Novel generic digital PCR-based method to detect and quantify replication-competent AAV genomes in AAV vector-based products

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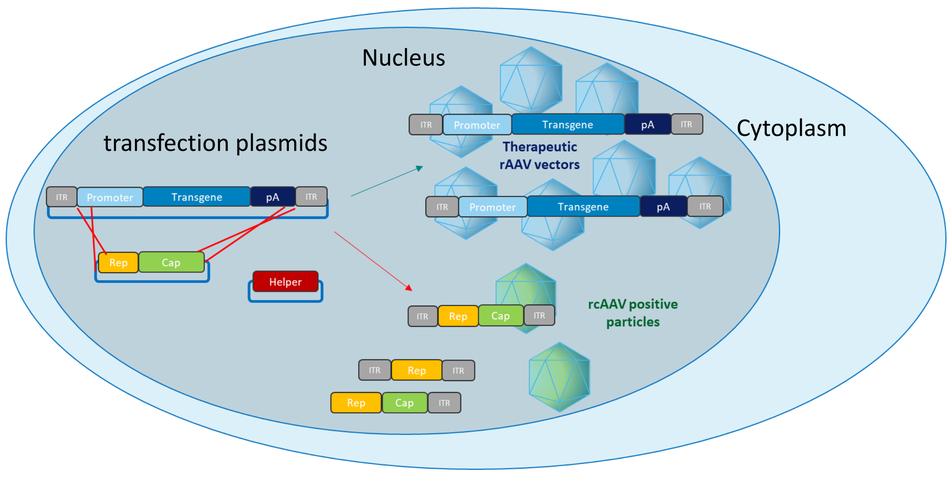
1 Context

Replication-competent AAVs (rcAAV) are gene therapy product-related impurities. They are composed of a viral vector capsid containing WT (wild-type) AAV-like genomes including the Rep and Cap genes with functional promoters, allowing their replication and generation of infectious particles in the presence of a helper virus. While WT AAV are not known to be pathogenic, unintended replication of AAV-like particles could present a risk of immunogenicity or genome integration, should a helper virus co-infect the AAV gene therapy target cells.

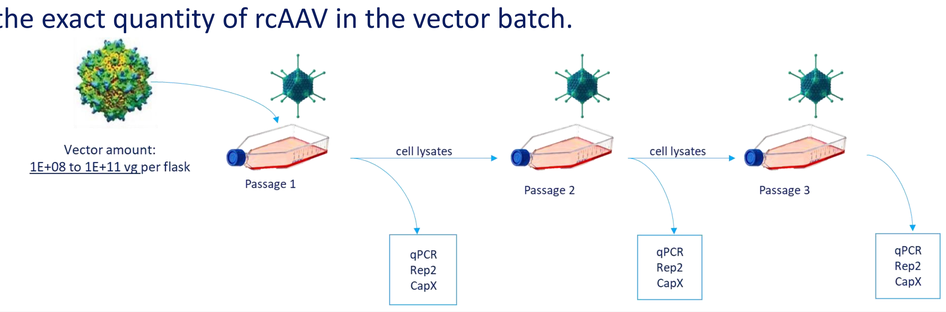
2 rcAAV generation

rcAAV are generated during the manufacturing of rAAV in producing cells. For instance, in HEK293 cells transfected with 3 plasmids, homologous and non-homologous recombination events could lead to rcAAV generation.



3 Detection of rcAAV in vector batches

The industry standard approach is a cell-based assay with serial infection. After one to three rounds of amplification, a qPCR analysis is performed to detect the presence of *Rep* (+/- *Cap).* The method uses engineered or WT positive controls (10-20 IU per flask) and a suitability testing is carried out to check for product interference. The method can determine if rcAAV are present above a certain level, but cannot determine the exact quantity of rcAAV in the vector batch.



