How to Multiplex With Color Combination Using ddPLEX CrystalTag and Droplet Digital™ PCR (ddPCR™) Technologies in Research Settings

Key Concepts

- Color combination enables the detection and quantification of up to 21 targets in a single reaction with the QX700 Droplet Digital PCR System
- Color combination's 21-target potential is viable in practice by leveraging CrystalTag technology: non-fluorescent CrystalTag Probes and a single set of universal CrystalTag Reporters avoid compounding synthesis costs and optimization complexity as plex number increases
- The population editor feature in QX700 ddPCR System Analysis Software supports single thresholding between positive and negative populations
- Successful assay design relies on application-specific design strategy, assay configuration, and a low co-encapsulation rate
- The color-combination and CrystalTag technology approach supports the development of hyperplexed assays in research settings, such as the ddPLEX CrystalTag ESR1 17 mut ddPCR Assay

Note

This study was originally carried out on the Nio+™ Digital PCR System, which supports seven optical detection channels with the following fluorophores: FAM, Yakima Yellow, ATTO 550, ROX, Cy5, ATTO 700/Cy5.5, and DY-521-XL. The QX700™ HT Droplet Digital PCR System (Bio-Rad Laboratories, Inc., catalog #17010628) also supports seven fluorophores, but with a different fluorophore set: FAM, HEX, ROX, ATTO 590, Cy5, Cy5.5, and ATTO 550. Despite these optical differences, the core digital PCR (dPCR) principles of droplet partitioning, endpoint fluorescence detection, and absolute quantification remain equivalent between platforms. Accordingly, the study's findings are applicable to research workflows implemented on the QX700 HT ddPCR System.

The assay referenced in this guide is identical to the ddPLEX CrystalTag ESR1 17 mut ddPCR Assay available from Bio-Rad Laboratories (catalog #12026887, #12026888). Note that the example data used Nio Crystal Universal Reporters, formulated to match the fluorescent channel specifications of the Stilla™ Nio Digital PCR System. For equivalent performance when running CrystalTag Assays across all seven channels of the QX700 Droplet Digital PCR System, QX700 CrystalTag Reporters 7 (Bio-Rad, #12027244, #12027265) should be used instead.

This guide describes research-use-only workflows and is not intended to establish clinical performance, diagnostic utility, or applications for patient management.

Introduction

Color-Combination Multiplexing

In color-combination multiplexing, a target is identified using two separate fluorophores detected in two different channels. By using two probes per sequence of interest, either competing or noncompeting, each population can be revealed in two colors, expanding the number of targets per assay detectable beyond the number of available channels (Figure 1A).

With the QX700 ddPCR System, up to 21 targets can be detected using its seven-color channels (Figure 2).

The QX700 HT Droplet Digital PCR System, CrystalTag Probes, CrystalTag Reporters, ddPLEX CrystalTag ESR1 17 mut ddPCR Assay, and QX700 CrystalTag Reporters 7 mentioned in this guide are for Research Use Only. Not for use in diagnostic procedures.

