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Analytic Selector Kit

Introduction

Integral membrane proteins present unique challenges to protein chemists and structural biologists. In their natural environment, these proteins exist within a mosaic lipid bilayer that is a dynamic and complex environment. Prior to structural study, however, these must be extracted from the cell membrane and solubilized in such a way as to satisfy the hydrophobic nature of the transmembrane regions while ensuring that hydrophilic domains are in contact with an aqueous phase. While a significant number of detergents may be capable of keeping the protein in a soluble form, the stability, homogeneity, and activity of any given membrane protein can be tremendously affected by the properties of the solubilizing component.

Deposits to the Protein Data Bank for membrane proteins comprise a broad range of unique detergents that were included in protein preparations leading to diffraction-quality crystals. Thus, while certain detergents (DM, DDM, or OG, for example) are known for their success in stabilizing integral membrane proteins for structural studies and are reported at high frequency, significant effort would be required to explore detergent space, even to cover detergents previously successful for protein crystallization.

The Analytic Selector Kit (Product No. AL-SEL) is a rapid, high throughput assay that explores detergent space to identify those best suited to stabilize the membrane protein of interest in advance of downstream protein crystallization trials. The assay uses differential filtration in 94 different detergents to generate information regarding both the stability and the size of the protein-detergent complex. The steps of this assay are simple: First, the protein is bound to an affinity resin, washed, and eluted in a new detergent. The eluate is then passed through two filter plates with differing molecular weight cutoffs. The filtration profiles report on stability (assessed by the degree of aggregation) and relative size of the protein-detergent complex. The sizing information from this assay correlates well with traditional size exclusion chromatography, which allows for the selection of detergents most likely to stabilize a protein in a manner that would be commensurate with crystallization and structure determination.

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Results from the Selector Kit can be obtained in approximately two hours using only a few hundred micrograms of protein. This kit is especially useful for membrane proteins which have undergone extensive crystallization trials with limited success. As the detergent used to solubilize the protein has a great effect on the propensity of a membrane protein to crystallize, exploring new detergents is an effective way to move membrane protein targets through the crystallization pipeline. The detergent panel used in the Selector Kit facilitates the testing of novel detergents for membrane proteins and offers particular benefit to those that are unstable in the small, commonly used, traditional set of detergents.

References
1. Vergis, J. M., *et al.* (2010) *Anal. Biochem.* **407(1)**, 1-11.
2. Vergis, J. M., *et al.* (2011) *Prot. Expr. Purif.* **78(2)**, 139-142.

Materials Needed

Kit Contents:

- One detergent screening plate containing 150 µl of 94 detergents at 2X working concentration, one blank and one position for the detergent currently stabilizing the protein sample
- One 0.22 µm filter plate with receptacle plate for detergent exchange
- One receptacle plate into which the proteins and newly exchanged detergents will be eluted
- One 100 MWCO filter plate with matched receptacle plate
- One 300 MWCO filter plate with matched receptacle plate

All filter plate-receptacle plate pairs provided in this kit are compatible with centrifugation. No adaptors or vacuum manifolds are required.

Materials Not Supplied:

In addition to the contents of this kit, you will need:

