

An Affinity-directed Protein Missile (AdPROM) System for Targeted Destruction of Endogenous Proteins

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[Abstract] We recently reported an Affinity-directed PROtein Missile (AdPROM) system for the targeted proteolysis of endogenous proteins of interest (POI) (Fulcher *et al.*, 2016 and 2017). AdPROM consists of the Von Hippel Lindau (VHL) protein, a Cullin 2 E3 ligase substrate receptor (Bosu and Kipreos, 2008), conjugated to a high affinity polypeptide binder (such as a camelid nanobody) that recognises the target protein in cells. When introduced in cells, the target protein is recruited to the CUL2 E3 ubiquitin ligase complex for ubiquitin-mediated proteasomal degradation. For target protein recruitment, we have utilised both camelid-derived VHH domain nanobodies as well as synthetic polypeptide monobodies based on the human type III fibronectin domain (Sha *et al.*, 2013; Fridy *et al.*, 2014; Schmidt *et al.*, 2016). In this protocol, we describe detailed methodology involved in generating AdPROM constructs and their application in human cell lines for target protein destruction. AdPROM allows functional characterisation of the POI and its efficiency of target protein destruction overcomes many limitations of RNA-interference approaches, which necessitate long treatments and are associated with off-target effects, and CRISPR/Cas9 gene editing, which is not always feasible.

Keywords: AdPROM, Proteolysis, VHL, Cullin2, Ubiquitination, Nanobody, Monobody, CRISPR/Cas9

[Background] This protocol enables one to design, build and express AdPROM VHL-nano/monobody constructs in mammalian cell lines to achieve the proteolytic destruction of the endogenous POI. In the original entries, we demonstrated the near-complete destruction of specific target proteins, by using nanobodies that recognise either green fluorescent protein (GFP) (Fridy *et al.*, 2014) or the inflamasomal protein ASC (Schmidt *et al.*, 2016) and two distinct monobodies that recognize the protein tyrosine phosphatase SHP2 (Sha *et al.*, 2013) as target probes, in a number of human cancer cell lines (Fulcher *et al.*, 2016 and 2017). This protocol provides details for the generation of AdPROM constructs, their expression in cells, and monitoring of target protein degradation and can be adapted for use with any nanobody and monobody, both for constitutive and inducible degradation of the POI. The focus of this protocol is not on the generation of nano/monobodies against POIs or CRISPR/Cas9 genome editing (to knockin GFP tags on POIs) but rather the latter steps to facilitate target protein destruction with the AdPROM system.

Materials and Reagents

1. Pipette tips (10 µl, 200 µl, 1,000 µl, alpha gel loading tips) (Greiner Bio One International, catalog numbers: 771290; STARLAB INTERNATIONAL, catalog number: S1111-1006; Greiner Bio One International, catalog number: 740295 and Alpha Laboratories, catalog number: LW1100 respectively)
2. 15 ml Falcons tube (Greiner Bio One International, catalog number: 188271)
3. 50 ml Falcon tube (Greiner Bio One International, catalog number: 227261)
4. 10-cm tissue culture dishes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 172931)
5. Micro tubes (1.5 ml) (SARSTEDT, catalog number: 72.706.400)
6. 0.45 µm sterile syringe filters (Sartorius, catalog number: 16555-K)
7. 0.22 µm sterile syringe filters (Sartorius, catalog number: 16532-K)
8. 96-well plates for tissue culture (Greiner Bio One International, catalog number: 655101)
9. Immobilon-®P PVDF membranes (Merck, catalog number: IPFL00005)
10. 10 ml plastic syringes (BD, BD Biosciences, catalog number: 302188)
11. Sterile disposable scalpels (Swann Morton, catalog number: 0503)
12. X-ray films (Konica Minolta, APLUS)
13. HEK-293 FT cells for retrovirus production (Thermo Fisher Scientific, Invitrogen™, catalog number: R70007)
14. Cell line of interest that expresses the target protein

Note: We used U2OS osteosarcoma, Human Embryonic kidney HEK-293, adenocarcinoma A549, and breast cancer MDA-MB-231 and MDA-MB-468 cells in the original entries (Fulcher et al., 2016 and 2017).
15. Cloning grade chemically competent DH5α cells (prepared in-house using a modified version of the Hanahan method (Sambrook and Russell, 2006))
16. pBABED (Dundee modified pBABE vector) Puro FLAG vectors containing controls and AdPROM reagents for retrovirus production

Note: These may be obtained from the MRCPPU <http://mrcppureagents.dundee.ac.uk/reagent-catalogues> (refer to AdPROM cloning procedure below for details).
17. pCMV-GAG/Pol (Cell Biolabs, catalog number: RV-111)
18. pCMV-VSVG (Cell Biolabs, catalog number: RV-110)
19. pRetroX-Tet-On Advanced system plasmids for Tet-inducible AdPROM expression (Takara Bio, Clontech, catalog number: 632104)
20. Nano/monobody cDNA with flanking EcoRI/NotI sites (the sequences for the nano/monobodies constructs used in the original entries were obtained from the literature (Fulcher et al., 2016 and 2017))

