Transient transfection and expression of eukaryotic membrane proteins in Expi293F cells and their screening on a small-scale: Application for structural studies

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Abstract

Cancers, neurodegenerative and infectious diseases remain some of the leading causes of deaths worldwide [1]. The structure-guided drug design is essential to advance drug development for these important diseases. One of the key challenges in the structure determination workflow is the production of eukaryotic membrane proteins (drug targets) of high quality. A number of expression systems have been developed for the production of eukaryotic membrane proteins. In the current chapter, an optimized detailed protocol for transient transfection and expression of eukaryotic membrane proteins in Expi293F cells is presented. Testing expression and purification on a small-scale allow optimizing conditions for sample preparation for downstream structural (cryo-EM) elucidation.

Keywords:

protein purification, mammalian expression system, Expi293F cells, transient expression, membrane proteins, GPCR, detergent screen, small-scale tests, FSEC

1. Introduction

Within the last decade, there was an obvious increase in the number of deposited structures of human membrane proteins linked to developments in both, cryo-EM imaging (see for reviews [2, 3] and recombinant protein production technologies [4] (Fig. 1A).

Membrane proteins account for up to 1/3 of proteins encoded in genomes [5, 6] and for humans, their number exceeds 6000 [6]. They perform a wide array of vital functions in the cell and remain the major category of targets for approved drugs [7-9]. While being attractive as pharmaceutical targets, proteins with transmembrane segments and large hydrophobic surface remain problematic for production, extraction, and purification. The classical studies of integral membrane proteins remain laborious and involve their overexpression in heterologous systems, detergent-mediated extraction from the cell and purification.

To get good quality samples for structural studies usually, multiple screenings are required. Nowadays, the quality of the samples used in research (e.g., yield, physical characteristics, functional activity, applicability for structural studies) largely depends on parameters of the protein production system [4, 10-14] (Fig. 1 and 2). Such parameters as expression system (cell line, expression medium, expression method), the design of expression construct (fusion tag and its position, codon optimization; truncations and point mutations), and the applied conditions for protein extraction and purification (buffer additives, ligands, lipids, detergents) are all important variables.

While the major class of human membrane proteins with deposited structures (~40 %), GPCR family members, were mostly produced in insect cells with the use of viruses, members of other protein families (immune receptors, ion channels, and transporters) were preferentially produced in mammalian cells (Figure 1B). Human Embryonic Kidney (HEK293) and Chinese Hamster Ovary (CHO) cells are the most popular mammalian expression systems for the production of target proteins [15]: CHO cells remain the main cell factory to produce pharmaceutical targets [16] and HEK293 cells are largely exploited to produce targets for

research. The typical yields of human membrane proteins are in the range of 3-200 μg for every litre of HEK293 cell culture [17-19]. In a very few cases, higher yields (~0.5–1 mg/L) were reported [20, 21].

With respect to the overexpression of human membrane proteins for structural studies, human cell lines (ATCC collection contains > 2000 human cell lines and hybridomas of different cell origin, cell type and application) excels in the production of complex proteins as they provide a native microenvironment for proper folding, processing, and post-translational modifications all essential for the functional integrity of proteins. The multiple available HEK293 derived cell lines can grow in both, adherent and suspension cultures, and they display high susceptibility to both, virus transduction (baculoviruses and lentiviruses) and transient transfection achieved with the aid of chemical compounds (cationic liposomes and cationic polymers) [22-24]. Therefore, the protein production design space in HEK293 cells is immense. While baculovirus transduction of mammalian cells (BacMam) is quite an established approach (e.g., BacMam transduction of suspension-adapted HEK293S GnTI- in [25] and was used to produce numerous human membrane proteins for structural studies, the transient transfection and expression approach remains much less used (Fig. 2A).

The recent progress in the field of recombinant protein production makes HEK293 system more attractive for protein production and may facilitate the production of human membrane proteins by transient approach. As such, new-engineered cell lines (Expi293F and Expi293E) are capable to grow to higher density in suspension (healthy to up to 6x 10^6 cells/mL) and perform required post-translational modifications. To obtain the aberrant protein glycosylation phenotype the HEK293S GnTI− cell line is available [26]. Cheaper alternatives to commonly used transfection agents can be purchased (e.g., cationic polyethylenimines, PEI) [23, 27]. Large work was done on improving promoters and their regulatory elements

and in-cell fluorescence detection approach for faster and better detection of produced proteins.

Despite there is currently no universal approach appropriate for eukaryotic protein production in HEK293 cells, there are few step-by-step protocols and guides on establishing expression in HEK293 cells and performing screening for proteins with a different application for research [28-32].

In the current chapter, we detail optimized step-by-step protocol to produce full-length eukaryotic membrane proteins using transient transfection and expression in small suspension cultures of Expi293F cells for higher yields and better quality of proteins at a lower cost. The most notable advantages of Expi293F cell line are high-density growth in suspension culture, ease of cell transfection, simple scale-up, versatile protein expression and production of proteins with required post-translational modifications. To enable the production of full-length toxic to cell proteins, we modified the cell pre-treatment procedure and used conditions of low hypothermia for cell growth after transfection. In our system, in-cell detection of expressed proteins with the use of fluorescence detection techniques allows fast analysis of targets and constructs at a low scale.

The protocol was established by a case study of six eukaryotic membrane proteins (Table1). The selected targets belong to different protein families, including two members of the GPCR family, and have different subcellular localization. To test whether different choices of promoters (CAG and CMV) and fusion tags (eGFP-His and mVenus-Strep) could affect the transient expression in Expi293F cells, we used two different vector systems and three expression construct variants (Figure 3). Namely, the protocol was tested with pOPINE 3C-eGFP-His6 vector (one of the pOPIN multi-target vectors available from our lab (https://www.oppf.rc-harwell.ac.uk/OPPF/) and pHR-CMV-TetO2 vector (transfer vectors suitable for both, transient expression in HEK293 cell lines and generation of lentiviruses for

transduction-mediated expression and production of stable HEK293S GnTI- and HEK293S GnT- TetR cell lines [33]). We show that for some particular targets (e.g., *Ntsr1* GPCR target) the N-terminal fusion of protein sequence to secretion signal peptide (in this case, RPTPσ – GILPSPGMPALLSLVSLLSVLLMGCVA) can improve protein expression in Expi293F cells.

In a few recent publications, the successful use of mixed detergents for protein extraction for downstream structural analysis was reported. Namely, DDM, GDN and SDS were used for extraction of voltage-gated sodium channel Nav1.2 [34]; (ii) CHAPSO and Digitonin was used for extraction of Intracellular protease γ-secretase [35, 36]; and (iii) DDM and C12E8 was used for extraction of ABC transporter Pgp [37, 38]. In our work, we also tested a set of mixed and single detergents for protein extraction and purification and found that CYMAL-6 and mixed micelles can be a good alternative to the most commonly used DDM detergent.

2. Materials

2.1 Sub-cloning genes of interest in pOPIN vectors using In-Fusion cloning technique

1. Gene of interest (cDNA, synthetic gene or other)
2. pOPINE 3C-eGFP-His6 vector digested with PmeI/NcoI restriction enzymes
3. Primers with 15 bp extensions overlapping with in-fusion entry sites
4. Phusion® High-Fidelity PCR kit (New England BioLabs)
5. DpnI restriction enzyme
6. AMPure XB Beckman Coulter magnetic beads
7. 96-well Magnetic Separator
8. As Alternative: NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL)
9. Elution TE Buffer (10 mM Tris-Acetate pH 8.0, 1 mM EDTA)
10. Vazyme ClonExpress II One Step Cloning kit
11. 37°C incubator
12. Stellar competent cells (Takara Bio)
13. Sterile (multi-well, not TC) plates with lids
14. Autoclaved LB medium
15. 50 mg/mL sterile filtered carbenicillin stock
16. LB-agar plates supplemented with 50 µg/mL carbenicillin, 1 mM IPTG and 20 µg/mL X-gal
17. QIAGAN Miniprep kit
18. Autoclaved 100% Glycerol stock

2.2 Preparation of transfection-grade plasmids

1. QIAGEN Plasmid Plus Midi kit
2. Vacuum manifold (such as Promega Vac-Man^{TM})

3. 1.5 mL sterile eppendorf tubes
4. Micro volume spectrophotometer such as NanoDrop™

2.3 Transient transfection and expression in Expi293F cells: General procedure

1. Laminar flow hood
2. Purified plasmid DNA of interest
3. Expi293F cells (cell line Catalogue number A14527)
4. Gibco Expi293™ Express medium
5. 125 and 500/1000 mL sterile plain bottom flasks with vented closure (ThermoFisher Scientific)
6. CO_2 orbital shaker
7. Tabletop centrifuge suitable for 50 mL Falcons tubes (Sorvall Legend Rt Plus)
8. Trypan blue stain (4% solution, Gibco)
9. Countess Automated Cell Counter (Invitrogen)
10. Countess cell counting chamber slides (Invitrogen)
11. 0.22 µM syringe sterile filters (Fisher scientific)
12. Sterile filtered 1 mg/mL Polyethylenimine PEI MAX 40K (water solution, pH titrated to 7.0 with NaOH) (Polysciences Inc, 24765-1)
13. Gibco OPTI-MEM Reduced Serum medium
14. Sterile filtered stock solutions of enhancers prepared in Expi293™ Express medium: 45% glucose, 0.3 M valporic acid and 1 M sodium propionate
15. 50 mL sterile Falcon tubes
16. Automatic Pipette Filler
17. Sterile serological pipettes (1, 5, 10, 25 and 50 mL)
18. Rainin Filter Tips

2.4 Screening of expression parameters on a small scale: 1 mL/12-well plate expression

1. 12-well Tissue culture treated plates with lid (Greiner CELLSTAR®)

2.5 Up-scaled expression

1. 500/1000 mL sterile plain bottom flasks with vented closure (ThermoFisher Scientific) or 1000 mL roller bottle (BIOFILL)

