The Quest for Clean Clusters: Probe Chemistry & Optimisation for Digital PCR

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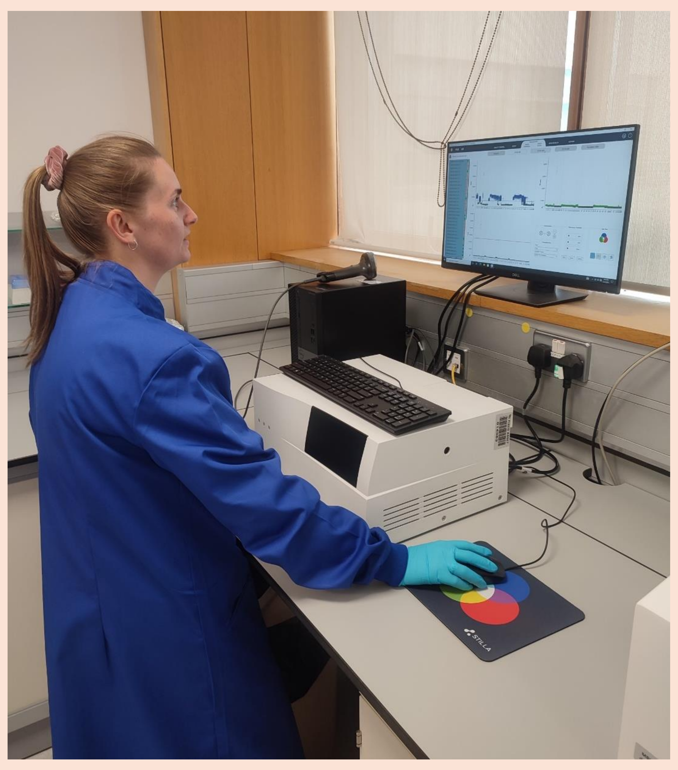
Background

Droplet digital PCR (ddPCR) provides absolute target quantification, improved sensitivity and enhanced multiplexing potential – making it a strong candidate for multi-pathogen detection in finfish diagnostics. However, successful transfer of real-time PCR assays to ddPCR depends heavily on probe chemistry, dye compatibility and additional optimisation.

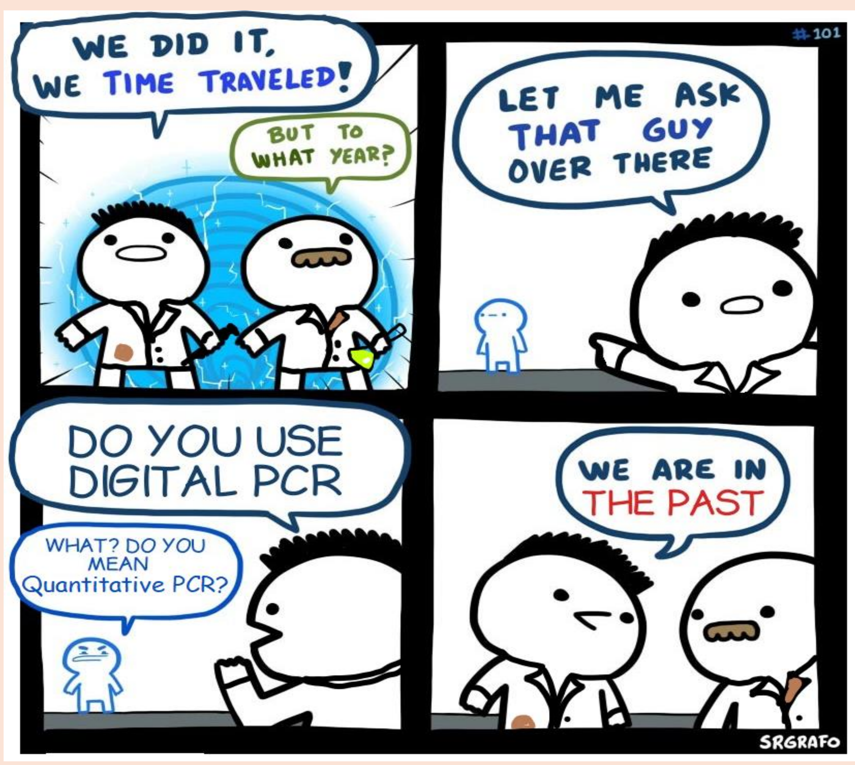


Objective

To transfer existing Taqman® real-time PCR assays to ddPCR by developing a robust multiplex ddPCR assay for relevant salmonid aquaculture viruses, and evaluate probe chemistries (MGB, QSY, RBO) to determine suitability for routine diagnostics.

