Takara Bio USA

AAVpro® CRISPR/SaCas9 Systems User Manual

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(120616)

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I. Introduction

The AAVpro CRISPR/SaCas9 Systems are used for the preparation of an adeno-associated virus (AAV) vector to deliver genes encoding the components necessary for CRISPR/SaCas9-mediated genome editing [i.e., single guide RNA (sgRNA) and SaCas9 nuclease] to mammalian cells. This AAV-based one-vector system uses Cas9 from *Staphylococcus aureus* (SaCas9), which has a similar editing efficacy to the more commonly used *Streptococcus pyogenes* Cas9 (SpCas9) but is about 1 kb shorter. By using the smaller SaCas9 it is possible to fit both *SaCas9* and the sgRNA sequence into a single vector and achieve efficient genome modifications in a wide-variety of mammalian cells both *in vitro* and *in vivo*. The AAVpro CRISPR/SaCas9 Helper Free System (AAV2) (Cat. No. 632619) is a complete system, containing reagents to construct custom-designed sgRNA expression plasmids and prepare AAV particles. The AAVpro CRISPR/SaCas9 Vector System (Cat. No 632618) contains the same components as Cat No. 632619 except for the packaging system; details are presented in Section II, List of Components.

The pAAV-Guide-it-1 vector is pre-linearized for the simple insertion of a target sequence for sgRNA expression from the human U6 promoter. **It is important to note that SaCas9 uses a different Proto-spacer Adjacent Motif (PAM) sequence than SpCas9.** This SaCas9 kit also functions best with target sequences that are 20–22 bp long. Please see Section V, Oligo Design for details. To construct the vector, a pair of user-provided oligos corresponding to the target genomic sequence of interest are annealed to form a 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. The AAVpro CRISPR/SaCas9 systems produce SaCas9 and sgRNA from a single vector. pAAV-Guide-it-1 is supplied pre-linearized and ready for insertion of the target sequence for your sgRNA expression (sgRNA shown in orange). When target cells are transduced with a virus packaged with the vector, the cells will express both SaCas9 and the sgRNA in order to achieve genome editing.

II. List of Components

- **AAVpro CRISPR/SaCas9 Helper Free System (AAV2) (Cat. No. 632619)**
  - pAAV-Guide-it-1 Vector (Linear) (Cat. No. 632621; not sold separately)
  - Guide-it™ Ligation Components v3 (Cat. No. 632620; not sold separately)
    - 50 µl DNA Ligation Mighty Mix
    - 1.5 ml Guide-it Oligo Annealing Buffer
    - 10 µl Guide-it Control Annealed Oligos v3 (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 x 100 µl)
    - 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)
  - pHelper Vector (Cat. No. 6282; not sold separately*)
    - 20 µl pHelper 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/SaCas9 Vector System (Cat. No. 632618)**
  - pAAV-Guide-it-1 Vector (Linear) (Cat. No. 632621; not sold separately)
  - Guide-it™ Ligation Components v3 (Cat. No. 632620; not sold separately)
    - 50 µl DNA Ligation Mighty Mix
    - 1.5 ml Guide-it Oligo Annealing Buffer
    - 10 µl Guide-it Control Annealed Oligos v3 (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 x 100 µl)
    - 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-1 Vector and Guide-it Ligation Components v3 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

- AAVpro 293T Cell Line (Cat. No. 632273)
- 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

Cat. No.	Product	Size
740588.50	NucleoSpin Plasmid	50 preps
740410.10	NucleoBond Xtra Midi	10 preps
740414.10	NucleoBond Xtra Maxi	10 preps

D. Transfection Reagent

We recommend using Xfect™ Transfection Reagent for transfection. The Xfect reagent provides high transfection efficiency for most commonly used cell types, including the AAVpro 293T cell line.

Cat. No.	Transfection Reagent
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)

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 (All Serotypes) (Cat. No. 6666) can be used to further purify AAV particles for transduction into individual animals or cultured cells.

V. Oligo Design

The CRISPR/SaCas9 system requires a custom sgRNA that contains a gene-specific targeting sequence and an SaCas9 nuclease-recruiting sequence. The targeting sequence is homologous to your target gene or genomic region of interest and will direct SaCas9 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 is an online tool, https://benchling.com, which can be helpful for determining the appropriate target sequence.

