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Analysis of the Adeno-Associated Virus (AAV) vector on the SeqStudio™ CE Platform

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ABSTRACT

We describe the successful use of Applied Biosystems Genetic Analyzer capillary electrophoresis instrumentation for the DNA analysis of Adeno-associated virus (AAV). AAV constructs for gene therapy consist of single-stranded DNA up to 4.5kb in length terminated at both ends by notoriously difficult to sequence GC-rich, inverted tandem repeat regions (ITRs). Both ITRs were sequenced on an AAV vector control plasmid DNA using dGTP BigDye™ Terminator v3.0 chemistry. Based on the high-quality sequence data two variants were detected in the plasmid DNA, a 4 bp deletion near the left –ITR and one single nucleotide mutation. For a fast confirmation of the integrity of the viral vector genome we generated LongAmp™ PCR amplicons up to 3kb in length for multi-color restriction mapping using SNaPshot™ chemistry, a fast, potentially single-tube assay. A commercial AAV8 preparation was used to demonstrate BigDye™ Terminator sequencing of the transgene in AAV virus preparations. AAV viral DNA was extracted in good yield (80%) using the Dynabeads™ SILANE Viral NA kit.

INTRODUCTION

Adeno-associate Virus (AAV) is a popular gene transfer vector in the field of gene therapy due to its lack of pathogenicity and its ability to infect humans without integrating into the genome of the host cell. Recent FDA approvals for the gene therapy drugs Luxturna(2017) for retinal dystrophy from Spark Therapeutics(Roche) and ZOLGENSMA(2019) for spinal muscular atrophy from Novartis testify to the power of the transgene approach to gene therapy. Both landmark treatments rely on AAV, a single-stranded DNA (ssDNA) virus, as the delivery vector for the transgene. AAV gene therapy is potentially also applicable to many other diseases such as Leber Congenital Amaurosis(LCA) a rare inherited eye disorder leading to sever vision loss at birth, Lipoprotein lipase (LPL) deficiency, Hemophilia A, Hemophilia B, Duchenne muscular dystrophy, Alzheimer's disease, Parkinson's disease, Rheumatoid arthritis (RA), GERT and Hepatitis.[1]
Reliable and automated nucleic acids analytical methods are required to meet the needs of the rapidly growing nucleic-acids biopharma industry. In the development, manufacturing and QC of AAV-based gene therapy products the DNA occurs in two forms, as double-stranded plasmid DNA carrying the target gene that is used in the manufacturing process by co-transfecting production cell culture and as single-stranded viral DNA packaged in the virus particles that are the actual therapeutic agent. AAV viral vectors for gene therapy contain ssDNA of up to 4.5kb in length terminated at both ends by GC-rich, inverted tandem repeat regions (ITRs)[2]. We demonstrate the analysis of both plasmid and viral DNA using dye-terminator sequencing and fragment analysis techniques on the SeqStudio™ Genetic Analyzer capillary electrophoresis instrument.

MATERIALS AND METHODS

Table 1. Reagent kits used in the study

Thermo Fisher Scientific Product	PN
AmpliTaq Gold™ 360 PCR Master Mix	4398881
Platinum™ II Hot-Start PCR Master Mix (2X)	14000013
BigDye™ Terminator v3.1 Sequencing Kit	4337456
dGTP BigDye™ Terminator v3.0 Sequencing Kit	4390229
ExoSAP-IT™ PCR Product Cleanup	78250
BigDye XTerminator™ Purification Kit	4376484
SNaPshot™ Multiplex Kit	4323161
Dynabeads™ SILANE Viral NA Kit	37011D

AAV control plasmid DNA (pAAV-400) was obtained from Cell Biolabs (San Diego, CA). The reference sequence of the plasmid DNA is available from the web site (cellbiolabs.com). AAV virus preparation (AAV8 CMV-GFP Control Vector ('Pre-made'), 2. 09 *10^13 vg/ ml) was obtained from Virovek (Hayward, CA). The sequence of the pFB-CMV-GFP plasmid used in the production of AAV8 CMV-GFP virus was kindly provided by Virovek.
Primer Blast (NCBI) was used to design PCR primers for the AAV plasmid and viral DNA. PCR primers were Custom Standard Oligos (desalted) from Thermo Fisher Scientific. The M13 -21 forward and reverse primers used for the sequencing reactions were Custom Standard Oligos (HPLC purified) .
LongAmp ™ Taq 2X Mastermix (NEB) was used for the generation of long PCR amplicons for the restriction digestion / SNaPshot™ DNA fingerprinting by fragment analysis. The EcoRI, BamHI, XbaI and XhoI restriction enzymes were obtained from Thermo Fisher Scientific.
Agarose gel electrophoresis was preformed using precast 2% E-gel™ (Cat # A45204) and Invitrogen Low DNA Mass Ladder (LDML, Cat # 10068013) marker.
Thermocycling was performed on a Veriti™ thermal cycler(Cat # 4375786). DNA Sequencing and Fragment analysis data was generated on a SeqStudio™ Genetic Analyzer. Sequencing data were aligned and variants identified using Applied Biosystems VariantReporter™ software.v3.0