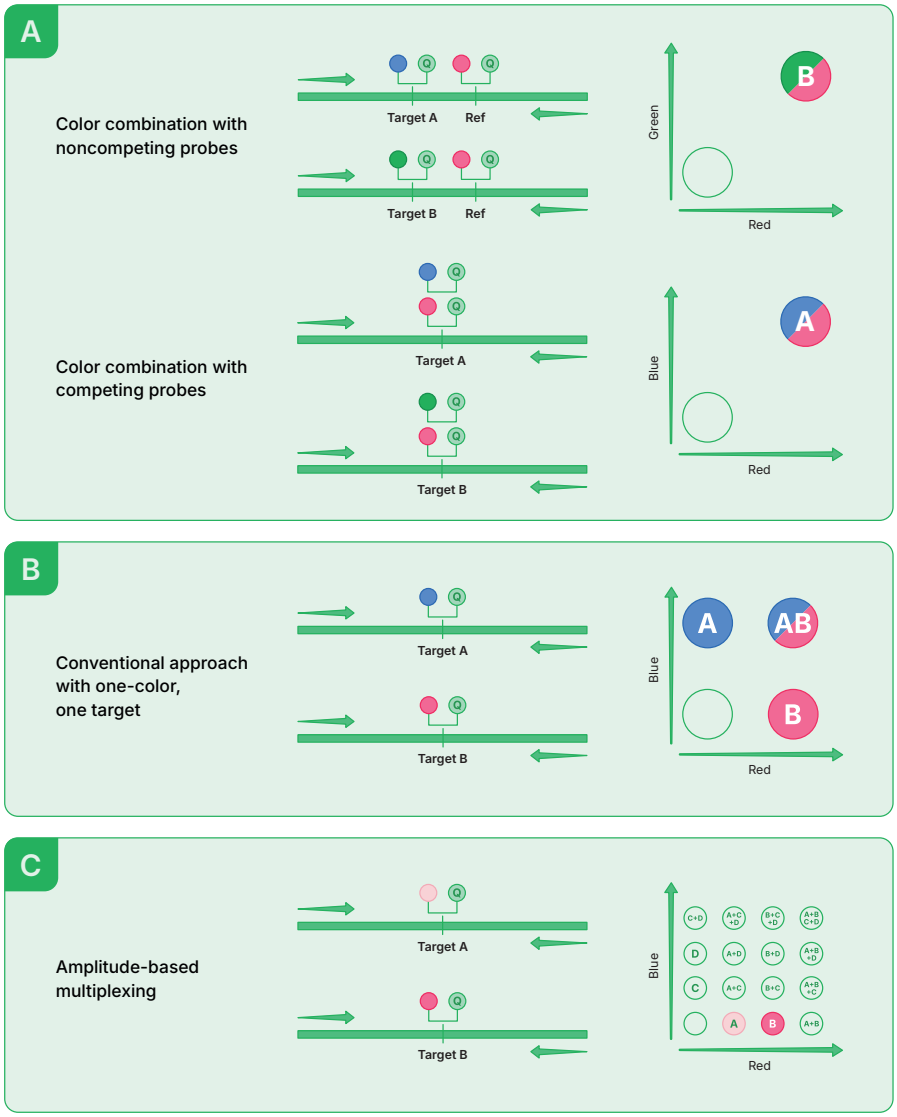


Fig. 1. Multiplexing approaches in ddPCR analysis. A, illustration of the color-combination approach, with two different design strategies, noncompeting and competing probes, that lead to equivalent fluorescence data. The cluster of droplets that contain target A is blue/red while the cluster of droplets that contain target B is green/red. **B,** illustration of the one-color, one-target multiplexing approach. For a blue/red two-plex assay, there are four clusters in the 2D dot plot: all negative partitions, blue partitions positive for target A only, red partitions positive for target B only, and blue/red partitions positive for both target A and target B. **C,** illustration of the amplitude-based multiplexing approach. Two distinct targets are detected using the red channel: one with a low-intensity cluster (target A) and one with a high-intensity cluster (target B). If two additional targets (target C and target D) are detected in another color, the resulting data include up to 16 different clusters in the 2D dot plot.

Multiplexing Approaches Using ddPCR Technology

Conventional approaches use target-specific fluorescent probes to detect a single target per detection channel (Figure 1B).

The multiplexing capacity is limited by the number of fluorescence channels available on the instruments, which cannot be increased beyond a certain number due to the limited bandwidth of optical detectors and the spectral overlap of commonly available fluorescent reporters.

Amplitude-based and ratio-based multiplexing approaches encode different targets with different fluorescence intensities using different concentrations of specific fluorescent probes.[1–5] These approaches make data analysis more complex than the conventional approach, as they require several levels of thresholding on a 2D dot plot to separate events according to their intensities (Figure 1C).

