

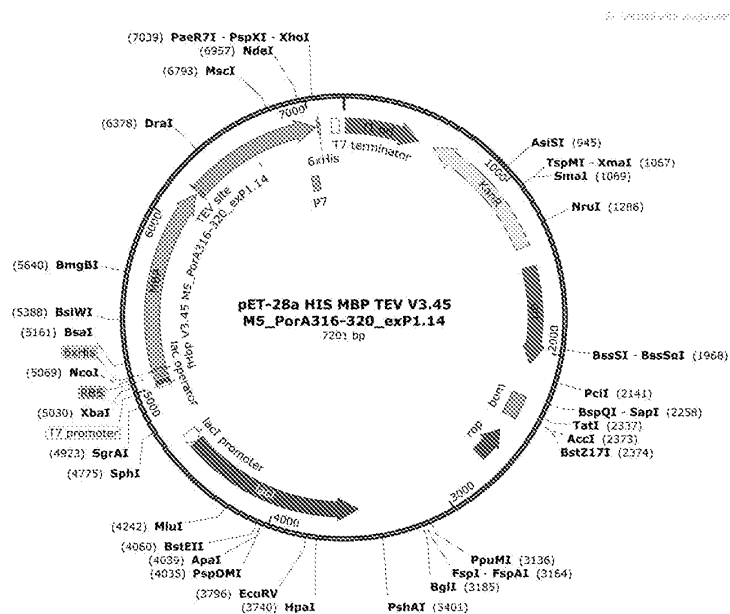


Figure-1

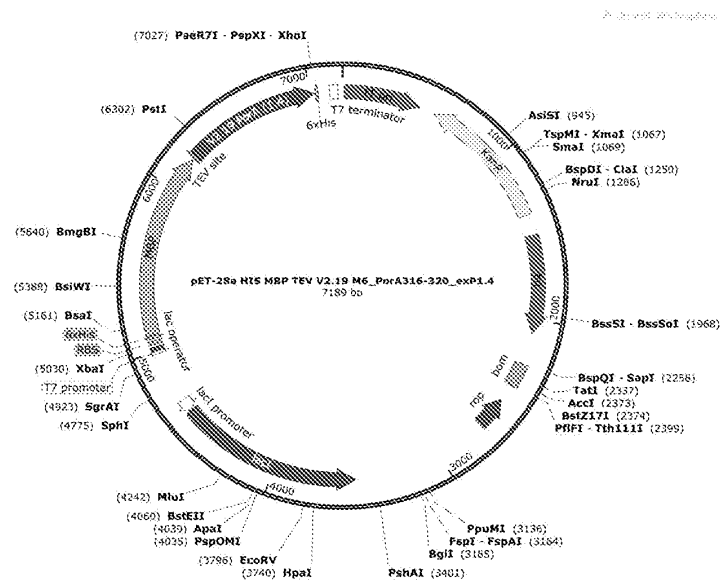
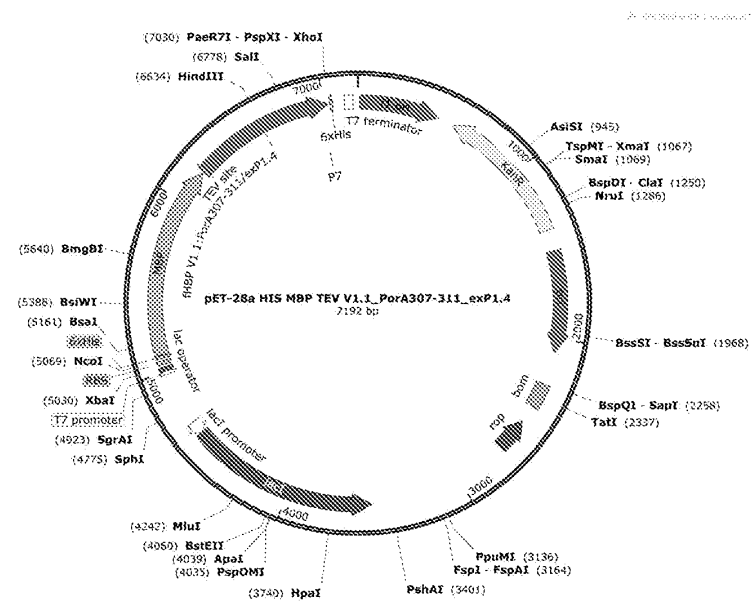
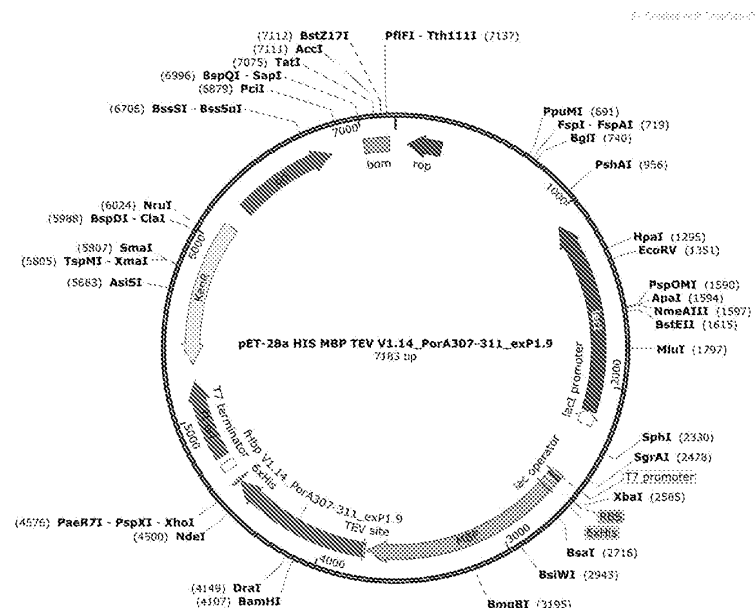
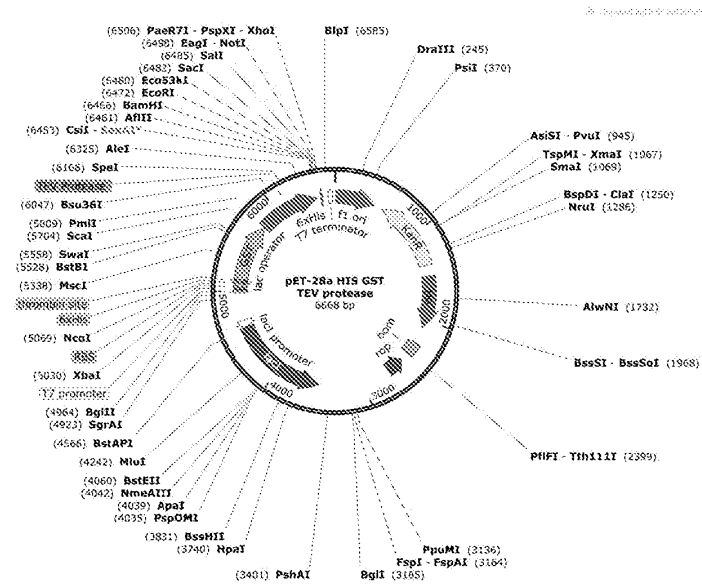
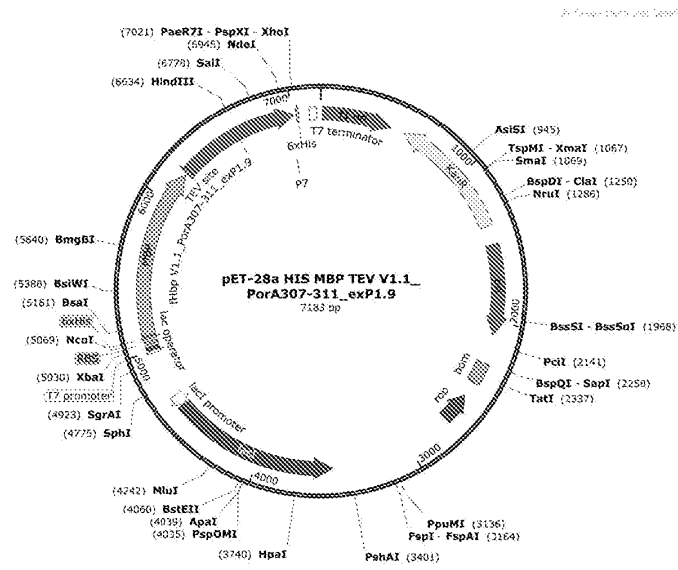