2.6 Visualization of expression by fluorescence microscopy

1. EVOS fluorescent microscope with 20x objective lens and EVOS™ Light Cube for GFP detection (Excitation/Emission wavelength = 470/525 nm)
2. Compatible and calibrated for imaging TC plates (96-, 24-, 12- and 6-well plates can be used)

2.7 Analysis of expression with Tali imaging system:

1. Tali™ Image-Based Cytometer (Invitrogen)
2. Tali™ Cellular Analysis slides (Invitrogen)

2.8 Analysis of expression using In-Gel GFP fluorescence of cell probes

1. 2x Loading Dye (100 mM Tris/HCl pH 6.8, 20% glycerol (v/v), 200 mM DTT, 4% SDS, 0.2% bromphenol blue)
2. Inhibitors cocktail for mammals (P8340, Sigma)
3. DNase I (SLBW0018, Sigma)
4. Tabletop centrifuge suitable for 1.5 mL eppendorf tubes (such as Beckman Coulter Microfuge® 16)
5. Optional: Sonic bath
6. Vertical rotating platform suitable for 1.5 mL eppendorf tubes (such as HulaMixer® Sample Mixer, Invitrogen)
7. 10% Bis-Tris NuPAGE™ Midi protein Gels (1 mm, 26-well)
8. 1x NuPAGE MOPS or 1x NuPAGE MES buffer

9. Bio-Rad Precision Plus Protein™ Dual Color Marker
10. Novex BenchMark™ Fluorescent Protein Marker
11. Bio-Rad PowerPac™ Basic Power Supply
12. Bio-Rad ChemiDoc MP Imaging system with excitation (485) and emission (525) filters

2.9 Membranes preparation

1. Low spin centrifuge (such as Beckman Coulter Allegra X-15R)
2. Ice cold Buffer1 containing 20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 5% glycerol (all required Buffers are summarized in Table 2)
3. Benchtop ultrasonic disintegrator (MSE Soniprep150 Plus Ultrasonic Disintegrator)
4. Floor standing Beckman Coulter Optima L-100 XP ultracentrifuge
5. Ti 45 rotor type and compatible tubes
6. Douncer homogenizer
7. Ice cold Buffer2 containing 20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 25 mM imidazole pH 8.0

2.10 Screening of detergents for protein extraction from membranes on a small scale

1. 100-200x cmc or/and 10% detergent stocks (water solutions)
2. Benchtop Beckman Coulter MX-XP ultracentrifuge
3. TLA 55 rotor
4. Beckman Coulter microcentrifuge 1.5 mL tubes

2.11 Purification of His-tagged targets in different detergents on a small scale

1. Buffer3 containing 20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 25 mM imidazole pH 8.0, 0.05% DDM
2. Ni-NTA agarose pre-equilibrated in Buffer3

3. Washing Buffer4 containing 20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 50 mM imidazole pH 8.0, 0.05% DDM
4. Elution Buffer5 containing 20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 400 mM imidazole pH 8.0, 0.05% DDM

2.12 Purification of Strep-tagged targets in different detergents on a small scale

1. MagStrep ‘’type3’’ XT Beads 5% suspension (IBA Lifesciences)
2. Biotin
3. Buffer6 containing 100 mM Tris pH 8.0, 150 mM NaCl, 10 mM MgSO4, 0.05% DDM
4. Buffer7 containing 100 mM Tris pH 8.0, 150 mM NaCl, 10 mM MgSO4, 50 mM biotin, 0.05% DDM
5. 24-well deep well plates
6. 24-well magnetic Separator

2.13 Quality control of His- and Strep-Tag purified Samples: FSEC

1. SRT-C-300 HPLC system column (20 mL)
2. HPLC system
3. Chromacol 0.3 mL Screw Top Fixed Insert Vial (ThermoFischer, 03-FISV) and Thermo Scientific™ 9 mm Autosampler Vial Screw Thread Caps
4. Freshly filtered and degassed running Buffer8 (20 mL for each sample) containing 10 mM Tris pH 8.0, 150 mM NaCl, 0.05% DDM

2.14 Purification from small-scale expression tests (3 mL/6-well plate)

1. 6-well Tissue culture treated plates with lid (Greiner CELLSTAR®)
2. 24 Tip Horn for use with ultrasonic disintegrator (if multiple samples will be analyzed)

3. Ammonium sulfate solution saturated in 50 mM Tris/HCl pH 8.0

2.15 Large scale affinity purification

Consumables will depend on results of test expressions and analytical purifications

3. Methods

3.1 Sub-cloning genes of interest in pOPIN vectors using In-Fusion cloning technique

The full list of available multi-target pOPIN vectors is available at https://www.oppf.rc-harwell.ac.uk/OPPF/protocols/cloning.jsp. In our studies, the good level of expression of full-length targets was achieved with pOPINE 3C-eGFP-His6 vector.

1. Design pairs of primers with 15 bp extensions overlapping with in-fusion entry sites (example in Table 3).
2. Amplify target gene in 50 µL PCR reaction using recommended for DNA polymerase settings.
3. PCR reaction must be followed by 1-hour DpnI digestion.
4. Purify resulting PCR fragments using AMPure XB Beckman Coulter magnetic beads (80 µL for each PCR reaction) and elute in 20 µL of TE buffer. As alternative spin column-based purification of PCR products can be used.
5. For 10 µL of In-Fusion reaction, mix 50-100 ng (1-3 µL) of purified PCR fragments, 100 ng (1-2 µL) of PmeI/NcoI double digested pOPINE 3C-eGFP-His6 vector, 1 µL Exnase II and 2 µL optimized buffer supplied with the cloning kit.
6. Incubate reaction 25 min at 37°C and then stop immediately by adding 20 µL of ice-cold TE buffer.
7. Use 5 µL of the resulting reaction mixture to transform 20 µL Stellar competent cells using standard heat shock transformation protocol [39].

8. To clone multiple constructs prepare and use sterile 2 mL LB agar/24-well plates with lids (not tissue culture treated).
9. For X-gal blue/white screening of recombinant plasmids use LB-agar plates supplemented with 50 µg/mL carbenicillin, 1 mM IPTG and 20 µg/mL X-gal. As positive clones pick only white colonies and culture them overnight in 10 mL of LB medium freshly supplemented with 50 µg/mL carbenicillin (*see* **Note 1**).
10. Use cell pellets from overnight cultures for plasmid preparation using QIAGAN Miniprep kit.
11. Confirm obtained DNA constructs by sequencing obtained clones with T7 fwd and GFP rev primers.
12. Produce 50% glycerol stocks and use them to grow larger scale cultures for transfection-grade plasmid preparation.

3.2 Preparation of transfection-grade plasmids

1. Purify transfection-grade plasmids (0.5-1 mg) from 150 mL overnight LB culture using QIAGEN Plasmid Plus Midi kit and if required, store plasmids at -20°C in sterile 1.5 mL eppendorf tubes (*see* **Note 2**).
2. Measure the purity and concentration of obtained DNA using a NanoDrop spectrophotometer. Plasmid DNA used for transfections should be of high purity. Good quality DNA with no protein and chemical contaminations should have the ratios of absorbance 260/280 between 1.8-2.0 and 260/230 between 2.0-2.2.
3. Calculate the overall amount of the DNA required for the transfection. Use 1 µg DNA per each 1 million of transfected cells (*see* **Note 3**).

3.3 Transient transfection and expression in Expi293F cells: General procedure

1. Perform all manipulations with Expi293F cells (subculture/expand/transfect/enhance/feed) in a laminar flow hood.
2. Aspirate and dispense cells using sterile serological pipettes and Automatic Pipette Filler. Pipettes should be discarded after a single use. Avoid vigorous mixing and pipetting of cells. Use the slow dispensing mode of pipette filler for handling cells and high-speed mode for dispensing medium.
3. Record passage number of cells and determine cell viability and total cells count during maintenance culture (*see* **Note 4**).
4. To check the cell number and viability by Trypan blue exclusion, take fresh 10 µL cells aliquots and mix them with 10 µL Trypan blue stain. Apply 10 µL of the resulting mixture on the cell chamber slide. Insert chamber slide in Countess Automated Cell Counter, focus the image and run the "Count'' program. Only cells showing ≥ 95% viability can be used for further transfection.
5. Maintain the suspension culture of Expi293F cells for at least three passages after defrosting (passage numbers 3-30 can be used in experiments) in a humidified (80%) incubator with 5-8% $C0_2$ at 37°C with 120 rpm in Gibco Expi293^{TM} Express medium at a cell density between 0.5-5.0x 10^6 cells/mL. Use 125 mL flask to maintain 30 mL Expi293F cells. To up-scale the culture, use 500 mL flask to maintain 100 mL Expi293F cells and 1000 mL flask to maintain 300 mL cells.
6. One day before transfection seed Expi293F cells at a cell density of 1x 10^6 cells/mL.
7. On the day of transfection transfer cells in sterile 50 mL Falcon tubes and shortly pellet (500 *g*, 10 min, RT), discard the supernatant and re-suspend cells in fresh pre-warmed expression medium by gentle pipetting to a final density of 2.0-2.5 x 10^6 cells/mL (see Note 5).

8. In the transfection mixture, dilute each 1 µg DNA with 100 µL OPTI-MEM serum-free medium and add 8 µg polyethylenimine PEI MAX 40K. After thorough mixing, incubate the mixture 10 min at RT and add gently (dropwise) to Expi293F cells (*see* **Note 6**).
9. Place cells immediately in shaking incubator and grow at 30°C, 125-150 rpm, 5-8% CO_2, 80% humidity.
10. To boost protein expression within 20 hours of post-transfection supplement the culture with the following final concentrations of enhancers: 5 mM valporic acid, 6.5 mM sodium propionate and 0.9% glucose (*see* **Note 7**).
11. After transfection, grow cells for another 1-6 days (we recommend to grow cultures 6 days).
12. High cell viability (≥ 80%) at the end of expression (Day 6) must be observed.

