nuclera

Guidance from Screen to Cryo-EM GPCR Samples

This guidance combines eProtein Discovery GPCR nanodisc screening with pilot fluorescence size-exclusion chromatography (F-SEC) to help identify the condition most likely to deliver an active, monomeric GPCR nanodisc population, prior to large-scale preparation for cryo-EM.

The key principle

Rather than scaling the highest-yielding condition for cryo-EM by default, this workflow uses F-SEC to evaluate multiple yield-enabling GPCR/nanodisc pairs and select the condition presenting the strongest monomeric, active peak prior to large-scale preparation. This minimizes misinserted GPCR material that can appear as “empty” nanodiscs in cryo-EM samples.

By combining this approach and ensuring rapid freezing (<2 hours) of samples on cryo-EM grids, we reduce pseudo-empty nanodiscs and produced example datasets for Neurotensin Receptor 1 (NTR1) and Turkey truncated thermostable β1-adrenergic receptor (ttsΔβ1AR) with a high percentage of GPCR-occupied particles in cryo-EM grid samples (63.6% and 50.1%, respectively).

Overview of the workflow

The recommended workflow has four stages:

1. Run an eProtein Discovery screen using the eProtein Discovery GPCR Nanodisc Panel to identify high yielding construct–nanodisc condition pairs.
2. Identify four GPCR-nanodisc conditions for pilot F-SEC.
3. Run pilot F-SEC and select the condition with the highest active, monomeric peak for cryo-EM.
4. Scale-up the selected condition to 1 mL, purify, concentrate, and freeze grids as quickly as possible (<2 hours).

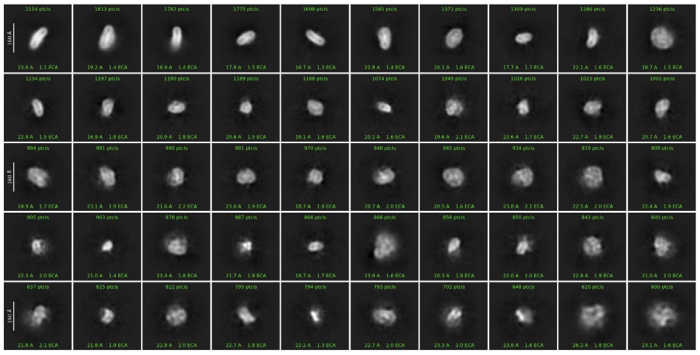
Example data

These examples are included to show the type of outcome that can be obtained when the workflow is followed. They should be used as reference datasets, not acceptance criteria.

Example 1. Apo NTR1 ~64% GPCR-occupied particles dataset

The sample consisted of 1 mg/mL apo NTR1 in MSP1E3D1-DOPG and was imaged *via* Talos Arctica™. From a dataset of 449 movies, 52,854 total particles were extracted. Analysis of the particles revealed 33,627 GPCR-occupied nanodiscs and 19,227 empty nanodiscs, representing a 63.6% occupancy rate (Figure 1).

(A)



(B)

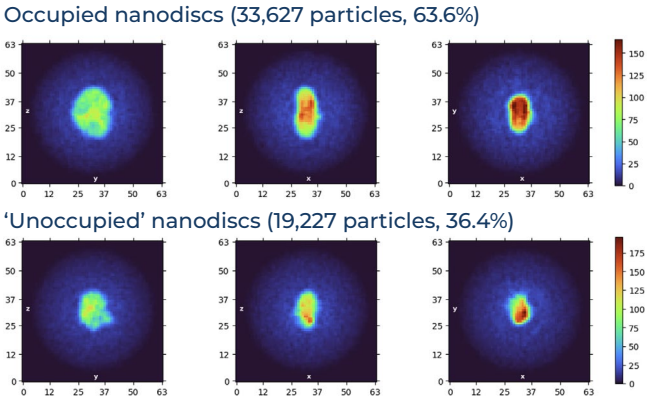
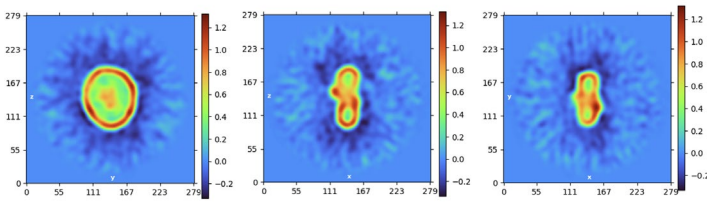


Figure 1. Results from Ntr1 grid 1. (A) 2D class average of apo NTR1 in MSP1E3D1-DOPG from 449 movies / 52,854 particles. **(B)** *Ab initio* contour map of the superposition of the particles distinguishing the occupied nanodiscs from empty nanodiscs.

