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I. Introduction

The AAVpro CRISPR/Cas9 Systems are used for the preparation of adeno-associated virus (AAV) vectors to deliver genes encoding the components necessary for CRISPR/Cas9-mediated genome editing [i.e., single guide RNA (sgRNA) and Cas9 nuclease] to mammalian cells. This AAV-based two-vector system overcomes the size restriction of AAV to produce the large Cas9 protein in target cells and allows efficient genome modifications in a wide-variety mammalian cells \textit{in vitro} and potentially \textit{in vivo}. The AAVpro CRISPR/Cas9 Helper Free System (AAV2) (Cat. No. 632608) is a complete system, containing reagents to construct an sgRNA expression plasmid and prepare AAV particles. Two AAV vectors are included to express Cas9 and a user-defined sgRNA. pAAV-Guide-it-Up and pAAV-Guide-it-Down each contain a truncated, upstream or downstream portion of the Cas9 gene with a 1.6-kb region of homology. Due to the nature of the AAV genome, when target cells are transduced with both viruses (AAV-Up and AAV-Down), recombination occurs at the region of homology to create a full-length Cas9 gene with an upstream CMV promoter (Figure 1).

A target sgRNA expressed from the human U6 promoter is introduced into the pAAV-Guide-it-Down plasmid. To construct the vector, a pair of user-provided oligos corresponding to the target genomic sequence of interest are annealed to form duplex. Then, the duplexed DNA is cloned into the pre-linearized vector using the included high-efficiency ligation mix. This kit also contains necessary controls and Stellar™ Competent Cells. Sufficient reagents are provided in this kit for construction of 10 different target (sgRNA) expression plasmids.

![Figure 1. AAV vector recombination produces a complete Cas9 gene](image)

pAAV-Guide-it-Up

1.6-kb overlap

pAAV-Guide-it-Down

Figure 1. AAV vector recombination produces a complete Cas9 gene. pAAV-Guide-it-Up and pAAV-Guide-it-Down each contain a truncated, upstream or downstream portion of the Cas9 gene with a 1.6-kb region of homology. When target cells are transduced with both viruses (Up and Down), recombination occurs at the region of homology to create a full-length Cas9 gene with an upstream CMV promoter.
II. List of Components

- **AAVpro CRISPR/Cas9 Helper Free System (AAV2) (Cat. No. 632608)**
  - AAVpro CRISPR/Cas9 Vector Set (Cat. No. 632610; not sold separately)
    - 20 µl pAAV-Guide-it-Up Vector (500 ng/µl)
    - 20 µl pAAV-Guide-it-Down Vector (Linear) (7.5 ng/µl)
  - Guide-it™ Ligation Components (Cat. No. 632605; not sold separately)
    - 50 µl DNA Ligation Mighty Mix
    - 1.5 ml Guide-it Oligo Annealing Buffer
    - 10 µl Guide-it Control Annealed Oligos (100 fmol/µl)
    - 10 µl Guide-it Sequencing Primer 1 (100 pmol/µl)
    - 1 ml PCR-Grade Water
  - Stellar Competent Cells (Cat. No. 636763)
    - 10 tubes Stellar Competent Cells (100 µl/tube)
    - 10 tubes SOC Medium (1 ml/tube)
    - 10 µl pUC19 Vector (0.1 ng/µl)
  - pRC2-mi342 Vector (Cat. No. 6281; not sold separately*)
    - 20 µl pRC2-mi342 Vector (1 µg/µl)
  - pHHelper Vector (Cat. No. 6282; not sold separately*)
    - 20 µl pHHelper Vector (1 µg/µl)
  - AAVpro Extraction Solution (Cat. No. 6283; not sold separately*)
    - 3 x 1.5 ml AAV Extraction Solution A
    - 3 x 150 µl AAV Extraction Solution B

* These components are available for separate purchase in larger quantities.