Figure-2





Seed development for 10 L scale fermentation batch

Thaw one vial of RCB in 30 mL Seed Media in 125mL shake flask

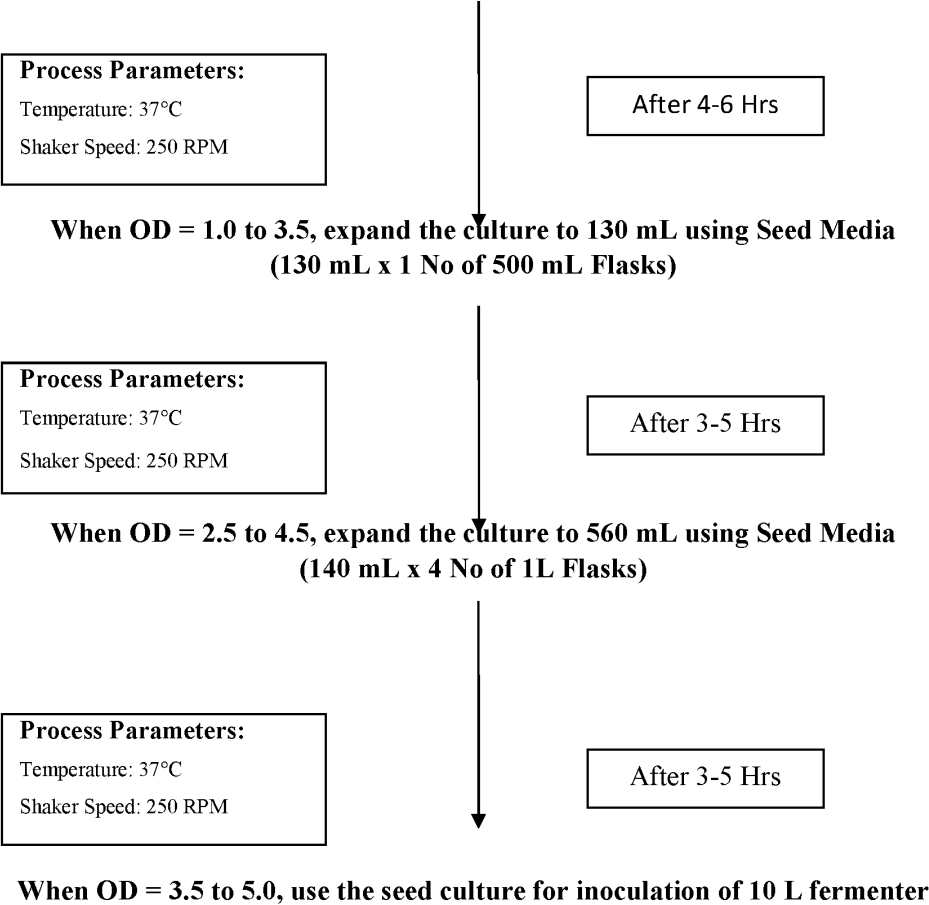


Figure-7

Production of tagged protein at 10L scale fermenter**Inoculation: Seed Culture 550 mL + Fermentation Media 5500 mL in 10L Fermenter****(During process maintain the L-Methionine concentration to 1-10 mM)****Process Parameters:**

Temp.: 37°C

pH: 7.0

DO: 30%

Gas Flow: 2-5 SLPM

Agitation: 300-1500 rpm

Oxygen % in total gas flow: 0-50%

Sampling interval: every Hr. check OD@
590nm

After 4-6 Hours

When glucose in the fermentation media gets depleted completely, start glucose feed

After 7-10 Hours

When OD at 590 nm is ~ 60-100 stop glucose feed and start glycerol feed. Induce the culture by adding lactose solution in to the culture (5-20 g/L final concentration). (At induction of tagged fHbp protein fHbp V3.45 M5: PorA 316-320/exP1.14 and fHbp V2.19 M6: PorA 316-320/exP1.4 shift the temperature set point of fermenter from 37°C to 33°C)

After 4-6 Hours of Induction

Harvest the Batch
(OD at 590 nm is ~ 90-180)**For cell separation, centrifuge the harvest broth at 4700 RPM (RCF= 7340) for 40 min at 4°C (Wet cell mass obtained ~ 150-350 g/L of harvest broth)****Handover Cell Pellet to DSP for protein purification**

