Development of novel high-resolution multiplex digital PCR (HR-mdPCR) approach for detailed evaluation and quantification of vector genome or DNA impurities sequence integrity

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Introduction

The integrity of the viral vector genome or other sequences is becoming an important quality attribute in CGT applications. If the complete viral genome is not present, lower potency can be expected; moreover, incomplete fragments, or presence of DNA impurities, may generate neoantigens in cells leading to unexpected immune responses. The integrity of the viral vector genome or other sequences can be assessed by several methods, each of which has its advantages and disadvantages, however long-read sequencing and multiplex digital PCR are the most commonly used sequence specific technologies. Nevertheless, long-read sequencing is not truly quantitative, and multiplex digital PCR does not provide information on presence/absence of sequences between the targeted regions. To enable sequence integrity analysis, which would overcome the time and price challenge of long-read sequencing, but at the same time provide an absolute quantification of longer targets, we have developed a novel approach called high-resolution multiplex digital PCR (HR-mdPCR).

Conclusions

- The developed 7-plex assay presents a proof-of-principle for high resolution integrity quantification by multiplex digital PCR (HR-mdPCR)
- 7-plex KanR assay provides information on plasmid quantity, together with quantitative information on presence of complete KanR genes and its fragments
- Next step will be to increase the multiplexing level to cover the whole length of AAV vector genomes

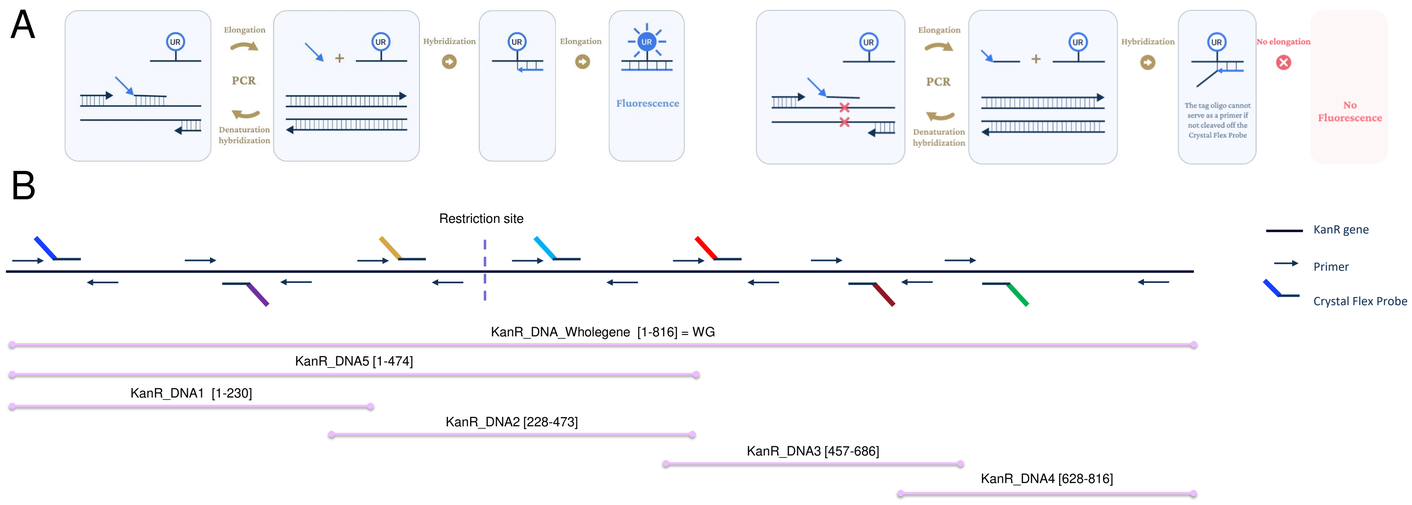
Methods

To show the proof-of-concept we have developed the 7-plex assay to cover the whole length of the nptII (kanamycin resistance; KanR) gene, thus providing high-resolution quantitative insight in the integrity of its sequence. We've used seven different Crystal Flex Probes (Stilla Technologies) for individual targets (Figure 1).
All of the experiments were conducted using Nio™+ Digital PCR system and analyzed using the Nio™ Analyzer software (Stilla Technologies).
A lot of effort was given into optimization of the assays in multiplex combination to obtain the best performance. The assay was further tested on complete or fragmented plasmid material, as well as on AAV samples as an impurity evaluation assay to show it performs well also in a background of AAV vector genomes.

