

Lentiviral Primary Fibroblast Immortalisation (hTERTing)

Reagents required

- PBS
- DMEM 10% FCS, L-Glutamine and pen/strep
- Optimem
- Fugene HD
- Transfer vector DNA (concentration approximately 0.5-1mg/ml)
- Packing vector DNA (psPAX, concentration approximately 0.5-1mg/ml)
- Envelope vector DNA (VSVG, concentration approximately 0.5-1mg/ml)
- Puromycin (Stock concentration 1mg/ml, final concentration 5µg/ml)
- Polybrene (Stock concentration 5mg/ml, final concentration 5µg/ml)
- HEK293T cells
- Target cell line

Safety considerations

- Care must be taken when working with lentiviruses.
- Use Virkon to decontaminate equipment etc.
- Use independent vacuum pump.
- Use double gloves and disposable labcoat.
- All waste must be autoclaved.
- Work in designated viral hood.

Lentivirus Production and Infection of Packing Cell line

Day 1: Transfect cells with vector DNA (this contains your gene of interest)

- Combine 4.5µg of packing and envelop vectors with 3µg of transfer vector in 1.5ml Optimem and mix gently.
- Dilute 36µl Fugene HD in 1.5ml Optimem and mix gently.
- Leave for 5min at RT.
- Combine both Optimem solutions, mix gently and leave at RT for 20min.
- During this time trypsinise HEK293T and collect 6×10^6 cells in 5ml of complete.
- Add transfection mix to 10cm dish and then add the 5mls of DMEM containing the cells. Rock gently to mix. Avoid swirling as this causes reagents to pool in the centre.

Day 3: Change the media on the HEK293T in the morning. This will help to prevent cell death.

Day 5: Change the media on the HEK293T and add puromycin. Final concentration of puromycin should be 0.75µg/ml.

Day 5 or 6: Transfer cells to a T75 or T175 flask for virus collection.

Day 10-15: Cells are considered stable if they have been under constant selection.

Production of Lentivirus Containing Media

Day 1:

- HEK293T cells should be approximately 60-70% confluent.
- Remove the media containing puromycin and add fresh DMEM with 10% FCS.
- Plate primary cells and one control cell line (e.g. 1BR) at 5×10^5 in a T25cm flask.

Day 2:

- If the cells have reached approximately 80% confluency the virus containing media can be collected.
- Remove media from HEK293T cells using a plastic pipette. Transfer to a falcon tube. Do not use a universal as the virus will adhere to the plastic.
- Centrifuge at 1500 rpm for 5min to pellet any cell debris.
- Collect the media using a disposal plastics syringe.
- Filter the media through a disposable $0.45\mu\text{m}$ filter into flacon tube.
- Add polybrene at a final concentration of $10\mu\text{g/ml}$ and mix gently.
- Remove media from the primary cells and wash with PBS. Wash with PBS two more times.
- Add the media containing the virus and polybrene to the primary cells.
- Incubate at 37°C overnight.

Day 3: Change media after 12-24h. If needed multiple infections can be done.

Day 5 -6: Add puromycin at a final concentration of $0.5\mu\text{g/ml}$.

Day 7-8:

- Change the media.

Continue to change the media every few days. If there is a lot of cell death increase the frequency of media changes and introduce washes.

Trouble Shooting

<u>Problem</u>	<u>Probable Cause</u>	<u>Advice</u>
Primary cells start dying after the addition of virus containing media	Media collected from the packing line was devoid of nutrients.	Ensure that the packing line doesn't reach confluency until the day the media is harvested. Ensure enough media is added to the packing line the day before harvesting. If using a T175 a minimum of 10mls of fresh media must be added the day before.
Packing line dies when puromycin added	Cells lack the puromycin resistance gene.	Ensure packing line still has resistance gene by transfecting with more plasmid. Or create new packing line
All the primary cells die when puromycin added, but controls survive	Cells do not contain the resistance gene.	<ul style="list-style-type: none"> • This could be because the virus was unable to enter the cells. Ensure polybrene is used. • Too much virus may have entered the cells. This can happen with rapidly dividing cells using the lentiviral method. See 'How to decide which hTerting method to use' • Puromycin was added too quickly after infection. Leave at least 48-72 hours between infection and addition of puromycin to allow the cells to produce the proteins needed for resistance. • Too high concentration of puromycin added. Begin with 0.5 µg/ml of puromycin. This can be increased to 0.75 µg/ml. Concentrations higher than this are not recommended.
Cells start dividing rapidly when puromycin is removed from the media	Cells are removing the resistance gene from their genome.	Keep the cells in puromycin for a minimum of 10-15 days. This should produce a stable cell line. If necessary repeat using a different infection method.
All cell lines die after puromycin is added	Cells do not contain the resistance gene.	Virus is not being produced by the packing line. Packing line may still have the transfer vector in its genome, but may have removed packing and/or envelop vectors from their genome. Remake the packing line.

