Rainbow™

universal nucleic acid detection probes for digital PCR

instructions for use

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Revision history

version	date	description
v1.0	June 26, 2023	• initial release
v1.1	Sept 01, 2023	• Rainbow primer and probe nomenclature added • updated results section
v1.2	Nov 07, 2023	• updated results section • added recommended thermocycling conditions • probe nmol equivalents added to number of reactions • extended FAQ section
v1.3	March 19, 2024	• updated results section • updated dPCR instrument compatibilities • recommendation which primer (forward or reverse) to convert to Rainbow primer

Overview Rainbow™ technology

Introduction

Rainbow™ primers and probes allow universal detection of nucleic acids using digital PCR. The technology is patent pending.

As a user, you no longer need to design and optimize a target-specific probe. To enable universal detection of a DNA target, we modify one of the oligonucleotides of an existing PCR primer pair (converting it into a Rainbow™ primer) so that it can be recognized by a Rainbow™ probe during amplicon generation.

Once you have Rainbow™ probes that are compatible with the fluorescent detection channels of your digital PCR instrument, you can quantify any target in a singleplex or multiplex assay.

Rainbow™ probes are ideally suited for applications that require many different probes (e.g. multiplexing or higher-order multiplexing) or applications that only need small amounts of probes (e.g. pilot experiments or patient-specific assays).

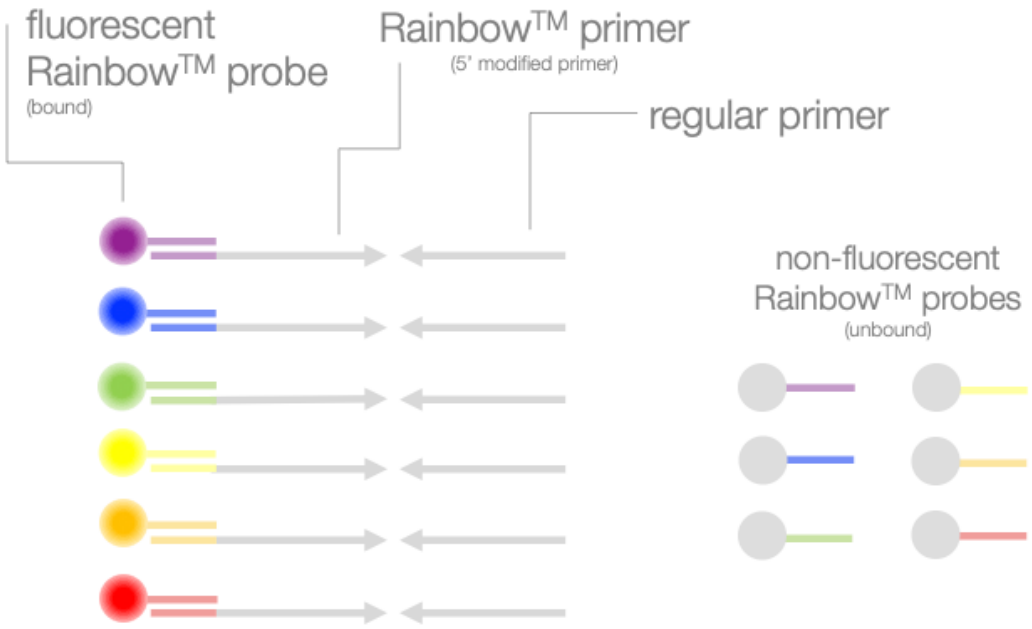


Figure 1: Principle of universal DNA detection using Rainbow™ technology. When unbound, the Rainbow™ probe is quenched. When bound to the amplicon generated by the Rainbow™ primer, the probe fluoresces.

