Experimental guidelines for assembling full-length adenoviral vector (AdV) molecular clones encoding CRISPR/Cas9 components

Adapted from: Holkers *et al*. Construction and characterization of adenoviral vectors for the delivery of TALENs into human cells. *Methods*, doi: 10.1016/j.ymeth.2014.02.017 (2014).

Materials:

- AdEasy® "shuttle" plasmids containing the transgene-of-interest, e.g., pAdSh.PGK.Cas9 (#58253), pAdSh.U6.gRNA^{S1} (#58252), pAdSh.U6.gRNA^{GFP} (#58255) or another pShuttle (#16402) derivative.

- AdEasy® "backbone" plasmid pAdEasy-1 (#16400) or a derivative.

- Electro-competent recombinogenic *E. coli* strain BJ5183 (Addgene bacterial strain 16398) containing the pAdEasy-1 "backbone" plasmid or a derivative.

- *E. coli* strain DH5α.

- 10 mg/ml transfer RNA (tRNA) carrier.

- Luria-Bertani broth (LB) medium and agar plates.

- 500 µg/ml kanamycin sulfate.

- PmeI or MssI restriction enzyme.

- Absolute ethanol.

- Table-top Eppendorf centrifuge.

- Electroporator, e.g., Gene Pulser electroporator (Bio-Rad).

- Agarose gel electrophoresis reagents and apparatus.

- Spectrophotometer, e.g., NanoDrop ND-1000 (Thermo Scientific).

Methods:

- Digest 4 µg of selected transgene-containing pAdShuttle plasmid, e.g., pAdSh.PGK.Cas9 (#58253), pAdSh.U6.gRNA^{S1} (#58252) or pAdSh.U6.gRNA^{GFP} (#58255) with the restriction enzyme PmeI (or MssI isoschizomer) in a total volume of 40 µl. Check the completeness of the digestion by subjecting a 10-µl sample to agarose gel electrophoreses. *Note*: Other AdEasy®-compatible shuttle plasmids can be generated by cloning into the MCS of pShuttle (#16402) the transgene(s) of interest.

- Precipitate the digested DNA by adding 20 µl of demineralized water, 25 µg of tRNA “carrier” molecules plus 2.5 volumes of ice-cold absolute ethanol, homogenize and centrifuge the mixtures at 20,000 ×g for 30 minutes at 4°C.

- Dissolve the recovered dried DNA pellet in 30 µl of demineralized water and use 10 µl to transform, in an ice-cooled 1-mm cuvette, a 90-µl suspension of electro-competent BJ5183 cells (Addgene bacterial strain: #16398) containing the pAdEasy-1 (#16400) “backbone” plasmid or a derivative of choice. BioRad Gene Pulser electroporation settings: 1.25 V, 200 W and 25 µFD with a time constant of about 4 seconds.

- Add 400 µl of LB broth medium without antibiotics, transfer the bacteria to a sterilized Eppendorf tube and shake for 45-60 minutes at 30°C.

- Plate the transformed cells onto two LB agar plates containing 50 µg/ml of kanamycin sulfate and, after an overnight incubation period at 30°C, select and grow small-sized colonies for 16-20 hours at 30°C in the presence of 50 µg/ml of kanamycin sulfate.

- Isolate the various plasmid clones by using a conventional alkaline lysis DNA extraction protocol and, on the basis of restriction enzyme analyses, select clones containing full-length AdV genomes for large-scale plasmid DNA purification. Optional extra

step: transform into and purify from a non-recombinogenic *E. coli* strain (e.g. DH5α) the full-length AdV molecular clones.

- Determine the concentration and purity (A260/A280) of the resulting large-scale DNA preparation(s) by spectrophotometry and confirm their integrity by restriction enzyme fragment length analyses. If warranted, complement these data via transgene-directed DNA sequence analyses. *Note*: the rescue and propagation of AdV particles in *E1*-complementing packaging cells (e.g. 293, 911 or PER.C6®) as well as their concentration and purification, are detailed in:

Holkers, M., Cathomen, T. and Gonçalves, M.A. Construction and characterization of adenoviral vectors for the delivery of TALENs into human cells. *Methods* (2014) doi: 10.1016/j.ymeth.2014.02.017. Epub ahead of print.
http://www.sciencedirect.com/science/article/pii/S1046202314000619

The constructs pAdSh.PGK.Cas9 (#58253), pAdSh.U6.gRNA^{S1} (#58252) and pAdSh.U6.gRNA^{GFP} (#58255), are described in:

Maggio, I., Holkers, M., Liu, J., Janssen, J.M., Chen, X. and Gonçalves, M.A. Adenoviral vector delivery of RNA-guided CRISPR/Cas9 nuclease complexes induces targeted mutagenesis in a diverse array of human cells. *Sci Rep*. (2014) 4:5105.
http://www.nature.com/srep/2014/140529/srep05105/full/srep05105.html