The analysis may become more complex as the number of fluorescence intensity levels increases and may require additional threshold setting during data interpretation.

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Advantages of Color-Combination Multiplexing

With the color-combination approach, more than one target per color can be distinguished within the same sample, expanding multiplexing capacity in research settings.

The implementation of the color-combination approach in quantitative PCR is limited to applications in which only one target is present in the sample. When more than one target is present, the resulting fluorescent signal comprises more than two colors and becomes ambiguous, preventing reliable target identification. Partitioning with ddPCR technology can facilitate the identification of fluorescence signals within individual partitions.

Advantages of Color-Combination Multiplexing With CrystalTag Technology

Color combination is compatible with any hydrolysis-based probe chemistry. In principle, any probe that generates a fluorescent signal upon cleavage can be configured across two detection channels per target. However, the practical ceiling for multiplexing is determined by probe architecture. With conventional fluorescent hydrolysis probes, every target-specific probe must carry its own fluorophore and quencher, making synthesis increasingly costly and optimization progressively harder as plex number grows. CrystalTag technology removes this constraint by decoupling target recognition from fluorescent signaling: the CrystalTag Probe is entirely non-fluorescent, with signal generated instead by a universal CrystalTag Reporter triggered by a short tag oligo released during amplification. Because the target-specific probe carries no fluorescent modification, probe synthesis workflows may be simplified compared with approaches requiring fluorophore-modified probes, and can be optimized independently of any fluorophore chemistry, supporting assay designs that may leverage up to 21 theoretical target combinations within a seven-channel system.

For more information on CrystalTag technology see bulletin 4021.

Conventional one-color, one-target approach, 7-plex

Target	FAM	HEX	ROX	ATTO 590	Cy5	Cy5.5	ATTO 550
1	✔						
2		✔					
3			✔				
4				✔			
5					✔		
6						✔	
7							✔

Color-combination approach, 21-plex

Target	FAM	HEX	ROX	ATTO 590	Cy5	Cy5.5	ATTO 550
1	✔	✔					
2	✔		✔				
3	✔			✔			
4	✔				✔		
5	✔					✔	
6	✔						✔
7		✔	✔				
8		✔		✔			
9		✔			✔		
10		✔				✔	
11		✔					✔
12			✔	✔			
13			✔		✔		
14			✔			✔	
15			✔				✔
16				✔	✔		
17				✔		✔	
18				✔			✔
19					✔	✔	
20					✔		✔
21						✔	✔

Fig. 2. Multiplexing capacity using conventional and color-combination approaches.

Using Color-Combination Multiplexing

Step 1: How to Define Populations

QX700 ddPCR System Analysis Software supports experimental configuration and data analysis of color-combination data. The population editor function enables the definition of negative and positive populations in a color-combination assay (Figure 3).

The ratio between positive and negative partitions forms the basis of quantification in ddPCR analysis, and the statistical framework for converting this ratio into a concentration estimate is based on the Poisson approximation. As co-encapsulation events are removed from the analysis (because they lead to ambiguous results), the number of positive droplets for a given target is underestimated if other targets are present in the sample. To counterbalance this bias in the number of positive partitions, a correction must also be applied to the number of negative partitions. Then, to support statistically consistent concentration estimates, partitions that are negative for the target but positive for another target must also be eliminated from the total number of partitions.

Additionally, in color-combination data, QX700 Software identifies single-positive droplets and removes them from the positive and negative populations during analysis.

The statistical framework underlying color-combination multiplexing has been described by Santos-Barriopedro et al.[6]

Through the population editor function, the analysis of a color-combination assay relies on adjusting a single threshold for each channel to separate positive from negative droplets. Based on this 1D thresholding, QX700 Software colocalizes fluorescence within the same droplet and thus assigns and quantifies a population previously defined with the corresponding color code in the population editing tool.

The analysis remains straightforward in the presence of fluorescence variations and follows a workflow similar to the conventional one-color, one-target approach.

