A Comprehensive ddPCR Portfolio for In-depth DNA Impurity Profiling

ascend

Sonya M Schermann, Sabine Geiger, Renée Kober, Felicia Thoennissen, Marina Magerl, Sophie Beer, Markus Hoerer

Abstract 890

Abstract

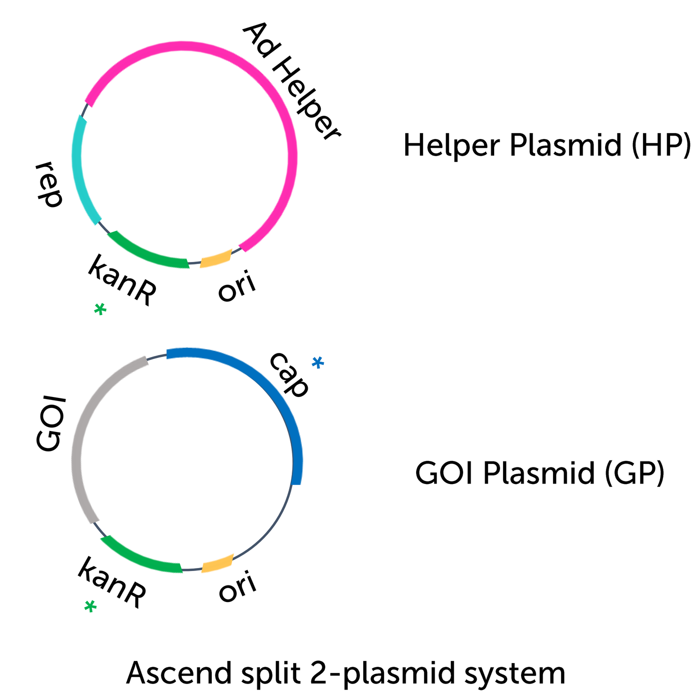
DNA impurities present in recombinant adeno-associated virus (rAAV) products pose a potential risk to patients and therefore need to be thoroughly understood. Methods are required for quality control (QC) of final product and also for extended product characterization. Here we discuss some of the ways in which multiplex digital droplet polymerase chain reaction (ddPCR) can be used in these two applications.

For QC release, multiplexing can be used to monitor multiple DNA impurities in a single assay, reducing the number of assays required for release of product and thereby minimizing the time and cost of batch release. Here we present a duplex method for monitoring residual DNA impurities of different origin and discuss aspects of method development and qualification/validation of such assays.

For extended product characterization, we show how multiplexing can be used in a variety of applications, for example in understanding the length profile of encapsidated plasmid or host cell DNA, which is an important attribute when considering potential risk to patients of such encapsidated sequences. Long read NGS sequencing established in-house is used as an orthogonal method to validate the results obtained. We also demonstrate applications in which multiplex ddPCR can be used to interrogate whether two different sequences are present in the same capsid, for example residual rep and cap DNA, an application which can be used to predict the potential for rAAV batches to contain replication competent AAV (rcAAV).

Taken together, such multiplex ddPCR methods represent powerful tools to determine not only the presence, but the amount, size and identity of encapsidated residual DNA and as such can be used both in QC and in extended characterization of rAAV products.

Two for one: a single QC assay to measure two plasmid impurities



Specificity is demonstrated by detection of cap only on the GOI plasmid and kanR on both plasmids

