Development and validation of a digital PCR assay for monitoring measles, mumps, and rubella in wastewater

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Background

86% of U.S. Measles Cases Have Occurred in Unvaccinated Individuals in 2024

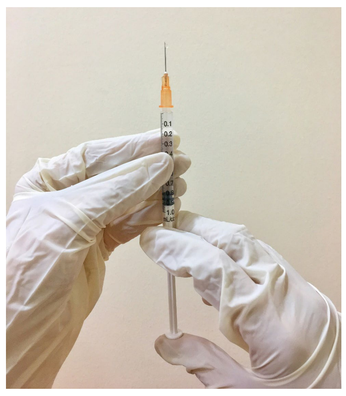
Centers for Disease Control and Prevention

Why measure vaccine preventable diseases?

Measles, mumps, and rubella (MMR) are highly contagious viruses that can cause serious health complications including deafness, encephalitis, and death among unvaccinated individuals. All three of these viruses can be prevented with the MMR vaccine.

Vaccination rates for the MMR vaccine in over 30 US states have dropped below the 95% threshold needed to create herd immunity and prevent these diseases from spreading. Decreasing vaccination rates have led to a rise in measles cases across the US. In this year alone, 13 outbreaks have been reported as of July 2024 (Centers for Disease Control and Prevention).

Wastewater-based surveillance of MMR could supplement public health surveillance of positive clinical cases to enhance outbreak detection and potentially identify high-risk areas for targeted immunization campaigns.



dPCR Assay Comparison

Methods

The performance of two MMR assays were tested on Qiagen's QIAcuity digital PCR (dPCR) platform:

- CDC Assay (designed for clinical testing)
- Houston Wastewater-Based Epidemiology Assay (designed for wastewater testing)

To compare the sensitivity of the assays, viral recovery (gene copies per liter) was measured for each Single-Plex assay by performing a dilution series of clinical specimen RNA extracts for measles, mumps, and rubella. The percent recovery was calculated by setting the repetition with the highest RNA concentration at 100% and dividing the RNA concentrations of the remaining repetitions into that value. Viral recovery and signal resolution were examined to determine the best dPCR outcome for each assay.

Results

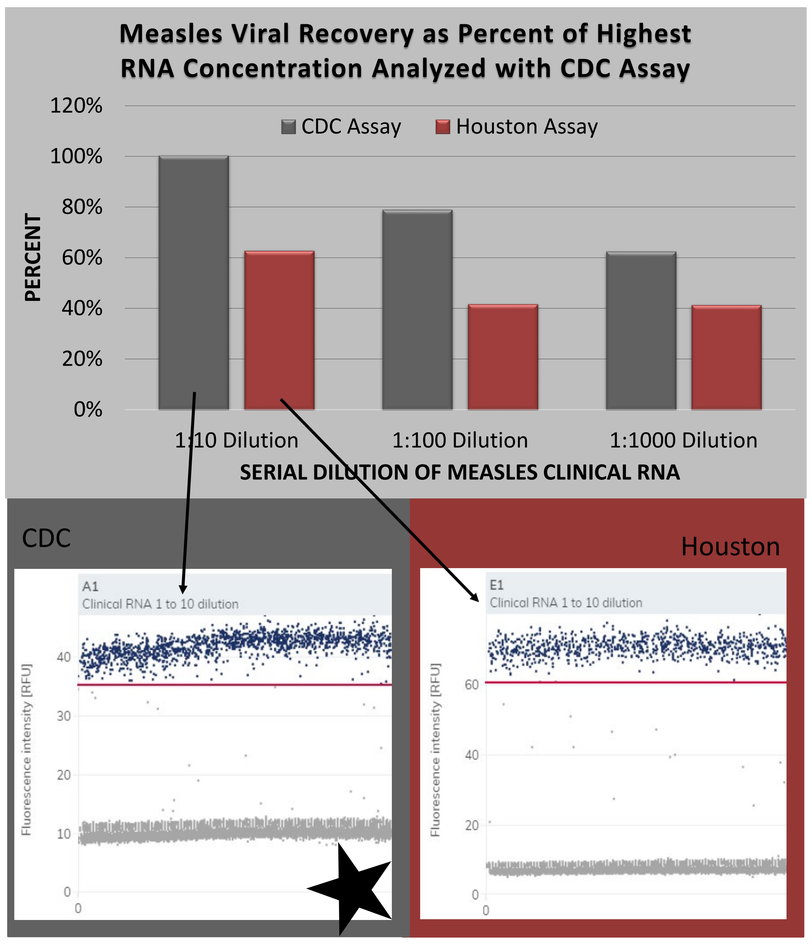
For measles, the Houston Assay produced wider separation between the positive and negative fluorescence intensity (RFU) clouds, but the CDC assay displayed much higher viral recovery. For mumps, the CDC Assay exhibited much higher viral recovery with minimal RFU separation, while the Houston Assay displayed better RFU separation but very inconsistent binding patterns. For rubella, the Houston Assay produced wider RFU separation, more consistent binding, and higher viral recovery compared to the CDC Assay.