21. Restriction enzymes (FastDigest)–*Bam*HI, *Eco*RI, *Dpn*I and *Not*I (Thermo Fisher Scientific, Thermo Scientific™, catalog numbers: FD0054, FD0274, FD1703 and FD0594 respectively)
22. Ultrapure agarose (Thermo Fisher Scientific, Invitrogen™, catalog number: 16500500)
23. Sequencing oligos (0.025 nM, Desalted) (Sigma-Aldrich)
24. QIAquick Gel Extraction Kit (QIAGEN, catalog number: 28704)
25. Rapid DNA Ligation Kit (Thermo Fisher Scientific, Thermo Scientific™, catalog number: K1422)
26. Ampicillin (ForMedium, catalog number: AMP25)
27. QIAprep Spin Miniprep Kit (QIAGEN, catalog number: 27104)
28. PureLink™ HiPure Plasmid Filter Maxiprep Kit (Thermo Fisher Scientific, Invitrogen™, catalog number: K210017)
29. KOD Hot Start DNA polymerase (Merck, catalog number: 71086-3)
30. Magnesium sulfate (MgSO₄) (Sigma-Aldrich, catalog number: M7506)
31. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)
32. Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Gibco™, catalog number: 11960085)
33. Foetal bovine serum (FBS) (Labtech, catalog number: FCS-SA/500)
34. L-Glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 25030024)
35. Penicillin/streptomycin (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
36. Opti-MEM (Thermo Fisher Scientific, Gibco™, catalog number: 31985062)
37. Cell culture grade trypsin (Thermo Fisher Scientific, Gibco™, catalog number: 25300054)
38. Polyethylenimine (PEI) (Polysciences, catalog number: 24765)
39. HEPES (Sigma-Aldrich, catalog number: H4034)
40. Polybrene (Sigma-Aldrich, catalog number: 107689)
41. Puromycin (Sigma-Aldrich, catalog number: P9620)
42. Phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Gibco™, catalog number: 14190169)
43. Non-fat dried milk powder (we use Marvel milk powder)
44. 4-12% Bis/Tris gradient gels (Novex)
45. Primary antibody that recognises the POI

*Note: We used in-house generated antibodies recognizing VPS34 and FAM83G in the first of the original entries (Fulcher *et al.*, 2016). Both antibodies can be made available upon request or purchased from the MRC-PPU Reagents Website (<http://mrcppureagents.dundee.ac.uk/reagent-catalogues>). For the second original entry (Fulcher *et al.*, 2017), we used anti-SHP2 (C-terminus (Cell Signaling Technology, catalog number: 3397); and N-terminus (Cell Signaling Technology, catalog number: 3752)) and anti-ASC (Martin Oeggerli, Adipogen, catalog number: AL177) antibodies.*

46. Primary antibodies that recognise GFP (an anti-GFP antibody from ChromoTek, catalog number: 3H9), and in-house generated anti-GFP antibody, which can be purchased from the MRC-PPU Reagents Website (<http://mrcppureagents.dundee.ac.uk/reagent-catalogues>)

47. Primary antibody that recognises VHL (Cell Signalling Technology, catalog number: 68547)
48. Primary antibody that recognises a house keeping gene (loading control) (We use anti-GAPDH (Cell Signaling Technology, catalog number: 2118))
49. Bovine serum albumin (BSA) powder (Sigma-Aldrich, catalog number: A7906)
50. Enhanced Chemiluminescence (ECL) reagent (GE Healthcare, catalog number: RPN2106)
51. Secondary antibodies for primary antibody identification
Note: We use anti-sheep IgG-HRP (Santa Cruz Biotechnology, catalog number: sc-2770); and anti-rabbit IgG, HRP-linked (Cell Signaling Technology, catalog number: 7074)
52. G418/Geneticin (Thermo Fisher Scientific, Gibco™, catalog number: 10131035)
53. Doxycycline (hydrochloride) (Sigma-Aldrich, catalog number: D3447)
54. Orange G (Sigma-Aldrich, catalog number: O3756)
55. Glycerol (VWR, catalog number: 24388.320)
56. Ethylenediaminetetraacetic acid (EDTA) (ForMedium, catalog number: EDTA250)
57. Tris (VWR, catalog number: 103157P)
58. Sucrose (VWR, catalog number: 27480.360)
59. Sodium chloride (NaCl) (VWR, catalog number: 27810.364)
60. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma-Aldrich, catalog number: E3889)
61. Sodium orthovanadate (Sigma-Aldrich, catalog number: 450243)
62. β-Glycerophosphate (Sigma-Aldrich, catalog number: G9422)
63. Sodium fluoride (Sigma-Aldrich, catalog number: S7920)
64. Sodium pyrophosphate (Sigma-Aldrich, catalog number: P8010)
65. Nonidet P-40 substitute (Sigma-Aldrich, catalog number: 74385)
66. β-Mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
67. Protease inhibitor cocktail tablet (Roche Diagnostics, catalog number: 11836170001)
68. Glycine (VWR, catalog number: 10119CU)
69. Methanol (VWR, catalog number: 20847.307)
70. Sodium dodecyl sulphate (SDS) (VWR, catalog number: 444464T)
71. Bromophenol blue (Sigma-Aldrich, catalog number: B0126)
72. Hydrochloric acid (HCl) for pH adjustment (VWR, catalog number: 20252.335)
73. Tween 20 (Sigma-Aldrich, catalog number: P1379)
74. Gelatin (from porcine skin) (Sigma-Aldrich, catalog number: G2500)
75. DNA loading buffer (see Recipes)
76. Lysis buffer (see Recipes)
77. Running buffer (10x) (see Recipes)
78. Transfer buffer (10x) (see Recipes)
79. Sample buffer (5x) (see Recipes)
80. TBS (10x) (see Recipes)
81. TBS-T (1x) (see Recipes)

