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Developing a Customizable Panel of Real-time qPCR Assays on Microfluidic TaqMan™ Array Cards® for Respiratory Tract Pathogen Detection

Kelly Li, Jisheng Li, Nicole Fantin, Bonnie Moy, Sunali Patel, Ioanna Pagani, Emily Zeringer, Toinette Hartshorne, Hong Ji, Nitin Puri, and Kamini Varma
Clinical R&D, Genetic Science Division, Life Science Group, Thermo Fisher Scientific, 180 Oyster Point Blvd, South San Francisco, CA 94080

INTRODUCTION

Uncomplicated respiratory tract infection account for ~25 million physician visits annually in the U.S. Respiratory infections (RI) are ranked as the greatest single contributor to the overall burden of disease in the world. A wide variety of bacteria and viruses are capable of causing upper and lower respiratory tract infections.

Panel based testing using molecular methods to identify these pathogens has clear advantages over non-molecular or single target molecular testing. However, many current methods are limited to either fixed panels or have low throughput. To address these unmet needs, we have developed a large collection of TaqMan assays for respiratory pathogens and leveraged TaqMan™ Array Card (TAC) technologies. The combination presents a robust research application for respiratory pathogen detection and allows customization of both the size and content of the test panel.

Here we describe development of the new application customized qPCR research panels for any subset of the targets can be built on either Applied Biosystems™ TaqMan™ Array Cards®. A novel set of TaqMan™ real-time qPCR Assays was developed to over 40 distinct respiratory pathogen targets, including bacteria, DNA viruses, RNA viruses, and fungi. We identified unique gene targets that are species-specific and designed TaqMan™ assays using the proprietary assay design pipeline. These assays were evaluated with plasmid templates and ATCC genomic DNA/gRNA (gDNA/gRNA) controls. Linearity and analytical sensitivity was studied and revealed that at least 5 log linear dynamic range (with R^2 >0.99) with limit of detection (LOD) down to 1-10 copies/ul. For specificity, each assay was tested against all the rest of plasmids and ATCC gDNA/gRNA controls and no significant cross-reactivity was observed

In summary, we have developed a large collection of TaqMan™ assays for respiratory pathogens. Using synthetic DNA and RNA templates and as well as ATCC gDNA/gRNA controls, we have demonstrated excellent assay performance of accuracy, sensitivity, specificity, and reproducibility. In conjunction with TaqMan™ Array Cards, the application enables researchers to study a large number of respiratory in a single reaction with a simple workflow, fast turnaround time, and high throughput yet flexible sample/target combinations.

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MATERIALS AND METHODS

A proprietary assay design pipeline was used to design TaqMan™ research assays for signature genes for targeted respiratory pathogens. These FAM™ dye-labeled assays were pre-loaded and lyophilized onto wells on a TaqMan® Array Card (TAC), a microfluidic device. The TAC platform offers a 384-well, single-plate real-time PCR in an 8 sample by 48-well format with a single target tested in each of 48 available wells (including controls, Fig 1A). Microfluidic technology distributes the sample into individual wells and real-time PCR detection takes place within a qPCR instrument (QuantStudio 7 or QuantStudio 12K, Fig 1B) with results generated in one hour. The process and individual steps involved in setting up a card are schematically outlined in Fig 1C.

An oligo pool containing a large multiplex set of primers was developed for use in a single tube reverse transcription and pre-amplification reaction. This reaction generates cDNA and amplifies both genomic DNA and cDNA for the full set of microbiota targeted by the qPCR assays.

Plasmids for synthetic DNA or RNAs templates that contain amplicon sequences were ordered from GeneArt. IVT was performed to generate synthetic RNA templates. Both synthetic DNA and RNA were quantified by digital PCR using QuantStudio™ 3D Digital PCR System. Genomic DNA/RNA controls were purchased from ATCC. Assays were first evaluated with DNA templates, then RNA templates and ATCC gDNAs/gRNA controls. Serial dilution studies were performed for dynamic range and limit of detection (LOD). A robust protocol was developed to using TaqMan Fast Virus 1-step Master Mix for RT-PreAmp step and Fast Advanced Master Mix on QuantStudio™ 7 or 12K Real Time PCR System™ for qPCR. Manufacture recommended conditions are followed.

