



Immortalized Cell Culture Protocol

We thank Dr Martin Dorf at Harvard University for generously generating and providing the immortalized cell lines to the PRF Cell & Tissue Bank. Thank you.
PRF cell lines were immortalized using the following protocol:

Retrovirus Infection

A. Producing Virus: 12-24 hr before transfection, packaging HEK293 cells were plated on a 100-mm plate at 60-80% confluency. Each plate was co-transfected with 12µg retroviral construct pBABE-puro (SV40T) and 12µg pCL-Ampho Retrovirus Packaging Vector. The culture medium was aspirated 8-10 hr after transfection, and replaced with 10ml of complete medium. The culture was incubated for an additional 48-72 hr to attain optimal viral titer.

B. Infecting Target Cells: The target cells were plated on a 100 mm plate 12-18 hr before infection. The medium from packaging cells was collected and filtered through a 0.45-µm cellulose acetate or polysulfonic filter. Once the cells were 40-60% confluent, 8 ml virus-containing medium with a final concentration of 10 µg/ml polybrene was added. Three rounds of infection were performed sequentially, ~12 hrs apart. The medium was replaced after 24 hr incubation.

C. Selection of Stable Cell Lines: 72 hr after infection, the cells were trypsinized, split at 1:3 and transferred to two 100 mm plates in the presence of 3 µg/ml puromycin, plus one control plate without antibiotic. The culture medium was changed every 2 or 3 days for approximately 2 to 4 weeks to produce stable cell lines.

Growth Media

DMEM- Invitrogen #11960-044 (high glucose without L-glutamine)

15% FBS Fetal Bovine Serum

1% (1x) Penicillin-Streptomycin (Invitrogen#15140-122)

1% (1X) L-glutamine (Invitrogen#25030-081)

0.75 µg/ml puromycin (InvivoGen #ant-pr-1)

Trypsin

Trypsin EDTA C .25% (Invitrogen#25200-056)

Hank's Balanced Salt Solution

HBSS- (Invitrogen#14170 (1X) (-) calcium Chloride, (-)magnesium chloride, (-) magnesium sulfate)

DMSO for Freezing

Cell Culture Grade DMSO

Cells will arrive frozen on dry ice at a concentration of approximately 5 x 10⁵ cells/vial

Protocol for thawing fibroblasts

1. Thaw cells rapidly in a 37°C water bath.
2. Wipe the outside of the vial with 70% ethanol.
3. Transfer the thawed cells to a T25 flask containing 5 ml of growth media without puromycin.
4. Place flask in 37°C incubator overnight.
5. The following day, observe under the microscope to ensure cells have attached.
6. Remove media and replace with fresh growth media.

Subculturing PRF cell lines

- 1) When cells are established and growing, begin to use media containing 0.75 µg/ml puromycin. Continue to maintain the cells in media containing 0.75 µg/ml puromycin.
- 2) Cells should be split when confluent.
- 3) To split cells (volumes given are assuming cells are in a T25):
 - A. Using sterile technique, remove media and rinse flask with 2-3 ml of sterile HBSS. Remove and discard HBSS.
 - B. Add 1 ml trypsin and incubate for 2-3 minutes. Cells should be checked under an inverted microscope to determine if they have begun to round up, lift and float. If cells are not detached after 3 minutes, you may incubate another 1-2 minutes
 - C. Tighten cap and gently tip flask from side to side to dislodge all cells. Flask may be tapped lightly on side to loosen cells if needed.
 - D. Add 4 ml of new media to flask as soon as cells are floating to inactivate the trypsin. Rinse down sides of flask several times with the new media to wash all cells off the plastic and in to the solution. Remove all liquid containing cells and transfer to a 15ml conical (or 50 ml if you are pooling 3 or more flasks).

- E. Using sterile technique, remove an aliquot for counting on a hemacytometer.
- F. While you are counting cells, the rest of the solution should be spun in the clinical centrifuge for 5 minutes at 1000 rpms.
- 4) After calculating your cell count, plate cells at 2.5×10^5 cells per T 25 filter top flask.
- 5) Cells should be fed every 2-3 days with fresh growth medium containing 0.75 µg/ml puromycin.

Freezing:

Cells should be frozen at no less than 5×10^5 cells /ml/cryovial in growth media containing 10% DMSO and 30% FBS **without puromycin** and subsequently placed in an isopropanol freezing chamber at -80°C overnight. Transfer to the liquid nitrogen the next day.

For Further Information Please Contact:

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