Application Note

Clara® AquaPlex Mixes: Overcoming spectral limitations and improving detection in multiplexed qPCR & RT-qPCR assays

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Introduction

Multiplex qPCR is a commonly used technique to identify multiple targets in one go (using spectrally distinct fluorophores). However, this can sometimes be limited by the selected probe dyes and instrument limitations. This application note describes a method to improve detection in multiplex qPCR using Clara® AquaPlex qPCR mixes.

One of the most common probe dyes in multiplex reactions is the Cyanine 5 dye (Cy5). It is a far-red-fluorescent dye with excitation/emission maxima at 635/665 nm. Cy5 is spectrally distinct from other common probe fluorophores, such as FAM or HEX dyes. However, probes labelled with the Cy5 dye will often display a lower fluorescence plateau compared to other dye-target combinations, which in turn is associated with a delay in target amplification. Although the precise mechanism underlying this effect is not well described, it is likely due to the lower quantum yield of Cy5[1] and fluorophores used in the same channel (*i.e.*, with similar excitation and emission peak) compared to other commonly used dyes.

It is possible to circumvent this issue by choosing different fluorophores, other than Cy5. A problem with this approach, however, is the limited number of detection channels available on many real time instruments. This is further limited by the fact that many instruments require the use of a passive reference dye to normalise the signal, which further reduces the number of available channels for probe-fluorophore detection.

Traditionally, ROX (6-carboxy-X-rhodamine) has been employed as a passive reference dye and is the most common option available for this purpose. With excitation/emission maxima of 578/604 nm, ROX occupies a detection channel that is suitable for use with other commonly available probe dyes, for instance, Texas Red (596/615 nm) and CalRed 610 (590/610 nm).

Clara® AquaPlex mixes do not contain ROX, but instead utilise a passive reference dye with excitation and emission maxima in the channel used for Cy5. Using these mixes presents an opportunity to refine qPCR assays, by occupying the Cy5 detection channel for passive reference data collection, while freeing up the ROX channel for use with other dyes which do not have a negative impact on target detection and fluorescence intensity.

In this application note, we demonstrate the clear benefits of using a passive reference dye in the Cy5 channel when running a multiplex qPCR. To investigate the impact of replacing Cy5-labelled probes with Texas Red-labelled probes in multiplex detection, we compared the performance of Clara® Probe 1-Step Mix No-ROX with Clara® Probe 1-Step Mix AquaPlex in various multiplex qPCR reactions on DNA and RNA samples.

Materials & Methods

Primer and template preparation

Primer sets including the oligonucleotides for 3 targets were prepared by mixing 400 µL of 10 µM primers (F + R) to 200 µL of 10 µM probes so that the

Name	Dye-Sequence	Concentration	Target
E_Sarbeco_F	ACA GGT ACG TTA ATA GTT AAT AGC GT	400 nM	SARS-CoV-2 E-Gene
E_Sarbeco_R	ATA TTG CAG CAG TAC GCA CAC A	400 nM	
E_Sarbeco_P1	**FAM**-ACA CTA GCC ATC CTT ACT GCG CTT CG-**BHQ1**	200 nM	
InfA-F	GAC CRA TCC TGT CAC CTC TGA C	400 nM	Influenza A
InfA-R	AGG GCA TTY TGG ACA AAK CGT CTA	400 nM	
InfA-P	**HEX**-TGC AGT CCT CGC TCA CTG GGC ACG-**BHQ1**	200 nM	
GAPDH-F	TTC ACC ACC ATG GAG AAG GC	400 nM	GAPDH
GAPDH-R	GGC ATG GAC TGT GGT CAT GA	400 nM	
GAPDH-P(Tex)	**Tex615**-TGC ATC CTG CAC CAC CAA CTG CTT AG-**BHQ2**	200 nM	
GAPDH-P(Cy5)	**Cy5**-TGC ATC CTG CAC CAC CAA CTG CTT AG-**BHQ2**	200 nM	
B2M-F	GGT CTT TCT GGT GCT TGT CT	400 nM	β-microtubulin
B2M-R	TAT GTT CGG CTT CCC ATT CTC	400 nM	
B2M-P(Tex)	**Tex615**-ACC GGC CTG TAT GCT ATC CAG AAA-**BHQ2**	200 nM	
B2M-P(Cy5)	**Cy5**-ACC GGC CTG TAT GCT ATC CAG AAA-**BHQ2**	200 nM	