82. TE buffer (pH 8.0) (see Recipes)

Equipment

1. Water baths 37 °C and 42 °C
2. Incubator/shaker 37 °C (Infors)
3. Desktop centrifuge (4 °C)
4. Desktop centrifuge (RT [room temperature])
5. 500 ml Erlenmeyer flask
6. Thermocycler (Thermo Fisher Scientific, Applied Biosystems, model: ProFLEX PCR System)
7. Vortex (Scientific Industries, model: Vortex-Genie 2)
8. Humidified incubator for cell culture
9. Sterile hood for tissue culture, suitable for category 2 work
10. Pipettes (P2, P20, P200, P1000)
11. Table-top heat block
12. Gel electrophoresis apparatus (Peqlab)
13. X-ray film developer
14. Autoclave
15. Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop™ 1000)

Procedure

A. Generation of AdPROM constructs for constitutive expression in cells

1. Preparation of inserts
 - a. Synthesise the specific nano/monobody binding domain using GeneArt (Life Technologies) with flanking 5'-EcoRI and 3'-NotI sites as follows:
GAATTGCCATG-(nano/mono)-TGAGCGGCCGC. If the sequence contains internal EcoRI or NotI sites then introduce silent changes to remove them such that the flanking sites are unique, it may also be useful to codon optimise according to the cell line to be targeted.
 - b. Re-suspend the lyophilised GeneArt vector in 50 µl d/dH₂O (distilled/deionised H₂O).
 - c. Digest 25 µl of the re-suspended GeneArt vector with EcoRI and NotI at 37 °C for 2 h; (25 µl DNA, 10 µl FastDigest Green Buffer, 2 µl EcoRI, 2 µl NotI, 61 µl d/dH₂O).
 - d. Run the entire digest on a 1% agarose gel (130 V, 35 min) (split between neighbouring wells as necessary). Two bands should be visible; the heavier vector band should run at approximately 2.3-2.5 Kb depending on the GeneArt backbone provided and the released insert band should run at the expected size of the synthesized nano/monobody binding

domain (see Figure 1 for a simulated DNA digest gel image). Excise the released cDNA insert (small band). For DNA loading buffer (see Recipes).

- e. Purify the digested insert using the QIAquick gel extraction kit according to the manufacturer's instructions and elute in 50 μ l d/dH₂O.

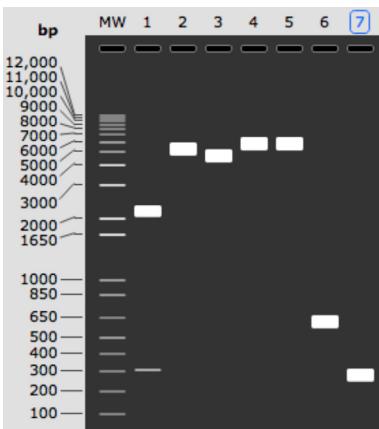


Figure 1. Simulated DNA agarose gel highlighting the expected products obtained in each digest. Molecular weights (MW) are in base pairs (bp). 1. (Step A1c) pMK-RQ AdPROM (GeneArt synthesized) \times *Eco*RI + *Not*I (upper band = vector, 2,286 bp, lower band = insert, 307 bp). 2. (Step A2b) pBABED P FLAG VHL 5xGly no stop (DU54795) \times *Eco*RI + *Not*I, 5,830 bp. 3. (Step A2b) pBABED P FLAG (DU37983) \times *Eco*RI + *Not*I, 5,176 bp. 4. (Step B2a) pRetroX-Tight Puro \times *Eco*RI + *Not*I, 6,536 bp. 5. (Step B2i; Step B3j) pRetroX-Tight Puro \times *Bam*HI + *Not*I, 6,560 bp. 6. (Step B3c) VHL PCR \times *Bam*HI + *Not*I, 664 bp. 7. (Step B2e) Nano/monobody PCR \times *Bam*HI + *Not*I, 307 bp.

