

Advancing AAV Analytics with a 4-Plex dPCR and Universal AAV Standard for Genome Integrity and Titer Assessment

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01 INTRODUCTION

Viral vectors, particularly adeno-associated viruses (AAVs), are widely used in gene therapy due to their safety profile and ability to support long-term gene expression. As the field advances, there is increasing need for standardized approaches to AAV quantification and characterization to improve method consistency and reproducibility¹. To address this need, Agathos Biologics developed a Universal AAV Reference Standard (UAS) containing multiple PCR target sites distributed across the AAV genome, including bGH, WPRE, CMV, eGFP, SV40, and hGH. Designed for compatibility with multiplex qPCR and dPCR workflows, the UAS supports vector genome titer determination, assay performance evaluation, and genome integrity assessment through analysis of multiple genomic regions².

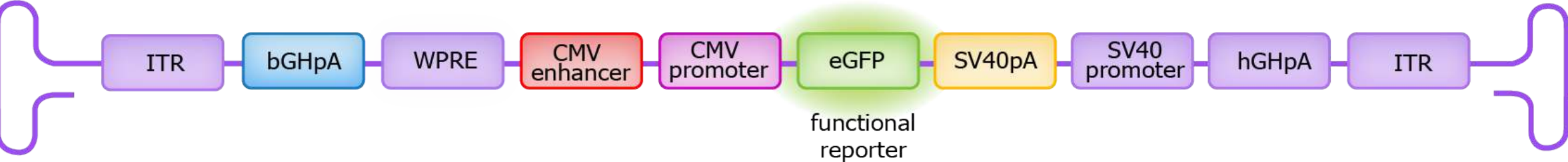


Figure 1. Graphical Representation of the Universal rAAV Standard and dPCR Assays Used. The figure shows the composition of the UAS genome, with ITRs flanking a sequence in which target sites derived from key regulatory and coding elements (e.g., CMV enhancer and promoter, eGFP, SV40 promoter, and multiple polyadenylation signals) are distributed across the genome. These are selected regions used as targets for PCR assays rather than full-length sequences. *Image is not drawn to scale.*

