

Membrane Protein Solubilization Protocol with Copolymers & Detergents

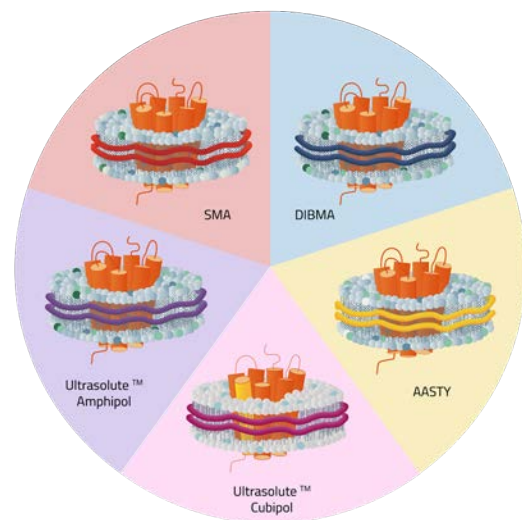
Introduction

Proteins of high stability and purity are of utmost importance for structural research and pharmaceutical drug development yet reaching that goal demands patience. It is riddled with challenges, especially for integral membrane proteins. From choosing the optimal buffer to affinity chromatography, many 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 challenge for 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 copolymers, that can solubilize membrane proteins yet retain native structures of the lipid bilayer. With these new techniques, we present an updated protocol to isolate any membrane protein successfully.

To optimize your screening process we recommend additionally using our [„Copolymer Purification Protocol for Screening Approach“](#) protocol. Which was created in cooperation with Greg Dodge, PhD (Imperiali Lab, MIT).

Please contact us if you have questions or need assistance optimizing the protocol for your application: contact@cube-biotech.com



Available Backbone classes for copolymers: SMA, DIBMA, AASTY, Ultrasolute™ Cubipol, Ultrasolute™ Amphipol

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 is 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 ion²⁺ resins. For higher resistance INDIGO resin is recommended.

^e Divalent cations might lead to precipitation when using certain copolymers.

Before you begin

To observe the efficiencies of the purification steps, it is advised to draw SDS-PAGE samples after important applications and visualize the proteins through Western 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 re-adjust pH value 0.01 mM Leupeptin
 - 0.01 mM E-64
 - 0.1 mM PMSF
 - 1 mM Phenanthroline
- Weigh pellet and resuspend in protein buffer (~ 15 mL buffer on 5 g pellet)
- Disrupt cells and pool lysate^{SDS}
 - Sonification

Note: Protease inhibitors interfere with UV/Vis measurements at 280 nm

- French Press
 - Or other lysis procedure of choice
4. For soluble proteins:
- Centrifuge at 20 000 rcf for 30 min at 4 °C
 - Discard pellet (cell debris), collect supernatant^{SDS}
5. For membrane proteins:
- 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), homogenize 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 x g supernatant		100 000 x g pellet	
Advantages	Disadvantages	Advantages	Disadvantages
Time efficient	Might contain interfering soluble proteins	Might lead to higher purity	10-50 mM pH 6.8-8.2 (pH 7.5 ideal) ^b
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-50mg/ml	up to 10% (v/v)
/	Protease inhibitor are discarded with supernatant, which might lead to more denaturation	/	Varied

Solubilization from integral cells or lysate might be faster but also often 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 that can be optimised for a higher solubilization efficiency.

Parameters include:

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

To decide whether detergents or copolymers 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.

Copolymer		Detergents	
Advantages	Disadvantages	Advantages	Disadvantages
Native lipid environment preserved	Dilution necessary to improve affinity binding especially when working with His-tag	Some detergents can solubilize even proteins with extreme high TM-counts	Native lipid environment could be stripped away
No need to modify buffer with detergent conc. above CMC ^a	Divalent cations might lead to precipitation of some copolymers	High solubilization efficiency	Buffer needs to be supplied with detergent conc. above CMC ^a
Preserves function and native conformation	AASTY and SMA interfere with 280 nm measurements	/	Detergents interfere with diagnostic assays
Solubilization and stabilization are combined into a simple one-step procedure	/	/	Can cause denaturation or change in quaternary structure
Solubilization and purification can be performed at higher temperatures	/	/	/
Solubilization and purification can be performed at higher temperatures	/	/	/

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

Copolymers form synthetic nanodiscs around the protein, thereby maintaining the native phospholipid environment and preserving the protein's native and, thus, functional properties. Detergents, on the other hand, form micelles around the hydrophobic belt, thus stripping the native lipid environment away.

