Impact of sample treatment on DNA length distribution analysis by duplex ddPCR

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M MAGERL, F THOENNISSEN, S BEER, RC FEINER, M HOERER, SM SCHERMANN, S GEIGER

Abstract 1438

Abstract

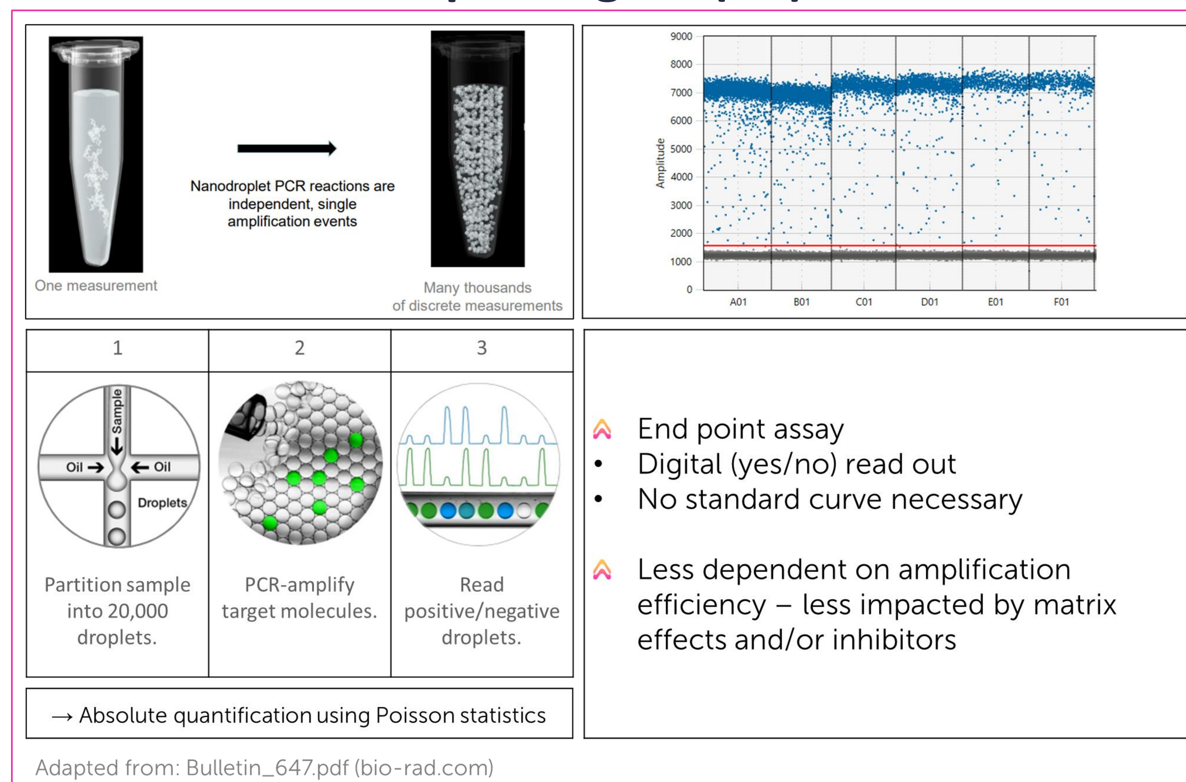
Recombinant adeno-associated virus (rAAV)-encapsidated plasmid impurities are an undesirable byproduct of vector manufacturing consisting of heterogeneous non-vector genome fragments of DNA arising from the production plasmids.

Horizontal gene transfer of manufacturing plasmid-derived resistance genes might be a potential risk when they are packaged as full-length genes.

