panels, and panels of clinical samples (positives and negatives). Other factors such as robustness, reproducibility and, if relevant, linearity and quantitation accuracy were also assessed. A test would only be deemed acceptable if it was shown to be identical (or superior) in performance to the individual tests.

Failure at this stage required further experiments using altered concentrations of primers or probes. If such changes did not result in an acceptable improvement in test performance, then the test was discarded. Such tests would then continue to be used as single assays until new tests could be identified from the literature that proved to be simpler to multiplex. Candidate assays would then be rigorously assessed in singleplex format before being assessed as part of a multiplex assay.

2.2. Assessing the performance of the test in the presence of more than one target

If the multiplex assay is unlikely to encounter mixed infections (such as a CSF) then the need to assess its performance in the presence of >1 target is not necessary so standard commercial PCR kits can be used.

However, if mixed infections are likely to be common in a sample (or if an internal control will be amplified in every sample), then it is important to study the relevance of competition.[17–19] The stronger target is preferentially amplified and uses up most of the available PCR reagents. This reduces sensitivity of a part of the multiplex (always the target that is not preferentially amplified) and can result in false negative results. For quantitative assays, this may result in incorrect viral load. Few commercial PCR kits have been designed specifically for multiplexing. Few are reliable when more than 1 pathogen is present, particularly if the concentration of each target is significantly different.

This test characteristic can be assessed using a chessboard technique, designed to provide a large number of wells each containing a known concentration of two PCR targets at different ratios (Table 3). Each well is then tested by the multiplex PCR and the results are compared to its performance on a dilution series of each target. Experiments such as this can determine whether competition is likely to be an issue and can also measure the ratio of each target above which competition can be expected.

Careful optimisation of PCR reagents can remove this problem.[18–22] For example, limiting the concentration of the

Table 3
The chessboard procedure used to determine how well a multiplex test performs on samples containing >1 target.

Pathogen 1 dilution series	Pathogen 2 dilution series						
	(a) 10^{-1}	(b) 10^{-2}	(c) 10^{-3}	(d) 10^{-4}	(e) 10^{-5}	(f) 10^{-6}	(g) 10^{-7}
(A) 10^{-1}	Aa	Ab	Ac	Ad	Ae	Af	Ag
(B) 10^{-2}	Ba	Bb	Bc	Bd	Be	Bf	Bg
(C) 10^{-3}	Ca	Cb	Cc	Cd	Ce	Cf	Cg
(D) 10^{-4}	Da	Db	Dc	Dd	De	Df	Dg
(E) 10^{-5}	Ea	Eb	Ec	Ed	Ee	Ef	Eg
(F) 10^{-6}	Fa	Fb	Fc	Fd	Fe	Ff	Fg
(G) 10^{-7}	Ga	Gb	Gc	Gd	Ge	Gf	Gg
(H) 10^{-8}	Ha	Hb	Hc	Hd	He	Hf	Hg

A dilution series of the extracted nucleic acid from pathogen 1 and pathogen 2 is made. Using a microtitre plate a volume of each dilution of pathogen 1 is added to each column so that, for example, dilution 1 of pathogen 1 (labelled here as (A)) is present in the first well of each column. Subsequently an equal volume of each dilution of pathogen 2 is added to each row so that, for example, dilution 1 of pathogen 2 (labelled here as (a)) is present in the first column only. The wells will now contain different concentrations of pathogens 1 and 2 and can be used to assess the multiplex PCR.

primers used by the PCR assay that is being preferentially amplified can prevent strong positive samples using up the available PCR reagents. However, this must be carried out without reducing the sensitivity of the assay. For assays that use an internal control a low concentration of the internal control should be included in each assay to ensure competition is kept to a minimum. The flipside of this is that strong positive samples can compete with the internal control leading to samples being wrongly labelled as inhibited. This can lead to doubt over whether the result given is accurate.

In recent years some commercial companies have attempted to rectify this problem via the use of PCR kits specifically developed for multiplex PCR. For example, we use the Qiagen Multiplex kit for our transplant screen which simultaneously detects and quantifies adenovirus, CMV and EBV (www.qiagen.com). This kit is designed specifically for 2–4 plex real time PCR and through various mechanisms has been shown to prevent test interaction/competition and therefore allows simultaneous detection and accurate quantitation to take place. The use of this kit has allowed us to develop a single set of five standards each containing a known concentration of all three viruses: adenovirus, CMV and EBV. These five standards provide linear and reproducible standard curves for all three pathogens. We have also used this kit in our STD screen and confirmation assay that is used to detect *C. trachomatis*, *Nessieria gonnorhoeae* and an internal control in a single tube.

