My Favourite Reagent

Real-time RT-PCR detection of 12 respiratory viral infections in four triplex reactions

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1. Introduction

Traditional viral culture, usually in combination with direct immunofluoresence (DIF), is the gold standard for the laboratory diagnosis of viral respiratory infection. However, these methods are insensitive, laborious, have prolonged turnaround times, and cannot detect all recognised viral respiratory pathogens. PCR is more sensitive and specific than traditional methods and can be used to detect fastidious viruses. Real-time PCR is at least as sensitive as nested gel-based PCR protocols and offers increased rapidity (results available within the working day). The use of specific labelled probes ensures easy interpretation when used in a multiplex format. We describe four triplex TaqMan[TM]-based RT-PCR methods adapted from published methods and further developed in-house for the diagnosis of 12 viral respiratory pathogens.

2. Selection of primers and probes

Primers and probes for each triplex are described in full (Table 1). Real-time RT-PCR assays for human metapneumovirus (hMPV) (Mackay et al., 2003), RSV A and B (van Elden et al., 2003), and coronavirus 229E and OC43 (van Elden et al., 2004) were published previously. Although the primers for the detection of influenza A and influenza B, rhinovirus, and parainfluenza 1 and 2 were from previously published methods (Bredius et al., 2004; Templeton et al., 2004), TaqMan[TM] probes for these pathogens were adapted from the original molecular beacons. In-house real-time RT-PCR methods were developed for coronavirus NL63 and parainfluenza 3 using Beacon designer 2.0 (Premier Biosoft International) and Primer Express (Applied Biosystems). Conserved target regions were identified using BLAST (www.ncbi.nlm.nih.gov/blast). Regions within the 1a and haemagluttinin genes were chosen for coronavirus NL63 and parainfluenza 3, respectively. The primers and probes were shown to detect all submitted NL63 and parainfluenza 3 sequences. No interfering secondary structures were observed using the mfold algorithm (www.bioinfo.rpi.edu).

3. Selection of flourophores

All real-time RT-PCR assays were developed for use on the ABI 7500 real-time PCR system. For multiplex real-time PCR, ABI recommend that probes are labelled with the dyes FAM and VIC, as they are distinguishable from each other because they have different emission wavelength maxima (518 nm and 554 nm). We chose CY5 as the third dye as its emission wavelength maxima is far removed from both FAM and VIC (670 nm). No cross-talk was observed.

4. Protocol

Respiratory controls and samples were extracted using the Qiagen blood minikit on the Biorobot 9604 using standard protocols. Amplification was carried out in a 25 μl reaction volume using the Invitrogen superscript III One step q-RT-PCR system containing 10 μl of extracted sample. Primers and probes were added to each PCR reaction at the concentration shown (Table 1). Reverse transcription was performed

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Table 1
Primers and probes used in triplex real-time RT-PCR assays