Example 2. Apo NTR1 structural reconstitution dataset

A second grid containing 1 mg/mL apo NTR1 in MSP1E3D1/DOPG was imaged with Talos Arctica™. From a dataset of 200 movies, 125,084 total particles were extracted. After refinement, 40,404 GPCR-occupied nanodiscs were identified and a 13 Å structural reconstitution image was generated to reveal the clear presence of an occupied nanodisc (Figure 2).

(A)



(B)

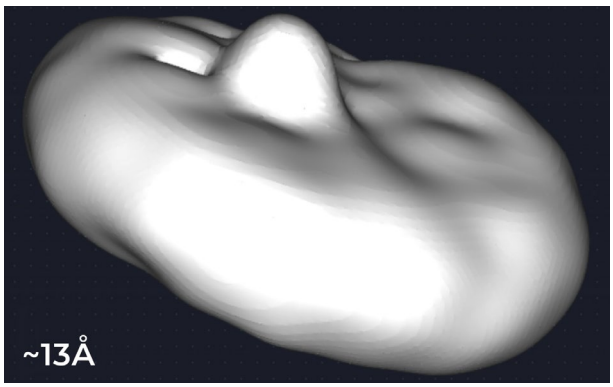
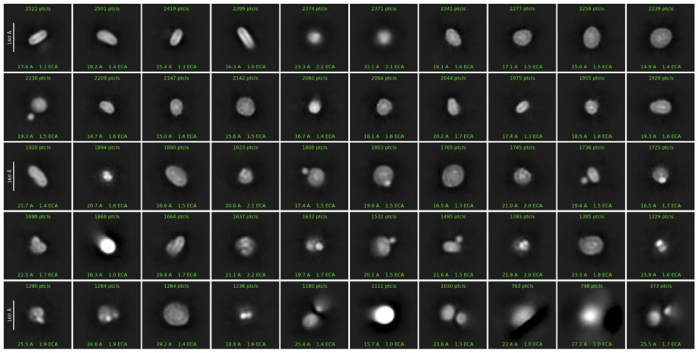


Figure 2. Results from NTR1 grid 2. (A) *Ab initio* contour map of the superposition of apo NTR1 occupied MSP1E3D1-DOPG nanodiscs from a dataset of 200 movies and 40,404 total particles. **(B)** Structural reconstitution of occupied particles produced a 13 Å image that reveals the presence of Ntrl in the nanodisc.

Example 3. Apo ttsΔβ1AR ~50% GPCR-occupied particles dataset

The sample consisted of 1 mg/mL apo ttsΔβ1AR in MSP1E3D1-DOPG and was imaged via Talos Arctica™. From a dataset of 325 movies, 88,312 total particles were extracted. Analysis of the particles revealed 44,215 GPCR-occupied nanodiscs and 44,097 empty nanodiscs, representing a 50.1% occupancy rate (Figure 3).

(A)



(B)

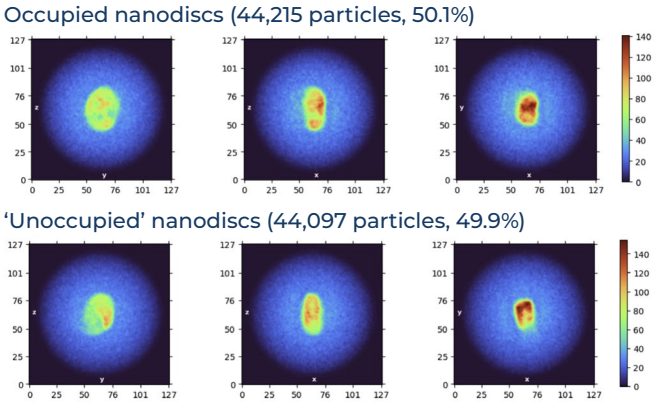


Figure 3. Results from ttsΔβ1AR. (A) 2D class average of apo ttsΔβ1AR in MSP1E3D1-DOPG from 325 movies / 88,312 particles. **(B)** *Ab initio* contour map of the superposition of the ttsΔβ1AR MSP1E3D1-DOPG particles distinguishing the occupied nanodiscs from empty nanodiscs.