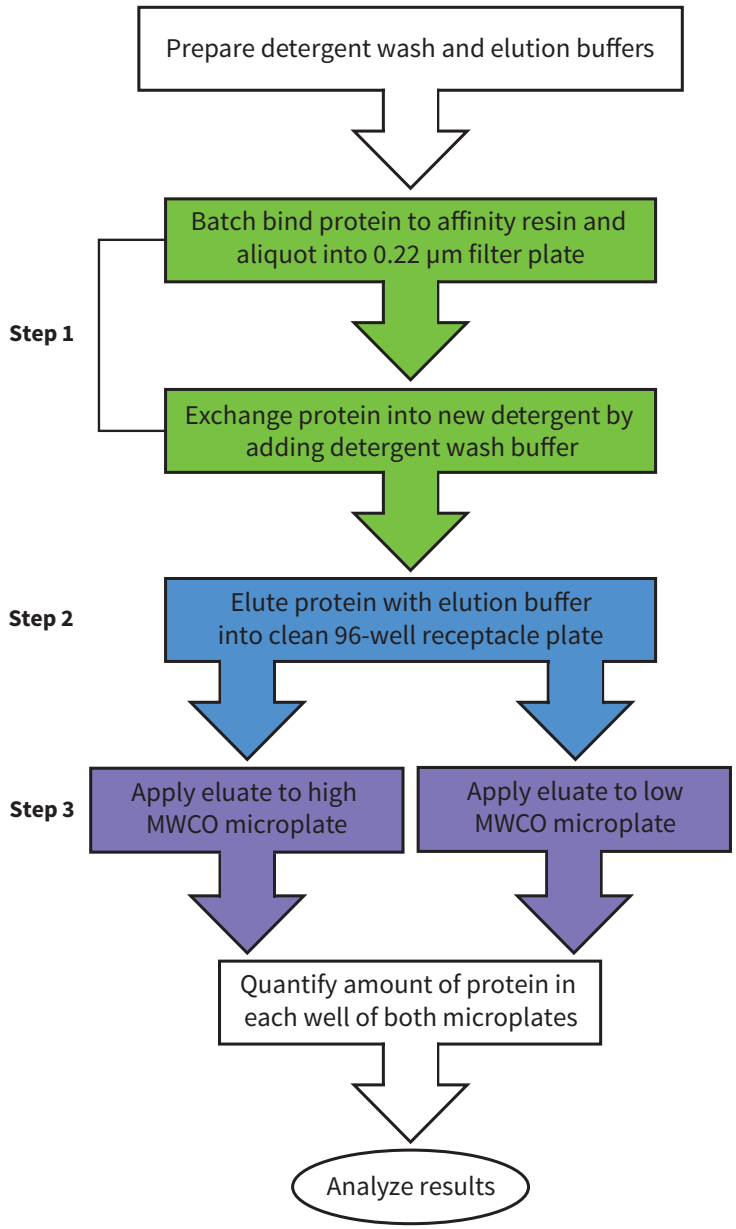
- Affinity resin
- 2X wash buffer
- 2X elution buffer
- Currently used detergents and buffers
- 96-well microplate
- Multi-channel pipette and basins
- Centrifuge with microplate adapters
- Appropriate method for detection and quantification of protein

Prerequisites

The input sample for this assay should preferably be a pure, detergent-solubilized protein with the affinity tag still attached.

If using an antibody-based detection method for your protein, the sample does not need to be fully purified, but it must not be proteolysed, as there will be no distinction between the proteolysed and non-proteolysed protein.

