Analytic Extractor Kit

Introduction

Integral membrane proteins (IMPs) insert into biological membranes such that the cell membrane, which is a lipid bilayer approximately 30 Å in thickness, shields the hydrophobic membrane-spanning regions of the protein. To purify IMPs, the protein must first be extracted from the bilayer through the replacement of cell lipids with solubilizing detergents. The selection of detergent for both the initial solubilization of the IMP and for downstream study is exceptionally important, as one must ensure that the selected detergent does not inhibit function, cause irreversible denaturation, or interfere with protein purification or crystallization.

Previous studies on the selection of detergents for the initial extraction of IMPs have demonstrated the importance of detergent screening[1-7]. One of the most exhaustive studies was performed by White *et al.*[1] in which 122 high-expressing predicted membrane proteins were screened in six commonly used detergents: Triton® X-100, LDAO, FC-12, C_8E_4 and DDM. In these tests, the zwitterionic detergents LDAO and FC-12 were the most efficient at solubilizing yeast membrane proteins; however, all of the detergents in the panel solubilized at least 35% of the proteins tested.

The Analytic Extractor Kit (Product No. AL-EXTRACT) contains a panel of the most successful detergents used for the solubilization of IMPs from the cell membrane. The detergents in this panel were chosen based on their prior effectiveness and use in the literature, and the working concentration of each detergent is dependent on the critical micelle concentration (CMC) of each detergent (*i.e.* higher concentrations of lower CMC detergents compared to higher CMC detergents)[2]. The results from this assay will aid in the selection of a detergent for further downstream studies, including X-ray crystallography, NMR, and biochemical assays.

It is important to note that regardless of the success of a particular detergent in extracting the IMP from the cell membrane, subsequent experiments may require the reduction, removal, or exchange of the detergent. For assistance in selecting optimal detergents for downstream experiments, please refer to the Selector Kit within the Analytic product line.

Analytic
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Kit Contents

The Analytic Extractor Kit contains 8 detergents commonly used for solubilization and/or crystallization of IMPs from the membranes of both prokaryotic and eukaryotic cells. Working concentrations of each detergent were chosen based on their CMC.

Common Name	Full Name	Anatrace® Catalog No.	CMC (% [w/v])	Provided Concentration (5X stock)	Working Concentration
Anapoe®-X-100	Triton X-100	APX-100	0.015%	5%	1%
C_8E_4	Tetraethylene Glycol Monooctyl Ether, Anagrade®	T350	0.25%	10%	2%
$C_{12}E_8$	Octaethylene Glycol Monododecyl Ether, Anagrade	O330	0.0048%	2.5%	0.5%
DM	n-Decyl-β-D-Maltoside, Anagrade	D322	0.087%	5%	1%
DDM	n-Dodecyl-β-D-Maltoside, Anagrade	D310	0.0087%	5%	1%
FC-12	Fos-Choline®-12, Anagrade	F308	0.047%	5%	1%
LDAO	n-Dodecyl-N,N-Dimethylamine-N-Oxide, Anagrade	D360	0.023%	5%	1%
OG	n-Octyl-β-D-Glucoside, Anagrade	O311	0.53%	10%	2%

Protocols

Membrane Preparation:

Small scale detergent extraction of membrane proteins can be performed using either membrane fractions or lysed cells. Membrane fractions can be prepared using any conventional method; however, a generic protocol is provided here. The specific buffer needed for the resuspension of cells and membranes is generally protein specific, and the addition of reducing agents and protease inhibitors is often necessary. Additionally, the use of high ionic strength buffers (up to 0.5 M NaCl) has been shown to improve extraction efficiency.

To prepare a membrane fraction, pelleted cells are resuspended in buffer and lysed via sonication, French press, or a microfluidizer. Lysed cells are then centrifuged to remove cell debris (typically 30,000 x g). The supernatant is then subjected to a high-speed centrifugation step (typically 120,000 x g in an ultracentrifuge) to isolate membrane fractions. This membrane fraction can be resuspended, and frozen until needed. Total protein content in resuspended membranes can be determined via Bradford assay or an A_{280} reading. For the Extractor Kit assay, total protein concentration in the resuspended membrane sample should be approximately 0.5 mg/ml.