Recommended workflow

Stage 1. eProtein Discovery Screening

Goal

Use eProtein Discovery to identify GPCR construct and nanodisc conditions that give sufficient purified yield to justify pilot F-SEC and larger scale-up for cryo-EM.

Guidance

Run a multiplex screen on the eProtein Discovery System using the eProtein Discovery GPCR Nanodisc Panel Screen Kit (NC3023) and the standard membrane protein workflow. This step is used to obtain yield and purifiability reports and identify:

- GPCR constructs worth carrying forward.
- Membrane scaffold protein/lipid combinations that maximize predicted yields for the target GPCR.
- Conditions that should be examined further by F-SEC.

Note: For GPCRs containing disulfide bonds, include PDI/GSSG during screening in all Cell-free Blends to support the correct redox environment.

For GPCR construct design it might be worth exploring:

- **Length variants:** Truncate flexible regions (e.g. N/C-termini, ICL3) to reduce aggregation and improve stability.
- **Thermostabilizing point mutations:** Improves receptor stability and homogeneity.

- **Solubility/fusion tags:** N-terminal or intracellular fusion tags (e.g. BRIL, mystic-tags) to improve GPCR yield and stability.
- **Orthologues:** GPCRs from other species can increase stability and purification yields.

Example results

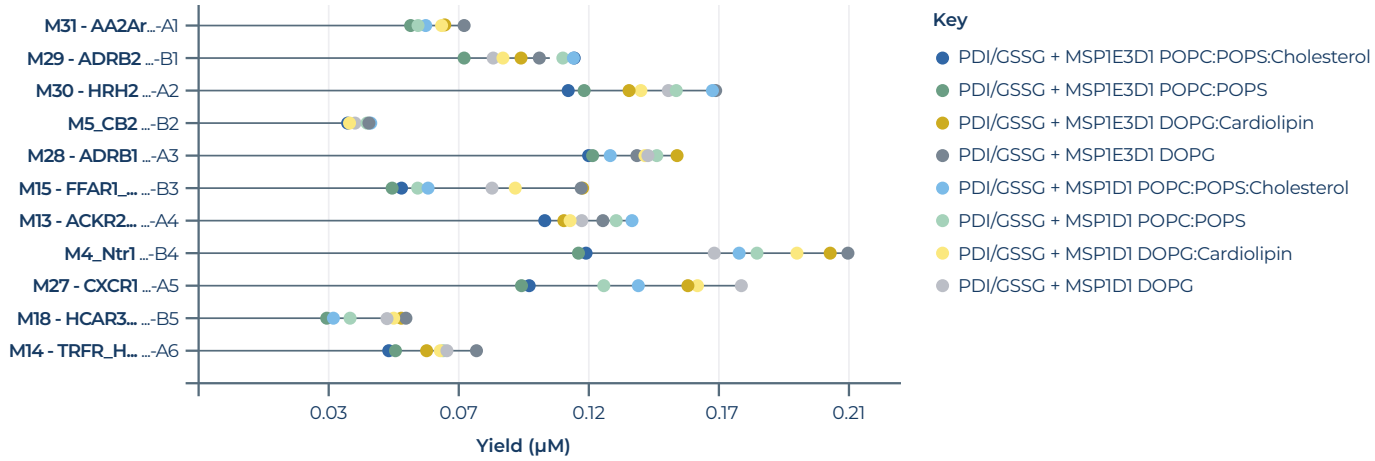


Figure 4. Example eProtein Discovery screen of 11 GPCRs against the GPCR Nanodisc panel for purified yield.

Stage 2. Select four conditions for pilot F-SEC

Goal

Choose four MSP-lipid nanodisc conditions for a target GPCR to perform a pilot F-SEC.

Guidance

Prior to large-scale expression, a small scouting experiment should be performed to ensure that the expressed protein shows a clear monomeric nanodisc peak in F-SEC.

