

Chapter 2

High Capacity Extrachromosomal Gene Expression Vectors

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Abstract

Extrachromosomal gene expression vectors that contain native genomic gene expression elements have numerous advantages over traditional integrating mini-gene vectors. In this protocol chapter we describe our work using episomal vectors where expression of a cDNA is controlled by a 10 kB piece of genomic DNA encompassing the promoter of the low density lipoprotein receptor. We explain methods to sub-clone large genomic inserts into gene expression vectors. We also illustrate various methods employed to ascertain whether expression from these vectors is robust and physiologically relevant by investigating their sensitivity to changes in cellular milieu. Delivery of gene expression vectors *in vivo* is also described using hydrodynamic tail vein injection, a high pressure, high volume tail vein injection used for liver-directed gene transfer.

Key words: Episomal, Hydrodynamic tail vein injection, LDLR, Familial hypercholesterolaemia, Live imaging, Cholesterol, Genomic promoter, Luciferase

1. Introduction

High capacity genomic DNA gene expression vectors where transgene expression is controlled by native expression elements have a strong advantage over mini-gene vectors where a heterologous promoter drives cDNA expression. When using an entire genomic locus it is possible to deliver a complete gene including all introns, exons and regulatory elements in the correct genomic context. This is important for many applications that require systems that do not lead to transgene over-expression (1, 2).

Working with gene expression vectors that deliver transgenic DNA into cells without integrating into the genome is becoming increasingly attractive. Integration of a vector does ensure long-term retention of the transgene, however, it can also lead to gene silencing through positional effects, and cellular transformation (3, 4).

In this chapter we describe the use of high capacity extrachromosomal vectors in vitro and in vivo in the context of our work in gene therapy for familial hypercholesterolaemia (FH) (5–8). FH is a condition caused by mutations in the low density lipoprotein receptor (*LDLR*) gene and is characterised by high circulating levels of cholesterol (9, 10). The condition represents a unique challenge in gene therapy as over-expression of *LDLR* leads to toxic intracellular accumulations of LDL (11, 12). In addition, any transduced population of cells will be required to clear large amounts of cholesterol from the plasma continuously as cholesterol synthesis is constitutively active in the liver. This means that the therapeutic *LDLR* transgene has to complement the loss of function of the endogenous gene by expressing the *LDLR* in a physiologically regulated manner. In this protocol chapter we include descriptions of functional analysis of *LDLR* transgene expression including expression of reporter genes from genomic promoter regions and analysis of LDL binding and internalisation by quantitative cell culture assay.

2. Materials

Unless otherwise stated all chemicals were obtained from Sigma (Dorset, UK).

2.1. Vector Design

2.1.1. BAC DNA Maxi-Prep

1. LB agar (e.g. Calbiochem) prepared as per manufacturer's instructions and autoclaved.
2. Antibiotics: Ampicillin (Amp): 50 mg/ml solution made up in MilliQ water and filtered through a 0.22 µm filter. Kanamycin (Kan): 25 mg/ml solution made up as for Ampicillin. Chloramphenicol (Chl): 15 mg/ml solution made up in 70% ethanol. All antibiotic solutions were stored in aliquots at -20°C.
3. LB Broth Miller (e.g. Novagen, VWR, Leighton Buzzard) made up as per manufacturer's instructions and autoclaved.
4. Qiagen Tip 50 Maxiprep kit (Qiagen, Crawley): all buffers are included with kit. Buffer P1 should have RNase added before storing at 4°C. Buffer P3 should also be stored at 4°C, buffer QF should be heated to 55°C before use.
5. Kimwipe tissues (Kimberly Clark, Fisher Scientific, UK).
6. All centrifugations were performed in a Beckman Avanti J-E centrifuge – Rotors: J10.5 and J17.
7. 250-ml centrifuge bottles (Beckman, High Wycombe, UK).
8. Oakridge tubes (Beckman, High Wycombe, UK).
9. Isopropanol (VWR, Leighton Buzzard, UK).

10. Tris–EDTA: TE, 10 mM Tris–HCl at pH 8, 1 mM EDTA.

11. Materials required for pulsed field gel electrophoresis.

*2.1.2. Sub-cloning
Genomic Fragments*

1. pSC101-BAD-gbA-tet plasmid (Gene Bridges, Heidelberg). This plasmid contains tetracycline resistance (tet), has a temperature sensitive origin of replication and the genes required for homologous recombination (recE, an exonuclease and recT) are under the control of an arabinose inducible promoter.
2. BioRad Gene Pulser Controller (BioRad, Hemel Hempsted, UK); unless otherwise stated all electroporation into bacterial host cells was performed at settings: 25 μ F, 1.8 kV and 200 Ω .
3. Electrocompetent cells containing genomic expression plasmid (e.g. BAC).
4. SOC medium (Invitrogen, Paisley, UK).
5. LB agar plates containing tetracycline (9 μ g/ml, pSC101) and chloramphenicol (15 μ g/ml, BAC plasmid).
6. L-arabinose.
7. Glycerol.
8. BioXact Long, long range polymerase (Bioline, London, UK).
9. Qiagen PCR purification kit (Qiagen, Crawley, UK).

*2.1.3. Retrofitting
Expression Plasmids with
Episomal Maintenance
Plasmids*

1. Cre enzyme/buffer (NEB, Hitchin, UK).
2. Dialysis membrane (Millipore, Watford, UK).
3. DH10B electrocompetent cells (Invitrogen, Paisley, UK).

2.2. Cell Culture

1. CHO a7 Ldlr –/– cell line.
2. Hams F12 medium (Invitrogen, Paisley, UK).
3. L-glutamine (L/G, Invitrogen, Paisley, UK).
4. Penicillin/streptomycin (P/S, Invitrogen, Paisley, UK).
5. Foetal bovine serum (FBS, Invitrogen, Paisley, UK).
6. Lipid depleted foetal bovine serum (LPDS, Biomedical Technologies, Stoughton, MA).
7. Tissue culture plasticware, e.g. 75 cm², 25 cm² flasks, 96/24/12/6 well plates.

*2.2.1. Establishment of
Episomal Clonal Cell Lines*

1. Lipofectamine (Invitrogen, Paisley, UK).
2. Opti-MEM, serum free medium (Invitrogen, Paisley, UK).
3. Trypsin/EDTA (Invitrogen, Paisley, UK).
4. G418 (neomycin analogue, Invitrogen, Paisley, UK).
5. Selection medium: Hams F12, 1% L/G, 1% P/S, 10% FBS, 600 μ M G418.

