Membrane Protein Solubilization Protocol with Polymers

Introduction

Proteins of high stability and purity are of upmost importance for structural research and pharmaceutical drug development, yet the process to reach that goal demands patience and is riddled with challenges, especially for integral membrane proteins. From choosing the optimal buffer to affinity chromatography, a lot of different purification steps – while traditionally established – are usually in need of optimization to accommodate the individuality of the target protein. The variety of proteins requires an increasingly more varied tool kit of methods to combat each new arising challenge for a successful purification.

Recently new tools have been added to the tool kit to create a novel path for the notoriously difficult purification of membrane proteins. While detergents already showed a wide use in membrane protein solubilization, here we expand the purification methods with the introduction of synthetic nanodiscs – polymers, that are able to solubilize membrane proteins yet retain native structures of the lipid bilayer. With these new techniques at hand, we present an updated protocol to successfully isolate any membrane protein you desire.

Choice of buffer

The purification of proteins is highly dependent on the buffers used for its isolation. Since proteins have varying characteristics such as ionic charge and solubility which can be influenced by buffer properties, the choice of the lysis buffer is paramount for successful isolation. In fact, if the amino acid sequence is available, the protein's pI can be calculated and thus the pH value can be adjusted to prevent a neutral net charge at which it is more

likely to precipitate. As a rule of thumb, a pH difference of 1 to the protein's pI can be advantageous.

If no information for specific buffers are available we recommend the following buffers as a starting point (see Table 1).

Table 1: Recommended buffer composition for protein purification. This buffer should be used as a starting point for further purification in case no information about the protein can be gathered from published literature.

Buffer for Rho1D4-tagged Proteins			Buffer for His-tagged Proteins		
Concentration	**Substance**	**Amount**	**Concentration**	**Substance**	**Amount**
20 mM	HEPES	4.77 g	20 mM	HEPES	4.77 g
100 mM	NaCl	5.84 g	100 mM	NaCl	5.84 g
			10 mM	Imidazole	0.68 g
	Water	1 L		Water	1 L
Adjust pH to pH 7.5			Adjust pH to pH 7.5		

The recommended buffer combines physiologically native conditions with a good buffering range capacity. However, individual proteins may need further optimization of the buffer to improve solubility, purity and stability. Buffer agents to consider are displayed in Table 2.

Table 2: Additives to consider for the optimization of buffer composition.

Agent	Example	Influence on	Amount
Salt	NaCl, KCl, etc.	Solubility, Binding efficiency, Aggregation	100 mM and up
Buffer agent and pH	HEPES, TRIS[a], Phosphate, etc.	Stability, Solubility, Aggregation	10-50 mM pH 6.8-8.2 (pH 7.5 ideal)[b]
Glycerol		Stabilization	up to 10% (v/v)

Reducing agents[d]	DTT, TCEP, β-mercaptoethanol	Oxidative damage protection	Varied
Other Additives	EDTA[d], Mg^{2+} [e], Ca^{2+} [e], ligands, etc.	Stability, Native function	Varied

[a] TRIS buffer pH is highly dependent on temperature. Always check pH before use!

[b] pH values with higher deviation to the protein's pI leads to higher solubility. Non-mild pH values could lead to denaturation.

[c] Reducing agents should not be used with binding matrices containing Rho1D4-antibodies.

[d] Reducing agents and EDTA might interfere with metal ion^{2+} resins. For higher resistance INDIGO resin is recommended.

[e] Bivalent cations might lead to precipitation when using certain polymers.

Before we begin

To observe the efficiencies of the purification steps, it is advised to draw SDS-PAGE samples after important applications and visualise the proteins through Wester Blotting. Key samples that have been determined to be of interest have been accentuated with ^{SDS}. Membrane proteins tend to aggregate during preheating and during the SDS-PAGE itself, so we recommend an incubation at 46°C for 30 min and a sample buffer with higher concentration of SDS.