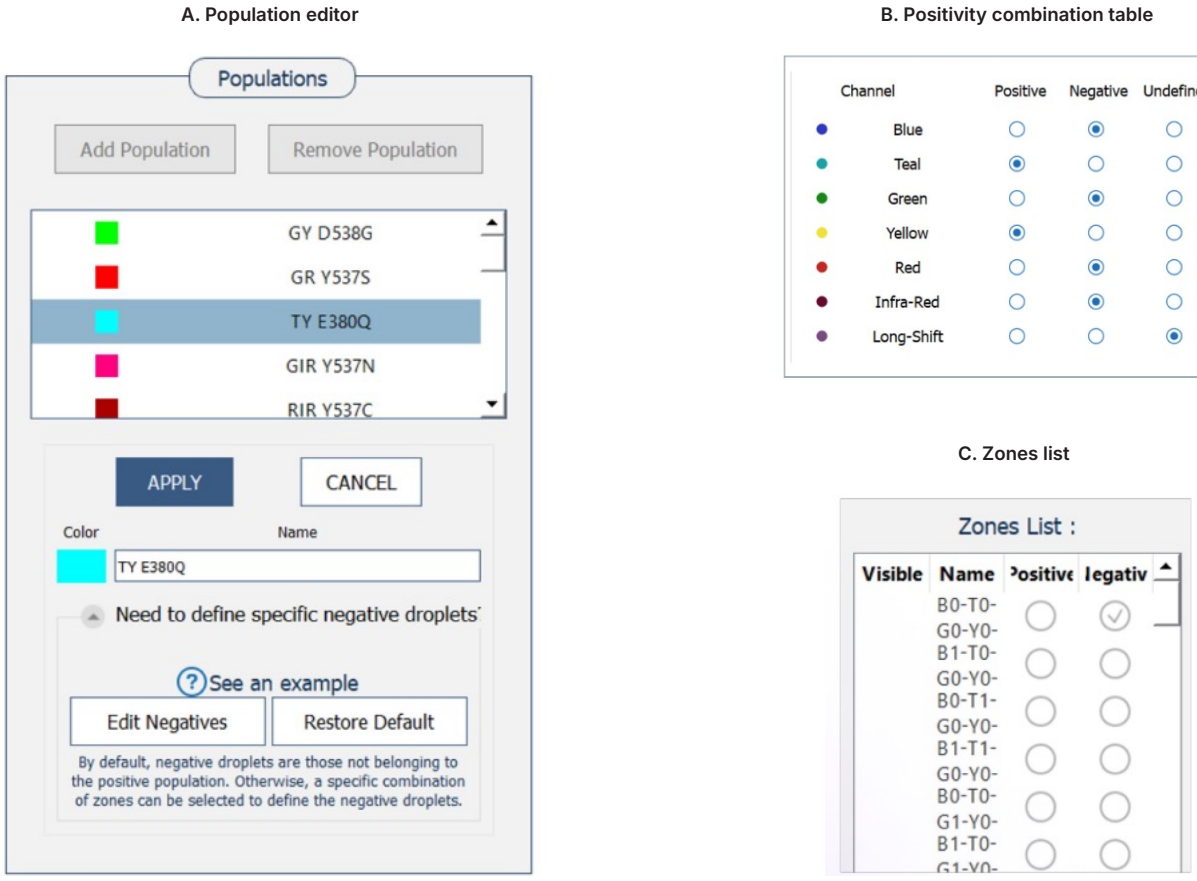


Fig. 3. Population editing in the QX700 ddPCR System Analysis Software.

Practical Research Example: Population Definition in an Estrogen Receptor 1 (*ESR1*) 18-plex ddPCR Assay

The *ESR1* E380Q mutation can be used to illustrate population definition in an oncology research assay developed using the color-combination approach (see assay configuration in Table 1).

