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Small-Scale Screening to Large-Scale Over-Expression of Human Membrane Proteins for Structural Studies

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Abstract

Membrane protein structural studies are frequently hampered by poor expression. The low natural abundance of these proteins implies a need for utilizing different heterologous expression systems. *E. coli* and yeast are commonly used expression systems due to rapid cell growth at high cell density, economical production, and ease of manipulation. Here we report a simplified, systematically developed robust strategy from small-scale screening to large-scale over-expression of human integral membrane proteins in the mammalian expression system for structural studies. This methodology streamlines small-scale screening of several different constructs utilizing fluorescence size-exclusion chromatography (FSEC) towards optimization of buffer, additives, and detergents for achieving stability and homogeneity. This is followed by the generation of stable clonal cell lines expressing desired constructs, and lastly large-scale expression for crystallization. These techniques are designed to rapidly advance the structural studies of eukaryotic integral membrane proteins including that of human membrane proteins.

Keywords

Membrane protein expression; Mammalian cell culture; FSEC; Large-scale expression

1 Introduction

Membrane proteins constitute an integral component of the cellular proteome and participate in several physiological processes. Their clinical importance is emphasized by the fact that the majority (~60 %) of prescription drugs act on membrane proteins. Despite their physiological importance, the success rate of structure determination of human

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membrane protein structures is low [1, 2]. Membrane protein crystallization requires extensive optimization of many parameters that are not critical for soluble proteins. A major bottleneck is the over-expression of proteins in order to obtain sufficient quantity of pure, homogeneous, and stable (PHS) proteins for structural studies [3].

Although prokaryotic expression systems are the most commonly utilized vehicles, they have not been very successful for over-expression of mammalian membrane proteins as they fail to provide the necessary folding machinery or posttranslational modifications. On the other hand mammalian cells, particularly human cells, offer inherent advantages for expressing human membrane proteins due to the endogenous translocation machinery, posttranslational modifications, and lipid environment that is most native to mammalian membrane proteins. In the past, mammalian expression systems were not extensively manipulated for structural studies primarily due to technical difficulties associated with large-scale cultures and limited utility of traditional constitutive promoters for toxic proteins. With recent advances in inducible promoters, new methods have been developed and employed for expressing such proteins [3–5].

It is a widely known fact in the structural biology field that one cannot predict from the beginning if a particular protein can be successfully crystallized, purified, or even expressed. Considering all the hurdles at every single step, we developed a simplified yet robust methodology that can be employed towards screening a multiple numbers of constructs without a need for milligram quantities of protein. Our strategy involves generation of several desired constructs for a particular gene. Such constructs may include N-terminal or C-terminal truncations alongside the full-length construct. Different N- or C-terminal tags are added to the construct to allow for purification. For small-scale screening of these constructs, we utilize FSEC [6], where the gene of interest is cloned into an EGFP vector (pACMV-tetO-EGFP). Based on FSEC results, one can roughly compare the expression level between different constructs and then might consider re-cloning the gene into an expression vector of choice without EGFP (pACMV-tetO) [7] or can continue with the same vector simply by cleaving the EGFP later on. FSEC can also be utilized for several other optimizations including of buffer, additives, and detergents for achieving stability and homogeneity (Fig. 1a). Once constructs are chosen, one can move towards generating a stable clonal cell to be utilized for further screening (Fig. 1b). Post-detergent screening, thermostability assays that provide a proxy for protein stability can be useful in the pursuit of ideal conditions by optimizing buffers, pH, salt concentrations, and ligands to facilitate crystallization of a membrane protein that is also more stabilized conformationally by the environment, ligands, lipids, detergents, and many other variables [8].

2 Materials

Prepare all solutions using distilled, deionized water and analytical grade reagents (unless specified otherwise). All reagents for cell culture work (media, antibiotics, chemicals, etc.) should be used of tissue culture grade. All prepared reagents should be stored at 4 °C (unless indicated otherwise). For cells and other waste disposal consult the material safety data sheets for all chemicals used and follow the safe procedures for handling and proper disposal of chemicals.

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2.1 Cloning of the Target Gene

1. pACMV-tetO mammalian expression plasmid (*see* Note 1).
2. Cloning primers: Forward primers F1 and F2, and reverse primers R1 and R2, diluted to 25 μM in nuclease-free water. Store at −20 °C (*see* Notes 2 and 3).
3. Deoxyribonucleotide (dNTP) mix: 10 μM Stock diluted to 2 μM in nuclease-free water. Store at −20 °C.
4. PCR Purification Kit (Qiagen): Follow the manufacturer's instructions. Store at room temperature (*see* Note 4).
5. Gel Extraction Kit (Qiagen): Follow the manufacturer's instructions. Store at room temperature (*see* Note 5).
6. Phusion DNA polymerase (New England Biolabs) (*see* Note 6).
7. Restriction Endonuclease: XbaI and XhoI supplied with appropriate buffers (New England Biolabs).

2.2 Transfection and Adherent Cell Culture

1. HEK293S GnTI^{-} cells (or another highly transfection efficient mammalian cell line).
2. Adherent DMEM (Dulbecco's modified Eagle's medium) medium: To 1 L DMEM high-glucose media add 1 % penicillin-streptomycin, and 10 % iron-supplemented bovine calf serum (BCS). We usually used the BCS provided by Hyclone (due to its premium quality).
3. Selection medium: 1 L DMEM high glucose, 1 % penicillin-streptomycin, 10 % FBS, 4 % Geneticin ®, and 0.1 % blasticidin S HCl.
4. 5 mg/mL Blasticidin solution: Weigh 50 mg of blasticidin S HCl, dissolve in 10 mL of water. Filter-sterilize, aliquot, and store at −20 °C for 6–8 weeks.
5. 20 mg/mL Doxycycline hyclate: Weigh 100 mg of doxycycline solution, dissolve in 5 mL of autoclaved water. Filter-sterilize, aliquot, and store at −20 °C for 6–8 weeks.
6. 20 % (wt/vol) Glucose: Weigh 100 g of glucose in 500 mL of autoclaved water. Filter-sterilize and store at 4 °C for 2–3 months.