Methodology

- Transfer and optimise singleplex assays to the Stilla Naica Prism 3 ddPCR machine.
- Assessment of probe chemistries:
  1). MGB (TaqMan®)
  2). QSY (TaqMan®)
  3). RBO (Universal Nucleic Acid Detection, Pxlence)
- Evaluate cluster separation, fluorescence intensity, relative uncertainty and multiplex performance.
- Initial experiments were carried out using manufacturers recommendations, then further annealing temperature and probe concentration were refined.



Results

Probe Chemistries

MGB Probes

- Strongest fluorescence (all channels).
- Clear positive/negative clusters.
- Reliable performance in 3-plex (Figure 1).
- MGB selected as primary chemistry for multiplex assay development.

QSY Probes

- Lower florescence intensity.
- Reduced cluster separation.
- Major optimisation needed for multiplexing.

RBO Probes

- Lower signal among all chemistries
- False positives observed in negative matrix.
- Poor separation in blue channel (Figure 2).

Examples of Multiplexing on ddPCR

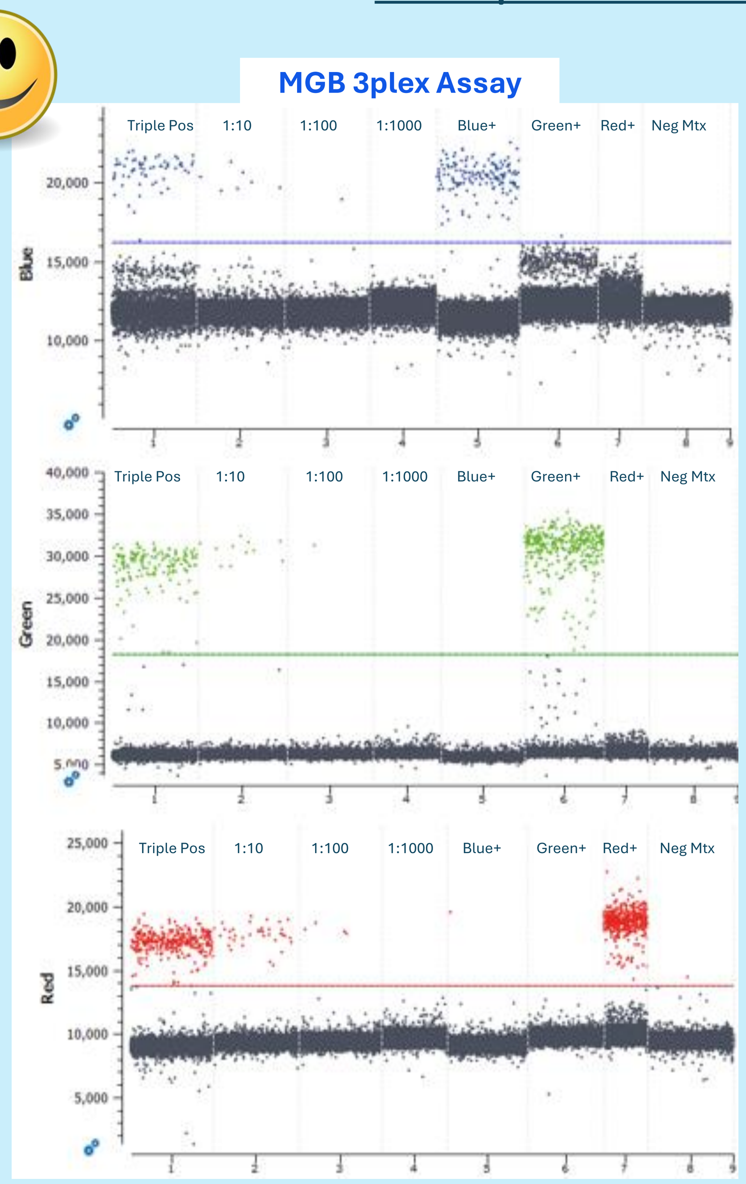


Figure 1. 1D droplet plots before optimisation, illustrating reliability to perform in a triplex reaction and clear positive-negative clusters.