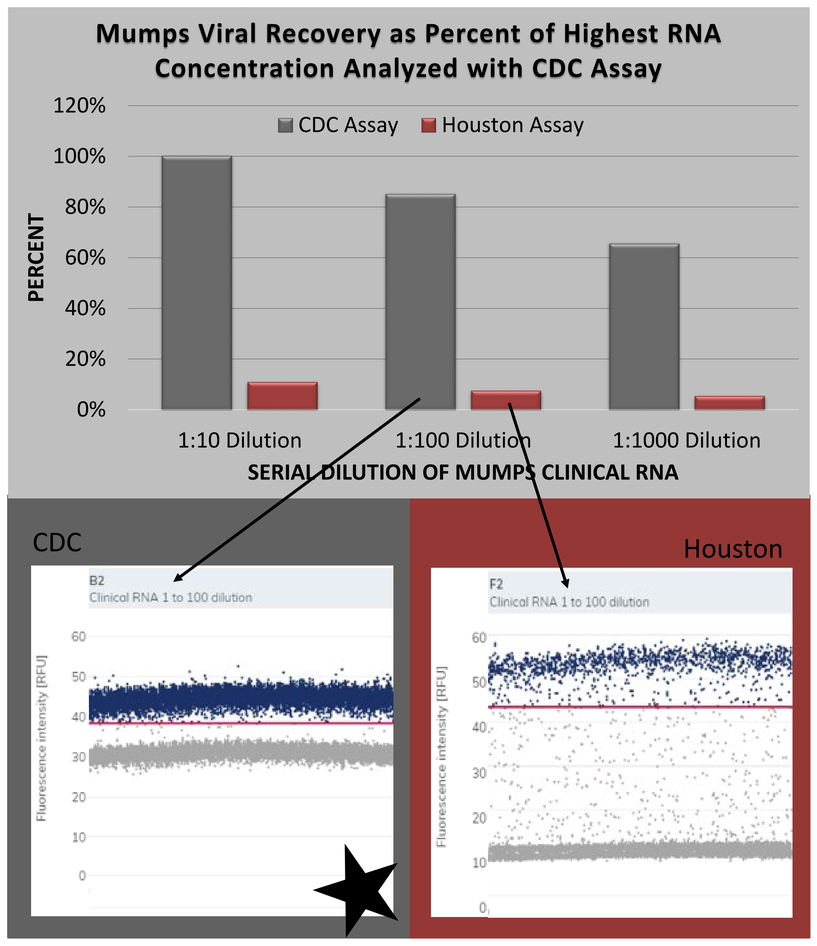
One assay was selected for each disease:

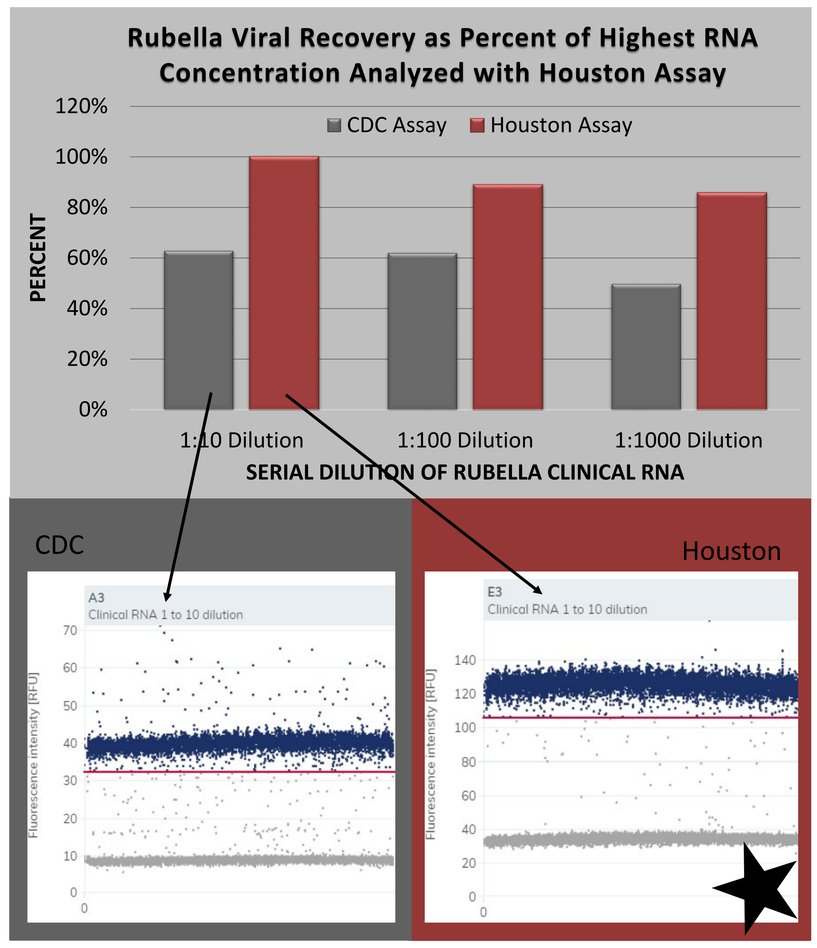
- Measles: CDC Assay
- Mumps: CDC Assay
- Rubella: Houston Assay

Denotes better dPCR outcome

Single-Plex Assay Comparison – Serial Dilution of Clinical RNA Extracts







The 1:100 Dilution is pictured above because the 1:10 Dilution for the CDC Assay was too saturated to make an accurate comparison.

Concentration Comparison

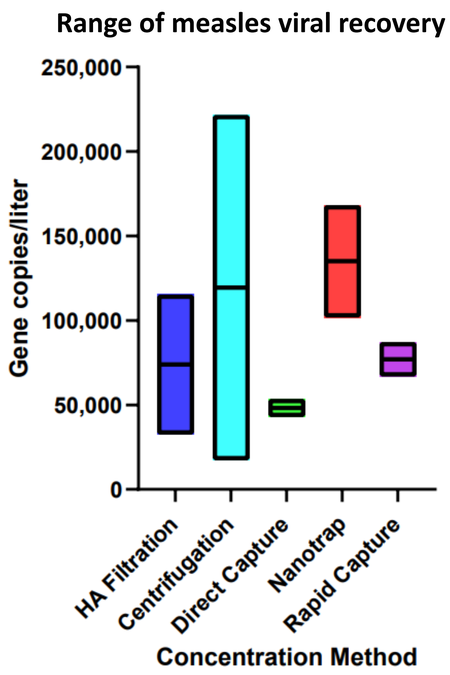
Methods

To select the most robust workflow, measles positive wastewater from a known outbreak was used to test five different viral concentration methods including

- HA Filtration
- Centrifugation
- Promega Direct Capture
- Ceres Nanotrap® Microbiome A Particles
- Promega Rapid Capture

Results

- HA Filtration and Centrifugation exhibited high variability.
- Promega Direct Capture and Promega Rapid Capture exhibited lower variability between repetitions but also lower viral recovery.
- Ceres Nanotrap® Microbiome A Particles displayed high viral recovery with moderate variability between repetitions.
- The optimal concentration method selected was Ceres Nanotrap® Microbiome A Particles.