Triplex	Pathogen	Primer sequences (concentration in nM)	Probe sequence (concentration in nM)	Target
1	Influenza A	AAAGCGAATTTCAGTGTGAT (1000) GAAGGCAAT GTGAGATTT (500)	6FAM-CCC TCT TCG GTG AAA GCC CT-BHQ (300)	NS1 gene
	Influenza B	GTCCATCAAGCTCCAGTTTT (1000) TCTTCTTACAGCTTGCTTGC (500)	CY5-CCTCCGTCTCCACCTACT TCGTT-BHQ (300)	Nucleoprotein gene
	Human metapneumovirus	AACCGTGTACTAAGTGATGCACTC (500) CATTGTTTGACCGGCCCCATAA (500)	VIC-CTTTGCCATACTCAATGAACAAAC-TAMRA (300)	Nucleocapsid protein gene
2	RSV A	AGATCAACTTCTGTCATCCAGCAA (1000) TTCTGCACATCATAATTAGGAG (250)	6FAM-CACCATCCAACGGAGCACAGGAGAT-BHQ (300)	Nucleocapsid protein gene
	RSV B	AAGATGCAAATCATAAATTCACAGGA (1000) TGATATCCAGCATCTTTAAGTA (1000)	CY5-TTTCCCTTCCTAACCTGGACATA-BHQ (300)	
	Rhinovirus	TGGACAGGGTGTGAAGAGC (1000) CAAAGTAGTCGGTCCCATCC (1000)	VIC-TCCTCCGGCCCCTGAATG-TAMRA (300)	Five untranslated region
3	Parainfluenza 1	ACCTACAAGGCAACAACATC (1000) CTTCCTGCTGGTGTGTTAAT (500)	CY5-CAAACGATGGCTGAAAAAGGGA-BHQ (300)	HN gene
	Parainfluenza 2	CCATTTACCTAAGTGATGGAA (1000) CGTGGCATAATCTTCTTTTT (1000)	VIC-AATCGCAAAAGCTGTTCAGTCAC-TAMRA (300)	HN gene
	Parainlfuenza 3	CCAGGGATATAYTAYAAAGGCAAAA (1000) CCGGGRCACCCAGTTGTG (1000)	6FAM-TGGRTGTTCAAGACCTCCATAYCCGAGAAA-BHQ (300)	HN gene
4	Coronvirus 229E	CAGTCAAATGGGCTGATGCA (1000) AAAGGGCTATAAAGAGAATAAGGTATTCT (1000)	6FAM-CCCTGACGACCACGTTGTGGTTCA-BHQ (300)	
	Coronavirus OC43	CGATGAGGCTATTCCGACTAGGT (125) CCTTCCTGAGCCTTCAATATAGTAACC (1000)	CY5-TCCGCCTGGCACGGTACTCCCT-BHQ (300)	
	Coronavirus NL63	ACGTACTTCTATTATGAAGCATGATATTAA (1000) AGCAGATCTAATGTTATACTTAAAACTACG (1000)	VIC-ATTGCCAAGGCTCCTAAACGTACAGGTGTT-TAMRA (300)	

for 15 min at 50 °C. Platinum taq polymerase was activated 95 °C for 2 min and 40 cycles of PCR performed at 95 °C for 8 s and 60 °C for 34 s using an ABI 7500 SDS. Total reaction time per triplex reaction was approximately 75 min.

5. Analytical sensitivity

All real-time PCR assays were previously assessed in their original single target or duplex formats using panels of known culture and DIF or nested PCR positive and negative samples. All of the real-time RT-PCR assays were more sensitive than traditional or nested RT-PCR methods (data not shown). All real-time RT-PCR assays (except coronavirus NL63 and parainfluenza 2) were also assessed using quality control molecular diagnostics (QCMD) respiratory panel (Forde et al., in press). Each real-time RT-PCR test detected the appropriate target at the appropriate end point dilution.

6. Sensitivity compared to current diagnostic methods

Four multiplex real-time RT-PCR assays were developed for the simultaneous detection of

1. influenza A, influenza B, and hMPV;
2. RSV A, RSV B, and rhinovirus;
3. parainfluenza 1, 2, and 3;
4. coronavirus 229E, OC43, and NL63.

Each triplex real-time RT-PCR assay was initially assessed using positive controls and compared to the previous duplicate or single target format. Triplex assays were then compared to panels of tissue culture and DIF and/or nested RT-PCR positive samples (where available). To determine whether mixed infections would reduce triplex sensitivity end point dilutions of each viral target were tested in both a single target and a pooled target (containing 10 different viral targets at the same endpoint dilution) format.

6.1. Multiplex real-time RT-PCR for influenza A, influenza B, and human metapneumovirus

Positive control samples for influenza A, influenza B, and hMPV were tested in triplicate wells using the triplex assay in parallel with the previous duplex or single target format (Table 2). There was no significant loss of sensitivity (as observed by cycle threshold (Ct)) between methods. The triplex assay was then tested on 11 influenza A positive samples. The triplex assay detected all the previous positive samples. The endpoint dilution of both the single target controls and pooled format controls were detected by the triplex assay showing that "mixed infections" would not reduce the sensitivity of this assay.

