High precision multiplexed quantification using 6-color Crystal Digital PCR™ on the naica® system

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Abstract

The genomic analysis of clinical samples, whether it be frozen tissue, FFPE or liquid biopsies, requires the ability to quantify low-level genetic aberrations in a complex DNA background with high precision and sensitivity. Stilla Technologies' 6-color naica® system is an ultrasensitive, easy-to-use digital PCR technology capable of quantifying multiple biomarkers in a single reaction. The naica® system workflow is comprised of optimized master mix reagents, microfluidic consumables, a 2-in-1 droplet generator and thermocycler, and a 6-color imager. For data analysis, the Crystal Miner software allows for flexible sample analysis and inciteful data visualization. Altogether, the naica® system workflow requires only a single pipetting step, thereby considerably limiting the variability contributed by the operator.

The naica® multiplex PCR MIX ensures optimal simultaneous quantification of multiple targets across the five-log dynamic range of the naica® system, as well as robust quantification of low target concentrations in a highly abundant and complex DNA background. R^2 scores > 0.99 were obtained for all targets of a 6-color detection panel ranging from 0.2 to 13000 copies (cp)/µL.

As repeatability is a key performance criterion to evaluate the quality of a digital PCR system and detection assay, we also assessed the intra- and inter-run repeatability of the naica® system with a 6-color assay. Three target concentrations from three different DNA sources were tested at 200, 2000 and 10,000 cp/µL. To allow robust statistical analysis on the means of concentration and variance of concentration, three runs of a total of 36 wells were performed for each target concentration using Stilla Technologies' highly sensitive Sapphire chip. Our results showed that at each of the three tested concentrations, the inter-run and intra-run variability of the 6-color naica® system was less than 6%. For each of the three tested concentrations, no significant inter-run or intra-run differences were found, neither on the concentration level ($p<0.05$, two-tailed Mann-Whitney statistical test) nor on the variance of concentration level ($p<0.01$, two-tailed Mann-Whitney statistical test). The absence of a significant difference implies that among multiple runs neither the well position in the chip nor the chip position in the Geode or Prism6 instrument has a significant impact on the results. Hence, any sample, including positive and negative control samples, can be loaded anywhere on the chips without significantly impacting the results, confirming high performance while ensuring ultimate flexibility in the experiment design.

Introduction

Digital PCR technologies are evolving to provide higher multiplexing capabilities, firstly by increasing the number of fluorescent detection channels available. In 2021, Stilla Technologies commercialized the first 6-color digital PCR platform, the naica® system, marking a milestone in digital PCR technology innovation. To support the development of highly multiplexed Crystal Digital PCR™ (cdPCR) assays, Stilla developed the naica® multiplex PCR MIX, a mastermix for TaqMan®-based assays specifically optimized for high multiplexing. This mix is available at 5x and 10x concentrations, saving reaction volume for increased sample and/or primer and probes input.

Here we show that the 6-color naica® system, including the naica® multiplex PCR MIX and the Prism6 instrument (the 6-color fluorescence reader) provide the user with:

- A robust simultaneous detection of 6 different targets across the 5-log dynamic range of the naica® system with Sapphire Chip.
- Very high precision on the results
- Great flexibility in the experiment design, specifically in the choice of the number of replicates and of the well positions for samples and controls, thanks to a strong repeatability

Methods and Materials

The 6-color Crystal Digital PCR™ workflow

The 6-color Crystal Digital PCR™ workflow **(Figure 1)** enables the highest multiplexing capacity in a single reaction saving both time and precious sample and providing ultrasensitive low-level detection of multiple reactions in parallel. By partitioning sample reactions into a large 2D array of droplets through a confinement gradient, homogeneity in the droplet size is ensured, and the need for oil flow is eliminated. Crystal Digital PCR™ technology can be used for absolute nucleic acid quantification in a wide range of assays including, but not limited to oncology (copy number variation, mutation detection, rare event detection, therapeutic monitoring). Crystal Miner software measures the concentrations of targeted nucleic acids, providing automatic identification of positive and negative droplets for all fluorescence channels and intuitive image analysis.