Probe and primer order workflow

1. Go to www.pxlence.com and select 'Rainbow probes' from the Products menu bar.
2. To order a Rainbow™ probe,
   a. select the fluorophore (FAM (default), Yakima Yellow, HEX, ATTO425, ATTO550, ATTO590, Texas Red, ROX, Cy5, Cy5.5, or ATTO700)
   b. select the number of reactions* (100 (default), 500, or 2500; equivalent to 0.25, 1.25, or 6.25 nmol)
   c. adjust the number of times you want to order this item (default is 1)
   d. click 'Add to cart' button (see Figure 2)
3. Repeat step 2 if you want to order additional probes.
4. To order a Rainbow™ primer,
   a. select the channel in which you want to detect this amplicon (use
   b. Table 1 below)

      For example: use purple' *for a Rainbow™ primer that should be detected with a FAM labeled Rainbow™ probe; or use 'blue' for a Rainbow™ primer that should be detected with a HEX labeled Rainbow™ probe.*
   c. paste the primer sequence (5'-3', only IUPAC nucleotides, no other characters and up to 30 nt long)
   d. give it a name (maximum 24 characters, only letters, numbers, and special characters - _ :)
   e. adjust the number of times you want to order this item (default is 1)
   f. click 'Add to cart' button.

      Note 1: At least 10 nmol of Rainbow™ primer is delivered (> 10 000 reactions)

      Note 2: Either the forward (sense or + strand) or reverse (antisense or – strand) primer (from a classic primer pair) can be modified as a Rainbow primer. We recommend using the primer with the lowest Tm.
5. Repeat step 4 if you want to order additional Rainbow primers.
6. Go to 'My cart' in the top menu and click on 'Go to checkout' button.

* Reactions are assumed 20 µL volume with 125 nm Rainbow™ probe and 100 nM Rainbow™ primer.

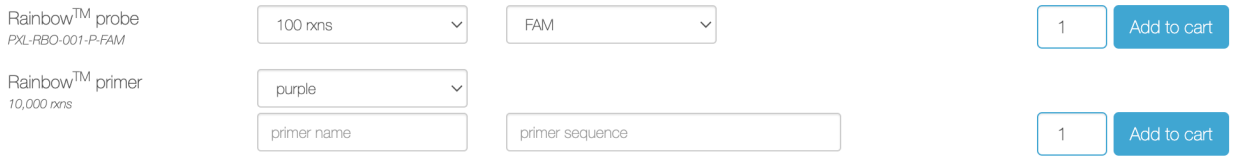


Figure 2: Order page for Rainbow™ probes and primers

Rainbow primer and probe nomenclature

A Rainbow™ PCR assay consist of 1) a Rainbow™ primer, 2) a regular primer, and 3) a compatible Rainbow™ probe. The Rainbow™ primer name consists of three parts, each separated by a hyphen: *target-orientation-probe*

- target: the name you entered for your primer during ordering (e.g. gene symbol)
- orientation: F for forward or R for reverse primer, must also be entered during ordering
- probe: the first letter of the color of the tube cap containing the compatible probe (Purple, Blue, Green, Yellow, Orange, or Red); this letter is also part of the probe name, and is automatically added during ordering of the Rainbow™ primer

The Rainbow™ probe name starts with PXL-RBO-xxx, followed by the first letter of the color of the tube cap (symbolizing the 6 fluorescent detection channels) and the specific fluorophore. For a given channel, we offer between 1 and 3 different fluorophores (e.g. we have both HEX and YakimaYellow for the Blue channel; the optimal choice depends on the instrument, see Table 1).

For example, the Rainbow™ PCR assay used to generate the 1-color plots below is named as follows. This assay detects wild-type human *BRAF* at position V600.

Rainbow™ primer	regular primer	Rainbow™ probe
BRAFV600E-WT-F-P	BRAFV600E-WT-R	PXL-RBO-001-P-FAM

Recommended primer and probe concentrations

The following final reaction concentrations are a good starting point for a singleplex assay:

Rainbow™ primer	100 nM
regular primer	300 nM
Rainbow™ probe	125 nM

We recommend adjusting these concentrations in function of the PCR master mix, oligonucleotide sequences, or multiplexing degree. Multiplexing may require higher probe concentrations (if needed, start with doubling the concentration). Importantly, the Rainbow™ primer concentration must be a bit lower than the Rainbow™ probe concentration, and 3-5x lower than the regular primer concentration.