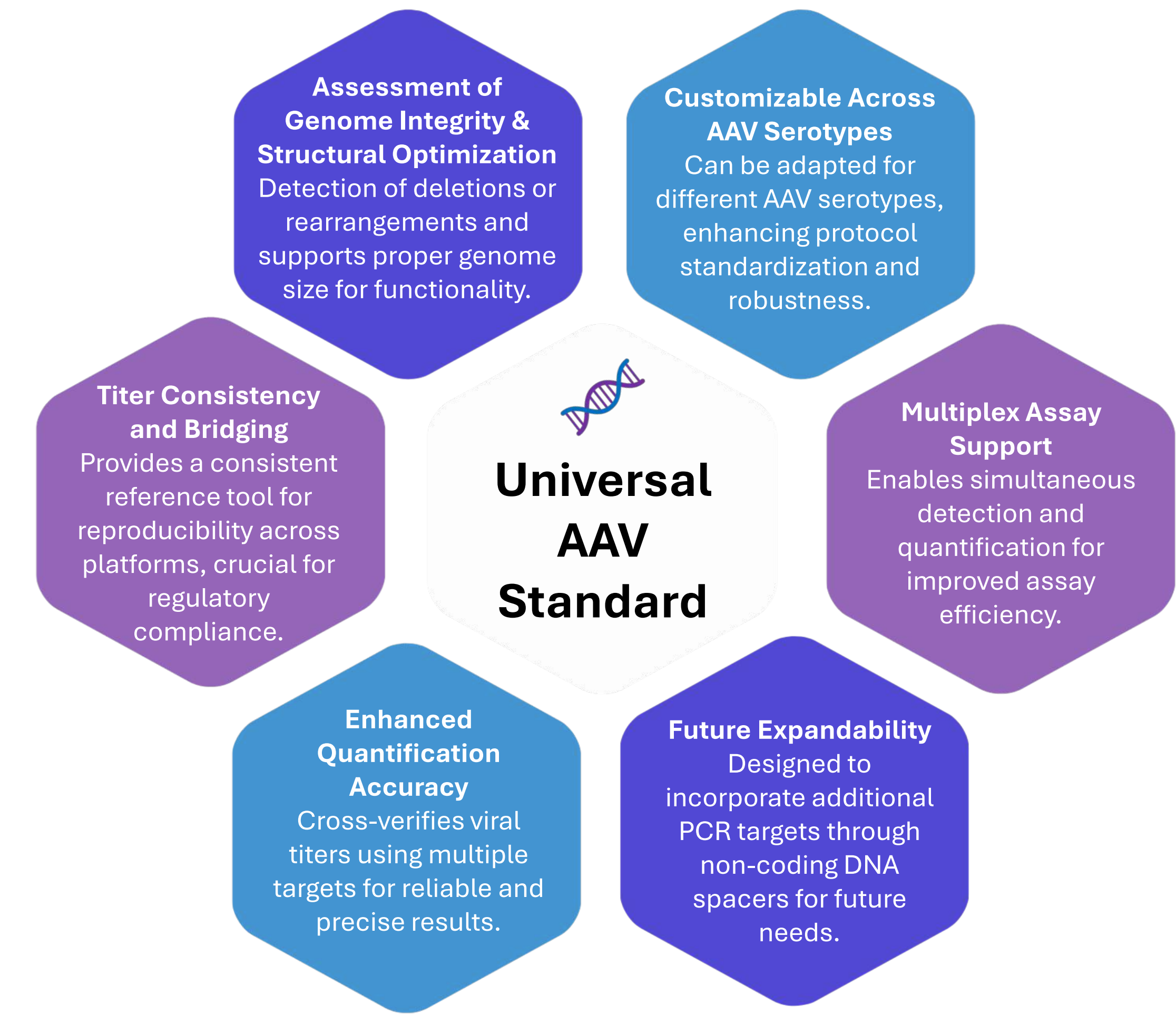


Figure 2. Universal AAV Standard Utility. Schematic overview of the Universal AAV Standard highlighting key features: genome integrity assessment, support for multiplexed digital PCR assays, quantification consistency across serotypes, assay bridging capability, structural optimization, and future expandability.

Beyond assay development, the UAS serves as a reference material for cross-laboratory comparisons, method qualification, and system suitability testing. Its multi-target design supports evaluation of assay performance and genome fragmentation patterns across analytical workflows. Building on this framework, multiplex dPCR approaches were implemented to enable simultaneous analysis of multiple genomic regions within a single reaction. In collaboration with NibaLabs, 4-plex assays were applied to the UAS to assess genome integrity alongside titer measurements, increasing analytical efficiency while reducing sample consumption. The UAS was evaluated across multiple production and analytical workflows, supporting its potential as a practical tool for improved standardization in AAV analytics^{3,4}.

04 CONCLUSIONS

- Both NibaPlex 4-plex dPCR assays produced comparable total genome titers (compared to Agathos/QIAGEN reference)
- Approximately half of detected genomes contained all target regions (46.85% and 52.67%), with the remainder distributed across assay-dependent intermediate and fragmented populations.
- This study demonstrates that assay design strongly influences the interpretation of genome integrity results, and fragment-class distributions should be interpreted within the specific assay framework.
- The Universal AAV Standard with NibaPlex 4-plex dPCR assays provides a robust workflow for VG titer and genome integrity assessment and supports improved cross-laboratory comparability.

02 MATERIALS & METHODS

The Universal AAV Standard packaged as rAAV8 (Cat. # AE1-CUS008.12, Lot # VIL745) was used to evaluate genome titer and genome integrity using multiplex dPCR assays. The rAAV8 preparation (VIL745), produced in the AE1-BHK cell line, contained the Universal AAV Reference Standard sequence as the AAV genome. Genome titer and integrity were assessed using the QIAcuity Digital PCR System (QIAcuity Eight, Cat. No. 911052) with QIAcuity High Multiplex Probe PCR Kit master mix (Cat. No. 25013). Prior to analysis, AAV samples were not additionally treated.

Two 4-plex dPCR assays were used to quantify genome titer and assess genome integrity by simultaneously targeting four genomic regions within a single reaction (Figure 3):

- NibaPlex 4.01: CMV enhancer, CMV promoter, GFP, and SV40 poly-A (Cat. No. 400011)
- NibaPlex 4.03: CMV enhancer, CMV promoter, GFP, and bGH poly-A (Cat. No. 400031)

Samples were analyzed at five dilution levels, each in triplicate wells. dPCR setup and cycling conditions followed standard CGT assay guidelines.

Genome titer (vg/mL) and genome integrity (%) were analyzed using QIAcuity Software Suite v3.1. Genome titer was calculated from positive partitions using Poisson statistics. Genome integrity was determined based on concordance of target detection within individual partitions, where partitions positive for all assay targets were considered intact genomes, while partitions positive for only a subset of targets were considered fragmented genomes.

