The Fab Four – validated GMP QC assays to more reliably assess DNA impurities for rAAV batch release

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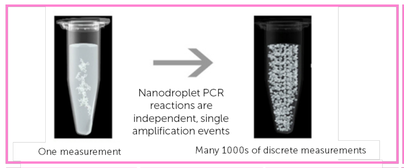
Poster 1323

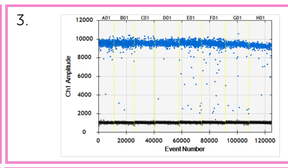
Introduction

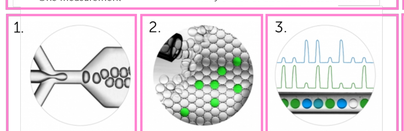
During recombinant adeno-associated virus (rAAV) production, the majority of capsids efficiently encapsidate the intended full-length transgene cassette flanked by AAV inverted terminal repeats (ITRs).
However, a small proportion of the packaged DNA consists of undesirable fragments which mainly originate from residual plasmid DNA or host cell genomic DNA. These impurities are an unavoidable byproduct of vector manufacturing, and regulatory authorities mandate their rigorous characterization and quantification to ensure that they do not compromise the safety profile and overall efficacy of the final therapeutic product.

In response to these regulatory requirements, we present a comprehensive panel of GMP-compliant QC assays specifically designed to assess DNA impurities in rAAV products.

Droplet digital (dd)PCR





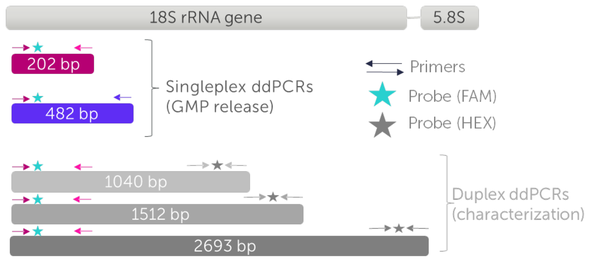


→ Absolute quantification using Poisson statistics

- End point assay
  - Digital (yes/no) read out
  - No requirement for standard curves, simplifying the assay, reducing costs & eliminating the need for bridging
- Less dependent on amplification efficiency – superior resistance to matrix interference/inhibitors

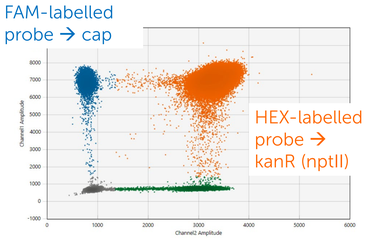
Adapted from: Bulletin_6407.pdf (bio-rad.com)

"Come Together"– Two 18S fragment length ddPCRs allow comprehensive profiling of host cell-derived DNA



- 45S ribosomal RNA gene as a surrogate for host cell-derived DNA
- Regulatory authorities require characterization of fragments > 200 bp
- Two singleplex assays for fragment lengths up to ~500 bp validated for GMP release
- Duplex assays for fragment lengths up to 2.7 kb for characterization in place
- Conversion of 18S copies/10^{12} vg to ng/mL possible – dependent on the producer cell line used

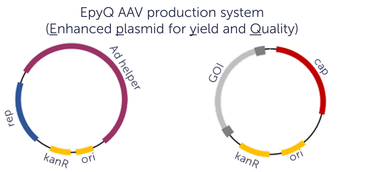
"Two of Us" – One assay, twice the insight: duplex ddPCR for cap and kanR impurities, saving costs, time and sample material



List of AAV serotpyes covered by our duplex ddPCR approach	
AAV1	AAV6
AAV2	AAV8
AAV3B	AAV9
LK03	hu37
AAV5	rh10 & rh74

- Simultaneous assessment of AAV cap and bacterial kanamycin resistance gene DNA impurities using a duplex ddPCR approach
- Duplex method fully validated under GMP; due to universal primer/probe design available for multiple serotypes – product-specific validation required

"Every Little Thing" – Reliable detection of rare E4ORF6 impurities



Contains **adenoviral helper genes (e.g. E4ORF6)** along-side **rep gene** essential for replication & capsid packaging

Contains **therapeutic gene of interest** alongside **cap gene** for essential proteins VP1, VP2, VP3

- Sensitive ddPCR assay enables detection of even minimal traces of plasmid-derived E4ORF6 DNA
- Critical impurity due to potential interactions with other oncogenic gene products
- Still largely absent from current release and characterization testing panels

Establishing meaningful and stringent validation criteria allows demonstration of high-quality assay performance

