An assay design pipeline for massively parallel, nano-scale syndromic panels: A case study using a viral RT-qPCR respiratory panel paired with virus-like particle (VLP) RNA quantitative controls

Nick Tang*, Mukesh Maharjan, Stephanie Nguyen, Ying Bao, Yusuke Fujino, Christopher Sontag, Tamara Fisher, Rohit Kadam, Jonathan Wang & Andrew Farmer

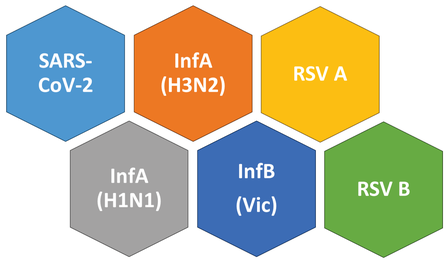
Takara Bio USA, Inc., 2560 Orchard Pkwy, San Jose, CA 95131 USA * CorrespondingAuthor

Introduction

The rise of respiratory infectious diseases, particularly highlighted by the SARS-CoV-2 pandemic, has prompted a surge in qPCR-based testing for the fast and accurate identification of multiple respiratory pathogens[1]. One challenge has been to design sensitive and specific assays within a large syndromic panel that exhibit no cross-reactivity[2,3]. To address this obstacle, we utilized Takara Bio's SmartChip® Real-Time PCR System, which is able to perform parallel singleplex qPCR reactions at the nanoliter scale across multiple samples and reactions without the need for complex degenerate or multiplex designs.

Methods

To complement the SmartChip system, we have developed a pipeline to streamline the process of designing large syndromic assay panels. Leveraging this pipeline, we successfully generated a comprehensive respiratory qPCR assay panel comprising six viral targets commonly associated with respiratory infections and several bacterial targets associated with co-infections and pneumonia. Notably, these targets include: SARS-CoV-2, Influenza A H1N1/H3N2, Influenza B, and Respiratory Syncytial Virus A/B.



The pipeline employs computational tools to optimize primer and probe selection and consists of several steps: "(1) retrieve relevant strain information from databases, (2) use strain filters based on clade and date of collection, and (3) use alignment-based assay filters encompassing strain-inclusivity, exclusivity, and other qPCR design criterie.

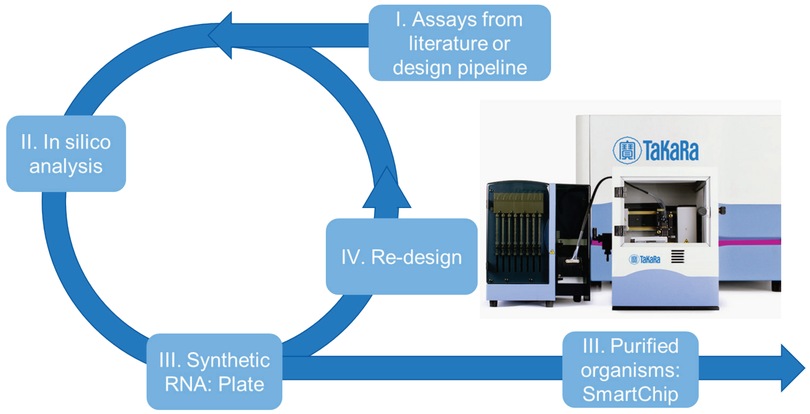


Figure 1. The SmartChip instrument and steps in our method streamlining the process of designing large syndromic panels for sensitive and specific detection of pathogens. This procedure consists of multiple iterations of in silico design and experimental validation. Ultimately, all assays are characterized on the SmartChip system with purified organisms.

References

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2. Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L, Verweij JJ, Taniuchi M, Sobuz SU, Haque R, Haverstick DM, Houpt ER. A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. J Clin Microbiol. 2013 Feb;51(2):472-80. doi: 10.1128/JCM.02658-12. Epub 2012 Nov 21. PMID: 23175269; PMCID: PMC3553916.
3. Babady NE. The FilmArray® respiratory panel: an automated, broadly multiplexed molecular test for the rapid and accurate detection of respiratory pathogens. Expert Rev Mol Diagn. 2013 Nov;13(8):779-88. doi: 10.1586/14737159.2013.848794. PMID: 24151847; PMCID: PMC7103684.