Table 1: Primers, probes and targets used

final concentration of each primer in the reaction was 400 nM and of each probe was 200 nM. The following 4 sets of primers and probes were prepared:

- Sars-CoV-2 E Gene (FAM), Influenza A (HEX), mouse GAPDH (Cy5)
- Sars-CoV-2 E Gene (FAM), Influenza A (HEX), mouse GAPDH (Texas Red)
- Sars-CoV-2 E Gene (FAM), Influenza A (HEX), mouse B2M (Cy5)
- Sars-CoV-2 E Gene (FAM), Influenza A (HEX), mouse B2M (Texas Red)

Template dilutions were used at 20,000, 4,000, 400, 40, and 4 copies of each target. No-template controls were also included. Three technical replicates were run for each sample.

Reaction setup

Reactions were set up using a Qiagen QIAgility robot in a final volume of 20 µL to include: 8.8 µL Milli-Q water, 5 µL 4x Clara® Probe Mix No-ROX or 4x Clara® Probe Mix AquaPlex, 1.2 µL of each primers/probes mix (described above) and 5 µL of sample (Table 2).

Cycling conditions

Amplification was carried out on a Bio-Rad CFX96 Touch qPCR machine as follows: 45 ˚C 20 min (reverse transcription, ommited for DNA targets), 95 ˚C 2 min (initial denaturation), followed by 50 2-step cycles at 95 ˚C 15 s (denaturation) and 60 ˚C 30 s (annealing-extension). Fluorescence measurements were acquired at the end of each cycle.

Reagent	Volume	Final conc.
Clara® Standard/AquaPlex Mix	5 µL	1x
Primer mix (10 µM)	0.8 µL	400 nM
Probe (10 µM)	0.4 µL	200 nM
RNA/cDNA template	5 µL	Variable
PCR grade dH_2O	8.8 µL	–

Table 2: Reaction setup and composition

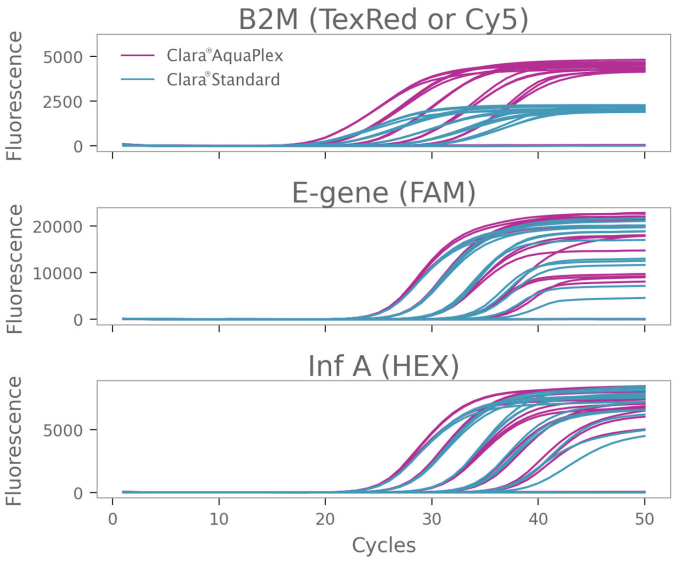

Figure 1. Improved amplification of RNA targets with Clara® Probe 1-Step Mix AquaPlex

Triplex amplification of the housekeeping gene B2M (β-microtubulin), SARS-CoV-2 E-Gene, and Influenza A. Amplification was carried out with Clara® Probe 1-Step Mix AquaPlex and with Clara® Probe 1-Step Mix No-ROX. Probes for B2M detection were labelled with Texas Red when using the Clara® AquaPlex Mix and with Cy5 when using the Clara® Standard Mix. Probe dyes for the E-Gene and Inf A remained the same. Five serial dilutions of total RNA template were used. The total reaction volume was 20 µL. Cycle conditions were 45 ˚C 20 min, 95 ˚C 2 min and 50 cycles of 95 ˚C 15 s, 60 ˚C 30 s. B2M amplification was improved when using a Texas Red probe in the Clara® AquaPlex Mix.