Probes are shipped at ambient temperature as 60x solution (7.5 µM) and are stable for 2 years when stored at -20 °C (recommended) or for at least 2 months when stored at room temperature. Limit the number of freeze-thaw cycles by aliquoting the (diluted) probe.

Primers are shipped at ambient temperature as lyophilized products. Resuspend with PCR-grade water to the desired concentration (we recommend a stock concentration of 100 µM). Resuspended primers are stable for at least 2 years when stored at -20 °C.

Recommended thermocycling conditions

The thermocycling program recommended by the instrument supplier is a good starting point. However, we experience better peak resolution (signal-to-noise) by using one or more of the following modifications:

- slow cool-down step at the end of cycling (after PCR, go to denaturation temperature for 5 seconds and slowly cool down to room temperature; slower is better)
- 5 more cycles than usual (also helps to resolve rain if present)
- short cycle times of 5-10 seconds for denaturation and 20 seconds for annealing/extension

*Table 1: Compatibility of Rainbow™ probes with digital PCR instruments**

catalogue number	probe channel (fluorophore)	Bio-Rad			Qiagen	Stilla Technologies			Roche	Sniper	ThermoFisher
		QX200	QX ONE	QX600	QIAcuity	naica 3	naica 6	Nio+	Digital LightCycler	DQ24	QuantStudio Absolute Q
PXL-RBO-001-P-FAM	purple (P) FAM	channel 1	channel 1	channel 1	green	blue	blue	blue	channel 2	channel 1	blue
PXL-RBO-001-B-YY	blue (B) YakimaYellow						teal	teal			
PXL-RBO-001-B-HEX	blue (B) HEX	channel 2	channel 2	channel 2	yellow	green			channel 3	channel 2	green
PXL-RBO-001-G-Atto425	green (G) ATTO425								channel 1	channel 6	
PXL-RBO-001-G-Atto550	green (G) ATTO550				orange		green	green			
PXL-RBO-001-G-Atto590	green (G) ATTO590			channel 6							
PXL-RBO-001-Y-TR	yellow (Y) Texas Red				red				channel 4		
PXL-RBO-001-Y-ROX	yellow (Y) ROX			channel 5	red		yellow	yellow		channel 3	red
PXL-RBO-001-O-CY5	orange (O) Cy5		channel 3	channel 3	crimson	red	red	red	channel 5	channel 4	dark red
PXL-RBO-001-R-CY5.5	red (R) Cy5.5		channel 4	channel 4					channel 6	channel 5	
PXL-RBO-001-R-Atto700	red (R) ATTO700						infra-red	infra-red			

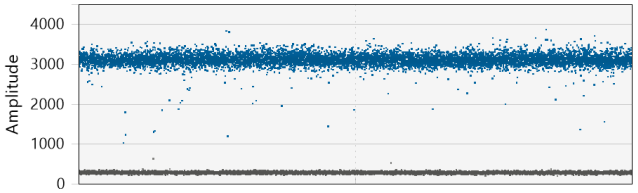
* Not all probes have been tested on all instruments. As such, this table provides guidance for selecting the right fluorophore based on the instrument specifications.

Representative 1-color plots

Below are representative 1-color singleplex results using different Rainbow™ probes on different digital PCR instruments. Each time, a BRAF V600E wild type assay was used with fragmented human genomic DNA as input (0.7 copies per partition (λ), resulting in an equal number of positive and negative partitions). Where mentioned, the peak resolution is calculated according to Lievens et al., 2016; primer/probe concentrations are mentioned as Rainbow™ primer / regular primer / Rainbow™ probe.