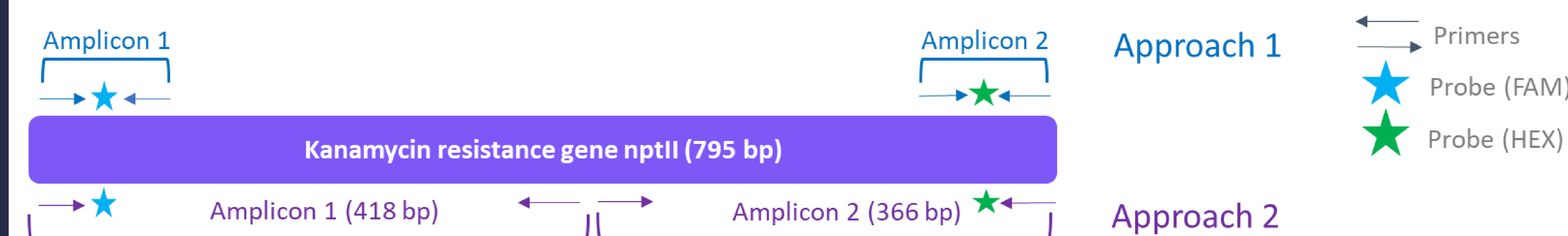
Thus, regulatory authorities require thorough characterization of these DNA impurities. We developed a novel duplex ddPCR method for assessing the length distribution of encapsidated nptII (kanamycin resistance) gene fragments.

Here we describe how typical sample treatments used to disrupt virus capsids prior to droplet formation can impact fragment length analysis performed by duplex ddPCR.

Droplet digital (dd) PCR



Assay setup – duplex ddPCRs for nptII kanamycin resistance gene (kanR)

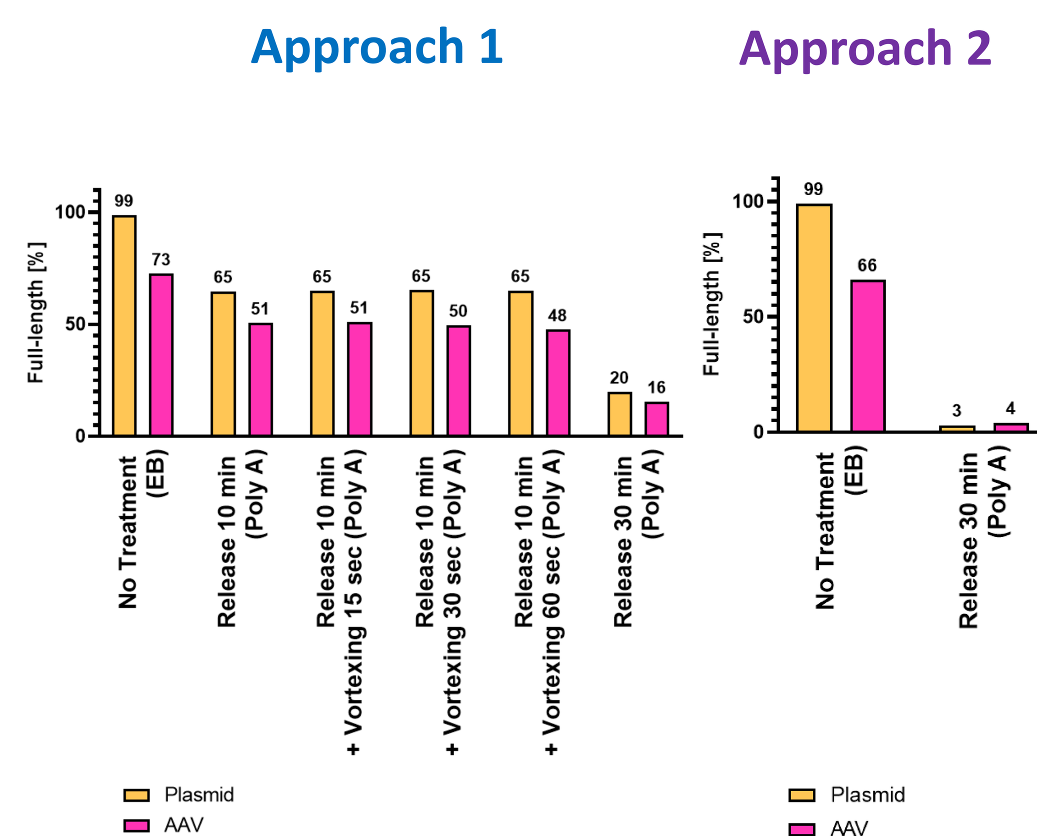


- Establishment of two duplex ddPCRs to assess full-length nptII gene species:
  - Approach 1: Two primer-probe sets with different fluorophores positioned 795 bp apart → risk of over-estimation and reporting of full-length species in CMC submissions due to co-packaging of two small nptII gene fragments in one capsid.
  - Approach 2: Two primer-probe sets with different fluorophores spanning approx. 400 bp fragments at each side of the gene → reduction of the risk of false double-positive droplets.