Figure-8

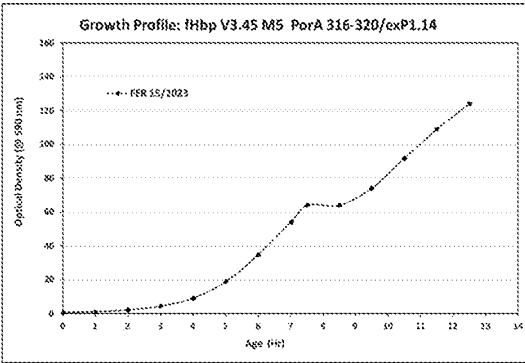


Figure-9a

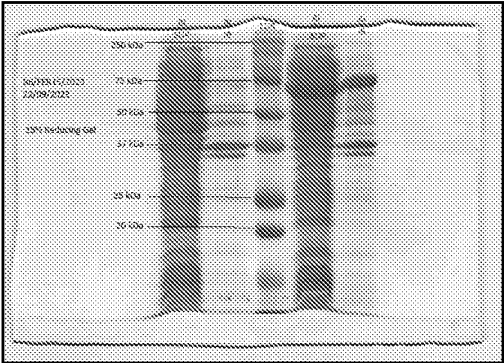


Figure-9b

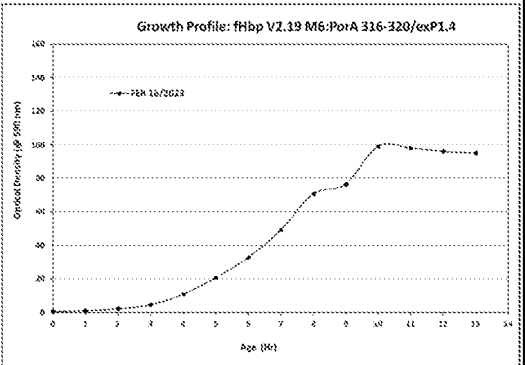


Figure-9c

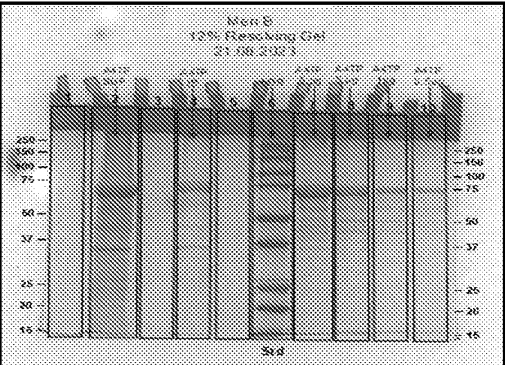


Figure-9d

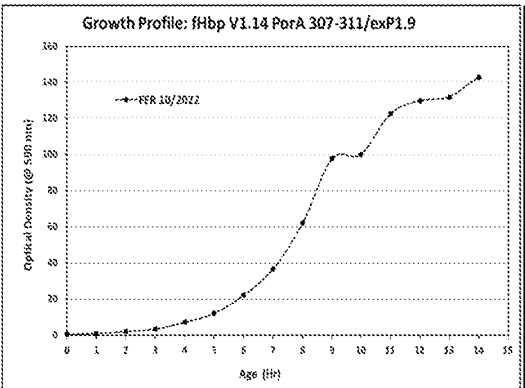


Figure-9e

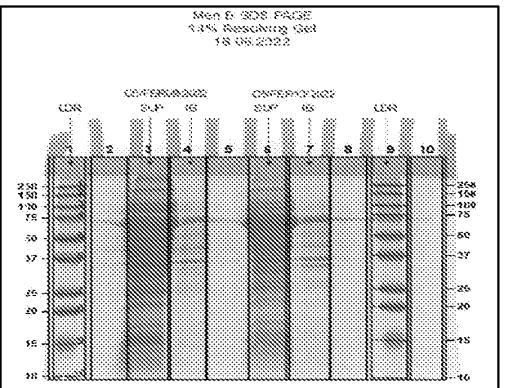
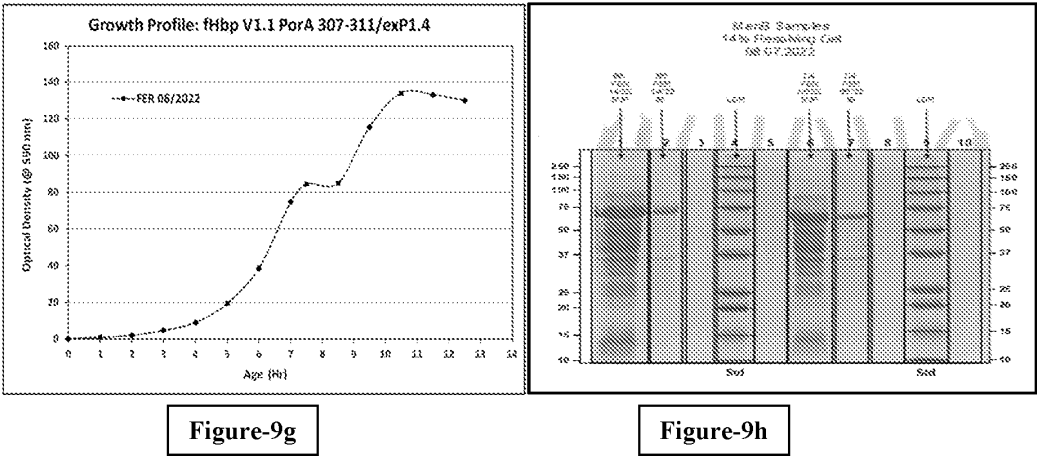


Figure-9f



Seed development for 10 L scale fermentation batch for the production of TEV protease