RESULTS

AAV plasmid DNA analysis

Figure 1. Map of the pAAV-400 control vector plasmid (Cell Biolabs)

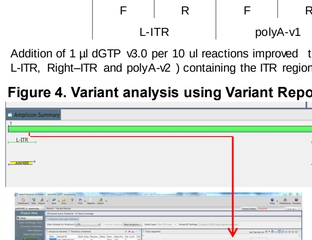
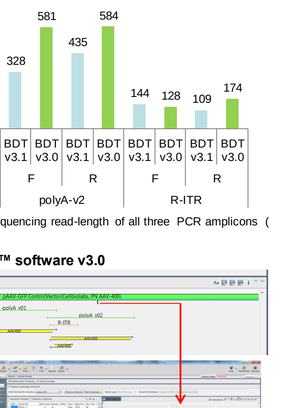


Figure 2. Agarose gel electrophoresis of PCR products using 2 PCR master mixes



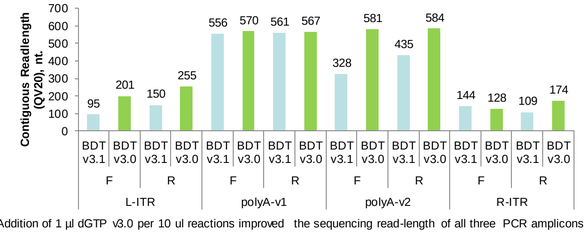
AmpliTaq Gold™ 360 Master Mix and Platinum™ II Hot-Start 2X Master Mix performed equally well for the four AAV PCR amplicons (see Table 1). All PCR reactions contained 5% GC Enhancer additive.

Table 2. PCR amplicons for BigDye® Terminator sequencing

Amplicon Name	FWD Primer	REV Primer	Length (bp)	Includes ITR
AAV400_L-ITR_v01	TTTGCTGGCCTTTTGCTCAC	GGCTATGAACTAATGACCCCGT	217	Yes
AAV400_R-ITR_v01	TTGTAGGTAACCACGTGCGG	GGAGAAAATACCGCATCAGGC	199	Yes
AAV400_polyA_v01	TAGAGTCGACCTGCAGAAGC	CATCACTAGGGGTTCCTGCG	585	No
AAV400_polyA_v02	CCTTCCCTGTCCTTCTGATTT	GGTTGAGTGTTGTTCCAGTTTG	608	Yes

All PCR amplicons were generated using 5'-tailed primers to enable the use of universal M13-21 forward primer (TGTAAAACGACG) and M13 Reverse primer (CAGGAAAC.AGCTATGA) in all sequencing reactions.

Figure 3. dGTP BigDye™ Terminator v3.0 improves sequencing data quality in the GC-rich ITR-regions



Addition of 1 µl dGTP v3.0 per 10 ul reactions improved the sequencing read-length of all three PCR amplicons (L-ITR, Right–ITR and polyA-v2) containing the ITR region.

Figure 4. Variant analysis using Variant Reporter™ software v3.0

Sequencing traces were analyzed for possible variants compared to the plasmid reference sequence. Two variants were found: a 4 base pair deletion near the Left-ITR and a single nucleotide mutation in the plasmid backbone (red arrows).

Figure 5. Workflow for multicolor restriction fingerprinting of the AAV transgene region using SNaPshot™ chemistry (adopted from Dvorak et al. [3])

Figure 6. LongAmp™ PCR products span the transgene region of the pAAV-GFP plasmid flanked by the Left- and Right –ITR (AB Variant Reporter view)

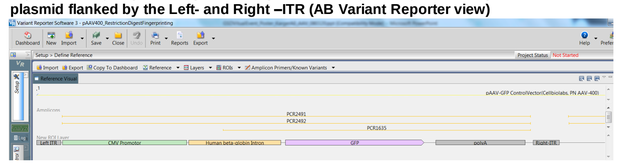
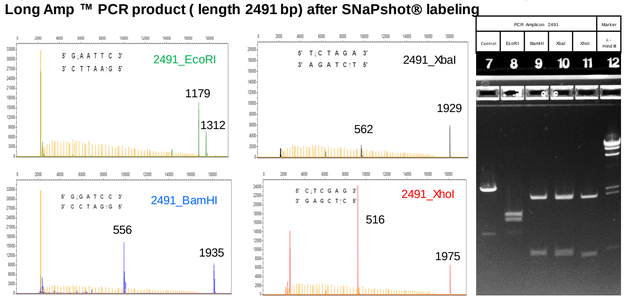


Figure 7. Agarose gel and SeqStudio™ Fragment analysis of 4 restriction digests of a Long Amp ™ PCR product (length 2491 bp) after SNaPshot® labeling



AAV virus analysis

Figure 8. Map of the AAV8 CMV-GFP virus vector expression plasmid (Virovek), virus DNA (2425 nt.) highlighted in blue

