**Lentivirus Packaging/Generation by Electroporation**

Material

* Transfer Vector:
  + Plasmid pXPR\_003
  + Insert: 20bp gRNA
* Packaging Mix
  + psPAX:
  + pMD2G (VSVG)
* Reagent:
  + DMEM complete: 10% heat-inactivated FBS, 100X glutamine, 100X NEAA
  + Anti-anti

Protocol

* Mix PM and TV
  + Take a PCR-tube strip
  + Add 1.5 uL PM (1000 ng/uL) to the bottom
  + Add 1.5 uL TV (1000 ng/uL) to the bottom
* Prepare HEK293T (or .S variant) packaging cells
  + Trypsinize
  + Count 50 million cells into 50-mL Falcon
  + Add 1.5-2.0 million cells per rxn.
  + Spin 200g for 2 min. Decant.
  + For each rxn, add 20 uL per rxn of BTX Electroporation solution to resuspend
  + For each rxn, add 16.4 uL SF + 3.6 uL Supplement 1 per rxn to resuspend
  + Add 20 uL cell suspension into wall of each PCR tube in strip containing PM + TV mixture
  + Then hand-flick to make sure everything is at the bottom.
* Electroporation Transfection into HEK293T cells
  + Prepare upfront a 24-well plate by filling 1.5 mL no-antibiotic media per well
    - Incubate plate in 37 to warm up
  + Set pipette to 21uL to distribute 1 rxn/tube 🡪 1 Lonza cuvette in strip
  + Use X unit
  + Choose program:
    - HEK-DG130 for BTX solution
    - CM130 for SF+Supplement solution
  + Immediately add to the pre-warm 24-well plates by sucking up some media to resuspend in cuvette
  + Check if cells survive the electroporation under the microscope
  + Put plate in lentiviral-packaging-specific incubator
  + 24 hours later, change the media with complete media (containing antibiotics)
  + 48 hours later, collect the supernatant by filtering it through 0.45 um filter using syringe. Add new complete media
  + 72 hours later, collect the supernatant again, if necessary. Virus can be aliquoted and stored in -80 for long-term storage.

**Lentivirus Transduction**

* Using 0.45 um Corning filters + syringes, collect 5mL media filter out HEK cell debris.
* Transfer viral media into cells to be transduced, prepared beforehand.
* In a 24-well plate: Distribute 500,000 cancer cells/well by adding 0.5mL into each well (10 million cells in 10 mL media)
* Plate Format: 16 wells getting viruses, 2 control wells (+) not getting selective antibio
* Final volume: 1-1.5 mL per rxn/well.
* Add 0.5-1 mL viral titer per well.
* Top off 2 control wells to final volume with media.
* After 24 hours, replace virus with selective antibiotic-containing media, except in (+)
* After ? days, when (+) is confluent and (-) all die, selection is complete
* Change to fresh puromycin-containing media as needed every few days.

<https://www.addgene.org/protocols/plko/#D>

**Plan:**

1. To compare transfection efficiency of each transfection method into HEK293T.S cells: Pratyusha suggests Xtreme vs. PEI by directly transfecting a plasmid containing GFP into one of the cancer cell lines. The readout may be images from a fluorescent microscope.

1. To shadow Pratyusha to see how she generates lentiviral particles for a control plasmid (sgRNA against GFP) from HEK293T.S cells and transduces into one of the 3 dual-reporter cell lines.

1. After, I can try to do this by myself in a pilot assay with a batch of 4 sgRNA against KRT20, SOX9, GFP, and mK2 in a parental cancer cell line (HT29). The readout for this is with RT-qPCR of ActB, Krt20, Sox9, and Prom1. Ideally Western as well but I have yet to do a Western in the lab yet.

1. After learning from the pilot study’s mistakes, I can proceed with the entire set of 25 sgRNA in dual-reporter HT29.

1. Separately, for determining the selective antibiotic’s concentration and time, could you tell me where to get these antibiotics?

1. Relative Gene Expression by RT-qPCR of LS180, HT115 after Merck60 treatment experiments at 24hpt and 48hpt.

* 1. Test Transfectio

Plan for Paul tries CRISPR validation of pooled screen (PART 1):

1. Transfection into HEK293T cells/Lentivirus Packaging:   
   Nilay & Pratyusha think that electroporation method of transfection into HEK293T cells are an overkill for transfecting so the suggested plan is to try using either \*Lipofectamine 3000, \*PEI, XtremeGene, or Calcium Phosphate. We ordered Lipofectamine and PEI.
   1. The thing I’m not sure about is which plasmid to use? 2 options:

                                                               i.      If I use the plasmid containing sgRNA against GFP and transduce it in a dual-reporter cell line, I’ll measure the decrease in GFP fluorescence in the dual-reporter cells?

                                                             ii.      Pratyusha suggests, for trying out transfection protocol for the first time, I can use a different GFP-expressing plasmid to transfect into HEK cells, then measure the increase in GFP fluorescence in HEK cells? If so, what plasmid can I use

* 1. Packaging Mix: I’m not sure what the ratio of these 2 plasmids are in the aliquot [@Spisak, Sandor, Ph.D.](mailto:Sandor_Spisak@DFCI.HARVARD.EDU) gave me?

                                                               i.      psPAX2

                                                             ii.      pMD2G

                                                           iii.      I’m probably going to need to plasmid-prep psPAX2 and pMD2.G separately unless the aliquot already has a good ratio.

* 1. Protocol using is one that Nilay shared in an email titled Transfection Protocol.

1. Transduction into cancer cells:
   1. Using 0.45 um filters, I’ll add the lentivirus into 24-well plate with seeding at 500,000 HT29 cells in 0.5 mL per well with 1 positive-control well (which gets no virus, no selective antibiotic)
   2. Still not sure the details
2. Validation that CRISPR did cut on-target and no off-target: ( need to choose a method)
   1. Mismatch cleavage assay: T7E1 or Surveyor – low-cost but erroneous according to literature
   2. PCR and Sanger sequencing – can only detect deletion???
   3. NGS – more expensive, good for high number of samples but accurate and able to detect off-target effects simultaneously
3. 4) Readout: RT-qPCR and Western blot against ActB/Housekeeping, KRT20, SOX9, Prom1

| **Assay** | **When to harvest cells?Days post-infection** |
| --- | --- |
| mRNA knockdown (quantitative PCR) | ≥3 days |
| Protein knockdown (western blot) | ≥4 days |
| Phenotypic assay | ≥4 days |

1. 5) After validating that the stable KO cell line is successfully created, create a frozen stock.