Thaw one vial of RCB in 30 mL Seed Media in 125mL shake flask

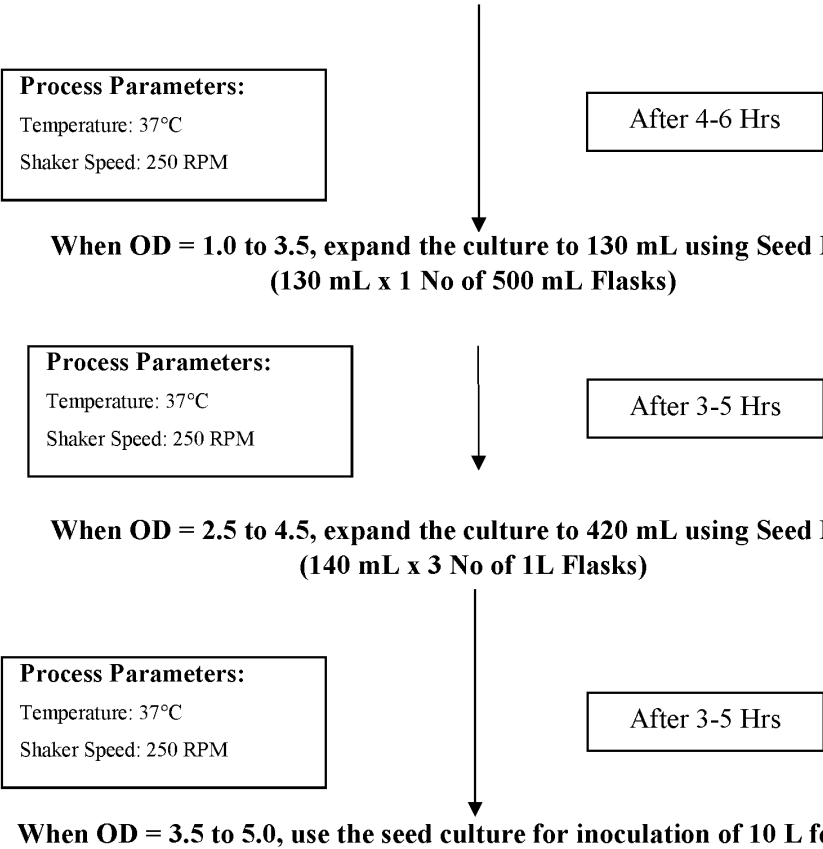
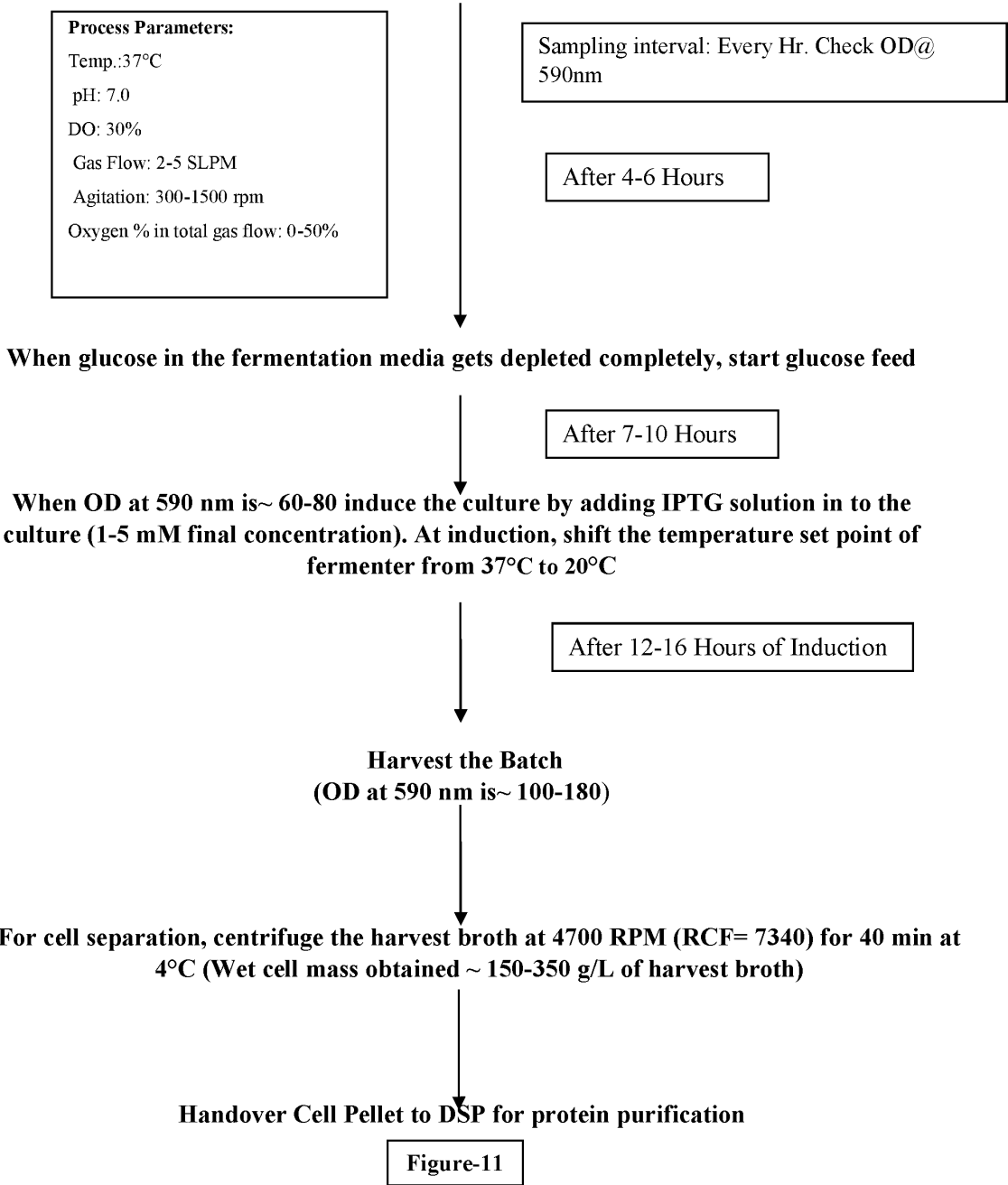


Figure-10

Production of TEV protease at 10 L scale fermenter

Inoculation: Seed Culture 420 mL + Fermentation Media 3600 mL in 10L Fermenter

(During process maintain the L-Methionine concentration to 1-10 mM)