Different detergents and copolymers 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 stabilize membrane proteins.

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

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

SMA ^a	SMALP BZ ^a	DIBMA	Ultrasolute™ Amphipol	AASTY ^a	Ultrasolute™ Cubipol
140	25	10	18	6-45	Cubipol
140-I	30	12	17	11-45	TRIS
200	35	Glucosamine	/	6-50	Sulfo
300	40	Glycerol	/	11-50	Fluorescein
502-E	/	6	/	6-55	Biotin
Sulfo	/	Fluorescein	/	11-55	/
/	/	Biotin	/	/	/
/	/	Glyco	/	/	/
/	/	Sulfo	/	/	/

^a Strong absorbance at 280 nm, which interferes with UV/Vis measurements.

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

A standard protocol is described as follows:

1. Measure the OD of the mix in a Nanodrop or UV-VIS at 280nm. 50 mg/ml is an adequate amount for further solubilization studies (1 mAu = ~1 mg/ml protein).
2. Add solubilization agent to the protein solution.
3. Ideal concentrations may vary, good starting points are:
 - 1 - 2 % detergent (with 1:10 CHS:detergent if necessary)
 - 0.5 - 5 % SMA/DIBMA/ SMALP BZ
 - 0.1 - 2.5 % Ultrasolute™ Amphipol

- 0.1 - 2.5 % AASTY
 - 0.1 - 2.5 % Ultrasolute™ Cubipol
4. Solubilize for 1 h to 16 h at 4 °C – 37 °C while stirring.
 - 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
5. Centrifuge at 100 000 rcf for 1 h at 4 °C
 - Discard pellet, collect supernatants^{SDS}

Preparing solution for Affinity Chromatography

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

Affinity Chromatography

1. Prepare binding matrix by discarding the ethanol-based storage buffer and washing the matrix with protein buffer.
2. 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 amounts 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.
3. Isolate the protein according to the product's 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
 - Co²⁺, while binding less, has a higher specificity for His-tagged proteins than Ni²⁺
- Discard flow-through^{SDS} and wash resin multiple times (3-10x) 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:

It is strongly advised to perform a full purification and analyse the elution samples when working with copolymers. Analyzing the solubilizate via Western-Blot is not an adequate method and will lead to misinterpretation of copolymer performance (see Figure 1.).

Analysis of isolation success via SDS-PAGE and Western Blot

During the workflow a lot of samples drawn for SDS-PAGE can be analyzed for the success of individual isolation processes. Samples are analyzed 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 30 min before use.

An example of a model purification is seen in Figure 1.