- **AAVpro CRISPR/Cas9 Vector System (Cat. No. 632609)**
  - AAVpro CRISPR/Cas9 Vector Set (Cat. No. 632610; not sold separately)
    - 20 µl pAAV-Guide-it-Up Vector (500 ng/µl)
    - 20 µl pAAV-Guide-it-Down Vector (Linear) (7.5 ng/µl)
  - Guide-it Ligation Components (Cat. No. 632605; not sold separately)
    - 50 µl DNA Ligation Mighty Mix
    - 1.5 ml Guide-it Oligo Annealing Buffer
    - 10 µl Guide-it Control Annealed Oligos (100 fmol/µl)
    - 10 µl Guide-it Sequencing Primer 1 (100 pmol/µl)
    - 1 ml PCR-Grade Water
  - Stellar Competent Cells (Cat. No. 636763)
    - 10 tubes Stellar Competent Cells (100 µl/tube)
    - 10 tubes SOC Medium (1 ml/tube)
    - 10 µl pUC19 Vector (0.1 ng/µl)
III. Storage

- Store the AAVpro Guide-it Vectors and Guide-it Ligation Components at –20°C upon receipt
- Store Stellar Competent Cells at –80°C upon receipt
- Store the AAVpro Extraction Solutions at room temperature after thawing

IV. Additional Materials Required

The following materials are required but not supplied.

A. Molecular Biology Supplies

- Target-specific oligos (see Section V.A)
- TE buffer or molecular-biology grade, nuclease-free water
- PCR reaction tubes
- Micropipette tips (with hydrophobic filters)
- 1.5-ml Eppendorf tubes
- Thermal cycler
- 42°C heat block
- 37°C incubator/shaker
- LB plates containing ampicillin (100 µg/ml)
- Bacteria spreader

B. Mammalian Cell Culture Supplies

- Medium for HEK 293 or HEK 293T Cells
  Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and sodium bicarbonate (Sigma-Aldrich, Cat. No. D5796); 10% Fetal Bovine Serum (FBS); 1% Antibiotic Antimycotic Solution (Sigma-Aldrich, Cat. No. A5955)
- Culture medium, supplies, and additives specific for your target cells
- 0.5 M EDTA (pH 8.0)
- Trypsin/EDTA (e.g., Sigma, Cat. No. T4049)
- 6-well, 12-well, 24-well, or 96-well cell culture plates, and 10-cm cell culture dishes

C. Plasmid Purification

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Product</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>740588.50</td>
<td>NucleoSpin Plasmid</td>
<td>50 preps</td>
</tr>
<tr>
<td>740410.10</td>
<td>NucleoBond Xtra Midi</td>
<td>10 preps</td>
</tr>
<tr>
<td>740414.10</td>
<td>NucleoBond Xtra Maxi</td>
<td>10 preps</td>
</tr>
</tbody>
</table>

D. Transfection Reagent

We recommend using Xfect™ Transfection Reagent for transfection. Xfect reagent provides high transfection efficiency for most commonly used cell types, including HEK 293 and HEK 293T cells.

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Transfection Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>631317</td>
<td>Xfect Transfection Reagent (100 rxns)</td>
</tr>
<tr>
<td>631318</td>
<td>Xfect Transfection Reagent (300 rxns)</td>
</tr>
</tbody>
</table>
E. Titer Determination
For accurate and consistent transductions, we highly recommend titrating your AAV stocks using the AAVpro Titration Kit (for Real Time PCR) Ver. 2 (Cat. No. 6233).

F. AAV Purification
The AAVpro Purification Kit (AAV2) (Cat. No. 6232) can be used to further purify AAV particles for transduction into individual animals or cultured cells.

V. Oligo Design
The CRISPR/Cas9 system requires a custom sgRNA that contains a targeting sequence and a Cas9 nuclease-recruiting sequence. The targeting sequence is homologous to your target gene or genomic region of interest and will direct Cas9 nuclease activity. Selecting the appropriate DNA sequence at the target region is critical for maximizing the potential for efficient cleavage at the target site and for minimizing non-specific cleavage events. There are several online tools (e.g., http://crispr.mit.edu/ or https://chopchop.rc.fas.harvard.edu/) that can be helpful for determining the appropriate target sequence.

Customizing the targeting sequencing of the sgRNA with the AAVpro CRISPR/Cas9 Vector System involves designing a pair of oligos that correspond to the target genomic sequence. When designing these oligos, use these guidelines:

1. The sense oligo (Oligo 1) should contain a 20-nucleotide sequence that is immediately adjacent to an NGG Proto-spacer Adjacent Motif (PAM) sequence at the 3’ end of the target sequence (do not include the PAM sequence). The antisense oligo (Oligo 2) is the complementary sequence of Oligo 1.
   - To minimize off-target cleavage, the entire target sequence including the PAM site should have at least three base mismatches with any other genomic sequence (Mali, P. et al., 2013; Hsu, P.D. et al., 2013); it is particularly useful if the mismatches are in the PAM site or adjacent to the PAM site.
   - For gene knockouts, it is recommended to choose a sequence that corresponds to the N-terminus of the protein.
2. For cloning, additional sequences should be included at the 5’ ends of the oligos. The sense oligo should include the 5’ overhang sequence, cegg; the antisense oligo should include the 5’ overhang sequence, aaac.