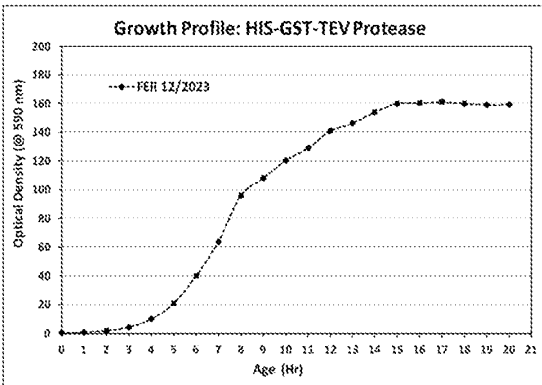


Figure-12a

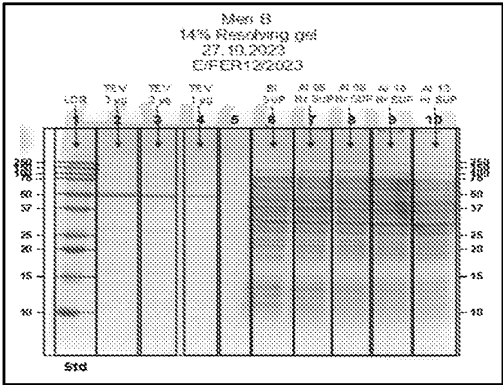


Figure-12b

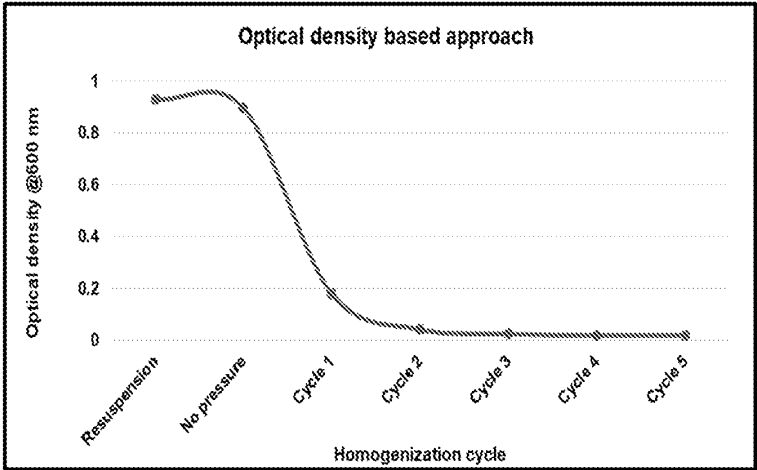


Figure-13

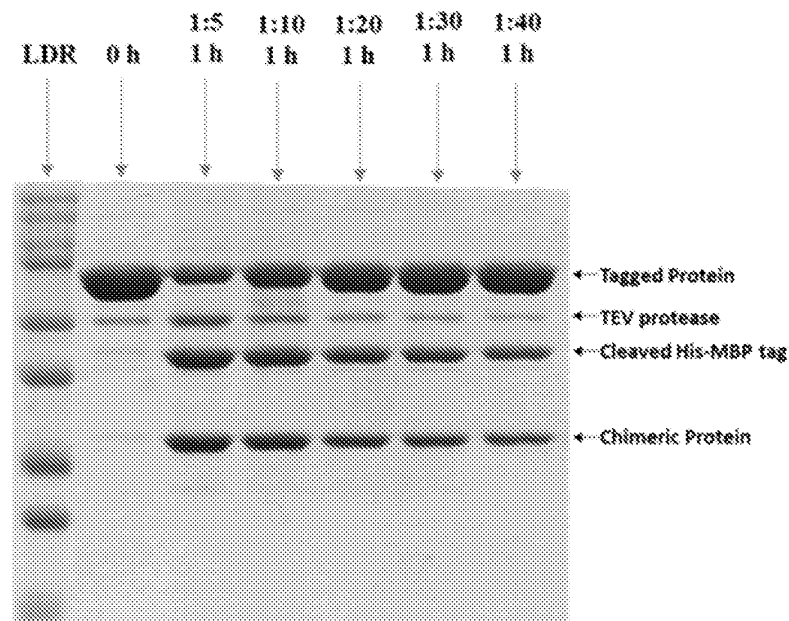


Figure-14a

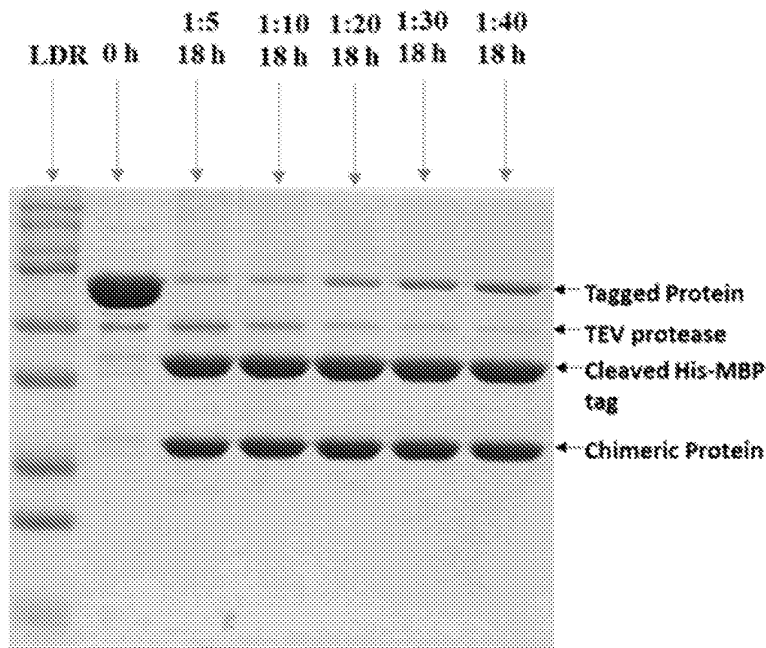


Figure-14b

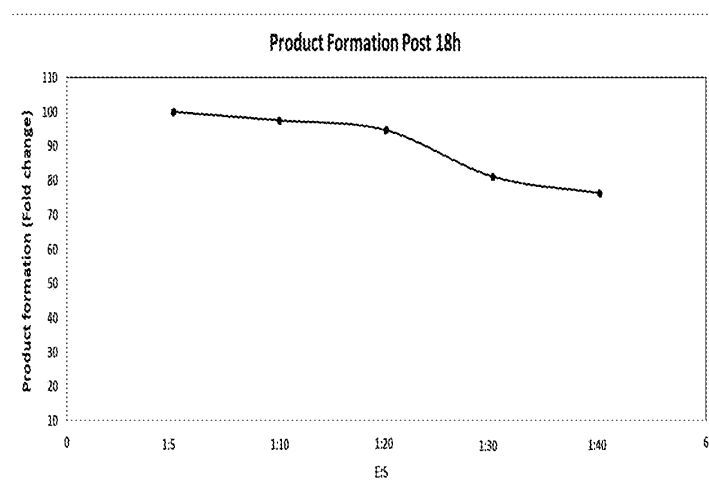


Figure-15

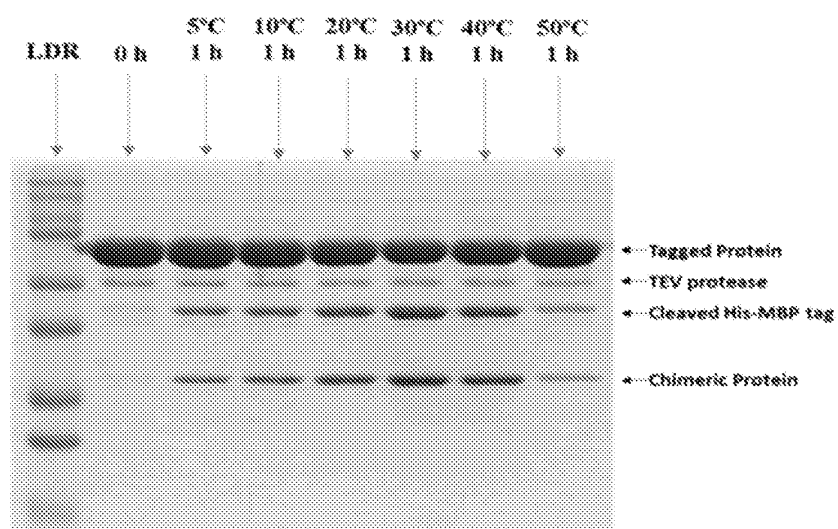


Figure-16

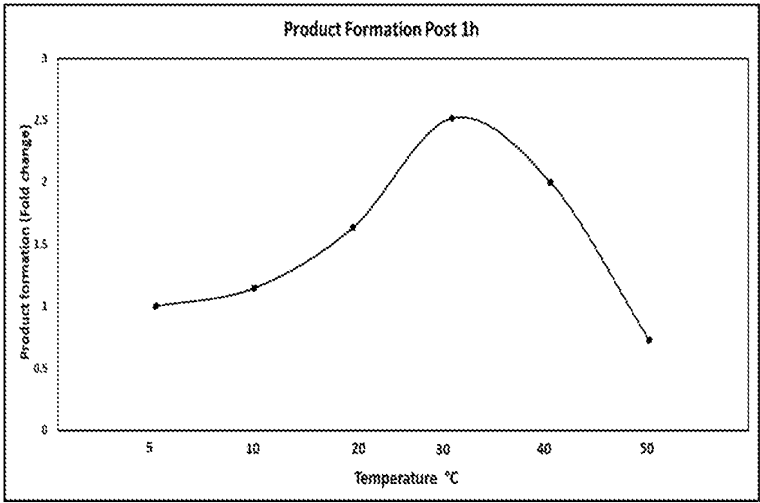


Figure-17

Purification of TEV protease by Ni-Sepharose Resin (pH 7.4)**Cell Lysis**

Harvested cell pellet 100 gram dissolved in ~2-3 L lysis buffer (25 mM Na-Phosphate buffer+100mM NaCl + 20mM Imidazol, pH 7.4) and Cell Lysed with Panda Homogenizer at 1000-1200 Bar for minimum 3 cycles and up to 5 Cycles, Centrifuge and collect supernatant, 0.45 µm filtration

**Ni-Sepharose 6FF Column**

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with lysis buffer and 0.45 µm filtered Post lysis supernatant loaded on the column



Wash 1 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl + 20mM Imidazol, pH 7.4); 5-6 CV



Wash 2 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 40mM Imidazol); 5-6 CV



Wash 3 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 80mM Imidazol); 5-6 CV



Wash4 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 120mM Imidazol); 5-6 CV



Elution with Buffer: (50mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 300 mM Imidazol)



After elution purified TEV protease concentrated and diafiltration carried out with 25mM Na-Phosphate buffer pH7.4 using 10kDa cassette and stored below -20°C with 20% glycerol until further use

Figure-18

Purification of TEV protease by Ni-Sepharose Resin (pH 8.5)**Cell Lysis**