Customizing the targeting sequencing of the sgRNA with the AAVpro CRISPR/SaCas9 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 20–22-nucleotide sequence that is immediately adjacent to an NNGRR(T) PAM sequence at the 3' end of the target sequence (do not include the PAM sequence), where N is any base and R is an A or G base, as shown in Figure 2. The T base in parentheses at the end is strongly preferred but not strictly required. The antisense oligo (Oligo 2) is the complementary sequence of Oligo 1.
    - To minimize off-target cleavage, we recommend having at least three base mismatches with any other genomic sequence in the entire sequence that includes the target and PAM sites. It is particularly useful if the mismatches are in the PAM site or adjacent to the PAM site (7–8 base "seed sequence" at the 3' end of target sequence) (Ran et al. 2015).
    - 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, **accg**; the antisense oligo should include the 5' overhang sequence, **aaac** (see also Figure 2).

    Oligo 1: 5'-accg XXX XXX XXX XXX XXX XXX XX-3' (20–22 nucleotide target sequence)

    Oligo 2: 5'-aaac YY YYY YYY YYY YYY YYY YYY-3' (complementary sequence to oligo 1)

NOTE: The guanine in the "accg" sequence of Oligo 1 functions as the transcription initiation site for sgRNA expression 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.

(Ran et al. 2015) demonstrate that for SaCas9, the optimal length of the target sequence is 21–23 bases, and that replacing the first base of the target sequence with guanine further improves SaCas9 activity. However, since the pAAV-Guide-it-1 vector already contains a guanine at the end of the cloning site, optimal target sequences for the CRISPR/SaCas9 system are 20–22 bases long.

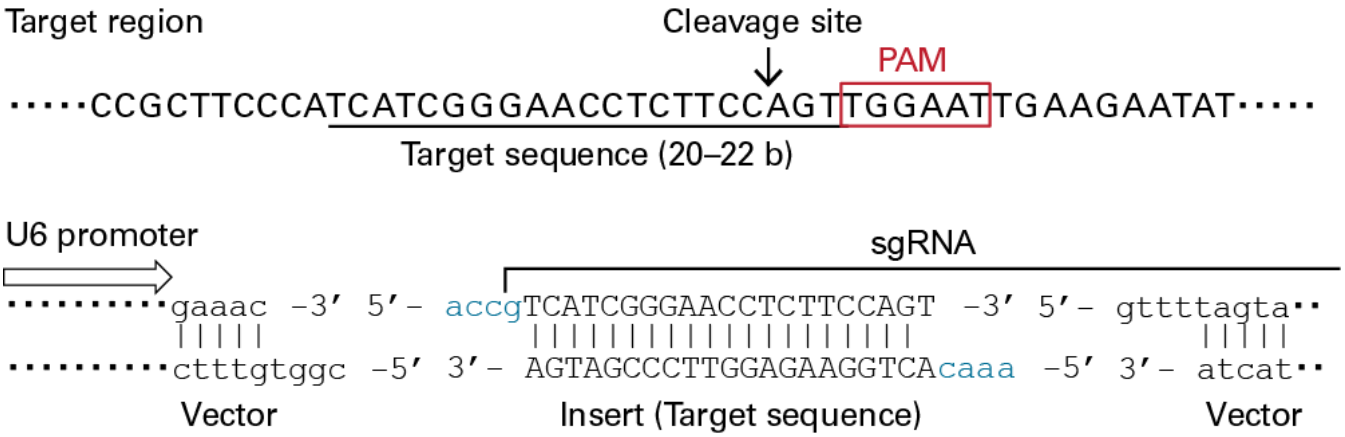

Figure 2. Example of cloning a target sequence using the AAVpro CRISPR/SaCas9 Vector System. This particular example corresponds to the sequence of the Guide-it Control Annealed Oligos v3, which are included in the kit.

VI. Cloning Target-Specific Oligos into pAAV-Guide-it-1 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:

1 µl	Oligo 1 (100 µM)
1 µl	Oligo 2 (100 µM)
8 µl	Guide-it Oligo Annealing Buffer
10 µl	Total Volume

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-1 Vector

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

2 µl	pAAV-Guide-it-1 Vector (Linear) (7.5 ng/µl)
1 µl	Target annealed oligos (100 fmol/µl; from Section V.A, Step 5) or Guide-it Control Annealed Oligos v3 (100 fmol/µl)
2 µl	PCR-Grade Water
5 µl	DNA Ligation Mighty Mix
10 µl	Total Volume

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.

