

## SOP for Preparation of the AAV Vector Standard

### 1. Scope

- 1.1 This procedure describes a method for preparation of the adeno-associated virus (AAV) vector standard that is used for measurements of the copy number of AAV vectors in the genomes of infected cells.
- 1.2 For the preparation of AAV vector-infected cells, see \_\_\_\_\_.
- 1.3 For the preparation of genomic DNA that includes the AAV vector, see \_\_\_\_\_.
- 1.4 For detail 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.

### 2. Principles

- 2.1 The AAV vector standard is prepared from a recombinant plasmid that is obtained from a cloned fragment of AAV vector in *E. coli* JM109 strain has been transformed with pT7Blue T-Vector (Novagen) and includes approximately 4 kbp of the product of PCR amplified from the genome of a recombinant line of 293 cells (2V8) by TA-cloning.

### 3. Reagents

- 3.1 pT7Blue T-Vector (NV004; Novagen), TaKaRa DNA Ligation Kit Ver. 2 (#6022; Takara Shuzo), *E. coli* JM109 competent cells (#9052; Takara Shuzo), TaKaRa Ex Taq DNA polymerase (5 U/μL; Takara Shuzo), 10x Ex PCR Buffer; (#RR001A; Takara Shuzo), TaKaRa LA Taq DNA polymerase (5 U/μL), 10x LA PCR Buffer (Mg<sup>2+</sup>-free; Takara Shuzo), 25 mM MgCl<sub>2</sub>, 2.5 mM dNTP mixture (#RR002A; Takara Shuzo), restriction enzyme PvuII (#151CS; New England BioLabs), QIAGEN Plasmid Midi Kit (#12145; QIAGEN), QIAquick™ PCR Purification Kit (#28104; QIAGEN), GenElute™ Minus EtBr Spin Column (#5-6510; Sigma, St. Louis, MO), molecular weight marker 1-kb Ladder (#15615-016; GIBCO BRL), 50x TAE Buffer (#24710-

030; GIBCO BRL), ethidium bromide (10 mg/mL; #15585-011; GIBCO BRL), agarose (#A6013; Sigma)

#### 4. Procedure for preparation of the AAV vector standard

4.1 Purify the amplified products of PCR approximately 4 kbp, which originates from 2V8, using the QIAquick™ PCR Purification Kit and introduce the fragment into pT7Blue T-Vector using the DNA Ligation Kit Ver. 2 from Takara Shuzo. Then transform *E. coli* JM109 with the resultant plasmid and isolate recombinant cells.

4.2 Select white colonies by "Blue/White" screening using X-gal and confirm the insertion of the DNA directly by PCR, as follows. Pick up a white colony and suspend the cells in 30 µL of sterile water in a 200-µL reaction tube. Add 20 µL of PCR mixture to the suspension and amplify the fragment of interest using a programmed thermal cycler as indicated below. Wear gloves and keep reagents on ice through out the procedure.

\*PCR mixture (per sample)

10x PCR buffer	5.0 µL
dNTP mixture (2.5 mM each dNTP)	4.0 µL
Primer M13 -47 (20µM)	0.5 µL
Primer M13 RV-M (20 µM)	0.5 µL
TaKaRa Ex Taq DNA polymerase (5 U/µL)	0.25 µL
Sterile water	9.75 µL
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	20.0 µL

\*Program for amplification by PCR

95°C, 1 min → 95°C, 30 sec → 55°C, 45 sec → 72°C, 3 min → 72°C, 7 min → 4°C, forever