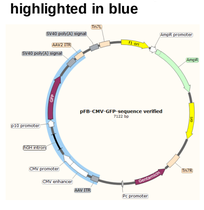
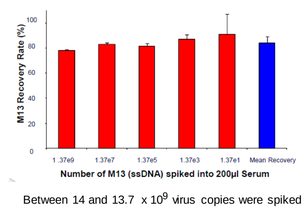
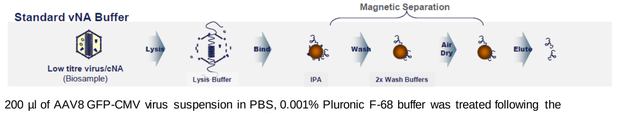


Figure 9. Recovery of M13 viral ssDNA using the Dynabeads ™ SILANE Viral NA Kit



Between 14 and 13.7 x 10^9 virus copies were spiked into artificial serum and DNA quantitated by qPCR after extraction

Figure 10. Dynabeads™ SILANE Viral NA Kit - workflow for DNA extraction



200 µl of AAV8 GFP-CMV virus suspension in PBS, 0.001% Pluronic F-68 buffer was treated following the protocol using a final elution volume of 100 µl of TE buffer..

Figure 11. Standard curve for Quant-iT OliGreen® ssDNA M5 Plate Reader Assay with 200 µl sample volume

Quantification of the AAV 8-GFP-CMV viral DNA using the Quant-iT™ OliGreen® ssDNA reagent (Cat. no. O7582) was performed in a 96-well plate reader format. Extracting viral DNA from 4 x 10^9 copies of the AAV8 preparation (nominally 5 ng ssDNA) we observed a 80% DNA recovery using the Dynabeads SILANE Viral NA kit.

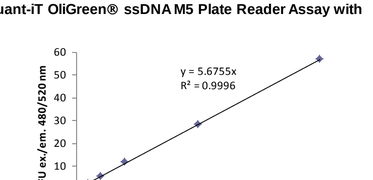
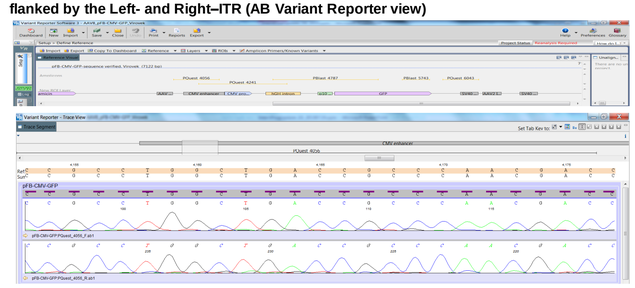


Figure 12. Confirmation sequencing of the transgene of the AAV8 CMV-GFP viral DNA flanked by the Left- and Right–ITR (AB Variant Reporter view)



PCR for confirmation sequencing was performed using AB AmpliTaq Gold Master Mix, 200 nmol each primer and 1 µl (4000 copies) of viral DNA per PCR reaction

CONCLUSIONS

- Using dGTP BigDye™ Terminator v3.0 dye-terminator chemistry we sequenced the GC-rich, difficult to sequence inverted Tandem Repeat regions (ITRs) of the pAAV-GFP control plasmid.
- PCR amplification of the ITR regions is equally effective with AmpliTaq Gold™ 360 and Platinum™ II Hot-Start 2X Master Mix
- Significant improvement in data quality of the GC –rich ITR was observed using the dGTP kit compared to standard BigDye™ Terminator v3.1. Sequencing reactions were analyzed on the SeqStudio™ Genetic Analyzer.
- Two variants from the published sequence were detected in the pAAV-GFP plasmid.
- For a fast characterization of the integrity of the AAV transgene in the plasmid we explored the use of a multi-color restriction mapping strategy by generating long PCR products up to 3 kb in length followed by restriction digestions and differential labeling of the 3'-overhang restriction ends using the SNaPshot™ chemistry kit.
- Multi-color restriction mapping is a fast, potentially single-tube assay for confirmation of the integrity of the AAV plasmid expression and viral genome constructs by capillary electrophoresis.
- A commercial AAV8 virus preparation was used to demonstrate extraction of viral DNA using the DynaBeads SILANE viral NA kit.
- Using the QuantiT OliGreen ssDNA kit we found 80% recovery of viral DNA extracted from 4x10^9 virus copies.
- DNA extraction and PCR yielded high quality traces confirming the integrity of the AAV8 viral vector DNA. After extracting viral DNA from 4x10^5 copies of the AAV8 control vector virus we used 4000 copies per PCR reaction for BigDye™ Terminator v3.1 sequencing. No variants were detected in the viral DNA.

REFERENCES

1. Naso, Michael et al., (2017) Adeno-Associated Virus (AAV) as a Vector for Gene Therapy (Review), *BioDrugs*, 31:317-334
2. Mroske, Cameron et al.,(2012). A Capillary Electrophoresis Sequencing Method for the Identification of Mutations in the Inverted Terminal Repeates of Adeno-Associated Virus. *Human Gene Therapy Methods*, 23:128-136
3. Dvorak, Jan et al., (2003) "High-throughput fingerprinting of bacterial artificial chromosomes using the SNaPshot labeling kit and sizing of restriction fragments by capillary electrophoresis." Elsevier: Genomics, 82, 378-389

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TRADEMARKS/LICENSING

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