NOTE: The Guide-it Control Annealed Oligos v3 are a positive control for cloning your target sequence into the pAAV-Guide-it-1 plasmid. They are not intended as a control for gene editing.

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).

   NOTE: Immediately inoculate colonies for the plasmid prep. If the plasmid is maintained for too long in *E. coli*, undesired vector rearrangements may occur.

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).
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:
  Freshney, R.I. (2005). Culture of Animal Cells: A Manual of Basic Technique, 5th Edition (Wiley-Liss, Hoboken, NJ).
- We recommend the AAVpro 293T Cell Line (Cat. No. 632273) for preparation of high-titer AAV. While many HEK 293 and HEK 293T cell lines are commercially available, not all of them are able to generate 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.

1. Transfect AAVpro 293T cells with the AAVpro Guide-it 1 Vector

1. Seed AAVpro 293T cells in a 100-mm dish in 10 ml of DMEM/10% FBS/1% Antibiotic Antimycotic Solution, and incubate at 37°C, 5% CO_2. Plate at a density such that the cells will be 50–80% confluent at the time of transfection.
2. Thaw and thoroughly vortex Xfect Polymer.
3. For each transfection sample, prepare the transfection mix in a microcentrifuge tube.

561 µl	Xfect Reaction Buffer
13 µl	pAAV-Guide-it-1 Vector containing your target sequence (1 µg/µl)
13 µl	pRC2-mi342 Vector (1 µg/µl)
13 µl	pHelper Vector (1 µg/µl)
600 µl	Total Volume

4. Vortex for 5 sec to mix thoroughly.
5. Add 11.7 µl of Xfect Polymer to the plasmid mixture, and vortex vigorously for 10 sec to mix.
6. 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.
7. 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.
8. Incubate the plate at 37°C, 5% CO_2.
9. 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% CO_2 for 2–3 days.

2. Isolate AAV Particles

1. 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.
2. Collect the medium containing detached cells in a sterile 15-ml centrifuge tube.
3. 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.
4. Loosen the cell pellet by tapping or vortexing the tube.
5. Add 0.5 ml of AAV Extraction Solution A and vortex for 15 sec to suspend the cells.
6. Let stand at room temperature for 5 min.
7. Vortex for 15 sec and centrifuge at 2,000–14,000 x g for 10 min at 4°C to remove cell debris.
8. 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), which provides rapid quantitation. The AAVpro Titration Kit (for Real-Time PCR) (Cat. No. 6233) measures AAV genome copy number using the viral AAV2 ITR domain as a target.

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 the AAV prep using a range of MOIs. We recommend first testing MOIs between 1 x 10^4 and 1 x 10^5. Rock the plate gently back and forth to spread the virus evenly.

   NOTE: 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 (All Serotypes) (Cat. No. 6666).

4. Continue to incubate and analyze the cells at appropriate time points. For most commonly used cell lines, we recommend incubating for at least 72 hr to observe sufficient genome modification using the Guide-it Mutation Detection Kit or the Guide-it Genome Confirmation Kit (Cat. No. 631443 or 632611 respectively) (Figure 3 on next page).