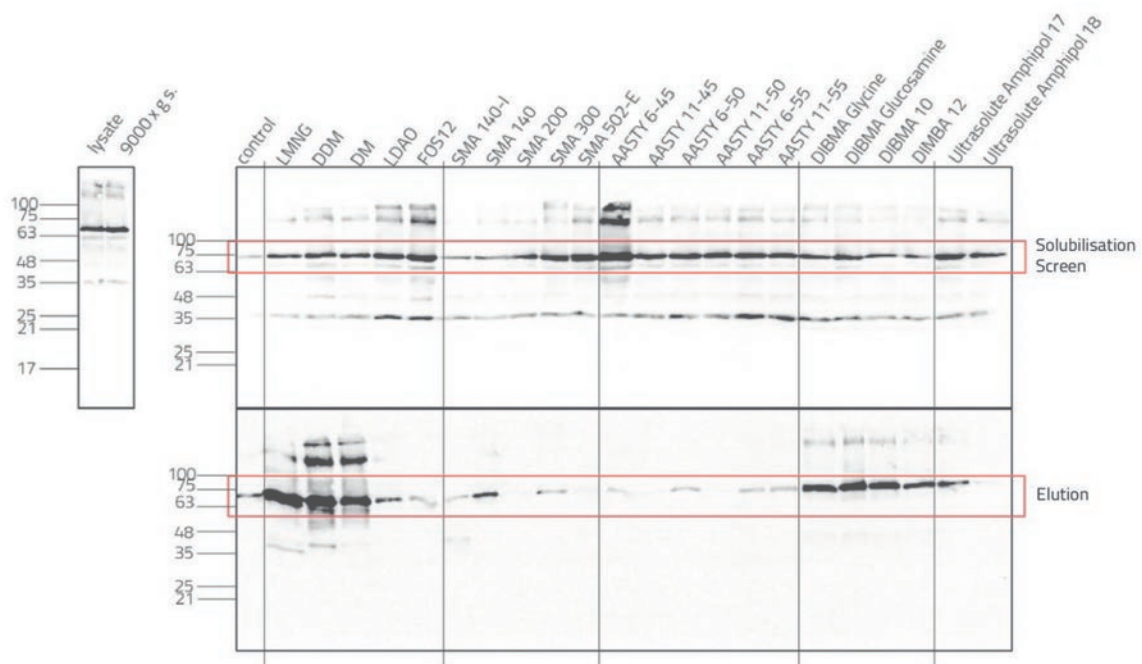


Figure 1: Isolation screening process of a MBP-tagged membrane protein at 68 kDa as tracked through SDS-PAGE with subsequent Western Blot analysis showing the importance of performing a screening until the elution step. In this example most solubilization agents seem successful in bringing the membrane protein into solution as shown by the upper Western Blot. However, as the lower Western Blot shows, only the samples isolated with the detergents, 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. The state of oligomerization 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:

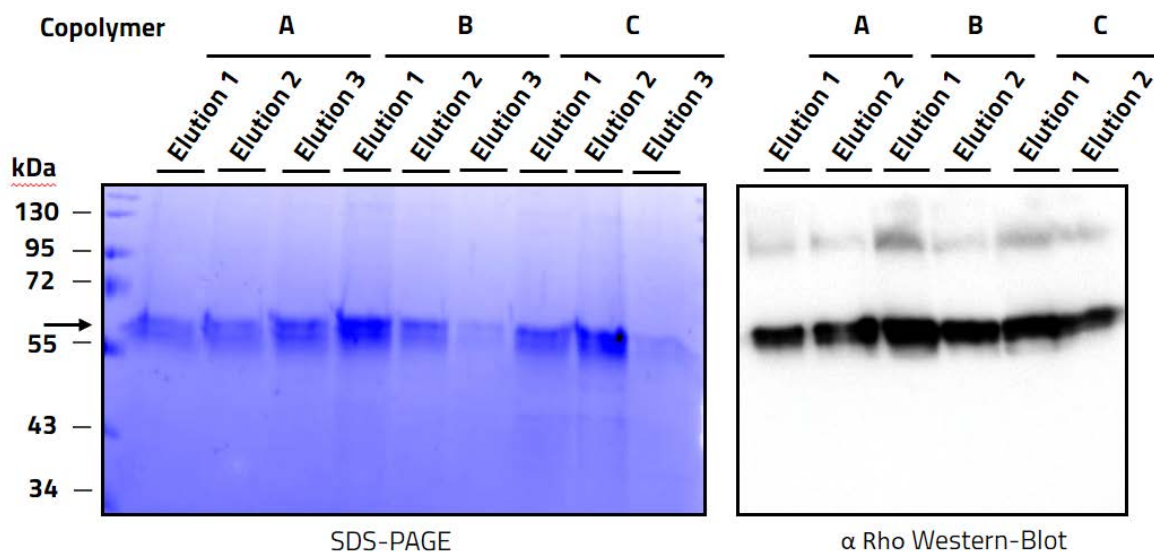


Figure 2: SDS-PAGE and Western Blot analysis of an upscaled Rho-tagged membrane protein isolation. The membrane protein was stabilized in three different Copolymers using optimized conditions as determined in a preceding screening process (as shown in Figure 1). The protein was purified with Rho MagBeads, after which multiple elutions could be collected.

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

- Equilibrate column with 1.5 column volume (CV) of protein buffer
- Meanwhile concentrate your 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 the 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 state.
- 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.