   Oligo 1: 5’-cegg XXX XXX XXX XXX XXX XX-3’
   Oligo 2: 5’-aaac YY YYYY YYYY YYYY YYYY YYYY-3’

**NOTE:** The first guanine in the “cegg” sequence of Oligo 1 functions as the transcription initiation site for sgRNA synthesis from the U6 promoter. When the target sequence is designed, it is not necessary to add an extra guanine or choose a guanine at the 5’ end of your target sequence.
VI. Cloning Target-Specific Oligos into pAAV-Guide-it-Down Vector

A. Protocol: Annealing Oligos

1. Resuspend each oligo completely in TE buffer or molecular-biology grade, nuclease-free water such that the concentration is 100 µM.

2. Mix the oligos in a 200-µl PCR tube as follows:

<table>
<thead>
<tr>
<th>µl</th>
<th>Oligo 1 (100 µM)</th>
<th>Oligo 2 (100 µM)</th>
<th>Guide-it Oligo Annealing Buffer</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** The final concentration of the oligos is 10 µM each.

3. Anneal the oligos using a thermal cycler to denature at 95°C and then reanneal by slowly reducing the temperature.

Program your thermal cycler with the following cycling conditions:

- 95°C, 2 min
- 10 min slope from 85°C to 30°C
- 25°C

4. Mix 1 µl of the annealed oligo solution with an additional 99 µl of Guide-it Oligo Annealing Buffer to make a 100 nM (100 fmol/µl) solution.

5. Store the annealed oligos at –20°C until use.
B. Protocol: Cloning the annealed oligos into pAAV-Guide-it-Down Vector

1. Thaw the necessary reagents at room temperature and set up the reaction as follows:

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>pAAV-Guide-it-Down (Linear) (7.5 ng/µl)</td>
</tr>
<tr>
<td>1</td>
<td>Target annealed oligos (100 fmol/µl; from Section V.A, Step 5) or Guide-it Control Annealed Oligos (100 fmol/µl)</td>
</tr>
<tr>
<td>2</td>
<td>PCR-Grade Water</td>
</tr>
<tr>
<td>5</td>
<td>DNA Ligation Mighty Mix</td>
</tr>
<tr>
<td></td>
<td>Total Volume (10 µl)</td>
</tr>
</tbody>
</table>

2. Incubate the reaction mix at 16°C for 30 min.
3. Meanwhile, thaw one vial of Stellar Competent Cells on ice.
4. Add the entire 10-µl ligation mixture to the competent cells and mix gently by tapping.
5. Allow the mixture to stand on ice for 30 min.
6. Heat shock the cells at 42°C for 45 sec and immediately place on ice. Incubate for 2 min.
7. Add 1 ml of SOC medium and incubate at 37°C for 1 hr with vigorous shaking.
8. Plate an appropriate amount of the culture on pre-warmed (37°C) LB plates containing ampicillin (final concentration 100 µg/ml).
9. Incubate the plates at 37°C overnight.

C. Protocol: Isolating and Analyzing Plasmids

1. Pick single colonies and inoculate into an appropriate amount of LB medium containing ampicillin (final concentration 100 µg/ml).
2. Incubate with shaking overnight at 37°C.
3. Purify plasmid DNA from bacteria. We highly recommend the NucleoSpin Plasmid kit (Cat. No. 740588.50) for rapid, high yield, and high purity purification. For transfection-grade plasmid preparation, use the NucleoBond Xtra Midi kit (Cat. No. 740410.10) or NucleoBond Xtra Maxi kit (Cat. No. 740414.10), respectively.
4. Determine the concentration of plasmid DNA and perform sequencing analysis using the included Guide-it Sequencing Primer 1 and your preferred sequencing protocol.

VII. Producing AAV

Please read the protocol completely before starting. Successful results depend on understanding and performing the following steps correctly.