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

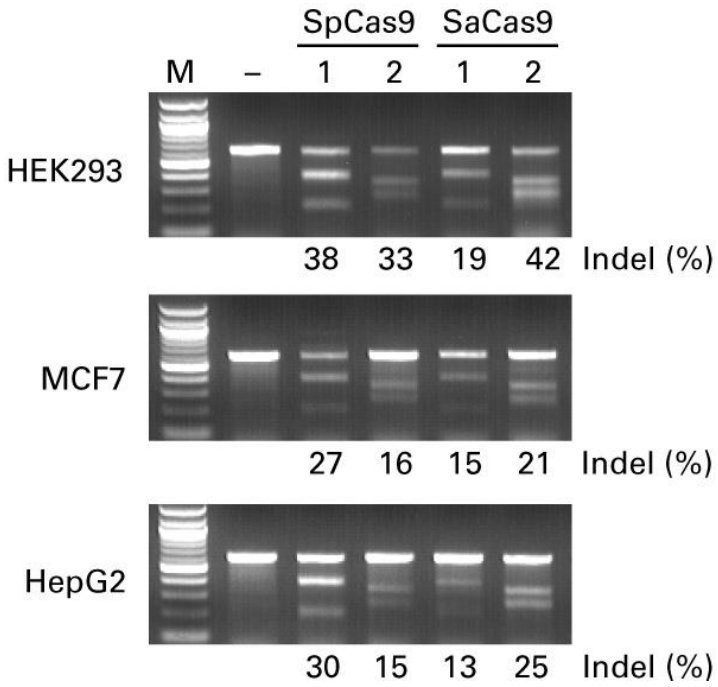

Figure 3. Guide-it Mutation Detection Kit for side-by-side genomic DNA modification comparison using AAVpro CRISPR/Cas9 System (SpCas9) and AAVpro CRISPR/SaCas9 System. 1 x 10^5 cells of three different cell lines were seeded in each well of 12-well plates one day before transduction. Cells were transduced with either the two-vector AAVpro CRISPR/Cas9 System (SpCas9) or the one-vector AAVpro CRISPR/SaCas9 System at 1 x 10^5 MOI for each vector as determined by genomic titer. After 72 hr, cells were harvested and analyzed using the Guide-it Mutation Detection Kit. Two different genomic regions were targeted and the results are shown in wells 1 and 2 for each enzyme. The indel percentage is indicated below each well for each cell type. For more details on this experiment, please see our website.

X. References

Ran, F. A. *et al.* In vivo genome editing using Staphylococcus aureus Cas9. *Nature* **520,** 186–191 (2015).

Appendix A. Troubleshooting Guide

Table 1. Troubleshooting Guide for the AAVpro CRISPR/SaCas9 Helper Free System (AAV2)

Problem	Possible Explanation	Solution
Vector Cloning		
Plasmid is difficult to grow or clone	Some viral vectors may undergo rearrangement between the 5' and 3' ITRs/LTRs when propagated in less-than-optimal *E. coli* host strains	Use freshly obtained transformant with Stellar Competent Cells (Cat. No. 636763, included) to produce high DNA yields and to minimize the potential for DNA rearrangements.
Virus Production		
Poor transfection efficiency	Cells plated too densely or not densely enough	Adjust the seeding density such that cells are 50–80% confluent at the time of transfection.
	Cell characteristics have changed	Use cells at a low passage number.
	Purity of plasmid is not transfection-grade	Prepare plasmid DNA using a plasmid purification kit, such as NucleoBond Xtra Midi/Maxi (Cat. No. 740410.10/740414.10, etc.).
Low titer	Poor transfection efficiency	See above.
	Virus was harvested too early or too late	Harvest virus 2–3 days after transfection.
	Transfection is toxic to cells	Use the optimized conditions provided in Section VII.
Transduction of Target Cells		
Poor transduction efficiency	Target cells cannot be infected by AAV2	Test infectivity of target cells using virus prepared with pAAV-ZsGreen1 Vector (Cat. No. 6231).
	Too little virus	Determine an exact virus titer using the AAVpro Titration Kit (for Real Time PCR) Ver. 2 (Cat. No. 6233) and/or use a higher MOI.
	Virus extract contains transduction inhibitors	Purify your virus prior to transduction using AAVpro Purification Kit (All Serotypes) (Cat. No. 6666).
	Cells plated too densely or not densely enough	Optimize density of cells when transducing.
Poor target cell viability	Culture conditions are not appropriate	Optimize the culture conditions for the target cells.
	Virus extract contains toxic substances	Purify your virus using the AAVpro Purification Kit (All Serotypes) (Cat. No. 6666).
	MOI is too high	Infect at a lower MOI.
	Excess viral extract is used	Use volumes no more than 1/10 of the total culture medium. Alternatively, the particles can be purified and concentrated using AAVpro Purification Kit (All Serotypes) (Cat. No. 6666).
Introducing Genome Modification		
No or little genome modification	Poor transduction efficiency	See Section C.
	Poor target cell viability	See Section C.
	Cells were analyzed too early or too late	Optimize the timeline for analyzing target cells.
	Genome modification detection assay/method is not working	Use Guide-it Mutation Detection Kit (Cat. No. 631443) to monitor mutation efficiency.

Appendix B: AAVpro CRISPR/SaCas9 Vector Information

For complete descriptions of the vectors provided with each system, refer to the Certificate of Analysis, which is available at www.takarabio.com.