Harvested cell pellet 100 gram dissolved in ~2-3 ltr lysis buffer (25 mM Tris-HCl buffer+100mM NaCl + 40mM Imidazol, pH 8.5) and Cell Lysed with Panda Homogenizer at 1000-1200 Bar for minimum 3 cycles and upto 5 Cycles, Centrifuge and collect supernatant, 0.45 µm filtration

**Ni-Sepharose 6FF Column**

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with lysis buffer and 0.45 µm filtered Post lysis supernatant loaded on the column



Wash 1 with Buffer: (25 mM Tris-HCl buffer+100mM NaCl + 40mM Imidazol, pH 8.5); 5-6 CV



Wash 2 with Buffer: (25 mM Tris-HCl buffer +100mM NaCl, pH 8.5+ 100mM Imidazol); 5-6 CV



Elution with Buffer:(25 mM Tris-HCl buffer +100mM NaCl, pH 8.5+ 300 mM Imidazol)

After elution purified TEV protease concentrated and diafiltration carried out with 25mM Tris-HCl buffer pH 8.5 using 10kDa cassette and stored below -20°C with 20% glycerol until further use

Figure-19

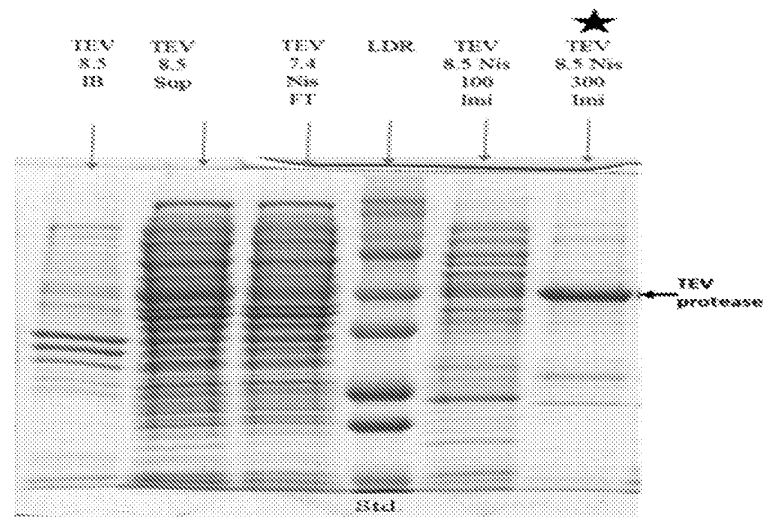


Figure-20a

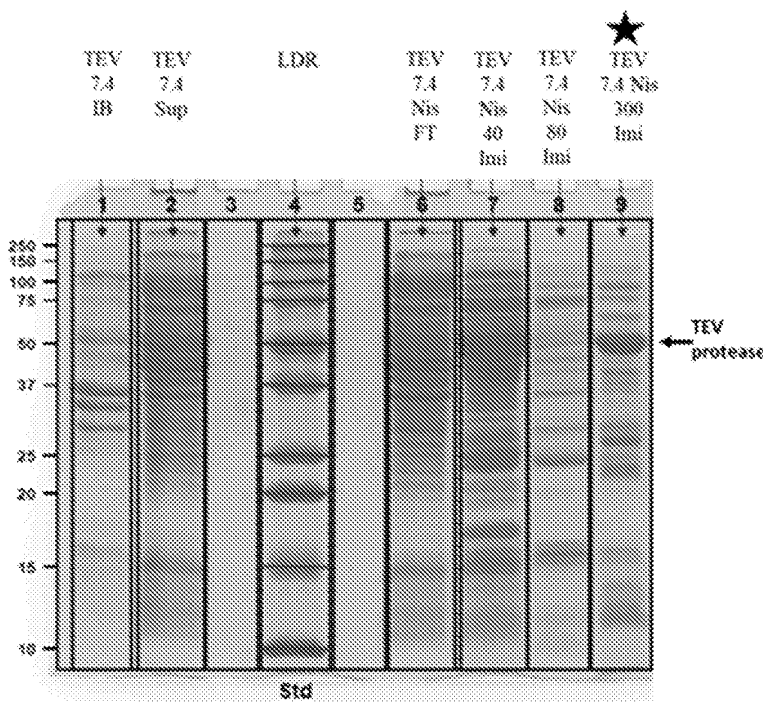


Figure-20b

Purification of 6×His-MBP tagged proteins by Ni-Sepharose Resin

Harvested cell pellet 100 gram dissolved in ~2-3 ltr lysis buffer (25 mM Na-Phosphate buffer+100mM NaCl+20mM Imidazol, pH 7.4) and Cell Lysed with Panda Homogenizer at 1000-1200 Bar for minimum 3 cycles and upto 5 Cycles, Centrifuge and collect supernatant, 0.45 µm filtration

**Ni-Sepharose 6FF Column**

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with lysis buffer and 0.45 µm filtered Post lysis Post lysis supernatant loaded on the column



Wash 1 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl+20mM Imidazol, pH 7.4); 5-6 CV