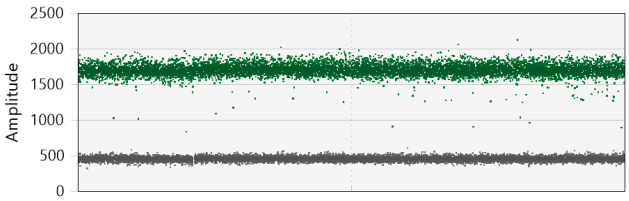
Bio-Rad QX200

PXL-RBO-001-P-**FAM**



4.64 (100/300/125)

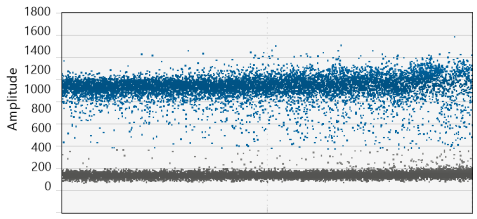
PXL-RBO-001-B-**HEX**



3.04 (100/300/125)

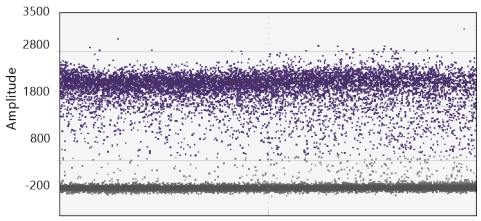
Bio-Rad QX600

PXL-RBO-001-P-**FAM**



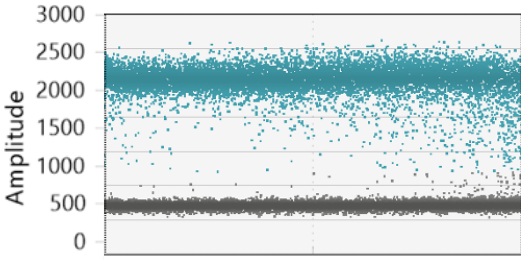
1.92 (100/300/125)

PXL-RBO-001-R-**CY5.5**



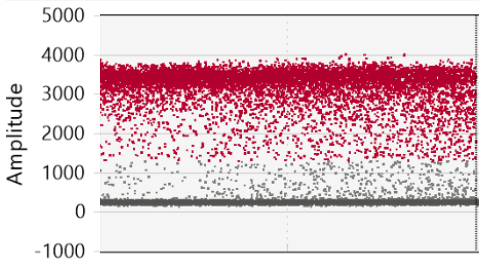
1.86 (100/300/125)

PXL-RBO-001-G-**ATTO590**



2.16 (100/300/125)

PXL-RBO-001-O-**Cy5**

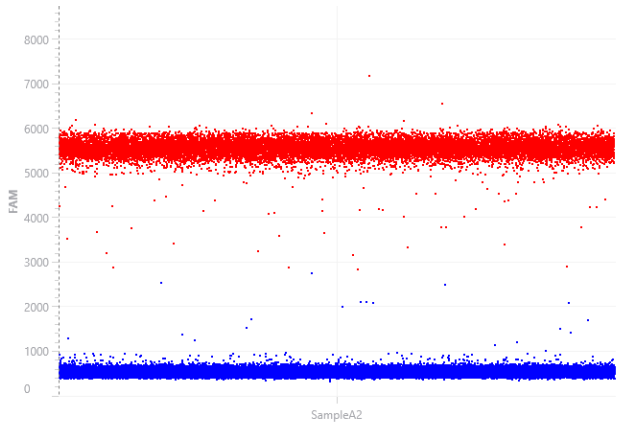


2.78 (100/300/125)

The QX600 results were obtained with unfragmented human genomic DNA, resulting in a bit of rain.