3.4 Screening of expression parameters on a small scale: 1 mL/12-well plate expression

1. Run expression on a small scale (1 mL cultures in 12-well plate) to pre-screen expression conditions (e.g., two temperatures (30° and 37°C), exchange of the medium before the transfection and expression time) and expression construct variants (affinity tags and their location) (Fig. 4).
2. One day before transfection seed cells at 1x 10^6 cells/mL.
3. On the day of transfection exchange medium and adjust cell density as it is described above.
4. Plate freshly suspended cells in 12-well Tissue culture treated plates (1 mL in each well).
5. Transfect each well with 2 µg of DNA diluted in 200 µL OPTI-MEM medium and supplement with 16 µg PEI MAX 40K.

6. In case protein production is toxic to cells, fast (within 2 days) cell proteolysis and massive protein fragmentation can be observed (example in Fig. 4). These conditions must be excluded from further experiments.
7. Results of the expression tests can be analyzed using one of the cell imaging systems (EVOS microscope or/and Tali imaging cytometer) and In-Gel GFP fluorescence of probes as it is described in sections 3.6-3.8.

3.6 Up-scaled expression

1. The optimal transfection and expression conditions were determined using six target eukaryotic membrane proteins and are summarized in Table 4.
2. Grow transfected cells in 500 mL (100 mL cells) or 1000 mL (300 ml cells) sterile flask with vented closure. As an alternative, 1000 mL roller bottle can be used to grow 100-300 mL transfected cells.

3.6 Visualization of expression by fluorescence microscopy

1. Use 12-well plate from small-scale experiments or collect fresh 1 ml cell probes from up-scaled expression and pipet them in a new 12-well plate.
2. Focus image using the white and green light detection options. Capture three fluorescent images from three randomly chosen locations under a 20x objective lens of EVOS fluorescent microscope.
3. In the case of successful transfection and expression, on average, a fluorescent image will contain several hundred green cells (Fig. 5).

3.7 Analysis of expression with Tali imaging system

1. Quantify cell viability and protein expression in GFP-containing cells (% of cells expressing GFP and GFP yield) in suspension cell-based assay using Tali™ Image-Based Cytometer.

2. Pipet 20 µL of freshly taken cell probes on slides supported by Tali™ Image-Based Cytometer.
3. Image cells using ≥ 9 fields.
4. Apply the RFU threshold to quantify the number of cells expressing GFP.
5. Plot and analyze data for % of cells expressing GFP and mean RFU signals of cells (Fig. 6A and 6B).
6. To correct data for cells and media background autofluorescence, use the negative control (cells transfected with construct without reporter gene).

3.8 Analysis of expression using In-Gel GFP fluorescence of cell probes

1. Perform all work with probes on ice or in a cold room to preserve GFP fluorescence of targets.
2. Pipet 1 mL cell probes in 1.5 ml eppendorf tubes.
3. Pellet cells (12500*g*, 10 min, 4°C) and discard the supernatant media.
4. Suspend pellets in 150 µL of 2x Loading Dye freshly supplemented with DNAseI and mixture of protease inhibitors for mammals. Mix with a pipette vigorously to get a homogeneous solution.
5. Optional: In addition, sonicate probes for 10 min in a sonic bath.
6. Mix probes 20 min at the vertical rotating platform in a cold room.
7. Spin down probes (12’500 *g*, 10 min, 4°C) and load 5 µL aliquots of the supernatants containing target proteins on 10% Bis-Tris gels. Do not boil samples before loading on SDS-PAGE. As a protein standard can be used 2 µL BenchMarck Fluorescent Protein Marker or 2 µL Precision Plus Protein™ Dual Colour Marker. Run the gel at 4°C in 1x MOPS or 1x MES buffer for 3.5 hours at 90V.

8. Visualize In-Gel GFP fluorescence of target proteins using Imaging system supplied with excitation (485) and emission (525) filters (e.g., Bio-Rad ChemiDoc MP Imaging system or other) (Fig 6C and 6D).

3.9. Membranes preparation

1. Prepare membranes from≥100 mL up-scaled expression of target proteins.
2. After expression transfer cells in two sterile 50 mL Falcon tubes and collect cell pellets by short (10 min) centrifugation at 3000 *g*, 4°C.
3. Suspend obtained pellets in 20 mL of ice-cold Buffer1 containing a freshly added mixture of protease inhibitors for mammals and DNase I.
4. Break cells on ice by 5 min sonication in 50% duty cycle with 10% amplitude and sonication pulse duration of 10 sec.
5. Remove unbroken cell debris by 35 min centrifugation at 3000 *g*, 4°C.
6. Subject obtained supernatant to 2 hours ultracentrifugation at 230000 *g*, 2 hours, and 4°C.
7. Mechanically re-suspend membrane pellets in 20 mL Buffer2 with Dounce homogenizer using 10-20 passes with a pestle.
8. Use obtained membranes for (i) small scale detergent/buffer screening or (ii) directly for large-scale purification.

3.10 Screening of detergents for protein extraction from membranes on a small scale

1. Both, single detergents from different classes and mixed micelles can be used (an example of the detergent screen is provided in Fig. 7E).
2. Make sure that equal volumes of detergents are added to each probe. To do so prepare 100 µL stock solutions of detergents: (i) To compare extraction efficiencies of detergents according to their cmc values, prepare 100-200x cmc stock solutions of

detergents of choice; (ii) To compare extraction efficiencies of 1% detergents, prepare 10% stock solutions of detergents to be to tested.

3. For each detergent probe, mix 0.9 mL of suspended membranes with 100 µL of prepared detergent stock.
4. Solubilise probes 1 hour on the vertically rotating platform in a cold room.
5. Transfer probes in 1.5 mL microcentrifuge tubes compatible with benchtop ultracentrifuge rotor.
6. Clear solubilizate by 1-hour centrifugation at 130000 *g*, 4°C in a benchtop ultracentrifuge.
7. From the obtained supernatant, load 10 µL of each probe on a gel.
8. Evaluate the efficiency of protein extraction from membranes based on the intensity of In-Gel GFP fluorescence signals of protein bands (Fig. 6) using the Imaging system supplied with excitation (485) and emission (525) filters (Fig. 7).
9. Use imager integrated software to measure the area of GFP-fused protein bands in each sample lane of the gel. Plot data and compare extraction efficiencies of different detergents (Fig. 7D).
10. In our screen, we compared the extraction efficiency of the most commonly used detergent, DDM, to several single and mixed detergents (Fig. 7D).

3.11 Purification of His-tagged targets from membranes in different detergents on a small-scale

1. Pre-equilibrate Ni-NTA agarose (100 µL resin for each probe) in Buffer3.
2. Apply solubilisate from the previous step on Ni-NTA agarose.
3. Bind proteins O/N at 4°C using a vertical rotating platform.
4. Do all subsequent purification steps in a batch mode on ice or in a cold room.
5. After binding sediment resin by gravity flow and discard the supernatant.

6. Wash resin 3 times in 1 mL Buffer3 and one time in 1 mL Buffer4.
7. For elution, add 150 µL of Buffer5 and incubate resin with gentle agitation 1 hour before collecting elution.
8. Load purified samples on (i) NuPAGE to assess protein purity and on (ii) FSEC column to assess homogeneity.
9. With the best detergent do large-scale extraction and purification.

3.12 Purification of Strep-tagged targets from membranes in different detergents on a small-scale

1. Aspirate required volume of MgStrep beads suspension (100 µL 5% suspension = 10 µL beads and is used for each 1 mL probe).
2. Separate MgStrep beads on Magnetic Separator and discard storage solution.
3. Equilibrate MgStrep beads suspension in 1 mL of Buffer6.
4. Apply solubilizate from the previous step on pre-equilibrated MgStrep beads. For more than 3 samples use 24-well deep-well block.
5. Shake plate at 400 rpm for ≥ 2 hours. For better results, leave suspension for overnight binding in a cold room.
6. Place a plate on Magnetic Separator and discard the supernatant. Rinse beads two times in 1 mL of Buffer6.
7. For elution apply 60 µL of Buffer7 and shake plate 1 hour before collecting the samples.
10. Load purified samples on (i) NuPAGE to assess protein purity and on (ii) FSEC column to assess homogeneity.
8. With the best detergent do large-scale extraction and purification.

3.13 Quality control of His- and Strep-tag purified Samples: FSEC

1. Monitor the monodispersity and stability of the purified target proteins in different detergents/buffers using FSEC (Fig. 8).
2. Centrifuge His-tag or Strep-tag purified samples (12500 *g*, 5 min, 4°C) and transfer 20-110 µL probes in 0.3 mL insert vials with a rubber closure. The injection of samples (10-100 µL) on SRT-C-300 HPLC system column (20 mL) can be done automatically using a high-throughput auto-sampler.
3. Run samples in Buffer8 at 0.5 mL/min flowrate.
4. Record both, GFP and tryptophan fluorescence.
5. Analyze FSEC traces in terms of peak area, elution profile and volume to get information on (i) expression level, (ii) the degree of monodispersity, and (iii) the approximate molecular mass.
6. Monodisperse and folded proteins will yield a single symmetrical peak and polydisperse, unstable, or unfolded proteins will yield multiple asymmetric peaks.

3.14 Purification from small-scale expression tests (3 mL/6-well plate)

1. To test protein construct variants (e.g., His, Strep or other tags fusions) run small-scale purifications using test plate expressions.
2. Expression volumes such as 3 mL (6-well plate experiment) can be used.
3. Spin down cells in 15 mL Falcon tubes (3000*g*, 10 min, 4°C).
4. Discard the supernatant and suspend cell pellets in 2 mL of ice-cold Buffer2 (suitable for His-tagged targets) or Buffer6 (suitable for Strep-tagged targets).
5. Transfer cell suspensions in 24-well deep-well block.
6. Use 24 Tip Horn for the sonicator to process numerous samples simultaneously.
7. Supplement broken cells with 1% DDM (or any other detergent of choice) and solubilise 1 hour at a vertical rotator in a cold room.
8. Run short centrifugation (12500g, 10 min, 4°C).