**2.2.2. Confirmation
of Plasmid Maintenance
(Plasmid Rescue)**

1. 10-cm cell culture plates.
2. STET buffer: 8% sucrose, 5% triton X-100, 50 mM EDTA, 50 mM Tris-HCl at pH 8.
3. Alkaline SDS: 1% SDS and 0.2 N NaOH.
4. 7.5 M ammonium acetate.
5. 1.5-ml Heavy Phase Lock Gel tubes (Eppendorf, Hamburg, Germany).
6. Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris-HCl at pH 8 and 1 mM EDTA.
7. Chloroform.
8. TE + RNase: 10 mM Tris-HCl at pH 8, 1 mM EDTA and 5 µg/ml RNase A.

**2.3. Functional Assays
In Vitro**

2.3.1. Luciferase Assay

1. Dynex luciferase plate reader with dual injectors (or similar).
2. Hams F12 medium P/S, L/G plus lipid depleted serum.
3. Transfection reagents (as in Subheading 2.2.1).
4. Cholesterol, make a 12 µg/ml working solution in 70% ethanol.
5. 25-Hydroxycholesterol, make a 0.6 µg/ml working solution in 70% ethanol.
6. Mevastatin (Merck, Nottingham, UK), working solution 1 mM made up in ethanol.
7. Sterol incubation medium: Hams F12 + LPDS, 1:1,000 cholesterol and 1:2,000 25-hydroxycholesterol.
8. Statin incubation medium: Hams F12 + LPDS and 1:1,000 Statin.
9. Luciferase lysis buffer: 25 mM Tris-PO₄ at pH 7.8, 0.2 mM 1,2-diaminocyclohexane tetraacetic acid, 1:10 glycerol, 1:100 Triton X-100 and 2 µM dithiothretol.
10. Luciferase Solution A – Luciferin solution: 0.3 mg/ml (Caliper Life Sciences, Hopkinton, MA).
11. Luciferase Solution B – Luciferase assay buffer: 15 mM MgSO₄, 15 mM KPO₄ at pH 7.8, 0.04 mM ethylene glycol tetraacetic acid at pH 7.8, 2 µM dithiothretol, 50 µl β-mercaptoethanol and 200 mg/ml adenosine triphosphate.
12. O-nitrophenyl-β-galactopyranoside (ONPG) assay buffer: 6 mM Na₂HPO₄, 4 mM Na₂H₂PO₄, 10 mM KCl, 1 mM MgSO₄, 20 mg/ml ONPG, 2 µM dithiothretol and 50 µl β-mercaptoethanol; 100 µl.
13. ONPG assay stop solution: 50 mM Na₂CO₃.

2.3.2. Dil-LDL Assay

1. Spectrofluorimeter, plate reader or similar. Excitation wavelength 520 nm and emission wavelength 580 nm.

2. HamsF12 medium P/S, L/G plus lipid depleted serum.
3. Transfection reagents (as in Subheading 2.2.1).
4. Cholesterol, make a 12 µg/ml working solution in 70% ethanol.
5. 25-Hydroxycholesterol, make a 0.6 µg/ml working solution in 70% ethanol.
6. Mevastatin (Merck), working solution 1 mM made up in ethanol.
7. Sterol incubation medium: HamsF12 + LPDS, 1:1,000 cholesterol and 1:2,000 25-hydroxycholesterol.
8. Statin incubation medium: HamsF12 + LPDS and 1:1,000 Statin.
9. DiI-LDL (AbD Serotech, Abingdon, UK).
10. Unlabelled human LDL (AbD Serotech, Abingdon, UK).
11. DiI-LDL medium: HamsF12 + LPDS and 10 µg/ml DiI-LDL.
12. DiI-LDL plus cold medium: HamsF12 + LPDS, 10 µg/ml DiI-LDL and 500 µg/ml human LDL.
13. DiI-LDL lysis buffer: 25 mM Tris-PO₄ at pH 7.8, 0.2 mM 1,2-diaminocyclohexane tetraacetic acid, 1:10 glycerol, 1:100 Triton X-100 and 2 µmol/l dithiothretol.
14. DiI-LDL standard curve solutions ranging from 0.016 to 2 µg/ml made up in lysis buffer.

2.4. Delivery and Analysis In Vivo

2.4.1. Liver-Specific Plasmid Delivery

1. Adult mice, 25–30 g.
2. Prewarmed sterile PBS.
3. Plasmid DNA 20–50 µg/animal in 2.5 ml PBS.
4. 27 g needles.
5. A 38–40°C heating box suitable for mice.
6. Isoflurane.
7. Oxygen.
8. Anaesthetic machine with an isoflurane vaporiser.
9. Warming pad set to 37°C.

2.4.2. Transfection Efficiency Analysis (Immunohistochemistry)

1. Cannula needle attached to a 50-ml syringe.
2. Phosphate buffered saline tablets (Sigma 79382).
3. 4% Paraformaldehyde w/v in PBS.
4. Ethanol: 70, 95 and 100%.
5. Xylene/histoclear.
6. Paraffin wax.
7. Automatic Tissue Processor.

8. Paraffin embedder.
9. Microtome.
10. Polysine slides (VWR, Leighton Buzzard, UK).
11. PAP pen (Abcam, Cambridge, UK) – creates a hydrophobic barrier around section keeping staining reagents on the section. This reduces the amount of reagent needed and also reduces cross-contamination between sections on the same slide.
12. Endogenous biotin blocking kit (Invitrogen, Paisley, UK) consisting of blocking solution A (streptavidin reagent) and blocking solution B (biotin reagent).
13. Immunohistochemistry blocking solution: 1% fish gelatin, 0.1% Triton X-100, 10% goat serum in Tris buffered saline – 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl.
14. Biotinylated anti-human LDLR monoclonal primary antibody (Fitzgerald Industries International, North Acton, MA).
15. Anti- β -galactosidase secondary antibody (Invitrogen, Paisley, UK).
16. 4',6-Diamidino-2-phenylindole (DAPI, Invitrogen, Paisley, UK) nuclear material counterstain that emits a blue fluorescence.
17. Mounting medium such as glycerol or Clearmount (Invitrogen, Paisley, UK).

*2.4.3. Transfection
Efficiency Analysis
(Live Imaging)*

1. IVIS 100 live imaging camera and software (Caliper Life Sciences, Hopkinton, MA).
2. Luciferin (Caliper Life Sciences, Hopkinton, MA).

*2.4.4. Transfection
Efficiency Analysis
(Plasmid Rescue)*

1. Genomic lysis buffer: 0.6% SDS, 100 mM NaCl, 50 mM Tris-HCl (pH 8), 20 mM EDTA.
2. Proteinase K 10 mg/ml working solution.
3. Phase lock gel (light, Eppendorf).
4. Ethanol 70 and 100%.
5. TE.
6. DH10B bacteria.
7. LB agar plates containing an appropriate antibiotic.