RESULTS

Fig. 1. TaqMan Array™ Card Technology (1A), QuantStudio™ 7 Real-time PCR System (1B) and Its Workflow (1C)

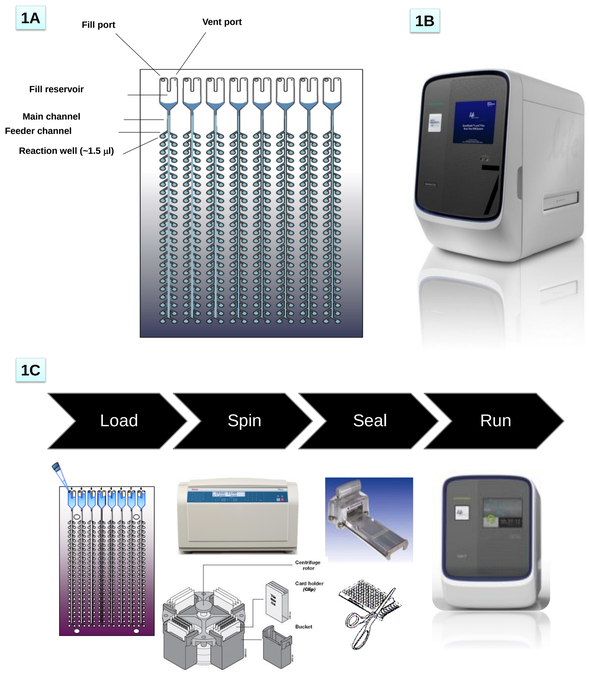
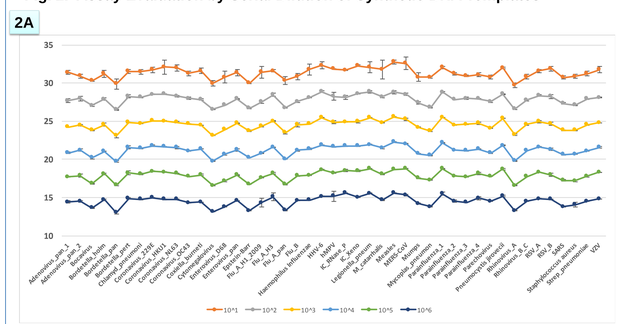


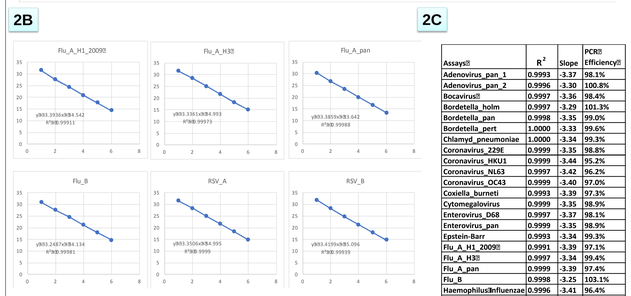
Table 1. Species List of Respiratory Tract Microbiota that TaqMan™ Assays Target

A collection of TaqMan assays that target respiratory tract microbiota was designed and validated (data not shown). Table 1 summarizes some of the targeted species with their available controls that are tested on TaqMan Array™ Card (TAC). The synthetic DNA and RNA templates and ATCC gDNA/gRNA controls used for their testing. Synthetic DNA template controls are available for all the targeted species and synthetic RNA templates are available for all the RNA viruses. ATCC gDNA and gRNA controls are available for most species except those that are uncultured and or hard to culture or restricted due to biosafety consideration.