We recommend selecting four conditions from the eProtein Discovery screen to scale-up and test *via* pilot F-SEC (all scale-up conditions should also contain PDI/GSSG):

1. Highest predicted yield condition from the screen.
2. The same lipid composition in the alternate MSP format.
   For example, if the highest-yielding condition is MSP1D1-POPC/POPS, also test MSP1E3D1-POPC/POPS.
3. MSP1E3D1-DOPG, unless already selected.
4. MSP1D1-DOPG, unless already selected.

This set gives a practical way to test:

- Whether the screen winner produces high yields of active, monomeric GPCR.
- If changing MSP while keeping lipid constant improves the fraction.
- Does DOPG-based conditions provide a better monomeric population even when they were not the highest-yielding screen hit.

Stage 3. Run pilot F-SEC

Goal

Perform F-SEC on small-scale crude expression mixtures to compare the four selected conditions and choose the one with the strongest active, monomeric GPCR nanodisc peak for larger scale-up and cryo-EM grid preparation.

Materials

To run the pilot F-SEC, the materials outlined in Table 1 are required, along with a SEC/UPLC system fitted with a fluorescence detector (see Table 2 as the example F-SEC instrumentation used). Note that not all reagents within the kits will be required.

Table 1. Materials required for pilot F-SEC of four samples.

Item / reagent	Order Quantity & details	Volume required
5 nM eGene™ of target GPCRs (Custom Linear eGene Prep Service)	1 x NC3022 (Nuclera) 1 mL scale-up	20 µL required
eProtein Discovery GPCR Nanodisc panel Screen kit	1 x NC3023 (Nuclera)	1.5 µL per nanodisc
Scale up Core Reagents Contains Wash Buffer and Cell-free core reagent	1 x NC3011-2 (Nuclera)	111.2 µL Wash Buffer 48 µL Cell-free core reagent
Scale-up PDI+GSSG Mix	1 x NC3016 (Nuclera)	6 µL
eProtein™ Quantification Kit Contains Detector Protein	1 x NC3014 (Nuclera)	6.8 µL Detector Protein
RNAse A (20 mg/mL)	E.g. 1 x T3018L (NEB®)	2 µL
200 µL PCR tubes	4 required	-
0.22 µm centrifugal filter	4 required	-
PCR thermocycler	-	-

Example F-SEC setup

Table 2. Example SE-UPLC system and conditions used by Nuclera.

Component	Examples
UPLC system	Waters ACQUITY® Premier UPLC system
Fluorescence detector	Waters ACQUITY® FLR set at (λ_{ex} = 488 nm; λ_{em} = 510 nm)

Component	Examples
SEC column	ACQUITY® Premier Protein SEC Column (250 Å, 1.7 µm, 4.6 x 150 mm)
Mobile phase	100 mM Tris and 150 mM NaCl (pH 7.5)
Sample injection volume	2 µL
Flow rate	0.4 mL/min

Pilot F-SEC protocol

1. Set up one 20 µL reaction for each selected condition in a 200 µL PCR tube as outlined in Table 3.

Table 3. Cell-free expression reaction setup for F-SEC.

Component	Volume (µL)
Cell-free core Reagent	12
5 nM eGene DNA construct for GPCR target	5
Selected Nanodisc	1.5
PDI/GSSG	1.5

2. Incubate at 29 °C overnight in a PCR thermocycler with a heated lid.

3. Following expression, add 0.5 µL of 20 mg/mL RNase A, 27.8 µL Wash Buffer, and 1.7 µL Detector Protein. This brings the final volume to 50 µL.

4. Incubate for a further 5 hours at 29°C. This step is used to digest residual RNA and complement Detector Protein with the DET tag on the GPCR to produce a GFP fluorescent signal.

5. Pass the samples through a 0.22 µm centrifugal filter and inject onto a SEC fitted with a fluorescence detector capable of detecting GFP (λ_{ex} = 488 nm; λ_{em} = 510 nm).

6. Review F-SEC traces and select the expression condition that gives the highest active fraction peak (monomeric GPCR) for 1 mL scale-up.