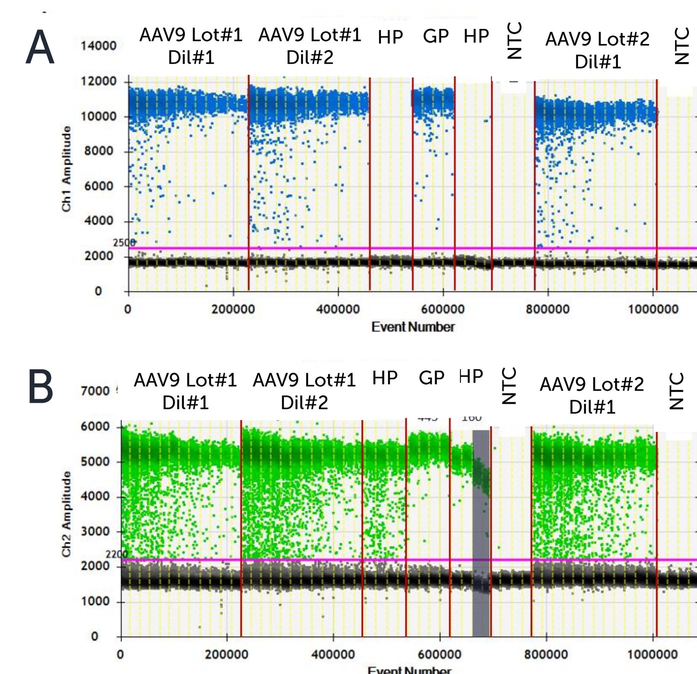


Figure 1. Duplex ddPCR assay with (A) FAM-labelled cap and (B) HEX-labelled kanR

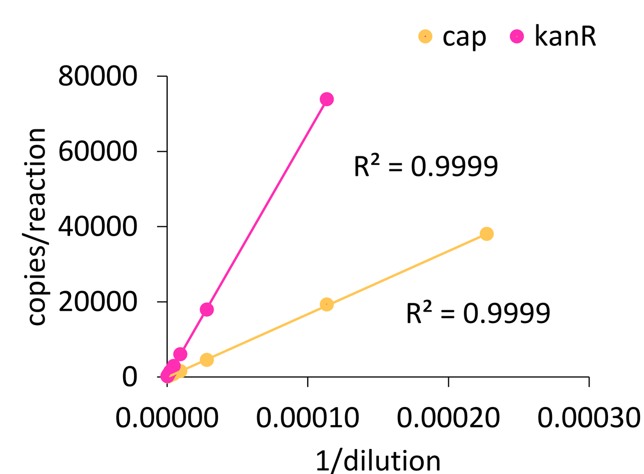


Figure 2. Linearity is demonstrated across a range of dilutions for both impurities

Parameter	Result	
	cap	kanR
Repeatability	≤ 1.5% CV	≤ 1.8% CV
Intermediate precision	≤ 5.1% CV	≤ 3.7% CV
Accuracy	93.4 – 98.9%	93.1 – 98.6 %

Table 1. Results for selected validation parameters

Key Considerations for Study Sponsors

- Measuring two impurities in a single assay lowers costs and reduces volume required for testing
- Primers for multiple cap serotypes and nptI or nptII kanR can be used in the assay
- Multiplex assays can be designed to monitor any combination of DNA impurities which are within the linear range of the ddPCR assay

Length profiling of encapsidated plasmid and host cell DNA

AAV containing DNA impurity corresponding to a full-length gene
→ Higher risk to patients

AAV containing small fragments (< 200 bp) of DNA impurities
→ Lower risk to patients

Characterising the length of mispackaged DNA and understanding the associated risks is a regulatory expectation

