Optimizing Expression & Purification Workflows for Membrane Proteins.

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Abstract – Membrane proteins are the gateway to cells and are involved in many critical functions. Each membrane protein is unique, and their complexity makes it difficult to fit into a standardized expression and purification workflow. While there is not a standardized solution to membrane proteins, there are best practices to achieve a streamlined and successful **Membrane Protein Expression** and **Purification**. Here, using various G protein-coupled receptors (GPCRs), a large family of transmembrane receptors, we demonstrate ways to optimize your workflows resulting in reduced timelines, enhanced protein yields and recovery and preservation of functional activity.

Membrane Protein Expression

Introduction – Choosing the appropriate expression system. Many factors need to be considered when choosing the appropriate host cell system for recombinant expression of membrane proteins including, ease of use, time-to-protein, yield, post translational modifications (PTMs), and functional activity. Using different host cell species can impact any of these considerations. While cell-free systems are fast and *E. coli* systems may yield more protein, mammalian systems are better suited to deliver proteins with native conformations, often necessary for functional and structural analysis (1). Traditional HEK293 cells are commonly for expressing membrane proteins. However, they are not without their limitations. Therefore, Thermo Fisher Scientific has designed the **Gibco Expi293™ Transient Protein Expression Systems** (**Table 1**) which allows for the rapid and robust production of various forms of proteins including difficult-to express and insoluble proteins such as membrane proteins.

Table 1. Expi293 Cell Line Systems.

Cell Line	Key Applications	Advantages
Expi293F™ Robust system that supports a wide variety of recombinant protein expression applications	• Expression of wide range of proteins to support biologics development, functional studies, target validation	• Native human level PTMs • Suitable to express secreted, soluble and insoluble proteins • Suspension, high-density cell line • High protein yields and fast time-to-protein (2-7 days)
Expi293F™ GnTI⁻ Derived from Expi293F cell line. N-acetyl-glucosaminyltransferase I (GnTI⁻) gene knockout	• **Expression of difficult-to-express and insoluble proteins** • Structural Biology – determination of glycoproteins via x-ray crystallography • Expression of hyper-glycosylated proteins	• Aids in expression of difficult-to-express and insoluble proteins • Uniform glycan profile leads to enhanced crystal formation • Suspension, high-density cell line • High protein yields and fast time-to-protein (2-7 days)

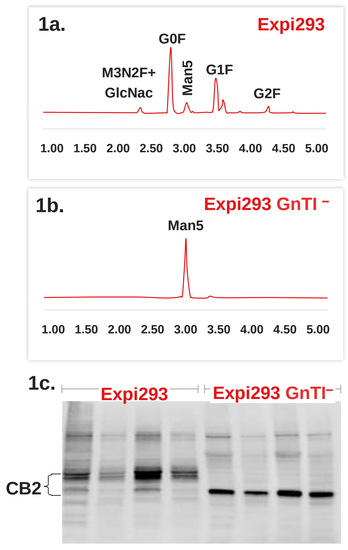


Figure 1. Variations in glycan profile patterns and membrane protein expression patterns in Expi293F™ and Expi293F™ GnTI⁻ cell lines. Glycan profile patterns for human IgG expressed in Expi293F™ **(a)** and Expi293F™ GnTI⁻ cell line **(b)**. **(c)** SDS-PAGE demonstrate heterogeneity of CB2 bands when expressed in Expi293F™ GnTI⁻ (right panel) compared to Expi293F™ cells (left panel).

Results – Expressing Cannabinoid Receptor II in Expi293F™ GnTI⁻.

Cannabinoid Receptor type II (CB2), is a member of the endocannabinoid system of mammals and some invertebrates (2). CB2 an important integral membrane protein primarily expressed during an active inflammatory event (3). Structurally, like most GPCRs, CB2 is a 45kDa protein made up of seven alpha-helical transmembrane-spanning domains (4) (**Diag. 1**). Here, a C-terminal GFP-fusion construct of CB2 [**Construct (a)**] is used as a model to optimize its expression in the **Expi293F™ GnTI⁻** cell line (5).