2. Preparation of vectors
 - a. Digest accepting vectors: for the AdPROM construct, cut pBABED Puro FLAG VHL (DU54795) and for the nano/monobody only control cut pBABED Puro FLAG (DU37983). An additional VHL only control vector is available if required (DU54477). All constitutive expression AdPROM reagents and vectors may be ordered online from the MRC-PPU (<http://mrcppureagents.dundee.ac.uk/reagent-catalogues>).
 - b. Digest each accepting vector with *Eco*RI and *Not*I at 37 °C for 2 h; (3-5 μ g vector, 10 μ l FastDigest Green Buffer, 2 μ l *Eco*RI, 2 μ l *Not*I, d/dH₂O to 100 μ l final volume). pBABED Puro FLAG VHL (DU54795) and pBABED Puro FLAG (DU37983) should produce single fragments of 5.9 Kb and 5.2 Kb respectively (Figure 1).
 - c. Run the entire digest on a 1% agarose gel (130 V, 35 min) (split between neighbouring wells as necessary) and excise the cut vector band (single large band).
 - d. Purify the digested vectors using the QIAquick gel extraction kit and elute in 50 μ l d/dH₂O.

3. Cloning, verification and Maxiprep

- a. Ligate the AdPROM insert into both the empty and VHL-containing accepting vectors using the Rapid DNA Ligation Kit (5 μ l insert, 1 μ l vector, 4 μ l 5x Rapid DNA Ligation Buffer, 1 μ l Ligase, 9 μ l d/dH₂O).
- b. Mix well and incubate for 5 min at RT.
- c. Add 5 μ l of the ligation reaction to 50 μ l competent DH5 α cells on ice and leave for 2 min.
- d. Move cells to 42 °C water bath for 45 sec to heat shock then place on ice for 1 min.
- e. Plate the entire contents of the transformation tube onto an LB (lysogeny broth) agar plate containing 100 μ g/ml ampicillin and place O/N (overnight) at 37 °C.
Note: Ampicillin is a slow-acting antibiotic thus there is no need for a recovery step before plating.
- f. The following morning, pick 4-6 colonies from the plate and add each to 4 ml LB (50 μ g/ml ampicillin) in 15 ml Falcons. Propagate O/N in a shaker incubator at 37 °C, 220 rpm.
- g. After 16 h growth, pellet cells by centrifugation at 2,000 $\times g$ for 10 min at 4 °C.
- h. Harvest plasmid DNA from each pellet using the QIAprep Spin Miniprep kit according to the manufacturer's instructions and elute in 50 μ l d/dH₂O.
- i. Set up a small 20 μ l diagnostic digest for each and incubate for 1 h at 37 °C; (6 μ l Plasmid DNA, 2 μ l FastDigest Green Buffer, 0.1 μ l EcoRI, 0.1 μ l NotI, 11.8 μ l d/dH₂O).
- j. Run the digested samples on a 1% agarose gel and look for the excision of the AdPROM insert. Two bands should be visible; the heavier vector band should run at approximately 4.3 Kb and the smaller released insert band should run at the expected size of the synthesized nano/monobody binding domain.
- k. Sequence 1 or 2 positives using primers pBABE F (5'-CCTCCTCTTCCTCCATCC) and pBABE R (5'-CCACACCTGGTTGCTGACTAATTGAG).
- l. Transform 50 μ l DH5 α cells with 5 ng of plasmid DNA from the confirmed clone and plate on an LB agar plate (100 μ g/ml ampicillin).
- m. The following morning pick a single colony and inoculate 10 ml LB (50 μ g/ml ampicillin) in a 50 ml Falcon tube and grow for 6-7 h at 37 °C, 220 rpm.
- n. Add the entire contents to a 500 ml Erlenmeyer flask containing 150 ml LB (50 μ g/ml ampicillin) and grow overnight at 37 °C, 220 rpm.
- o. Harvest plasmid from the culture using the PureLink™ HiPure Plasmid Filter Maxiprep Kit according to the manufacturer's instructions and elute the purified plasmid DNA in 1 ml TE buffer (pH 8.0).
- p. Measure the DNA concentration using a NanoDrop instrument in preparation for downstream transfection procedures; and verify the Maxi-prepped vector(s) by both restriction digestion and DNA sequencing.

B. Generation of AdPROM constructs for Tetracycline (Tet)-inducible expression in cells

1. Nanobody generation and primer design

- a. Synthesise the specific nano/monobody using GeneArt (Life Technologies) with flanking 5'-EcoRI and 3'-NotI sites as follows:
GAATTCCGCCATG-(nano/monobody)-TGAGCGGCCGC. If the sequence contains internal *Bam*HI, *Eco*RI or *Not*I sites then introduce silent changes to remove them such that the flanking sites are unique, it may also be useful to codon optimise according to the cell-line to be targeted.
- b. The multiple cloning site of the pRetroX-Tight Puro destination vector is limited, thus it is necessary to order additional primers to PCR amplify the AdPROM insert with flanking *Bam*HI and *Not*I sites: *gctaGGATCCGCCATG-(nano/monobody)-TGAGCGGCCGCgact* (the lower case bases indicate overhangs to allow direct digestion of PCR product). A T_m of 60 °C is recommended for PCR primers; if lower then alter the annealing temperature of any reactions accordingly.
- c. Design oligonucleotides to amplify VHL (variant 1, [NM_000551.3](#)) with a 3'-5xGLY linker, no stop codon and flanking 5'-*Bam*HI and 3'-*Eco*RI restriction sites; *gctaGGATCCGCC-(VHL)-GGTGGAGGCAGGTGAATT**Cgact* (the lower case bases indicate overhangs to allow direct digestion of the PCR product). A holding vector (DU52906) containing this cloned fragment is available from the MRC-PPU (<http://mrcppureagents.dundee.ac.uk/reagent-catalogues>).