2.3 Expression of the Target Protein

1. 1× Solubilization buffer or lysis buffer: Mix 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, and 10 % (vol/vol) glycerol. Immediately before use, add 1 mM PMSF

1. Gene of interest can be cloned in between Kpn1 and XhoI restriction sites of pACMV-tetO vector.
2. Remember to include a stop codon immediate downstream of the fusion tag on the reverse primer to prevent read-through.
3. Primers can be synthesized or ordered from any vendor.
4. Any PCR purification kit can be utilized though we have used the one from Qiagen.
5. Any gel extraction kit can be used though we have used the one from Qiagen. One should take proper care during single-band excision from gel.
6. For PCR step, any polymerase can be utilized though we had good results using Phusion ® high-fidelity DNA polymerase.

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and one complete EDTA-free protease inhibitor cocktail tablet. Chill to 4 °C and discard any unused buffer.

2. Octyl-β-d-glucopyranoside (OG), *N*-dodecyl- β-d-maltopyranoside (DDM) or other detergents to test protein solubilization: Prepare appropriate concentration of respective detergents in ddH_2O. We order detergents from Anatrace.

3. 1 mM Phenylmethanesulfonyl fluoride (PMSF) in isopropanol. Prepare just before use.

4. Complete EDTA-free protease inhibitor cocktail tablets: Prepare the inhibitor cocktail as prescribed in the manufacturer's protocol.

5. 2× Solubilization buffer: Mix 40 mM Tris–HCl pH 7.4, 200 mM NaCl, and 20 % v/v glycerol. Immediately before use, add 1 mM PMSF) and one complete EDTA-free protease inhibitor cocktail tablet. Chill the buffer to 4 °C and discard any unused buffer.

2.4 Fluorescence Detection Size-Exclusion Chromatography (FSEC)

1. Gel filtration column: Use either TSK-GEL G3000SW or Superdex200 10/300 GL, from GE Healthcare. Equilibrate the column with 1× solubilization buffer.

2.5 Suspension Culture

1. Suspension medium (for spinner flask): 1 L DMEM-high glucose without calcium salts, 1 % penicillin-streptomycin, 10 % iron-supplemented BCS, 1 % of Pluronic, 0.3 g Primatone RL/UF, 3.7 g sodium bicarbonate.

2. Suspension medium (for WAVE bioreactor): Prepare 10 L DMEM-high glucose without calcium salts, to which add appropriate volume for getting a final concentration of 1 % penicillin-streptomycin, 10 % iron-supplemented BCS, 1 % Pluronic, 3 g of Primatone RL/UF, and 37 g of sodium bicarbonate.

3. 500 mM Sodium butyrate: Weigh 27.5 g of sodium butyrate and dissolve in autoclaved water to a final volume of 500 mL. Filter-sterilize and store at room temperature (20–25 °C) for 6–8 weeks.

4. 10 % (wt/vol) Primatone RL/UF: Weigh 3 g of Primatone RL/UF and dissolve in 30 mL of autoclaved water. Filter-sterilize and use immediately.

5. 10 % (wt/vol) Pluronic: Weigh 50 mg Pluronic and dissolve in 500 mL of autoclaved water. Filter-sterilize and store at 4 °C for 6–8 weeks.

6. Spinner flask: Vigorously clean the spinner flask, by detaching the various components. For cleaning purpose, use 10 % v/v glacial acetic acid (with water) and allow stirring overnight at room temperature. Following day, discard the glacial acetic acid and rinse flask thoroughly in order to remove all traces of acid. Perform two rounds of liquid autoclaves, for 30 min each filled with distilled water. Finally, perform a dry autoclave for another 30 min. Allow the flask to cool down before use.

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7. WAVE bioreactor: Assemble the 20 L WAVE cellbag in a tissue culture room, following the manufacturer's protocols.

2.6 Affinity and Size-Exclusion Chromatography

1. SEC buffer: 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10 % (vol/vol) glycerol, and 40 mM OG (or 0.5 mM DDM). Store at 4 °C for up to 1 week.
2. FLAG resin: Equilibrate the resin with SEC buffer, following the manufacturer's protocol.
3. Tris(2-carboxyethyl)phosphine (TCEP).
4. Glass Econo-column: Use the column for loading the resin onto the column, followed by washing of the resin with SEC buffer and elution of the protein with SEC buffer, supplemented with FLAG peptide.
5. Superdex 200 10/300 GL: Equilibrate the column with three column volumes of SEC buffer.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Expression Construct Design

Design the multiple constructs in a manner to introduce N- and C-terminal tags along with the protease cleavage site (Fig. 2). In an experiment, we normally utilize a FLAG tag with 3C cleavage site at the N-terminal and octa or deca-His tag with thrombin cleavage site at the C-terminal via a two-step PCR (Fig. 3). Introduce a five-amino-acid Gly/Ala repeat spacer sequence in between the N/C-terminus of the transgene and the thrombin and 3C site, to facilitate tag removal. Different tag sequences are given in Table 1.

1. PCR1: Perform first PCR step using F1 and R1 primers to insert 3C and thrombin at the N- and C-terminal of the transgene, respectively. Resolve PCR1 on agarose gel and carry out the agarose gel extraction to purify the first PCR product, to be used as a template for the second PCR.
2. PCR2: Use F2 and R2 primers for the second-step PCR to add the start codon-FLAG tag at the N-terminal and octa/deca-His-stop codon at the C-terminal along with the desired proteases.

3.2 EGFP-Tagged Expression Vector

In order to rapidly assess the suitability of human membrane protein expression constructs, yielding the pure and homogeneous proteins, transiently transfect the glycosylation-deficient GnTI-deficient HEK293S cells. For quick screening purpose, introduce enhanced green fluorescence protein (EGFP) tag at the C-terminal of pACMV-tetO vector. PCR amplify the EGFP sequence along with five-amino-acid (Gly/Ala) spacer.

1. Design oligo with the N-terminal consisting of spacer-thrombin-EGFP (N), and a C-terminal consisting of EGFP (C)-$His_{8–10}$.

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2. Following standard protocols amplify PCR; digest the EGFP insert and pACMV-tetO vector.
3. Post-ligation, follow the manufacturer's protocol to transform the ligation products. Confirm the vector sequence.
4. Sub-clone the transgene of interest into pACMV-tetO-EGFP vector for rapid screening.