Results

Two different sets of three targets were amplified from RNA templates using Clara® Probe 1-Step Mix No-ROX and Clara® Probe 1-Step Mix AquaPlex. The same sets of three targets were amplified from cDNA templates in separate triplex qPCR reactions with Clara® Probe Mix No-ROX and Clara® Probe Mix AquaPlex (Table 1). The first set of RNA targets included β-microtubulin (B2M), the SARS-CoV-2 E-Gene (E-Gene), and Influenza A (Inf A), the second set comprised glyceraldehyde phosphate dehydrogenase (GAPDH), along with E-Gene and Inf A.

The E-gene and Inf-A targets were detected with probes labelled with FAM and HEX fluorophores, respectively, regardless of whether amplification was conducted using a standard Clara® Probe (1-Step) Mix (Clara® Standard) or Clara® Probe (1-Step) Mix AquaPlex (Clara® AquaPlex). B2M and GAPDH were detected with Cy5-labeled probes when using Clara® Standard mixes or with Texas Red-labelled probes when using Clara® AquaPlex. This was the case both with RNA and with cDNA templates. Importantly, the sequences of the probes for GAPDH and B2M were not altered and only the fluorophores were changed.

When amplifying the first target set from RNA templates all dilutions gave acceptable amplification

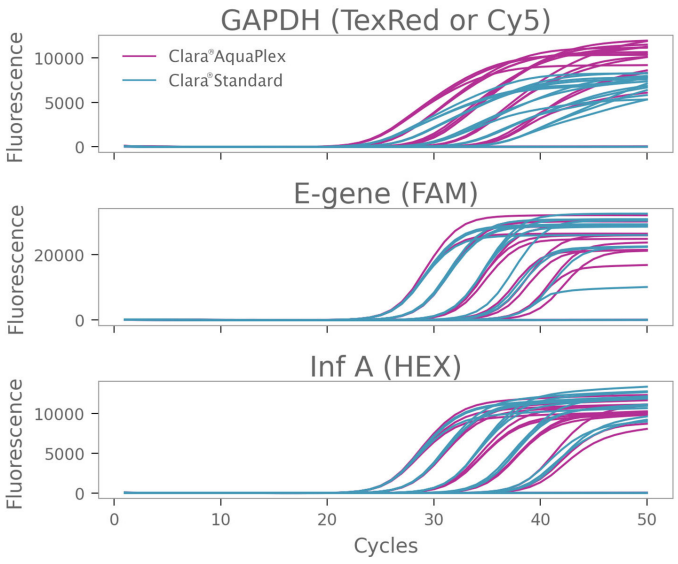

Figure 2. Improved amplification of RNA targets with Clara® Probe 1-Step Mix AquaPlex

Triplex amplification of the housekeeping gene GAPDH, SARS-CoV-2 E-Gene, and Influenza A. Amplification was carried out with Clara® Probe 1-Step Mix AquaPlex and with Clara® Probe 1-Step Mix No-ROX. Probes for GAPDH detection were labelled with Texas Red when using the Clara® AquaPlex Mix and with Cy5 when using the Clara® Standard Mix. Probe dyes for the E-Gene and Inf A remained the same. Five serial dilutions of total RNA template were used. The total reaction volume was 20 µL. Cycle conditions were 45 ˚C 20 min, 95 ˚C 2 min and 50 cycles of 95 ˚C 15 s, 60 ˚C 30 s. GAPDH amplification was improved when using a Texas Red probe in the Clara® AquaPlex Mix.