Cell lysis and centrifugation

- Add protease inhibitors (PI) to buffer and readjust pH value (Note: protease inhibitors interfere with UV/Vis measurements at 280 nm)
    - 0.01 mM Leupeptin
    - 0.01 mM E-64
    - 0.1 mM PMSF

  - 1 mM Pepstatin
  - 1 mM Phenanthroline
- Weight pellet and resuspend in protein buffer (~ 15 mL buffer on 5 g pellet)
- Disrupt cells and pool lysate[SDS]
  - Sonification
  - French Press
  - Or other lysis procedure of choice
- <u>For soluble proteins:</u>
  - Centrifuge at 20 000 rcf for 30 min at 4 °C
  - Discard pellet (cell debris), collect supernatant[SDS]
- <u>For membrane proteins:</u>
  - Option 1: Solubilize from integral cell
  - Option 2: Solubilize from lysate
  - Option 3: Centrifuge at 9 000 rcf for 30 min at 4°C
    - Discard pellet (cell debris), collect supernatant[SDS]
    - Solubilize from supernatant
  - Option 4: Centrifuge at 9 000 rcf for 30 min at 4°C
    - Discard pellet (cell debris), collect supernatant[SDS]
    - Centrifuge supernatant at 100 000 rcf for 1 h at 4°C
    - Discard supernatant[SDS] (soluble proteins), homogenise pellet[SDS]
    - Solubilize from pellet
  - Solubilization from different fractions may have specific advantages and/or disadvantages as seen in Table 3.

Table 3: Advantages and disadvantages of solubilization at different purification steps.

9000 rcf supernatant		100 000 rcf pellet	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Time efficient	Might contain interfering soluble proteins	Might lead to higher purity	Time inefficient
Less likely to lead to protein loss through denaturation	Protein concentration only modifiable through dilution	Protein concentration modifiable through used buffer volume. Ideally: 10mg/mL-30mg/mL	Possible loss of protein
	Protease inhibitor are discarded with supernatant, which might lead to more denaturation		Sufficient homogenisation important for short solubilizations

Solubilization from integral cells or lysate might be faster but also more impure in their yield. It is recommended to start a solubilization from the other two options.

Solubilization of membrane proteins

The solubilization of membrane proteins is dependent on a number of different parameters most of which can be optimised for a higher solubilization efficiency. Parameters include:

- Buffer conditions (salt, pH etc.)
- Choice of detergent or polymer
- Protein-to-solubilization agent-ratio
- Temperature
- Duration

To decide whether detergents or polymers should be used for the solubilization, it is important to consider individual advantages and disadvantages of each agent as well as what application the solubilized membrane proteins shall be used with [see Table 4].

Table 4: Advantages and disadvantages of solubilization agent choice.

Co-polymer		Detergents	
Advantage	**Disadvantage**	**Advantage**	**Disadvantage**
Native lipid environment preserved	Dilution necessary to enable affinity binding	Some detergents able to solubilize even proteins with high TM-counts	Native lipid environment could be stripped away
No need to modify buffer with detergent conc. above CMC[a]	Bivalent cations might lead to precipitation	High solubilization efficiency	Buffer needs to be supplied with detergent conc. above CMC[a]
Mostly no interference with assays	Short-term solubilization unfeasible	Short-term solubilization possible (2 h or less)	Detergents interfere with diagnostic assays
Preservers function and native conformation	May interfere with 280 nm measurements		Can cause denaturation or change in quaternary structure
Solubilization and stabilization are combined into a simple one-step procedure			

[a] Critical Micelle Concentration. Concentrations above the CMC lead to micelle formation needed to stabilise membrane proteins.

Polymers form synthetic nanodisc around the protein, thereby maintaining the native phospholipid environment and preserving the native and thus functional properties of the

protein. Detergents on the other hand form micelles around the hydrophobic belt, thus removing the lipids from the surrounding. Different detergents and polymers are listed in Table 5 and Table 6.

Table 5: Overview of commonly used detergents for solubilization of membrane proteins.

Non-ionic	CMC[a]	Zwitterionic	CMC[a]
DM	0.087 % / 1.8 mM	LDAO	0.023 % / 1-2 mM
DDM	0.0087 % / 0.17 mM	CHAPS	0.5 % / 8-10 mM
LMNG	0.001 % / 0.01 mM	FOS12	0.047 % / 1.5 mM
Brij® 35	0.11 % / 0.092 mM		
Digitonin	0.02-0.03 % / 0.25-0.5 mM		
TRITON™ X-100[b]	0.01 % / 0.2 mM		
TWEEN® 20	0.7 % / 0.06 mM		

[a] Critical Micelle Concentration. Concentrations above the CMC lead to micelle formation needed to stabilise membrane proteins.

[b] Contains phenyl group. Strong absorbance at 280 nm, which interferes with UV/Vis measurements.

Table 6: Overview of commonly used polymers for solubilization of membrane proteins.

