**Production and purification protocols for Ectodomain of SARS-CoV-2 Spike (S) protein**

**I. Production protocol for S-Ectodomain**

**Materials, Reagents and Equipment**

1.Expi293F™ Cells (Life Techn., Cat. #A14527)

2. Growth medium: Expi293™ Expression Medium 6 \*1L (Life Techn. Cat. # A1435102)

3. Opti-MEM I Reduced-Serum Medium (Gibco, Cat. #1850613)

4. FectoPRO® DNA transfection Kit 10mL (Polyplus-transfection® SA, Cat. #116-010)

5. Penicillin-streptomycin (Thermo Fisher Scientific, Gibco™, Cat# 15070063)

6. Sodium Butyrate (Sigma, Cat. # 303410)

7. Pierce BCA Protein Assay (Cat. #23225)

8. Microbiological Safety Cabinet - Class II

9. CO2 shaking incubator

10. Ultra Yield™ Flasks 2.5L Flask. (Thomson Instrument Company Cat. #: 931136-B)

**Procedure**

**Cell preparation:**

* Four days prior to transfection calculate the total number of cells required for the scheduled volume of production. Subculture Expi293F™ cell to the final cell’s density 0.3-0.5mln/ml
* One day before transfection, split healthy dividing cells to the density of 1.3 × 106 viable cells/ml and maintain 18 – 24 hours prior to transfection to ensure that cells are at the log-phase growth at the time of transfection with a viability >95%. Shake flasks overnight on an orbital shaker (95-125 rpm when using a shaker with a 2 cm orbital throw depending of the size of cell culture vessels) at 37°C, 8% CO2
* On the day of transfection, check cell density and viability using an automated cell counter or using the trypan blue dye exclusion method. Do not proceed with transfection unless cells are >95% viable.

**Transient transfection procedure:**

1. For each 1L of transfection, prepare reagent-DNA complexes as follows:
   * In a sterile tube, dilute 700ug of sterile plasmid DNA in 80ml of Opti-MEM® I Reduced Serum Medium (Life Technologies). Mix gently but thoroughly.
   * In a second sterile tube add 840ul of FectoPro transfection reagent
   * Add diluted DNA to the tube containing transfection reagent, invert several times and incubate at room temperature for 10 min to allow transfection complexes to form.

1. Add 10 ml of sodium butyrate (from a 1 M stock solution) to 1L of the prepared cells
2. After the DNA and FectoPro Transfection Reagent complex incubation is complete, add slowly transfection mix to each 1L of cells in the shaker flask.
3. Shake flasks on an orbital shaker (95 rpm when using a shaker with a 2 cm orbital throw) at 30°C, 8% CO2.
4. Start monitoring a cells density and viability after 72 hours of post-transfection time. Media for secreted proteins may be harvested beginning at approximately 72 hours post-transfection and assayed for recombinant protein expression. The incubation time for optimal protein expression depends on the nature of the recombinant protein.

**II. Purification protocol for S-Ectodomain**

**Materials, Reagents and Equipment**

1. Ni Sepharose® 6Fast Flow (GE Healthcare, Cat #17-5318-01)

2. Amicon™ Ultra Centrifugal Filter Units, 15ml, 100kDa (Millipore Sigma™ Cat# UFC910024)

3. Superose 6 Increase 10/300 GL (GE Healthcare Cat # 29-0915-96)

4. Gel Filtration Standards (Bio-Rad, Cat#1511901)

**Note:** Day before of purification, prepare two sets of the clean centrifuge bottles, make sure no chemical residues left in it:

* Rinse already washed bottles with dH20
* Fill again with dH20 and leave for at least 3 hours. Discard water and let air-dry

**Purification Buffers:**

**Note:** All buffers should be passed through a 0.22 µm filter before use.

10x buffer: 0.2M Hepes 7.5, 2M NaCl , 1.5% Glycerol

1st Wash buffer: 20mM Hepes 7.5, 200mM NaCl, 5% glycerol, 0 mM Imidazole

2nd Wash buffer: 20mM Hepes 7.5, 200mM NaCl, 5% glycerol, 25mM Imidazole

Elution buffer: 20mM Hepes 7.5, 200mM NaCl, 5% glycerol, 300mM Imidazole

SEC Buffer: 20mM Hepes 7.5, 200mM NaCl

**A. Histag purification**

1. Spin down cultured cells in centrifuge bottles @ 1500 RPM, 15 minutes at RT
2. Transfer cleared supernatant to centrifuge bottles, spin again @6500 RPM for 30min at RT
3. Transfer 1L of cleared supernatant into 2.8L Erlenmeyer flask, adjust pH by 10X times buffer, add Ni Sepharose beads (2ml per L, 4ml slurry) and proceed to the batch absorption
4. Batch absorption (BA) of cultured medium with Ni Sepharose beads could be performed by using 2.8L Erlenmeyer flasks in Innova shaker at RT for 2-3 hours, shaking at ~75 RPM
5. Let bound resin to settle by tilting flask on the ice for 30 min. Carefully decant the supernatant with minimal disturbance to the beads into another flask, add fresh Ni Sepharose beads and proceed with a second batch absorption (proceed with a 3rd batch absorption if needed)
6. Collect all of the bound resin in a gravity flow column
7. Wash with 40-50CV buffer without imidazole
8. Wash with 40CV buffer with 25mM imidazole, apply no more than 2-3 bed volumes at once (at this stage start checking on the Bradford assay, a protein might come out with even lower % of imidazole, collect washes)
9. Elute with 300mM Imidazole:

* Don’t apply all EB at once; use not more than one bed volume each time and apply very slowly to avoid disturbance of the beads.
* Incubate for 3-5 min after a first elution buffer. Collect small fractions.
* The volume of elution buffer required to elute the protein may vary and hence the eluate should be constantly assayed by adding 10ul of the eluate to 100ul of Bradford reagent to determine the point at which no more protein is being eluted

1. Combine purest peak fractions of eluate, concentrate at 160C and run SEC

**B. Purification procedure: SEC**

1. Equilibrate Superose 6 10/300 column with SEC buffer

2. Inject into the pre - equilibrated column 0.5ml of the IMAC eluate

3. Set flow rate to 0.4 mL/min.

4. Run gel with peak fractions on SDS-PAGE. Combine only the monomeric fractions, which elute at ~ 13.7ml on a Superose 6 Increase 10/300 column

5. Concentrate protein in an Amicon™ Ultra Centrifugal Filter Units, 15ml, 100kDa, check concentration, make aliquots and flash freeze, before placing into -800C