3. Methods

3.1. Vector Design

3.1.1. Construction of Retrofitting Plasmids

Extrachromosomal vector maintenance requires the inclusion of elements that will promote the maintenance of a plasmid vector as a replicating, episomal gene expression unit. Mammalian cells being either; the Epstein–Barr virus (EBV) derived episomal system, the S/MAR system or human artificial chromosomes (2).

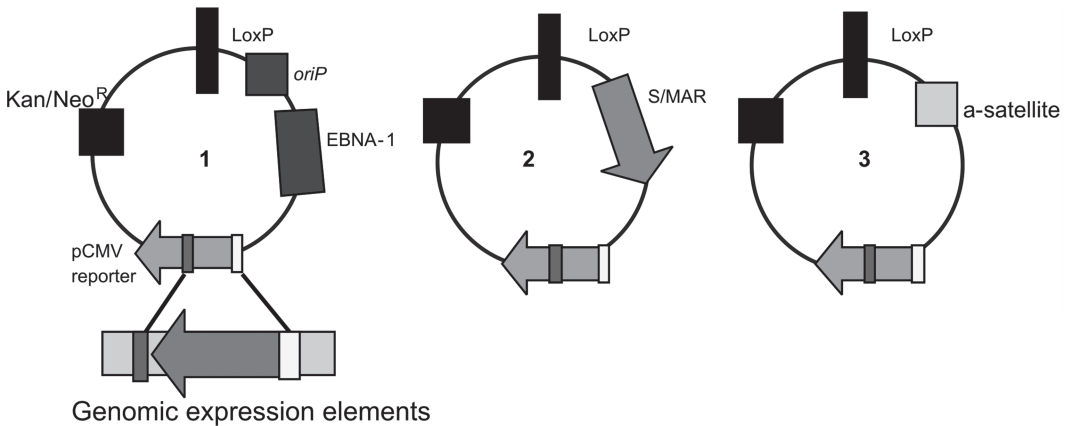


Fig. 1. Gene expression plasmid vectors for the promotion of extrachromosomal vector maintenance may contain one of three systems. (1) The EBV system requires the inclusion of *OriP* and EBNA-1. (2) The S/MAR system requires the inclusion of S/MAR sequences. (3) Human artificial chromosomes are produced from vectors containing alpha-satellite DNA. Also included in these vectors are pCMV-reporter gene expression cassettes which can be converted to genomic promoter-reporter gene expression cassettes through homologous recombination. Kanamycin/neomycin resistance (Kan/neo^R) is essential for selection in bacterial and mammalian cells. A *LoxP* site is included so that plasmids can be retrofitted to BAC plasmids or other *LoxP* containing plasmids.

Each maintenance system will require specific modifications to any plasmid. The EBV system requires the addition of the *trans*-acting Epstein-Barr virus Nuclear Antigen-1 (EBNA-1) protein and the *cis*-acting *oriP* origin of replication. The S/MAR system requires the S/MAR sequence from the pEPI-based vectors. Human artificial chromosomes require the inclusion of α -satellite DNA (Fig. 1). It is also important for expression analysis and clonal cell establishment to include a reporter gene under a constitutive promoter and a mammalian selection cassette (Fig. 1). For *in vivo* use a reporter gene such as luciferase is particularly useful if you are able to utilise live imaging technology. If this is not possible β -galactosidase is an excellent and versatile reporter with very little background expression. All vectors need to include a *loxP* site, which facilitates Cre-mediated recombination.

3.1.2. BAC DNA Maxi-Prep

The following protocol uses Qiagen Tip 500 maxi-prep kits with a modified protocol. It is highly efficient for the purification of large plasmids, but can also be used to obtain high yields from smaller plasmids.

1. On a LB agar plate containing the appropriate antibiotics, streak a small amount of bacterial stock and incubate overnight at 37°C.
2. Grow a small starter culture of a single colony in 1.5 ml LB containing the appropriate antibiotics for a minimum of 6 h shaking at 37°C. At this point, the media should appear slightly cloudy.

3. Tip the 1.5 ml culture into 250 ml of LB+antibiotics and grow overnight at 37°C shaking at 225 rpm.
4. The following morning harvest the bacterial cells by centrifugation; $6,000 \times g$ for 10 min.
5. Tip off the media and resuspend the bacterial pellet with 15 ml of cold (4°C) P1 (resuspension) solution containing RNase. To resuspend, completely leave the tubes to shake in the incubator for 10 min at 225 rpm.
6. Lyse the bacteria with 15 ml of P2 (lysis) solution. Incubate the cells for precisely 5 min, mixing every 1 min by gentle swirling.
7. Neutralise the lysis by adding 15 ml of P3 (neutralisation) solution and swirl to mix.
8. Incubate in P3 for 20 min on ice by gently inverting the tube at 2-min intervals. This step is important because the BAC DNA can be precipitated with the bacterial genomic DNA and is a major cause of low yields.
9. Pellet the flocculate by centrifugation; $15,000 \times g$ for 35 min at 4°C.
10. Prepare the Tip-500 columns for DNA binding
 - Equilibrate with 15 ml of QBT (equilibration) buffer
 - Insert a double layer of “kimwipe” tissue into the column by pushing in with a finger. This tissue acts a filter to prevent bacterial flocculate from clogging the column.
11. Pour supernatant containing the plasmid DNA through the tissue and let it run through the column. Gently squeeze out the tissue being careful to avoid any precipitate falling onto the column.
12. Wash the column twice with 30 ml of QC (wash) buffer.
13. Elute DNA into Oakridge tubes using 15 ml of prewarmed (55°C) QF (elution) buffer.
14. Precipitate the DNA by adding 10.5 ml isopropanol and pellet by centrifugation at $27,000 \times g$ for 30 min at 4°C. Be aware that isopropanol pellets tend to be glassy in appearance and so may not be visible.
15. Carefully tip off supernatant into a clean 50-ml plastic tube. At this point check to make sure the supernatant does not contain anything that looks like a pellet.
16. Wash the pellet in 3.5 ml of 70% ethanol without mixing. Centrifuge at $27,000 \times g$ for 30 min at 4°C.
17. Very carefully decant the supernatant and leave to air dry.
18. Resuspend the pellet by gentle flicking in 250 µl of TE buffer overnight at 4°C.

19. The following day, flick the tube again and spin briefly to collect the solution. Transfer to a fresh microcentrifuge tube and store at 4°C.
20. Check the quality of DNA preparation by restriction enzyme digestion of 400–500 ng of DNA. Digest should be separated using pulsed field gel electrophoresis.