Wash 2 with Buffer: (25mM Na-Phosphate buffer+300mM NaCl+20mM Imidazol, pH 7.4); 5-6 CV



Wash 3 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 40mM Imidazol); 5-6 CV



Wash 4 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 80mM Imidazol); 5-6 CV



Elution with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 300 mM Imidazol)



After elution purified 6×His-MBP tagged proteins concentrated and diafiltration carried out with 12.5 mM Na-Phosphate buffer pH7.4 using 10 kDa/30kDa cassette and stored at 2-8 °C until further use

Figure-21

Purification of tagged proteins expressed in inclusion bodies**Cell Lysis**

Harvested cell pellet 100 gram dissolved in ~2-3 ltr lysis buffer (25 mM Na-Phosphate buffer+100mM NaCl, pH 7.4) and Cell Lysed with Panda Homogenizer at 1000-1200 Bar for minimum 3 cycles and up to 5 Cycles, Centrifuge and collect supernatant and Post lysis pellet

Ammonium sulphate precipitation

Slowly add appropriate amount of Ammonium sulphate to make final concentration to 30% in supernatant. Allow it to stand for 20-30 min for complete precipitation. Centrifuge and collect precipitate

Protein Unfolding in Urea

Slowly dissolve ammonium sulphate precipitate/ IB's in to appropriate volume of 25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 8M Urea until it dissolves completely. Filter with 0.22 µm filter and load on the column

**Ni-Sepharose 6FF Column (on column refolding and purification)**

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with 25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 8M Urea and sample loaded on column



Refolding- wash the column with reducing concentration of 25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ reducing concentration of Urea i.e 8M, 6M, 4M, 2M, 1M (each 5-6 CV)



Wash 1 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4); 5-6 CV



Wash 2 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 40mM Imidazol); 5-6 CV



Wash 3 with Buffer:(25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 80mM Imidazol); 5-6 CV



Elution with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 300 mM Imidazol)



After elution purified 6×His-MBP tagged proteins concentrated and diafiltration carried out with 12.5 mM Na-Phosphate buffer pH7.4 using 10 kDa/30kDa cassette and stored at 2-8 °C until further use

Figure-22

His-MBP tag from purified protein was removed using TEV protease enzyme as follows-

6×His-MBP tagged protein in 12.5 mM Na-Phosphate buffer, pH7.4



Add appropriate volume of 100mM DTT so to make final concentration of 1mM



Thaw TEV protease frozen below -20 °C to RT and add 1 part of enzyme in mg per 20 or below parts of tagged protein in mg as substrate (Enzyme: Substrate should be 1:20 and below)



Adjust pH to 7.4 and Incubate at 30°C for 16 to 18 Hrs with gentle mixing



Purify Chimeric protein from digestion reaction with Ion exchange followed by affinity chromatography

Figure-23

Part 1- Ion exchange (Strong anion exchange Capto Q) chromatography

Equilibration and Sample Loading: Charged Capto Q column equilibrated with 12.5 mM Na-Phosphate buffer pH 7.4 and digestion reaction was loaded on the column. Collect Flow through



Elution 1 with Buffer: (12.5mM Na-Phosphate buffer+ 30mM NaCl, pH 7.4); 5-6 CV



Elution 2 with Buffer: (12.5mM Na-Phosphate buffer+ 60mM NaCl, pH 7.4); 5-6 CV



Elution 3 with Buffer: (12.5mM Na-Phosphate buffer+ 90mM NaCl, pH 7.4); 5-6 CV

FHBP V1.1 : PorA 307-311/exP1.4 and FHBP V1.1 : PorA 307-311/exP1.9 are present in flow through fraction. Whereas FHBP V3.45 M5 : PorA316-320/exP1.14, FHBP V2.19 M6 : PorA 316-320/exP1.4 and FHBP V1.14 : PorA307-311/exP1.9 are eluted in elution 2.

Fractions of interest are concentrated and diafiltered with 12.5mM Na-Phosphate buffer + 100mM NaCl + 5mM Imidazole using 5 kDa cassette and stored at 2-8 °C until further use

Figure-24

Part 2- Affinity chromatography Ni-Sepharose 6FF Column

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with 12.5mM Na-Phosphate buffer + 100mM NaCl + 5mM Imidazole and purified chimera from Ion exchange chromatography loaded on the column to further purify Chimeric proteins



Elution 1: (12.5 mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 30mM Imidazol); 5-6 CV



Elution 2: (12.5 mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 60mM Imidazol); 5-6 CV



Elution 3: (12.5 mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 300 mM Imidazol)



All the Chimeric proteins are eluted in Elution 1.

Elution 1/ Elution 2 containing final Drug substance i.e. Chimeric protein pooled if needed and concentrated and diafiltration carried out with 12.5 mM Na-Phosphate buffer pH7.4 using 5 kDa cassette.

Drug substance i.e. Chimeric protein sterile filtered and stored at 2-8 °C until further use/formulation