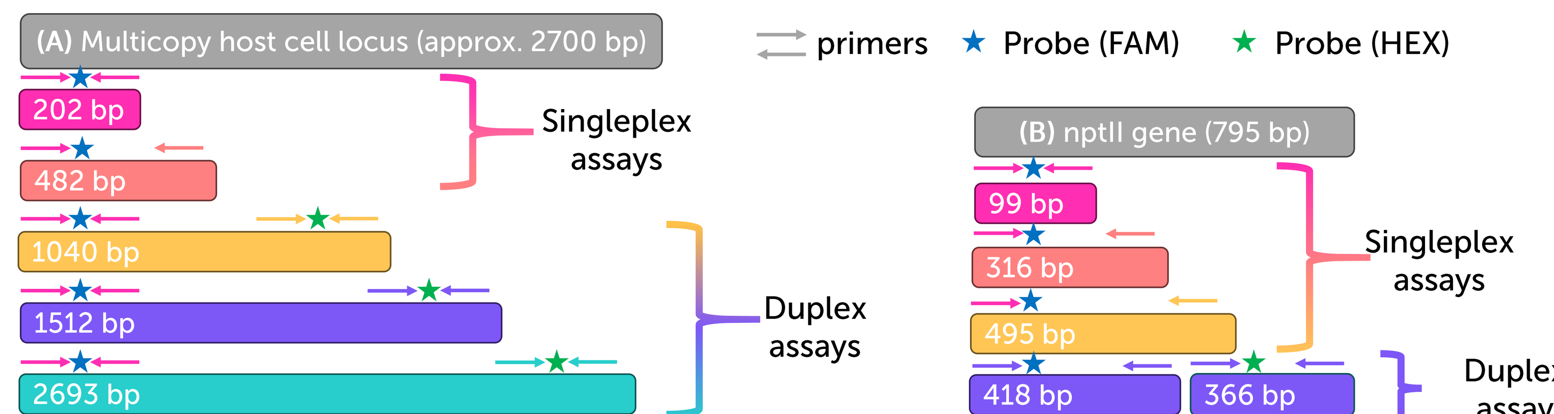


Figure 3. Assay setup for monitoring length of (A) large, e.g. certain host cell-derived genes and for (B) smaller genes, e.g. nptII (kanamycin resistance).
Singleplex assays are used for fragments up to approx. 500 bp and longer fragments can be monitored using duplex assays with small (<200 bp) amplicons at either end of a long gene or lager (up to 500 bp) amplicons to cover the full length of a smaller gene *

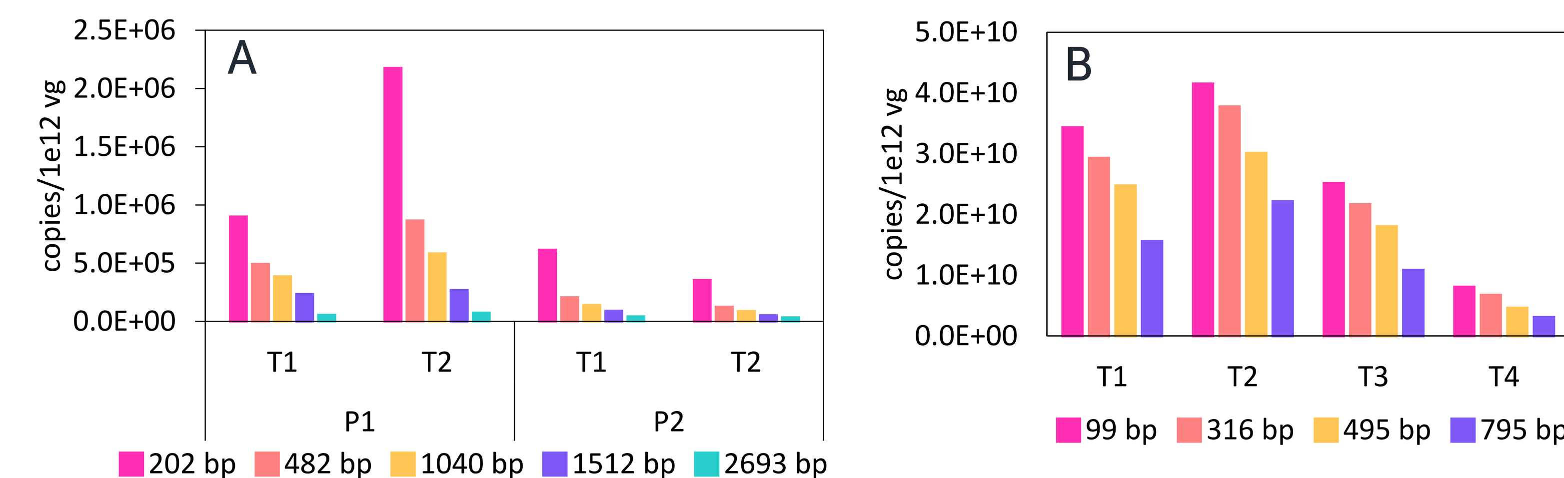
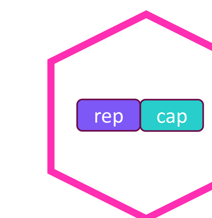