A. General Considerations

- This user manual provides only general guidelines for mammalian cell culture techniques. For users requiring more information on mammalian cell culture and transfection, we recommend the following general reference:
- Several HEK 293 and HEK 293T cell lines are commercially available, but some lines are unable to generate high-titer AAV. Once a HEK 293 cell line that results in high-titer AAV is identified, use that cell type consistently. HEK 293T/17 (ATCC, CRL-11268) are recommended for preparation of high-titer AAV.
B. Safety Guidelines for Working with Recombinant AAV

The protocols in this User Manual require the handling of AAV vectors. It is imperative to fully understand the potential hazards of, and necessary precautions for, laboratory use of these vectors. Viruses produced with AAV-based vectors could, depending on your gene insert, be potentially hazardous. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes in vivo. For these reasons, due caution must be exercised in the production and handling of any recombinant viruses. Follow all applicable guidelines for research involving recombinant DNA. Take appropriate safety measures when producing or handling recombinant AAV, including working in a biological safety cabinet and wearing protective laboratory coats, face protection, and gloves.

C. Protocol: Producing AAV Particles

NOTE: This protocol can be completed in 3–4 days.

A. Transfect HEK 293 or HEK 293T Cells with the AAVpro Guide-it Vectors

a) Seed HEK 293 or HEK293T cells in a 100-mm dish in 10 ml of DMEM/10% FBS/1% Antibiotic Antimycotic Solution, and incubate at 37°C, 5% CO₂. Plate at a density such that the cells will be 50–80% confluent at the time of transfection.

b) Thaw and thoroughly vortex Xfect Polymer.

c) For each transfection sample, prepare the transfection mix in a microcentrifuge tube. Two reactions should be prepared, one for the Up vector and one for the Down vector.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>561 µl</td>
<td>Xfect Reaction Buffer</td>
</tr>
<tr>
<td>13 µl</td>
<td>pAAV-Guide-it-Up or pAAV-Guide-it-Down containing your target sequence (1 µg/µl)</td>
</tr>
<tr>
<td>13 µl</td>
<td>pRC2-mi342 Vector (1 µg/µl)</td>
</tr>
<tr>
<td>13 µl</td>
<td>pHelper Vector (1 µg/µl)</td>
</tr>
<tr>
<td>600 µl</td>
<td>Total Volume</td>
</tr>
</tbody>
</table>

d) Vortex for 5 sec to mix thoroughly.

e) Add 11.7 µl of Xfect Polymer to the plasmid mixture, and vortex vigorously for 10 sec to mix.

f) Spin down for 1 sec to collect the contents at the bottom of the tube and incubate for 10 min at room temperature to allow nanoparticle complexes to form.

g) Add the entire DNA-Xfect solution dropwise to the cell culture medium. Rock the plate gently back and forth to mix.

NOTE: It is not necessary to remove serum from your cell culture medium. It is normal for the medium to change color slightly upon addition of the DNA-Xfect solution.

h) Incubate the plate at 37°C, 5% CO₂.

i) After at least 6 hr, replace the transfection medium with 10 ml fresh DMEM/2% FBS/1% Antibiotic Antimycotic Solution and incubate at 37°C, 5% CO₂ for 2–3 days.
B. Isolate AAV Particles
   a) Add 125 μl of 0.5 M EDTA (pH 8.0) to the 10-ml culture (1/80 volume). Rock the plate back and forth to thoroughly mix and let stand at room temperature for 10 min.
   b) Collect the medium containing detached cells in a sterile 15-ml centrifuge tube.
   c) Centrifuge at 1,750 x g for 10 min at 4°C and completely remove the supernatant.
      NOTE: Confirm that the supernatant has been completely removed before proceeding; particle isolation may be affected by residual supernatant.
   d) Loosen the cell pellet by tapping or vortexing the tube.
   e) Add 0.5 ml of AAV Extraction Solution A and vortex for 15 sec to suspend the cells.
   f) Let stand at room temperature for 5 min.
   g) Vortex for 15 sec and centrifuge at 2,000–14,000 x g for 10 min at 4°C to remove cell debris.
   h) Collect the supernatant in a new sterile microcentrifuge tube, add 50 μl of AAV Extraction Solution B, and mix.
      NOTE: The mixture can be used for viral titer determination using the AAVpro Titration Kit (for Real Time PCR) Ver. 2 (Cat. No. 6233) or for transduction of target cells. Otherwise, the mixture can be stored at –80°C until use. When necessary, thaw quickly in a 37°C water bath before use.

VIII. Determining AAV Titer

Determining the viral titer is necessary to obtain the following information:

- Confirmation that virus was produced.
- The proper transduction conditions for your particular cell type based on the multiplicity of infection (MOI) for the desired transduction efficiency (MOI = number of infectious virus particles per target cell). An excessively high MOI can be toxic to cells, while an extremely low MOI may not lead to efficient genome editing.
- The maximum number of target cells that can be transduced by a given virus volume.