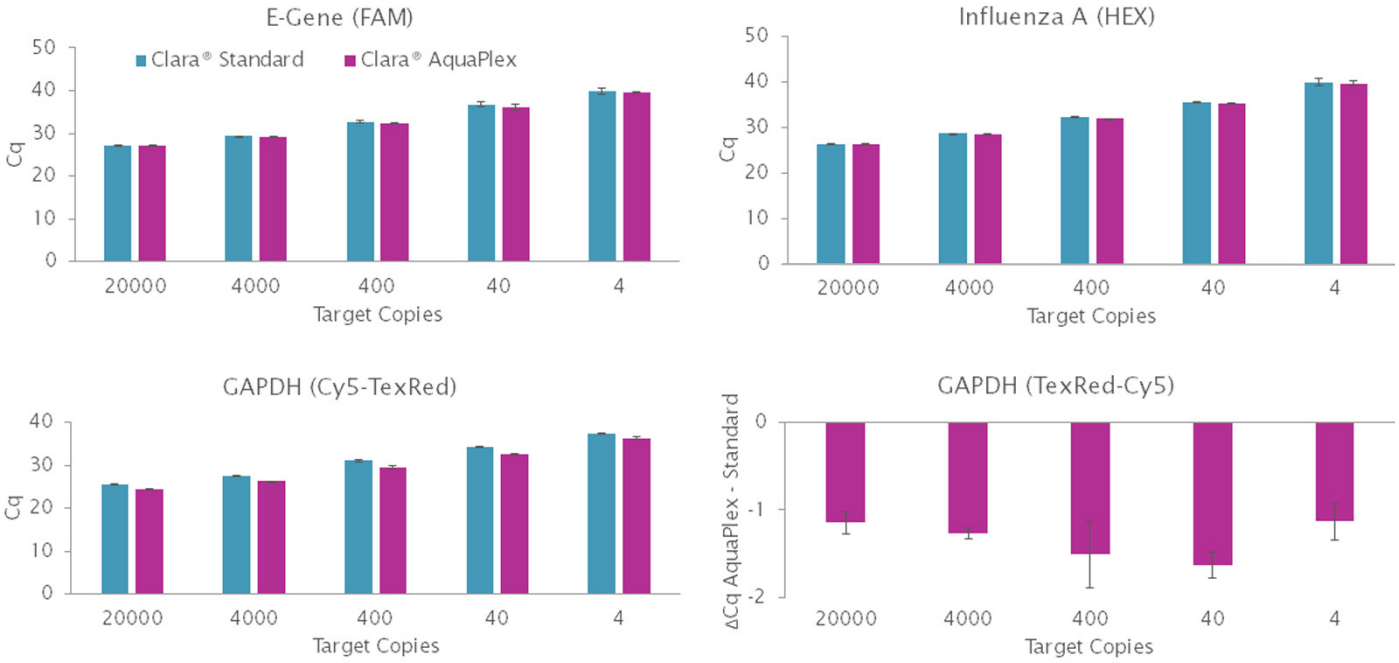

Figure 3. Clara® Probe 1-Step Mix AquaPlex improves target detection with ROX-channel probe dyes.

Cq values extracted from amplification plots shown in Figure 1 plotted against target copy number of B2M (β-microtubulin), SARS-CoV-2 E-Gene, and Influenza A. The difference in Cq values for B2M detection with the Texas Red-labelled probe (Clara® AquaPlex) minus the Cy5-labeled probe (Clara® Standard) are shown in the lower right graph. Using the Texas-Red probe in Clara® AquaPlex led to earlier detection, by more than 1 cycle, at all target concentrations.

curves. E-Gene and Inf-A average Cqs were practically identical across all template dilutions with a comparable efficiency, regardless of which mix was used for amplification. However, B2M showed markedly improved amplification and much stronger fluorescence in the amplification plateau phase when using the Texas Red-labelled probe in the Clara® AquaPlex mix, compared to the Cy5-labelled probe in the Clara® Standard mix. (Figure 1).

A similar effect was observed when amplifying the second target set. With GAPDH showing lower Cqs and higher fluorescence when using the Texas Red-labelled probe in the Clara® AquaPlex compared to the Cy5-labelled probe in the Clara® Standard mix. E-Gene and Inf-A detection was unchanged, regardless of the mix used for amplification (Figure 2).

Unsurprisingly, when comparing normalised fluorescence intensities between the Texas Red and Cy5 signals, they revealed that the improvement in Cq detection was due to the higher fluorescence of the Texas-Red dye when detecting B2M. However, in the case of GAPDH, the Cq values were also lower with the Texas-Red dye, suggesting that the probe-mediated detection of targets may be improved using a dye other than Cy5 in some instances, although this happens in a target-specific manner (not shown).