SMALP[a]	DIBMA	UltraSolute™ Amphipol	AASTY[a]
140	10	C_8C_0	6-45
140-I	12	C_6C_2	11-45
200	Glucosamine		6-50
300	Glycerol		11-50
502-E			6-55
			11-55

[a] Strong absorbance at 280 nm, which interferes with UV/Vis measurements

If solubilization efficiency is low it is advised to screen the aforementioned of parameters to improve the yield of total solubilized protein.

A standard protocol is described as follows:

- To start, the membrane protein concentration should be at around 10-30 mg/mL
- Add solubilization agent to the protein solution
    - For screening purposes distribute 2 mL per agent
- Ideal concentrations may vary, good starting points are:
    - 1 - 2 % detergent (with 1:10 CHS:detergent if necessary)
    - 0.5 - 5 % SMALP/DIBMA
    - 0.1 - 2.5 % UltraSolute Amphipol
    - 0.1 - 2.5 % AASTY

- Solubilize for 3 h to 24 h at 4 °C while stirring
    - Polymer solubilization for 12-24 h only (multiple solubilization steps possible)
    - Higher temperatures can be screened for optimization
        - Note: this can lead to better solubilization but also to higher degradation
        - Purified proteins need to be tested on their biophysical properties
- Centrifuge at 100 000 rcf for 1 h at 4 °C
    - Discard pellet, collect supernatants[SDS]

Preparing solution for Affinity Chromatography

- Polymers can interfere in the binding of protein to the binding matrix, therefore a reduction of polymer concentration after solubilization can be advantageous
- A dilution of the sample 1:10 before loading onto resin/magbeads^{SDS} showed improved yield after binding with binding matrices for some detergents a similar improvement could be observe
- For sufficient binding a polymer concentration of 0.25 % is essential. A further decrease down to 0.025 - 0.050 % polymer may be advantageous for an improved binding efficiency

Affinity Chromatography

- Prepare binding matrix by discarding the ethanol-based storage buffer and washing the matrix with protein buffer.
- From this point forward protein buffer will be modified according to previous conditions:
    - If protein is His-tagged, the washing buffer contains an increased concentration of imidazole that is deduced individually for the protein of interest. The elution buffer contains high amount of imidazole starting at 250 mM (17 g in 1 L). Exact concentrations have to be determined.
    - If the protein is solubilized in detergent every wash and elution buffer need to contain diluted detergent above the CMC.
- Isolate the protein according to the products' protocol. Commonly used binding affinities are displayed in Table 7.

Table 7: Tags and binding affinities commonly used for protein purification. Binding capacity and elution are displayed as cited by the product's protocol.

Tag	Binding matrix	Binding capacity	Elution with…
His	Ni-NTA Co-NTA INDIGO	60 mg/mL 12 mg/mL 80 mg/mL	250 mM imidazole or more
Rho1D4	Anti-Rho1D4 matrix	2.0 mg/mL	200 µM Rho1D4 peptide
FLAG® 3xFLAG®	Anti-FLAG® matrix	0.6 mg/mL	200 µg/mL FLAG® peptide 200 µg/mL 3xFLAG® peptide
MBP	Amylose	6-8 mg/mL	10 mM maltose
Strep®	StrepTactin®	5 mg/mL	2.5 mM desthiobiotin

- Load protein buffer onto the resin/magbeads
    - Using less amount of resin for His-tagged proteins can be advantageous for purity but may influence the yield
    - Co^{2+}, while binding less, has a higher specificity for His-tagged proteins than Ni^{2+}
- Discard flow-through[SDS] and wash resin multiple times (2-5x) with washing buffer
- Elute protein with elution buffer multiple times for 30 - 60 min[SDS]
    - For first test isolations a small elution volume can be used to get a highly concentrated eluate sample (long elution time up to 4 h)
- **Important note!** A good solubilization of protein does not always correlate with a favourable isolation process. Similarly, a polymer that shows a weak solubilization could still lead to better binding and thus more isolated protein. It is advised to screen polymers not only for solubilization but the whole isolation process.

Analysis of isolation success via SDS-PAGE and Western Blot

During the workflow a lot of samples drawn for SDS-PAGE can be analysed for the success of individual isolation processes. Samples are analysed with a specific Western Blot detection using corresponding tag specific antibodies to determine and track a possible loss of protein during any given purification step. Eluates should also be Coomassie stained to visualise their purity. As membrane proteins are prone to aggregation at high temperatures even in the vicinity of SDS, it is recommended to heat the SDS samples at about 46 °C for 20 min before use. An example of a model purification is seen in Fig.1.

