

Five Reporter Multiplex Detection using the Constellation Digital PCR (dPCR) System

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ABSTRACT

The ability to detect and quantitate multiple nucleic acid targets within the same reaction is advantageous in a number of biological applications, including copy number variation, pathogen detection, detection of allelic variants, and simultaneous detection of multiple transcripts^{1,2,3,4}. Researchers attempting to increase the number of assay targets that can be detected in both real-time and digital PCR systems have adopted several strategies for both detection using intercalating dye assays as well as conventional exonuclease probe assays. For intercalating dyes, where the detection of the reporter dye is limited to a single optical channel, the use of multiplexed assays with different amplicon lengths – hence different endpoint fluorescence – has demonstrated successful⁵.

Similarly, for fluorogenic exonuclease assays, the use of the assays with the same reporter dye but altered concentrations⁶ has been useful when the number of optical channels is limited. An extension of this concept – using another optical channel and using mixtures of probe sequences with different reporter ratios – has been successful⁷ as well. Lastly, maximizing the number of optically distinguishable reporters within a single reaction is a highly desirable strategy for increasing the number of detectable assay targets has been successfully demonstrated for up to five different reporters in a real-time PCR instrument⁸.

Although the Constellation plate reader is configured to read up to four optical channels in a single imaging run (one being reserved for ROX passive reference to monitor fluid loading within the partitions), filters for the Constellation plate reader are readily accessible and easily exchanged. Because the Constellation digital PCR system reads assays in 96 well digital PCR plates at the endpoint of the reaction, simply adding filters for additional dyes and re-reading the same endpoint reactions can be accomplished. In an effort to demonstrate the high optical multiplex capability of the Constellation system, five assays with different fluorescent reporters were tested alone or in combination to assess system performance.

METHODS AND MATERIALS

Genomic DNA from CEPH sample NA17245 was obtained from Coriell Cell Repositories and digested at an approximate concentration of 4,000 copies/ μ l with 4 U CviQI (New England Biolabs, cat # R0639S) for 16 hours at 25° C. Extracted, undigested genomic DNA from *Pseudomonas aureus* was supplied as a generous gift from the laboratory of Dr. Vaughn Cooper at the University of New Hampshire, Durham, NH, USA. Custom PrimeTime qPCR assays containing ZEN/IBFQ (from Integrated DNA technologies, Coralville, IA) were designed against human genomic DNA targets for NF2, JUN and GAPDH with FAM, HEX and TET reporters, respectively. A Custom PrimeTime qPCR assay containing a Cy5 reporter and IBRO quencher (from Integrated DNA technologies, Coralville, IA) was designed against the bacterial LacZ gene. A custom assay designed against the human CCND1 gene and containing a NED reporter and MGB quencher was obtained from Life Technologies Foster City, CA. TaqMan Fast Advanced Master Mix (Life Technologies Foster City, CA) was used with either human or bacterial genomic DNA at a final concentration of either 460 copies/ μ L or 205 copies/ μ L respectively.



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Constellation digital PCR system

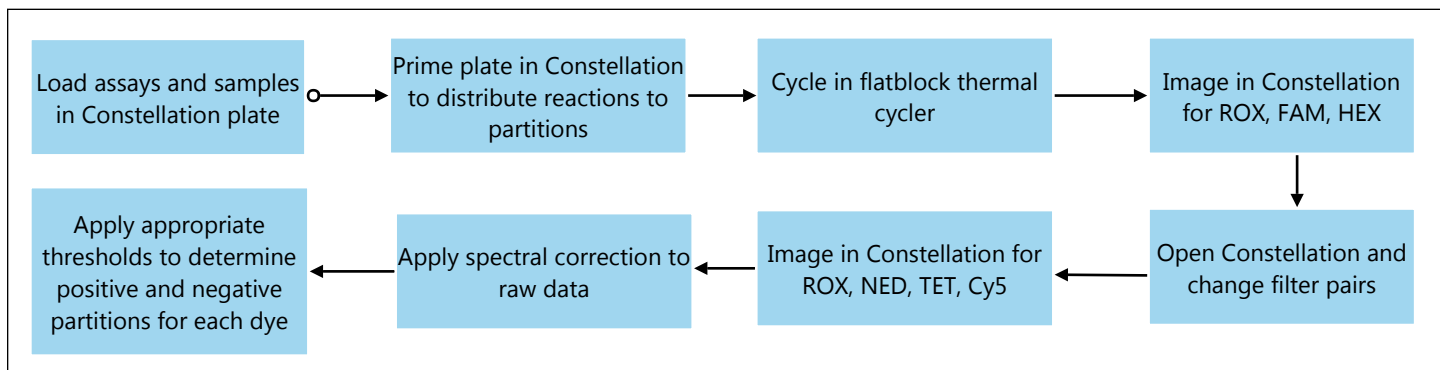


Figure 1. Flowchart of the workflow for multiplex detection of five distinct dye targets using the Constellation digital PCR instrument.

Samples were run (at a final concentration of 1x according to the manufacturer’s instructions) either as a single isolated assay, or in multiplex reaction with every other assay in eight individual wells on a single Constellation plate. Partitions were primed using the Constellation instrument and the entire plate was thermal cycled for 40 cycles in approximately one hour. The plate was imaged in Constellation using filters and exposure times appropriate for detection of dyes ROX, FAM, HEX, NED, TET and Cy5 (Fig. 1). Correction for spectral overlap was done using a correction matrix generated using assessments of the contributions of individual pure dyes for each reporter and optical filter combination. Copy number quantities for each digital PCR sample were determined by the Constellation software for individual wells and the average and standard deviation for replicate reactions are reported.