This improvement in target detection is also evident when comparing average Cqs for each target at each dilution. Comparing average Cqs for the first target set showed that amplification of E-gene and Inf-A resulted in a Cq difference (delta Cq) of less than 0.5 when using Clara® AquaPlex and Clara® Standard mixes, while this same difference in Cq was over 1 at all target dilutions for GAPDH amplification (Figure 3).

Practically the same results were also obtained when amplifying both target sets from cDNA template. GAPDH showed greatly improved amplification (Figure 4), while B2M amplification was marginally better (Figure 5) when these targets were amplified with Clara® AquaPlex and detected with Texas Red-labelled probes, compared to amplification with the Clara® Standard mix and detected with Cy5-conjugated probes. The E-gene and Inf-A showed similar amplification patterns regardless of which mix was used (Figures 4 and 5).

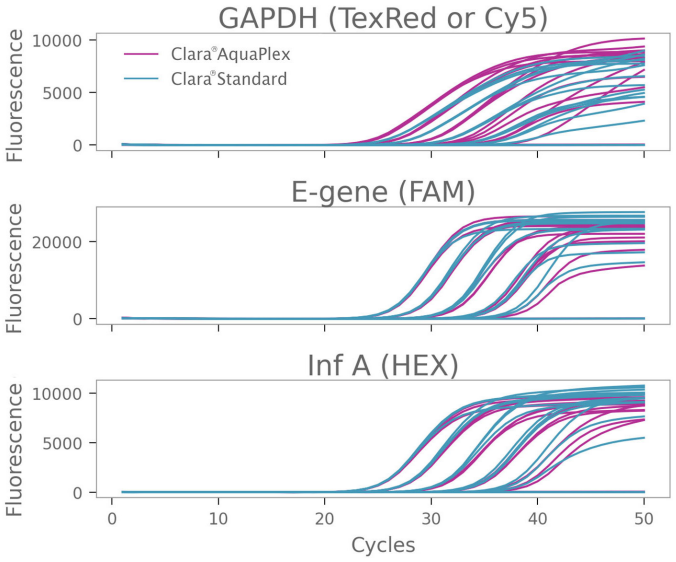

Figure 4: Improved amplification of DNA targets with Clara® Probe Mix AquaPlex

Triplex amplification of the housekeeping gene GAPDH, SARS-CoV-2 E-Gene, and Influenza A. Amplification was carried out with Clara® Probe Mix AquaPlex and with Clara® Probe Mix No-ROX. Probes for GAPDH detection were labelled with Texas Red when using the Clara® AquaPlex Mix and with Cy5 when using the Clara® Standard Mix. Probe dyes for the E-Gene and Inf A remained the same. Five serial dilutions of total RNA template were used. The total reaction volume was 20 µL. Cycle conditions were 95 ˚C 2 min and 50 cycles of 95 ˚C 15 s, 60 ˚C 30 s. GAPDH amplification was improved when using a Texas Red probe in the Clara® AquaPlex Mix.