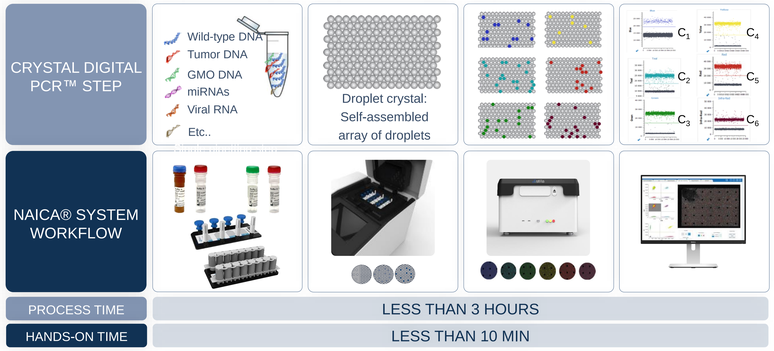

Figure 1. The naica® system: Absolute quantification of multiple genetic targets in a single run The 6-color naica® system is an easy-to-use dPCR platform that harnesses cutting-edge microfluidic technology to integrate the dPCR workflow onto a single consumable chip. The technology, known as Crystal Digital PCR™, partitions samples into a large array of thousands of individual droplet crystals – each its own reaction compartment – before amplifying nucleic acid molecules in each droplet crystal. These reactions are tagged with fluorophores to be read using up to six different fluorescence light channels, maximizing multiplexing capacity. The naica® system makes for a fast and simple workflow that can be completed with less than 10 minutes of hands-on time.

The 6-color assay

- TaqMan®-based
- Six target sequences, each located on a different DNA source
- Different DNA types (human genomic DNA, plasmid DNA, phage) are used to demonstrate the robustness of the system
- The PCR can be assembled with the concentration of each of the six targets tuned independently, with each targeted detected in one of six fluorescent detection channels **(Table 1).**

Target name	DNA Type	Dye	Detection Channel
Lambda	Phage	FAM	Blue
PhiX174	Plasmid	YY	Teal
pUC18	Plasmid	ATTO550	Green
pBR322	Plasmid	ROX	Yellow
pUC57	Plasmid	CY5	Red
ALB	human genomic	ATTO700	InfraRed

Table 1. Target names, DNA sources, fluorophores and detection channels of the 6-color assay.

Results

Robust quantification of one to six targets across the full dynamic range of the naica® system

A DNA sample containing the six targets of the 6-color assay **(Table 1)** all at the same concentration was prepared and serial diluted from 13000 to 0.2 copies (cp)/µL (expected concentrations in the cdPCR reaction).

- R^2 scores > 0.99 for all six targets highlight the reliable simultaneous quantification of six different targets in six independent fluorescence channels **(Figure 2A)**
- At the highest concentration tested (13000 cp/µL for each target), droplets contain an average of 48 amplifiable targets and 99% of droplets contain at least 33 amplifiable targets, showing the system allows high multiplexing and complex amplification in a single reaction.

Serial dilutions from 13000 to 0.2 cp/µL of pUC18 plasmid or pUC57 plasmid were quantified in triplicate in 25 µL Sapphire chip reactions in a background of the five remaining targets each at 3000 cp/µL or in the absence of other DNA targets. In the condition with background DNA, droplets contain on average nine amplifiable targets from the five background targets, yet coefficients of determination $R^2 > 0.99$ for both pUC18 **(Figure 2B and 2C)** and pUC57 (data not shown) showed reliable results for all targets independent of the background context.

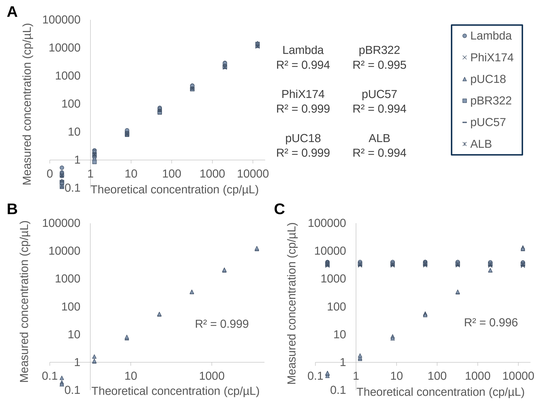

Figure 2. Robust quantification of one to six targets across the dynamic range of the naica® system. A) Serial dilution of a DNA sample containing the six targets of the 6-color assay all at the same concentration showing linear (all R^2>0.99) and sensitive 6-color Crystal Digital PCR™ results across the full dynamic range of Sapphire chips using the 10X naica® multiplex PCR MIX. Serial dilution of pUC18 alone (B) or in a background DNA of the five other targets at 3000 cp/µL each (C) showing the robustness of the linear detection in the multiplexing context. Serial dilutions targeted final concentrations in the cdPCR reaction of 0.2, 1.5, 8.0, 50, 320, 2050 and 13000 cp/µL. Each dilution point was assessed in triplicate in 25 µL Sapphire chip reactions. The amplification of each of the six targets was individually detected in blue, teal, green, yellow, red, and infra-red channels.