Figure-25

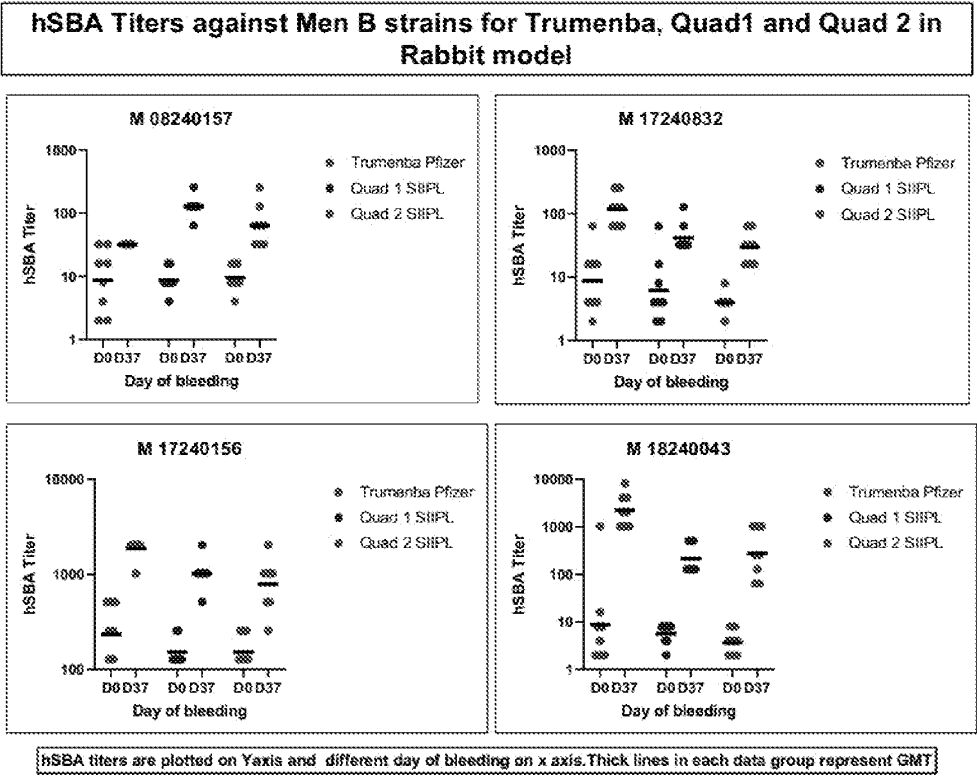


Figure-26

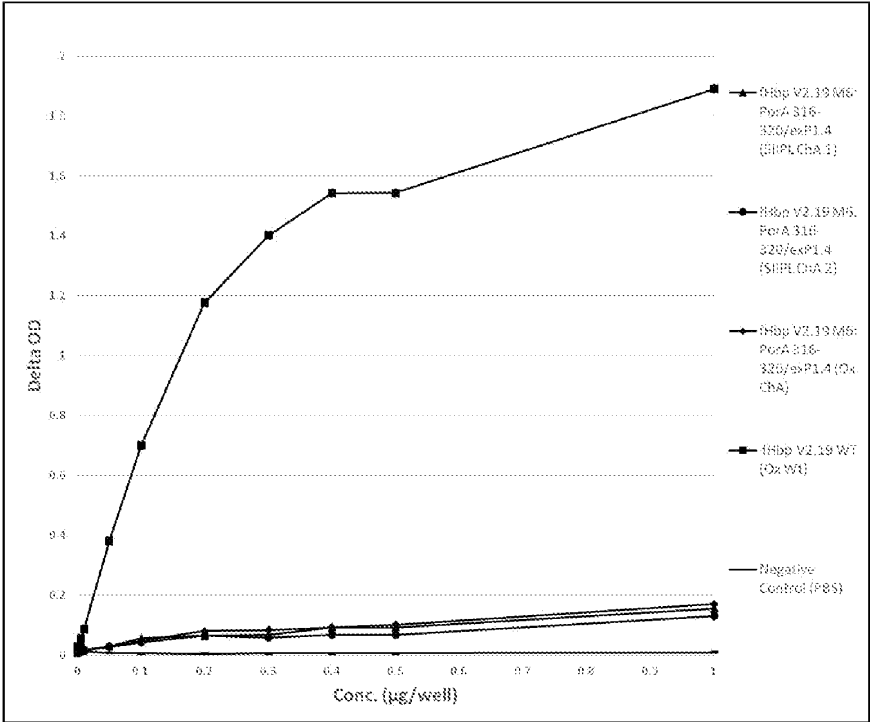


Figure-27

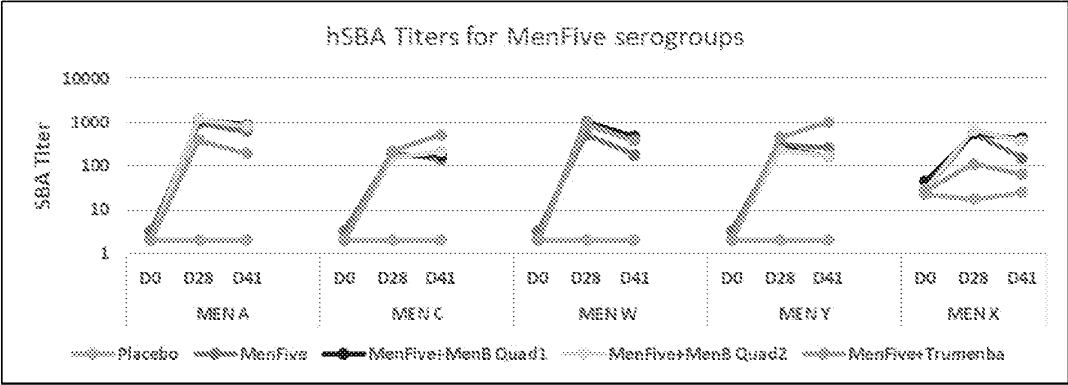


Figure-28

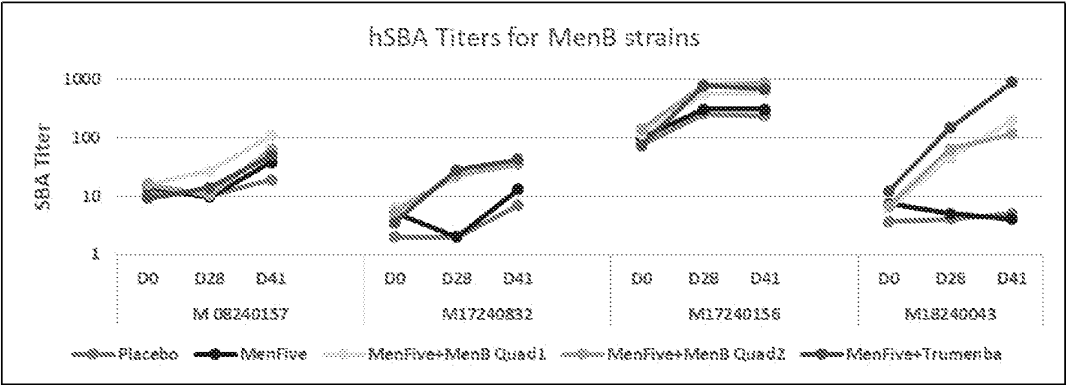


Figure-29

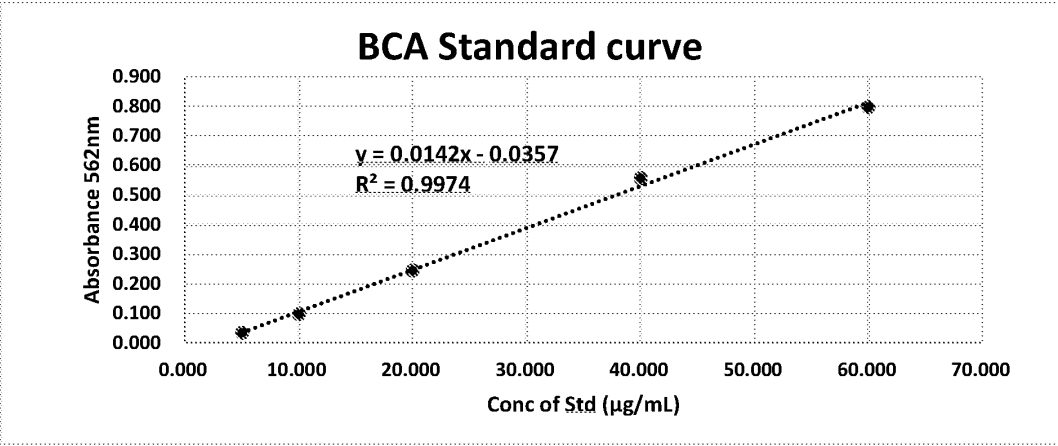


Figure-30