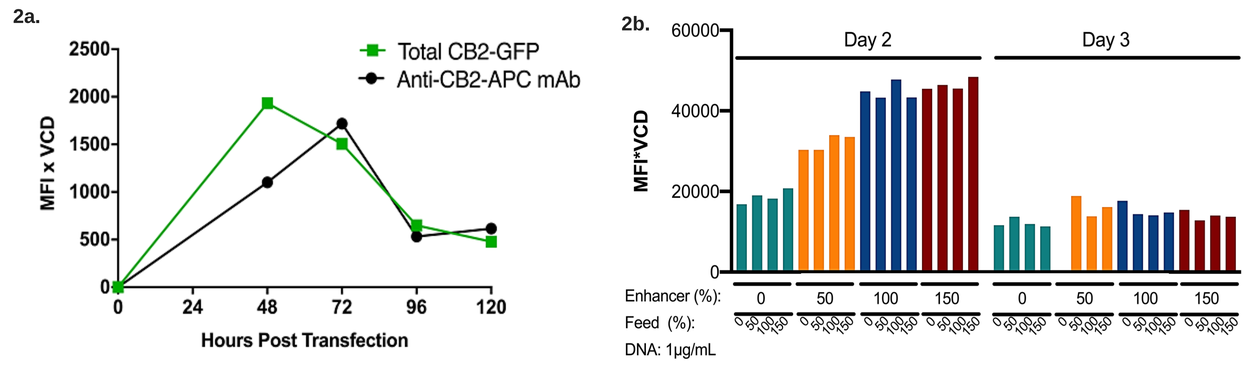


Figure 2. Expressing Cannabinoid Receptor II in Expi293F™ GnTI⁻. (a) GFP serves as suitable marker for determining membrane expression of CB2. To validate whether CB2 expressed as a GFP fusion protein [(**Construct (a)**] exhibited a similar profile as untagged CB2 [(**Construct (b)**] , Expi293F™ GNTI- cells were transfected with the two respective constructs. Presence and abundance of plasma membrane-associated CB2-GFP signal (total CB2-GFP; green) or un-tagged CB2 (anti-CB2-APC mAb; black) was determined by flow cytometry by measuring the median fluorescence intensity (MFI) at various time points. CB2 ligand CP-55,940 (5 uM) was added during expression run as a stabilizer and samples were standardized by multiplying by viable cell density (VCD) to correlate results. Membrane-associated CB2 staining was performed using anti-CB2 specific monoclonal antibody (mAb) conjugated to allophycocyanin (APC) that recognizes extracellular N-terminus domain of CB2. Note APC exhibits far-red fluorescence. **(b) Optimizing membrane protein expression conditions.** Expi293F™ GnTI⁻ cells were transfected to express CB2-GFP. Various concentrations of Enhancer 1 (Enhancer) and Enhancer 2 (Feed) were tested at percentages (%) at or above those recommended by the Expi system guide. Cell membrane-associated CB2 was examined by flow cytometry as described in (a) at Day 2 or Day 3 post transfection.

Membrane Protein Purification

Introduction – Protein Solubilization, Extraction and Stabilization

Maintaining solubility and native conformation of overexpressed GPCR constructs throughout the downstream purification process is challenging. Optimization of the affinity purification protocol and incorporation of stabilization factors into the buffers may be required to maintain receptor functionality throughout the sample preparation (6, 7, 8) (**Table 2.**). Here, using **Thermo Scientific™ Pierce™ GPCR Extraction and Stabilization Reagent**, we report an optimized method of extraction and purification of adenosine receptor type 2A (A2AR) and CB2 (both expressed in Expi293F™ GnTI⁻) that yield highly purified, stable and functional proteins ready for downstream analyses and application.

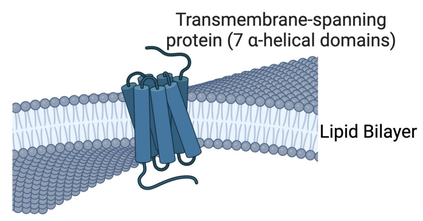
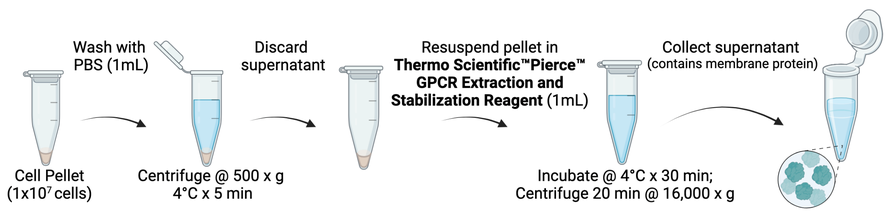


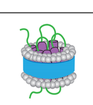
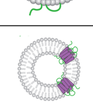
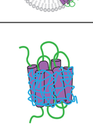
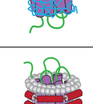
Diagram 1. Schematic representation of a GPCR-like transmembrane-spanning protein with 7 alpha-helical domains.