- The positive population is first defined in the positivity combination table (Figure 3B). Teal and yellow channels are set as positive channels, while all other channels are defined as negative (blue, green, red, and infra-red channels) or as undefined (the purple channel reserved for the reference gene)
- Negative populations for this target are then defined in the zones list (Figure 3C) and include droplets negative for all channels (B0, T0, G0, Y0, R0, IR0, LS0, where 0 indicates a negative signal in the corresponding channel and LS refers to the purple reference-gene channel) that contain no target, and droplets positive only in the purple channel (B0, T0, G0, Y0, R0, IR0, LS1, where 1 indicates a positive signal) that contain the wild-type DNA of exon 8

Step 2: How to Set Up the Fluorescence Compensation Matrix

Experimental Setup

With the conventional one-color, one-target approach, single-color controls are performed for all the channels by introducing a single target into the PCR mix, thus activating a single probe. The software then calculates the corrections needed to compensate for spillover from one probe into the other detection channels.

Given that two colors are activated per target in a color-combination assay:

1. One target is selected for each color used in the assay. For any color-combination assay, a mono-color control is required for each color to generate the compensation matrix. The target selected for each color is assay dependent.

Table 1. Genes and mutations targeted by the ddPLEX CrystalTag ESR1 17 mut ddPCR Assay and assay configuration in research settings.

Target	Mutation	Exon	Base Changes	COSMIC ID	Detection Channel 1	Detection Channel 2
1	*ESR1* exon 8 reference	8	N/A	N/A	Purple	N/A
2	E380Q	5	c.1138G>C	COSM3829320	Teal	Yellow
3	V422del	6	c.1265_1267del	COSM1074628	Blue	IR
4	S463P	7	c.1387T>C	COSM4771561	Teal	Red
5	D538G	8	c.1613A>G	COSM94250	Green	Yellow
6	D538N	8	c.1612G>A	N/A	Blue	Red
7	L536H	8	c.1607T>A	COSM6843697	Yellow	IR
8	L536P	8	c.1607T>C	COSM6906109	Yellow	Red
9	L536Q_delinsAG	8	c.1607_1608delinsAG	COSM4766050	Blue	Teal
10	L536R	8	c.1607T>G	COSM4774826	Teal	Green
11	Y537C	8	c.1610A>G	COSM1074637	Red	IR
12	Y537D	8	c.1609T>G	COSM6918757	Teal	IR
13	Y537H	8	c.1609T>C	COSM7449624	Blue	Yellow
14	Y537N	8	c.1609T>A	COSM1074635	Green	IR
15	Y537N_delinsTA	8	c.1608_1609delinsTA	N/A	Blue	Green
16	Y537S	8	c.1610A>C	COSM1074639	Green	Red
17	Y537S_delinsAG	8	c.1609_1610delinsAG	COSM6948665	Red	N/A
18	Y537S_delinsCA	8	c.1610_1611delinsCA	COSM6971270	Red	N/A

IR, infra-red; N/A, not applicable.

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To illustrate, in a 21-plex assay, the mono-color control for blue can be generated using one of targets 1–6, as all are detected in blue. Similarly, the mono-color control for teal can be generated using target 1 or targets 7–11, as all are detected in teal. The same principle applies to the remaining colors in the assay.

Example of target selection for generation of mono-color controls used to create a compensation matrix for a 21-plex assay:

Blue: target 1	Red: target 4
Teal: target 7	Infra-red: target 21
Green: target 2	Purple: target 21
Yellow: target 3	



2. For each selected target, a primer/probe pool is prepared using only one of the two target-specific probes, thereby activating a single color. All other reagents are included except the target sequences.
3. Individual targets are added to the chamber corresponding to the appropriate mono-color control.
4. A no template control (NTC) with no target template is added to determine the negative background fluorescence signal that can emanate from the probes, even in the absence of the target sequences.