Overview



Detergent Preparation and CMC

Row	Column	Number	Detergent	Abbreviation	Anatrace Prod. No.	CMC (mM)	[Stock Plate] (mM)	Working Conc. (mM)
A	1	1	Water					
A	2	2	Blank (for solubilizing detergent)					
A	3	3	Anzergent® 3-10	Z3-10	AZ310	39	156	78
A	4	4	Anzergent 3-12	Z3-12	AZ312	2.8	16.8	8.4
A	5	5	Anzergent 3-14	Z3-14	AZ314	0.2	20	10
A	6	6	n-Decyl-N,N-Dimethylglycine	DMG	D352	19	76	38
A	7	7	n-Dodecyl-N,N-Dimethylglycine	DOMG	D350	1.5	9	4.5
A	8	8	n-Decyl-N,N-Dimethylamine-N-Oxide	DDAO	D365	10.5	42	21
A	9	9	n-Undecyl-n,n,-Dimethylamine-Oxide	UDAO	U360	3.2	19.2	9.6
A	10	10	n-Dodecyl-N,N-Dimethylamine-N-Oxide	LDAO	D360	1	6	3
A	11	11	Cyclofos™-3	CF-3	C510	43	172	86
A	12	12	Cyclofos-4	CF-4	C512	14	56	28
B	1	13	Cyclofos-5	CF-5	C514	4.5	27	13.5
B	2	14	Cyclofos-6	CF-6	C516	2.68	16.08	8.04
B	3	15	Cyclofos-7	CF-7	C518	0.62	12.4	6.2
B	4	16	Fos-Choline®-12	FC-12	F308	1.5	9	4.5
B	5	17	Fos-Choline-13	FC-13	F310	0.75	15	7.5
B	6	18	Fos-Choline-14	FC-14	F312	0.12	12	6
B	7	19	Fos-Choline-15	FC-15	F314	0.07	14	7
B	8	20	Fos-Choline-16	FC-16	F316	0.013	2.6	1.3
B	9	21	Fos-Choline-ISO-9	FC-I9	FCI09	32	128	64
B	10	22	Fos-Choline-ISO-11	FC-I11	FCI11	26.6	106.4	53.2
B	11	23	Fos-Choline-UNSAT-11-10	FC-U10-11	FCU110	6.2	31	15.5
B	12	24	1,2-Diheptanoyl-sn-Glycero-3-Phosphocholine	DHPC	D607	1.4	8.4	4.2
C	1	25	LysoPC-12	LPC-12	L212	0.7	14	7
C	2	26	LysoPC-14	LPC-14	L214	0.036	7.2	3.6
C	3	27	CHAPS	CHAPS	C316	8	40	20
C	4	28	CHAPSO	CHAPSO	C317	8	40	20
C	5	29	Ph-Tripglu	Ph-Tripglu	T380	3.6	21.6	10.8
C	6	30	Cy-Tripglu	Cy-Tripglu	T385	1.8	10.8	5.4
C	7	31	LAPAO	LAPAO	L360S	1.6	9.6	4.8
C	8	32	Tripao	TRIPAO	T370	4.5	27	13.5
C	9	33	Anapoe®-20 (Tween 20)	T-20	APT020	0.059	11.8	5.9
C	10	34	Anapoe-35 (Brij 35)	Brij-35	APB035	0.091	18.2	9.1
C	11	35	Anapoe-X-100	TX-100	APX100	0.23	23	11.5
C	12	36	Anapoe-X-114	TX-114	APX114	0.2	20	10
D	1	37	Anapoe-X-305	TX-305	APX305	0.65	13	6.5
D	2	38	Anapoe-X-405	TX-405	APX405	0.81	16.2	8.1
D	3	39	[Octylphenoxy]Polyethoxyethanol	NID-P40	APND40	0.3	30	15
D	4	40	Lauryl Maltose Neopentyl Glycol	LMNG	NG310	0.01	2	1
D	5	41	Octyl Glucose Neopentyl Glycol	OGNG	NG311	1.02	6.12	3.06
D	6	42	Decyl Maltose Neopentyl Glycol	DMNG	NG322	0.036	7.2	3.6
D	7	43	CYMAL®-5 Neopentyl Glycol	CYMAL-5-NG	NG325	0.058	11.6	5.8
D	8	44	CYMAL-6 Neopentyl Glycol	CYMAL-6 NG	NG326	0.02	4	2
D	9	45	GDN101 - GDN	GDN	GDN101	0.018	3.6	1.8
D	10	46	Triethylene Glycol Monohexyl Ether	C_6E_3	T330	23	92	46
D	11	47	Tetraethylene Glycol Monohexyl Ether	C_6E_4	T340	30	120	60
D	12	48	Pentaethylene Glycol Monohexyl Ether	C_6E_5	P360	37	148	74

Continued on next page.