Organism Name	Multi Target Synthetic Plasmid	In Vitro Transcript (IVT) Product	ATCC Samples
Bocavirus	✓	✓	
Human Coronavirus 229E	✓	✓	✓
Human Coronavirus HKU1	✓	✓	
Human Coronavirus NL63	✓	✓	
Human Coronavirus OC43	✓	✓	✓
Human Enterovirus	✓	✓	✓
Human Enterovirus D68 Strain	✓	✓	✓
Human Metapneumovirus	✓	✓	
Human Parainfluenza Virus 1	✓	✓	✓
Human Parainfluenza Virus 2	✓	✓	✓
Human Parainfluenza Virus 3	✓	✓	✓
Human Parainfluenza Virus 4	✓	✓	✓
Human Parechovirus v2	✓	✓	
Human Rhinovirus A	✓	✓	✓
Human Rhinovirus B/C	✓	✓	
Influenza A H1 Seasonal	✓	✓	✓
Influenza A H3	✓	✓	✓
Influenza A	✓	✓	✓
Influenza B	✓	✓	✓
Measles Virus	✓	✓	✓
MERS	✓	✓	
Mumps Virus	✓	✓	✓
RSV A specific	✓	✓	✓
RSV B specific	✓	✓	✓
SARS	✓	✓	✓
Adenovirus	✓		✓
Bordetella bronchiseptica/parapertussis/pertussis	✓		✓
Bordetella holmesii	✓		✓
Bordetella pertussis	✓		✓
Chlamydophila pneumoniae	✓		✓
Coxiella burnetii	✓		
Haemophilus influenzae	✓		✓
HHV3 (VZV)	✓		✓
HHV4 (EBV)	✓		
HHV6	✓		
Legionella pneumophila	✓		✓
Moraxella catarrhalis	✓		✓
Mycoplasma pneumoniae	✓		✓
Pneumocystis jiroveci	✓		
Staphylococcus aureus	✓		✓
Streptococcus pneumoniae	✓		✓

Fig. 2. Assay Evaluation by Serial Dilution of Synthetic DNA Templates





2C

Assays	R^2	Slope	PCR Efficiency
Adenovirus_pan_1	0.9993	-3.37	98.1%
Adenovirus_pan_2	0.9996	-3.30	100.8%
Bocavirus	0.9997	-3.36	98.4%
Bordetella_holm	0.9997	-3.29	101.3%
Bordetella_pan	0.9998	-3.35	99.0%
Bordetella_pert	1.0000	-3.33	99.6%
Chlamyd_pneumoniae	1.0000	-3.34	99.3%
Coronavirus_229E	0.9999	-3.35	98.8%
Coronavirus_HKU1	0.9999	-3.44	95.2%
Coronavirus_NL63	0.9997	-3.42	96.2%
Coronavirus_OC43	0.9999	-3.40	97.0%
Coxiella_burneti	0.9993	-3.39	97.3%
Cytomegalovirus	0.9999	-3.35	98.9%
Enterovirus_D68	0.9997	-3.37	98.1%
Enterovirus_pan	0.9999	-3.35	98.9%
Epstein-Barr	0.9993	-3.34	99.3%
Flu_A_H1_2009	0.9991	-3.39	97.1%
Flu_A_H3	0.9997	-3.34	99.4%
Flu_A_pan	0.9999	-3.39	97.4%
Flu_B	0.9998	-3.25	103.1%
Haemophilus_Influenzae	0.9996	-3.41	96.4%
HHV-6	0.9999	-3.43	95.7%
hMPV	0.9994	-3.33	99.5%
Legionella_pneumoniae	0.9997	-3.32	100.0%
M_catarrhalis	0.9999	-3.41	96.5%
Measles	0.9989	-3.41	96.3%
MERS-CoV	0.9987	-3.39	97.3%
Mumps	0.9999	-3.31	100.6%
Mycoplas_pneumoniae	0.9988	-3.33	99.7%
Parainfluenza_1	1.0000	-3.31	100.4%
Parainfluenza_2	1.0000	-3.34	99.4%
Parainfluenza_3	0.9994	-3.35	99.0%
Parainfluenza_4	0.9999	-3.24	103.7%
Parechovirus	0.9998	-3.25	102.9%
Pneumocystis_jiroveci	0.9998	-3.35	99.0%
Rhinovirus_A	0.9998	-3.30	100.8%
Rhinovirus_B_C	0.9996	-3.29	101.5%
RSV_A	0.9999	-3.35	98.8%
RSV_B	0.9994	-3.42	96.1%
SARS	0.9999	-3.37	98.1%
Staphylococcus_aureus	0.9993	-3.34	99.4%
Strep_pneumoniae	1.0000	-3.35	98.7%
VZV	0.9996	-3.35	98.9%

