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PRODUCTION OF RETROVIRUS FOR GENE TRAP FLIPPING: CALCIUM-PHOSOHATE TRANSFECTION OF PACKAGING CELLS AND PRODUCTION OF VIRUS STOCKS

Adapted from Johannes Zuber, IMP, Vienna

MATERIALS AND REAGENTS

- Retroviral packaging cells (PlatE, Cellbiolabs)
- Cre/Flp-expressing plasmid with a FACS-able color and/or a selection marker (e.g. MLP-mCherry-Cre-puro)
- Optional: second plasmid as "negative control" (e.g. MLP-GFP-puro)
- RV-Helper plasmid

2 x HBS (1000ml) *italic substances are optional !*	280 mM NaCl (16.4g) 50 mM HEPES MW 238.31 (11.92g) 1.5 mM Na_2HPO_4 (0.21g anhydr./ 0.58g x12 H_2O) *12 mM Dextrose (2.0g) or Glucose (2.2g)* *10 mM KCl (0.74g)* H_2O (ad 1000ml) - distribute into 4 x 250ml and adjust pH to **to 6.96, 7.00, 7.04, 7.08 (!)** with 0.5M NaOH - perform a test precipitation with 15µg GFP+ and look for fine precipitate in hemocytometer - with those yielding a fine precipitate perform independent test transfections - working buffers should be filtered through a 0.2µm filter and stored in aliquots (10 ml) in –20°C - thaw always in room temperature (not in 37°C !)
1M $CaCl_2$ (20ml)	• 2.94g $CaCl_2$ add 20ml H_2O • filter through a 0.2µm filter • store aliquots at -20°C

CULTURE OF PlatE PACKAGING CELLS

- Medium: ESCM without LiF
- For the first couple passages, culture in ESCM without LiF, with puromycin 1:10'000 and with blasticidin 1:1'000, switch to medium without puromycin/blasticidin prior to transfection
- Culture in 5% CO_2, passage ~1:4 every 2-3 days, cells detach easy from plate!
- Always discard cells after >20 passages and avoid long episodes of confluence
- 4h prior to transfection, plate ~19 x 10^6 in 35ml media per 15cm plate. No further medium changes before transfection. Transfect as soon as they are adherent and 75-85% confluent.

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CALCIUM-PHOSPHATE TRANSFECTION OF PlatE PACKAGING CELLS

Quantities for one 15cm plate:

Solution A (total 1500µl, keep pipetting order):

plasmid DNA	60 µg
helper DNA	20 µg
H_2O (1125 – vol. of DNA)	... µl
1M $CaCl_2$	375 µl

Solution B (total 1500µl):

2 x HBS	1500µl

- Prepare both solutions in separate tubes or flasks
- Mix dropwise (A) to (B): Use a vortex as a means to "blow" bubbles through solution B. Use a pasteur pipet for drop-by-drop adding solution A to solution B while the vortex "blows" bubbles. The mixture should turn turbid.
- Leave mixture at RT for 15min
- If precipitate in the end gets flocculent re-suspend it by pipetting up and down or shearing through a 21G needle
- Add precipitate to cells dropwise while moving the plate in circular motions (caution: be gentle as the packaging cells easily detach from the plate)
- Optional: look for a fine precipitate on the cells 15 min after addition
- 8–14h (i.e. overnight) post transfection change media for recovery and/or 8-12h prior (first) infection change media to target cell media (ESCM for ESCs). Choose the volume needed for infection (e.g. 3ml per target 6well).
- Optional: check transfection efficiency under a fluorescence microscope
- Virus collection time: 24-72h post transfection; highest titers are at 32-60h post transfection
- Carefully aspirate viral supernatant from packaging cells, filter through 0.45µm filter and use for infection of ESCs (please also see *Gene Trap Flipping Protocol* at https://www.haplobank.at/ecommerce/control/haplobank_resource)

PRODUCTION OF VIRUS STOCK

To have some virus at hand you can produce a batch and store it at -80˚C for approximately half a year.

First day after transfection:

- am: change medium of PlatE to 20mL ESCM for 1st pm harvest
- Optional: check transfection efficiency under a fluorescence microscope
- pm (24h post transfection), 1st harvest: carefully suck off supernatant and filter through 0.45µm filter, store on ice overnight in cold room; add another 20ml ESCM to plates for next harvest

Second day after transfection:

- am, 2nd harvest: combine with 1st harvest; add ESCM to plates for next harvest
- Transfer combined supernatant to centrifuge tubes (approx. 35ml per tube) and concentrate by centrifugation (4h @ 24'000rpm), resuspend viral pellets in 0.5ml ESCM/centrifuge tube, pool and store on ice in cold room

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- Wash/rinse empty centrifuge tubes with PBS and keep in hood over night for virus inactivation
- pm, 3rd harvest: store on ice overnight in cold room; add ESCM to plates for next harvest third day after transfection:
- am, 4th harvest: combine with 3rd harvest and concentrate as before
- Discard PlatE
- Aliquot concentrated virus stocks (250µl)
- Freeze down and store at -80˚C

To determine future dilutions of the virus, do a test-infection on ES cells with several virus dilutions and analyze infection efficiency by FACS 48h post infection.