Diagram 2. General workflow for membrane protein extraction using Thermo Scientific™ Pierce™ GPCR Extraction and Stabilization Reagent. Reagent is suitable for downstream applications including protein quantitation, SDS-PAGE, western blot, binding assays and structural studies. This workflow was performed for experiments depicted in Fig. 3, 4, 5.



Pro-Tip | Whole cell lysate can be stably stored in **Thermo Scientific™ Pierce™ GPCR Extraction and Stabilization Reagent** for up to 1 week at 4ºC or 1 month at -20ºC for future protein purification. It is recommended to add a 10-fold dilution of the **Reagent** into wash and elution buffers to enhance receptor stability throughout the entire purification process.

Table 2. Different techniques for extraction and stabilization of membrane proteins.

Schematic	Name	Method	Description	Tips
	Detergent	Extraction	Disrupts the lipid bilayer and incorporates membrane proteins into a detergent micelle.	Choosing the best detergent for specific membrane protein requires screening and additional stabilizers like cholesterol or target specific ligands to maintain function.
	SMALP/ Polymer	Extraction/ Stabilization	Spontaneous encapsulation from a membrane into a nanoscale styrene maleic acid lipid particle (SMALP).	SMALP or polymer extraction provides a native environment, but often results in lower protein yields. It is ideal for understanding protein lipid interactions.
	Liposome	Stabilization	Systems that mimic lipid membranes (liposomes) to which a protein has been incorporated or inserted.	Liposomes require lipid screening to determine the best environment for the target protein to maintain function.
	Amphipol	Stabilization	Short amphipathic polymers designed to adsorb tightly onto the hydrophobic transmembrane surface of MPs and cover it with a thin interfacial layer of surfactant.	The target protein can be exchanged into an amphipols after detergent extraction and do not require lipids for stabilization.
	Nanodisc	Stabilization	Nanometer-scale planar discs of lipid bilayer stabilized by encircling membrane scaffold protein.	The target protein can be exchanged into a nanodisc after detergent extraction. Lipid screening is required to determine the optimal environment.

Pro-Tip | Remember to check compatibility between the selected extraction & stabilization reagents and desired downstream application. Screening of reagents is recommended. Exchange into a compatible format, such as a more advantageous detergent, amphipol, or membrane mimetic (i.e., liposome or nanodisc) may be required based on your desired downstream application.

Results – Extraction & Purification of two GPCR's.

