Adapting a Replication Competent Adeno-Associated Virus (rcAAV) Assay for Commercial Manufacturing

Oxana M. Tsygankova, PhD[1], Leann Walsh[1], Lana Sweet[1], Brian Tomkowicz, PhD[2]
[1]Department of Virology, Testing & Analytical Services, [2]Viral Vector Research and Development, SK Pharmteco – US, King of Prussia, PA 19406

ABSTRACT

Recombinant Adeno-Associated viruses (rAAVs)-based gene therapies are widely used for clinical purposes due to their unique characteristics. Unlike lentiviruses, they do not integrate into the host genome. Most manufacturing platforms for rAAVs includes a transient, triple-transfection of plasmid DNA into cells, mainly HEK-293, to produce viruses capable of infecting, but not self-propagating, in humans. However, the ability of otherwise replication incompetent viruses to acquire replication competence AAV (rcAAVs) has been observed. The assumption is that a recombination event occurs which swaps the *rep* and *cap* genes on one plasmid between the ITRs on the cis-plasmid. Subsequent packaging of these newly formed ITR-RepCap-ITR sequence will support replication of AAV *in vivo*.

A universally applicable assay to monitor the emergence and estimate the frequency of recombination events during commercial manufacturing is required. Herein, we report on the development and application of a cell-based qPCR assay to monitor rcAAVs. Cell-based assays with a PCR endpoint are efficient assays and routinely used for rcAAV detection. Because all recombinant AAVs are use the AAV2-derived *rep* gene, our assay is built around Rep-targeting primer/probe sets to analyze test articles derived from transduced cells. To validate the assay, we use *cap* gene-specific qPCR. When applying this method for rcAAV detection on cells transduced with AAV1 or AAV2 serotypes we are able to detect rcAAV events at a limit of detection (LOD) of 10 Infectious Units. The incorporation of this assay to monitor the emergence of rcAAV for product release is necessary to ensure patient safety and provides a rapid assay for timely execution during manufacturing.

RESULTS

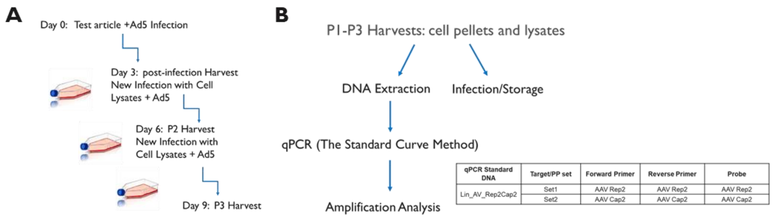


Figure 1. Schematic Diagram Outline rcAAV dPCR workflow. A) Amplification of rAAV in the presence of helper virus and B) qPCR Endpoint Assay

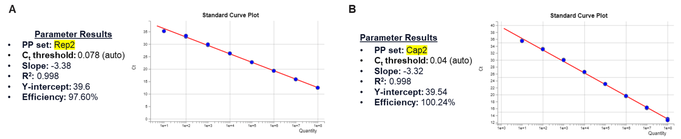


Figure 2 – Establishment of Parameters for qPCR Standard Curve. A) Parameter results demonstrated for A) AAV Rep2 and B) AAV Cap2 specific probe sets.

Rep2

Sample Name	C_t P1	C_t P2	C_t P3	Quantity P1 (copies /well)	Quantity P2 (copies /well)	Quantity P3 (copies /well)	Fold Change
NC1	Undetermined	Undetermined	Undetermined	0	0	0	N/A
NC2	Undetermined	37.6	Undetermined	0	4.9	0	N/A
TA10	32.5	33.52	33.96	166.2	82.4	60.8	0.37
TA10	32.77	33.43	34.12	138.1	87.9	54.4	0.39
10 TA10 1	25.35	21.54	17.07	2.30E+04	3.18E+05	6.95E+06	302.35
10 TA10 2	31.31	21.94	17.91	378.1	2.43E+05	3.88E+06	1.03E+04
10 TA10 3	31.81	26.54	23.58	268.2	1.02E+04	7.78E+04	290.09
PC10 1	24.26	19.68	15.88	4.90E+04	1.15E+06	1.57E+07	321.52
PC10 2	25.92	20.88	16.04	1.55E+04	5.02E+05	1.41E+07	911.99
PC10 3	29.18	23.77	21.73	1.64E+03	6.83E+04	2.80E+05	170.44
100 TA10 1	23.5	18.27	14.28	8.23E+04	3.04E+06	4.76E+07	577.74
100 TA10 2	25.18	18.66	14.54	2.60E+04	2.33E+06	3.97E+07	1.53E+03
PC100 1	25.32	17.97	13.57	2.35E+04	3.73E+06	7.76E+07	3.30E+03
PC100 2	24.93	18.56	13.79	3.07E+04	2.48E+06	6.65E+07	2.16E+03