   Example of the preparation of a mono-color control pools using the user selection of targets for mono-color controls of step 1:

   Blue: target 1 primers + target 1 blue probe only (without target 1 teal probe) + all other primers and probes for remaining targets + target 1 DNA, thus all primers and probes except teal probe for target 1 + target 1 DNA

   Teal: all primers and probes except green probe for target 7 + target 7 DNA

   Green: all primers and probes except blue probe for target 2 + target 2 DNA

   Yellow: all primers and probes except blue probe for target 3 + target 3 DNA

   Red: all primers and probes except blue probe for target 4 + target 4 DNA

   IR: all primers and probes except purple probe for target 21 + target 21 DNA

   Purple: all primers and probes except IR probe for target 21 + target 21 DNA

   NTC: all target primers and probes without DNA
5. Single-color experiments are then performed to compensate for fluorescence spillover among channels.
6. In most cases, removing one of the two probes for a given target does not affect the generation of the compensation matrix because the composition of the remaining pool is unchanged. If removal of a probe results in a fluorescence background level different from that of the NTC, another target detected in the same fluorescence channel can be selected and the process repeated.

   Example: For a background level different from the NTC in the blue channel, another target detected in blue can be selected.

   New blue: target 2 primers + target 2 blue probe only (without target 2 green probe) + all other primers and probes for remaining targets + target 2 DNA

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Assay Design Considerations for Color-Combination Multiplexing

Design Strategy (see Figure 1A)

- Noncompetitive approach: two probes, one per color, are designed for each target in different regions of the sequence. This approach increases design complexity but provides greater separation between positive and negative events than the competitive approach
- Competitive approach: two probes, one per color, are designed for the same target region. Separation between positive and negative droplets is typically reduced because of competition between the two probes

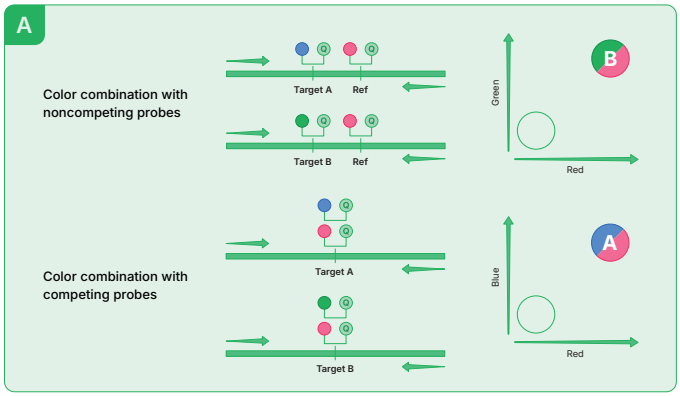


Fig. 1A. Multiplexing approaches in ddPCR analysis. (See Fig. 1, page 2, for all approaches.) **A**, illustration of the color-combination approach, with two different design strategies, noncompeting and competing probes, that lead to equivalent fluorescence data. The cluster of droplets that contain target A is blue/red, while the cluster of droplets that contain target B is green/red.

Assay Configuration

The elimination of co-encapsulation reduces the volume available for target quantification, which can affect assay sensitivity. The impact on sensitivity is more significant when one of the detected targets is highly abundant. To improve sensitivity, the abundant target (for example, a reference target or wild-type DNA) can be detected using a single color (one channel), while the remaining six channels are used in color-combination mode to detect the target mutations. In this configuration, multiplexing capacity is 17 targets per assay with a seven-color system.

DNA Input and Co-Encapsulation

In color combination, it is recommended to test the maximum DNA input to maintain assay performance and reduce co-encapsulation events, which can affect separation between negative and positive populations. When co-encapsulation involves variants present on the same amplicon, positive partitions with lower fluorescence intensity may be observed, generating rain below the main positive cluster. This effect may result from competition between probes and the different amplicons generated during amplification.

Insights: For the *ESR1* 18-plex assay, this limit was set at 3 ng/µL, corresponding to 18 ng in the 6 µL of PCR mix prepared (5 µL + 1 µL margin), thus 15 ng in the 5 µL of PCR mix loaded in the RDG16 Cartridge (Bio-Rad, #12025252).

