Plasmid impurity sizing of the *nptII* kanamycin resistance gene in rAAV by ddPCR

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Introduction

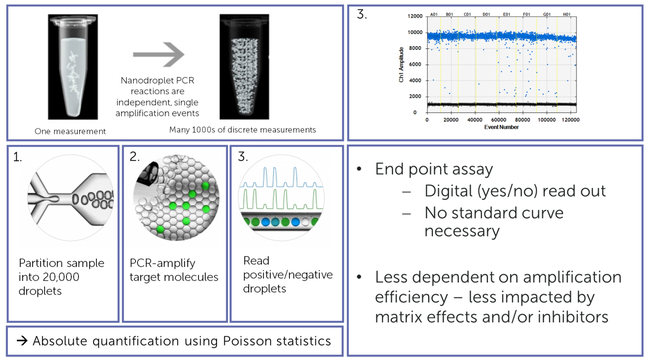
Recombinant adeno-associated virus (rAAV)-encapsidated plasmid impurities are an undesirable byproduct of vector manufacturing consisting of heterogeneous (non-vector genome) fragments of DNA arising from the production plasmids.

Regulatory authorities require thorough quantification of DNA impurities, but current qPCR methods used to detect impurities are based on quantification of small fragments of the sequence of interest. Therefore, these methods provide very limited information on the size distribution of mispackaged DNA species.

Horizontal gene transfer of manufacturing plasmid-derived resistance genes is a potential risk when they are packaged as full-length genes. However, this risk may not be identified by currently available analytical methods. To achieve a comprehensive understanding of the impact of packaged DNA sequences, we developed a panel of orthogonal methods such as LR-NGS or transcriptional profiling.

Here, we describe a novel ddPCR method for assessing the size profile of plasmid-derived antibiotic resistance gene impurities in rAAV products.

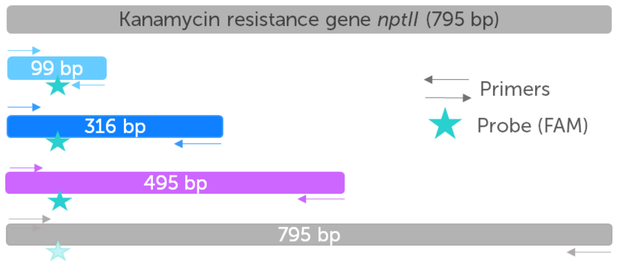
Droplet digital (dd) PCR



- End point assay
  - Digital (yes/no) read out
  - No standard curve necessary
- Less dependent on amplification efficiency – less impacted by matrix effects and/or inhibitors

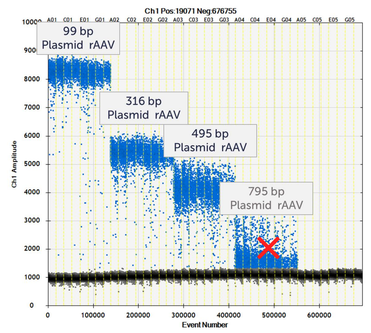
Adapted from: Bulletin_6407.pdf (bio-rad.com)

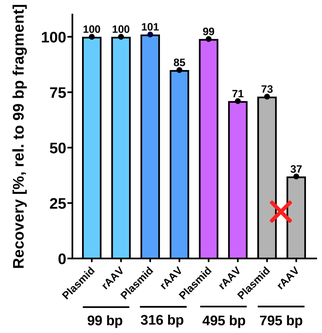
Assay setup – singleplex ddPCR



- Establishment of four singleplex ddPCRs
- Same forward primer and FAM-labeled probe used in all sets
- Reverse primer progressively moved towards 3' end of the gene to increase fragment length
- Linearized plasmid used as a control to assess recovery (>90%)

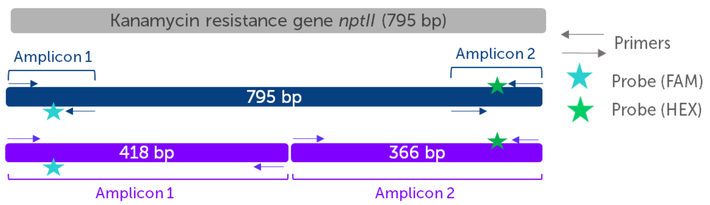
Good recovery of fragments up to 495 bp using a full-length plasmid control and lower proportion of longer fragments in rAAV by singleplex ddPCR





- Good droplet separation and recovery of plasmid for fragments up to 495 bp
- Singleplex approach not feasible for full-length (795 bp) analysis (low recovery of plasmid)

Assay setup – duplex ddPCR



- Double-positive droplets are indicative of DNA fragments containing both amplicons („full-length" species)
- Linearized plasmid used as a recovery control (>90% linked DNA species)
- Probable over-estimation of full-length species due to co-packaging of two small *nptII* gene fragments in one capsid (approach 1)
- Assessment of two longer amplicons reduces risk of false double-positive droplets (approach 2)

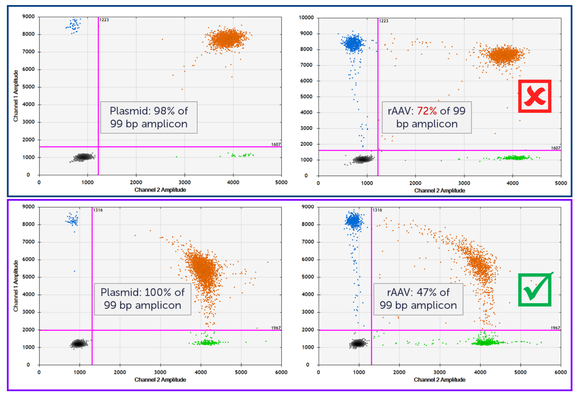
Good recovery of full-length species using a plasmid control and lower proportion of full-length species in rAAV by long amplicon duplex ddPCR

Approach 1

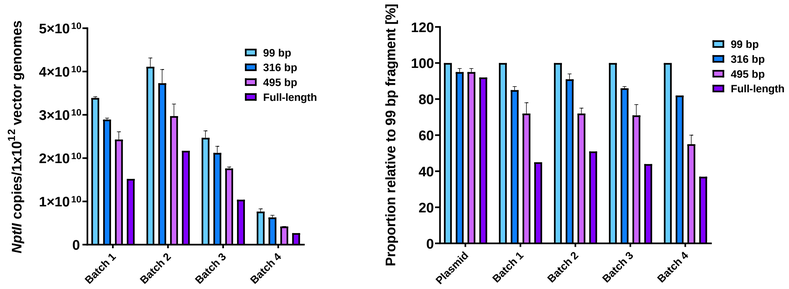
Co-packaged small fragments and full-length genomes contribute to double-positive droplets

Approach 2

Only full-length genomes contribute to double-positive droplets



Sizing assay allows impact assessment of biological starting materials and process parameters on nptII impurity profile of four large-scale batches



- Favorable impurity levels and size profile observed for one batch (Batch 4) that was produced with an improved manufacturing system (>80% reduction in absolute *nptII* copy numbers and up to 15% reduction of larger fragments when compared to Batch 2)

Summary

The method described here meets requirements for analysis of the size profile of packaged kanamycin (*nptII*) resistance gene impurities. Furthermore, the assay enables the assessment of the effects of different biological starting materials and process parameters on the *nptII* impurity size profile.

The data generated from this method can form part of a comprehensive assessment of the potential risk from packaged plasmid impurities. Learnings from this study are readily transferable to sizing assays for DNA of all kinds derived from starting materials used for rAAV manufacturing.

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