Cap2

Sample Name	C_t P1	C_t P2	C_t P3	Quantity P1 (copies /well)	Quantity P2 (copies /well)	Quantity P3 (copies /well)	Fold Change
NC1	Undetermined	Undetermined	Undetermined	0	0	0	0
NC2	Undetermined	Undetermined	Undetermined	0	0	0	0
TA10	31.29	33.39	34.57	413.6	98.5	44.1	0.11
TA10	31.27	34.02	34.74	418.4	64	39.4	0.09
10 TA10 1	28.74	23.99	17.97	2.36E+03	6.07E+04	3.68E+06	1.56E+03
10 TA10 2	28.09	32.62	23.04	3.67E+03	166.6	1.15E+05	31.44
10 TA10 3	25.54	21.51	15.64	2.09E+04	3.28E+05	1.82E+07	867.14
PC10 1	33.7	25.12	18.84	79.9	2.79E+04	2.04E+06	2.55E+04
PC10 2	26.63	25.53	19.91	9.98E+03	2.12E+04	9.82E+05	98.35
PC10 3	33.7	26.84	21.18	79.7	8.62E+03	4.11E+05	5.16E+03
100 TA10 1	25.19	22.34	17.08	2.67E+04	1.87E+05	6.78E+06	253.74
100 TA10 2	25.72	23.27	15.33	1.85E+04	9.91E+04	2.24E+07	1.21E+03
PC100 1	32.32	28.18	18.04	204.3	3.47E+03	3.52E+06	1.72E+04
PC100 2	30.77	29.09	15.02	590	1.86E+03	2.77E+07	4.70E+04

Table 1. Replication Competent AAV Validation Results. Application and Validation of rcAAV assay for Rep2- (top panel) and Cap2- (bottom panel) specific primer/probe sets. **Legend**: NC: Ad5 only; PC: wtAAV; TA: test article; TA10: $1x10^{10}$ GC; PC10: 10 IU; PC100: 100 IU; Fold Change: Change between passage 1(P1) and passage 3 (P3); C_t: cycle threshold. Indicates positive signal.

Rep

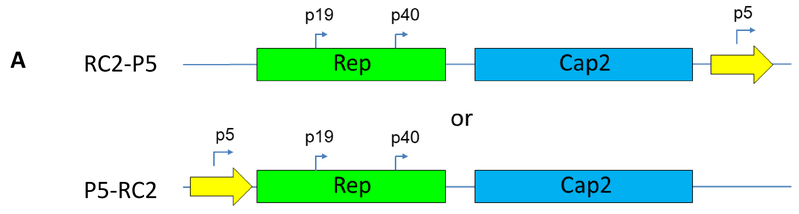
	Sample Name	C_t P1	C_t P2	C_t P3	Quantity P1 (copies /well)	Quantity P2 (copies /well)	Quantity P3 (copies /well)	Fold Change
MVB	MVB NC	Undetermined	Undetermined	Undetermined	0	0	0	N/A
	MVB TA9	31.66	21.96	11.17	238.7	1.76E+05	2.73E+08	1.14E+06
	MVB TA10	28.37	28.55	17.65	2.25E+03	1.99E+03	3.31E+06	1.47E+03
	MVB 1 TA9	31.02	33.97	32.30	368.6	49.5	154.4	0.42
	MVB 1TA10	27.54	10.81	7.61	3.95E+03	3.48E+08	3.07E+09	7.78E+05
	MVB 10TA9	26.37	7.99	6.99	8.77E+03	2.37E+09	4.69E+09	5.36E+05
	MVB 10TA10	28.05	11.04	6.75	2.79E+03	2.98E+08	5.53E+09	1.98E+06
	MVB PC1	30.73	29.96	29.02	448.6	759.9	1.44E+03	3.22
	MVB PC10	19.54	6.77	6.61	9.13E+05	5.47E+09	6.07E+09	6.64E+03
Purified Ad5	Pure NC	Undetermined	Undetermined	Undetermined	0	0	0	N/A
	Pure TA9	31.41	35.48	36.84	262.79	17.05	6.80	0.03
	Pure TA10	28.19	21.28	6.47	2.29E+03	2.38E+05	5.04E+09	2.20E+06
	Pure 1TA9	29.03	13.95	5.58	1.30E+03	3.30E+07	9.21E+09	7.06E+06
	Pure 1TA10	26.35	21.79	10.96	7.88E+03	1.69E+05	2.46E+08	3.12E+04
	Pure 10TA9	20.58	6.37	6.45	3.82E+05	5.41E+09	5.10E+09	1.34E+04
	Pure 10TA10	26.38	8.46	6.76	7.73E+03	1.32E+09	4.15E+09	5.37E+05
	Pure PC1	20.63	6.99	7.84	3.69E+05	3.56E+09	2.00E+09	5.43E+03
	Pure PC10	19.02	6.42	6.37	1.09E+06	5.22E+09	5.40E+09	4.96E+03