Example results: How to interpret pilot F-SEC data

A typical GPCR F-SEC trace shows up to three main peaks:

- **Aggregate peak:** This peak elutes in the void volume and corresponds to aggregated material.
- **Misinserted GPCR peak:** This peak appears at larger apparent molecular weight than the correctly folded GPCR-nanodisc complex. It is typically associated with misinserted or lower-quality GPCR-nanodisc populations in the range of ~300-600 kDa (depending on the protein and SEC instrumentation).
- **Active peak:** This is the lower-molecular-weight peak corresponding to the expected size of the correctly folded GPCR-nanodisc complex. For monomeric GPCRs in MSP1E3D1-DOPG, this is typically in the range of ~180–250 kDa.

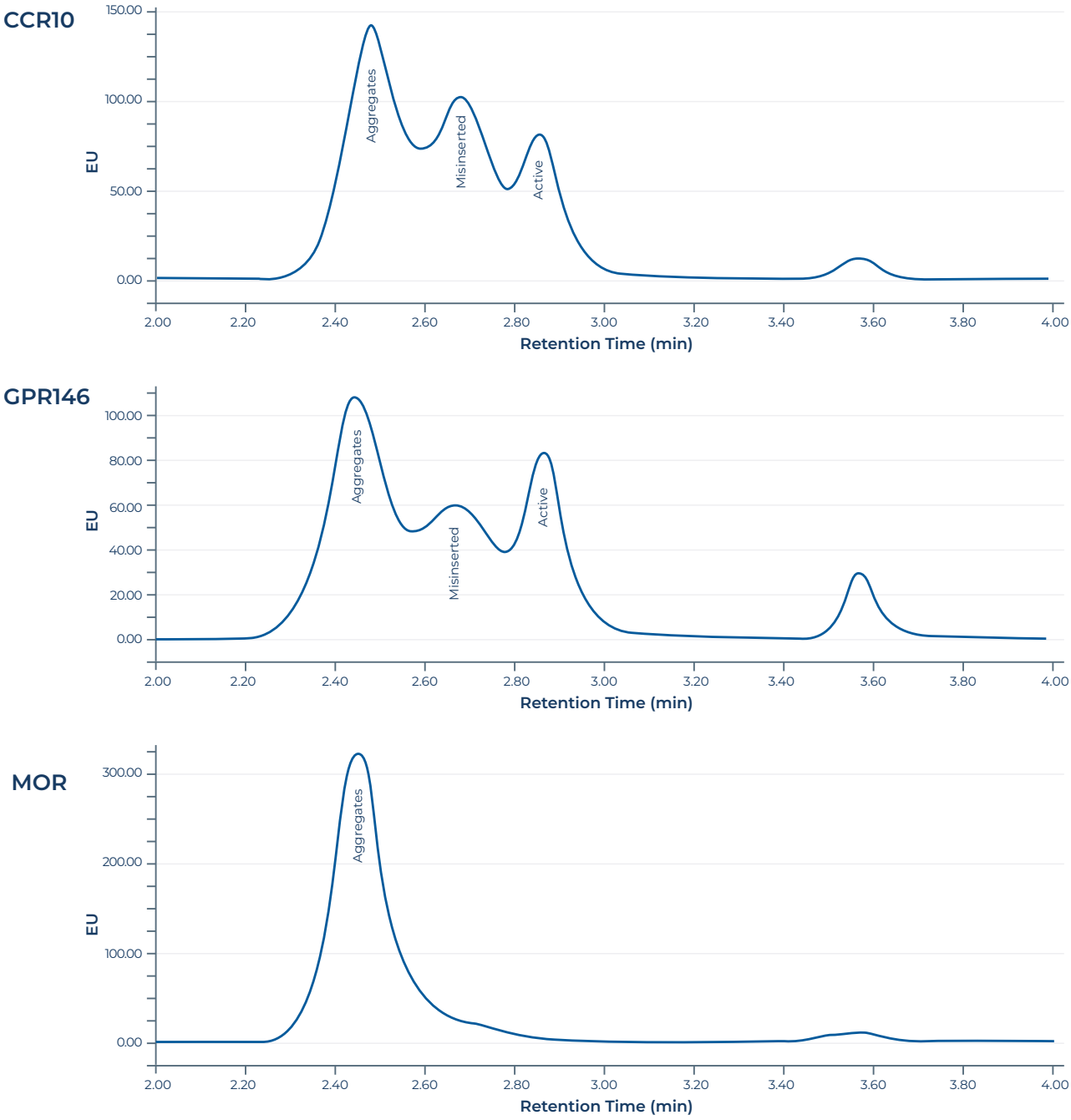


Figure 5. Example F-SEC of three monomeric GPCRs (CCR10, GPR146, and MOR) scaled up in MSP1E3D1-DOPG and PDI/GSSG. **Note:** F-SEC traces will differ for multimeric GPCRs. Retention times and peak widths can vary depending on the SEC column, mobile phase buffer composition and flow rate used.