Results

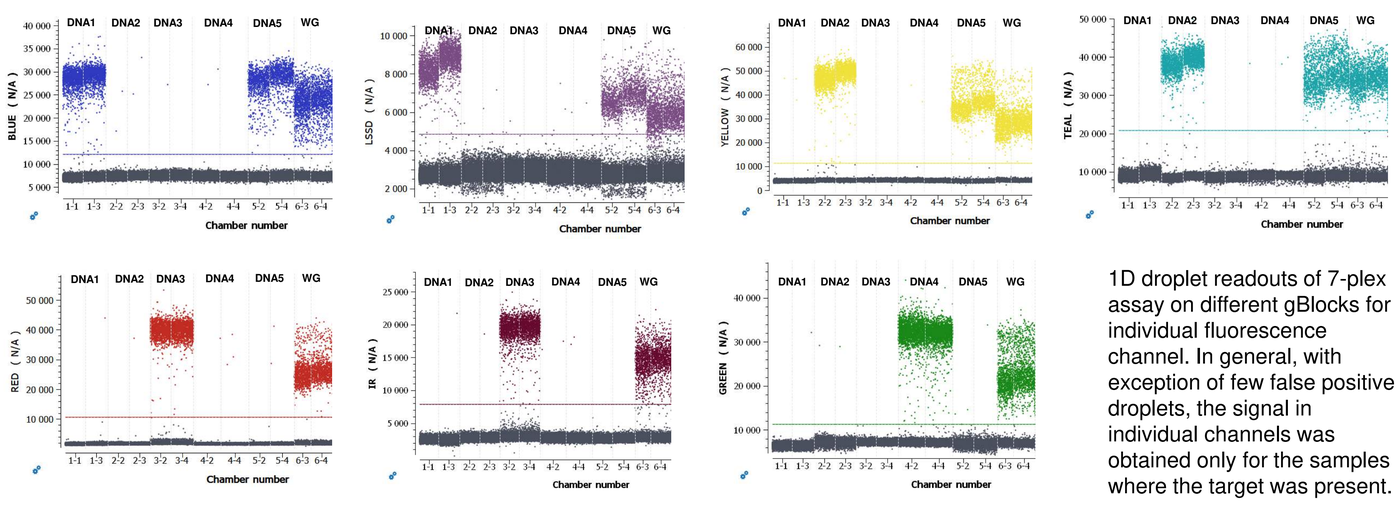
- Specific signal for present targets for each of the individual assays (Figure 2). In case of controlled fragmentation (restriction) the integrity of the sequence produced expected results (Figure 3). Triple and quadruple positive droplets were detected if KanR gene was restricted after the position of third assay (Figure 1B, approximately 2.5 % of the gene remained unrestricted).
- In general, approximately 5-10 copies per reaction were detected for unexpected fragments (to be determined if these are false positive signals or actual fragments present in the sample).
- Complete and incomplete KanR genes were detected and quantified in AAV sample. Complete genomes represented 61 % of all detected copies (Table 1). 7 out of 127 possible combinations represented additional 26 % of copies.
- When AAV samples were spiked with additional KanR genes (plasmid or gBlocks) the quantified concentration corresponded to the quantity of fragments present in AAV sample together with the spike sample (Table 1).
- The number of possible combinations of positive signals is complex and will need to be automated in the next step to facilitate the analysis.