1 Assays are designed to detect circulating strains

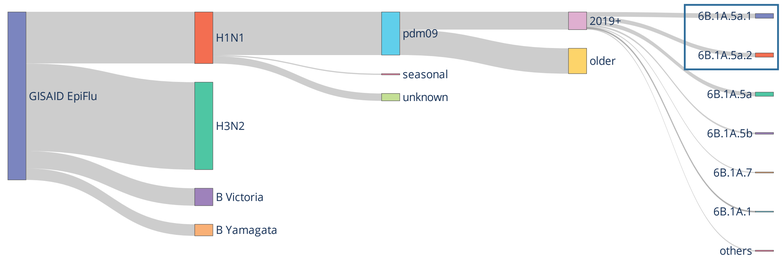

Figure 2. We use Nextclade to classify and exclude non-circulating strains. For example, high-quality sequences of recently circulating clades of H1N1 (6B.1A.5a.1-2, outlined) are a small subset (7,691 sequences) of the GISAID EpiFlu database (146,586 sequences), as shown by the above Sankey plot.

2 Assays are designed to be specific: inclusivity

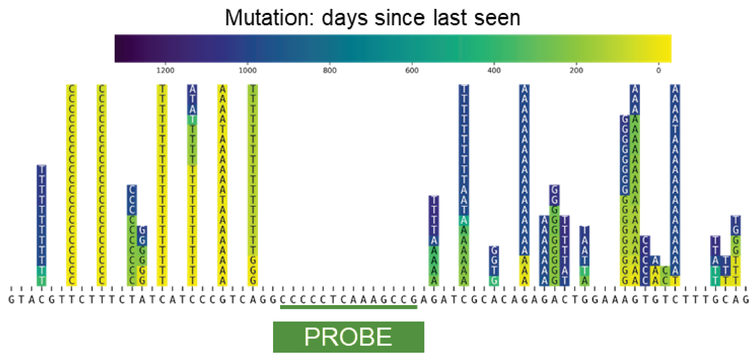

Figure 3. Overall, our assays cover greater than 99% of the reference strains without mismatches. Alignment-based design heavily penalizes the inclusion of non-conserved bases. For example, the above illustration shows base mismatches aggregated from various strains of H1N1 (colored by days since last seen) against the consensus sequence (bottom). Optimally, the probe (green) for the H1N1 assay lies on a region that has no mismatches (blank). When tradeoffs are necessary, base-positions with recent mutations (yellow) are penalized more than older ones (blue). Also, base-positions with more mutations (higher stack) are penalized.

3 Assays are designed to be specific: exclusivity

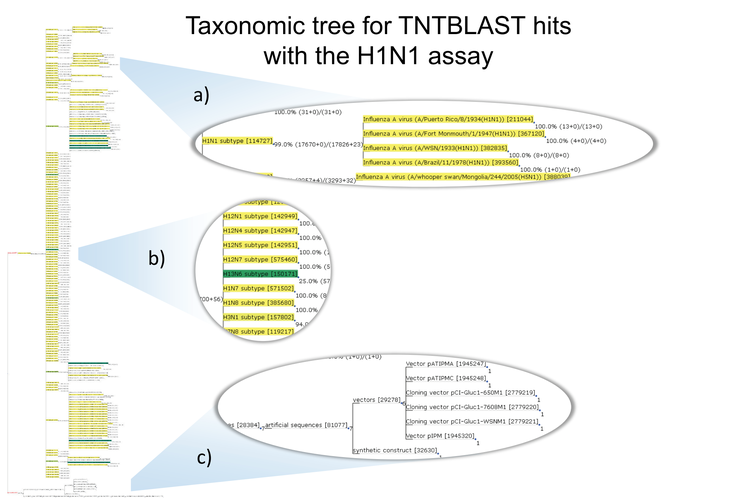

Figure 4. In silico, our assays exhibited no cross-reactivity. We searched the entire GenBank database for potential nonspecific amplifications using thermonucleotideBLAST (TNTBLAST). For example, this taxonomic tree for hits with the H1N1 assay demonstrated **inclusivity** within Influenza A, with high coverage of the GenBank entries with the H1N1 subclassification, colored yellow (a), and varying levels of coverage of GenBank entries with other uncommon low-pathogenic Influenza A subclassifications, colored green to yellow depending on coverage (b). Outside of Influenza A, only synthetic constructs exhibited cross-reactions (c), demonstrating favorable **exclusivity**.