2.3. Choosing the correct fluorescent dyes

The preferred dyes for triplex assays are FAM, VIC and Cy5.[22] These dye combinations work on most assay platforms and are commonly used by researchers. These dyes are chosen because the excitation and emission wavelengths are different enough to allow accurate detection of each, reducing the risk of crosstalk. Crosstalk is when the fluorescence increase associated with one dye spills over into another channel that is being used to detect another dye. This leads to two positive results: one real and one not. Sometimes a strong FAM signal can also be detected in the VIC channel resulting in a false positive result. Some PCR platforms can also confuse large increases in VIC-Tamra signals as increases in the background calibration dye ROX. Subsequently, the platform will reduce the fluorescence levels of the dyes detected in all wells which on some occasions can result in false negative traces or strange traces. In most cases crosstalk can be eliminated with appropriate test optimisation (e.g. reducing or increasing the amount of dye/probe present without any loss in test sensitivity) or PCR platform re-calibration.

However, when developing 4 plex assays the choice of dye for the fourth assay is limited. It is dependent upon the PCR platform and whether the PCR kit uses ROX as a reference dye. As a result researchers often have to use dyes with similar excitation and emission spectra to FAM, VIC and Cy5, increasing the risk of crosstalk.

Two of our current assays (the conjunctivitis and the non-norovirus gastroenteritis multiplexes) utilise four different florescent dyes. Our laboratory tests are carried out on either the ABI7500 or the Rotorgene PCR platforms. On the ABI 7500, Tamra bidopy & NED are the recommended 4th dyes whereas Texas Red & ROX are suggested for the Rotorgene 3000. However, despite this guidance significant crosstalk was encountered. For example, when using Tamra biodopy positive traces were found in all dye channels (Fig. 1A) and positive Texas Red traces on the Rotorgene were found to appear in the Cy5 channel. Careful optimisation could not rectify this issue and the machine calibration was not at fault. This was eliminated by using ROX as the 4th dye on both platforms (Fig. 1B). It should be noted that using ROX as a probe dye is not recommended for some ABI PCR platforms as it is commonly used as a reference dye. However, this function can be "switched off" and we have found no problems when using this as the fourth dye in our qualitative tests. It should also be noted that some PCR kits will come with ROX already added and therefore may not be of use in this situation.

3. Outcomes of multiplexing on the routine service

The use of multiplex PCR has resulted in several positive outcomes for the laboratory service.[22] For example, the ability to add targets, without increasing the number of assays needed