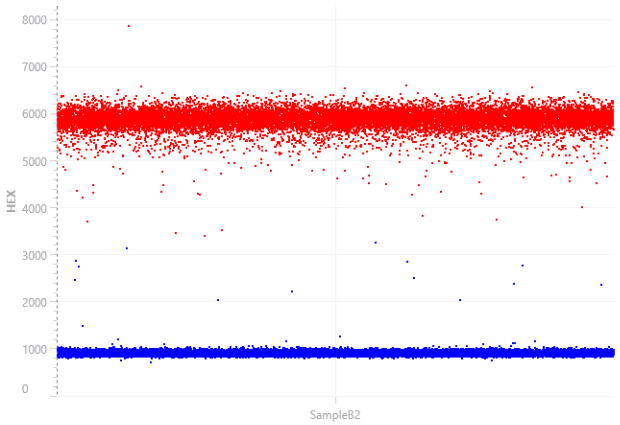
Roche Digital LightCycler

PXL-RBO-001-P-**FAM**



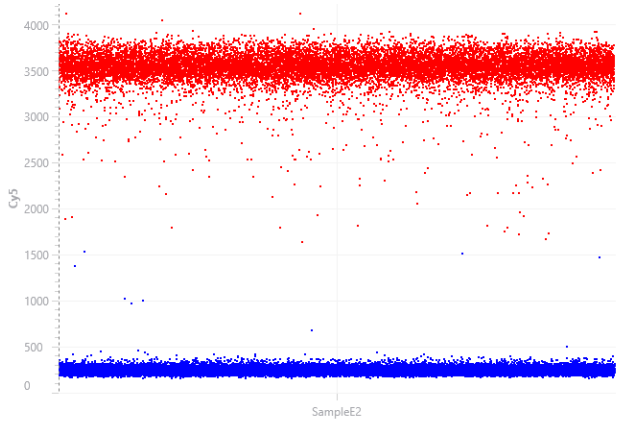
(100/300/125)

PXL-RBO-001-B-**HEX**



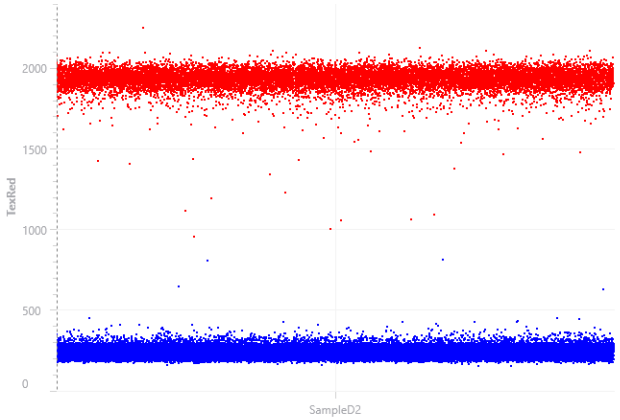
(100/300/125)

PXL-RBO-001-O-**Cy5**



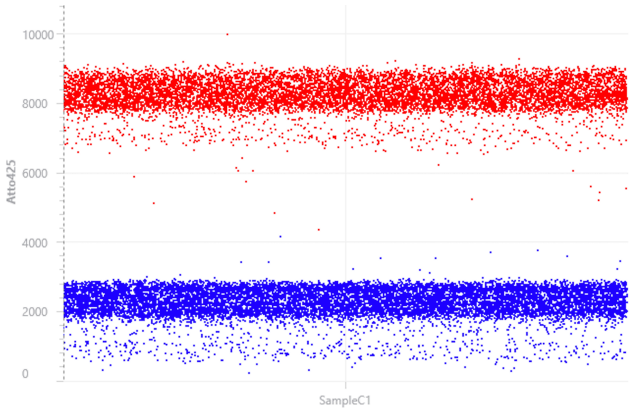
(100/300/125)

PXL-RBO-001-Y-**Texas Red**



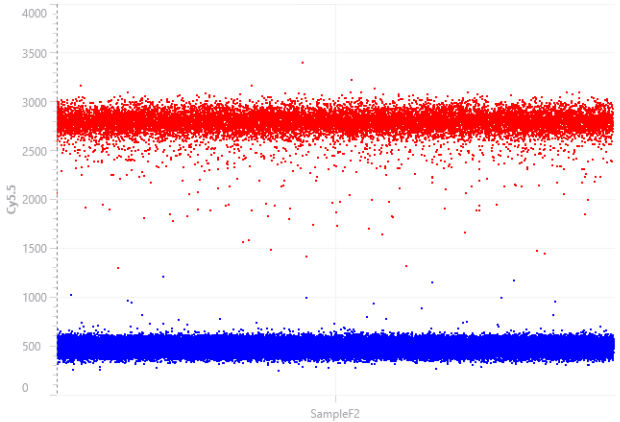
(100/300/125)

PXL-RBO-001-G-**ATTO425**



(100/300/125)