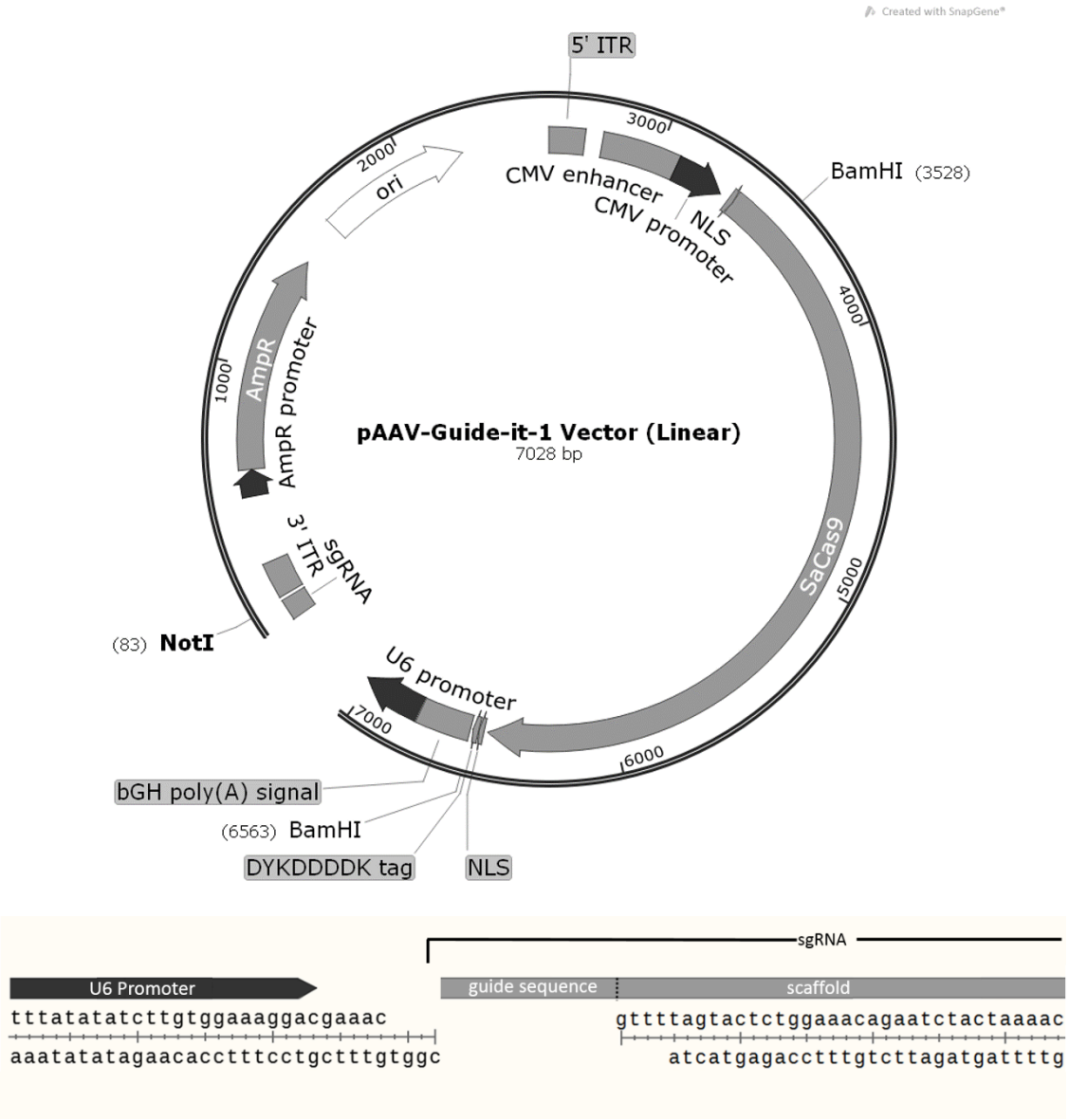

Figure 4. pAAV-Guide-it-1 Vector (Linear) map (top) and cloning site for user's guide sequence (bottom).