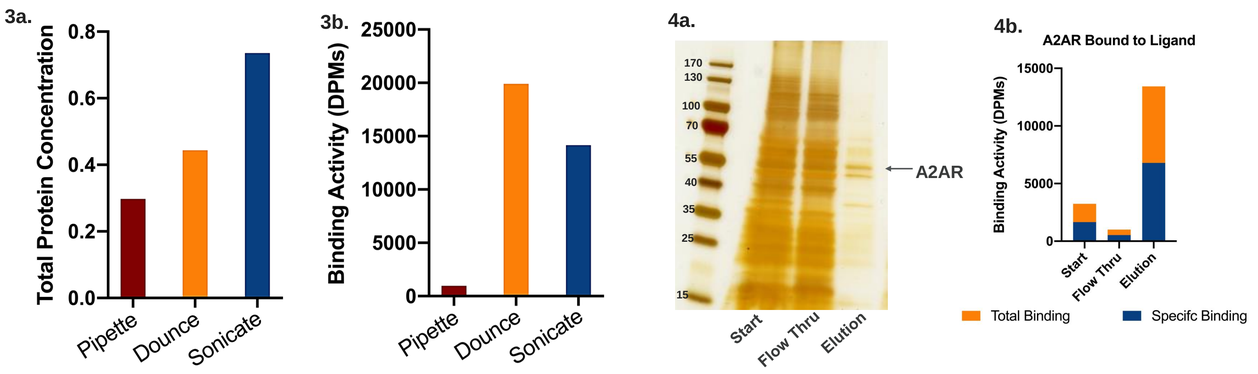


Figure 3. Protein solubilization by moderate mechanical cell disruption is better at preserving A2AR activity. Moderate mechanical forces, such as Dounce homogenization, may solubilize less A2AR protein **(a)** but it is better at preserving activity **(b)**. Activity was assessed by radioligand binding assay. 5mg of each sample were incubated for 75 minutes at room temperature with either radiolabled adenosine (^{3}H-Adenosine) alone (total binding) or unlabeled adenosine and ^{3}H-Adenosine (competitive binding assay; to determine non-specific binding). Free radioligand was removed via gel filtration. **Construct (c)** was used for this study.

Figure 4. Purification strategy for A2AR. (a) Silver stained SDS-PAGE of A2AR-GFP-His solubilized in **Thermo Scientific™ Pierce™ GPCR Extraction and Stabilization Reagent** and purified using **Thermo Scientific™ Pierce™ Ni-NTA Magnetic Agarose Beads**. Note the efficient results during elution. **(b)** As determined by radioligand binding, affinity purification-enriched A2AR-GFP-His remains highly functional. 5ug ^{3}H-Adenosine served as ligand for A2AR. **Construct (c)** was used for this study.

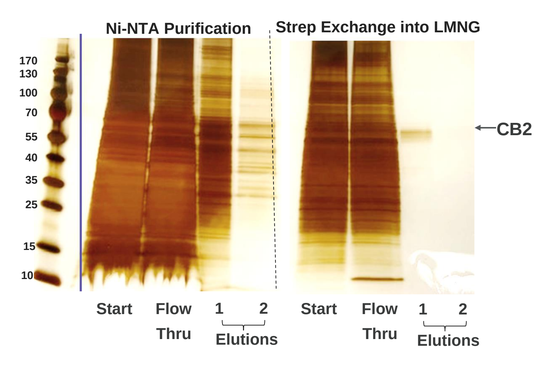


Figure 5. Double affinity purification supplies optimal purity level for membrane proteins and facilitates buffer exchange. Silver stained SDS-PAGE of double affinity-purified CB2 (His-CB2-GFP-Strepavidin). The protein was initially purified using Pierce™ Ni-NTA Magnetic Agarose Beads (100 µL of a 25% slurry). Bound protein was eluted from beads using standard protein elution buffer supplemented with **Thermo Scientific™ Pierce™ GPCR Extraction and Stabilization Reagent** (9:1). The subsequent eluate was bound to MagStrep® Strep-Tactin®XT beads, then eluted and exchanged in to LMNG, a buffer more amenable to cryoEM. **Construct (d)** was used for this study.

Conclusions

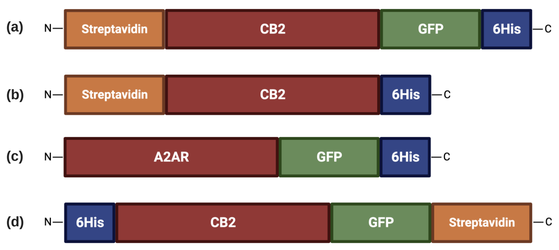
Expression

- Expi293 GnTI⁻ line cell systems is suitable for expressing various GPCR membrane proteins.
- GFP fusion can assist in narrowing down conditions optimal for expressing CB2 and likely applied to other membrane proteins of interest.
- Day 2 post transfection is optimal for harvest of CB2. Higher percentage of feed beyond recommended did not present a meaningful impact likely due to short length of protein expression run used for membrane protein expression.
- While additional DNA concentrations were tested (data not shown), 1ug/mL was determined to be optimal.

Purification

- High levels of GPCR overexpression may require additional mechanical forces to aid in solubilization. While moderate mechanical forces, such as Dounce homogenization, may solubilize less A2AR protein, it is better at retaining protein functionality/preserving protein activity.
- Double affinity purification is useful for obtaining high purity proteins and facilitates buffer exchange into formulation more suited for certain downstream applications such as cyro-electron microscopy (cryo-EM).

Constructs



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