Application of Color-Combination Multiplexing in an 18-Plex *ESR1* RUO Assay

Through color combination powered by CrystalTag technology, the developed 18-plex ddPLEX CrystalTag ESR1 17 mut ddPCR Assay allows for the simultaneous detection and quantification of 18 targets within a single reaction in research settings. Fifteen somatic *ESR1* mutations are detected in color combination across four exons: exon 5 (E380Q), exon 6 (V422del), exon 7 (S463P), and exon 8 (D538G, D538N, L536H, L536P, L536Q, L536R, Y537C, Y537D, Y537H, Y537N, Y537NdelinsTA, Y537S). Two additional mutations of exon 8 coding for the same protein change are co-detected in single color (Y537SdelinsAG and Y537SdelinsCA detected in the red channel). Finally, a reference probe is detected on an unmutated portion of exon 8 (codons 543–547), allowing the total DNA quantification and the estimation of the mutant allele frequency (MAF). This reference is isolated in one color (purple channel) to maintain the assay's sensitivity. The targeted mutations and the assay configuration are depicted in Table 1.

The 18-plex *ESR1* assay illustrates why CrystalTag technology is designed to support high-order color-combination assay configurations. With conventional fluorescent probes, each additional target compounds synthesis cost, spectral validation burden, and optimization complexity. With CrystalTag technology, all mutation-specific CrystalTag Probes are non-fluorescent: fluorescent output is generated uniformly by the CrystalTag Reporter, meaning that adding a target requires only probe sequence design, not a new labeled reagent. The result is a panel that reaches high-plex capacity without a proportional increase in reagent complexity, cost, or development time.

The ability of the 18-plex *ESR1* assay to detect 18 targets simultaneously in the same sample was first assessed with synthetic DNA using the QX700 HT ddPCR System and RDG16 Cartridge (Figure 4). The 18-plex *ESR1* assay was characterized on serial dilutions of seventeen DNAs, each bearing one of the targeted *ESR1* mutations in a background of WT DNA (500 cp/µL, 1.5 ng/µL). Mutant allele fractions ranging from 10% to 0.3% were detected. The coefficient of determination of linear regressions performed between input and measured copies per reaction ranged from R^2 = 0.9936 to R^2 = 0.9996, depending on the mutation targeted.