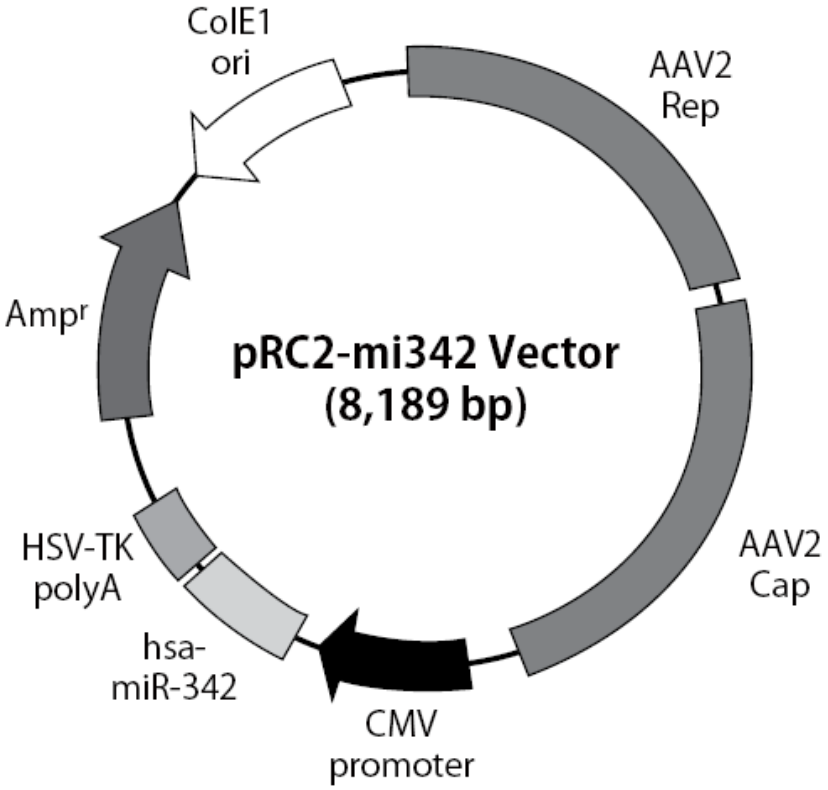

Figure 5. pRC2-mi342 Vector map.

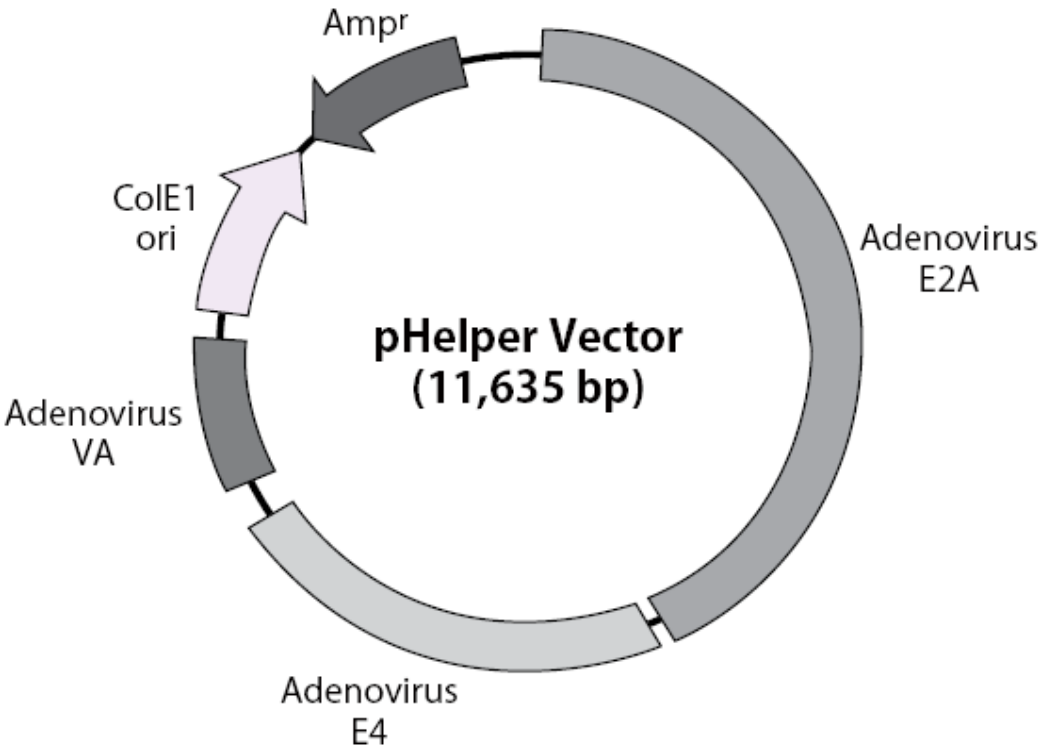

Figure 6. pHelper Vector map.

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