Protocol for transient expression and purification of secreted proteins from Expi293™ cells

1. Cell maintenance

Thawing cells

1. Gently aspirate cells from a cryovial and seed a 125 ml Erlenmeyer containing 30 ml of Expi293™ Expression Medium. Cells only reach high cell densities in this medium but can be grown in other serum-free media.
2. Incubate cells in a 37 °C incubator with a humidified atmosphere of 8% CO_2 in air on an orbital shaker at 125 rpm.

Passaging cells

1. Expi293™ cells can reach up to 8 X 10^6 /ml with 100% viability at times in which case split the cells down to 3X10^5 /ml on housekeeping days.
2. Otherwise, cells are seeded between 4-5 X 10^5 /ml on Mondays and Thursdays.
3. Expi293™ cells can be seeded as 30 ml, 50 ml, 100 ml & 300 ml cultures without any problems.

Freezing of cells

1. Spin 30 ml Expi293™ cells in sterile Falcon tubes at 1200 rpm for 10 min.
2. Decant media and resuspend cells in 10 ml of commercial cryo freezing media.
3. Place 2 ml of cells per cryovial and store at -80 °C.

2. Transfection

Preparation of reagents

1. Dissolve 100 mg of PEI Max 40K in 90 ml of water. Stir using a PTFE-coated stirring bar. It should take less than 5 min.
2. pH using NaOH or HCl to pH 7.00. Add water to 100 ml. Filter 0.2 µm under the hood, aliquot, store in aliquots at -20 °C.
3. Dissolve 500 mg valproic acid (Sigma P4543) in 10 ml of cell media or HBSS, filter 0.2 µm, store in aliquots at -20 °C.

4. Dissolve 1 g sodium propionate (Sigma P1880) in 10 ml of cell media or HBSS, filter 0.2 µm, store in aliquots at -20 °C.
5. Dissolve 4.5 g glucose in 10 ml of cell media or HBSS, filter 0.2 µm, freeze (or buy from Sigma G8769).
6. Prepare 100X stock of biotin (if biotinylation of target protein required): dissolve 0.82 g in 40 ml of 125 mM NaOH, filter 0.2 µm, store at 4 °C.

Transfection

NB Transfections are typically performed around 4 pm, with enhancers added before 10 am the following morning.

1. On the day before transfection, seed Expi293™ cells between 1.0-1.5 X 10^6 /ml at ≥ 95% viability.
2. The following day, determine cell count and viability: if cells are between 1.5-2 X 10^6 /ml and ≥ 95% viable, proceed with transfection.
3. For 30 ml culture volume: add 30 µg plasmid DNA to 3 ml Opti-MEM™ media in a 15 ml Falcon and gently shake. Then add 160 µl of PEI Max 40K transfection reagent.

   NB For larger transfection volumes scale-up components proportionally; see volume calculator table below.

   NB If biotinylation of target protein required, use 15 µg BirA plasmid alongside 15 µg plasmid of interest for transfection (total 30 µg DNA; scale according to culture volume).
4. Mix well and incubate for 10 min at RT before adding to cells; return to incubator.
5. If inhibition of N-glycan processing is required, add 30 µl Kifunensine (1 mg/ml) to the culture; scale according to culture volume.
6. The following day (16-18 h post transfection), add enhancers: 500 µl of valproic acid, 200 µl of Na propionate and 550 µl of glucose; return to incubator.

   NB If biotinylation of target protein required, add 300 µl 100X biotin; scale according to culture volume.

   NB At this stage, the incubator temperature can be reduced to 30 °C if necessary.
7. On day 5 after transfection, determine cell count and viability. Harvest media and spin for 20 min at 6000 *g*.

8. Filter sterilise the supernatant using 0.45 µm bottle top filter and add an equal volume of sterile PBS.
9. Proceed to purification using standard mammalian purification protocol.

Volume calculator table:

Culture volume	Opti-MEM™	PEI Max 40K	Kifunensine*	Valproic acid	Sodium propionate	Glucose	Biotin*
30 ml	3 ml	160 µl	30 µl	500 µl	200 µl	550 µl	320 µl
100 ml	10 ml	540 µl	100 µl	1700 µl	650 µl	2000 µl	1.1 ml
170 ml	17 ml	920 µl	170 µl	2.9 ml	1.1 ml	3.4 ml	1.7 ml
300 ml	30 ml	1.6 ml	300 µl	5 ml	3 ml	5.5 ml	3.2 ml
500 ml	50 ml	2.7 ml	500 µl	8.5 ml	3.25 ml	10 ml	5.5 ml

*If required.