Figure 4. Exemplary data for (A) HCD sizing, showing results from two transgenes (T1 and T2) and two processes (P1 and P2) and for (B) kanR sizing, showing results from four transgenes (T1 to T4)

Key Considerations for Study Sponsors

- Sizing of mispackaged DNA and an understanding of the associated risks is a regulatory expectation
- While both small and large fragments pose an immunogenicity risk, larger fragments may additionally pose a risk due to expression of e.g. oncogenes
- NGS sequencing capabilities complement the multiplex ddPCR data to generate a robust assessment of risk to patients

Presence of rep and cap in a single capsid



Replication competent AAV (rcAAV), containing functional AAV rep and cap genes flanked by ITR sequences in a single capsid, is considered a critical quality attribute for AAVs

A duplex ddPCR design can be used to determine how many capsids contain both rep and cap gene fragments, to estimate likelihood of rcAAV formation *

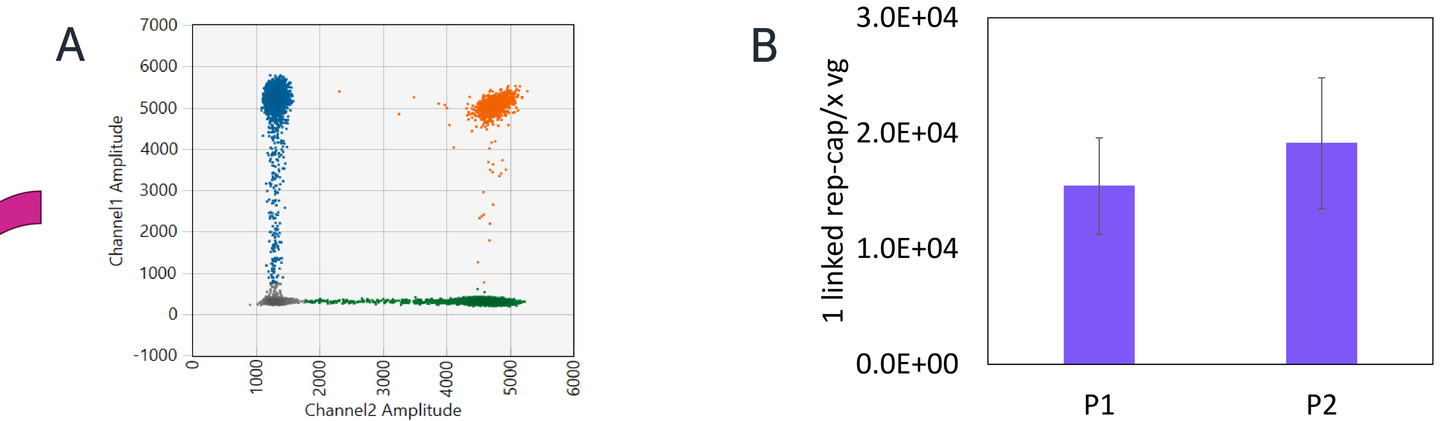


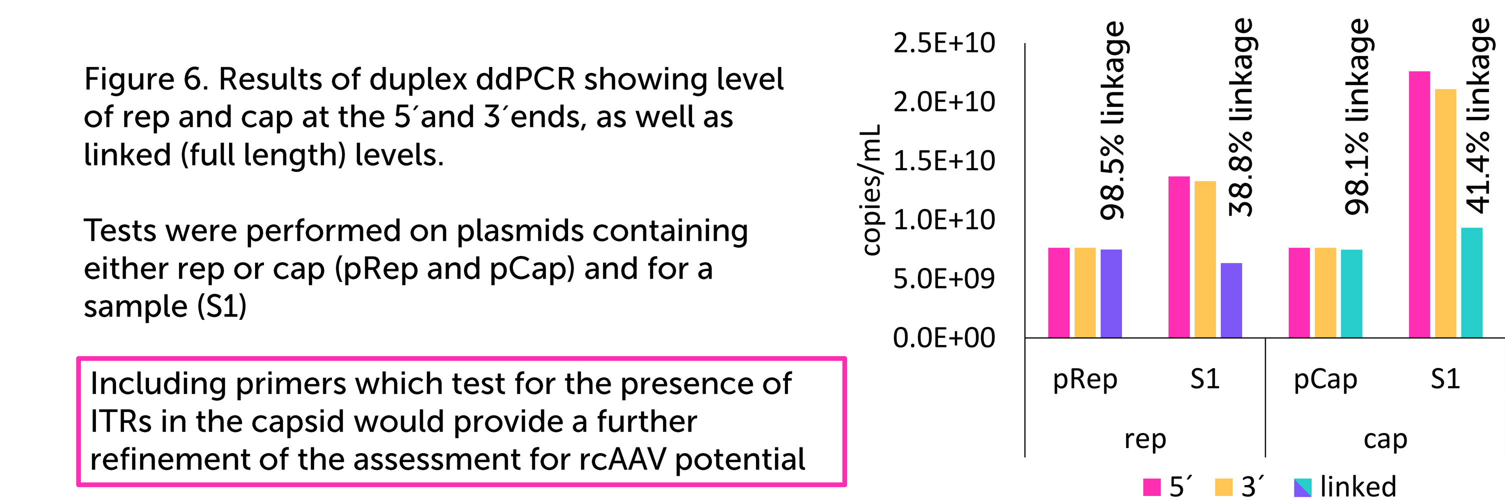
Figure 5. (A) Droplet clusters for an assay in which small (< 150 bp) amplicons for rep (FAM) and cap (HEX) are tested for linkage (B) Results for two different processes, expressed as 1 linked rep-cap/x vg, i.e. higher values indicate a higher risk for rcAAV

Because data are based on small amplicons only, presence of linked rep-cap only indicates potential for rcAAV. Sizing data (below) can further be used to refine assessment of rcAAV potential.

Figure 6. Results of duplex ddPCR showing level of rep and cap at the 5´and 3´ends, as well as linked (full length) levels.

Tests were performed on plasmids containing either rep or cap (pRep and pCap) and for a sample (S1)