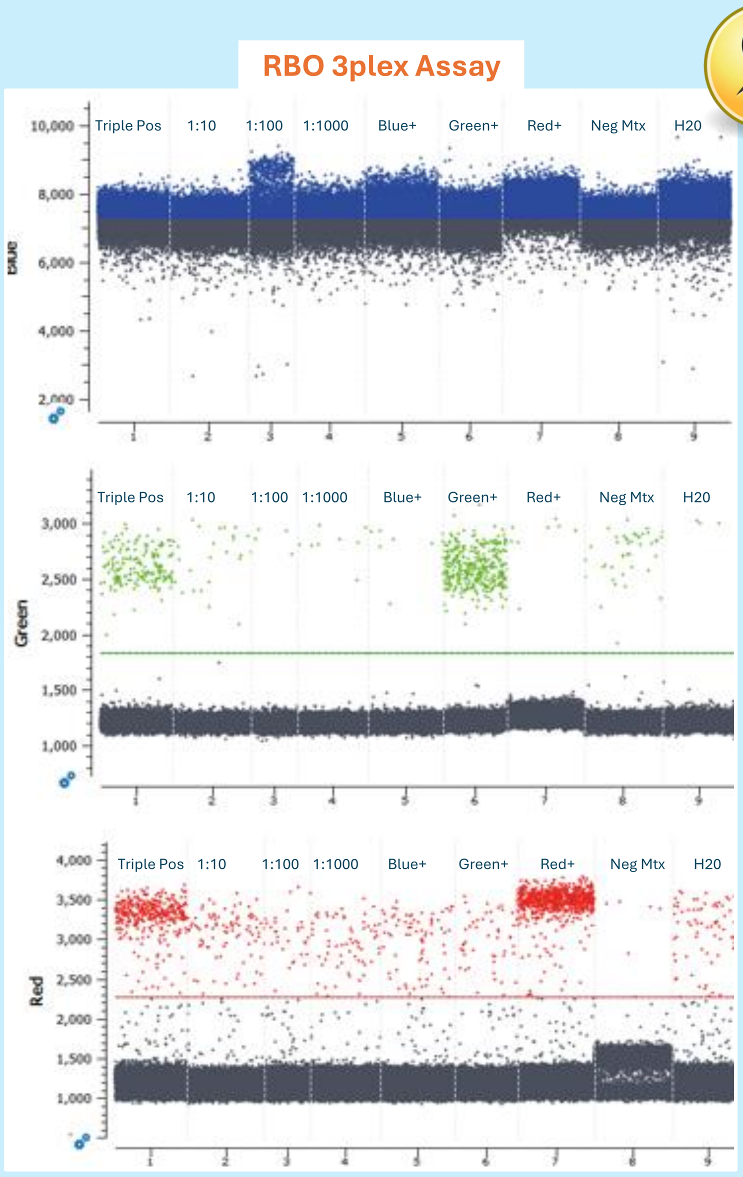


Figure 2. 1D droplet plots of the RBO triplex assay demonstrating limited fluorescence amplitude and inconsistent cluster distinction.

Probe Concentration Optimisation

- Each probe was tested at 100, 150, 200 & 250nM (ending at the optimum values).
- Optimal probe was selected using target spiked matrix with tight positive/negative clusters and clear separation, reduced rain, highest separability score, lowest relative uncertainty and the most economical probe concentration.
- The optimum probe concentration combination selected was Blue @ 150nM, Green @ 100nM, Red @ 200nM shown in 1D and 2D plots (Figure 3 & 4).