3. Purification of secreted proteins

This protocol is for an Äkta Xpress platform using programmes described in Nettleship et al. Methods Mol Biol. 2009;498:245-63. This article also includes a transcript of the "Mammalian Affinity Gel Filtration" programme.

Buffers

- Wash buffer: PBS + 20mM imidazole pH 7.4 – 2L
- Elution buffer: PBS + 300mM imidazole pH 7.4 – 1L
- Gel filtration buffer: 1xPBS pH 7.4 – 2L
- 1 empty bucket for the Flow through (2L)
- A bottle for the supernatant + PBS

Equilibration of GF columns

It is recommended to start column equilibration the day before purification as this takes several hours.
Ensure the appropriate gel filtration (GF) column (see note 1) is attached to the ÄKTAXpress system in position 5. See 'Changing Superdex Columns' for instructions on how to do this.

Column wash steps are performed using the program: Instant Run → Gel Filtration Equilibration
GF columns are stored in 20% EtOH and must be washed with water prior to equilibration into purification buffers to prevent salt precipitation (see column care flowchart).
All buffers and samples must be filtered through 0.45 μm and degassed before use.

The ÄKTXpress system should be cleaned with water prior to column equilibration:

- Transfer lines A1, A2, A3, A4 into water
- Ensure the waste lines are in an empty container and that no HisTrap columns are attached to the system
- Run programme: Method Run → Mammalian Cleaning System
- Run program: Instant Run → Gel Filtration Equilibration (see note 2)
- Transfer the A1 and A4 lines to Gel Filtration Buffer
- Run program: Instant Run → Gel Filtration Equilibration

The GF column is now equilibrated and ready for use.

<u>Cells</u>

1. Harvest the mammalian cells. Pour the culture in conical sterile centrifuge pots, and centrifuge in the rotor JS 5.3, 6000g for 20min, 4C.

2. Filter the supernatant using a 0.8um filter. Add same volume of PBS 1x filtered (if 1.2L supernatant, add 1.2L PBS 1x). Add a magnetic stirrer and increase the pH with NaOH to pH 7.4.

<u>Preparing the ÄKTXpress system</u>

1. Transfer the lines into buffers as follows:
    a. A1 and A2 lines into Wash Buffer
    b. A3 line into Elution Buffer
    c. A4 line into Gel Filtration Buffer (the line should already be here following column equilibration
    d. F3 line into empty Duran bottle (at least 1.5x the volume of sample being loaded)
2. Run program: Method Run → Mammalian Prepping System

3. Follow the program instructions: it will pump wash A1 and A2 into Wash Buffer, clean the inlets, and then will ask you to screw a 5 ml HisTrap column into position to equilibrate it (see note 3)
4. Once the HisTrap is attached and has been equilibrated, the program will remind you to check the waste bottle, which should be emptied.

The system is now ready to start the purification.

Purification

1. Transfer the A2 line into the sample, slowly to avoid air bubbles in the line. Ensure the line is right at the bottom of the sample container, using a bung if necessary.
2. Run program: Method Run → Mammalian Affinity Gel Filtration
    a. Name the run with the date, sample name and any other relevant details, and save to an appropriate folder.
3. The program will ask ‘have you placed a block?’ prior to starting. Ensure a 2 ml 96-well block has been placed in the fraction collector and click ‘continue’.
4. The programme will load the sample onto the HisTrap column via the A2 line until air is detected in the line, at which point the HisTrap will be washed and bound protein eluted. The UV peak from elution is used to guide loading of the protein directly onto the GF column. 2 ml fractions will be collected from the SEC run.
5. Run SDS-PAGE on fractions corresponding to UV peaks in the resulting chromatogram to confirm which contain the purified protein of interest:
    a. Mix 10 µl protein solution with 10 µl Laemmli buffer and heat at 95 °C for 5 min
    b. Run an SDS-PAGE gel of the samples and stain using Instant Blue®
    c. Concentrate the protein as required; aliquot and plunge freeze in liquid nitrogen for storage at −80 °C