Experimental results

4 Designed assays are sensitive: synthetic RNA experiments

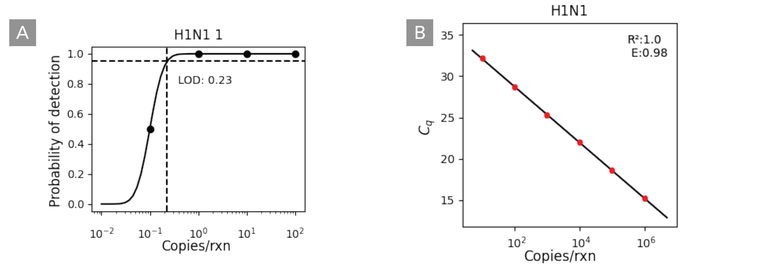

Figure 5. Experiments with synthetic and VLP-encapsulated RNA controls containing target sequences demonstrated good sensitivity. (**Panel A**) An example of probit analysis for H1N1 assay estimates an LOD of approximately 1 copy/rxn at 95% detection rate (n ≥ 20). Linear fit of quantification cycles (Cq) for a dilution series of synthetic H1N1 controls shows efficiency (E) of 90–110% with 5+ dilutions. In this example (**Panel B**), we achieved an E of 0.98 within the range of 10–10^6.

5 Designed assays are sensitive and specific: purified organism experiments

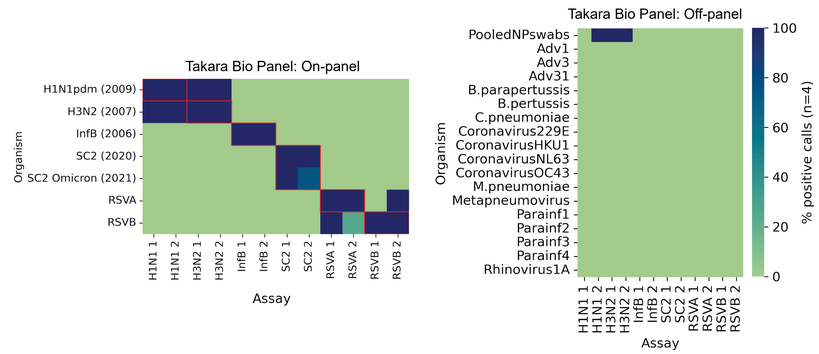

Figure 6. Tests using purified intact organisms and pooled nasopharyngeal swabs on the SmartChip system demonstrate proficiency in target pathogen detection. Heat maps for on-panel (left) and off-panel (right) organisms show the % positive calls for six targets from 12 assays (2 assays per target) for 24 purified organisms. **Green**: no positive calls; **blue**: 100% positive calls. Consistently positive calls from assays for their designated targets (**on-panel: blue** along the outlined diagonal) demonstrate sensitivity in detections. The absence of positive calls from assays for related viruses other than their targets shows no on-panel or off-panel cross-reactivity between unrelated organisms (**on-panel: green** off the outlined diagonal; **off-panel:** completely **green**). Unexpectedly, pooled nasopharyngeal swabs (PooledNPswabs) exhibited positive calls for Influenza A (**blue**), indicating the possibility of the detection of an asymptomatic infected individual within the pool.

Conclusions

- Our study showcases the implementation of an assay design and characterization pipeline combining in silico analyses and experimental evaluation using the SmartChip® Real-Time PCR System to perform parallel singleplex qPCR reactions
- Our pipeline uses data from comprehensive, frequently updated databases to support optimal primer and probe selection
- Assay panel design excludes non-targets (exclusivity) while detecting all intended target strains/isolates (inclusivity)
- A case study generating a comprehensive respiratory qPCR assay panel targeting viral pathogens illustrates successful application of this pipeline combining in silico analyses and experimental evaluation

Meeting details and poster download:
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