To prepare a small sample of lysed cells for the Extractor Kit assay, resuspended cells can be lysed using glass beads of an appropriate size (Biospec) followed by centrifugation to remove the beads. The supernatant can then be transferred to a clean tube and frozen at -80°C until later use. Total protein content in the supernatant can be determined via Bradford assay or an A_{280} reading. For the Extractor Kit assay, total protein

concentration in the resuspended membrane sample should be approximately 0.5 mg/ml.

Detergent Extraction:

1) Preparation of detergents:
   a. Each detergent in the Extractor Kit is provided as a 5X stock solution, and each ampule contains enough detergent for five assays. After opening each ampule, make 5 aliquots of 40 µl each using air tight, screw top tubes and store at -20°C.
   b. To prepare the detergent solution for the Extractor Kit assay, transfer 40 µl of each detergent to a clean, labeled, Eppendorf tube.
   c. To each tube, add 160 µl of buffer (this should be the same buffer used to resuspend membranes or cell pellets) to create a 1X detergent solution for each detergent in the Extractor Kit.
2) Solubilization of cell membranes:
   a. Starting with resuspended membranes or lysed cells, add a 9 µl aliquot (~4.5 µg total protein) to each of nine clean, labeled, Eppendorf tubes.
   b. To each tube, add 141 µl of each 1X detergent solution. To one tube, add 141 µl of buffer solution as a negative control.
   c. Incubate samples at room temperature for 1 hour. Samples should be continually agitated during the solubilization process by placing on a roller-mixer, tube-rotator, or similar device.
   d. Spin samples at >100,000g for one hour in an ultracentrifuge to pellet insoluble material. For preparative ultracentrifuges, we recommend the Beckman-Coulter Ti 42.2 rotor. Alternatively for tabletop ultracentrifuges, a number of small volume rotors are available such as the TLA-100 and TLA-120.1. If no small volume ultracentrifuge rotors are available, samples can be centrifuged in a refrigerated tabletop microcentrifuge at the maximum speed; however, this is typically not recommended.
3) Evaluating extraction efficiency:
   a. From each tube, remove 16 µl of supernatant (~0.5 µg) and mix with 4 µl SDS loading buffer.
   b. Load 20 µl of each sample on an SDS-PAGE gel, and run gel using standard protocols (See notes regarding heating membrane protein samples prior to loading on a gel).
   c. As a positive control, it is recommended that ~0.5 µg of resuspended membranes or cell lysate (starting material from step 2a) also be loaded on the gel.
   d. Transfer gel to a membrane and perform an immunoblot (Western) using an appropriate antibody to the protein of interest.
   e. Upon examination of the developed membrane, compare intensities of the bands corresponding to the protein of interest in each lane to one another. Higher intensity bands correspond to a detergent with a greater extraction efficiency.

Notes:

1) The choice of buffer often has an effect on extraction efficiency. As with soluble proteins, buffers should be chosen based upon the isoelectric point of the protein of interest. For buffers in the neutral pH range, HEPES is a commonly used buffer for membrane proteins. Furthermore, polyols stabilize solubilized membrane proteins. Typical glycerol concentrations range from 5-50% (v/v). High concentrations of glycerol will increase the viscosity of the preparations that can cause problems in the ultracentrifuge and on chromatographic columns[3].
2) Solubilization of membranes can be performed at room temperature or at 4°C. Solubilization is typically more effective at room temperature; however, if stability issues arise, longer solubilzation times at 4°C can be used as an alternative.
3) In practice, membrane proteins prepared for analysis by SDS-PAGE are typically not heated prior to loading on the gel. Upon heating, membrane proteins will often form insoluble aggregates that do not enter the gel[4].
4) Oftentimes, membrane proteins will not resolve to their exact molecular weight on an SDS-PAGE gel. Protein detergent complexes will often have different properties which will cause the protein to resolve to a higher or lower molecular weight.
5) As an alternative method to the Western blot protocol, detergent extraction efficiency can also be determined by fluorescence SEC (fSEC). For this assay, membrane proteins of interest are fused with a fluorescent protein, typically GFP, and subjected to size-exclusion chromatography. For more information on the fSEC assay, please refer to references 5-7.

Troubleshooting

The buffer concentration can affect the solubility and stability of proteins. Especially phosphate buffers are known for their solubilizing properties. Concentrations of 0.1-0.5 M phosphate buffer increase the solubility of membrane proteins[4].

References

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