3.3 Transient Transfection and Induction of HEK293S GnTI^{-} -Deficient Cells

Transiently transfect HEK293S GnTI $^{-}$ cells with pACMV-tetO plasmid containing the gene of interest (Fig. 4). Over-expression of human membrane proteins is favorable while using an inducible cell line as high cell density can be achieved before induction, thereby attenuating the toxicity.

1. Seed three 10 cm plates (~40 % confluence) with HEK293S cells (DMEM + 10 % FBS) (*see* Notes 7 and 8).
2. Next day, visualize cells under the microscope to confirm cell confluence (~70–80 %).
3. Gently mix 10 μg of plasmid DNA (Eppendorf tube A) in 1.5 mL of Opti-MEM I (*see* Note 9).
4. Simultaneously, following the manufacturer's instructions, dilute 60 μL of Lipofectamine 2000 in 1.5 mL of Opti-MEM I medium (Eppendorf tube B).
5. Incubate both reactions (Eppendorf tube A and tube B) at room temperature for 10 min.
6. Mix diluted DNA samples (from tube A) to the diluted Lipofectamine 2000 (tube B).
7. Incubate at room temperature for 30 min.
8. In the meantime, aspirate off media and add 7 mL of Opti-MEM I.
9. Gently add DNA-liposome complex to the plate containing Opti-MEM I media and mix by gently swirling the plate.
10. Incubate at 37 °C in a CO_2 incubator.
11. Post-4–6 h aspirate off media and add fresh adherent medium (DMEM + 10 % FBS).
12. Incubate at 37 °C overnight.
13. Post-24 h, use 2 μg/mL doxycycline to induce the cells.

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7. For each individual transfection include positive and negative controls. For a positive control typically a pACMV-tetO construct containing a well-expressed transgene is suitable, whereas a transfection without a plasmid serves as a negative control.
8. For a 6-well tissue culture plate add 2 mL DMEM medium per well and 500 μL of trypsin, for a 10 cm plate we add 10 mL DMEM medium and 1 mL of trypsin, and for a 15 cm plate we add 25 mL DMEM medium and 2 mL of trypsin.
9. For transfection purpose we use the plasmid eluted with pre-warmed ddH_2O instead of elution buffer.

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14. 32–36 h post-induction, wash cell monolayer on plate with PBS (twice) and trypsinize cells.

15. Harvest the cells and perform the whole-cell membrane solubilization.

3.4 Expression Assessment

1. Resuspend cells in 1 mL of 2× solubilization buffer by pipetting up and down gently.

2. For solubilization, based on total number of detergents to be screened, split resuspended cells into equal-volume aliquots and transfer to 1.5 mL ultracentrifuge tube.

3. Add 2× detergent solution (final 1× concentration).

4. Routinely, we screen expression levels in four different detergents per sample: β-octylglucoside (β-OG) (400 mM), β-dodecylmaltoside (β-DDM; 40 mM), lauryldimethylamine-oxide (LDAO; 200 mM), and fos-choline 14 (FC-14; 40 mM) (*see* Notes 10 and 11).

5. Solubilization time might vary with different detergents. For screening purpose solubilize by 2-h stirring at 4 °C.

6. To compare the extent of solubilization, save 12 μL as pre spin aliquot per detergent solubilization.

7. Remove insolubilized material by centrifugation in TLA45 ultracentrifuge rotor at high speed (100,000 × *g*) for 1 h.

8. After transferring supernatant to 1.5 mL microfuge tube, take out 12 μL as post-spin sample.

9. Perform western blot with pre-spin and post-spin samples to analyze detergent solubilization. We follow standard western blotting protocols to compare pre-spin and post-spin samples.

3.5 Fluorescence Detection Size-Exclusion (FSEC) Chromatography

1. Wash a gel filtration column (TSK-GEL G3000SW or Superdex200 10/300 GL) connected to Shimadzu chromatography system with 2× column volume with water to remove the 20 % ethanol used to store column.

2. Equilibrate the column with 1× solubilization buffer.

3. Use RF-10AXL Shimadzu fluorescence detector (λ_{ex} = 488 nm and λ_{em} = 509 nm) with initially set to a sensitivity of “medium” and a gain of “16×.” Use SPD-10A UV-Vis Shimadzu detector to detect absorbance at 280 nm (A280).

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10. One can analyze the expression of different constructs solubilized in a couple of detergents to remove the possibility of expression being hindered due to the insolubility in a particular detergent itself.

11. From our experience, for crystallography purpose most of the membrane proteins are solubilized in DDM and OG. FC14, being a harsh detergent, solubilizes the membrane proteins but might leave the protein inactive.

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4. Following the manufacturer's instructions, inject ~100 μL of 0.45 μm filtered solubilized sample. Perform each experiment in triplicates.

5. Compare the expression levels and profile among the different constructs over FSEC chromatogram (*see* Note 12).

3.6 Antibiotic Kill Curve

1. One day prior to performing antibiotic selection, seed cells in 6-well plates containing 0.5 mL complete growth medium per well. At the time of antibiotic selection, cell density should be nearly ~60–80 % (0.8–3.0 × 10^5 cells/mL).

2. Add increasing amounts of the G418 to duplicate wells of cells. Maintain one well as a control and do not add any antibiotic. We add 0, 100, 200, 400, 800, and 1000 μg/mL selection antibiotic in duplicate wells. Plasmids with different antibiotic markers can also be utilized. An optimal concentration range for different selection markers is given in Table 2.

3. Examine cultures for 1 week for toxicity signs while replacing media (containing selection antibiotic) on alternate days (*see* Note 13).

4. The antibiotic concentration at which the cells are dead post-1 week of antibiotic selection is considered as an optimal dose. Once the optimal antibiotic dose is decided, seed cells for stable transfection.

3.7 Stable Clone Generation

Based on FSEC analysis, one can select the construct(s) providing the best expression and profile on chromatogram among all others. Alternatively, one can also go back and reclone the best expressing construct into any other vector of choice without EGFP. After selecting the best construct, proceed towards stable clonal cell generation (Fig. 5) (*see* Note 14).

1. One day prior to transfection, plate cells in a 6-well tissue culture plate so that the cell density is nearly ~70–80 % at the time of transfection.