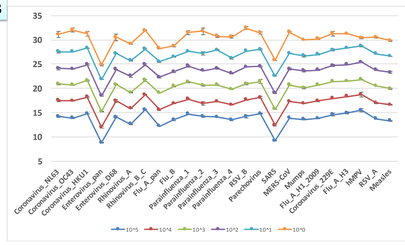
Serial dilution was performed with a multi templates plasmid from 10^6 copies/µl stock to 10 copies/µl on TAC. Volume of 10ul of synthetic DNA control was used in each port. Roughly 3.3 Ct differences were observed in adjacent dilution cross all the targets (**2A**). Linear dynamic range of these assays was plotted in scatter plots (**2B**), X-axis is $\log_{10}$ of plasmid template concentration (copies/µl) and Y axis is Ct values. The R square and slope for each assay are summarized in the table (**2C**) and PCR efficiency are calculated .

Fig. 3. Assay Evaluation by Serial Dilution of Synthetic RNA Templates

3A

Assay	R^2	Slope	PCR Efficiency
Coronavirus_229E	1.000	3.337	99%
Coronavirus_HKU1	1.000	3.317	100%
Coronavirus_NL63	1.000	3.363	98%
Coronavirus_OC43	0.997	3.546	91%
Enterovirus_D68	1.000	3.271	102%
Enterovirus_pan	1.000	3.22	104%
Flu_A_H1_2009	0.999	3.254	103%
Flu_A_H3	1.000	3.283	102%
Flu_A_pan	0.999	3.229	104%
Flu_B	0.995	3.08	111%
hMPV	0.993	3.089	111%
Measles	1.000	3.311	100%
MERS-CoV	0.998	3.471	94%
Mumps	1.000	3.283	102%
Parainfluenza_1	0.999	3.334	99%
Parainfluenza_2	0.994	3.483	94%
Parainfluenza_3	0.999	3.377	98%
Parainfluenza_4	0.997	3.36	98%
Parechovirus	1.000	3.309	101%
Rhinovirus_A	1.000	3.303	101%
Rhinovirus_B_C	0.998	3.226	104%
RSV_A	1.000	3.36	98%
RSV_B	0.996	3.566	91%
SARS	1.000	3.331	100%

3B



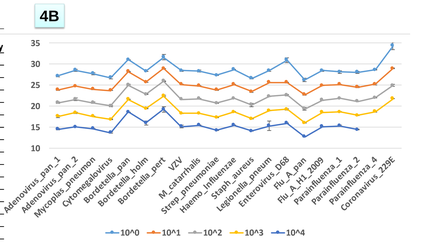
Assays targeting RNA viruses were tested with synthetic RNA templates. Reverse transcription was performed with these IVT (*in vitro* transcription) products. Serial dilution was performed with synthetic RNA pool from 10^5 to 10 copies/µl and. 10ul of RNA pool was used in each port. The R square, slope and PCR efficiency are summarized (**3A**). Roughly 3.3 Ct differences were observed in adjacent dilution cross all the targets (**3B**).

