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

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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.

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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...
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, vzz    | 2                        | hsv-1/2/vzz    | 1                         |
| Genital                    | hsv-1/2         | 1                        | hsv-1/2/lymphis | 1                         |
| Stool (non-outbreak)       | Adenovirus, astrovirus, rotavirus, sapovirus | 4                  | Adenovirus/astrovirus/rotavirus/sapovirus | 1                        |
| Eye swab                   | Adenovirus, hsv-1/2, C trachomatis, vzz | 4                | Adenovirus/hsv-1/2/C trachomatis/vzz | 1                        |
| Blood (transplant)         | cmw, ebv, adenovirus | 3                | Adenovirus/cmw/ebv | 1                        |
| CSF                        | Enterovirus, mumps, hsv-1/2, vzz, cmw, ebv, hhv6/ | 7                | Enterovirus/parechovirus, mumps, hsv-1/2/vzz, cmw/ebv/hhv-7 | 4                        |
| Total                      |                 | 21                       |                | 9                         |

Each line represents a well.

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                   | (a) 10⁻¹                   |
| (B) 10⁻²                   | (b) 10⁻²                   |
| (C) 10⁻⁻                   | (c) 10⁻⁻                   |
| (D) 10⁻⁻                   | (d) 10⁻⁻                   |
| (E) 10⁻⁻                   | (e) 10⁻⁻                   |
| (F) 10⁻⁻                   | (f) 10⁻⁻                   |
| (G) 10⁻⁻                   | (g) 10⁻⁻                   |
| (H) 10⁻⁻                   | (h) 10⁻⁻                   |
| (I) 10⁻⁻                   | (i) 10⁻⁻                   |
| (J) 10⁻⁻                   | (j) 10⁻⁻                   |

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, Nesseria 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. 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 fluorescent dyes. Our laboratory tests are carried out on either the ABI 7500 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. For example, the ability to add targets, without increasing the number of assays needed...
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, parainfluenza 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.\(^2\)

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.

### References

1. Babbaraju K, Wong S, McMillan T, Lee BE, Fox JD. Diagnosis and epidemiological studies of human metapneumovirus using real-time PCR. J Clin Virol 2007;40:November (3):186–92.
2. Pilger DA, Cantarelli VV. Human metapneumovirus and human coronavirus NL63. Pediatrics 2008;121(Febuary (2)):445–6 [author reply 446–7].
3. Jiang X, Wang J, Graham DV, Estes MK. Detection of Norwalk virus in stool by polymerase chain reaction. J Clin Microbiol 1992;30(October (10)):2529–34.
4. Gunson RN, Collins TC, Carman WF. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. J Clin Virol 2006;35(April (4)):255–67.
5. Arya M, Shergill IS, Williamson M, Gomsmrell I, Arya N, Patel HR. Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn 2005;5(March (2)):209–19.
6. Aslanzadeh J. Preventing PCR amplification carryover contamination in a clinical laboratory. Ann Clin Lab Sci 2004;34(Autumn (4)):389–96.
7. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biol Mol Tech 2004;15(September (3)):155–66.
8. Mackay IM. Real-time PCR in the microbiology laboratory. Clin Microbiol Infect 2004;10(March (3)):190–212.
9. Tan BH, Lim EA, Liaw JC, Seag SG, Yap EP. Diagnostic value of real-time capillary thermal cyclers in virus detection. Expert Rev Mol Diagn 2004;3(March (2)):219–30.
10. Gunson RN, Collins TC, Carman WF. The real-time detection of sapovirus. J Clin Virol 2006;35(March (3)):321–2.
11. Gunson RN, Carman WF. Comparison of two real-time PCR methods for diagnosis of norovirus infection in outbreak and community settings. J Clin Microbiol 2005;43(April (4)):2030–1.
12. Gunson RN, Collins TC, Carman WF. Real-time RT-PCR detection of 12 respiratory viral infections in four triplex reactions. J Clin Virol 2005;33(August (4)):341–4.
13. Miller I, Gunson R, Carman WF. Norwalk like virus by light cyclcr PCR. J Clin Virol 2005;25(August (2)):231–2.
14. Ratcliff RM, Chang G, Kool T, Slots TP. Molecular diagnosis of medical viruses. Cur Issues Mol Biol 2007;9(August (2)):87–102.
15. Wittwer CT, Herrmann MG, Gundry CN, Elenitoba-Johnson KS. Real-time multiplex PCR assays. Methods 2001;25(December (4)):430–42.
16. Mackay I. Real time PCR in microbiology: from diagnosis to characterisation. Caister Academic Press; 2007.
17. Persson K, Hamby K, Ugozzoli LA. Four-color multiplex reverse transcription polymerase chain reaction—overcoming its limitations. Anal Biochem 2005;344(September (1)):33–42.
18. Ishii T, Sootome H, Shao L, Yamashita K. Validation of universal conditions for duplex quantitative reverse transcription polymerase chain reaction assays. Anal Biochem 2007;362(March (2)):201–12.
19. Ishii T, Sootome H, Yamashita K. Practical evaluation of universal conditions for four-plex quantitative PCR. Anal Bioanal Chem 2007;388(May (1)):271–8.
20. Engel H, Kueppers C, Koenig M, Leoffert D. Successful gene expression analysis by multiplex, real-time, one-step RT-PCR, irrespective of the targets amplified. Biotechniques 2007;43(August (2)):230–1.
21. Coleman JW, Johnson JE, Clarke DK. Simultaneous quantification of four RNA targets by multiplex, real-time RT-PCR without optimization. Biotechniques 2007;43(September (3)):369–71.
22. Cirino NM, Tavakoli NP, Madison-Anettrucci S, Egan C. Multiplex RT-PCR in microbiology. In: Mackay IM, editor. Real time PCR in microbiology: from diagnosis to characterisation. Caister Academic Press; 2007. p. 183–221.

### Table 4

| Sample          | Annual total | Number of tests carried out (pre-multiplex) | Number of tests carried out (post-multiplex) | Total difference (%) |
|-----------------|--------------|-------------------------------------------|---------------------------------------------|----------------------|
| Eves            | ∼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                 |