Row	Column	Number	Detergent	Abbreviation	Anatrace Prod. No.	CMC (mM)	[Stock Plate] (mM)	Working Conc. (mM)
E	1	49	Pentaethylene Glycol Monoheptyl Ether	C_7E_5	P370	21	84	42
E	2	50	Tetraethylene Glycol Monooctyl Ether	C_8E_4	T350	8	40	20
E	3	51	Pentaethylene Glycol Monooctyl Ether	C_8E_5	P350	7.1	35.5	17.75
E	4	52	Hexaethylene Glycol Monooctyl Ether	C_8E_6	H350	10	50	25
E	5	53	Pentaethylene Glycol Monodecyl Ether	$C_{10}E_5$	P340	0.81	16.2	8.1
E	6	54	Hexaethylene Glycol Monodecyl Ether	$C_{10}E_6$	H360	0.9	18	9
E	7	55	Polyoxyethylene(9)decyl Ether	$C_{10}E_9$	APO109	1.3	7.8	3.9
E	8	56	Heptaethylene Glycol Monododecyl Ether	$C_{12}E_7$	H370	0.069	13.8	6.9
E	9	57	Octaethylene Glycol Monododecyl Ether	$C_{12}E_8$	O330	0.09	18	9
E	10	58	Polyoxyethylene(9)dodecyl Ether	$C_{12}E_9$	APO129	0.05	10	5
E	11	59	Polyoxyethylene(10)dodecyl Ether	$C_{12}E_{10}$	AP1210	0.1	20	10
E	12	60	Polyoxyethylene(8)tridecyl Ether	$C_{13}E_8$	APO138	0.1	20	10
F	1	61	Big CHAP	CHAP	B300	2.9	17.4	8.7
F	2	62	Big CHAP, Deoxy	CHAP-D	B310	1.4	8.4	4.2
F	3	63	n-Heptyl-β-D-Thioglucopyranoside	HTG	H301	29	116	58
F	4	64	n-Octyl-β-D-Thioglucopyranoside	OTG	O314	9	25	12.5
F	5	65	n-Octyl-β-D-Glucopyranoside	OG	O311	18	72	36
F	6	66	n-Nonyl-β-D-Glucopyranoside	NG	N324	6.5	32.5	16.25
F	7	67	CYGLU®-3	CYGLU-3	C323G	28	112	56
F	8	68	HECAMEG	Anameg-7	A340	19.5	78	39
F	9	69	Hega-9	HEGA-9	H109	39	156	78
F	10	70	Hega-10	HEGA-10	H110	7	35	17.5
F	11	71	Mega-9	M9	M325	25	100	50
F	12	72	Mega-10	M10	M320	6	12	6
G	1	73	2-Hydroxyethyloctylsulfoxide	OHES	Bachem - P1105	24.3	96.8	48.4
G	2	74	CYMAL-3	CYMAL-3	C323	30	120	60
G	3	75	CYMAL-4	CYMAL-4	C324	7.6	38	19
G	4	76	CYMAL-5	CYMAL-5	C325	2.4	14.4	7.2
G	5	77	CYMAL-6	CYMAL-6	C326	0.56	11.2	5.6
G	6	78	CYMAL-7	CYMAL-7	C327	0.19	19	9.5
G	7	79	2,6-Dimethyl-4-Heptyl-β-D-Maltoside	DMHM	DH325	27.5	110	55
G	8	80	2-Propyl-1-Pentyl-β-D-Maltopyranoside	PPM	P310	42.5	170	85
G	9	81	n-Octyl-β-D-Maltopyranoside	OM	O310	19.5	78	39
G	10	82	n-Nonyl-β-D-Maltopyranoside	NM	N330	6	30	15
G	11	83	n-Decyl-α-D-Maltopyranoside	DαM	D322HA	1.6	9.6	4.8
G	12	84	n-Decyl-β-D-Maltopyranoside	DM	D322	1.8	10.8	5.4
H	1	85	n-Undecyl-α-D-Maltopyranoside	UDαM	U300HA	0.58	11.6	5.8
H	2	86	n-Undecyl-β-D-Maltopyranoside	UDM	U300	0.59	11.8	5.9
H	3	87	ω-Undecylenyl-β-D-Maltopyranoside	ωUDM	U310	1.2	7.2	3.6
H	4	88	n-Dodecyl-α-D-Maltopyranoside	DDαM	D310HA	0.15	15	7.5
H	5	89	n-Dodecyl-β-D-Maltopyranoside	DDM	D310	0.17	17	8.5
H	6	90	n-Tridecyl-β-D-Maltopyranoside	TDM	T323	0.03	3	1.5
H	7	91	n-Octyl-β-D-Thiomaltopyranoside	OTM	O320	8.5	42.5	21.25
H	8	92	n-Nonyl-β-D-Thiomaltopyranoside	NTM	N350	3.2	19.2	9.6
H	9	93	n-Decyl-β-D-Thiomaltopyranoside	DTM	D335	0.9	18	9
H	10	94	n-Undecyl-β-D-Thiomaltopyranoside	UDTM	U342	0.21	21	10.5
H	11	95	n-Dodecyl-β-D-Thiomaltopyranoside	DDTM	D342	0.05	10	5
H	12	96	Sucrose 12	S-12	S350	0.3	30	15

Protocol

Protein Preparation:

1. Perform batch binding of your protein sample to the affinity resin of choice. A total of 10 µl of affinity resin is needed in each well of the 0.22 µm filter microplate. The Talon resin protocol below has been provided as a reference.
   a. Add 2.4 ml of 50% Talon slurry to a 15 ml tube, and equilibrate resin with the current detergent buffer.
   b. Add a total of 500 µg of protein sample to the tube and bring the total volume up to 6 ml using the current detergent buffer. This yields a 20% slurry.
   c. Batch bind your sample to the affinity resin overnight at 4°C.