2. Post-18–24 h of seeding, perform transfection following the protocol as explained under Fig. 4.

3. On day three, aspirate off overnight DMEM, gently wash with PBS, and add 500 μL trypsin. Detach cells from the plate surface by tapping it against the hood surface. Neutralize trypsin by adding 1.5 mL DMEM (*see* Note 8).

4. Prepare five 10 cm plates for different dilutions (1:400, 1:200, 1:80, 1:40, and 1:20).

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12. FSEC normally is used as a quality measure but can also be used as a quantity measure with or without combination of western blotting.

13. For obtaining the stable transfection, cells go under selection media resistance/pressure for 4–6 weeks. At the beginning of first week we notice a lot of cell death while changing the media on every alternate day; during rest of the selection pressure media is changed twice to thrice per week until resistant foci can be identified. Cell death decreases post-2–3 weeks and single colonies start appearing. Pick several foci and expand cells in 24-well plate.

14. Since our technique relies upon random integration of the gene of interest into the genome, the expression levels obtained are strongly dependent on where the transgene integrates. Selection of clonal cells is then required to identify clones with high expression that are stable under prolonged culture.

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5. Transfer 50 μL (1:400 dilution), 100 μL (1:200), 250 μL (1:80), 500 μL (1:40), and 1 mL (1:20) to the plates and add adherent DMEM media to bring the final volume to 10 mL accordingly. Incubate overnight at 37 °C in a CO_2 incubator.

6. Next day (on day 4), replace media with 10 mL of selection media containing the selection marker quantity calculated from antibiotic kill curve per 10 cm plate (*see* Note 14). Incubate overnight at 37 °C in CO_2 incubator.

7. On day 5, replace overnight media with 10 mL of selection media containing the selection marker quantity selected based upon antibiotic kill curve per 10 cm plate (*see* Note 14). Incubate overnight at 37 °C in CO_2 incubator.

8. Once the single and isolated colonies appear, mark them. Out of all the colonies from five plates, we choose only 24 individual colonies.

9. Aspirate off media from the plate and gently wash the cells with D-PBS.

10. Based on colony diameter, using a sterile forceps, pick a sterile cloning cylinder, and gently press it over the autoclaved grease for applying grease layer at the bottom of cylinder.

11. Move the greased cylinder over a colony of cells, firmly press it, add trypsin to the cloning cylinder depending upon cylinder's size, and gently pipette up and down to detach cells.

12. Transfer the detached cells to one well of a 24-well culture plate and make up volume to 1 mL with adherent DMEM medium. If required, rinse the area covered by cloning cylinder with media to collect the cells left after first transfer. Incubate cells overnight at 37 °C in CO_2 incubator.

13. Start selection post-24 h by replacing the DMEM media with selection media. Till cells reach ~80–90 % confluence stage, aspirate off old media and provide cells with fresh selection media on every alternate day.

14. First expand the colonies to 6-well plate after trypsinization with 100 μL of trypsin and post-attainment of cell confluence, expand each to 10 cm plate containing 10 mL media (*see* Note 8).

15. At 80–90 % confluence, expand cells from one 10 cm to two 15 cm plates (plate A and plate B) containing 15 mL media (*see* Note 8).

16. At this step, plate A is used to freeze cells for future use while plate B is used to compare expression level among all 24 clonal cells (*see* Note 15).

[15]On a routine basis, we perform western blotting by following standard protocols to find the best expression clones among all the 24 stable clones. We perform all the screening at the small scale by solubilizing whole cells, without preparing membranes as explained under Expression Assessment section. For detergent screening, we again take the advantage of EGFP fusion tag and employ FPLC to check membrane protein homogeneity in a particular detergent as explained under fluorescence detection size-exclusion chromatography section.

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3.8 Solubilization of Membrane Proteins

Membrane protein purification from yeast and insect cells requires membrane preparation while in our experience with HEK 293S cells whole-cell solubilization provides comparatively higher yield of pure protein and eliminates the prerequisite of membrane preparation. Based on detergent used for solubilization, cell pellets are solubilized for 1–2 h at 4 °C in solubilization buffer. Once the best expressing clone is identified (via either western blotting or FSEC), expand the particular clone from 10 cm plate to 1 L suspension culture.

3.9 Suspension Culture

Following standard protocols, thaw frozen cell vials of best expression clone and for expansion move from adherent towards suspension cultures (Fig. 6). For 1 L suspension culture, use twelve 15 cm plates at 70–50 % confluence.

1. Pre-warm 1 L suspension medium at 37 °C water bath.
2. Inside the hood, aseptically add about 800 mL pre-warmed suspension medium to the spinner flask through side arm.
3. Out of 12 plates aspirate off DMEM from four 15 cm plates at a time, gently wash with DPBS, and trypsinize cells. Add 8 mL DMEM suspension to resuspend cells very well.
4. Carefully, transfer the cells to the spinner flask via side arm and follow the same procedure for transferring cells from all twelve 15 cm plates to the same spinner flask.
5. Transfer the leftover suspension media to the flask and close the side arm lid. Post-moving the flask to 37 °C incubator, loosen both side arm lids for gas exchange.
6. Take cell count on a daily basis and once the suspension cell density reaches at ~70–80 % confluence, split cells by transferring half of the cells to the new spinner flask containing 500 mL of freshly prepared pre-warmed suspension medium. Incubate the spinner flask at 37 °C incubator.
7. Determine the total number of cells and percent viability using a hemocytometer and trypan blue exclusion on a daily basis.

For suspension culture using large-scale WAVE Bioreactor Cellbags, we commonly use 20 L wave bag for 10 L culture volume. For bringing the final volume to 10 L, pre-warm 7 L of suspension medium at 37 °C water bath, and thaw 700 mL of bovine calf serum and antibiotics accordingly.

1. Take the WAVE cellbag inside the hood and using a sterile razor remove the plastic wrapping and tighten all the inlets and outlets.
2. Aseptically, transfer suspension media to cellbag and following the manufacturer’s instructions secure the cellbag on the holder tray of a rocking unit, close off the outlet air filters, and with 10 % CO_2/air inflate the cellbag

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bioreactor. Rock at 15 rpm for 20–30 min till the bag is completely inflated. Once bag is completely inflated, open the outlet air filters and rock for another 1–2 h for the complete equilibration of pH and temperature.