Fig. 4. Assay Evaluation by Serial Dilution using ATCC gDNA/gRNA Controls

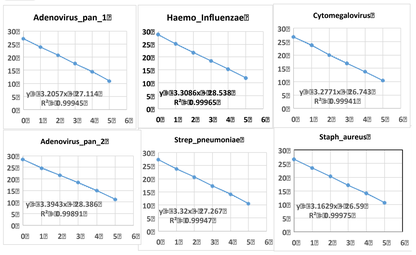
4A

Assay	R^2	Slope	PCR efficiency
Adenovirus_pan_1	0.9994	-3.21	105.1%
Adenovirus_pan_2	0.9989	-3.39	97.1%
Mycoplasma_pneumoniae	0.999	-3.26	102.8%
Cytomegalovirus	0.9994	-3.28	101.9%
Bordetella_pan	0.9962	-3.30	100.8%
Bordetella_holm	0.9985	-3.09	110.8%
Bordetella_pert	0.9994	-3.21	105.1%
Legionella_pneum	0.998	-3.29	101.3%
VZV	0.9963	-3.52	92.2%
M_catarrhalis	0.9991	-3.29	101.4%
Strep_pneumoniae	0.9995	-3.32	100.1%
Haemo_Influenzae	0.9997	-3.31	100.6%
Staph_aureus	0.9997	-3.16	107.1%
Coronavirus_229E	0.9959	-3.60	89.6%
Enterovirus_D68	0.9993	-3.33	99.9%
Flu_A_pan	0.9998	-3.34	99.4%
Flu_A_H1_2009	0.999	-3.30	100.9%
Parainfluenza_1	0.9997	-3.21	104.9%
Parainfluenza_2	0.9999	-3.39	97.2%
Parainfluenza_4	0.9999	-3.29	101.3%

4B

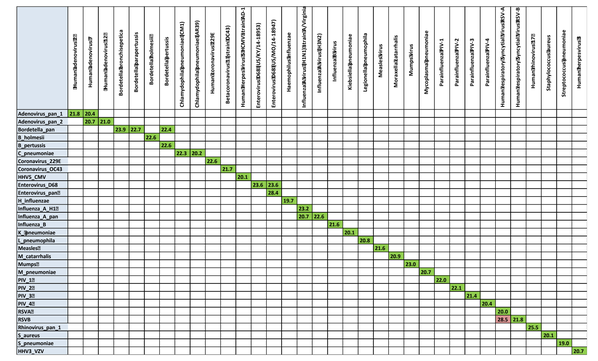


4C



ATCC gDNA/RNA controls were tested with serial dilution from 10^4 copies/µl stock to 1 copies/µl. RT-PreAmp was done and then followed by qPCR on TAC. Excellent linear dynamic range and PCR efficiency are summarized (**4A**) plotted. LOD of all tested assays close to 1 copies/ul with at least 5 logs linearity (**4B** and **4C**).

Fig. 5. Assay Evaluation by Serial Dilution using ATCC controls of gDNA/gRNA



Assays were also evaluated for their on-target and off-target performance using gDNA/gRNA from ATCC. Columns represent assay tested and rows represent ATCC controls. The desired on-target Ct values were highlighted in green and unexpected off-target Ct values are in red. The result demonstrate excellent assay specificity.

CONCLUSIONS

1. A large collection of TaqMan™ assays was developed for high throughput detection of respiratory pathogen.
2. These research assays were evaluated on TAC with DNA/RNA synthetic templates and gDNA/gRNA controls. Excellent sensitivity and specificity are demonstrated- LOD) is down to ~1-10 copies/ul (in the sample) and at least 5 logs of linearity with R^2 >0.99.
3. TaqMan array Card™ technology allows customization of both the size and content of the panel. This application enables researchers to study many respiratory pathogens simultaneously and test various numbers of samples with a customizable panel. Therefore, it not only provides a simple workflow and fast turnaround time but is also high throughput with flexible sample/target combinations.