3.1.3. Sub-cloning Genomic Fragments Using Homologous Recombination

Here we describe the sub-cloning of large genomic fragments into plasmids using RecE/RecT or ET recombination. In our work we used recombination to create a plasmid that contained a 10 kb piece of genomic DNA encompassing the LDLR genomic promoter driving either luciferase or LDLR cDNA (8).

1. Generate an expression plasmid containing (Fig. 2); trans-gene expression cassette driven by heterologous promoter, human origin of replication, a polyadenylation site and anti-biotic resistance. Kanamycin/neomycin is useful as it allows for selection in both bacterial and mammalian cells and a loxP site for retrofitting.
2. Identify genomic region for subcloning.
3. Design recombination primers (Fig. 2); at least 55 bp homologous to genomic sequence and at least 25 bp homologous to the vector sequence

Primer A – 55 bp arm homologous to the genomic DNA 10 kb down stream of LDLR start codon. 25 bp arm homologous

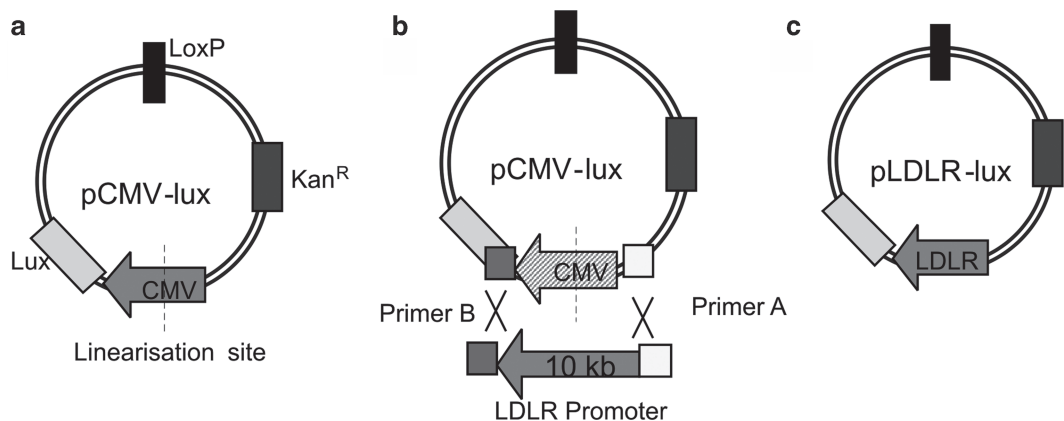


Fig. 2. Subcloning of genomic inserts into plasmid vectors using ET recombination is shown here schematically. The plasmid is designed that contains an expression cassette (a). The plasmid is linearised in the middle of the pCMV sequence. Primer A and Primer B are designed with homology arms that equate to 25 bp homology to the plasmid and 55 bp to genomic DNA (b). A PCR is performed using the linearised pCMV-lux plasmid. This results in a PCR product that incorporates the entire plasmid minus the pCMV and containing 5' and 3' homology arms homologous to genomic DNA. This product is electroporated into bacteria containing the *LDLR* BAC. Following recombination the resulting plasmid has the *LDLR* promoter in place of the pCMV promoter (c).

to the region immediately upstream of CMV promoter on the expression plasmid.

Primer B – 55 bp arm homologous to 55 bp up stream of LDLR start codon. The 25 bp arm is homologous to either LDLR or luciferase cDNA and includes the start codon.

Production of ET recombination electrocompetent cells

4. Thaw on ice a vial of electrocompetent DH10B cells containing specific genomic expression plasmid (e.g. LDLR BAC).
5. Electroporate 10 ng of pSC101-BAD-gbA-tet plasmid.
6. Add 450 μ l of SOC medium; mix. Transfer cell suspension to a 5-ml tube and shake at 30°C for 1 h.
7. Plate out bacteria on Chl/Tet LB agar plates and grow overnight at 30°C.
8. To confirm presence of pSC101-BAD-gbA-tet plasmid and BAC plasmid in bacteria, pick a single colony using a sterile inoculation needle. Dip the needle first into 1.5 ml of LB media containing chl then the same needle into 1.5 ml of LB media containing tet. Grow shaking overnight at 30°C (tet), or 37°C (chl). The following day extract plasmid DNA using an appropriate mini-prep method and check for intact plasmids using restriction enzyme digestion.
9. To create recombination ready cells; pick single clones from LB (tet/chl) plates and grow overnight in 1.5 ml LB (tet/chl) at 30°C.
10. The following morning tip the small starter culture into 100 ml LB (tet/chl) media and grow (30°C) to an OD of 0.1–0.15.
11. Add 1.5 ml of 10% L-arabinose to 100 ml culture and continue to grow with shaking at 37°C to an OD of 0.35–0.40 at A_{600} .
12. At this point stop the cells growing further by incubating on ice in the cold room (4°C) for 40 min.
13. Centrifuge to pellet bacteria (6,000 $\times g$ for 15 min at 4°C).
14. Pellet is then washed three times in 100 ml of ice cold 10% glycerol (6,000 $\times g$ for the first wash, 8,000 $\times g$ for the subsequent washes, all for 15 min at 4°C).
15. The pellets are resuspended in about 0.5–1 ml of the remaining supernatant from the final wash, and aliquoted into 50- μ l aliquots. Aliquots are snap frozen and stored at –80°C.

Sub cloning (see Note 1)

It is important to optimise annealing temperature for the recombination primers. A gradient of temperatures between 55 and 72°C should be sufficient to obtain the most efficient annealing temperature.

16. Set-up 6× long range PCR reactions as follows:

10× ^a OPTi buffer	2.5 µl
MgCl ₂ ^a (50 mM)	0.875 µl
dNTP (8 mM)	1.6 µl
DNA ^b	50 ng
Forward primer (1 µM)	2.5 µl
Reverse primer (1 µM)	2.5 µl
BioXact long	0.25 µl
MilliQ Water	Up to 25 µl

^aReagents included with BioXact long polymerase

^bLinearised at an appropriate site located between the 25 bp homology arms (see Note 2)

17. Perform the polymerase chain reaction on the six reactions using the following protocol.
18. Once PCR programme has run, make two pools of PCR mix containing reactions 1–3 and 4–6. Purify DNA from reaction components using a PCR purification kit (Qiagen) eluting DNA in 50 µl of milliQ water. Elute a second time in 40 µl of milliQ water and pool with 50 µl of eluate.