Stage 4. Scale the Optimized Condition for Rapid Grid Freezing

Goal

Perform a larger 1 mL scale-up of the condition that performed best based on predicted yield (from the eProtein Discovery screen) and proportion of the expressed protein in the active peak (from pilot F-SEC), then move quickly through purification, concentration, and grid freezing to preserve sample quality. Minimizing sample time and handling steps between protein scale-up and cryo-EM grid freezing (<2 hours) can also reduce pseudo-empty nanodiscs.

Materials

The reagents listed in Table 4 assume the eProtein Discovery predicted yield for the GPCR target is over 100 µg/mL, a larger scale-up volume may be required if this is not the case (please review step 5 of the protocol for larger scale-up and grid freezing below).

Table 4. Materials required for 1 mL scale-up and grid-freezing.

Item	Order Quantity & details
5 nM eGene of target GPCR (250 µL required)	1 x NC3022 (Nuclera) 1 mL scale-up
Scale-up Core Reagents Contains wash buffer and cell-free core reagent.	1 x NC3011-2 (Nuclera)
Scale-up Bead	1 x NC3011-1 (Nuclera)
Scale-up PDI-GSSG	1 x NC3016 (Nuclera)
Scale-up Buffers contains Additive Buffer	1 x NC3021 (Nuclera)
Scale-up selected nanodisc. Select 1 between: ▹ **NC3024:** Scale-up MSP1D1-DOPG ▹ **NC3025:** Scale-up MSP1D1-DOPG/Cardiolipin ▹ **NC3026:** MSP1D1-POPC/POPS ▹ **NC3027:** MSP1D1-POPC/POPS/Cholesterol ▹ **NC3028:** MSP1E3D1-DOPG ▹ **NC3029:** MSP1E3D1-DOPG/Cardiolipin ▹ **NC3030:** MSP1E3D1-POPC/POPS ▹ **NC3031:** MSP1E3D1-POPC/POPS/Cholesterol	1 x NC3024– NC3031 (Nuclera)
RNAse A (20 mg/mL)	E.g. T3018L (NEB). 10 µL required.
30 kDa Amicon® Ultra microconcentrator	E.g. UFC5030 (Merck). 2 required.
1.5 mL Lo-bind microcentrifuge tube	1 required
CryoEM grids (e.g. Quantifoil 1.2/1.3 Cu 300 mesh)	-
Cryogenic prep instrument (e.g. Vitrobot™ Mark IV System)	-

Protocol for larger scale-up and grid freezing

1. Assemble the 1 mL scale-up reaction as described in Table 5 in a 1.5 mL Lo-bind microcentrifuge tube.

Table 5. Cell-free expression reaction setup for 1 mL scale-up.

Component	Volume (µL)
Cell-free core reagent	600
5 nM eGene DNA construct for GPCR target	250
Selected Nanodisc	37.5
PDI/GSSG	75
Additive Buffer	37.5

2. Incubate overnight at 29 °C.

3. Add 10 µL of 20 mg/mL RNase A and incubate for 30 mins at 29 °C.

4. Purify with strep magbeads according to the scale-up protocol.

5. Concentrate the 1000 µL elution to approximately 100 µL using a 30 kDa Amicon® Ultra microconcentrator, following the manufacturer's guidance. The target concentration is approximately 1 mg/mL.

> ⚠ **Do not use concentrators with < 30 kDa molecular weight cut off, as they will retain and concentrate the surfactant present in our elution buffer and impact the quality of recovered concentrated protein.**

Note: If the predicted yield from the eProtein Discovery screen suggests that 1 mg/mL is not achieved by a 10x concentration, further concentrate to reach 1 mg/mL. If concentrating to 1 mg/mL reduces volume below requirements for cryo-EM, use the screen predicted concentration to calculate the appropriate volume of scale-up to perform.

6. Freeze samples onto cryo-EM grids as soon as possible, ideally within 2 hours of concentration. Loaded grids can then be stored indefinitely in liquid nitrogen. See Table 6 for example cryo-EM grid preparation conditions.