9. **Optional:** To concentrate sample for SDS-PAGE analysis and to reduce the detergent concentration before affinity purification, do short Ammonium Sulphate precipitation of probes. To do so, (i) collect supernatant after low spin centrifugation and measure its precise volume, (ii) add slowly an equal volume of saturated Ammonium Sulphate solution and mix 2-3 minutes at RT, (iii) for better precipitation leave on the bench for another 5 min; (iv) spin down 20 min at 12'500g. Pellet will contain target protein and can be re-suspended in ≥ 150 µL of Buffer3 or Buffer6.
10. Load sample aliquots from steps 7 and 8 on SDS-PAGE and analyse in-gel GFP fluorescence signals after run is completed (Fig. 9).
11. With remaining supernatant do purifications using magnetic beads as it is described above in sections 3.11 and 3.12.

3.15 Large-scale affinity purification

1. Use the best detergent to run large-scale extraction.
2. Large-scale purification must include overnight on-column removal of recombinants fusion and reverse IMAC.

4. Notes

1. Multi target pOPIN vectors carry lacZ gene upstream of the reading frame allowing blue-white selection in *E. coli*.
2. A suitable mammalian expression vector with an appropriate expression promoter and translational signal (minimal (ACCATG) or full (GCCACCATG) Kozak consensus sequence) should be used for this protocol.
3. Our protocol is suitable for any scale of expression: 1-3 mL plate experiments and 30-300 mL up-scaled expression in flasks. Scale provided volumes and quantities of reagents proportionally to the used volume of transfected cells.
4. Do not use high-density cells (>5-6x 10^6) for routing sub-culturing as it may reduce protein titer.
5. Higher starting cell density is essential, as at 30°C the proliferation of cells will be reduced.
6. Do not mix DNA and PEI directly as they will precipitate immediately.
7. Valporic acid and sodium propionate are known as histone deacetylase inhibitors (iHDACs) [40, 41] and are used to cope with transcriptional repression of transfected plasmids. The use of valporic acid, sodium propionate and glucose feed in combination helps substantially enhance gene expression.

Acknowledgements

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Figure legends

Fig. 1 Annual deposition of the human membrane protein structures 1997-2019 and expression hosts used for protein production.

(A) A number of human membrane protein (MPs) structures released annually (state on 5.01.2020). In the last two years, more than 120 structures of human MPs were deposited to the Protein Data Bank (PDB) archive. (B) Expression hosts used to produce human MPs for downstream structural analysis. The majority of targets were produced in insect and HEK293 expression systems. Up to now, a high prevalence of using insect cells system to express GPCR family members is observed.

Fig. 2 Approaches used for the production of human MPs in HEK293 derived cell lines and protein purification strategies.

Within the last decade (2010-2019) numerous human MPs were successfully produced in HEK293 cells, purified and used for atomic-level structural determination (over 75 structures are currently deposited in PDB archive). The protein production and extraction strategies for individual proteins within this group differ. (A) Statistics on HEK293 derived cell lines and DNA delivery systems used for protein production. The HEK293S GnTI- cell line is the most commonly used host cell line and BacMam system-based transduction of HEK293 cells remains prevalent in human MPs production pipelines. (B) Detergents used for the extraction of human MPs. At the initial step of protein solubilization, when protein extraction in the

native oligomeric and functional state is essential, in most cases detergents of choice (DDM, LMNG, MNG-3, Digitonin) were supplemented with cholesteryl hemisuccinate (CHS) to increase protein stability in solution. The use of single detergents (DDM, Digitonin, CHAPSO, GDN, MNG-3, DMNG, OG and Triton X-100) or mixed micelles also leads to the extraction of multiple stable proteins. (C) Statistics on affinity tags used to purify human MPs. Different single and tandem tags were used for the purification of target proteins. To enable in-cell detection of expression and for further purification, numerous protein targets were expressed fused to fluorescent proteins (FP).

Fig. 3 Overview of used expression constructs.

Three different C-terminal protein construct variants were used for expression and purification of selected targets. In all cloned constructs, the gene of interest (GOI) was fused to a fluorescent protein (eGFP or mVenus) and affinity tag (His6 or Twin Strep) through cleavable linkage. With one of the constructs (N3), the effect of the signal sequence (RPTPσ) on the expression of membrane proteins was assessed.

Fig. 4 Optimization of the transient expression of targets on a small-scale

Miniaturizing test expressions using 1 mL/12-well plate allows parallel processing of greater breadth of variables. (A) The in-gel fluorescent protein signals at 48 hours post-transfection. Under conditions of mild hypothermia (30°C), the quality of expressed proteins improves for most of the targets (no fragmentation). (B) Representative anti-His western blot of *SLC10A1* target expressed at two different temperatures. (C) Comparison of in-cell GFP yields of targets produced in old and freshly exchanged expression medium. A full exchange of the medium shortly before transfection increases protein yield at both temperatures, 37 and 30°C,

and allows express proteins longer (summarized in (D)). (E) The in-gel fluorescent protein signals obtained for three different construct variants expressed under optimal conditions (30°C, fresh expression medium, and 6 days). All targets fused to GFP-His and some of the targets fused to mVenus-Strep are amenable to expression in Expi293F cells. (F) In-cell fluorescence signals indicate higher versatility of GFP-His tag and better expression of targets fused to GFP-His (summarized in (G)).

Fig. 5 Visualization of GFP-fused protein expression

In-cell GFP fluorescence signals images of targets expressed in Expi293F cells at 30°C at different time points captured with EVOS fluorescent microscope (scale bar, 125 μm). Already at 24 hours post-transfection, the GFP signals for target proteins are detected. With enhancers and after longer expression (2-6 Days in total) the progress in GFP fluorescence is observed.

Fig. 6 Analysis of GFP-fused protein expression

Left panel: Quantification of GFP fluorescence in living cells using Tali imaging system. (A) Progression of the number of cells expressing GFP-fused targets and (B) GFP yield over days are shown. The highest GFP signals are observed at the end of expression (day 6 after transfection). Right panel: Checking GFP fluorescence of target proteins after 6 days of expression. (C) GFP fluorescence of harvested cell pellets. (D) GFP fluorescence of treated cell probes before and after SDS-PAGE analysis.

Fig. 7 Screening of detergents for protein solubilisation and purification

(A) In-Gel GFP fluorescence signals of target proteins solubilised in different detergents (20x cmc). (B) In-Gel GFP fluorescence signals and (C) Coomassie-stained protein bands of target proteins purified in different detergents via Ni-NTA beads. (D) Plotted GFP intensities of targets extracted in different detergents. Despite CYMAL-6 did not provide the best solubilisation for most of the protein targets, CYMAL-6 was the only detergent that extracted all targets with good efficiency. Mixed micelles provided very good solubilisation for most of the non GPCR targets. Most of the detergents that provided good solubilisation for targets, were also good for protein purification via Ni-NTA. (E) Example of detergent screen composition used in our studies.

Fig. 8 FSEC analysis of protein stability in different detergents

(A) FSEC analysis of *SLC6A1* target purified in different detergents via Ni-NTA beads. Few detergents and detergent mixtures from our list provide good extraction and maintain protein stable across purification, including commonly used for membrane protein DDM + Digitonin mixture. (B) Comparison of intensities of probes obtained in different detergents. (C) Representative In-gel fluorescence and Coomassie staining of *SCL6A1* samples purified via Ni-NTA and Strep Tactin beads indicate the high quality of samples at the end of the purification. (D) According to FSEC traces, both constructs (GFP-His and mVenus-Strep C-terminal fusions) are stable dimers at the end of the purification. (E) The yield of GFP-His and mVenus-Strep fused target differs: While GFP-His fused target is better expressed and more protein is obtained at the end of the purification, the mVenus-Strep fusion provides higher specificity for binding.

Fig. 9 Small-scale analytical protein purification

In-gel GFP fluorescence signals and Coomassie staining of samples purified using cell pellets from 3 mL/6-well plate test expressions. In this set of experiments, broken cells were solubilised in DDM+LDAO mixture, precipitated with 50% AS and re-suspended material was used for affinity purification on MagStrep beads. Full-length proteins were detected for all three human MPs targets: T4 (*SLC6A1*), T5 (*SLC35D1*) and T6 (*SLC35D2*).

Tables

Gene Name	**Localization in cell**	**Source**	**TM**	**kDa**
Ntsr1	Plasma membrane	*Rattus norvegicus*	7	47
SLC10A1	Plasma membrane	*Bos taurus*	9	41
ADORA2A	Plasma membrane	*Homo sapiens*	7	45
SLC6A1	Plasma membrane	*Homo sapiens*	12	67
SLC35D1	ER membrane	*Homo sapiens*	8	39
SLC35D2	Golgi apparatus membrane	*Homo sapiens*	10	37

Table 1: Overview of tested eukaryotic membrane proteins.

NN	Composition
Buffer1	20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 5% glycerol
Buffer2	20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 25 mM imidazole pH 8.0
Buffer3	20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 25 mM imidazole pH 8.0, 0.05% DDM
Buffer4	20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 50 mM imidazole pH 8.0, 0.05% DDM
Buffer5	20 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM MgS04, 400 mM imidazole pH 8.0, 0.05% DDM
Buffer6	100 mM Tris pH 8.0, 150 mM NaCl, 10 mM MgSO4, 0.05% DDM
Buffer7	100 mM Tris pH 8.0, 150 mM NaCl, 10 mM MgSO4, 50 mM biotin, 0.05% DDM
Buffer8	10 mM Tris pH 8.0, 150 mM NaCl, 0.05% DDM

Table 2: Composition of Buffers.