Figure 1. Crystal Flex Probe principle and amplicon positions on KanR gene



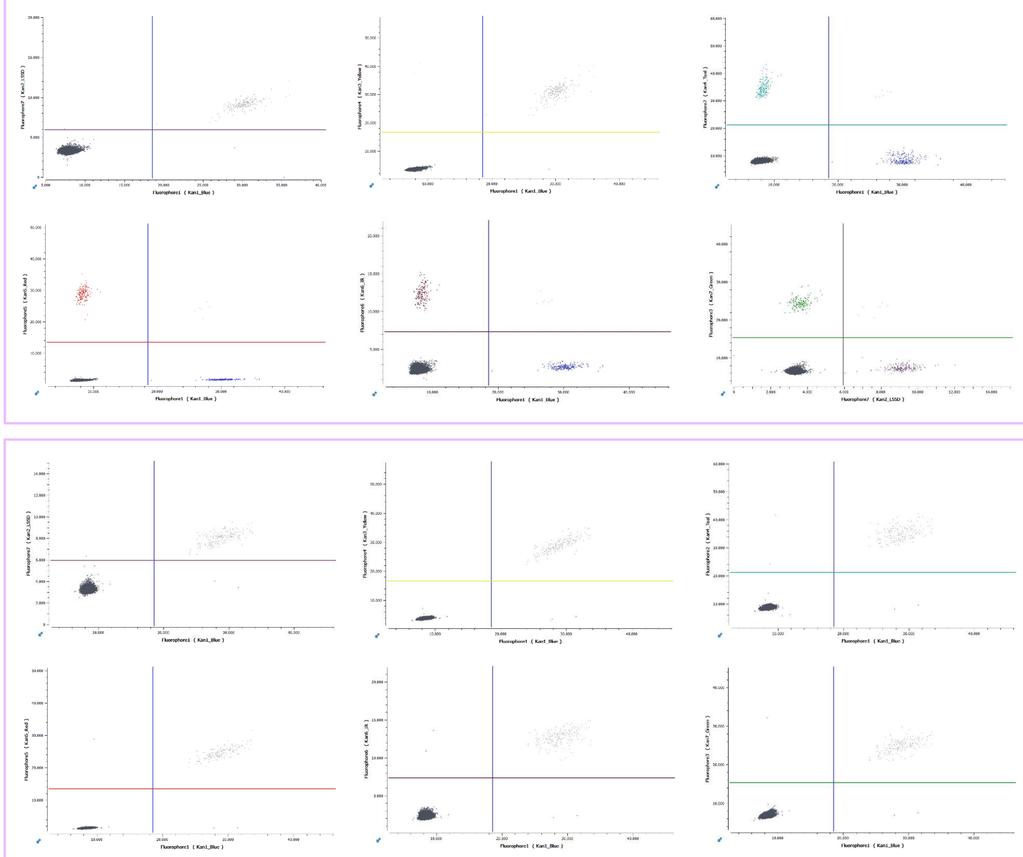
A) Principle of Crystal Flex Probes, where part of Flex probe is compatible to the reporter. Once target is amplified the tail is released and acts as a primer for the reporter. Reporter can emit fluorescence only if the second strand was elongated. B) Positions of primers and probes on the KanR gene to enable coverage of the whole gene. gBlocks that were used for optimization are shown below the scheme.

Figure 2. Droplet readouts of 7-plex assay on a set of different DNA fragments



1D droplet readouts of 7-plex assay on different gBlocks for individual fluorescence channel. In general, with exception of few false positive droplets, the signal in individual channels was obtained only for the samples where the target was present.

Figure 3. 2D droplet readouts of 7-plex assay on complete and restricted KanR gene



Restricted KanR gene

2D droplet readout combinations of Blue assay with all other assays from closest to the most distant. Double positive droplets were observed only in the case when the assays were targeting the same fragment. After the restriction site between Yellow and Teal assay single positive clusters are present (some double positive droplets indicate incomplete restriction).

Complete KanR gene

2D droplet readout combinations of Blue assay with all other assays from closest to the most distant. Double positive droplets were observed in all combinations since targets were present on the same molecule (KanR gene).

Table 1: Percentage of fragments after spiking of AAV sample

	AAV spiked with:	DNA1	DNA2	DNA3	DNA4	DNA5	plasmid	none
signal in channel	Green	2	0	0	**40**	0	1	0
	Yellow, Teal	0	**28**	0	0	4	0	0
	RED, IR	0	0	**29**	0	0	0	0
	Blue, LSSD	**28**	1	1	1	2	1	1
	Blue, LSSD, Yellow, Teal	1	1	1	0	**23**	1	1
	All (complete gene)	30	37	38	38	36	**71**	**61**

Nio™ Digital PCR all-in-one dPCR system