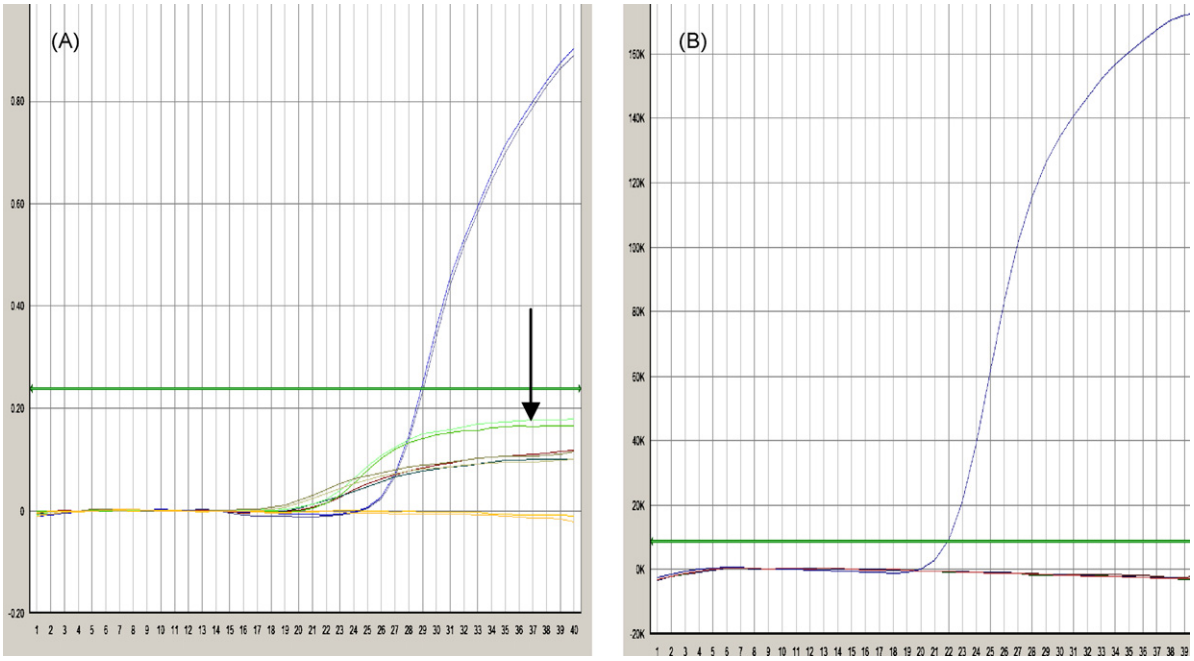

Fig. 1. ABI 7500 trace showing appearance of crosstalk in the Cy5 channel (shown by arrow) that appeared when using tamra-bidopy as the fourth dye (A). The un-arrowed trace is the Cy5 positive control. Diagram (B) shows that the crosstalk is gone when ROX is used as the fourth dye (the remaining trace is the Cy5 positive control).

Table 4
Expected test savings (in terms of mastermix) for each disease syndrome/sample type.

Sample	Annual total	Number of tests carried out (pre-multiplex)	Number of test carried out (post-multiplex)	Total difference (%)
Eye	~2,000	8,000	2,000	75
Stool (non-outbreak)	~1,600	6,400	1,600	75
CSF	~1,600	11,200	6,400	43
Vesicle fluid	~2,000	4,000	2,000	50
Genital	~2,000	2,000	2,000	0
Blood (TX)	~3,000	9,000	3,000	66
Total	~12,200	40,600	17,000	58.2

to be set up, has allowed the inclusion of pathogens previously not tested for including new discoveries (e.g. human metapneumovirus and coronavirus NL63). The inclusion of the parechovirus test will increase the detection rate in patients with enterovirus like illnesses whereas the inclusion of human metapneumovirus, influenza C, parainlfuenza 4, the coronaviruses NL63, OC43, 229e has lead to increases in the overall viral detection rate in respiratory samples. Such improvements will have positive outcomes on clinical management, infection control and public health since our tests are now more likely to provide a relevant diagnostic result.

This has not been associated with increased cost. With multiplexing fewer tests are carried out and less reagents (in particular PCR mastermix-the most expensive component of a PCR test after extraction costs) are required (Table 4). Significant cost savings may also be achieved due to the fact the service requires less staff hands on time.[22]

Multiplexing by sample type/disease syndrome simplifies the routine service. Reductions in sample turn around time have been achieved by reducing the amount of separate tests needed to be carried out. This ensures more samples can be tested within the working day. In future, such simplifications in the test procedure will also aid the development of automated testing systems which in turn will make the service less error prone and more rapid.

Test selection has also become more straightforward as it is based on disease syndromes rather than on pathogen type, mirroring the multiplex test design. Very little senior staff input is now required in test selection.

The reporting of results is more streamlined since all relevant results are now more available at the same time, rather than sequentially. Consequently, the virologist will be able to advise based on a more complete picture. Combined with the quicker turn around time this should lead to improved patient management.

There are disadvantages associated with adopting such a system. Firstly, the more we multiplex the less able we are to provide single pathogen testing. Consequently, some samples may get additional test results that were not initially requested. This will lead to a small extra unnecessary cost but may also provide unexpected results that may cause interpretation difficulties. For example, testing a BAL for CMV will result in additional testing for adenovirus and EBV. In a number of cases we have detected EBV at high viral loads. It is unclear what role, if any, this virus was having on the clinical picture. Further, if laboratories use a PCR kit that is not designed for multiplexing then they may fail to detect mixed infections.

4. Final comment

An increasing number of virology laboratories are now utilising in house real time PCR assays as the frontline diagnostic tests. As the number of tests on offer increases the natural progression from this will be to rationalise their service via multiplexing. This process is, at present, only limited by the number of fluorescent dyes available from probe manufactures and the number of channels available to detect these on PCR platforms. A further limitation is the lack of commercial kits specifically designed for multiplex PCR. Once these factors are addressed, laboratories should be able to multiplex much more simply bringing with it significant service benefits.

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