4 Limitations of the cell-based assay for rcAAV testing

Cell line used	Contrary to *in-vivo*, AAV2 is 100 to 1000-fold more infectious than AAV8 in cultured cell lines.	Assay sensitivity is serotype-dependent
	Mostly HEK293 used but cell lines are different between CROs	Assay sensitivity is cell-line dependent
Endpoint analysis	Detection of Rep2 or Rep2/CapX	Endpoint analysis and LOD are different
	Positivity defined at round 3 : LOD based on copies/reaction or copies/cell (depending on CRO)	
vg load	vg titers vary among CROs/CDMO	Different vg loads can lead to different results
Positive control	Determination of infectivity of positive control can vary between CROs due to a difference in quantification method	Bias in assay sensitivity evaluation

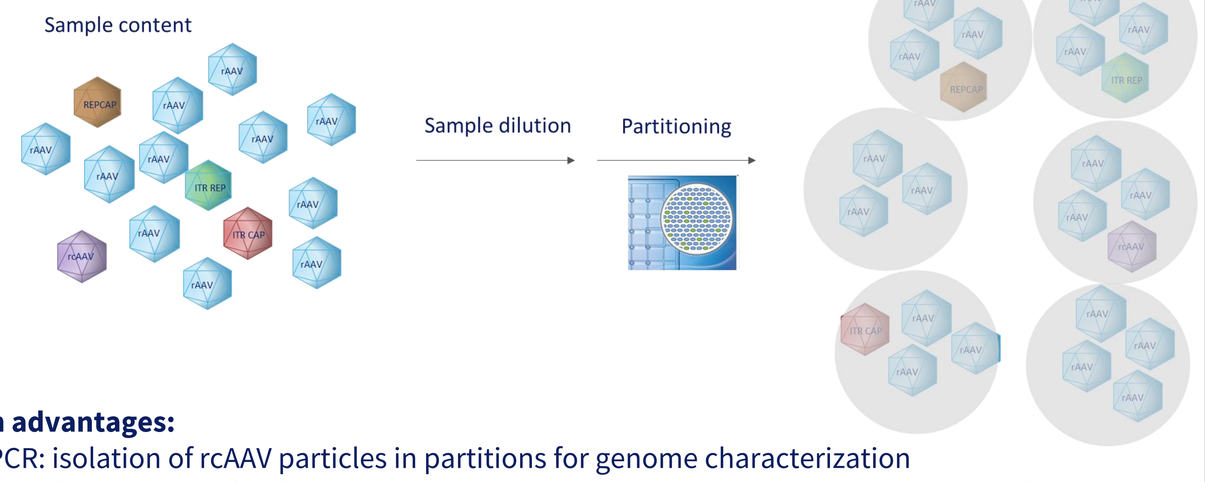
→ Discrepancies in rcAAV detection results

5 Triplex dPCR: a new assay for accurate rcAAV genome quantification

To be replication competent, a recombinant AAV genome must consist of the ITR sequences flanking, Rep and Cap genes arranged spatially as presented in the Figure below, mimicking a WT AAV genome.

Variable nucleotide sequences and sizes due to recombination events between rAAV and Rep/Cap plasmids

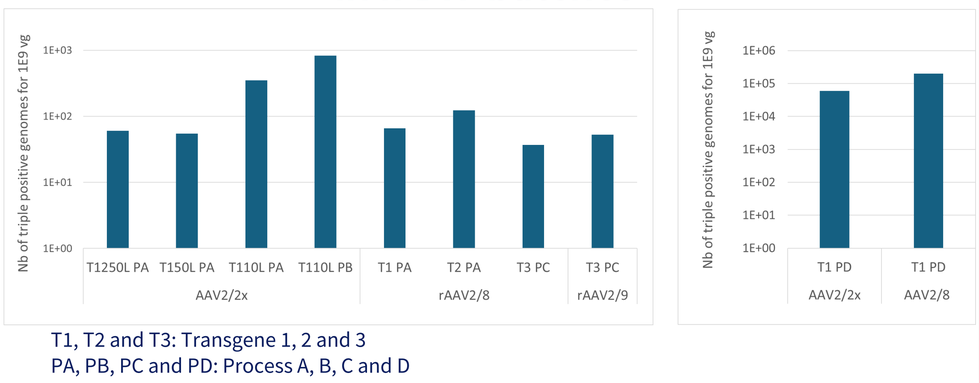
The detection and quantification by triplex digital PCR (dPCR) of simultaneous occurrence of ITR-REP, REP-CAP and CAP-ITR junction fragments is an unbiased method to evaluate the presence of rcAAV genomic entities in vector preparations. Triple positive partitions represent putative rcAAV genome, whereas particles containing 1 or 2 targeted junctions out of 3 represent partial genomes are not considered replication competent.