95°C	15 min	30–50 Cycles
95°C	30 s	
55–65°C	30 s	
72°C	1 min/kB to amplify	
72°C	1 min/kB to amplify	

19. Digest 85 µl of DNA with Dpn1.
20. PCR purify using PCR purification kit eluting in 20 µl.
21. Electroporate 8 µl into ET recombination electrocompetent cells (produced in step 4).
22. Following electroporation add 550 µl of LB media with no antibiotics, transfer cell suspension to a 5-ml tube and shake at 37°C for 75 min.
23. On a 10-cm agar plate containing appropriate antibiotics, spread 2 µl of bacteria. On a second 15-cm agar plate, spread the remaining bacteria and grow at 37°C overnight. The antibiotics used here should correspond to the plasmid, not the BAC, i.e. if the plasmid is kanamycin resistant then grow the cells on kanamycin plates that do not contain chloramphenicol.
24. Pick single clones for mini-prep analysis of recombination.

3.1.4. Retrofitting
Expression Plasmids with
Episomal Maintenance
Plasmids

In this section we describe Cre/loxP mediated retrofitting. This is a highly efficient way of combining expression cassettes with episomal maintenance elements.

1. Prepare the following recipe (see Note 3).

Sub-cloned plasmid	1 µg
Retrofitting plasmid	50 ng
10× Cre buffer	3 µl
Cre enzyme	1 unit
MilliQ water	Up to 30 µl

2. Incubate samples at 37°C for 30 min followed by 10 min at 75°C to inactivate the Cre enzyme.
3. Dialyse sample against water for 3 h to remove salts.
4. Electroporate 15 µl into DH10B cells.
5. Plate onto LB agar containing the appropriate antibiotic combination and incubate overnight at 37°C.
6. Analyse retrofitting using restriction enzyme digestion.

3.2. Cell Culture

3.2.1. Establishment of
Episomal Clonal Cell Lines

Described here is a protocol for the establishment of clonal cell lines in CHO a7 *Ldlr*^{-/-} cells (8).

1. Seed 1×10^5 – 1×10^6 cells per well of a 6-well dish. Leave to grow for 24 h.
2. Make up the transfection mix in a 15-ml tube. For a 6-well dish use up to 4 µg plasmid DNA and 10 µl of lipofectamine in a total of 1.5 ml Opti-MEM. Leave mix to complex for about 10 min.
3. While waiting for the DNA/lipofectamine to complex, wash cells three times with Opti-MEM.
4. Apply 1.5 ml of transfection mix to cells and swirl gently.
5. Incubate cells in transfection mix for 4–6 h.
6. Remove transfection mix and wash cells three times in Opti-MEM.
7. After the final wash apply 3 ml of normal growth media to each well and leave cells for 48 h.

For each transfected well:

8. Wash 1× with PBS.
9. Apply 0.75 ml of trypsin and leave for 2 min.
10. Apply 0.75 ml of selection media (Subheading 2.2.1) and mix up and down to dislodge cells from plate.
11. Dispense all 1.5 ml of media plus cells into 13.5 ml of selection media (tube A).

12. Perform a serial dilution of cells; take 1 ml from tube A and dispense into tube B containing 14 ml of selection media and mix. Take 1 ml from tube B and dispense into tube C containing 14 ml of selection media.
13. Seed the cells from the serial dilutions into 6-well plates. Three wells per dilution should be sufficient.
14. In addition, seed a control well containing untransfected cells in selection media.
15. The cells should now be left until single clones have grown. It is important that cells are left as long as it takes for the untransfected cells to be completely killed by the selection antibiotics and for large, well defined colonies to form. It is normal for this to take up to 15 days.

Clones should be reasonably large, about 2 mm, and completely isolated from surrounding cells to avoid contamination of clonal populations. Once clones have reached a reasonable size that is, they are visible as small dots on the base of the plate, they can be picked. Clones can be picked using plastic clone rings (Sigma); however, in our experience, it is easier to pick the clones by hand as we will describe.
16. Looking at the plate from underneath, circle clones to be picked with a marker.
17. Check circled clones under the microscope; they should be discreet cell clones.
18. Remove selection media and wash cells with PBS before applying 1.5 ml of trypsin.
19. Take the plate to the microscope and using a 4× objective identify clone to be picked and using a P20 pipette, aspirate clonal cells from the plate (see Note 4).
20. Dispense cells into a single well of a 96-well plate containing 100 µl of selection media. Leave cells to grow to confluency.
21. Once cells are confluent, transfer them to progressively larger wells until they are growing in 25 cm² flasks.

3.2.2. Confirmation of Plasmid Maintenance (Plasmid Rescue)

1. Plate $2-5 \times 10^6$ clonal cells into 10-cm tissue culture dishes.
2. When confluent, extract episomal plasmid DNA using alkaline lysis. Scrape cells into 1.5 ml of PBS and centrifuge for 3 min at $5,000 \times g$.
3. Resuspend the cell pellet in 60 µl of STET buffer.
4. Lyse cells with 130 µl of alkaline SDS.
5. Neutralise with 110 µl of ammonium acetate and incubate on ice for 5 min.
6. Centrifuge at $13,000 \times g$ for 30 min at 4°C.

7. Transfer supernatant to Phase Lock gel Heavy tube that has been centrifuged at maximum speed for 1 min.
8. Pipette 500 μ l of Phenol:Chloroform onto supernatant and mix well.
9. Centrifuge at $13,000\times g$ for 2 min at room temperature. Remove the upper aqueous phase and repeat.
10. Extract twice with 400 μ l of Chloroform using phase lock Eppendorfs.
11. Precipitate DNA using 2.5 times the volume of absolute ethanol. Centrifuge at $13,000\times g$ for 30 min at 4°C to pellet DNA.
12. Wash pellet with 70% ethanol.
13. Resuspend DNA in 20–50 μ l of TE/RNase.
14. Confirm circular plasmid status by restriction enzyme digestion.

3.3. Functional Assays *In Vitro*

3.3.1. Luciferase Assay

Functional analysis of expression can be undertaken using luciferase reporter gene expression. Here we describe an assay that is used to assess the expression from a 10 kB piece of genomic DNA encompassing the LDLR promoter. We use sterols and statins to investigate the expression dynamics from the promoter region (Fig. 3) (8).