Table 2
Comparison of Ct of positive controls in triplicate for influenza A, influenza B, and HmPv in single, duplex, and triplex RT-PCR reactions

	Mean Ct (±S.E.M.)
Flu A in duplex	29.91 (0.1)
Flu A in triplex	30.46 (0.32)
Flu B in duplex	25.90 (0.21)
Flu B in triplex	26.22 (0.53)
hMPV in single	29.83 (0.38)
hMPV in triplex	28.93 (0.18)

6.2. Multiplex real-time RT-PCR for RSV A, RSV B, and rhinovirus

Positive samples for RSV A, RSV B, and rhinovirus were tested in a triplex format in triplicate and compared to duplex or single target format (Table 3). No loss in sensitivity was observed for RSV A and RSV B. The sensitivity of the rhinovirus real-time RT-PCR improved when incorporated in the triplex assay (as shown by a reduction in the Ct). This increase in sensitivity was sample specific (only occurred with the positive control) as no reduction in Ct was observed when the testing the rhinovirus positive samples. The triplex assay was then assessed on 42 RSV positive samples (NPA samples positive by DIF and nested gel-based PCR) and 11 rhinovirus positive samples. The triplex assay detected all previously positive samples. The endpoint dilution of both the single target controls for RSV A, RSV B, and rhinovirus and pooled format controls (containing 10 viral targets) were detected by the triplex assay showing that "mixed infections" would not reduce the sensitivity of this assay.

6.3. Multiplex real-time RT-PCR for parainfluenza 1, 2, and 3

Positive control samples for parainflueza 1, 2, and 3 were tested in a triplex format, either with the new PF3 assay or the original test. The introduction of the new PF3 assay had no effect on the sensitivity of the PF1 and PF2 RT-PCR assays (Table 4). However, the new PF3 RT-PCR was more sensitive than the previous method. The new triplex assay was then compared to the published method on 19 DIF PF3 positive samples. The new method detected one additional sample (19 versus 18). The endpoint dilution of both the single target controls and pooled format controls were detected

Table 3
Comparison of Ct for RSV A, RSV B, and rhinovirus in single, duplex, and triplex RT-PCR reactions

	Mean Ct (±S.M.E.)
RSV A duplex	17.43 (0.28)
RSV A triplex	17.19 (0.15)
RSV B duplex	22.06 (0.22)
RSV B triplex	21.17 (0.28)
Rhino single	26.05 (0.13)
Rhino triplex	23.85 (0.02)

Table 4
Comparison of published parainfluenza triplex assay with triplex parainfluenza assay incorporating new PF3 RT-PCR assay

	Ct of PF1 control	Ct of PF2 control	Ct of PF3 control
Published parainfluenza triplex assay	28.34	24.17	28.37
Published parainfluenza triplex assay with new PF3 PCR	27.75	25.02	24.24

Table 5
Comparison of Ct for single, duplex, and triplex RT-PCR assays for corovirus 229E, OC43, and NL63

	Ct of OC43 control	Ct of 229E control	Ct of NL63 control
OC34/229E/NL63 assay	23.76	31.9	26.67
OC43/229E assay	23.63	31.3	
NL63 assay			26.23

by the triplex assay showing that "mixed infections" would not reduce the sensitivity of this assay.

6.4. Multiplex real-time RT-PCR for coronavirus 229E, OC43, and NL63

Positive samples for coronavirus 229E, OC43, and NL63 were tested in a triplex format and in the duplex or single target format (Table 5). No significant changes in Ct were observed. The endpoint dilution of both the single target controls and pooled format controls were detected by the triplex assay showing that "mixed infections" would not reduce the sensitivity of this assay.

We have shown these triplex real-time RT-PCR assays to be at least as sensitive our previous RT-PCR assays. The rapidity, stability, and ease of use of these triplex real-time RT-PCR assays results in improved turn-around-times (12 pathogens within the working day), easier interpretation, and increased cost effectiveness. The implementation of these assays will no doubt improve patient management, infection control procedures, and the effectiveness of surveillance systems.

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