Gene Name	Oligonucleotide Sequence 5'→3' (fwd primer)	Oligonucleotide Sequence 5'→3' (rev primer)
Ntsr1	AGGAGATATACCATGCACCTCAA CAGCTCCGTGC	CAGAACTTCCAGTTTAGGACAAA GGCAGGCCAGCG
SLC10 A1	AGGAGATATACCATGGAGGCCTT CAACGAATCTTCC	CAGAACTTCCAGTTTGTTTGCCAT GTTGAGTTGCTC
ADOR A2A	AGGAGATATACCATGCCCATCAT GGGCTCCTCG	CAGAACTTCCAGTTTGTCCGTGG CGTAGGTCTGG
SLC6A 1	AGGAGATATACCATGGCGACCAA CGGCAGCAA	CAGAACTTCCAGTTTGATGTAGG CCTCCTTGCTGG
SLC35 D1	AGGAGATATACCATGGCGGAAGT TCATAGACG	CAGAACTTCCAGTTTCAACACTG CTCCTTTCCCCT
SLC35 D2	AGGAGATATACCATGACGGCCGG CGGCCAGGC	CAGAACTTCCAGTTTGCTCTTCA AATCCAAACAGA

Table 3: Primers for In-Fusion cloning of targets in PmeI/NcoI restriction sites of pOPINE 3C-eGFP-His6 vector

Step	Parameter	Recommendation	
I. Transfection **(10 min)**	**Cell volume**	100 mL	300 mL
	Amount of DNA	200-250 µg	600-750 µg
	Transfection agent	PEI MAX 40K	
	Transfection medium	OPTI-MEM Serum Reduced	
	Duration	10 min	
	Temperature	RT	
II. Expression **(3-6 Days)**	**Starting cell density**	2.0-2.5x 10^6	
	Cells Viability	≥ 95%	
	Expression medium	Freshly Exchanged Gibco Expi293™	
	Flask type	Vented, 500 mL	Vented, 1000 mL
	Temperature	30°C	
	Shake speed	125 rpm	150 rpm
	Duration	3-6 Days	
	Detection	(GFP)-fluorescence	
III. Supplements	**Time of addition**	≤ 20 hours post-transfection	
	Enhancer1 (Valproic acid)	1.7 mL	5.0 mL
	Enhancer2 (Sodium propionate)	0.65 mL	2.0 mL

	Feed (Glucose)	2.0 mL	5.5 mL

Table 4: Optimized conditions for up-scaled transient expression of targets in Expi293F cells

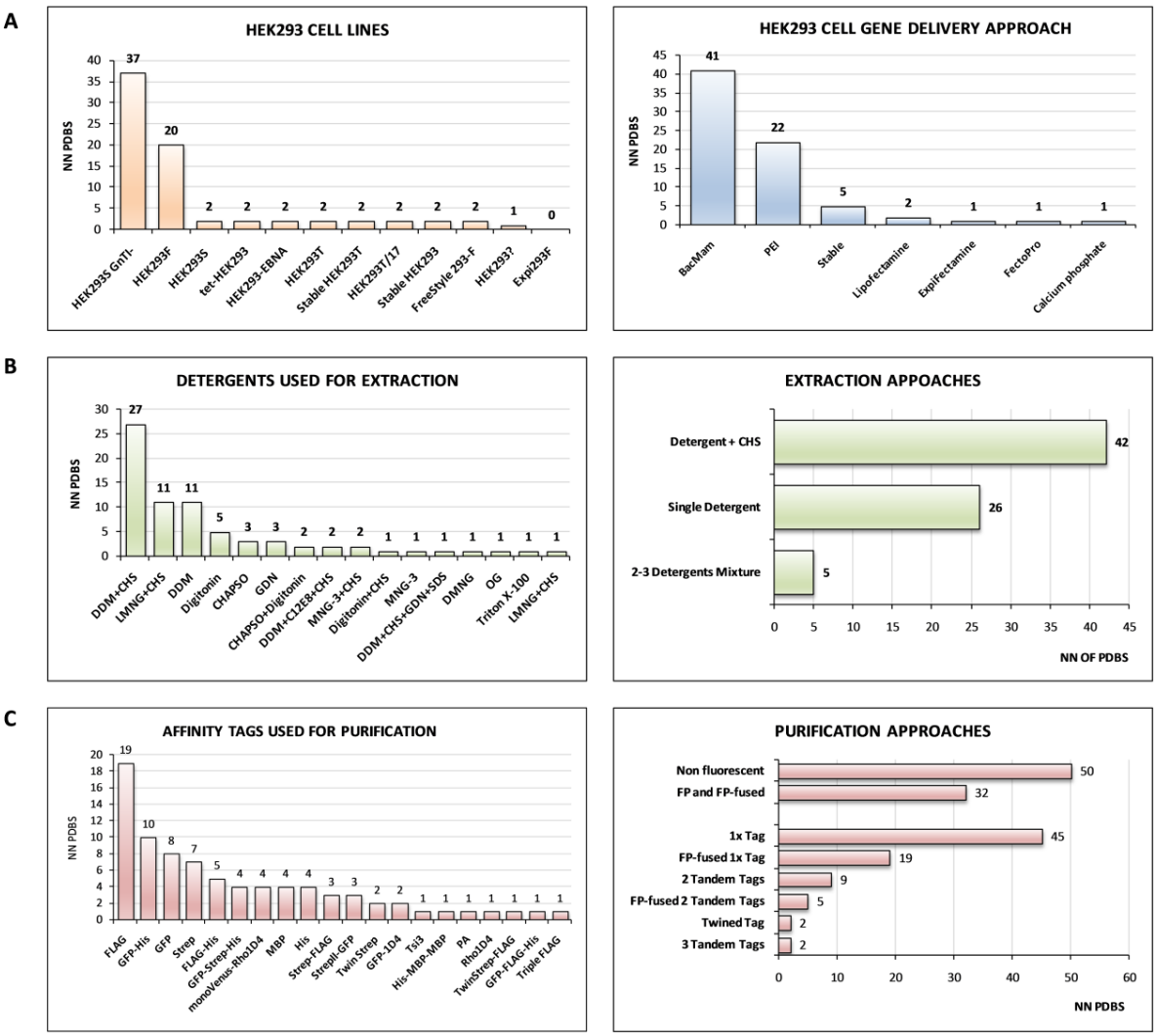
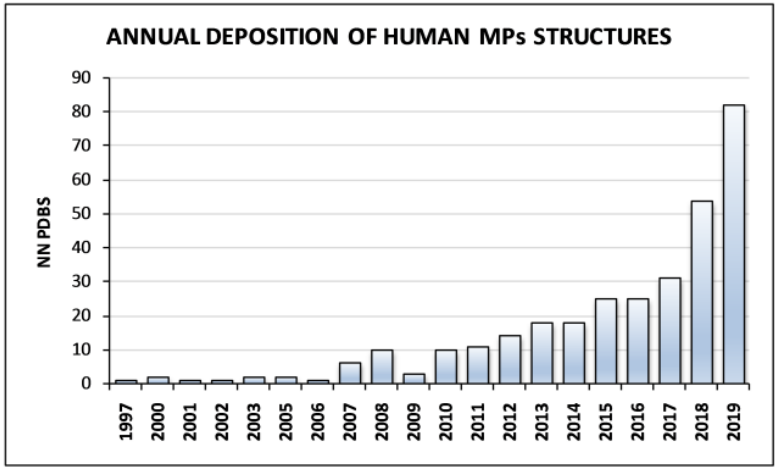
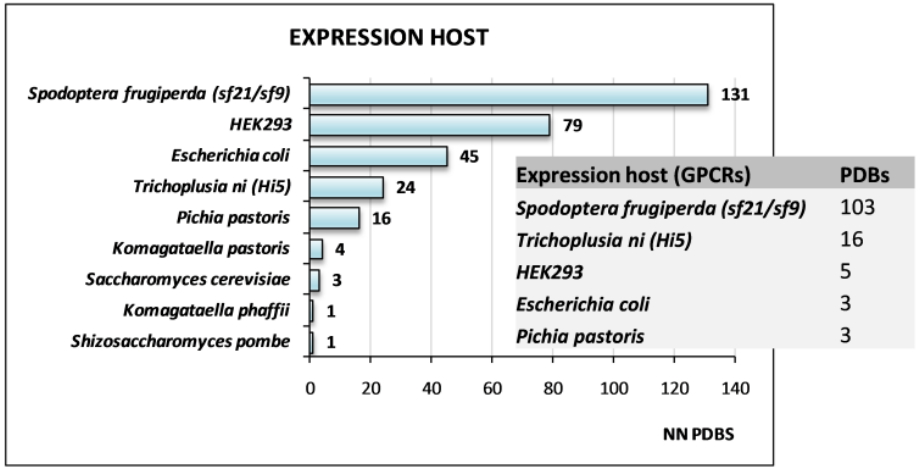

A
HEK293 CELL LINES
NN PDBS
HEK293S GnTI-
HEK293F
HEK293S
tet-HEK293
HEK293-EBNA
HEK293T
Stable HEK293T
HEK293T/17
Stable HEK293
FreeStyle 293-F
HEK293?
Expi293F
HEK293 CELL GENE DELIVERY APPROACH
BacMam
PEI
Stable
Lipofectamine
ExpiFectamine
FectoPro
Calcium phosphate
B
DETERGENTS USED FOR EXTRACTION
DDM+CHS
LMNG+CHS
DDM
Digitonin
CHAPSO
GDN
CHAPSO+Digitonin
DDM+C12E8+CHS
MNG-3+CHS
Digitonin+CHS
MNG-3
DDM+CHS+GDN+SDS
DMNG
OG
Triton X-100
LMNG+CHS
EXTRACTION APPOACHES
Detergent + CHS
Single Detergent
2-3 Detergents Mixture
NN OF PDBS
C
AFFINITY TAGS USED FOR PURIFICATION
FLAG
GFP-His
GFP
Strep
FLAG-His
GFP-Strep-His
monoVenus-Rho1D4
MBP
His
Strep-FLAG
Strep-GFP
Twin-Strep
GFP-1D4
Tsi3
His-MBP-MBP
PA
Rho1D4
TwinStrep-FLAG
GFP-FLAG-His
Triple FLAG
PURIFICATION APPROACHES
Non fluorescent
FP and FP-fused
1x Tag
FP-fused 1x Tag
2 Tandem Tags
FP-fused 2 Tandem Tags
Twined Tag
3 Tandem Tags
NN PDBS