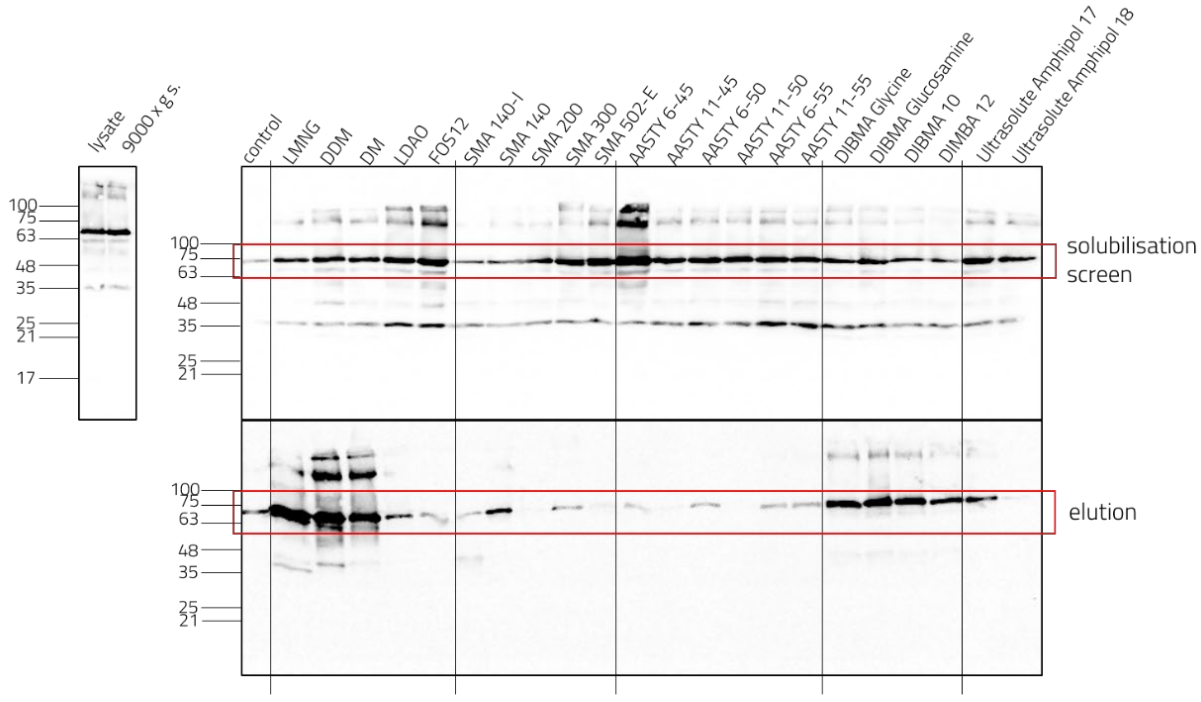

Figure 1: Isolation screening process of a MBP-tagged membrane protein at 68 kDa as tracked through SDS-PAGE with subsequent Western Blot analysis. In this example most solubilization agents were successful in bringing the membrane protein into solution as evidenced by the upper Western Blot. However, as the lower Western Blot shows, only the samples isolated with the detergents and DIBMAs as well as Ultrasolute™ Amphipol 17 retained the protein until the elution stage

Analysis of oligomeric state

After purifying the protein through affinity chromatography, it is important to correctly assign whether it is conserved as a monomer or an oligomer, since most of the time proteins show functionality only in their oligomeric form. The state of oligomerisation can be deduced through measurements of size, for example:

- Dynamic Light Scattering
- Native PAGE/SMA PAGE
- Size Exclusion Chromatography (SEC)

SEC in particular is a popular method as it also allows separation of oligomers from monomers as well as truncated fragments. This allows for further purity and functional analysis of possible native conformations. A protocol for a SEC procedure is as follows:

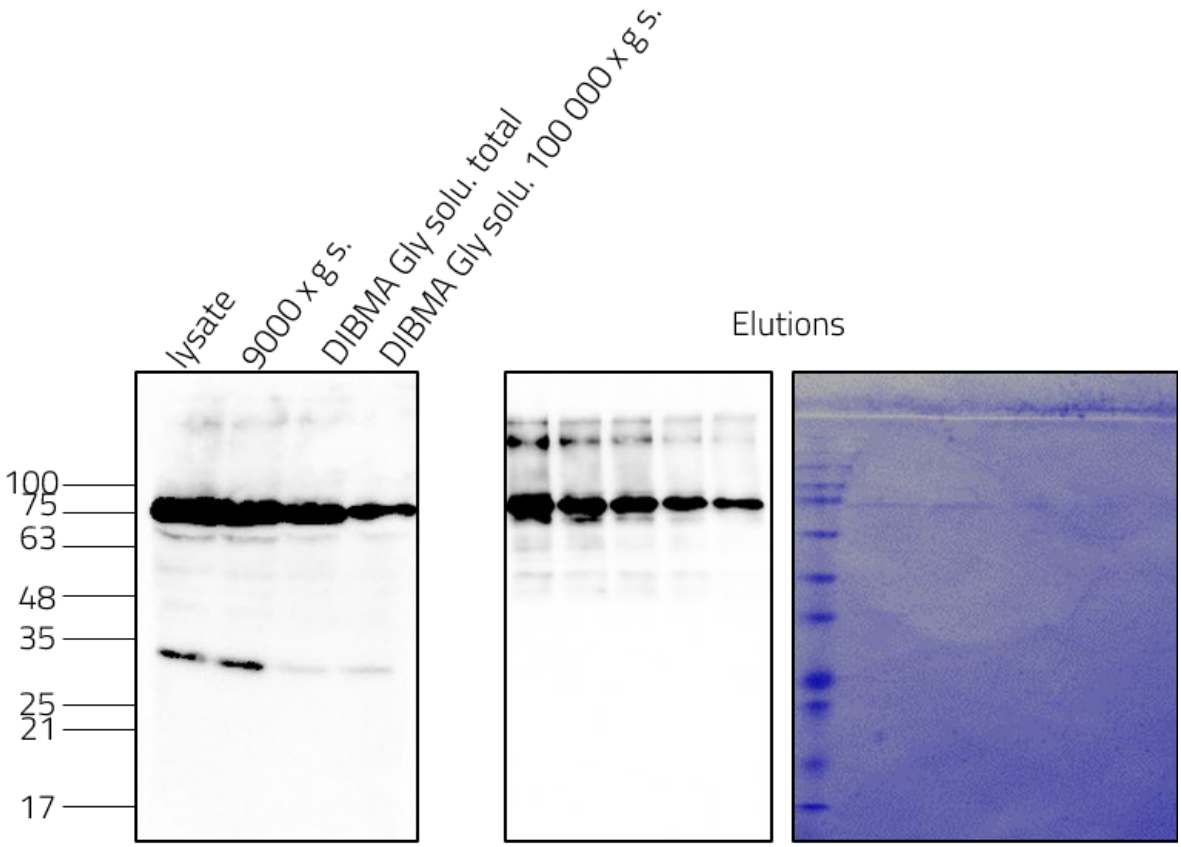

Figure 2: Upscaled isolation of MBP-tagged membrane protein at 68 kDa as tracked through SDS-Page with subsequent Western Blot analysis using optimized conditions as determined in a preceding screening process (see Figure 1). Here, the protein was solubilized with DIBMA Glycerol and further purified with Amylose Resin, after which multiple elutions could be collected. The SDS-PAGE analysis furthermore reveals the elutions to be pure and Western Blot positive.

Choose column with good separation range and resolution for the target protein

- Equilibrate column with 1.5 CV (column volume) of protein buffer
- Meanwhile concentrate protein solution to a final volume matching your column properties
    - Centrifuge repeatedly at max. 3500 rcf for 5 min and 4 °C
    - To prevent aggregation gently mix solution by pipetting up and down between centrifugation steps
- Slowly load protein sample onto the loop of the chromatograph

  - For maximal protein recovery do not use more than half of loop volume
  - For maximal reproducibility use 2-4x the loop volume
- Inject loop solution onto the column and continue run with 1.5 CV protein buffer
  - Fractionate the elution: 1 mL/fraction or 0.5 mL/fraction is a good start for test isolations
- Compare peak position (retention volume) with theoretical protein MW and if necessary verify oligomerization
- Collect SDS samples of every fraction corresponding to the peak and verify purity and identity of target protein with SDS-PAGE/native PAGE and Western Blot.
- Merge samples considered pure and concentrate the solution until target concentration is reached