3. Following the manufacturer's protocol, clamp the inlet and outlet air filters, carry cellbag to the biosafety cabinet, and inoculate with cells by aseptically pouring all the cells.

4. Bring bag back to bioreactor, secure it on holder tray, and inflate it following **step 2**. Once bag is completely inflated unclamp the outlet air filters and rock at 22 rpm.

5. On a daily basis, monitor the cell density and at ~1.0×10^6 cell count feed the cells with 20 % w/v glucose and 10 % w/v Primatone RL/UF.

6. Post-24 h of feeding, induce cells with 2 μg/mL doxycycline and 5 mM sodium butyrate.

7. Harvest cells post-36 h (or optimized time) for centrifugation for 10 min at 5000 × *g* at 4 °C (*see* Note 16).

3.10 Affinity Chromatography

Post-whole-cell lysis and protein solubilization, based on the tag choice affinity chromatography is performed (Fig. 7) (FLAG affinity purification discussed below) (*see* Note 17). All procedures should be performed on ice or at 4 °C.

1. Pre-equilibrate FLAG resin with solubilization buffer following the manufacturer's protocols and incubate for binding at 4 °C for 2–3 h.

2. Post-binding transfer the resin with supernatant to Econo-column and collect the flow-through fractions.

3. Wash the column with SEC buffer (10 column volume).

4. Elute target protein with 1 mL of 100 μg/mL FLAG peptide (in SEC buffer). Collect 10 elutes of 1 mL each and add 3 mM final concentration of TCEP, a reducing agent (or as per the manufacturer's instructions) right away.

5. For separation on SDS-PAGE, load maximal amount of sample onto an SDS-PAGE gel and run for desired amount of time for adequate separation.

6. We routinely separate the recovered material by SDS-PAGE and pool overexpressing elution fractions.

7. At this step we cleave off the tag(s) (including EGFP tag in case of EGFP constructs) with appropriate proteases, using the manufacturer's recommended concentrations or proceed for overnight dialysis in SEC buffer.

16. Post-induction harvest time should be optimized for each protein. In our experience, 36-h incubation post-induction gave the best results.
17. We normally prefer N-terminal FLAG and/or C-terminal HIS tag. Here, we explored the FLAG purification.

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3.11 SEC Chromatography

1. One can choose a column for size-exclusion chromatography based on protein size or resolution requirement. We routinely use Superdex 200 10/300 GL size-exclusion chromatography column.
2. Pre-equilibrate the column with two column volumes of degassed SEC buffer, inject the filtered sample, and collect the pool fractions of desired peak.
3. Based on protein molecular weight, use a centrifugal filtration device to concentrate a sample to the desired protein concentration, and proceed to crystallization trials.

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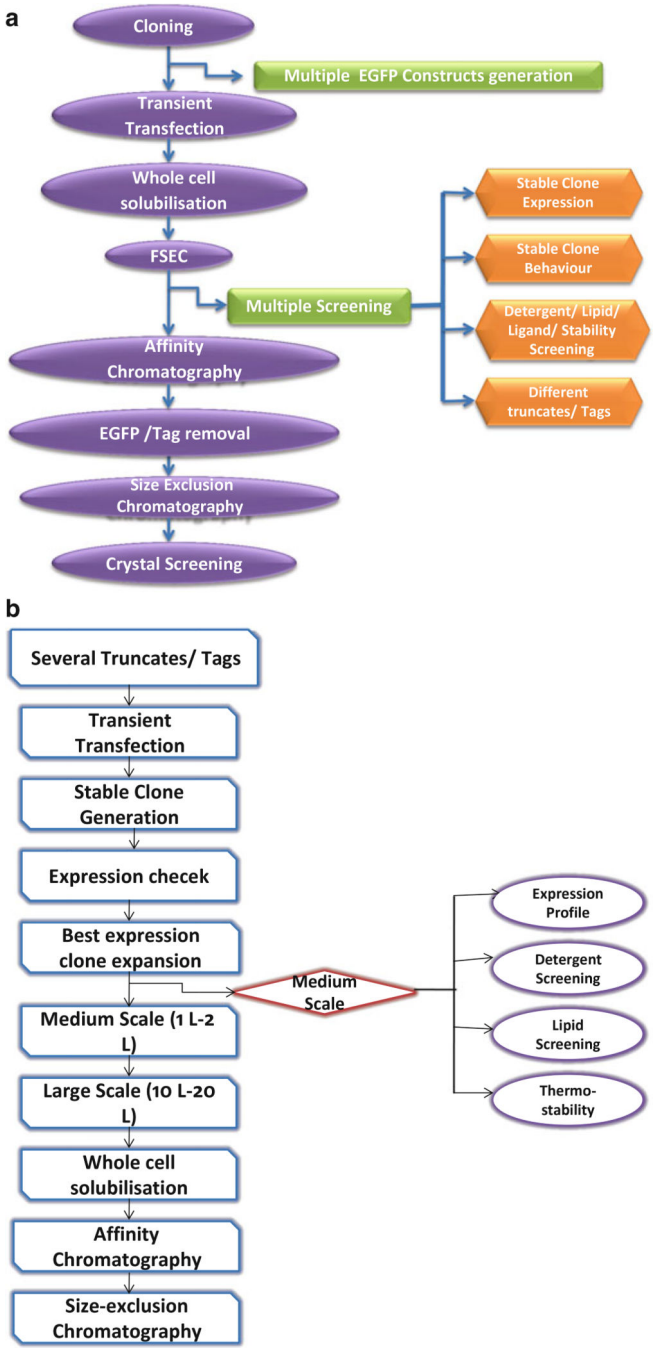

Fig. 1.