2. Generation of Tet-inducible nano/monobody control
 - a. Digest the vector pRetroX-Tight Puro with *Bam*HI and *Not*I to produce a single fragment of 6.6 Kb (Figure 1), purifying as shown above (step A2) and eluting in 50 µl d/dH₂O.
 - b. Re-suspend the lyophilised GeneArt nano/monobody vector in 50 µl d/dH₂O.
 - c. PCR amplify the nano/monobody with *Bam*HI and *Not*I primers using KOD Hot Start DNA polymerase (1 µl template [10 ng/µl GeneArt cDNA], 5 µl 10x PCR buffer, 5 µl dNTP mix [2 mM each], 3 µl 25 mM MgSO₄, 3 µl DMSO, 1.5 µl each F&R primers [10 µM], 1 µl KOD polymerase, 29 µl d/dH₂O).
 - d. PCR: 2 min 94 °C then 30 cycles of 15 sec 94 °C, 30 sec 60 °C, and 25 sec 72 °C followed by a single elongation step of 7 min 72 °C.
 - e. Run 5 µl of the PCR reaction with 15 µl loading buffer on a 1% agarose gel to determine success. The PCR band should match the size of the nano/monobody template (Figure 1).
 - f. Clean 40 µl of the confirmed PCR reaction (step B2d) using the QIAquick PCR purification kit according to the manufacturer's instructions but eluting in 85 µl d/dH₂O.
 - g. Cut the entire cleaned PCR product by adding 10 µl 10x FastDigest buffer, 2 µl *Bam*HI, 2 µl *Not*I and 1 µl *Dpn*I to the eluate from step B2f. Incubate at 37 °C for a minimum of 2 h.
 - h. Repeat the PCR cleanup step, this time eluting in 50 µl d/dH₂O to yield the digested AdPROM insert.

- i. Ligate the AdPROM insert into pRetroX-Tight Puro following the cloning, verification and Maxiprep steps shown previously (section A3) except use *Bam*HI and *Not*I in step A3i (Figure 1), sequence with pBABE F (5'-CCTCCTCTTCCTCCATCC) and pRetroX R (5'-TGCTCCAGACTGCCTTGG) in step A3k and inoculate 250-400 ml of LB (50 µg/ml ampicillin) in step A3n.
3. Generation of Tet-inducible AdPROM vector
 - a. Set up a VHL PCR using KOD Hot Start DNA polymerase; (1 µl template [VHL containing vector, section A3 above]), 5 µl 10x PCR buffer, 5 µl dNTP mix (2 mM each), 3 µl 25 mM MgSO₄, 3 µl DMSO, 1.5 µl each F&R primers (10 µM), 1 µl KOD polymerase, 29 µl d/dH₂O.
 - b. PCR: 2 min 94 °C then 30 cycles of 15 sec 94 °C, 30 sec 60 °C, and 50 sec 72 °C followed by a single elongation step of 7 min 72 °C.
 - c. Run 5 µl of the PCR reaction with 15 µl loading buffer on a 1% agarose gel to determine success. The expected band size for VHL and linker should be 0.7 Kb (Figure 1).
 - d. Clean 40 µl of the confirmed PCR reaction (step B3b) using QIAGEN's QIAquick PCR purification kit according to manufacturer's instructions but eluting in 85 µl d/dH₂O.
 - e. Cut the entire cleaned PCR product by adding 10 µl 10x FastDigest buffer, 2 µl *Bam*HI, 2 µl *Eco*RI and 1 µl *Dpn*I to the eluate from step B3d. Incubate at 37 °C for a minimum of 2 h then repeat the PCR cleanup step, this time eluting in 50 µl d/dH₂O to yield the digested VHL insert.
 - f. Digest 25 µl of the re-suspended GeneArt nano/monobody vector with *Eco*RI and *Not*I at 37 °C for 2 h (25 µl DNA, 10 µl 10x FastDigest Green Buffer, 2 µl *Eco*RI, 2 µl *Not*I, 61 µl d/dH₂O).
 - g. Run the entire digest on a 1% agarose gel (split between neighbouring wells as necessary) and excise the released cDNA insert (small band).
 - h. Purify using the QIAquick gel extraction kit and elute in 50 µl d/dH₂O to yield the AdPROM insert.
 - i. Perform a 3-way ligation (see Figure 2) by simultaneously inserting the VHL and AdPROM inserts into pRetroX-Tight Puro using the Rapid DNA Ligation Kit; (2.5 µl VHL insert (*Bam*HI/*Eco*RI), 2.5 µl AdPROM insert (*Eco*RI/*Not*I), 1 µl pRetroX-Tight Puro vector (*Bam*HI/*Not*I), 4 µl 5x Rapid DNA Ligation Buffer, 1 µl ligase, 9 µl d/dH₂O).
 - j. Follow the cloning, verification and Maxiprep steps shown previously (section A3) with some minor changes; allow 10 min for the ligation reaction in Procedure A3b, add 10 µl of ligation reaction to 100 µl cells in step A3c, perform diagnostic digests in Procedure E using *Bam*HI and *Not*I (Figure 1), sequence with pBABE F (5'-CCTCCTCTTCCTCCATCC) and pRetroX R (5'-TGCTCCAGACTGCCTTGG) in step A3k and inoculate 250-400 ml of LB (50 µg/ml ampicillin) in step A3m.