> ⚠ **Avoid freeze-thaw before applying the sample to the grid as this will impact protein quality.**

Table 6. Example cryo-EM grid preparation details used in datasets.

Component	Examples
Cryogenic prep instrument	Vitrobot™ MkIV
Grid type	Quantifoil™ 1.2/1.3 Cu 300 mesh
Glow discharge	Yes, 25 mA for 60 seconds
Charge polarity	Negative
Target sample concentration	1 mg/mL
Sample volume	3 µL

Additional guidance

- **Preparative SEC:** While we have not validated preparative SEC specifically for Cryo-EM samples, be aware that it reduces yield; increase your initial scale-up volume to compensate. For general GPCR purification, we use a Superdex® 200 Increase 10/300 column (Cytiva) in SEC buffer (100 mM Tris pH 7.5, 150 mM NaCl) at 0.75 mL/min. Concentrate and quantify the final sample using the eProtein Quantification kit (NC3014).

- **G-protein complexing:** While not validated internally, external KOL guidance advises that GPCR and G-protein coupling can require low salt concentrations (e.g. 10 mM NaCl) because sodium ions stabilize

the GPCR inactive state (especially in Class A GPCRs). Since our Cell-free core reagent (>200 mM NaCl) and purification buffers (150 mM NaCl) exceed this, consider complexing after desalting or dialysis of the purified GPCR. If testing of ligands or G-proteins directly in the 1 mL scale-up mix is still desirable, replace the 37.5 µL Additive Buffer and maintain all other conditions (Table 5).

Conclusion

This guidance is intended to help structural biology users move from screen to grid with a clearer decision process. The recommended approach is to use eProtein Discovery screening to identify promising construct–nanodisc pairs, then use pilot F-SEC to select the condition with the strongest active, monomeric GPCR nanodisc population before 1 mL scale-up and cryo-EM grid preparation. Example datasets for NTR1 and ttsΔβ1AR show the type of outcome that can be achieved when this workflow is followed.

Appendix

A list of all reagents to order from Nuclera for the pilot F-SEC and 1 mL scale-up grid freezing protocol is located in Table A1. Customer sourced reagents and materials are provided in Table A2.

Table A1. Reagents to order from Nuclera for pilot F-SEC and 1 mL scale-up for cryo-EM.

Catalog. No.	Item	Order Quantity
NC3022	5 nM eGene 1 mL scale-up format from Linear eGene Services	2
NC3023	eProtein Discovery™ GPCR Nanodisc panel Screen kit	1
NC3011-2	Scale up Core Reagents	2
NC3016	Scale-up PDI+GSSG Mix	2
NC3014	eProtein™ Quantification Kit	1
NC3011-1	Scale-up Bead	1
NC3021	Scale-up Buffers	1
NC3024–NC3031	▹ **NC3024:** Scale-up MSP1D1-DOPG ▹ **NC3025:** Scale-up MSP1D1-DOPG/Cardiolipin ▹ **NC3026:** MSP1D1-POPC/POPS ▹ **NC3027:** MSP1D1-POPC/POPS/Cholesterol ▹ **NC3028:** MSP1E3D1-DOPG ▹ **NC3029:** MSP1E3D1-DOPG/Cardiolipin ▹ **NC3030:** MSP1E3D1-POPC/POPS ▹ **NC3031:** MSP1E3D1-POPC/POPS/Cholesterol	1 (Select one based on pilot F-SEC and screen results)

Table A2. User-sourced reagents and consumables required for pilot F-SEC and 1 mL scale-up for cryo-EM.

Item description	Quantity
RNAse A (20 mg/mL)	12 µL
200 µL PCR tubes	4
0.22 µm centrifugal filter	4
30 kDa Amicon® Ultra microconcentrator	2
1.5 mL Lo-bind microcentrifuge tube	1
CryoEM grids (e.g. Quantifoil 1.2/1.3 Cu 300 mesh)	-

Table A3. User-supplied equipment required for pilot F-SEC and 1 mL scale-up for cryo-EM.

Item description
PCR thermocycler
SEC/UPLC system fitted with a fluorescence detector suitable for GFP detection (see Table 2 for an example)
Cryogenic prep instrument (e.g. Vitrobot™ Mark IV System)

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