Workflow illustrating multiple construct screening for crystallization via FSEC (**a**) and stable clone generation (**b**)

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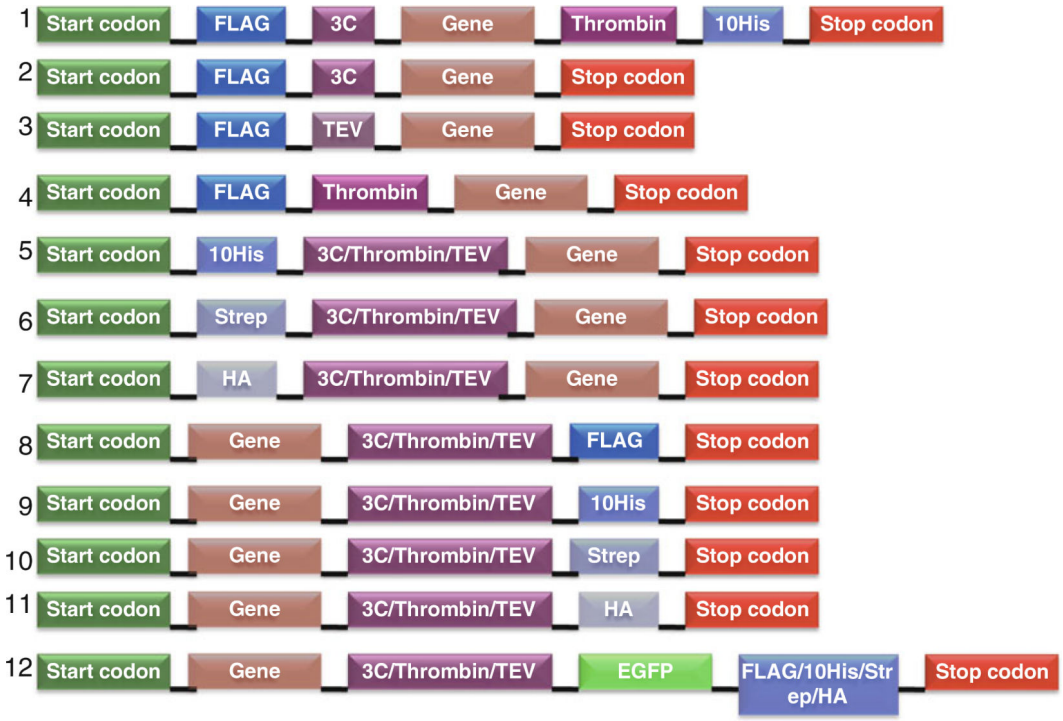

Fig. 2.

Schematic representation of various possible constructs introducing the N-terminal and C-terminal tags along with the protease cleavage site. The affinity tags and protease cleavage sites can be introduced either at the N-terminal or C-terminal of the gene. Construct with EGFP tag can also be designed in for rapid screening through FSEC and EGFP can be cleaved along with tag post-affinity purification

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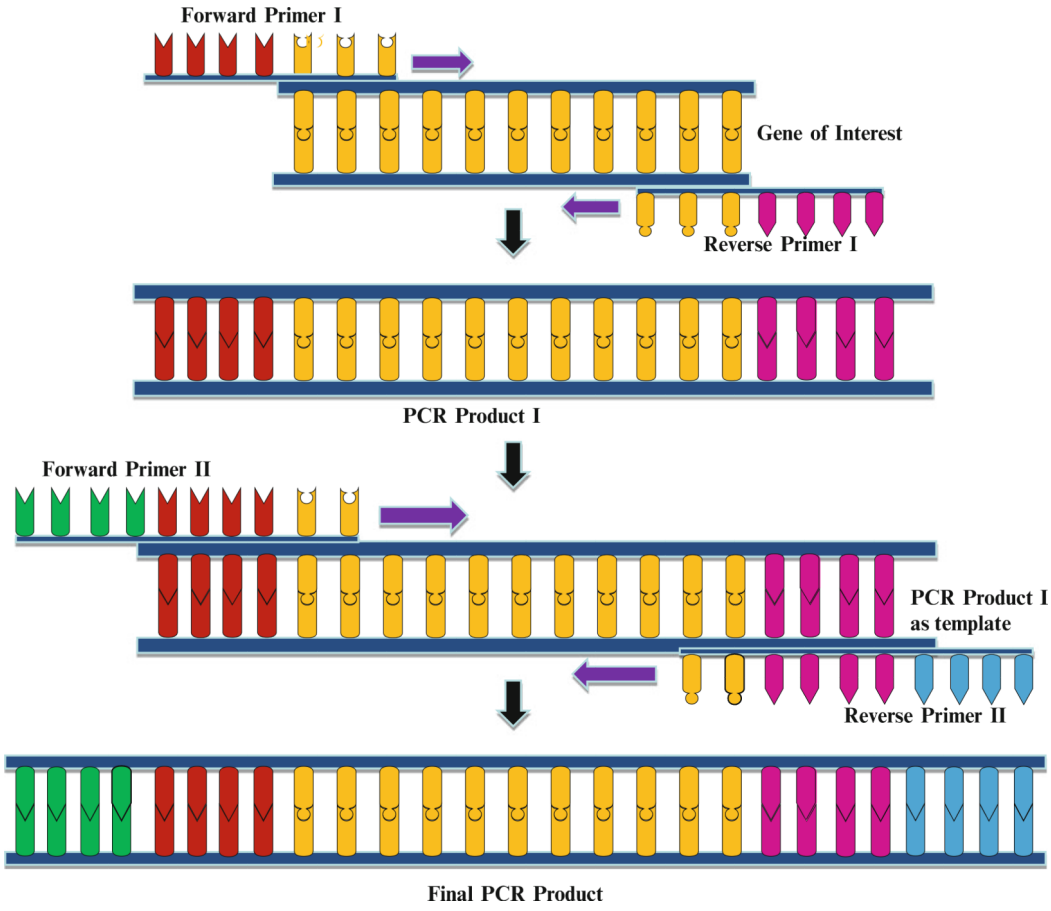

Fig. 3.

