shRNA Lentivirus Production using HEK293T cells and FuGENE

Biohazard Concerns

Lentivirus is a modified HIV virus and although unable to replicate in a host, it must be handled with caution. When working with these viruses, work only in BL2+ designated hoods or viral vector rooms. All handling, storage and disposal of biohazard waste must be in accordance with Institute rules and regulations, OSHA, EPA and MWA.

Notes

All rules of the virus room must be followed. Be sure you receive the proper training on policies and procedures of the virus room before use.

Materials

1.) 293T packaging cells (ATCC # CRL-11268)

2.) Plasmid DNA for:

   a.) shRNA-pLKO.1 plasmid
   b.) pCMV-dR8.91 (Delta 8.9) plasmid containing *gag*, *pol* and *rev* genes
   c.) VSV-G expressing envelope plasmid
   d.) pLKO.ps control plasmid

3.) FuGENE 6 (Fisher # NC9666789)

4.) 100mm Corning CellBIND tissue culture plates (Fisher # 07-202-516)

5.) DMEM media (ATCC #30-2002)

6.) OptiMem Reduced Serum Media (Invitrogen # 31985-070)

7.) FBS (ATCC #30-2020)

8.) Pen/Strep (ATCC #30-2300)

9.) 15mL Centrifuge Tubes (Fisher # 05-538-53F)

10.) 50mL Centrifuge Tubes (Fisher # 07-203-510)

Procedure

1 day before transfection:

Before Starting: Make and warm DMEM media supplemented with 10% FBS. No antibiotic!

1.) Split 293T cells into twelve 100mm culture dishes at a density of 5x10^6 cells per plate using 10ml of DMEM media supplemented with 10% FBS per plate.

2.) Be sure that each plate gets a consistent number of cells, and they are evenly distributed throughout the plate.

3.) Incubate for the next 24 hours undisturbed at 37ºC and 5% C02.

Day of transfection:

Before Starting: Warm OptiMem media in a 37ºC water bath.

1.) To a 15mL centrifuge tube, add:

   a.) 3096µl OptiMem media
   b.) 504µl FuGENE 6
   c.) Mix *gently* by swirling the pipet tip around or tapping with a finger (do not pipet or vortex to mix)
   d.) Incubate at room temperature for 5 minutes.

2.) Meanwhile, to another 15mL centrifuge tube, add:

   a.) 7.2µg VsVg (1 tube, frozen at -20C)
   b.) 36µg of delta 8.9 (1 tube, frozen at -20C)
   c.) 72µg of DNA expression vector (1 tube, frozen at -20C)
   d.) Bring volume up to 3600µl with OptiMem media
   e.) Mix *gently* by swirling with the pipet tip or tapping with a finger (do not pipet or vortex to mix)

Note: The ratio of Fugene to DNA is 7:1
The ratio of delta 8.9 to VsVg is 5:1

3.) Combine the tubes from steps 1 and 2 and mix *gently* by flicking to mix the contents. Total volume will be 7200µl.

4.) Incubate the combined mixture at room temperature for 30-45 minutes.

5.) Remove the 12 plates of 293T cells from incubator and bring them into the hood. Add 600ul of the DNA mix to the dish ***drop wise*** and evenly to each plate.

Note: 293T cells detach very easily from the plate. Take care in adding the complex mix.

6.) Swirl the plate very gently to mix. Bring the plates into the virus room.

7.) Incubate the cells overnight in the virus room incubator at 37ºC and 5% C02.

Day 1 after transfection:

Before Starting: Warm DMEM media supplemented with 10% FBS and 1% Pen/Strep in the 37ºC water bath.

1.) Change the media to fresh 10ml DMEM media supplemented with 10%FBS and 1% Pen/Strep.

Note: 293T cells can detach easily from the plate and you do not want to disturb them, particularly at this point. Add the fresh media carefully.

Day 2 after transfection:

Before Starting: Warm DMEM media supplemented with 10% FBS and 1% Pen/Strep in a 37C water bath.

Note: Place SW-28 centrifuge tubes, SW-28 rotor buckets and caps into the hood to be sterilized by UV light overnight.

1.) Collect viral harvest at ~40 hours after transfection into six 50mL centrifuge tubes (2 plates of virus into one 50mL tube). Place the 50mL tubes into the cold room.

2.) Add 10ml of fresh DMEM media supplemented with 10% FBS and 1% Pen/Strep.

Note: 293T cells can detach easily from the plate and you do not want to disturb them. Add the fresh media carefully.

Day 3 after transfection:

Note: Look at any GFP control plates under the fluorescent microscope to be sure the cells are expressing GFP. If they are, you can assume the cells have taken in the DNA and are producing virus.

1.) Collect viral harvest at ~60 hours post transfection into the 50mL tubes used the day before. Each 50mL tube will have approximately 40mL of virus.

2.) Discard the 293T cells.

3.) Proceed with virus purification and concentration (see protocol "Lentivirus Concentration and Titer").