1. Seed 1×10^4 cells per well in a 24-well plate allowing for 4 wells per condition and leave for 24 h.

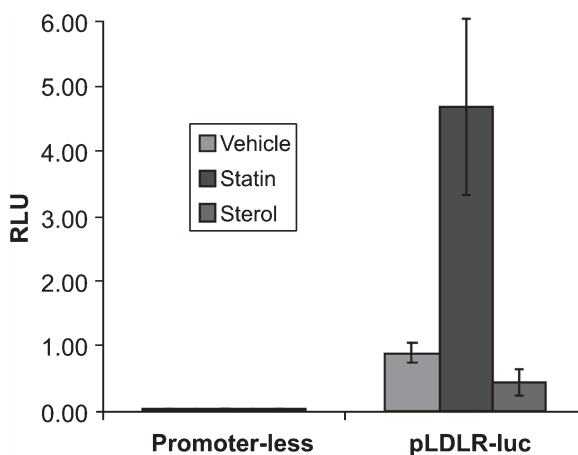


Fig. 3. Luciferase expression in CHO A7 *Ldlr* $-/-$ cells expressing luciferase under the control of the LDLR genomic promoter is sensitive to regulation by sterols and statins. The CHO a7 *Ldlr* $-/-$ cells respond in a physiologically relevant manner to cellular stimuli with a 50% reduction in luciferase expression seen with the addition of sterols and a fivefold increase in luciferase expression seen with the addition of statin.

2. The following day transfect plasmid DNA as described in Subheading 2.2.1 with the exception that the amount of DNA should be no more than 1 µg and using 1 µl lipofectamine.
3. Following the incubation period wash off transfection mix and incubate the cells in 250 µl of either sterol incubation media, statin incubation media, or HamsF12 + LPDS with 10 µl of vehicle (ethanol) control for 24–72 h.
4. Wash cells twice with PBS and apply 100 µl of luciferase lysis buffer, incubate for 20 min at room temperature. Luciferase lysis buffer can be made up in advance and stored at room temperature with the exception of the DTT, which should be added just before use from frozen aliquots.
5. Make up Solution A and Solution B. Solution A should be made up fresh each time. Solution B can be made up and stored at room temperature with the exception of the addition of ATP and DTT, which should be added fresh from frozen aliquots.
6. Set-up luciferase plate reader such that 50 µl of Solution A and 100 µl of Solution B will be dispensed into each well immediately prior to the luciferase value being read.
7. Transfer the entire contents of each well into a well of a black 96-well assay plate. Remove 3 µl of lysate to a separate colourless assay plate.
8. Run luciferase assay.
9. In the separate assay plate apply 100 µl of ONPG assay buffer and incubate at 37°C for 1–10 min checking at regular intervals.
10. Once the colour has changed to a very light yellow, apply 50 µl of ONPG stop solution and read the OD at A_{460} .
11. The luciferase value divided by the ONPG value gives a luciferase expression level corrected for transfection efficiency. Alternatively, luciferase can be normalised to total well protein using established protein assay methods.

3.3.2. DiI-LDL Assay

Functional analysis of LDL receptor activity in vitro was undertaken using a fluorescently labelled LDL analogue called DiI-LDL (7, 8).

1. Prepare cells as in items 1–3 of Subheading 2.3.1.
2. For each condition incubate 3 wells with DiI-LDL media and one well with DiI-LDL plus cold media for 5 h.
3. Wash cells twice with PBS (1% Bovine Serum Albumin).
4. Wash cells three times with PBS (Fig. 4).

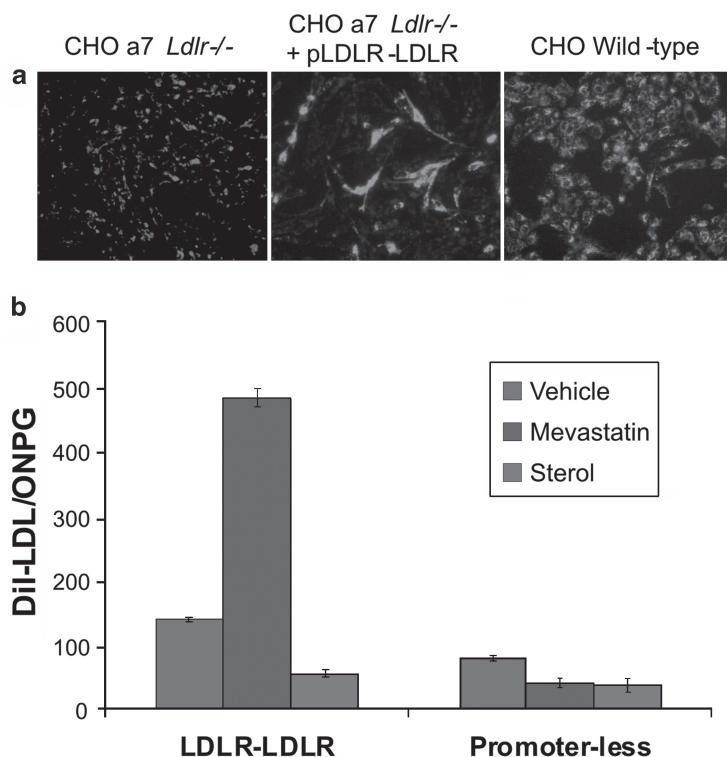


Fig. 4. Binding and internalisation of LDL can be analysed using a fluorescent analogue of LDL, DiI-LDL. (a) Incubation of CHO cells with fluorescently labelled LDL (DiI-LDL) demonstrates efficiency of expression of *LDLR* cDNA from the genomic promoter. In CHO a7 *Ldlr*^{-/-} cells no binding of LDL is seen. CHO a7 *Ldlr*^{-/-} cells expressing LDLR from the genomic promoter exhibit binding and internalisation of DiI-LDL that is comparable to the untransfected wild-type CHO cells. (b) The binding and internalisation seen in CHO cells expressing LDLR from the genomic promoter is quantifiable and is sensitive to cellular stimuli. Incubation of infected cells with sterols leads to a reduction in functional LDL receptors and therefore a 50% reduction in binding and internalisation of DiI-LDL. Whereas incubation with statins leads to a twofold increase in binding and internalisation of DiI-LDL.

5. Lyse cells with lysis buffer and transfer to spectrofluorimeter plate/cuvette.
6. Remove 3 μ l of sample and perform ONPG assay as per items 9–11 of Subheading 2.3.1.
7. Read DiI-LDL fluorescence levels using spectrofluorimeter and normalise to ONPG (Fig. 4).

3.4. Delivery and Analysis In Vivo

3.4.1. Liver-Specific Plasmid Delivery

Here we describe the protocol for liver-specific plasmid delivery using hydrodynamic tail vein injection. This is a particularly efficient means of transfecting the liver in vivo using plasmid DNA (13–15). The premise of the hydrodynamic injection is a combination of high pressure and large volume. A bolus of fluid equalling the total blood volume is injected into the tail vein of a mouse in less than 10 s. This bolus of fluid travels up the vena cava and is stopped by the heart causing retrograde flow into the hepatic

portal vein. The large volume of fluid flowing into the capillaries of the liver causes them to swell, which results in the breaking apart of cell adhesions thus making holes in the membranes allowing the DNA to flow in. The transfection efficiency can be as high as around 60% of hepatocytes and is very well tolerated by the mice.