Diagrammatic representation of a two-step PCR method utilized to introduce N-terminal and C-terminal affinity tags and protease cleavage site

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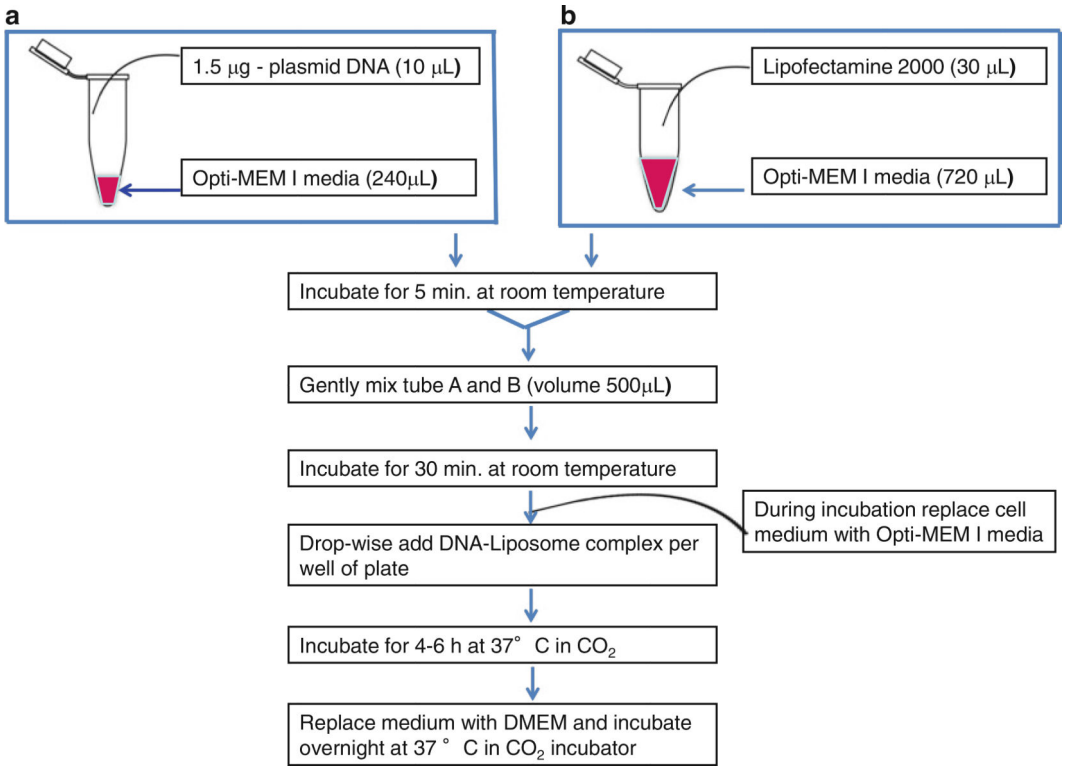

Fig. 4.
Transient transfection

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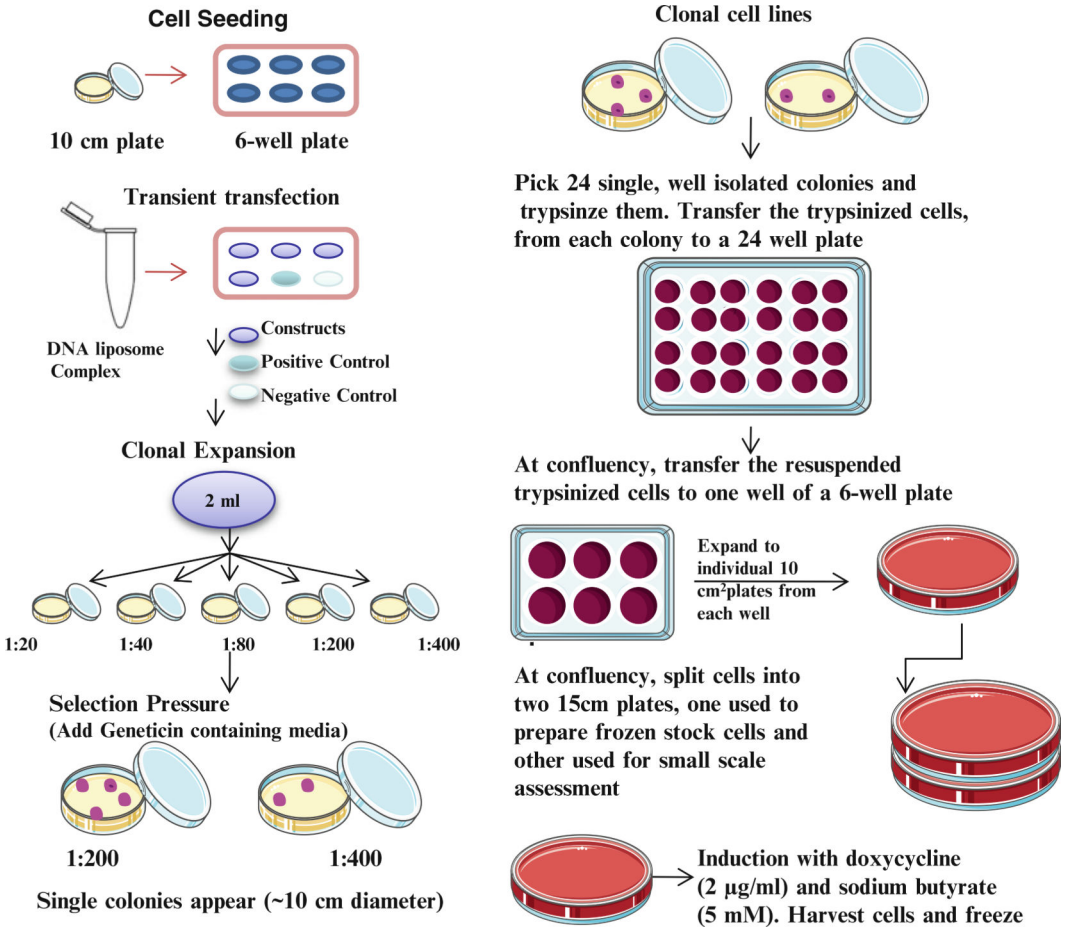

Fig. 5.