Assay setup – sample treatment

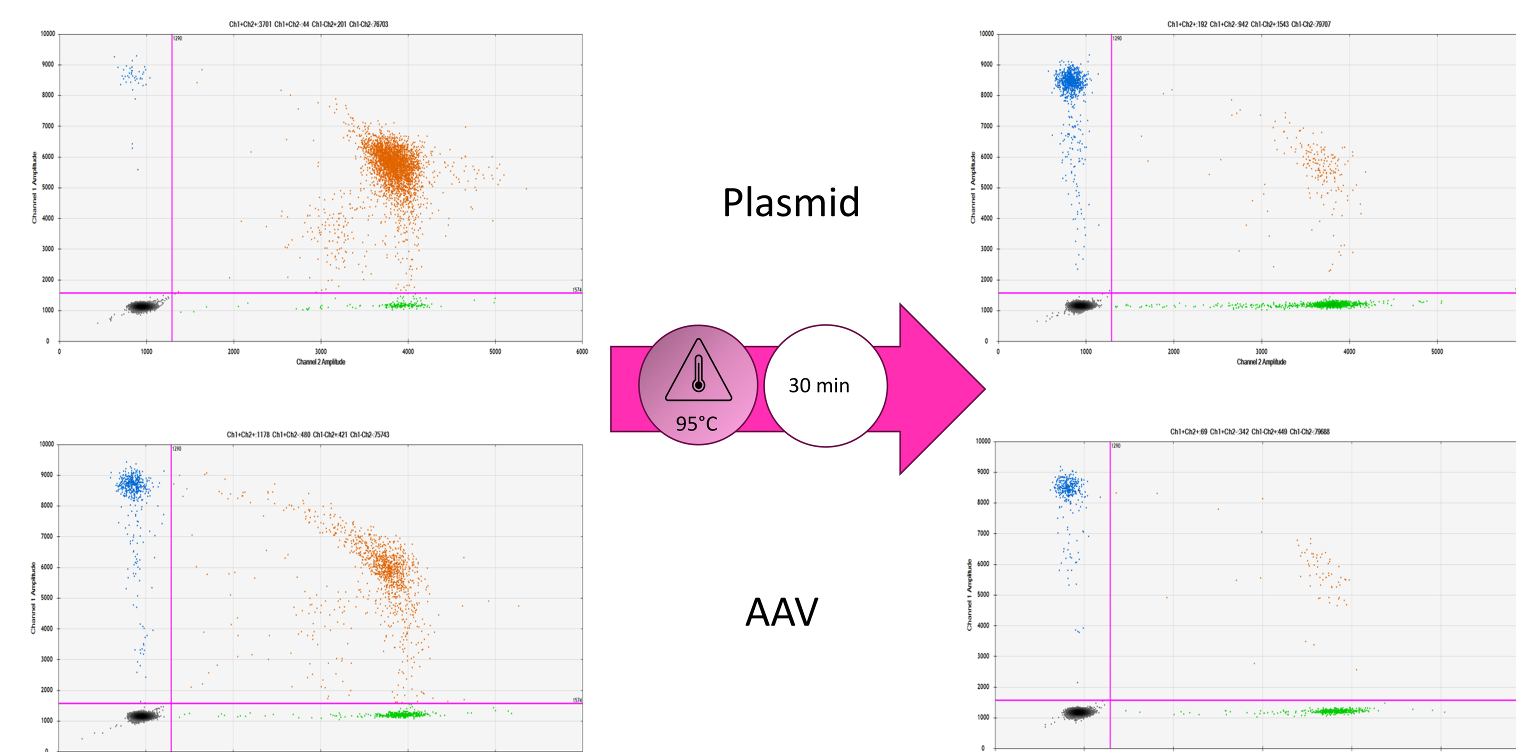
Prolonged heat treatment distorts DNA size analyses

- Samples without treatment showed the expected results.
- Dramatic decrease in full-length species after heat treatment for 30 minutes.
- Heat treatment for 10 minutes caused less fragmentation.
- Mechanical manipulation (vortexing) did not cause DNA fragmentation.