PXL-RBO-001-R-**CY5.5**

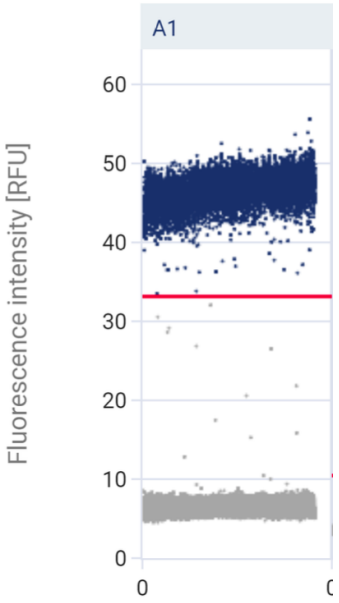


(100/300/125)

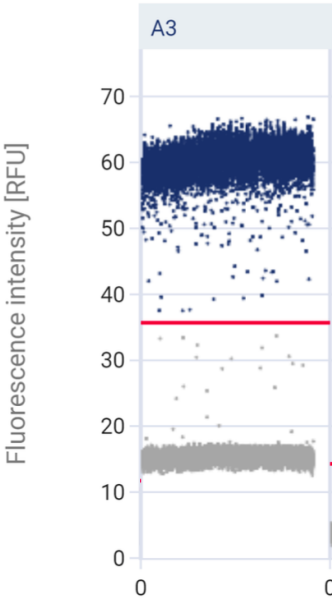
The Digital LightCycler results were generated with a Universal plate.

Qiagen QIAcuity

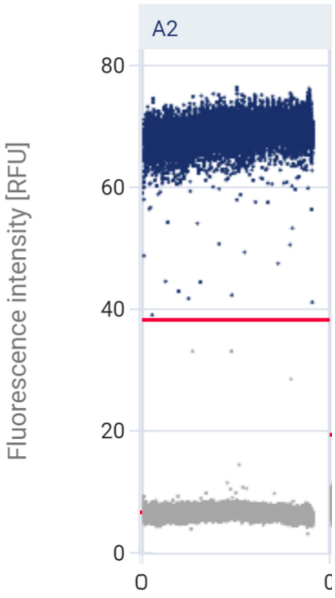
PXL-RBO-001-P-**FAM**



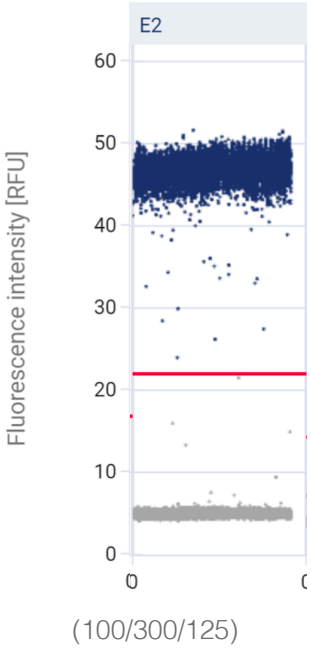
PXL-RBO-001-B-**HEX**



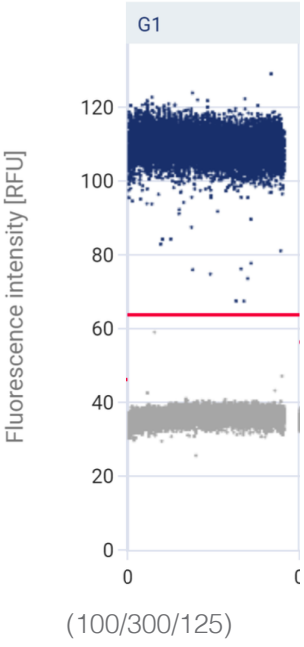
PXL-RBO-001-G-**ATTO550**



PXL-RBO-001-Y-**Texas Red**



PXL-RBO-001-O-**Cy5**



*The QIAcuity results were generated with a Nanoplate 26K 24-well and the **OneStep Advanced Probe Kit** (which gives superior results in our hands compared to the other mix, even for DNA targets; just omit the reverse transcriptase and RT step when not quantifying RNA).*

Higher-order multiplex quantification using digital PCR

Digital PCR is very well suited for multiplex quantification, because of the partitioning of the template molecules. As a result, there is much less competition among the different assays, enabling the accurate quantification of rare events, or analytes of very different concentration.

