

SOP for Measurement of the Copy Number of the AAV Vector in Genomic DNA Using the AAV Vector Standard

1. Scope

- 1.1 This procedure describes a method for the measurement of the copy number of adeno-associated virus (AAV) vector in genomic DNA using the AAV vector standard.
- 1.2 For preparation of AAV vector-infected cells, see _____.
- 1.3 For preparation of genomic DNA that contains the AAV vector, see _____.
- 1.4 For detailed instructions about the thermal cycler (PTC-200; MJ Research), see _____, and for details of the densitograph (AE-6920M-03; ATTO), see _____.
- 1.5 For the high-sensitivity detection of the AAV vector by PCR, see Virus Bank SOP-AAV-001.
- 1.6 For preparation of the AAV vector standard, see Virus Bank SOP-AAV-002.

2. Principles

- 2.1 The copy number of the AAV vector is determined from a standard curve prepared using the AAV vector standard and PCR.

3. Reagents

- 3.1 TaKaRa LA Taq DNA polymerase (5 U/ μ L; Takara Shuzo), 10x LA PCR buffer (Mg²⁺-free; Takara Shuzo), 25 mM MgCl₂, 2.5 mM dNTP mixture (#RR002A; Takara Shuzo), Molecular Weight Marker 1-kb Ladder (#15615-016; GIBCO-BRL), 50x TAE buffer (#24710-030; GIBCO-

BRL), ethidium bromide (10 mg/mL; #15585-011; GIBCOBRL), agarose (#A6013; Sigma, St. Louis, MO).

4. Preparation of serial dilutions of the AAV vector standard

- 4.1 Just before use, make serial 10-fold dilutions of 10^7 - 10^4 copies/ μ L of the AAV vector standard that has been prepared by the TA-cloning method. Do not freeze and store the serially diluted solutions (sensitivity can decrease as a result of adsorption of DNA in dilute solutions to the inner surfaces of tubes).

5. Preparation of samples

- 5.1 For measurements of the copy number of the AAV vector in genomic DNA, prepare serial dilutions of your sample so that values fall within the linear range on the standard curve prepared with the AAV vector standard. Preliminary assays may be required to determine the optimal dilutions and then three appropriate sequential 10-fold serial dilutions of the sample should be prepared.

6. Procedure

- 6.1 Perform amplification by PCR using as template of the AAV vector standard in 1 μ L of the original solution and in serially diluted samples, in duplicate. For PCR, place 49- μ L aliquots of PCR mixture (see below; prepared without template DNA) in 200- μ L reaction tubes. Add 1 μ L of solution of template DNA. Perform PCR in a programmed thermal cycler. Wear gloves and keep reagents on ice during the entire procedure.

*PCR mixture (per sample)

10x LA PCR Buffer	5.0 μ L
Template DNA (diluted)	1.0 μ L
ITR-AD30 (100 μ M)	0.5 μ L
dNTP mixture (2.5 mM each dNTP)	4.0 μ L
TaKaRa LA Taq DNA polymerase (5 U/ μ L)	0.25 μ L
Sterile water	39.25 μ L

	50.0 μ L

*Program for the thermal cycler

94°C, 1 min → 94°C, 1 min → 64°C, 30 sec → 72°C, 3 min → 72°C, 7 min → 4°C, forever

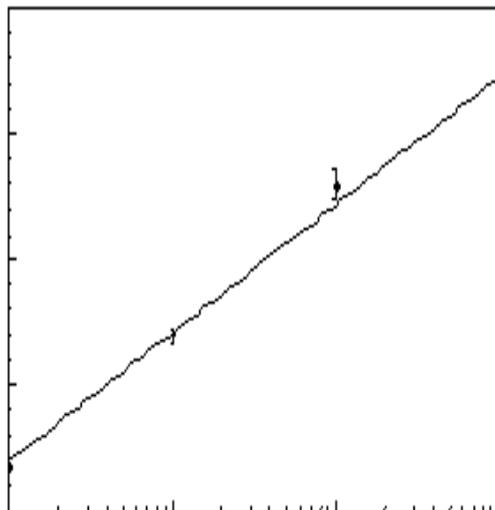


Total of 30 cycles

- 6.2 The product of PCR is detected by agarose gel electrophoresis. Fractionate all reaction mixtures after PCR on the same gel. To 5 μL of each reaction mixture after PCR, add 1 μL of 6x gel-loading buffer (0.25% bromophenol blue/0.25% xylene cyanol/30% glycerol). Load 5 μL of each mixture on the gel and fractionate by electrophoresis in 1x TAE Buffer, for 30 min, at 100 volts at room temperature.
 - 6.3 Soak the gel in a solution of ethidium bromide in Milli-Q water (2 $\mu\text{g}/\text{mL}$) for 5 min at room temperature. Then wash the gel with tap water for 5 min. Visualize bands of DNA under UV light (312 nm), and record the image on the densitograph (AE-6920M-03; ATTO). Analyze images with "Zone Densitometry" software and determine the intensity of fluorescence of the band of the product of PCR of approximately 4 kbp. Save the data as a text file using computer software "Microsoft Excel 5.0".
7. Calculation
 - 7.1 Calculate the copy number of the AAV vector in the sample using the Excel program and a standard curve prepared with the data obtained by "Zone Densitometry" that correspond to the amounts of the product of PCR amplified from known numbers of copies of the AAV vector standard as template.
 - 7.2 Print the picture and data, and record all necessary information.

7.3 Examples of measurements

Standard conc.	Density 1	Density 2	AVERAGE	SD				
1.00E+07	17172	16320	16746	602.45				
1.00E+06	12564	13368	12966	568.51				
1.00E+05	6720	7200	6960	339.41				
1.00E+04	1536	1896	1716	254.56				
1.00E+03	372	624	498	178.19				
Genome	Density 1	Density 2	AVERAGE	SD	Copies/μL	AVERAGE	SD	
1V5 x0.05	8436	7308	7872	797.62	2.91E+06	3.01E+06	2.84E+05	
x0.025	6048	6432	6240	271.53	2.79E+06			
x0.0125	6120	4080	5100	1442.50	3.33E+06			
2V8 x1	3336	3252	3294	59.40	1.85E+04	2.80E+04	8.34E+03	
x0.5	2640	3252	2946	432.75	3.16E+04			
x0.25	1380	1764	1572	271.53	3.40E+04			



$$x \ 10 \ Y = - 18506 + 5109.6 \text{Log}(x) \quad R=0.99626$$

$$1V5 : 3.01 \times 10^6 \text{ copies}/\mu\text{L} \ (\pm 0.28 \times 10^6)$$

$$2V8 : 2.80 \times 10^4 \text{ copies}/\mu\text{L} \ (\pm 0.83 \times 10^4)$$