Each bar represents the viral RNA concentration of two replicates for each method. The bar represents the data range and the middle line displays the average of the two repetitions. All five methods were quantified with dPCR CDC Assay for measles.

Assay Optimization

Temperature Gradients

- Temperature gradient experiments were performed to select the optimal PCR cycling conditions for each assay when using Qiagen's QIAcuity dPCR instrument.
- The temperature of the annealing and extending step of dPCR was evaluated at 60.7°C, 59.6°C, 58.5°C, 57.5°C, 56.4°C, 55.3°C, and 54.2°C for all six Single-Plex assays.
- All of the assays performed well across all the temperatures and exhibited slightly higher viral recovery at the lower temperatures. (Data not shown.)
- A temperature of 58°C was selected for the final assay to limit the chance of non-specific binding that can occur at lower annealing temperatures.

MMR Tri-Plex Cross-reactivity

- When using primer concentrations at 400nM and probe concentrations at 200nM, the measles CDC Assay and mumps CDC Assay produced strong results, but the rubella Houston Assay viral recovery decreased significantly compared to the Single-Plex Assay results.
- To determine the optimal performance of the MMR Tri-Plex Assay, the concentration of the rubella Houston Assay oligonucleotides were tested in five different variations. The results of the following Tri-Plex variations were compared against the rubella Single-Plex Houston Assay.
  - MMR 1: 400nM primers and 200nM probes
  - MMR 2: 900nM primers and 200nM probes
  - MMR 3: 900nM primers and 400nM probes
  - MMR 4: 900nM primers and 100nM probes
  - MMR 5: 1200nM primers and 200nM probes
- MMR 1 exhibited lower viral recovery than the Single-Plex rubella Houston Assay, and MMR 3 generated much higher variability compared to the other Tri-Plex variations. (Data not shown.)
- A second experiment was conducted to further examine the performance of MMR 2, MMR 4, and MMR 5. The rubella viral recovery for each Tri-Plex variation was compared to the rubella viral recovery of the Single-Plex Houston Assay by calculating the average percent difference of five replicates. MMR 2 displayed lower viral recovery than MMR 4 and MMR 5, and the average viral recovery of MMR 4 and MMR 5 was very similar. MMR 5 had slightly better positive and negative RFU separation compared to MMR 4. (Data not shown.)
- Due to the similar results produced by MMR 4 and MMR 5, MMR 4 was selected to save materials by using lower primer and probe concentrations.

Final Oligonucleotide Concentrations

- Measles and Mumps Primers – 400nM
- Measles and Mumps Probes – 200nM
- Rubella Primers – 900nM
- Rubella Probes – 100nM

PCR Cycling Conditions:			
Step		Time	Temp ºC
Reverse Transcription		40 min	50
DNA polymerase activation		2 min	95
40 cycles	Denaturation	15 sec	95
	Anneal/Extend	1 min	58

CONCLUSIONS & NEXT STEPS

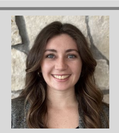
Conclusions

- The measles CDC Assay, mumps CDC Assay, and rubella Houston Assay exhibited the best results on Qiagen's QIAcuity dPCR platform. The MMR Tri-Plex assay produces optimal results at an annealing/extending temperature of 58°C and when using oligonucleotide concentrations of 400nM for measles and mumps primers, 900nM for rubella primers, 200nM for measles and mumps probes, and 100nM for rubella probes.
- The Ceres Nanotrap® Microbiome A Particles were the most robust concentration method for the MMR Tri-Plex assay workflow.

Future Experiments

- Testing to determine the level of blank (LOB), level of detection (LOD), and level of quantification (LOQ) of the final MMR Tri-Plex assay is underway.
- To verify the most robust workflow was selected, further repetitions of the concentration comparison with more replicates will occur after development of the final MMR Tri-Plex assay is complete.

Contact



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