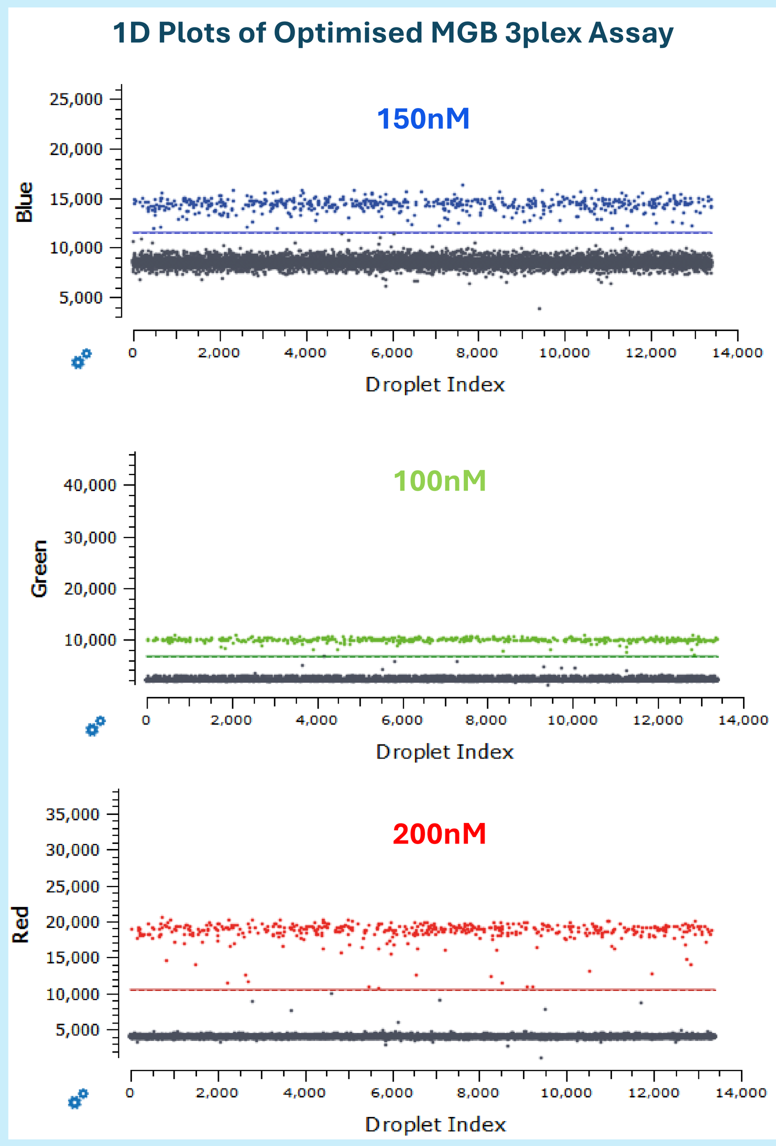


Figure 3. 1D droplet plots displaying tighter positive-negative clusters with stronger separation in blue, green and red channels using the most economical probe concentration after optimisation of the triplex ddPCR assay.