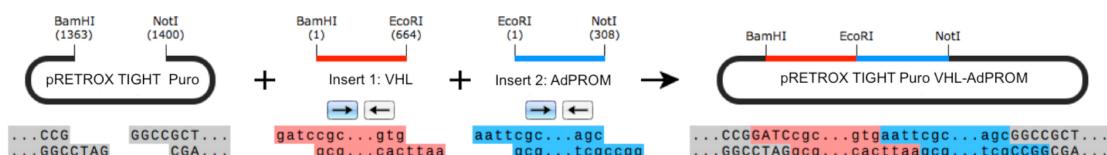


Figure 2. Schematic illustrating a typical 3-way ligation as described in step B3i. The diagram shows the Tet-inducible pRetroX Tight plasmid (see Procedure E), however, the principles for pBABED plasmids remain the same.

C. Retrovirus production and target cell infection for the constitutive expression AdPROM system

1. Culture HEK-293 FT cells and the desired target cell lines following the recommended conditions. For HEK-293 FT cells, cells are grown in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin (hereafter referred to as complete medium). Cells are maintained at 37 °C in a humidified incubator at 5% CO₂.
2. Seed 5 × 10⁵ HEK-293 FT cells per one 10-cm culture dish in complete medium and incubate at 37 °C in a humidified incubator at 5% CO₂ overnight. The next day the cells should be ~70% confluent. Prior to transfection, replace medium with 9 ml of complete medium. One 10-cm dish of ~70% confluent HEK-293 FT cells is enough for the generation of an individual retrovirus. For the constitutive expression AdPROM system we recommend the following control and AdPROM retroviruses: Flag-tagged Nano/monobody alone, Flag-tagged VHL alone, Flag-tagged VHL-nano/monobody fusion protein (see Figure 3 for a flow diagram describing the process).

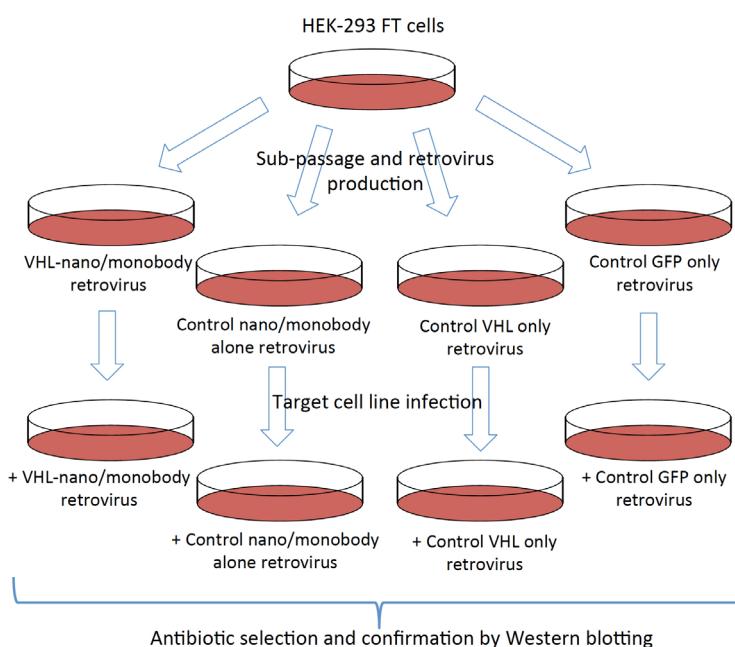


Figure 3. Schematic flow diagram illustrating the process by which target cell lines are infected with AdPROM and control retroviruses as outlined in Procedure C. Briefly, HEK-293 FT cells are sub-passaged and individually transfected with control or AdPROM

constructs, along with the GAG/Pol and VSVG constructs required for retroviral production. Following incubation, retrovirus-containing medium from each cell dish is collected, filtered and applied to the target cells for 24 h. Infected target cells are selected in appropriate antibiotic-containing medium, and successful retrovirus integration is confirmed through Western blotting.