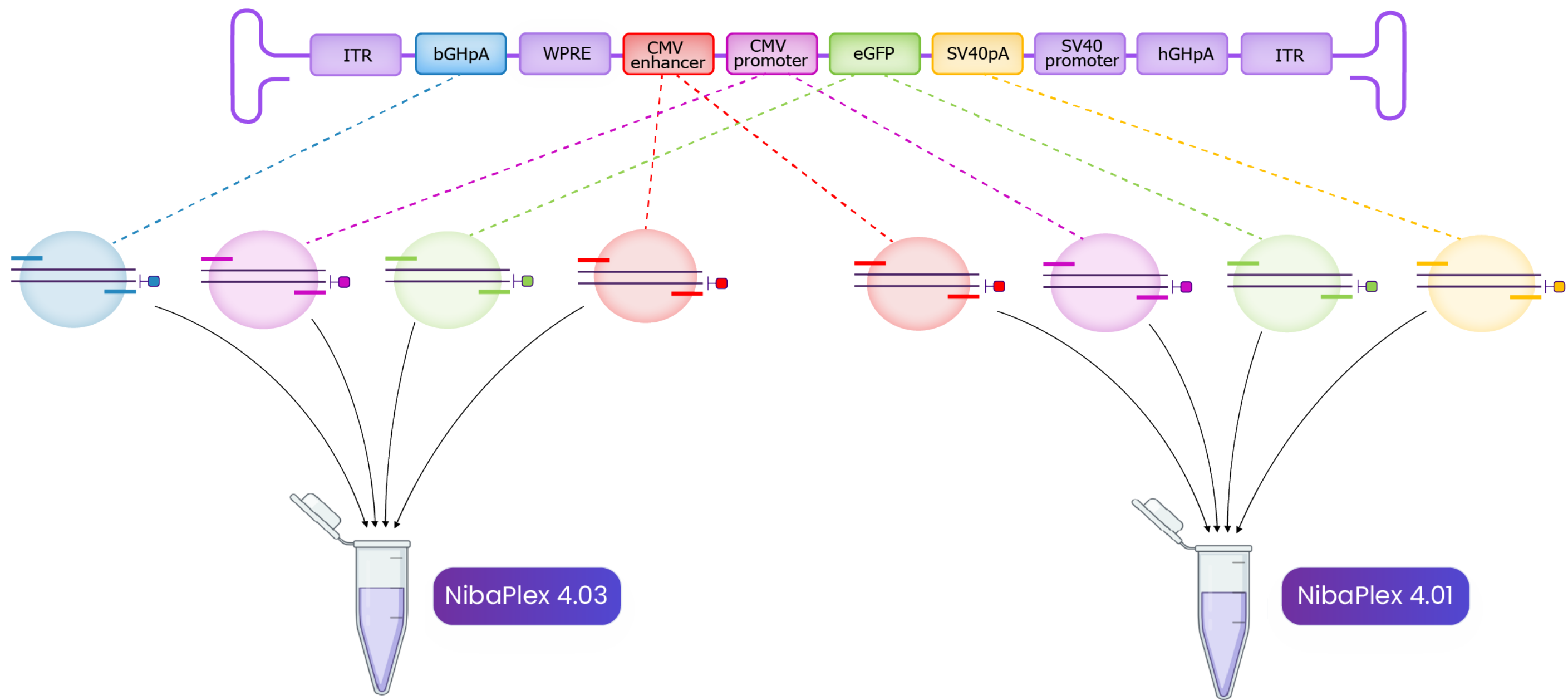


Figure 3. Graphical representation of UAS vector target regions and the NibaPlex 4.01 and 4.03 four-plex dPCR assays. The upper schematic shows the UAS vector architecture, including ITRs, polyadenylation elements, WPRE, CMV regulatory elements, eGFP, and SV40/bGH-associated regions. Dashed colored lines indicate the target regions interrogated by each assay. The lower panels illustrate the two four-plex dPCR assay mixes.

03 RESULTS & DISCUSSION

The mean AAV genome titer was $1.13 \times 10^{13} \pm 2.98 \times 10^{12}$ vg/mL (Agathos Biologics, COA), with consistent performance across Duplex 1, 3, and 4. Targets near the ITR regions (bGHpA and hGHpA) showed slightly lower titers than targets located within the central portion of the genome. Genome integrity analysis showed the highest percentages of intact genomes is observed for shorter fragments (1.5 and 1.9 kb) near the GFP transgene and lower integrity near the ITRs. Intra-assay precision was <20% CV, while cross-site precision was 11%, 4%, and 53% CV for Duplex 1, 3, and 4, respectively².

