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Production of Retrovirus for gene trap flipping: Calcium-phosphate transfection of packaging cells and production of virus stocks

Adapted from Johannes Zuber

Materials & Solutions

- retroviral packaging cells (PlatE, Cellbiolabs)
- Cre/Flp-expressing plasmid w/ 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 $CaCl_2$ ad 20ml H_2O · filter through a 0.2µm filter · store aliquots at -20°C

Culture of PlatE packaging cells

- medium: ESCM w/o LiF
- for the first couple passages, culture in ESCM w/o LiF + puro 1:10'000 + blast 1:1'000, switch to medium w/o puro/blast prior to transfection

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- 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 change before transfection. Transfect as soon as they are adherent and 75-85% confluent.

Calcium-phosphate transfection of packaging cells

quantities for one 15cm plate:

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

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

Solution B (total 1500µl): - 2 x HBS 1500µl

- prepare both solutions in separate tubes or flasks
- mix dropwise (A) to (B): Use a vortexer 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 vortexer "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 gently 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. o/n) 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 (pls see protocol 'Gene trap flipping')

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Production of virus stock

To always have some virus at hand you can produce a batch and store it at -80deg 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 virus harvest: carefully suck off supernatant and filter through 0.45µm filter, store on ice o/n in cold room; add another 20mL ESCM to plates for next harvest

second day after transfection:

- am, 2nd harvest: combine w/ 1st harvest; add ESCM to plates for next harvest
- transfer combined supernatant to centrifuge tubes (appr. 35mL per tube) and concentrate by centrifugation (4h @ 24'000rpm), resuspend viral pellets in 0.5ml ESCM/centrifuge tube and pool, store on ice in cold room
- wash/rinse empty centrifuge tubes w/ PBS and keep in hood o/n for virus inactivation
- pm, 3rd harvest: store on ice o/n in cold room; add ESCM to plates for next harvest

third day after transfection:

- am, 4th harvest: combine w/ 3rd harvest and concentrate as before
- discard PlatE
- aliquot concentrated virus stocks (250µL)
- freeze down and store at -80deg

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

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