3. Pipette 300 μ l of serum-free Opti-MEM into two 1.5 ml Eppendorf tubes. To one of these tubes, add 6 μ g of the cDNA of interest (e.g., Flag-VHL alone) in the pBABED puro vector, 3.2 μ g of the pCMV-GAG/Pol, and 2.4 μ g of the pCMV-VSVG. To the other tube, add 24 μ l of 1 mg/ml PEI (diluted in 25 mM HEPES pH 7.5). A transfection control with pCMV5-GFP (10 μ g) is also recommended.
4. Allow the tubes to incubate for 5 min at room temperature (RT), before combining both tubes and incubating for a further 20 min at RT.
5. Following the incubation period, add the resulting infection mix drop-wise on to the 10-cm dish of 70% confluent HEK-293 FT cells.
6. Place cells in the 37 °C incubator and incubate for 16 h.
7. Collect the medium from cells and dispose safely (by following appropriate disposal guidelines). Replace with fresh complete medium and incubate cells for 24 h.
8. Collect the retrovirus-containing medium and filter through 0.45 μ m sterile syringe filters into sterile tubes. Either store the filtered viral media at -80 °C, or add desired amount drop-wise onto the target cells (50-70% confluent at the time of infection) for infection. Typical dilutions range from 1:2 to 1:200 and need to be tested to achieve desired expression of AdPROM. Add polybrene (8 μ g/ml, diluted in water) to aid infection. Dispose of the HEK-293 FT cells safely.
9. Infect the target cells for 24 h.
10. Remove the viral media, and wash the cells once in sterile PBS. Supply 10 ml of complete medium onto the target cells and allow them to recover for 24 h.
11. Select the infected cells in appropriate antibiotic. For pBABED Puro vectors, puromycin (2 μ g/ml) is applied to cells for 2 days (concentrations and length of antibiotic treatment sufficient to kill each target cell should be determined). As a control, include a dish of non-infected cells, to monitor the effectiveness of the antibiotic cytotoxicity.
12. When control cells are all dead, collect the antibiotic-resistant cells and expand them for analysis by Western blotting.

D. Testing the effectiveness of target protein degradation by constitutive expression AdPROM

1. For lysis of AdPROM infected cells, wash cells twice in ice-cold PBS, and lyse cells in (200 μ l-1 ml) ice-cold lysis buffer (see Recipes) using cell scrapers. Transfer extracts into 1.5 ml Eppendorf tubes and store on ice. Include non-infected wild type cells as a negative control.
2. Following 10 min incubation on ice, clarify extracts by centrifugation (13,000 $\times g$, 20 min, 4 °C).

3. Collect the supernatant and perform a Bradford assay (Bradford, 1976) to quantify the protein concentration in each sample.
4. Normalise the protein concentrations in each sample by diluting extracts in lysis buffer so that the protein concentration in each extract is the same.
5. Add 5x SDS sample buffer (see Recipes) to each sample for a final concentration of 1x SDS sample buffer. Denature proteins by heating at 95 °C for 5 min. Samples can be stored at -20 °C for future use.
6. Following a brief centrifugation (13,000 x g, 2 min, RT), extracts are ready to load onto SDS polyacrylamide gels.
7. Resolve extracts (typically 20 µg protein per lane) by SDS-PAGE and transfer onto PVDF membranes. Include molecular weight markers on end lanes on both sides.
8. For Western blotting, membranes are first blocked in 5% milk in TBS-T (1x) (see Recipes). Membranes are then incubated with primary antibodies diluted in either milk or BSA in TBS-T (1x) at 4 °C overnight, or for 1 h at RT (the dilution and milk vs. BSA need to be optimised for each antibody). To monitor target protein levels, use an antibody that recognises the POI, in addition to an antibody that recognises a house-keeping gene such as GAPDH or actin, for use as a loading control. Additionally, to confirm successful retrovirus integration, blot for the Flag-tag that was fused to the control and AdPROM retroviruses.
9. Membranes are then washed in TBS-T, before incubation with HRP-conjugated secondary antibodies for 1 h at RT. Subsequently proteins of interest are detected by using ECL, and exposing the membranes to X-ray films for different exposure times.

E. Adaptation of AdPROM for Tet-inducible POI degradation

As described above (Procedure C-Procedure D) for constitutive POI degradation section, with the following changes:

1. The pRetroX-Tet-On advanced system is a two-vector system, comprised of the pRetroX-Tet-On transactivator (G418 resistant) and the pRetroX-Tight gene of interest (puromycin resistant). These vectors are treated as per the pBABED puro vector system (6 µg added to the infection mix). One can either infect the target cells with both retroviruses at the same time and select for infection with both G418 and puromycin; or firstly infect target cells with the pRetroX-Tet-On transactivator, and select cells in G418 (200 µg/ml), and when non-infected cells are all dead, G418-resistant cells can be re-infected with the pRetroX-Tight AdPROM constructs and selected in puromycin (2 µg/ml).
2. The viral dilutions for these retroviruses will need to be optimised. Ideally one wants to see no detectable AdPROM expression in the absence of Tet/doxycycline, but robust expression in the presence of Tet/doxycycline.
3. For inducible AdPROM expression purposes, we recommend using 0.5-2 µg/ml doxycycline for the initial characterisation.