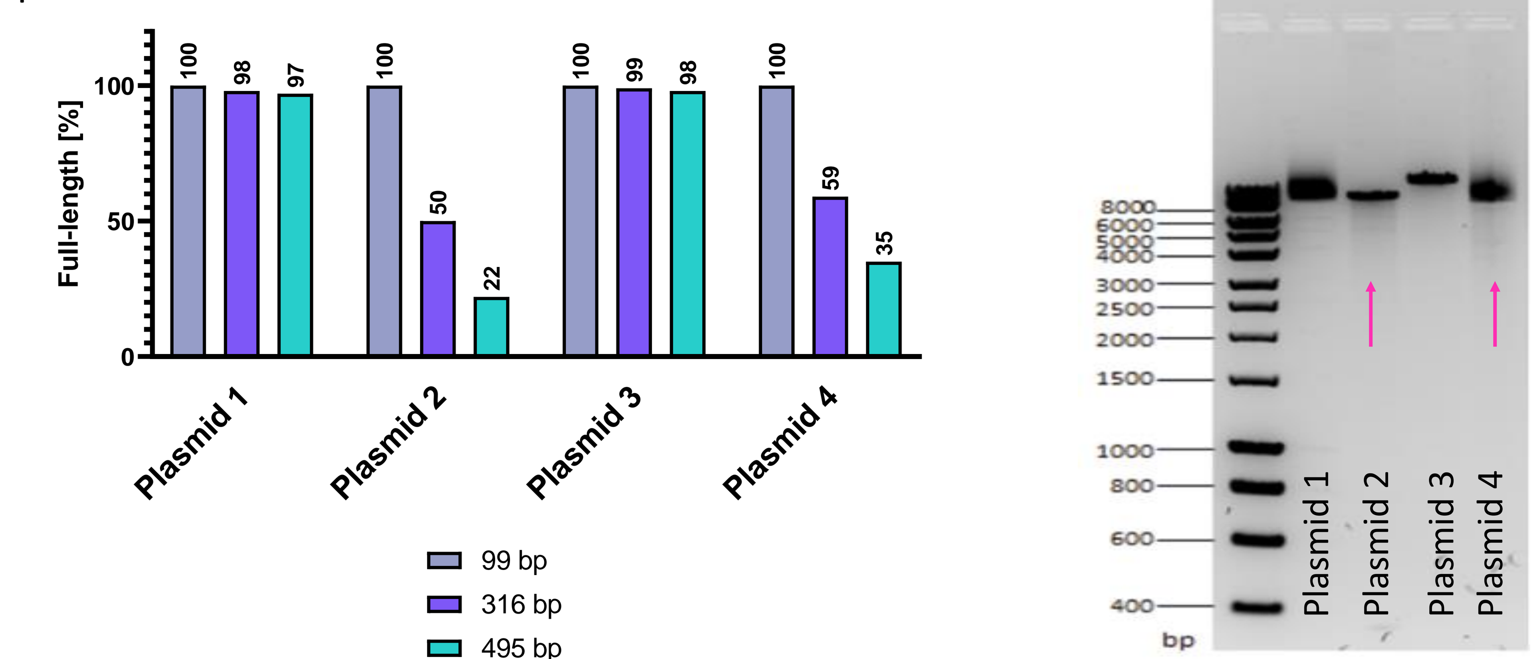
2D plots - pre and post heat treatment

- Heat treatment causes fragmentation of DNA as shown by a dramatic reduction of linked species in 2D cluster plot.



Quality control of plasmid standards

- Plasmids used as standards for PCR assays can degrade unnoticed over time.
- Degradation of Plasmids 2 and 4 observed in ddPCR sizing assay was confirmed by agarose gel analysis.
- The ddPCR sizing assay can be used to generate meaningful data on the quality of plasmid standards.



Summary

Care must be taken during sample treatment since commonly used methods such as heat treatment can quickly result in fragmentation of DNA, leading to an underestimation of full-length impurity species. Separation of intact capsids into droplets prior to heat disruption ensures that DNA fragments are distributed into individual droplets in one piece. With the second duplex ddPCR approach (using primer/probe sets spanning approximately 400 bp at each side of the gene) we have developed a robust assay for highly reliable reporting of DNA sizing data that are requested by regulatory bodies in risk assessments.

Aim higher

Ascend Advanced Therapies GmbH | Fraunhoferstraße 9b | 82152 Planegg | Germany

www.ascend-adv.com
Marina.Magerl@ascend-adv.com