Virus titer can be measured by real-time PCR (vector genome assay) or by infection assay (biological titer measurement). Real-time PCR analysis of vector genomes provides rapid quantification, whereas determining titer by infection of cells is generally more accurate. There are other titration methods for AAV2 vectors that involve assay of viral capsid proteins, but these methods may detect nonfunctional (empty) particles.

A. Vector Genome Assay

The AAVpro Titration Kit (for Real Time PCR) (Cat. No. 6233) can be used to measure virus titer by real-time PCR analysis using the viral ITR domain as a target.

B. Biological Titer Measurement Method

Determine titer by measuring the expression of the gene of interest. An example protocol for quantification of an AAV2 vector expressing the fluorescent protein ZsGreen1 (pAAV-ZsGreen1 Vector, Cat. No. 6231) is provided below.

a) Prepare HT1080 cells at a concentration of 4 x 10⁴ cells/ml in DMEM/10% FBS/1% Antibiotic Antimycotic Solution.

b) Inoculate several wells of a 24-well plate with 0.5 ml of the cell suspension (2 x 10⁴ cells) and culture overnight.

c) Prepare serial dilutions of the prepared AAV2 particle solution using DMEM/10% FBS/1% Antibiotic Antimycotic Solution. The dilution ratio depends on the virus titer, but serial dilutions in the 1,000–100,000-fold range are recommended.

d) Three days after infection, detach the cells using Trypsin/EDTA, and analyze ZsGreen1 expression by flow cytometry.
IX. Introducing Genome Modification with the Prepared AAV Vectors

A. General Considerations

Successful transduction and genome modification depends on the type of target cells or tissues used. Therefore, optimization of experimental conditions will be required.

B. Example Protocol: Transduction and Genome Modification in Dividing Cells

NOTE: This protocol can be completed in 4 days.

1. Prepare enough plates/wells for target cells in complete growth medium 12–18 hr before transduction. The seeding density will depend on the growth characteristics of your cell line and the incubation time after transduction. 

2. Thaw your AAV stocks, or use virus freshly prepared from packaging cells (Section VII).

3. Transduce your target cells with both AAV preps (AAV-Up and AAV-Down) in a 1:1 ratio (virus genomic titer), using several MOIs. We recommend first testing MOIs between 2 x 10⁴ (1 x 10⁴ AAV-Up + 1 x 10⁴ AAV-Down) and 2 x 10⁶ (1 x 10⁶ AAV-Up + 1 x 10⁶ AAV-Down). Rock the plate gently back and forth to spread the virus evenly.

NOTE: Make sure that the titers (genomic titer) of AAV-Up and AAV-Down are in a 1:1 ratio to promote efficient recombination of viral genomic DNA in target cells. Also, the total volume of viral extract used should be below 1/10 of the total culture medium volume. Depending on the cell type, contaminants in the AAV extract may be toxic to the cells. If necessary, the particles can be purified using the AAVpro Purification Kit (AAV2) (Cat. No. 6232).

4. Continue to incubate and analyze the cells at appropriate time points. For most commonly used cell lines, we recommend incubating for 72 hr to observe sufficient genome modification using the Guide-it Mutation Detection Kit (Cat. No. 631443) (Figure. 3). 

NOTE: It is not necessary to remove the virus from the medium after transduction, but it may be necessary to replace the medium after long incubation (>72 hr).

Figure 3. Genome modification using AAVpro CRISPR/Cas9 System. 1 x 10⁵ cells were seeded in 12-well plates one day before transduction. Cells were transduced with an MOI of 1 x 10⁵ (genomic titer) of AAV-Up and AAV-Down particles targeting the CCR5 gene. After 72 hr, cells were harvested and analyzed using the Guide-it Mutation Detection Kit. For comparison, cells were transfected with a Cas9/sgRNA plasmid targeting the CCR5 (P).

