Fast Optimization of a Multiplex Influenza Identification Panel Using a Thermal Gradient

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Introduction

The year 2009 was marked by the emergence of a novel influenza A (H1N1) virus that infects humans. There is a need to identify the different strains of influenza virus for purposes of monitoring the H1N1 strain pandemic and for other epidemiological and scientific purposes. In preparation for an accurate and rapid identification of H1N1, a laboratory in Singapore established a multiplex influenza identification panel that detects H1N1 among influenza A subtype H1 (flu A), subtype H3 (H3N2), and influenza B (flu B) viruses. The panel uses a 5' nuclease (TaqMan) probe–based four-target multiplex real-time PCR assay.[1]

In this tech report we illustrate the use of the thermal gradient feature on the CFX96™ real-time PCR detection system to optimize reverse transcription (RT) and fast PCR conditions for a four-target multiplex PCR assay.

Methods

Viral RNA

The following virus strains were used: influenza A (H1N1), influenza A subtype H1 (flu A), influenza A subtype H3 (H3N2), and influenza B (flu B). Input concentration ranged between 10 and 1,000 copies.

Optimization of RT

Optimization of RT was performed with the four-target multiplex RT-qPCR MDX kit (Experimental Therapeutic Centre). The TaqMan probes were tagged with the following fluorophores: H1N1, HEX; flu A, FAM; H3N2, Cy5; and flu B, Texas Red.

RT-qPCR reactions were set up as indicated in Table 1 using the iScript™ one-step RT-PCR kit for probes (Bio-Rad Laboratories, Inc.).[2] Reactions were incubated in the CFX96 system (Bio-Rad) for 30 min at 45, 45.6, 47, 48.9, 51.3, 53.3, 54.4, and 55°C (temperature gradient), followed by 2.5 min at 95°C, then subjected to 42 three-step cycles of 95°C for 17 sec, 55°C for 31 sec, and 68°C for 32 sec. Fluorescence data were collected at the 55°C annealing step. Positive control RNAs equivalent to approximately 1,000 viral copies were used for optimization.

Multiplex RT-qPCR

RT-qPCR reactions were set up as indicated in Table 1. Reactions were incubated at 50°C for 30 min followed by 2.5 min at 95°C, then subjected to 42 three-step cycles of 95°C for 17 sec, 55°C for 31 sec, and 68°C for 32 sec. Fluorescence data were collected at the 55°C annealing step.

Table 1. Reaction setup for ten reactions.

Components	Volume, µl
2x RT-qPCR reaction buffer	125.0
H1N1 primer pair mix (7.5 µM each)	5.0
H1N1 probe–HEX (10 µM)	2.5
Flu A primer pair mix (7.5 µM each)	5.0
Flu A probe–FAM (10 µM)	2.5
H3N2 primer pair mix (7.5 µM each)	5.0
H3N2 probe–Cy5 (10 µM)	2.5
Flu B primer pair mix (7.5 µM each)	5.0
Flu B probe–Texas Red (10 µM)	2.5
RNA template (range from 0.01 pg–1 µg)	Up to 90
Reverse transcriptase and Taq polymerase mix	5.0
RNase-free H_20	Top up to 250

Optimization of Fast qPCR

RT-qPCR reactions were set up as indicated in Table 1, incubated for 10 min at 50°C followed by 2.5 min at 95°C, then subjected to 42 two-step cycles of 95°C for 17 sec and 31 sec at 55, 56, 58, 60.9, 64.5, 67.5, 69.2, and 70°C (temperature gradient). Fluorescence data were collected at the gradient range of the 55–70°C annealing-extension step. Positive control RNAs equivalent to about 1,000 viral copies were used for fast qPCR optimization.

Results and Discussion

RT Optimization Using the Thermal Gradient

The temperature gradient feature of the CFX96 real-time PCR system was used to determine the optimal RT temperature for the four-target multiplex RT-qPCR assay. The CFX96 system was programmed to perform RT at temperatures ranging from 45 to 55°C, followed by a three-step PCR protocol (denaturation-annealing-extension). Results from the RT gradient in Table 2 show that the optimal RT temperature range (in blue) was between 45.0 and 51.3°C for all primer-probe sets, with influenza A reactions working as high as 53.3°C. Selecting higher RT temperatures improves the specificity of the assay but compromises the assay sensitivity. In order to maintain both specificity and sensitivity, we have selected 50°C RT for the rest of the four-target multiplex assay validation.

Table 2. Optimization of RT reaction temperature for the four-target multiplex influenza RT-qPCR assay.

	C_T*			
RT Reaction Temperature, °C	H1N1 (HEX)	Flu A (FAM)	H3N2 (Cy5)	Flu B (Texas Red)
55.0	32.68	33.70	32.41	31.71
54.4	32.34	33.70	32.46	30.67
53.3	32.09	32.50	31.81	30.57
51.3	31.67	32.48	31.70	29.26
48.9	31.45	32.66	31.04	28.89
47.0	31.18	32.02	31.09	29.45
45.6	31.16	32.24	31.15	29.47
45.0	30.93	32.97	30.98	29.43

* Threshold cycle (C_T) values in blue reflect optimal RT temperatures yielding the lowest C_T ±0.5°C for each primer-probe set, while C_T values in red indicate suboptimal temperatures.