1. Warm the animals in a heating unit for 5–10 min at 38–40°C taking care to not let the animals get too hot (see Note 5).
2. Induce animal with 5% isofluorane in 2 l/min oxygen.
3. Maintain anaesthesia with 2% isofluorane in 2 l/min oxygen.
4. Locate the tail vein and inject 2.5 ml of injection solution (see Note 6).
5. Allow the animal to recover in home cage.

*3.4.2. Transfection
Efficiency Analysis
(Immunohistochemistry)*

Here we describe immunohistochemical protocol to detect β -galactosidase and human LDLR in injected mice (6, 8).

1. Administer an overdose of anaesthetic to the animal.
2. Once death is confirmed, through cessation of respiration and absence of reflexes, pin out the animal exposing its abdomen. Make a mid-line incision in the skin on the abdomen and fold back.
3. Cut through the abdominal muscle layer exposing the organs. Cut up to the thorax.
4. Cut through the diaphragm and the ribcage on either side exposing the thoracic cavity.
5. Using a cannula needle pierce the apex of the left ventricle and make a nick in the right atrium (see Note 7).
6. Using a large syringe attached to the cannula needle push through at least 10 ml of PBS until the fluid leaving the heart at the atrium is clear.
7. Once the fluid is running clear switch to PFA, pushing through at least 10 ml (see Note 8).
8. Remove required organs, chop into 5 mm² pieces for processing and incubate in PFA for at least 48 h, but no more than 1 week.
9. The tissue is now ready for processing. The tissue is dehydrated by incubation in increasing concentrations of ethanol, cleared of ethanol using Xylene and infiltrated with paraffin wax (see Note 9).
10. Embed the tissue in paraffin blocks and cut 5 μ m thin sections.
11. Float onto coated slides and store at room temperature.
12. Rehydrate sections to prepare for staining; HistoClear (2 $\times$ 1 min), 100% ethanol (2 $\times$ 1 min), 95% ethanol (1 $\times$ 1 min), 70% ethanol (1 $\times$ 1 min), water (2 $\times$ 1 min), PBS (2 $\times$ 5 min) and leave sections in PBS.

13. Using a PAP pen draw circles around each section and apply blocking solution A. Place slide in humidified container and incubate at 37°C for 10 min.
14. Flick off solution A, but do not wash before applying a drop of solution B to the section. Place in a humidified container and incubate at 37°C for 10 min.
15. Wash 3 × 5 min in PBS.
16. Apply 50 µl of IHC solution containing 1:50 dilution of anti-LDLR and a 1:500 dilution of anti-β-galactosidase to each section and incubate overnight at 4°C. Control sections – anti-LDLR only, anti-β-galactosidase only, primary antibody only and secondary antibody only.
17. Wash 3 × 5 min in PBS.
18. Apply secondary antibody for not more than 1 h. Do not expose to light.
19. Wash 3 × 5 min in PBS (in a light-tight box).
20. Incubate in DAPI solution for 10 min (in a light-tight box).
21. Wash 3 × 5 min in PBS (in a light-tight box).
22. Perform a wet mount and analyse using a fluorescence microscope (Fig. 5).

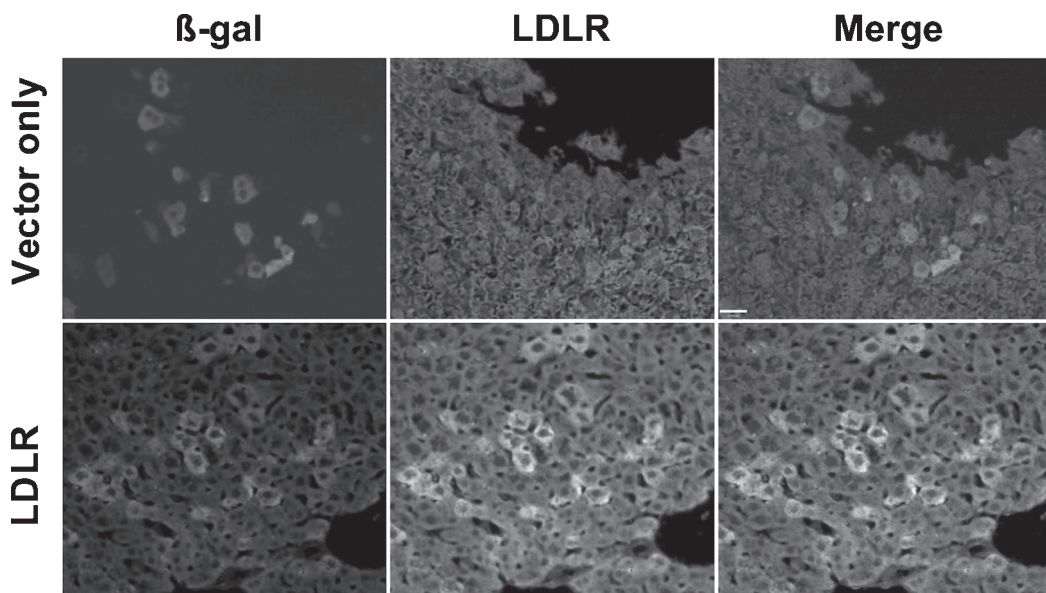


Fig. 5. Hydrodynamic tail vein injection of a plasmid expressing β-galactosidase from a CMV promoter and LDLR from the *LDLR* genomic promoter results in efficient transfection of hepatocytes in vivo. Here we show that expression of human LDLR protein is detectable and co-localises with β-galactosidase. Sections are co-stained with antibodies specific to β-galactosidase and human LDLR and counterstained with the DAPI nuclear stain. The livers show co-localisation of staining for human LDLR and β-galactosidase. This co-localisation is absent in livers from animals injected with a plasmid that only expresses β-galactosidase. This liver is only positive for β-galactosidase expression. For colour image, see Ref. (8).

3.4.3. Transfection Efficiency Analysis (Live Animal Imaging)

This protocol describes live animal imaging using an IVIS 100 luciferase imaging camera (Caliper Life Sciences, Hopkinton, MA) (8).

1. Anaesthetise animals with 5% isofluorane in 2 l/min oxygen.
2. Once induced dose animals with 100 μ l of luciferin via intra-peritoneal injection.
3. Place the animals inside the chamber and maintain anaesthesia at 2% isofluorane in 2 l/min oxygen.
4. After a 4-min incubation period from the time of luciferin injection, image the animals for luciferase expression (Fig. 6).