Parameter (ICH Q2)	Acceptance criterion
Specificity – no interference from buffer compounds	• Confirmation of dilutional linearity
Specificity – no interference from DNA impurities	• Recovery 80 – 120% compared to same non-spiked sample • CVs ≤ 15% (dilution replicates and overall) • Copy number < LLOQ when adding the genomic or plasmid DNA spike to the NTC
Range (linear response, validation of lower range limits)	• $r^2 \geq 0.98$ across at least 5 dilutions • Recovery 80 – 120% compared to sample with highest concentration • CVs ≤ 15% (dilution replicates and overall)
Accuracy	• Recovery of plasmid copies = 80 – 120% compared to orthogonal spectrophotometric measurement • CVs ≤ 15% (dilution replicates and overall)
Precision – repeatability	• CV ≤ 15% of 3 replicate mean values per sample
Precision – intermediate precision	• CV ≤ 20% of assay mean values across all sample measurements • CVs ≤ 15% (across sample dilutions in one run)

The Fab Four GMP QC release assays meet all pre-defined acceptance criteria

Parameter (ICH Q2)	Results HCD 202 bp	Results HCD 482 bp	Results cap	kanR	Results E4ORF6
Specificity ✓	• Recovery + spike = 100% • CVs ≤ 4% • NTC + spike = 1 gc/reaction	• Recovery + spike = 104% • CVs ≤ 10% • NTC + spike = 0 gc/reaction	• Recovery + spike = 100% • CVs ≤ 2% • NTC + spike = 0 gc/reaction	• Recovery + spike = 100% • CVs ≤ 3% • NTC + spike = 37 gc/reaction	• Recovery + spike = 99% • CVs ≤ 2% • NTC + spike = 0 gc/reaction
Linearity ✓	• r^2 =1.00 (10 dil.) • Recovery = 89% – 101% • CVs ≤ 14%	• r^2 =1.00 (5 dil.) • Recovery = 105% – 115% • CVs ≤ 10%	• r^2 =1.00 (8 dil.) • Recovery = 85% – 100% • CVs ≤ 12%	• r^2 =1.00 (8 dil.) • Recovery = 93% – 105% • CVs ≤ 13%	• r^2 =1.00 (7 dil.) • Recovery = 100% – 103% • CVs ≤ 6%
LLOQ	• 28 gc/reaction	• 198 gc/reaction	• 40 gc/reaction	• 437 gc/reaction	• 58 gc/reaction
Accuracy ✓	• CVs ≤ 4% • Recovery = 98% - 99%	• CVs ≤ 4% • Recovery = 93% - 95%	• CVs ≤ 2% • Recovery = 98% - 101%	• CVs ≤ 2% • Recovery = 97% - 101%	• CVs ≤ 3% • Recovery = 81% - 85%
Repeatability	• CV = 9%	• CV = 6%	• CV = 2%	• CV = 2%	• CV = 3%
Intermediate precision (2 samples) ✓	• CV S1 = 5% CV S2 = 2% • CVs S1 ≤ 6% CVs S2 ≤ 4%	• CV S1 = 4% CV S2 = 3% • CVs S1 ≤ 9% CVs S2 ≤ 15%	• CV S1 = 2% CV S2 = 4% • CVs S1 ≤ 3% CVs S2 ≤ 5%	• CV S1 = 2% CV S2 = 4% • CVs S1 ≤ 3% CVs S2 ≤ 4%	• CV S1 = 1% CV S2 = 2% • CVs S1 ≤ 12% CVs S2 ≤ 4%

Strengthening assay control through appropriate system and sample suitability tests

Parameter	Acceptance Criterion
Sample Acceptance Criteria	
Accepted droplet count/well	≥ 10,000
Valid replicates/dilution	2 out of 2 valid replicates
Valid dilutions/sample	2 out of 3 valid dilutions
CV [%] of replicates/dilution	CV ≤ 15%
CV [%] across all dilutions and replicates	CV ≤ 15%
Working range	Result must be within working range determined during validation
System Suitability Acceptance Criteria	
Trending Control (TC)	Target value ± 20% of mean TC value determined during validation CV (of replicates and across all determinations) ≤ 15%
Mean Value NTC	≤ 4 copies/20 µL reaction

Summary

Our Fab Four assay panel, developed in accordance with ICH Q14 and validated following ICH Q2(R2), ensures reliable and accurate impurity profiling for batch release while reducing sample volume, time and costs for release testing. This work also addresses key challenges in impurity testing, particularly in establishing appropriate assay acceptance criteria. It highlights the critical role of robust impurity profiling for rAAV gene therapy products and complements our EpyQ platform, which was designed to minimize DNA impurities in AAV batches. Together, these efforts provide a practical framework for developers and manufacturers to enhance the quality, safety, and regulatory compliance of their therapeutic candidates, facilitating their successful progression toward clinical and commercial stages.

Aim higher

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