Fig.1

A


ANNUAL DEPOSITION OF HUMAN MPs STRUCTURES
NN PDBS
0
10
20
30
40
50
60
70
80
90
1997
2000
2001
2002
2003
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019

B


EXPRESSION HOST
Spodoptera frugiperda (sf21/sf9)
HEK293
Escherichia coli
Trichoplusia ni (Hi5)
Pichia pastoris
Komagataella pastoris
Saccharomyces cerevisiae
Komagataella phaffii
Shizosaccharomyces pombe
131
79
45
24
16
4
3
1
1
0
20
40
60
80
100
120
140
NN PDBS
Expression host (GPCRs) PDBs
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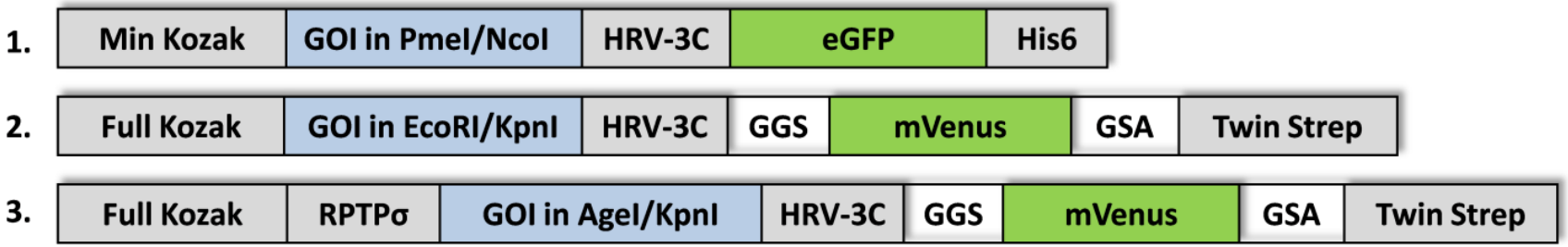
Trichoplusia ni (Hi5) 16
HEK293 5
Escherichia coli 3
Pichia pastoris 3

Fig. 2


1.
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GOI in PmeI/NcoI
HRV-3C
eGFP
His6
2.
Full Kozak
GOI in EcoRI/KpnI
HRV-3C
GGS
mVenus
GSA
Twin Strep
3.
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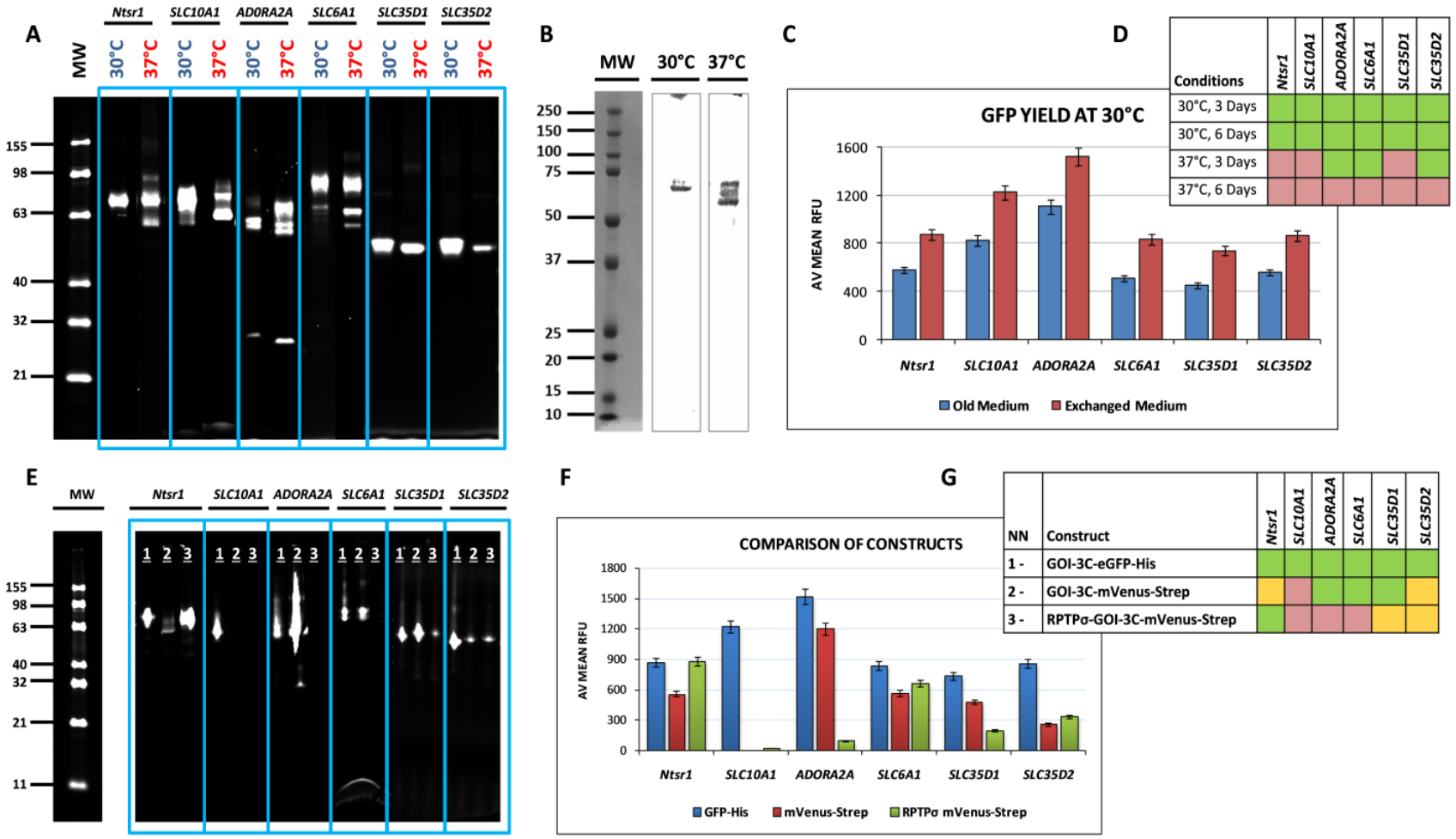
RPTPσ
GOI in AgeI/KpnI
HRV-3C
GGS
mVenus
GSA
Twin Strep

Fig. 3


A
Ntsr1
SLC10A1
ADORA2A
SLC6A1
SLC35D1
SLC35D2
MW
30°C
37°C
155
98
63
40
32
21
B
MW
30°C
37°C
250
150
100
75
50
37
25
20
15
10
C
GFP YIELD AT 30°C
AV MEAN RFU
1600
1200
800
400
0
Ntsr1
SLC10A1
ADORA2A
SLC6A1
SLC35D1
SLC35D2
Old Medium
Exchanged Medium
D
Conditions
Ntsr1
SLC10A1
ADORA2A
SLC6A1
SLC35D1
SLC35D2
30°C, 3 Days
30°C, 6 Days
37°C, 3 Days
37°C, 6 Days
E
MW
Ntsr1
SLC10A1
ADORA2A
SLC6A1
SLC35D1
SLC35D2
1 2 3
155
98
63
40
32
21
11
F
COMPARISON OF CONSTRUCTS
AV MEAN RFU
1800
1500
1200
900
600
300
0
Ntsr1
SLC10A1
ADORA2A
SLC6A1
SLC35D1
SLC35D2
GFP-His
mVenus-Strep
RPTPσ mVenus-Strep
G
NN
Construct
Ntsr1
SLC10A1
ADORA2A
SLC6A1
SLC35D1
SLC35D2
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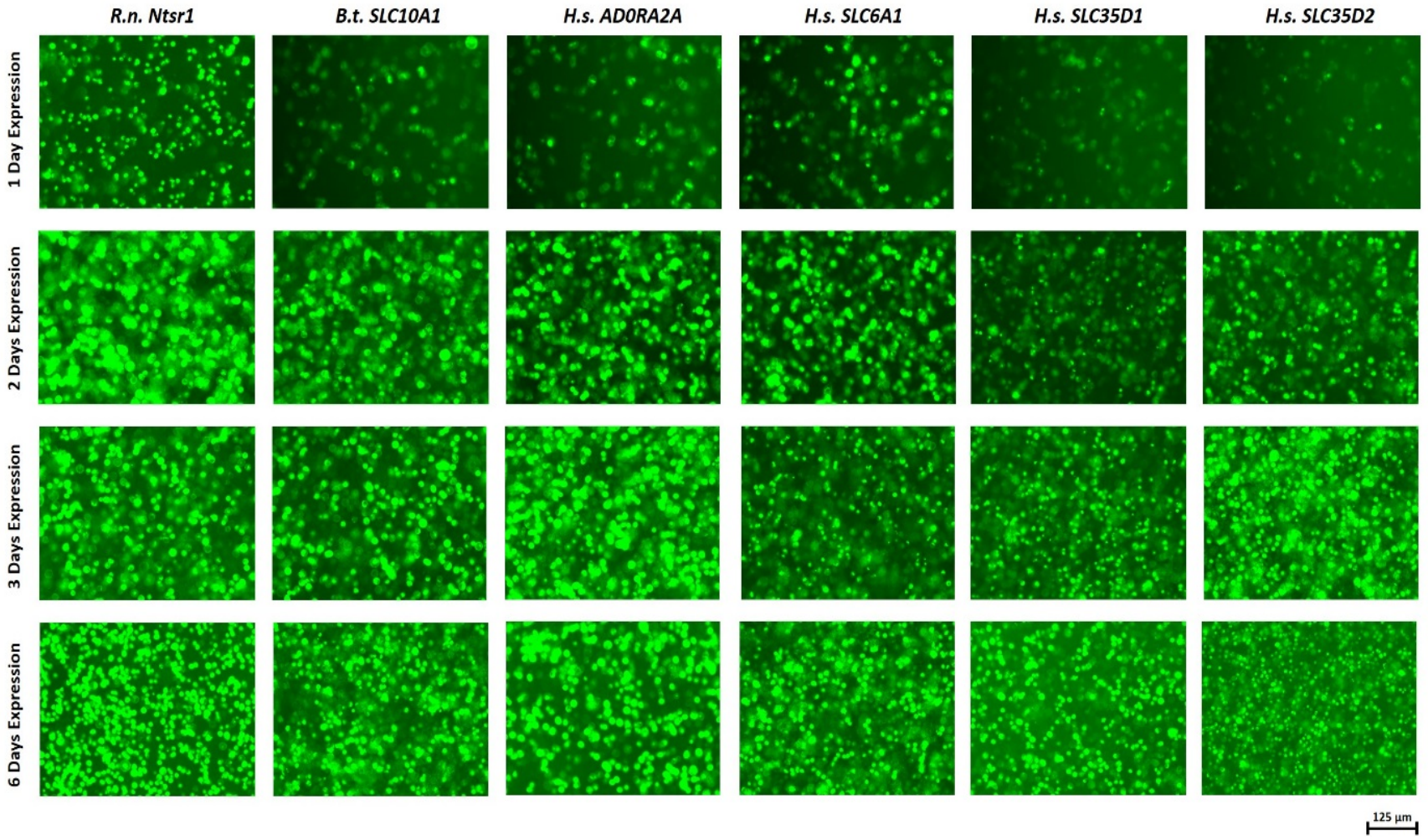
2 -
GOI-3C-mVenus-Strep
3 -
RPTPσ-GOI-3C-mVenus-Strep