Detergent Plate Preparation:

2. The detergent stock plate has been prepared under argon. We strongly recommend that the detergents be stored at or below 4°C prior to use.
3. Thaw detergent stock plate
   a. You may need to resuspend detergents by gentle heating.
   b. Centrifuge plate prior to removing seal.
4. Remove seal from detergent stock plate and label plate "wash plate".
5. Add 150 µl of the current stabilizing detergent, at 2X concentration, to well A2.
   Example: If the protein is currently stabilized in a buffer containing 1% DDM, add 150 µl 2% DDM to well A2.
6. Dispense 40 µl of 2X elution buffer into a 96-well microplate (not included). Label this plate "elution plate".
7. Add 40 µl of 2X detergent stock to the elution plate, mixing the solutions by pipetting up and down several times.
8. Add 110 µl of 2X wash buffer to the remaining 110 µl of 2X detergent stock plate (labeled wash plate in step 4), mixing the solutions by pipetting up and down several times.
9. Upon completion of the above steps, the "wash plate" should contain 220 µl of 1X detergent solutions, and the "elution plate" should contain 80 µl of 1X detergent solutions.

Step 1: Detergent Exchange

10. Using a multi-channel pipette, dispense 50 µl of the protein-bound affinity resin slurry (20%) into each well of the 0.2 µm filter plate. This plate is prepackaged with a snap-fit receptacle plate that can be used directly for centrifugation.
    Recommended: Cut the ends of the pipette tips to create a wider bore to easily pipette resin.
11. Wash resin and remove unbound protein by adding 150 µl of the current detergent buffer to each well of the 0.22 µm filter plate, and centrifuging at 2000 x g for 2 minutes. Discard flow-through. Repeat this step 5 times.

12. Add 30 µl of the prepared detergent wash buffer from the "wash plate" (step 8) to each well of the 0.22 µm filter plate. Centrifuge at 2000 x g for 2 minutes and discard flow-through. Repeat this step 6 times. On the final addition of the wash buffer, use the remaining amount of buffer.

Step 2: Protein Elution

13. Replace the used receptacle plate with a new elution receptacle.
14. Add 70 µl of the prepared detergent elution buffer from the "elution plate" (steps 6 and 7) to each well of the 0.22 µm filter plate and spin the plate at 2000 x g for 2 minutes. Save the flow-through.

Step 3: Differential Filtration

15. The Selector Kit includes 100K MWCO and 300K MCWO filter plates that have each been preassembled with a receptacle plate in which to collect the filtrate. The eluate from step 14 will be applied in equal volumes to these plates.
16. Add 30 µl of the elution to both MWCO filter plates.
17. Spin both plates at 2000 x g for 4 minutes and save the flow-through. If the elution volumes of the 100K MWCO plate seem low, spin again for another 2 minutes.

The eluate in both receptacle plates is now ready for protein detection. If a stopping point is needed, the receptacle plates can be sealed and stored at 4°C. The precise duration of storage will be protein-specific.

Protein Detection:

To determine stability and size information about the protein of interest, the relative amount of protein in each well of both elution microplates needs to be quantified. This can be accomplished by multiple methods, which have been briefly outlined below. Due to the sensitivity of antibody-based detection methods, we recommend the use of dot blot or ELISA methods for the Selector Kit assay. Additionally, the presence of fluorescent tags or co-factors can also be detected (*e.g.* GFP). These methods lessen the protein requirement and also enable detection from non-purified or partially purified samples. Other spectroscopic methods are provided as alternatives; however, there are some detergents in the panel that are not compatible with these methods, as outlined below.

Dot Blot

This assay takes advantage of the affinity tag used for purification to perform a Western blot or an antibody specific for protein of interest. In order to perform this assay, one must have the appropriate primary and secondary antibodies for their protein, as well as a dot-blot apparatus, and a method to quantify intensities of each spot. We recommend that the user applies 10 µl of each filtrate to the wells of the dot blot apparatus, ensuring that the deposited solution is well mixed, evenly spread

around the well, and washed thoroughly. Antibody dilution and incubation will depend on the chosen antibodies. Quantification of spot intensities should follow the manufacturer's recommendations depending on the available system.