High repeatability achieved on the 6-color naica® system

The straightforward workflow, requires a single pipetting step, thereby considerably limiting the variability contributed by the operator. Overall, there are two main sources of dPCR measurement variability:

- Theoretical Poisson uncertainty, an inevitable component that accounts for both sampling error and partitioning error.
- Experimental dPCR measurement variability, inherent to each dPCR workflow (e.g. pipetting step).

Here, we assessed the intra- and inter-run repeatability with a 6-color assay **(Table 1)** having the same expected final target concentration in each color.

- Final target concentrations tested: 200, 2000 and 10,000 cp/µL
- Three runs of three Sapphire chips (= 36 wells) for each concentration
- Controlled conditions: using a single Geode, a single Prism6, on the same day, by the same operator, using the same pipets to depose a single PCR reaction mix for a given concentration.

For each concentration tested and for each channel, the relative standard deviation (RSD) on measured target concentrations was computed. These RSD values are a measure of the total variability obtained when assessing the same sample in 36 replicates. In the described experimental conditions, total variability remains low (RSD <6.3%), with experimental sources of variability representing, on average, half of the total variability (Figure 3).

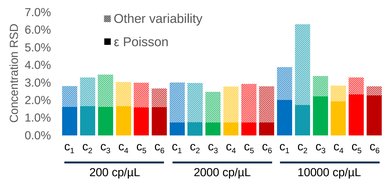

Figure 3. Total measurement variability of the naica® system per channel and for each targeted concentration.

Total measurement variability (full bar and striped bar) is given by the experimental RSD measured on the 36 replicate wells of each condition; the Poisson error is the average of the Poisson RSD estimation obtained on each of the 36 wells.

The questions we asked were:

- Does the well position in a run impact the results on a given sample? In other words, does the system give reproducible results from chip to chip and from well to well?
- Does the system give reproducible results from run to run?

Strong reproducibility would provide flexibility in experimental design by not having to multiply the number of wells dedicated to controls and by being able to place the different samples and their replicate wells in any fashion desired by the experimenter.

To analyze the reproducibility of the system, the 36 wells—all replicates of a given sample—were divided in subgroups in six different ways called D1 to D6 **(Table 2).**

Name of dividing way	Description	# of sub-groups	wells / sub-groups
D1	All the wells of a given run	3	12
D2	All the wells of a given chip in a given run	9	4
D3	All the wells of a given row in a given run	12	3
D4	All the wells of a given chip position in all the runs	3	12
D5	All the wells of a given position (row and chip) in all the runs	12	3
D6	All the wells of a given row in all the runs	4	9

Table 2. Description of the six dividing ways

The average concentrations obtained for the six targets and their variances were computed for each subgroup. For each metric (average concentration or variance of concentration), a Shapiro-Wilk test, using a right-tailed normal distribution determined that all the datasets were not normally distributed at 95% confidence level. For each metric, the datasets from D1, D2 and D3 (intra-run), and then datasets from D4, D5, and D6 (inter-run), were compared pairwise to assess statistical significance via a two-tailed Mann-Whitney statistical test using the following calculator:

https://www.socscistatistics.com/tests/mannwhitney/default2.aspx

No statistically significant (p-value above 0.05 for all comparisons) difference between the average concentration datasets was found. No statistically significant (p-value above 0.01 for D1-D3 of the 200 cp/µL sample and D1-D2, D1-D3, and D4-D5 of the 2000 cp/µL sample; p-value above 0.05 for all other comparisons) difference between the variance of the concentration datasets was found. In other words, the measured variability does not depend on the subgroup division, meaning the naica® system provides very good reproducibility between chips, wells, and runs, in this controlled experimental setting.

Conclusion

- The 6-color naica® system enables an excellent linear quantification of six independent DNA targets across its full dynamic range.
- Quantification of a target DNA is extremely robust to highly multiplexed assays and to a high abundance of background DNA.

Great flexibility in the experiment design:

- Well position in the chip and in the geode among multiples runs has no significant impact on the results → need to dedicate only one well to a control sample for multiple runs.
- A sample can be loaded anywhere in several runs performed in controlled conditions with no significant impact on the results.

For more information about 6-color Crystal Digital PCR™ and other naica® products, visit Stilla Technologies at https://www.stillatechnologies.com/ and posters 534, 527, 75, 2932 and 2942

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