Fig. 4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B.t. SLC10A1
H.s. AD0RA2A
H.s. SLC6A1
H.s. SLC35D1
H.s. SLC35D2
1 Day Expression
2 Days Expression
3 Days Expression
6 Days Expression
125 µm

Fig. 5

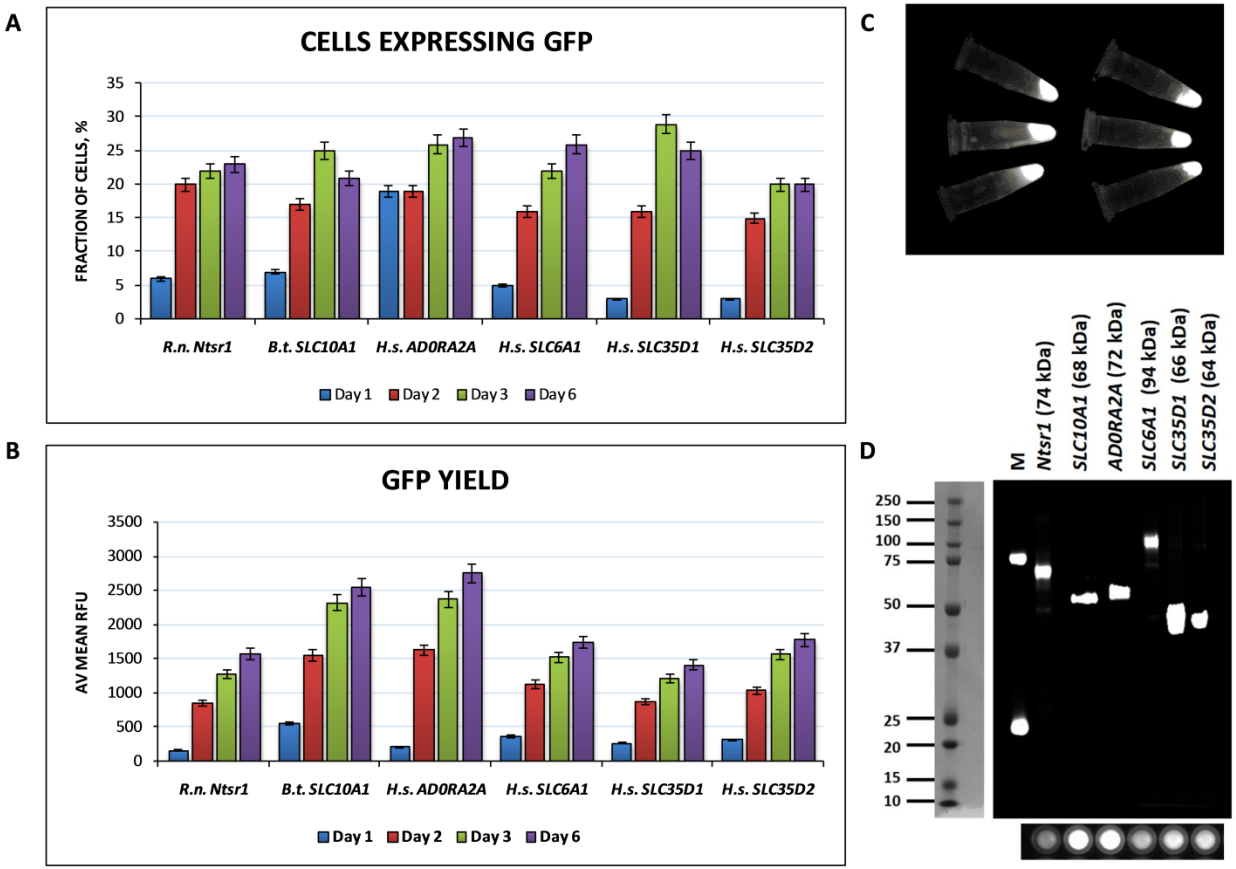
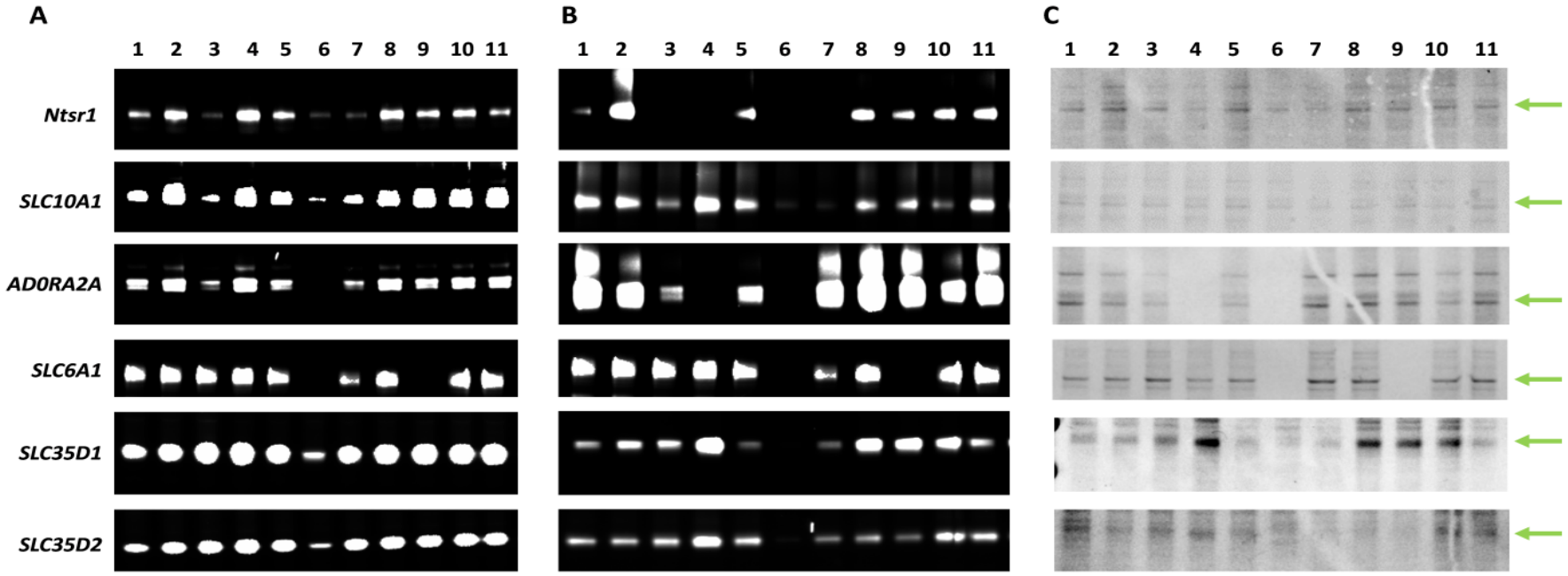
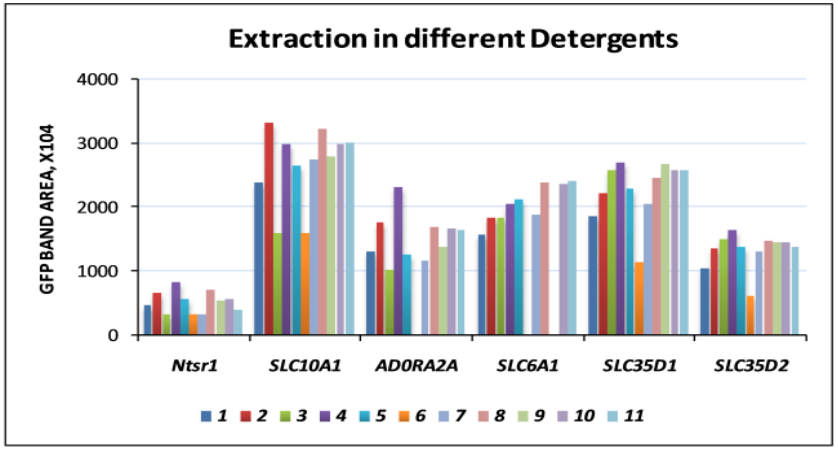