X. References

# Appendix A. Troubleshooting Guide

Table 1. Troubleshooting Guide for the AAVpro CRISPR/Cas9 Helper Free System (AAV2)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Explanation</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Vector Cloning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid is difficult to grow or clone</td>
<td>Some viral vectors may undergo rearrangement between the 5’ and 3’ ITRs/LTRs when propagated in less-than-optimal <em>E. coli</em> host strains</td>
<td>Use Stellar Competent Cells (Cat. No. 636763, included) to produce high DNA yields and to minimize the potential for DNA rearrangements.</td>
</tr>
<tr>
<td>B. Virus Production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor transfection efficiency</td>
<td>Cells plated too densely or not densely enough</td>
<td>Adjust the seeding density such that cells are 50–80% confluent at the time of transfection.</td>
</tr>
<tr>
<td></td>
<td>Cell characteristics have changed</td>
<td>Use cells at a low passage number.</td>
</tr>
<tr>
<td></td>
<td>Purity of plasmid is not transfection-grade</td>
<td>Prepare plasmid DNA using a plasmid purification kit, such as NucleoBond Xtra Midi/Maxi (Cat. No. 740410.10/740414.10, etc.).</td>
</tr>
<tr>
<td>Low titer</td>
<td>Poor transfection efficiency</td>
<td>See above.</td>
</tr>
<tr>
<td></td>
<td>Virus was harvested too early or too late</td>
<td>Harvest virus 2–3 days after transfection.</td>
</tr>
<tr>
<td></td>
<td>Transfection is toxic to cells</td>
<td>Use the optimized conditions provided in Section VII.</td>
</tr>
<tr>
<td>C. Transduction of Target Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor transduction efficiency</td>
<td>Target cells cannot be infected by AAV2</td>
<td>Test infectivity of target cells using virus prepared with pAAV-ZsGreen1 Vector (Cat. No. 6231).</td>
</tr>
<tr>
<td></td>
<td>Too little virus</td>
<td>Determine an exact virus titer using the AAVpro Titration Kit (for Real Time PCR) Ver. 2 (Cat. No. 6233) and use a higher MOI.</td>
</tr>
<tr>
<td></td>
<td>Virus extract contains transduction inhibitors</td>
<td>Purify your virus prior to transduction using the AAVpro Purification Kit (AAV2) (Cat. No. 6232).</td>
</tr>
<tr>
<td></td>
<td>Cells plated too densely or not densely enough</td>
<td>Optimize density of cells when transducing.</td>
</tr>
<tr>
<td>Poor target cell viability</td>
<td>Culture conditions are not appropriate</td>
<td>Optimize the culture conditions for the target cells.</td>
</tr>
<tr>
<td></td>
<td>Virus extract contains toxic substances</td>
<td>Purify your virus using the AAVpro Purification Kit (AAV2) (Cat. No. 6232).</td>
</tr>
<tr>
<td></td>
<td>MOI is too high</td>
<td>Infect at a lower MOI.</td>
</tr>
<tr>
<td></td>
<td>Excess viral extract is used</td>
<td>Use volumes no more than 1/10 of the total culture medium. Alternatively, the particles can be purified and concentrated using the AAVpro Purification Kit (AAV2) (Cat. No. 6232).</td>
</tr>
</tbody>
</table>

Clontech Laboratories, Inc. A Takara Bio Company
## D. Introducing Genome Modification

<table>
<thead>
<tr>
<th>Issue</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or little genome modification</td>
<td>Poor transduction efficiency</td>
</tr>
<tr>
<td></td>
<td>See section C.</td>
</tr>
<tr>
<td>Poor target cell viability</td>
<td>See section C.</td>
</tr>
<tr>
<td>Cells were analyzed too soon or too late</td>
<td>Optimize the timeline for analyzing target cells.</td>
</tr>
<tr>
<td>Viruses (AAV-Up and AAV-Down) not used in a 1:1 ratio</td>
<td>Determine exact viral titer using the AAVpro Titration Kit (for Real Time PCR) Ver. 2 (Cat. No. 6233) and use in 1:1 ratio.</td>
</tr>
<tr>
<td>Genome modification detection assay/method is not working</td>
<td>Use Guide-it Mutation Detection Kit (Cat. No. 631443) to monitor mutation efficiency.</td>
</tr>
</tbody>
</table>
Appendix B: AAVpro CRISPR/Cas9 Vector Information

For complete descriptions of the vectors provided with each system, refer to the Certificate of Analysis, which is available at [www.clontech.com](http://www.clontech.com).

![pAAV-Guide-it-Down Vector (Linear) map and cloning site for user’s guide sequence.](image)

Figure 4. pAAV-Guide-it-Down Vector (Linear) map and cloning site for user’s guide sequence.
Figure 5. pAAV-Guide-it-Up Vector map.
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This document has been reviewed and approved by the Clontech Quality Assurance Department.