While in principle as many targets can be simultaneously quantified as fluorescent detection channels available in the digital PCR instrument at hand (i.e. classic multiplexing), there are various methods to quantify more targets than detection channels, a strategy called higher-order multiplexing (Huggett et al., 2020). At pxlence, we use the ratio-based or color-combination strategy.

Classic multiplexing

If you are new to digital PCR, we recommend starting with classic multiplexing, i.e. quantifying maximally the same number of targets as fluorescent detection channels in your instrument. In a 6-, 5-, 4-, 3- and 2-color instrument, you can thus quantify up to 6, 5, 4, 3 and 2 targets in the same reaction.
According to the dMIQE guidelines (Huggett et al., 2020), we recommend inspecting 2-color plots during assay optimization and as quality control in the final experiments (see Figure 3). Target A is detected with a 100% channel 1 probe; target B with a 100% channel 2 probe; the double-positive cluster AB contains partitions with both A and B targets and has equally high fluorescence intensity as single positive clusters for a non-competing assay (top) or half the fluorescence intensity for a competing assay (bottom) (Whale et al., 2016).

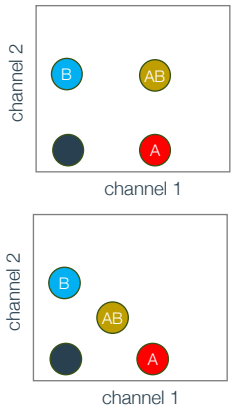


Figure 3: Classic multiplexing with non-competing (top) and competing (bottom) assay.

Ratio-based higher-order multiplexing

This strategy enables the quantification of 3 (or more) targets in 2 channels, using a mixture of probes for the additional target(s). As in classic multiplexing, target A and B are detected using pure channel 1 or 2 probes, respectively, but an additional target (here C) is detected with a 50:50 mixture of probes for channel 1 and 2 (Figure 4). In principle, up to 5 targets can be quantified by using probe mixtures of 25:75, 50:50, and 75:25 (Whale et al., 2016). With 50:50 probe mixtures (3 targets in 2 channels), n x (n - 1) x 3/2 targets can be simultaneously quantified using an n-color dPCR instrument.
For accurate quantification, the double- and triple positive clusters can be safely ignored, albeit with some loss of precision (depending on the target concentration).

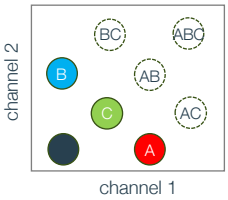


Figure 4: Ratio-based multiplexing (here using 50:50 probe mixture)

Color-combination higher-order multiplexing

When using dual-color combinations, each target is detected with a 50:50 mixture of probes, whereby no single-positive partitions exist; hence, target A is quantified as a double-positive cluster. This strategy has been applied in qPCR (e.g. Marras et al., 2019), and is perfectly suitable for dPCR as well. In principle, n x (n - 1) / 2 targets can be simultaneously quantified using an n-color dPCR instrument. For accurate quantification, the higher-order positive clusters (e.g. AX, AY, AXY, resulting from partitions in which other targets detected in the same channels are co-amplified) can be safely ignored, albeit with some loss of precision (depending on the target concentration). Three or more colors can also be combined.

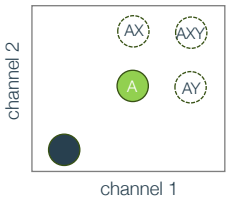


Figure 5: Dual-color combination multiplexing

Frequently Asked Questions

1. Question: *Should a DNA detection probe not be target specific?*

   Answer: It is a common misunderstanding that a probe is absolutely required for an assay to be specific. In fact, a double-strand specific dye (e.g. EvaGreen or SYBR Green I) is perfectly suitable for selective amplification of a target of interest. Crucially important is the selectivity of the primer pair, which can be predicted using *in silico* tools (e.g. primerBLAST, or BiSearch) and verified empirically (through electrophoresis or amplicon sequencing). Sometimes, a probe may mask co-amplification of an unwanted amplicon, severely impacting analytical sensitivity (Vandenbroucke et al., 2001). We therefore strongly recommend enforcing – where possible – that a primer pair is specific on itself.