Cap

Sample Name	Ct P1	Ct P2	Ct P3	Quantity P1 (copies /well)	Quantity P2 (copies /well)	Quantity P3 (copies /well)	Fold Change
MVB NC P1	Undetermined	Undetermined	Undetermined	0.00	0.00	0.00	N/A
MVB TA9 P1	33.98	23.51	11.72	168.24	1.47E+05	3.03E+08	1.80E+06
MVB TA10 P1	31.09	31.07	20.09	1.10E+03	1.11E+03	1.35E+06	1.23E+03
MVB 1 TA9 P1	35.72	38.16	35.58	54.90	11.3	59.8	1.09
MVB 1TA10 P1	31.56	11.86	10.55	808.02	2.77E+08	6.49E+08	8.03E+05
MVB 10TA9 P1	28.10	7.91	9.75	7.60E+03	3.57E+09	1.09E+09	1.43E+05
MVB 10TA10 P1	30.67	12.92	12.10	1.43E+03	1.40E+08	2.37E+08	1.65E+05
MVB PC1 P1	34.99	32.09	31.96	87.86	573.7	622.4	7.08
MVB PC10	21.51	7.41	11.53	5.38E+05	4.93E+09	3.44E+08	6.39E+02

Sample Name	Ct P1	Ct P2	Ct P3	Quantity P1 (copies /well)	Quantity P2 (copies /well)	Quantity P3 (copies /well)	Fold Change
Pure NC	Undetermined	Undetermined	Undetermined	0	0	2.0	N/A
Pure TA9	33.26	38.94	Undetermined	80.14	1.84E-5	0	N/A
Pure TA10	30.65	22.48	7.92	452.77	1.03E+05	1.63E+09	3.61E+06
Pure 1TA9	30.36	15.25	6.78	550.18	1.25E+07	3.47E+09	6.31E+06
Pure 1TA10	28.24	23.18	12.32	2.24E+03	6.48E+04	8.75E+07	3.91E+04
Pure 10TA9	21.86	6.71	8.46	1.55E+05	3.63E+09	1.14E+09	7.35E+03
Pure 10TA10	31.18	10.66	8.67	319.5	2.64E+08	9.89E+08	3.10E+06
Pure PC1	22.56	7.84	8.48	9.76E+04	1.72E+09	1.13E+09	1.15E+04
Pure PC10	21.36	7.74	10.23	2.16E+05	1.84E+09	3.52E+08	1.63E+03

Table 2. Bridge Study for rcAAV2 testing. rcAAV detection using MVB (left panel) or Purified Ad5 (right panel) using Rep or Cap2-specific primer probes as indicated. . Indicates positive signal.



B

Sample Name	Ct P1	Ct P2	Ct P3	Quantity P1 (copies /well)	Quantity P2 (copies /well)	Quantity P3 (copies /well)	Fold Change
NC	27.5	26.4	25.5	4.53E+03	9.49E+03	1.77E+04	3.90
P5-RC2 1	26.9	25.5	9.7	7.10E+03	1.73E+04	7.94E+08	1.12E+05
P5-RC2 2	28.2	29.3	28.9	2.84E+03	1.36E+03	1.79E+03	0.63
P5-RC2 3	28.9	31.1	28.1	1.89E+03	3.96E+02	3.08E+03	1.63
RC2-P5 1	27.2	33.5	25.6	5.73E+03	7.99E+01	1.69E+04	2.95
RC2-P5 2	26.5	28.6	28.6	8.90E+03	2.23E+03	2.16E+03	0.24
RC2-P5 3	28.7	28.8	27.9	2.08E+03	1.91E+03	3.60E+03	1.73
PC10 + P5-RC2	28.4	19.8	6.5	2.54E+03	8.73E+05	6.35E+09	2.50E+06
PC10 + RC2-P5	23.9	8.4	9.7	6.40E+04	1.79E+09	7.92E+08	1.24E+04
PC10	21.5	8.2	10.1	2.74E+05	1.96E+09	5.67E+08	2.07E+03

Figure 3. Effects of P5 promoter location of rcAAV formation. A) Schematic diagram showing the AAV genome configuration used in this study, demonstrating 5' (P5-Rep2Cap2) and 3' (Rep2Cap2-P5) promoter locations. B) Summary of results that show rcAAV formation and positive controls ( ).

SUMMARY

- We demonstrate the robustness of a rcAAV assay that can detect recombination event in all conditions tested.. The location of the P5 confirms these findings.
- This assay is GMP-ready and available for use in commercial GT therapy manufacturing as a clinical, GMP release assay.