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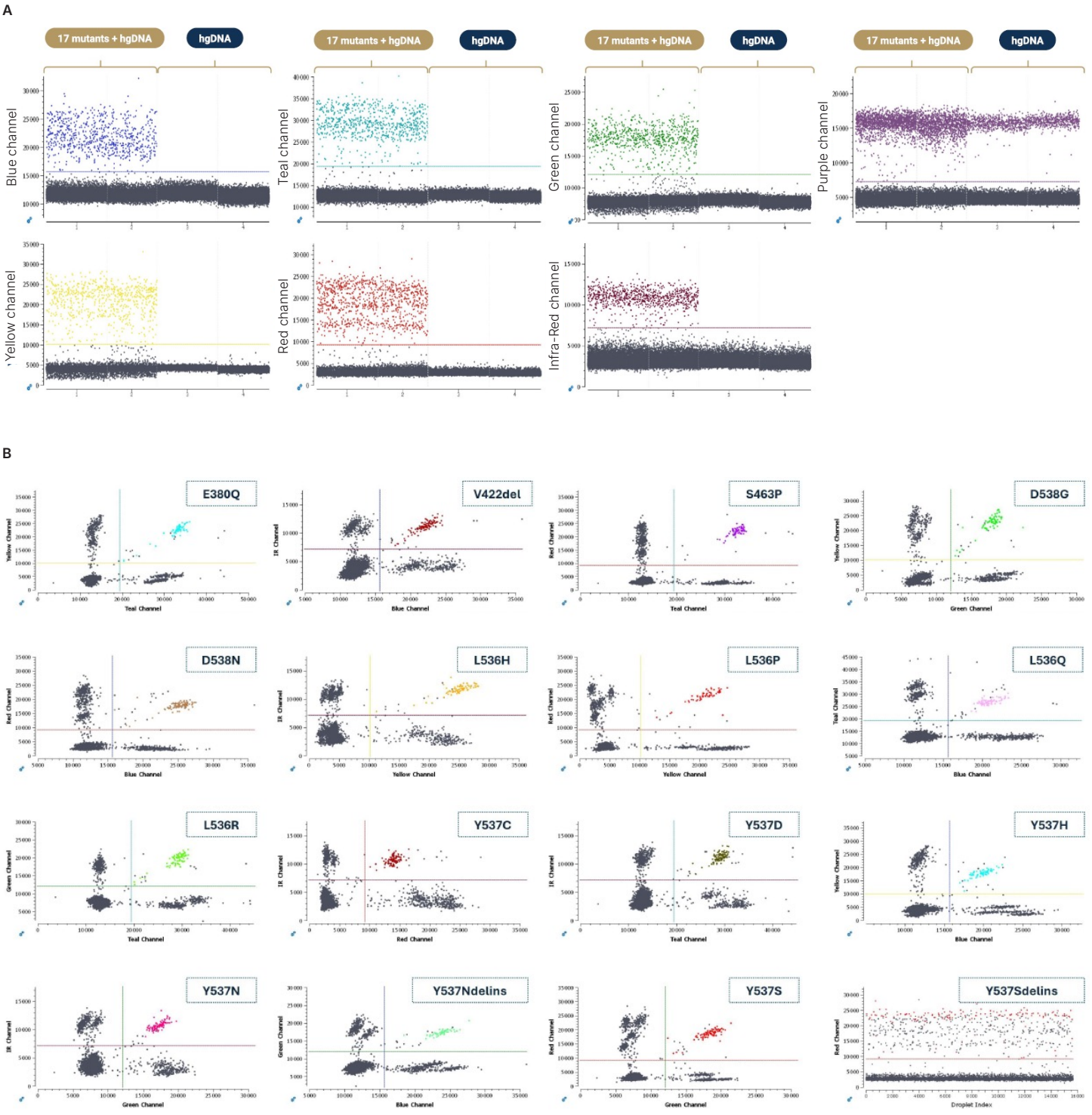


Fig. 4. Analytical validation of the ddPLEX CrystalTag ESR1 17 mut ddPCR Assay with synthetic DNA in research settings. A, DNA 1D plots in the seven detection channels. Chambers 1–2: positive control = hgDNA (≈150 cp/µL) + the 17 mutants (≈25 cp/µL each) targeted in this assay. **B,** visualization of the populations in 1D and 2D plots using QX700 ddPCR System Analysis Software. cp, copies.

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Conclusion

Color-combination multiplexing enables higher-order multiplexing in ddPCR research workflows by extending target detection beyond the number of available fluorescence channels. Combined with population-based analysis and fluorescence compensation workflows in QX700 ddPCR System Analysis Software, this approach supports the identification and quantification of multiple targets within a single reaction in research settings.

The ddPLEX CrystalTag ESR1 17 mut ddPCR Assay demonstrates the feasibility of high-order multiplexing using a color-combination approach under the conditions evaluated in this research study. The enabling factor is CrystalTag technology: non-fluorescent CrystalTag Probes paired with a single set of QX700 CrystalTag Reporters mean that panel expansion requires only sequence-level probe design, not new labeled reagents, which may reduce the need for additional fluorescently labeled probe designs when expanding multiplex panels. As multi-target detection demands grow, CrystalTag technology provides the architecture to meet them without sacrificing the simplicity and quantitative rigor that define ddPCR analysis in research settings.

Visit **bio-rad.com/QX700Family** for more information.

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