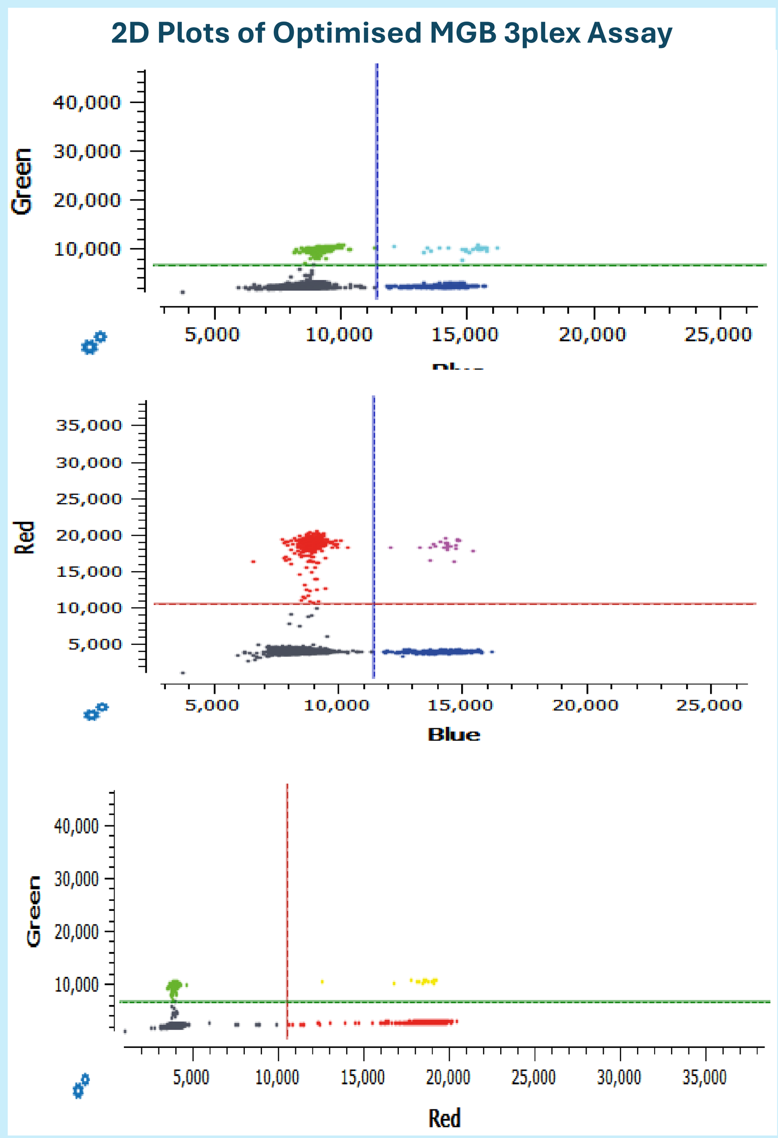


Figure 4. 2D fluorescence amplitude plots of the optimised MGB triplex ddPCR assay. Each panel displays droplet separation across two channels, showing clear negative, single-positive and multi-positive clusters.

Cluster Colour Coding:

Blue +	Green +	Red +	Negative -
Blue-Green +	Blue-Red +	Green-Red +	Triple +

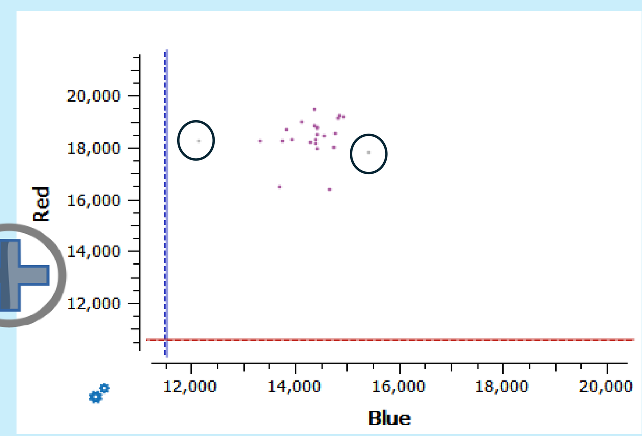


Figure 5. in the 2D plot are circled regions highlighting the triple-positive droplet cluster, confirming clear multi-channel discrimination in the optimised triplex assay.

Key Findings

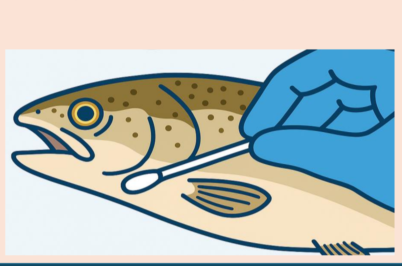
- Probe chemistry = main determinant for successful ddPCR transfer.
- MGB probes produced the cleanest cluster separation.
- QSY & RBO showed reduced signal and would require major optimisation to multiplex.
- ddPCR transfer requires re-optimisation and not simple one-to-one transfer of real-time PCR conditions.

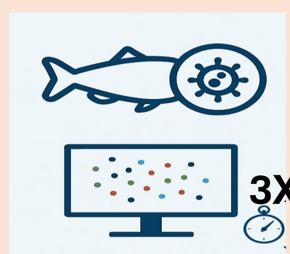
Next Steps...

1. Establish sensitivity of assay: Limit of Blank (LOB), Limit of Detection (LOD) & Limit of Quantification (LOQ) for each target.
2. Evaluate specificity, reproducibility and repeatability of assay.
3. Validate detection in gill swabs as an early detection method.

Impact for Fish Health & Aquaculture Industry:

- Faster detection of common finfish viruses, even at low levels.
- Absolute quantification to support disease monitoring.
- Non-lethal sampling for virus detection reducing unnecessary stock loss.
- Improved laboratory efficiency as multiple pathogens detected in one test.





Images generated with Microsoft Copilot.

Long-Term Goal:

To produce a robust, validated, multiplex ddPCR assay for the detection and quantification of relevant finfish viruses in salmonid aquaculture, enabling improved and efficient pathogen surveillance.

References

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