   Obviously, primer dimer formation is problematic when using a universal probe or generic DNA binding dye. Therefore, we recommend a) to include no-template controls, and b) keep primer concentrations (especially in multiplex reactions) as low as possible (in general, there is no need to go beyond 300 nM for end-point detection in digital PCR). Several tools exist to predict primer dimer formation (e.g. PrimerDimer)

2. Question: *Can I detect the same target with two different fluorophores?*

   Answer: Yes. In general, we recommend ordering one primer as two different Rainbow™ primers (see below) and using each at half of the normal concentration. At pxlence, we use this strategy in a ratio-based or dual-color combination strategy to achieve higher-order multiplexing.

   For example, order the same primer in 2 different Rainbow™ versions, e.g. modify the primer with the PXL-RBO-099-P tag sequence (to be detected with a FAM-labeled probe) and modify the same primer with the PXL-RBO-099-B tag sequence (to be detected with a HEX-labeled probe).

3. Question: *Can I use my existing dPCR primers with the universal Rainbow™ probes?*

   Answer: Absolutely. One of the attractive features of the Rainbow™ probes is that any good primer assay can be converted into a Rainbow™ assay by modifying one of the primers. Simply upload your primer sequence as instructed in the 'Probe and primer order workflow' section and you will receive a modified primer whose amplicon can be detected with the intended probe. We recommend converting the primer with the lowest Tm (from a given primer pair).

4. Question: *Can I use the universal Rainbow™ probes for (RT-)qPCR?*

   Answer: The Rainbow probes were developed for digital PCR where fluorescence measurements are done at the of the PCR, typically below the annealing temperature (e.g. room temperature). In contrast, during real-time PCR, fluorescence measurements are done at the annealing temperature (e.g. 60 °C). Therefore, the probes that are currently available (with catalog numbers starting with PXL-RBO-001) are not suitable for qPCR. However, we have a different set of probes suitable for qPCR (starting with PXL-RBO-002). Please enquire about their availabilities.

5. Question: *Can the universal probe technology be licensed from pxlence?*

   Answer: Yes. Please contact info@pxlence.com to enquire about licensing options.

References

Huggett et al., Clinical Chemistry, 2020: The digital MIQE guidelines update: minimum information for publication of quantitative digital PCR experiments for 2020

Paper with minimal information guidelines for designing, executing, analyzing and reporting on digital PCR experiments. The paper comes with a checklist spreadsheet and also provides recommended nomenclature.
One of the pxlence co-founders is co-author on this paper.

Lievens et al., Plos ONE, 2016: Measuring digital PCR quality: performance parameters and their optimization

Paper reporting on various strategies to evaluate and improve dPCR assay performance. Amongst others, an R script to calculate peak resolution (i.e. separability score, how well positive and negative partitions can be separated) is provided.

Marras et al., Plos ONE, 2019: Color-coded molecular beacons for multiplex PCR screening assays

Paper describing a dual-color-combination strategy in qPCR to detect 15 bacterial species using 6 fluorescent channels.

Vandenbroucke et al., Nucleic Acids Research, 2001: Quantification of splice variants using real-time PCR

Paper that documents the danger of relying on a probe to differentiate specific from non-specific amplification. While the probe may be specific for the target of interest, non-specific primers may co-amplify off-targets. If these off-targets are abundant, they may compromise the analytical sensitivity of the target of interest, or even prevent detection at all.
One of the pxlence co-founders is co-author on this paper.

Whale et al., Biomolecular Detection and Quantification, 2016: Fundamentals of multiplexing with digital PCR

Excellent review paper describing all you need to know about multiplexing with digital PCR.