Figure 4 summarizes genome integrity and VG titer analysis of the UAS using the NibaPlex dPCR 4-plex assays (4.01 and 4.03). Total titers were 1.08×10^{13} and 1.03×10^{13} vg/mL, respectively, comparable to the Agathos reference (Qiagen assays), confirming reliable quantification of the AAV8 standard.

Variation in individual genome fragment profiles revealed a complex mixture of full-length genomes, structured intermediate deletions, and low-abundance highly fragmented regions. Both assays identified a dominant all-four-target-positive population, representing 52.67% (5.41×10^{12} vg/mL) for NibaPlex 4.03 and 46.85% (5.07×10^{12} vg/mL) for NibaPlex 4.01, indicating that approximately half of detected genomes contained all assayed target regions. The remaining signal was distributed among fragmented, truncated, and rearranged genome species.

Together, these findings demonstrate that multiplex dPCR can resolve genome integrity profiles by distinguishing intact genomes from structured deletion, truncation, rearrangement, and fragmentation populations, providing information beyond total genome titer alone.

NibaPlex 4.03						% of individual genome fragment	
bGH	CMV/enh	CMV/prom	GFP			[%]	[vg/mL]
+	+	+	+			52.67	5.41E+12
+	+	-	+			2.55	2.62E+11
+	+	+	-			1.33	1.37E+11
+	+	-	-			0.26	2.68E+10
-	+	+	+			8.98	9.23E+11
-	+	-	+			0.28	2.89E+10
-	+	+	-			0.63	6.43E+10
-	+	-	-			0.30	3.07E+10
+	-	+	+			2.53	2.60E+11
+	-	-	+			0.11	1.14E+10
+	-	+	-			0.28	2.85E+10
+	-	-	-			1.94	1.99E+11
-	-	+	+			22.32	2.29E+12
-	-	-	+			3.38	3.47E+11
-	-	+	-			2.45	2.51E+11
SUM						100	1.03E+13

NibaPlex 4.01						% of individual genome fragment	
CMV/enh	CMV/prom	GFP	polyA SV40			[%]	[vg/mL]
+	+	+	+			46.85	5.07E+12
+	-	+	+			1.04	1.13E+11
+	+	-	+			10.28	1.11E+12
+	-	-	+			0.98	1.06E+11
+	+	+	-			3.55	3.84E+11
+	-	+	-			3.39	3.67E+11
+	+	-	-			3.00	3.25E+11
+	-	-	-			1.88	2.03E+11
-	+	+	+			19.74	2.14E+12
-	-	+	+			2.84	3.07E+11
-	+	-	+			3.24	3.50E+11
-	-	-	+			1.64	1.77E+11
-	+	+	-			0.37	3.96E+10
-	-	+	-			0.54	5.86E+10
-	+	-	-			0.66	7.10E+10
SUM						100	1.08E+13

Figure 4. Distribution of individual genome fragments determined using the NibaPlex 4.01 and 4.03 4-plex dPCR assays. The tables show positive and negative signal combinations for individual target regions within the UAS vector. For each signal combination, the percentage of each individual genome fragment population [%] and the estimated concentration in copies per mL [vg/mL] are shown. The summed total of all detected fragments is indicated in the bottom row.

Although multiplex dPCR enables absolute quantification of detectable genome fragments, fragment-class distributions are inherently assay-dependent. Because NibaPlex 4.01 and 4.03 interrogate different target configurations (e.g., SV40 vs bGH inclusion), identical DNA fragments may be assigned to different classes depending on target coverage and assay design. As a result, fragment populations represent assay-specific detectable spaces rather than absolute genome composition. For example, partially overlapping fragments may be fully resolved in one assay but reclassified into alternative intermediate categories in another. While total target titers remained comparable across assays, fragment distributions differed due to target selection, assay architecture, and detection. These findings highlight that genome integrity metrics from multiplex dPCR should always be interpreted within the context of the specific assay design.

PATENT APPLICATION:
U.S. Patent Application No. 18/649,859, titled "A Recombinant Adeno-Associated Virus (rAAV) Universal Reference Standard for Determining rAAV Genome Copy Titer by Quantitative PCR," filed by James Brown, on behalf of Agathos Biologics, April 29, 2024.

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 - Khan, S., Van den Berghe, N., Henckaerts, E. Standardisation in the Analytical Characterization of Adeno-Associated Virus (AAV) Vectors. *J. Vis. Exp.* (233), e71152, doi:10.3791/71152 (2026).

MORE INFORMATION:



A Q&A on Viral Vector Integrity Analysis: Advancements in Gene Therapy Development



NibaLabs NibaPlex® genome integrity solution



Universal AAV Standard for digital PCR (dPCR)



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