PEI Transfection of 293pTP Cells for Making Recombinant Adenoviruses

(Edited and Commented by TCH @ 07/09/2019)

I. Plating 293pTP cells in T-25 flasks

About 293 cells: *293 cells are derived from **h**uman **e**mbryonic **k**idney cells (a.k.a., HEK 293 cells) that are stably expressing human adenovirus E1A and E1B genes. As you know, most recombinant adenoviruses are missing the E1A and E1B genes. 293 cells provide the E1A and E1B proteins and allow recombinant adenoviruses to replicate and produce more virus particles. Thus, 293 cells are also referred as the **packaging line** for recombinant adenoviruses. Although there are several other packaging lines available, **293 cells are the most commonly used line for generating and amplifying adenoviruses**. In addition, 293 cells are also a **popular choice** for many transient gene expression studies because of its high transfection efficiency.*

Why 293pTP cells: *293pTP cell line was engineered by our lab and stably expresses Ad5 Precursor Terminal Protein (pTP), leading to significant acceleration of adenovirus packaging and amplification, compared to HEK293 cells. For more information, please read Wu N et al. **Gene Therapy** 21(7): 629-637, PMID: 24784448.*

Plate 293pTP cells in T-25 flasks at subconfluency (30-40%) 2 ~ 4 hours prior to transfection (i.e., plating cells in early morning if you want to finish transfection within the same day). This approach has been working very well in our lab, and is particularly useful for those who are concerned about potential over-confluence of the overnight plated culture.

Note: *A lower confluence could achieve higher transfection efficiency but may yield a lower absolute number of transfected cells. The following procedure can be scaled up or down proportional to the surface area of plate, dish, or well with little or no change in the results.*

II. Pac I digestion of adenoviral recombinant plasmids (i.e., pAd-YFG)

Rationale: *The purpose of Pac I digestion is to liberate the two ITRs of adenoviral genome so that viral DNA replication (i.e., adenovirus production) can be initiated effectively by adenoviral pTP.*

Pac I digestion: Perform 100µl digestion reaction, using **3µl** Pac I (NEB). You will need to use **3-5µg** DNA for Pac I digestion and to transfect one T-25 flask.

***Note**: **1)** We usually use alkaline miniprep DNA, and thus one miniprep or 2/3 of one miniprep should be sufficient to transfect one T-25 flask (as the concentration of a typical miniprep is 0.1~0.2µg/µl). **2)** The miniprep DNA must be treated with RNase A (~50µl @ 37C for 30min) to remove any residual RNAs, which should be checked on DNA gels (using 5µl of the digestion mix), followed by PC-8 extractions and ethanol precipitation. **3)** The Pac I digestion reaction should only last 30 min (>1 hour or overnight digestion is ridiculous and will hurt you!). [**Optional**: You can check 5-10ul of your digestion reaction mix (**NOT** your precipitated DNA) on gel to make sure that the Pac I digestion is complete].*

Perform ethanol precipitation, and wash the pellet twice with 70% ethanol. Resuspend DNA in **30µl** sterile ddH2O. Ready for transfection (see **Stage III**).

***Note**: **1**) You should always check 1-2ul of your miniprep DNA on an agarose gel (Caution: Nanodrop reading is **less** reliable and optional). **2**) If the concentration of*

your miniprep DNA concentration is low, you can first precipitate DNA because the miniprep is usually dirty, and the impurities could inhibit your Pac I digestion. ***3)*** *There is* ***no need*** *to perform PC-8 (phenol/chloroform) extraction and/or gel purification of the Pac I digested pAdYFG DNA (If you do, you will get more hurt than helped).*

III. Transfection of 293pTP cells with PEI

1. Prepare a 1.5 ml microfuge tube, add the **30µl** of Pac I-digested DNA solution, and **200µl** of OptiMEM or plain DMEM (i.e., DMEM **without FBS**) per transfection.
2. Mix **200µl** of OptiMEM or plain DMEM, **30µl** of Pac I digested DNA, and **5~8µl** of PEI stock solution (**MOLab homemade PEI stock = 3µg/µl;** usually 5~7µg PEI/µg DNA) per transfection. Let the mix sit in the cell culture hood for **10 min**.
3. Meanwhile, carefully aspirate the complete medium (**containing FBS**) from T-25 flask.

   [***Optional***: gently wash cells by adding **5 ml** of OptiMEM or serum-free DMEM to the side of the flask, rocking slowly to allow the medium to cover the cells, and aspirating the medium]

   [***Note****: It is* ***NOT*** *desirable to wash the less adherent cells, such as* ***293pTP cells***].
4. Add **2.5 ml** OptiMEM or plain DMEM to the flask. Return flask to **37°C** incubator until DNA/PEI mix is ready.
5. Add the DNA/PEI mix to one side of the flask. Rock gently and return flasks to the incubator.
6. After **2-6 hours**, remove the OptiMEM or plain DMEM and replace with **8-10 ml** of complete DMEM medium.

   [***Note****: longer transfection time could result in higher transfection efficiency, as long as the cells are healthy. Thus, you should check the health status of the cells every 20~30min after 2 hours*].

   [***Note****: If you observe significant amounts of cells are floating at the end of transfection, please* ***DO NOT*** *change the medium. Instead, add 6ml serum-containing DMEM to terminate the transfection and allow the floaters to reattach to the flask. Change medium after 4~6 hours or next morning].*
7. Check GFP or RFP signal at **24~48 hours** after transfection to assess transfection efficiency. Keep checking GFP/RFP signal for about 5-10 days. Collect the transfected cells when apparent adenoviral package appears (comet-like foci), usually at 5-10 days depending transfection efficiency.
8. Perform the freeze-thaw cycles to release adenoviruses. The cleaned viral lysate will be used for subsequent amplifications in HEK293 or 293pTP cells.

VI. General comments on transfection and adenovirus making in 293pTP cells:

1) The transfected cells usually become confluent at 2-3 days after transfection, and will stay confluent until you collect them. The culture medium usually becomes **yellowish**. **Do not panic**! There is **No Need** to change the medium [*If you do, you will get hurt as the packaged adenoviruses are usually released to the medium and infect neighboring 293pTP cells, leading to*

higher virus titers]. Instead, you can add 1-2ml of the complete DMEM medium every 3-5 days. We usually do not add any fresh medium or only add 2ml fresh complete DMEM at day 4 or 5.

2) As confluent 293pTP cells are very easy to detach, please handle the flask very gently and do not leave the flask at room temperature for too long. *Whenever 293pTP cells peel off, the game is over!*

3) You should see some intensified GFP or RFP foci usually at 5-7 days after transfection. If your transfection efficiency is not very high (i.e., <10%), you may want to leave the transfected cells for up to **10 days** in order to obtain higher titers of the initial virus lysate. Waiting for 1-2 extra days makes a big difference in terms of enhancing initial virus titers. **Patience will pay off** as it takes much longer time to amplify low titer viral lysate!