Multiplex RT-qPCR Assay Sensitivity

To determine the multiplex RT-qPCR assay sensitivity, we tested different concentrations of the four viral RNAs ranging from 1,000 to 10 copies. Results shown in Figure 1 clearly show that the primer-probe sets were able to detect all the viral RNA controls with no cross talk between channels.

Optimization of Fast qPCR Conditions

Optimization of fast qPCR conditions was achieved by combining the annealing and extension steps. Combining these steps is essential for 5' nuclease (TaqMan) probe–based assays because the probes need to anneal to the target while the product is being extended.

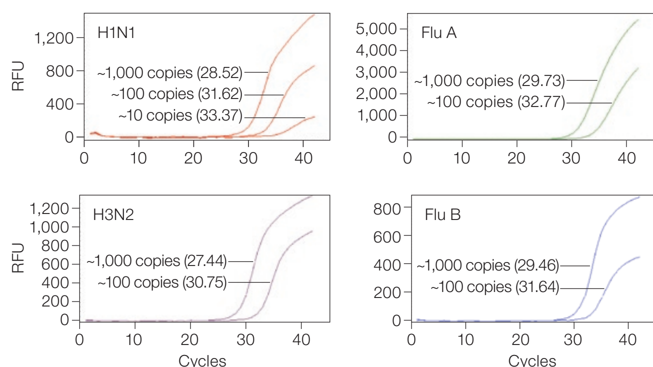

Fig. 1. Multiplexing of four-target influenza RT-qPCR. Amplification profiles of H1N1, flu A, flu B, and H3N2. Viral RNA isolates ranging from 1,000 to 10 copies were detected. RFU, relative fluorescence units.

Additional timesavings were achieved by reducing the RT time from 30 min to 10 min. C_T values in Table 3 and amplification plots in Figure 2 illustrate the optimal fast qPCR annealing-extension temperature range for H1N1 (58–60.9°C), flu A (60.9–64.5°C), flu B (55–58°C), and H3N2 (58–64.5°C). We selected 58°C for annealing-extension to perform the fast four-target multiplex RT-qPCR assay validation (total run time for this reaction was shortened from 128 to 73 min). The results generated using fast cycling conditions in Table 4 show that all viral RNA controls tested were detected with a 1–2 C_T delay compared to the standard 30 min RT using three-step qPCR cycling conditions.

Table 3. Optimization of the four-target multiplex influenza RT-qPCR assay for fast PCR.

	C_T*			
Annealing Temperature, °C	H1N1 (HEX)	Flu A (FAM)	H3N2 (Cy5)	Flu B (Texas Red)
70.0	—	—	—	—
69.2	—	41.98	—	—
67.5	—	31.11	—	—
64.5	—	29.47	27.67	—
60.9	30.81	29.79	27.55	31.68
58.0	31.26	30.01	27.78	30.54
56.0	31.83	30.15	28.18	30.54
55.0	31.53	30.39	28.44	30.06

* C_T values in blue reflect optimal annealing-extension temperatures yielding the lowest C_T ±0.5°C for each primer-probe set, while C_T values in red indicate suboptimal temperatures.

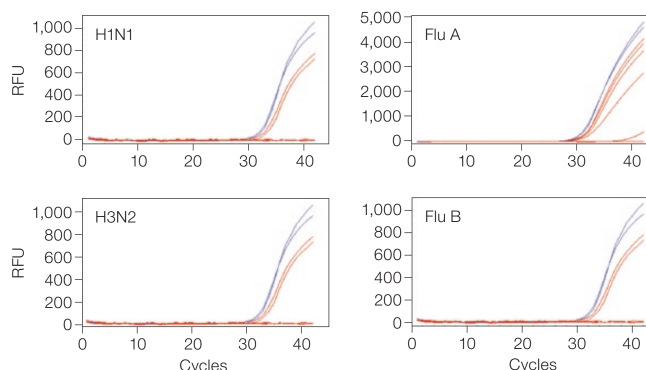

Fig. 2. Optimization of fast qPCR for the four-target multiplex influenza RT-qPCR. Amplification profiles of PCR reactions listed in Table 3. Blue and red traces indicate PCR reactions performed at optimal and suboptimal annealing temperatures, respectively. RFU, relative fluorescence units.

Table 4. Comparison of standard PCR and fast PCR efficiency.

		C_T		
Viral RNA	Copy Number	Standard Run (128 min)	Fast Run (73 min)	ΔC_T
H1N1	1,000	28.52	27.85	–0.67
	100	31.62	32.32	0.70
	10	33.37	34.42	1.05
Flu A	1,000	29.73	30.99	1.26
	100	32.77	34.32	1.55
H3N2	1,000	27.44	28.72	1.28
	100	30.75	32.38	1.63
Flu B	1,000	29.46	31.45	1.99
	100	31.64	33.54	1.90

Conclusions

We have demonstrated that the gradient feature of the CFX96 system dramatically accelerates the optimization of the RT and PCR steps for multiplexing RT-qPCR assays. The thermal gradient also improved the ease of converting cycling conditions from a three-step to a two-step PCR assay, thus significantly reducing PCR run times while maintaining assay specificity and sensitivity.

[1]The test protocol described in this paper has not been cleared or approved by the FDA or any other regulatory agency for human diagnostic or other clinical use.

[2]This product may be used for scientific research purposes only. Please refer to the product insert for additional information.

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