A
CELLS EXPRESSING GFP
FRACTION OF CELLS, %
35
30
25
20
15
10
5
0
R.n. Ntsr1
B.t. SLC10A1
H.s. AD0RA2A
H.s. SLC6A1
H.s. SLC35D1
H.s. SLC35D2
Day 1
Day 2
Day 3
Day 6
B
GFP YIELD
AV MEAN RFU
3500
3000
2500
2000
1500
1000
500
0
R.n. Ntsr1
B.t. SLC10A1
H.s. AD0RA2A
H.s. SLC6A1
H.s. SLC35D1
H.s. SLC35D2
Day 1
Day 2
Day 3
Day 6
C
D
M
Ntsr1 (74 kDa)
SLC10A1 (68 kDa)
AD0RA2A (72 kDa)
SLC6A1 (94 kDa)
SLC35D1 (66 kDa)
SLC35D2 (64 kDa)
250
150
100
75
50
37
25
20
15
10

Fig. 6



D

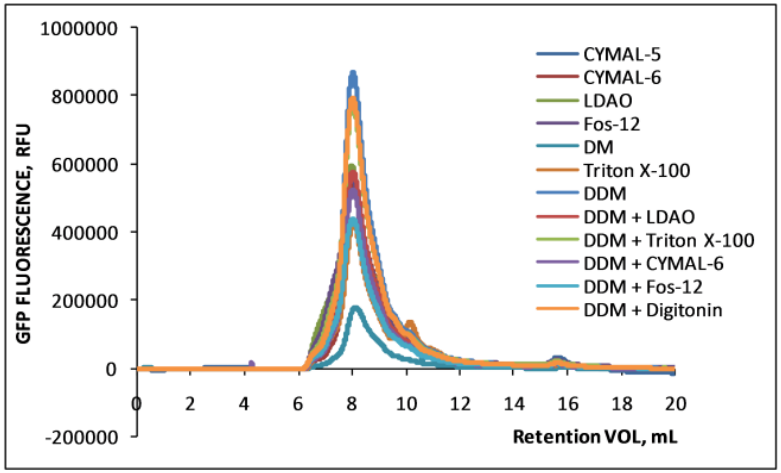


E

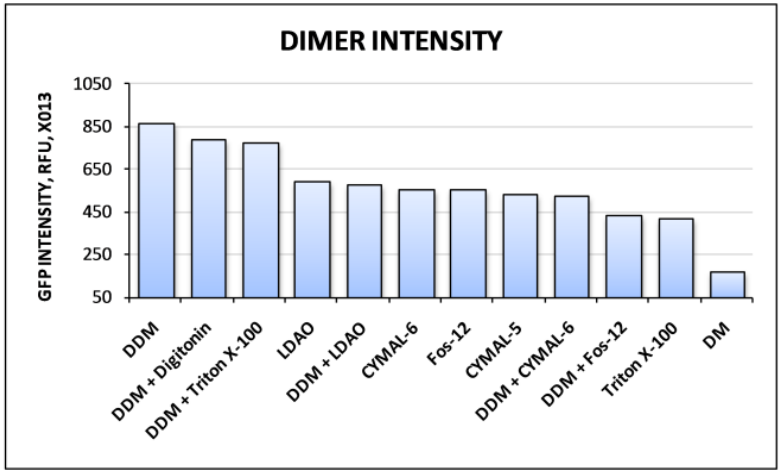
N	Detergent	cmc, %	Detergent type
1	CYMAL-5	0.12	Nonionic
2	CYMAL-6	0.028	Nonionic
3	LDAO	0.023	Zwitterionic
4	Fos-12	0.047	Zwitterionic
5	DM	0.087	Nonionic
6	OG	0.53	Nonionic
7	Triton X-100	0.01	Nonionic
8	DDM	0.01	Nonionic
9	DDM + DM	n/d	Mixed
10	DDM + LDAO	n/d	Mixed
11	DDM + Triton X-100	n/d	Mixed

Fig. 7

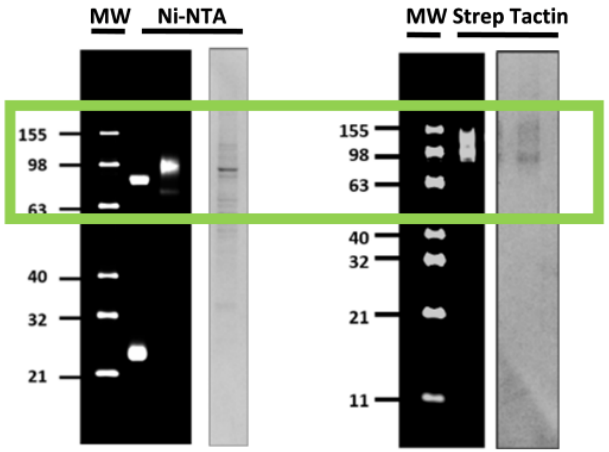
A



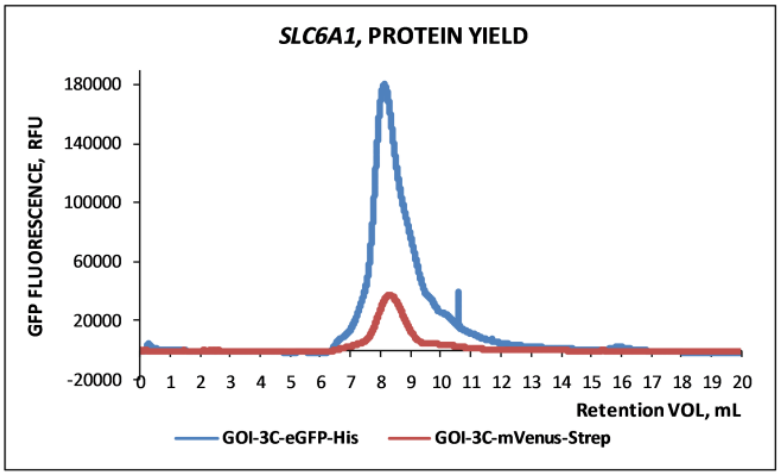
B



C



D



E

	In-cell fluorescence, RFU	FSEC (DM) fluorescence, RFU
GOI-3C-eGFP-His	836.4	179779
GOI-3C-mVenus-Strep	569.2	33914
Ratio	1.5	5.3

Fig. 8

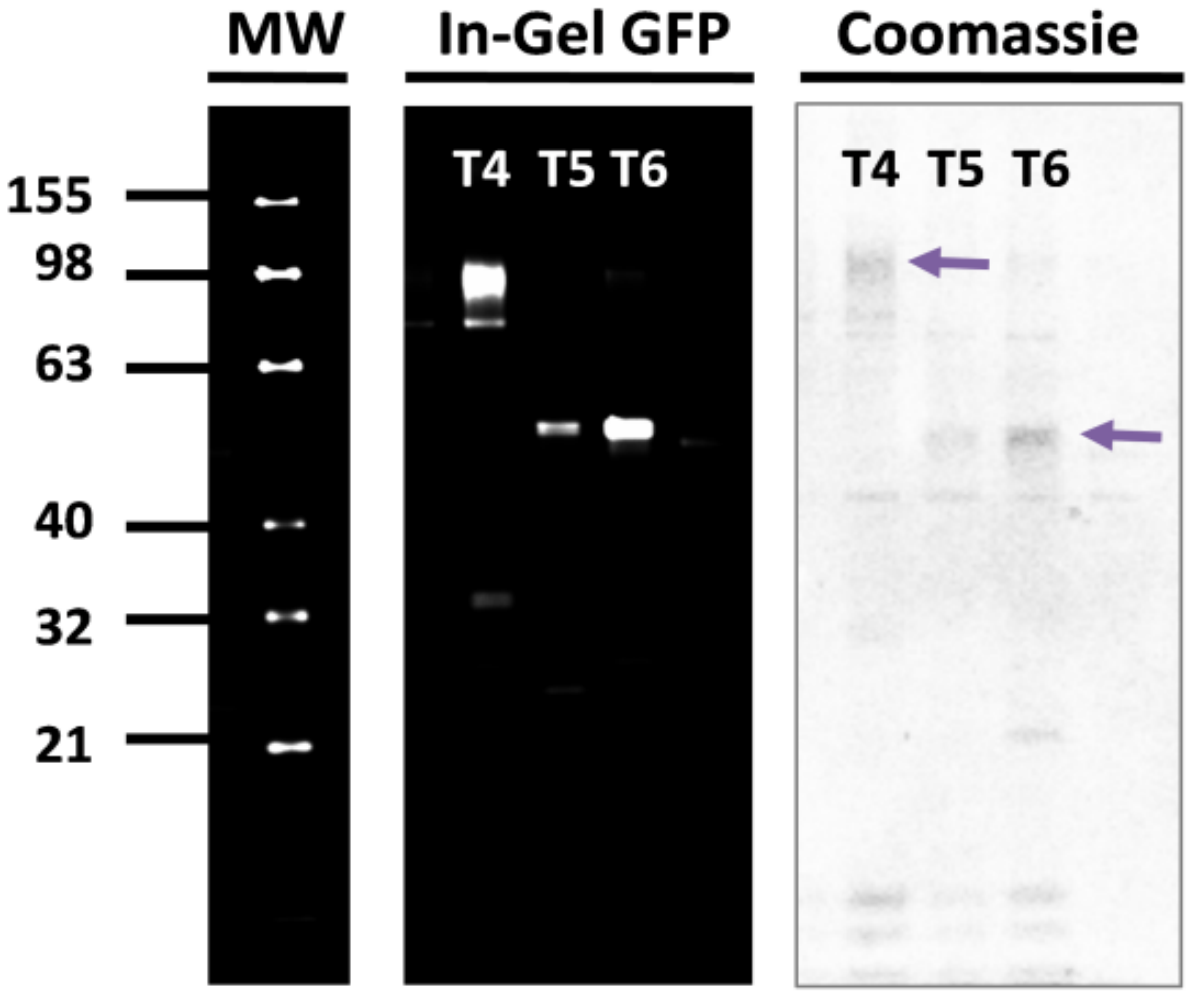


Fig. 9