RESULTS

Using the Constellation digital PCR system, samples of known human and bacterial genomic copies were assayed for using exonuclease assays with different fluorescent reporters as shown in table 1.

The results are shown in Figure 2, with the observed copy number for either each assay tested in isolation or when tested as a component of a multiplexed reaction in competition with each other assay.

For all five assays tested, either as a single independent assay or when in direct competition with all other assays, the expected quantity of input DNA was detected, either approximately 460 copies/μL for human genomic targets or 205 copies/μL for the LacZ genomic assay.

CONCLUSIONS

Using multiplexed fluorogenic exonuclease assays with many as five optically distinguishable reporter dyes can accurately and precisely determine the appropriate copy number using the Constellation dPCR system. Since assays detecting human targets of identical ploidy were chosen the results for each target were expected to be, and were observed to be identical. A spiked in target was chosen specifically to be both of distinct genomic origin and copy number relative to the human assay targets. The expected number of target copies for the bacterial LacZ assay was observed to be both identical to the expected value as well as distinct from the values

Assay Target	Reporter	Quencher	Organism	Expected copies/μL
NF2	FAM	ZEN/IBFQ	Human	460
JUN	HEX	ZEN/IBFQ	Human	460
CCND1	NED	MGB	Human	460
CCND1	TET	ZEN/IBFQ	Human	460
LacZ	Cy5	IRFQ	P. aureus	205

Table 1. Assay information and expected target copies.

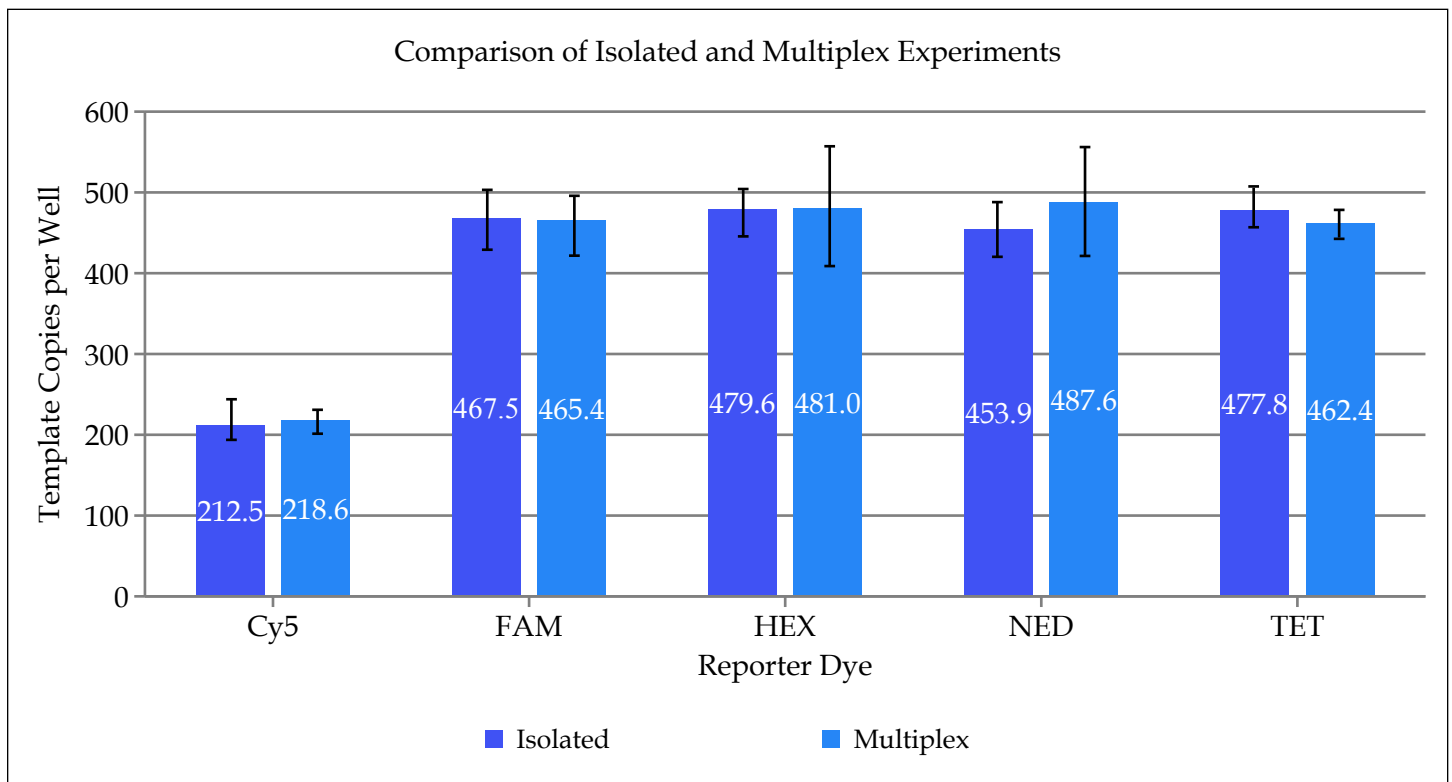


Figure 2. Constellation digital PCR target copy determination for individual and multiplexed assays. Copied detected by assays targeting human genomic DNA, NF2 (FAM reporter), JUN (HEX reporter), CCND1 (NED reporter), GAPDH (TET reporter) were expected to have 460 copies/ μ L while the bacterial LacZ assay (Cy5 reporter) was expected to have 205 copies/ μ L.

observed for the human genomic DNA assays. These observations clearly demonstrate the capability of the Constellation digital PCR system to accurately and precisely detect as many as five optically distinct assay targets within a single reaction, extending the utility of the system for users desiring the ability to simultaneously detect multiple targets within a single reaction.

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