Pictorial representation of stable clone cell line generation

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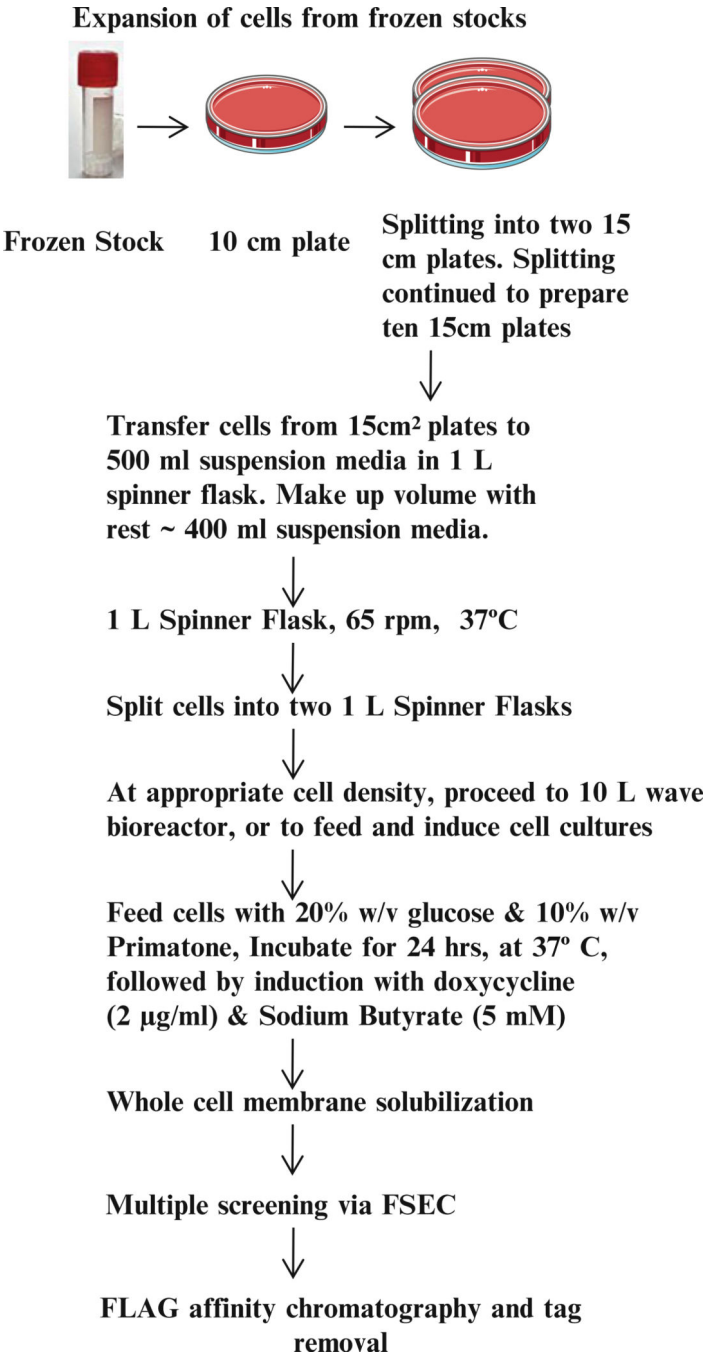

Fig. 6.
Expansion of frozen stocks to medium- and large-scale suspension culture

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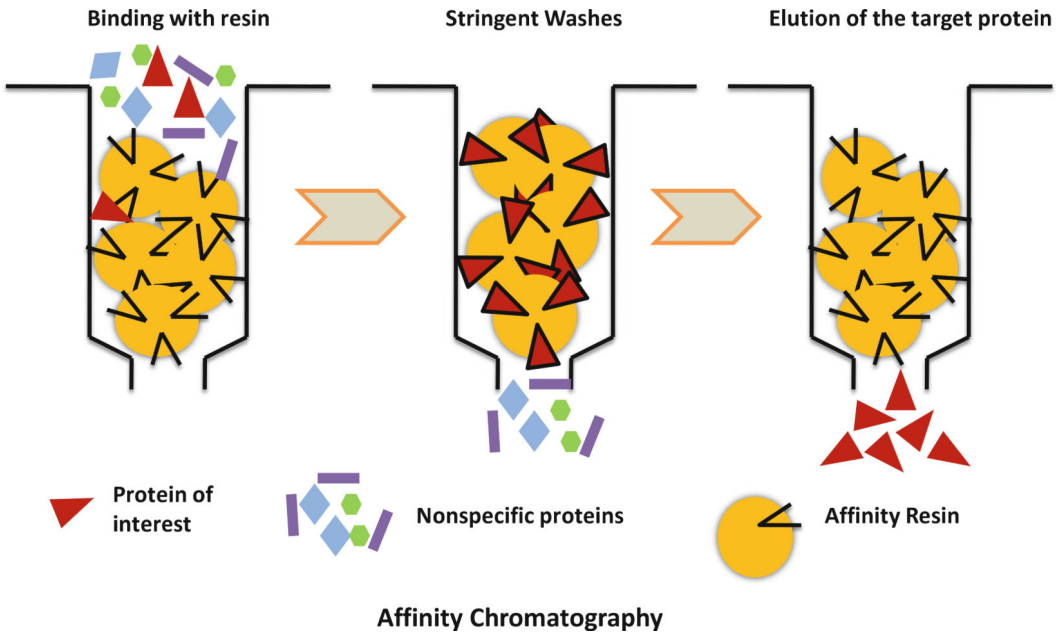

Fig. 7.

Principle behind affinity chromatography has been shown in the above figure. The first step defines the binding of protein with the resin, followed by the removal of nonspecific proteins with the help of stringent washes. Finally, the target resin-bound protein is eluted out

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Table 1

List of protein affinity tags and protease cleavage sites with their amino acid sequences, used for designing of several constructs

TAG (N)	Amino acid sequence
FLAG	Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys
10His	His-His-His-His-His-His-His-His-His-His
Strep	Trp-Ser-His-Pro-Gly-Phe-Glu--Lys
HA	Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala
Protease	Amino acid sequence
Thrombin	Leu-Val-Pro-Arg-Gly-Ser
3C	Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro
TEV	Glu-Asn-Leu-Tyr-Phe-Gln-Gly

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Table 2

Various antibiotics used for the purpose of screening for optimal antibiotic dose. Selection concentrations of these antibiotics have also been listed above

Selection marker	Formula	FW (g/mol)	Selection conc. (μg/mL)
Geneticin	$C_{20}H_{40}N_4O_{10}.2H_2SO_4$	496.6•196.1	600–800
Zeocin	$C_{55}H_{83}N_{19}O_{21}S_2Cu$	1137.41	200–400
Blasticidin S	$C_{17}H_{26}N_8O_5HCl$	458.9	5–10
Hygromycin B	$C_{20}H_{37}N_3O_{13}$	527.5	100–200