4. Prior to cell lysis, expression of the AdPROM and control constructs will need to be performed, and ideally a Tet/doxycycline induction time course performed to monitor both AdPROM expression and target protein degradation.

F. Degradation of proteins for which no nano/monobody is available

In the event that no nano/monobody exists for your POI, we have shown that it is possible to first introduce an endogenous GFP knockin tag onto the gene encoding the POI using CRISPR/Cas9 gene editing technology, to enable targeting of the GFP-tagged target protein with high affinity anti-GFP nanobodies.

Following successful GFP integration onto the target protein gene locus, the protocol for degradation of the GFP-tagged endogenous protein either constitutively or inducibly is the same as above (Procedure C-Procedure E).

Data analysis

1. Have the retroviruses been integrated correctly? Blotting for the epitope-tagged VHL alone, nano/monobody alone, and the VHL-nano/monobody fusion protein will confirm successful AdPROM and control cDNA construct expression. Note that there will be a band shift corresponding to the molecular weight of the nano/monobody (around 100 amino acids) for the VHL-nano/monobody fusion protein relative to the VHL alone construct (see the original entries for representative Western blots highlighting the VHL mobility shift (Fulcher *et al.*, 2016 and 2017)).
2. Does AdPROM expression result in degradation of the POI? In uninfected cells and cells expressing VHL alone or the nano/monobody alone, there should be no degradation of the POI relative to the loading control. However, if target protein degradation is observed in cells expressing the VHL-nano/monobody polypeptide, with no detectable change in the levels of the loading control relative to the other samples, then the AdPROM system has been integrated successfully (see the original entries for representative Western blots highlighting the degradation of the POI with no concurrent proteolysis of the loading control protein (Fulcher *et al.*, 2016 and 2017)).
3. For the Tet-inducible system, if a time course experiment has been performed, blotting for VHL-nano/monobody polypeptide expression will determine the time for AdPROM expression. Blotting for the AdPROM-targeted POI will show the time taken for inducible proteolysis of the target protein. The degradation of the POI should succeed the AdPROM fusion protein expression.

Notes

1. In some cases, expression of the nano/monobody alone retroviruses may inadvertently stabilise the target protein through binding to it and e.g., blocking ubiquitination sites present on the target protein.
2. If, despite successful expression of the AdPROM system, no target protein degradation is observed, confirm nano/monobody:POI interaction through co-IP experiments. If the nano/monobody does not interact with the POI, or if the interaction is low-affinity, this could explain the lack of AdPROM-induced proteolysis of the target protein.
3. For essential genes, constitutive AdPROM-mediated degradation of the target protein can still lead to lethality. In this instance, consider using the inducible AdPROM system to temporally control the proteolysis of the target protein.
4. If the inducible AdPROM system is leaky, we recommend further dilution of the pRetroX-Tight gene of interest vector. Alternatively, replacing the FBS with Tet-free FBS may be helpful.

Recipes

1. DNA loading buffer
 - 100 mg Orange G
 - 15 ml glycerol
 - 12 ml 0.5 M EDTA pH 7.5-8.0
 - 23 ml d/dH₂O
2. Lysis buffer
 - 50 mM Tris-HCl pH 7.4
 - 0.27 M sucrose
 - 120 mM NaCl
 - 1 mM EDTA pH 8.0
 - 1 mM EGTA pH 8.0
 - 1 mM sodium orthovanadate
 - 1 mM sodium glycerophosphate
 - 50 mM sodium fluoride
 - 10 mM sodium pyrophosphate
 - 1% Nonidet P-40
 - 0.1% β-mercaptoethanol (add fresh)
 - Protease inhibitor cocktail tablet (add fresh)
 - Diluted in d/dH₂O
3. Running buffer (10x)
 - 250 mM Tris
 - 1.9 M glycine

- 1% (v/v) SDS
- Diluted in d/dH₂O
- 4. Transfer buffer (10x)
 - 250 mM Tris
 - 1.9 M glycine
 - 10% (v/v) final methanol
- 5. Sample buffer (5x)
 - 312.5 mM Tris-HCl, pH 6.8
 - 50% (w/v) glycerol
 - 10% (w/v) SDS
 - 0.10% (w/v) bromophenol blue
 - 5% (w/v) β-mercaptoethanol
- 6. TBS (10x)
 - 200 mM Tris
 - 1.5 M NaCl
 - Diluted in d/dH₂O
 - pH to 7.6 with HCl
- 7. TBS-T (1x)
 - Dilute 10x TBS in d/dH₂O
 - Add 0.1% Tween-20
- 8. TE buffer (10x)
 - 100 mM Tris-HCl pH 8.0
 - 10 mM EDTA pH 8.0
 - Diluted in d/dH₂O

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