Main advantages:

- dPCR: isolation of rcAAV particles in partitions for genome characterization
- Detection of recombination events between the rAAV genome and Rep/Cap plasmids for rcAAV genome specificity
- Detection of multiple targets (3) to evaluate genome integrity for accurate quantification

6 Triple-positive events in rAAV batches



T1, T2 and T3: Transgene 1, 2 and 3
PA, PB, PC and PD: Process A, B, C and D

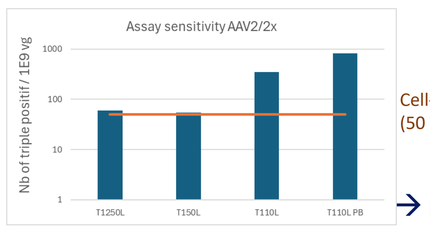
rAAV vector batches of various serotypes (2, 8, 9) and transgenes cassettes (T1, T2, T3) produced by 2 different plasmid-DNA triple-transfection processes (A and B) led to 1E1 to 1E3 triple-positive genomes/1E9 vg across the board, showing **that rcAAV genome formation is independent of capsids, genome structure or manufacturing process in HEK293 cells.** The use of synthetic DNA (PD) devoid of plasmid backbone sequences did not reduce the number of triple positive partitions.

7 Serotype with low in vitro infectivity results in higher input of triple positive genomes in rcAAV cell-based assay

Positive Control (cell-based assay)	IP/mL	vg/mL	Nb of triple positif genome/mL (% of TPg/vg)	**Number of vg for 10 IP**	**Number of TPg for 10 IP**
AAV2/2x	2.7E10	2.1E12	1.4E11 (6.6%)	**8E2**	**5E1**
AAV2/8	1.7E7	3.1E12	8.7E10 (2.8%)	**2E6**	**5E4**

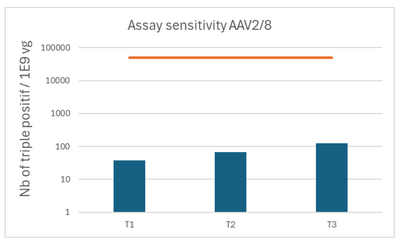
Positive controls of 10 to 20 infectious particles (IP) are used in the cell-based assay for engineered (WT)AAV2/2x and (WT)AAV2/8.
10 IP correspond to 5E1 and 5E4 Triple Positive genomes (TPg) for AAV2/2x and AAV2/8 respectively.
This results in a lower sensitivity of the cell-based assay for detecting rAAV2/8 compared to rAAV2/2x (1000-fold less sensitive).

8 Low in vitro infectivity serotype does not show evidence of rcAAV in cell-based assay despite high levels of triple positive genomes



Cell-based assay positive control 10 IP (50 Triple Positive genomes)

→ rcAAV were detected by the cell-based assay for the 4 rAAV2/2x batches



Cell-based assay positive control 10 IP (5E+04 Triple Positive genomes)

→ No rcAAV were detected by the cell-based assay for the 3 rAAV2/8 batches

9 Conclusion

Triplex dPCR provides an unbiased method for detection and quantification of putative rcAAV genome in rAAV vector preparation, as a complementary method to the cell-based assay.

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