Including primers which test for the presence of ITRs in the capsid would provide a further refinement of the assessment for rcAAV potential



Key Considerations for Study Sponsors

- Duplex cap/rep assays are a higher throughput alternative to rcAAV testing during development
- The success of starting material strategies with the potential to decrease rcAAV, such as the Ascend split-plasmid system, can be demonstrated using the method
- Multiplexing could be used to determine presence of both full length rep, full length cap and ITRs within the same capsid

* See also Poster 1438 on May 10th for important considerations in sample preparation for duplex methods

Summary

This poster has demonstrated the versatility of duplex ddPCR for profiling DNA impurities in AAV preparations. Three examples have been demonstrated: 1. A QC assay using duplex ddPCR to monitor two impurities, thereby reducing costs and sample usage. 2. A characterization assay for determining the length of packaged impurities, a parameter which is important for assessing the potential risk of those sequences to patients. 3. An assay which can estimate the potential for production of rcAAV which can be used as a higher throughput alternative to rcAAV testing to develop starting materials and processes which minimize this impurity. Study sponsors can use these assays to provide regulatory authorities with confidence that they have a good understanding of the amounts, size and identity of the DNA impurities packaged within their AAVs. The information generated by the assays can become a key component of the assessment of the risk that packaged DNA impurities pose to patients.

Aim higher

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

www.ascend-adv.com
sonya.schermann@ascend-adv.com

Poster downloads