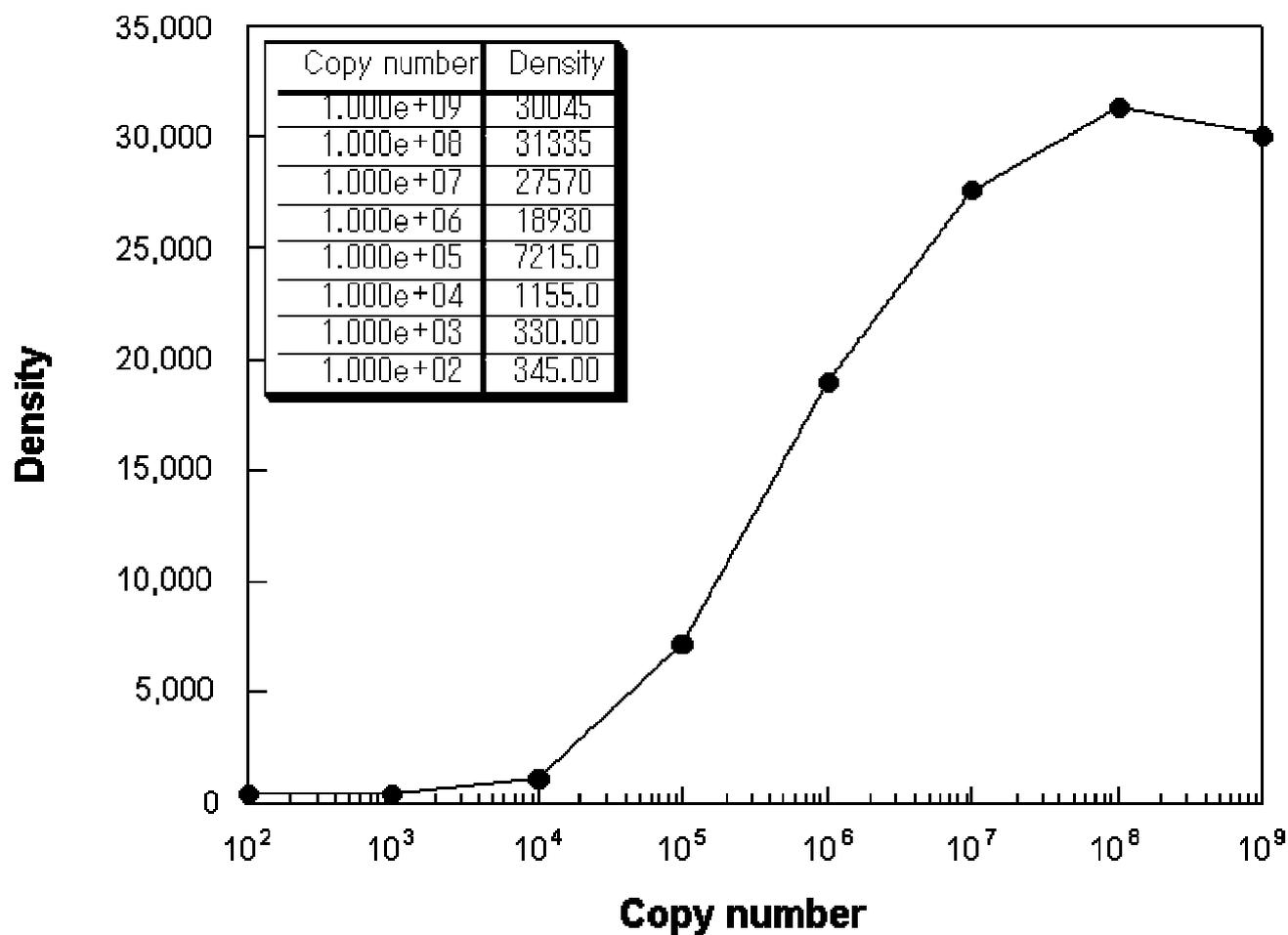


Total of 25 cycles

4.3 After PCR, the product is detected by agarose gel electrophoresis. To 5 µL of the 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 the mixture on a 0.7% agarose gel and fractionate it by electrophoresis in 1x TAE buffer, for 30 min, at 100 volts at room temperature.

- 4.4 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). Record and save the image using the densitograph.
- 4.5 Culture cells that harbor the AAV vector fragment of approximately 4 kbp . Prepare a sufficient amount of plasmid that includes the AAV vector using Plasmid Midi Kit (QIAGEN). Digest the plasmid with the restriction enzyme *PvuII* and fractionate the digest by electrophoresis on a 0.7% agarose gel. Cut out a segment of the gel that includes the inserted DNA of approximately 4 kbp. Purify the AAV vector standard with a GenElute™ Minus EtBr Spin Column (Sigma). Measure the concentration of DNA at 260 nm and calculate the copy number from the length of the AAV vector fragment and the molecular weight (1 b=330).
- 4.6 Make serial 10-fold dilutions of  $10^9$ - $10^2$  copies/ $\mu\text{L}$  of the AAV vector standard. Perform PCR with 1  $\mu\text{L}$  of solution at each dilution. For measurements of the copy number of the AAV vector in genomic DNA, determine the concentrations of that correspond to the lower and upper limits of the linear range on the standard curve derived from quantitation of the AAV vector.

#### 4.7 Example of measurements



**Standard curve of AAV vector copy number by PCR**