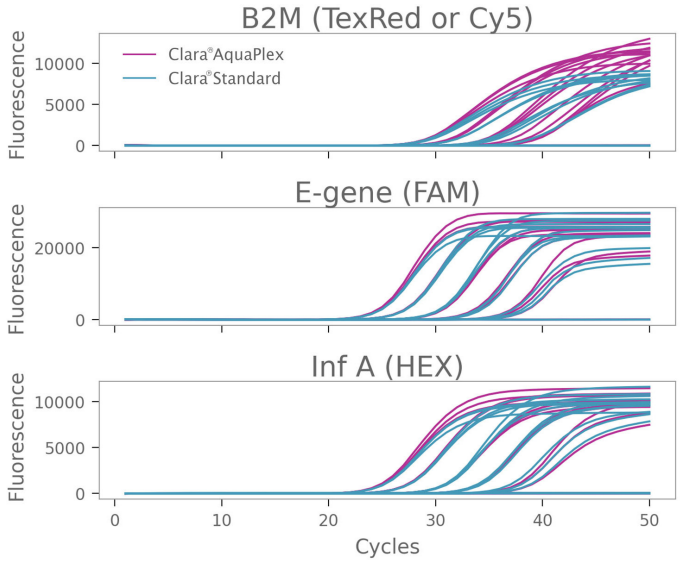

Figure 5: Improved amplification of DNA targets with Clara® Probe Mix AquaPlex

Triplex amplification of the housekeeping gene B2M (β-microtubulin), SARS-CoV-2 E-Gene, and Influenza A. Amplification was carried out with Clara® Probe Mix AquaPlex and with Clara® Probe Mix No-ROX. Probes for B2M detection were labelled with Texas Red when using the Clara® AquaPlex Mix and with Cy5 when using the Clara® Standard Mix. Probe dyes for the E-Gene and Inf A remained the same. Five serial dilutions of total RNA template were used. The total reaction volume was 20 µL. Cycle conditions were 95 ˚C 2 min and 50 cycles of 95 ˚C 15 s, 60 ˚C 30 s. B2M amplification was improved when using a Texas Red probe in the Clara® AquaPlex Mix.

Discussion

These results provide compelling evidence for the enhanced performance of multiplex qPCR assays when substituting Cy5- with Texas Red (or similar dye)-labelled probes. This substitution is facilitated by using Clara® AquaPlex mixes instead of ROX as a passive reference and leads to significant improvement in the detection sensitivity and fluorescence intensity of targets like B2M and GAPDH.

One of the key findings is the consistent improvement observed in the Cq values and fluorescence plateaus for targets detected with Texas Red-labelled probes in the AquaPlex mix. Most of this improvement is due to the higher fluorescent levels (due to the higher quantum yield versus that of Cy5). However, other mechanisms (such as probe stability or interaction with other reaction components) may be involved, and being different when Cy5-labelled probes are used.

The use of Clara® AquaPlex mixes allows for the reallocation of the ROX channel to probes conjugated with fluorophores with the same excitation/emissions (Texas Red, CalRed610, ROX itself) which do not exhibit the same detection issues as Cy5. This reconfiguration significantly enhances the detection performance of multiplexed qPCR assays, addressing one of the major limitations of using Cy5 in qPCR.

Moreover, the performance benefits were consistent across both RNA and cDNA templates, indicating that the advantages of using Clara® AquaPlex mixes are robust and applicable across different template types. This consistency is crucial for clinical diagnostics and research applications where reliability and sensitivity are paramount. Another advantage of Clara® AquaPlex mixes is that unlike for the ROX versions, there is no requirement for different concentrations for use with different instruments.

Conclusion

In conclusion, Clara® Probe and Probe 1-Step AquaPlex Mixes demonstrated a significant improvement in multiplex qPCR and 1-step RT-qPCR assays by mitigating the challenges associated with Cy5-labelled probes. The substitution of Texas Red- for Cy5-labelled probes, enabled by using Clara® AquaPlex mixes, resulted in higher fluorescence intensity and (in a target-dependent manner) in better target amplification, and ultimately in improved sensitivity and reliability of detection. This offers a direct solution to the issue or poorer detection with Cy5 probe dyes.

The transition to Clara® AquaPlex mixes offers a practical solution for overcoming the spectral limitations of current qPCR setups, thereby potentially reducing the need for expensive upgrades to equipment with additional detection channels.

Implementation Considerations

Not all instruments enable alteration of passive reference dye settings. Users are encouraged to refer to their instrument product manual or manufacturer to ensure that this is possible. Suitable passive reference dyes may be labelled as “Cy5” or “Mustang Purple” on compatible instruments. Our online selection tool indicates some of the most common instruments in circulation with which Clara® Probe Mix and AquaPlex Mixes are compatible.

References

1. R. B. Mujumdar 1, L. A. Ernst, S. R. Mujumdar, C. J. Lewis, A. S. Waggoner, Cyanine dye labelling reagents: sulfoindocyanine succinimidyl esters, 1993 Mar-Apr;4(2):105-11. doi: 10.1021/bc00020a001.