Enzyme-linked immunosorbant assay (ELISA)
ELISAs are a plate based assay where the protein of interest is immobilized to a 96-well plate either through direct adsorption to the polystyrene plate or through interaction with an antibody coated on the plate surface. Once immobilized, detection of the protein can be performed using an enzyme-conjugated primary antibody (direct detection), or a primary antibody and enzyme-conjugated secondary antibody (indirect detection). ELISAs are protein specific, so it is recommended that the experimental procedures for detecting your protein of interest be worked out prior to using this assay for detection in the Selector Kit.

Detection of GFP-tagged proteins
In recent years, the use of Green Fluorescent Protein (GFP) fused to one termini of the membrane protein has been exploited to facilitate the selection of effective detergents by an alternate method, fluorescence detection size exclusion chromatography. As a result, numerous GFP-tagged protein constructs are readily available in many membrane protein laboratories. The GFP fluorophore emits green fluorescence light at 509 nm on excitation at 395 nm or 498 nm. The fluorescence of GFP can be used to detect and quantify the protein of interest in the Selector assay.

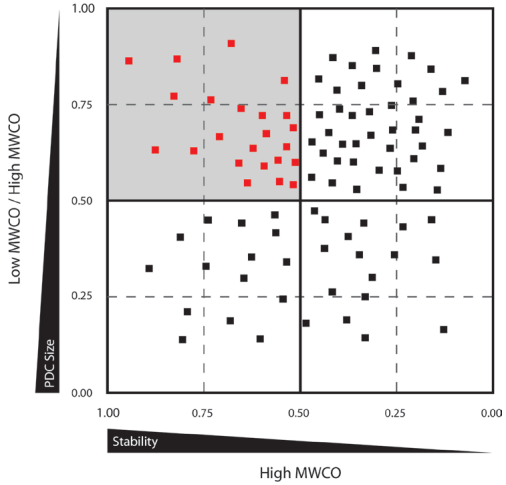
Alternative spectroscopic methods
Direct detection of the protein using absorbance at 280 nm can be performed; however some of the detergents do absorb at this wavelength. Additionally, if the protein of interest has any co-factors or tags that can be detected by spectroscopic methods (*e.g.* heme groups), they may be used for protein detection. Full absorbance scans (200 nm – 800 nm) of each detergent in the panel are available upon request.

When excited at 280 nm, most proteins will emit fluorescence at 320 nm. This detection method is applicable to the Selector assay since only the relative amounts of protein present in each well of the elution plates need to be quantified. Please note that five of the detergents in the panel (C11 – D3) do emit 320 fluorescence when exited at 280 nm, and should be removed from the final analysis if using this detection method.

Data Analysis
Import the quantified protein values into the **Selector Analysis Kit spreadsheet** available for download from **www.microlytic.com**. This will create a "quad-plot" in which assay results are sorted into quadrants based on the stability and size of the protein-detergent complex.

Upon entry, the analysis spreadsheet will normalize the values in each well to the well with the highest amount of protein. The normalized values from the high MWCO filter plate will be plotted on the horizontal axis, while the ratios of low:high normalized values are plotted on the vertical axis.

The high MWCO values report on protein stability, with values approaching 1 indicating a stable (non-aggregated) protein and values approaching 0 indicating a less stable (aggregated) protein. The ratio of the low MWCO to high MWCO values are calculated to indicate the relative size of the protein-detergent complex (PDC). This ratio is inversely proportional to the PDC size, with values approaching 1, indicating a small PDC size, and values approaching 0, indicating a large PDC size.



The readout of this analysis may help identify detergents that are suitable for crystallization and other downstream applications. Detergents in the upper left quadrant are shown to stabilize the protein and reduce the size of the PDC. Both of these properties correlate to the crystallizability of membrane proteins.

Additional Support
If problems arise, please contact Technical Support for assistance at 1-781-214-6827 or **www.microlytic.com/support**. A corresponding technical video is available on our website at **www.microlytic.com/videos**.