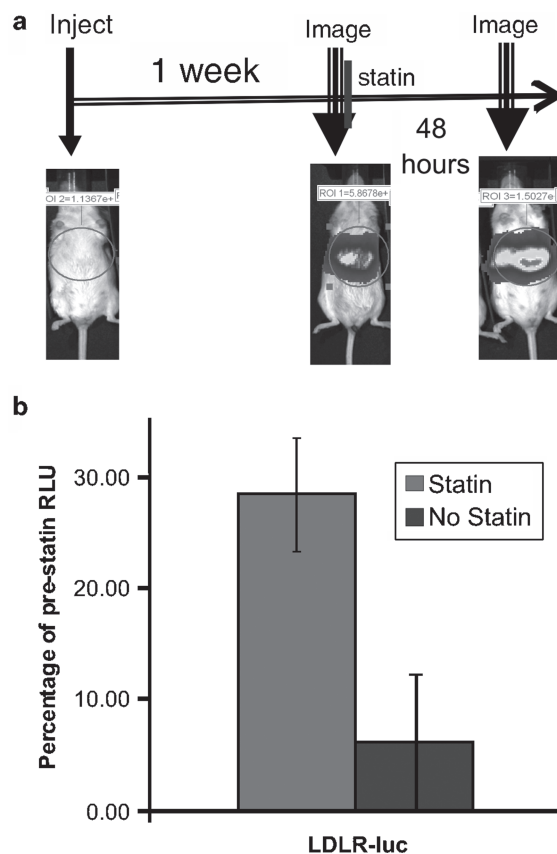


Fig. 6. Luciferase expression in vivo following hydrodynamic tail vein injection is robust and sensitive to drug administration. **(a)** Experimental time-line of a statin administration protocol showing representative luciferase activity images, hydrodynamic injection (black line), luciferase expression imaging (triple line) and statin administration (large arrow). **(b)** Administration of a single dose of 600 mg/kg of pravastatin resulted in five-fold more luciferase expression. Luciferase levels are expressed as a percentage of the luciferase levels calculated from the pre-statin administration imaging.

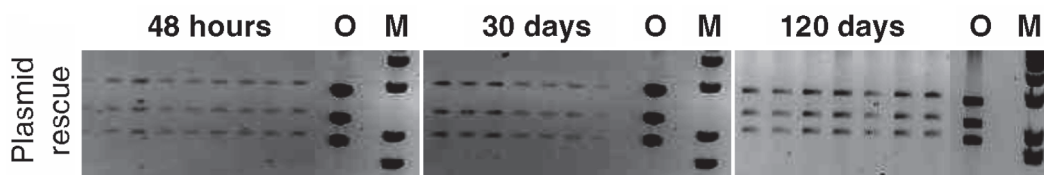


Fig. 7. Plasmid rescue from genomic DNA isolated from animals injected with an EBV containing plasmid. Plasmid retention is demonstrated by the presence of the plasmid as a circular episomal element that can be rescued 48 h, 30 days and 120 days following injection. O, original injected plasmid; M, marker.

5. Analyse luciferase expression levels using LivingImage software that allows you to quantify amount of luciferase in photons/s (Fig. 6).
6. Allow animals to recover in home cage.

3.4.4. Plasmid Rescue from Tissue

1. Homogenise small pieces of frozen liver in genomic lysis buffer.
2. Add 100 µl of proteinase K and incubate for at least 24 h at 37°C.
3. Perform a phenol:chloroform extraction as per items 6 and 7 in Subheading 2.2.1 using light phase lock gel tubes (Eppendorf).
4. Precipitate DNA in absolute ethanol.
5. Wash in 70% ethanol and leave DNA pellets to air dry.
6. Resuspend DNA in 200 µl of Tris-EDTA at room temperature for about 48 h.
7. Electroporate 1 µl of genomic DNA into DH10B bacteria and plate onto appropriate LB agar plates.
8. To confirm circular plasmid DNA make small cultures from single colonies, purify plasmid DNA using alkaline lysis, and perform restriction enzyme digestion (Fig. 7).

4. Notes

1. This protocol can be particularly problematic and requires careful optimisation. There are a variety of things that can be altered to optimise the protocol. Annealing temperature is a key variable, also extension time; most protocols recommend 1 min per kilobase. Addition of agents such as Q solution (Qiagen) or glycerol can result in better long-range products.
2. We have found that linearising the plasmid between the homology arms results in a more efficient recombination. This step can however, be left out.

3. The amount of each plasmid used in the retrofitting protocol will be dictated by the relative sizes of the plasmids. If you are retrofitting a large BAC plasmid that is 100 kB with a small retrofitting plasmid that is 10 kB you will want to have a tenfold excess of BAC plasmid.
4. The easiest way to pick a clone is to identify the cell population down the microscope using the 4× objective. Then, depress the plunger of the pipette and, still looking down the microscope, position the tip of the pipette tip in the media over the top of the clone. Once you can see the shadow of the tip down the microscope slowly manoeuvre it to be immediately adjacent to the cells. Slowly release the plunger aspirating the clonal cells.
5. It is very important that the animals are adequately heated before injection. Heating the animals properly will not only allow easy visualisation of the vein, in our experience the injection is more effective and the recovery of the animal is quicker. The animals should not however be allowed to over-heat. For this reason it is important to not allow the heating chamber to get above 40°C and to not leave the animals in the chamber for longer than 10 min. If an animal does start to show signs of overheating such as sweating around the neck and face, remove the animal from the chamber to its home cage, allow free access to water and wait at least 24 h before reattempting injection.
6. Hydrodynamic tail vein injection is a high speed high pressure tail vein injection. Once the needle is in the vein the entire 2.5 ml injection solution should be injected in no more than 10 s. Slow injection speed is a major cause for poor transfection efficiency.
7. It is important that the cannula is correctly placed in the ventricular cavity and not in the wall or pushed too far through into the atrium. If the needle is poorly placed, the perfusion will not work. If the needle is placed correctly when the PBS is pushed through there should be an obvious flow from the nick in the right atrium and you will notice organs such as the liver turning increasingly pale.
8. Once the flow of PFA begins it is normal for the muscles to involuntarily contract.
9. The process of tissue processing is lengthy and takes about 8 h. For this reason it is easier to use an automatic tissue processor such as the Leica TP1020 tissue processor (Leica, Milton Keynes, UK). However, if this is not available, use the following protocol; 70% ethanol (2×20 min), 95% ethanol (2×20 min), 100% ethanol (2×20 min), Xylene (2×20 min), paraffin (65°C, 2×30 min). Take care not to leave the tissue blocks in hot paraffin for longer than the specified time as this can dry out the block and make it very difficult to cut.

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