Proteus Detergent Anion Exchange Mini Spin Columns

User Guide

Neo-Biotech novel Proteus Detergent Anion Exchange (DetEx) Mini Spin Columns designed for rapid and effective removal of free detergents micelles and complete detergent exchange. Optimized for membrane proteins with pI<8 in complex with non-ionic or zwitterionic detergents.

Simple and adaptable to your protein requiring only a microfuge for operation. Faster and more effective than other methods of detergent removal or exchange such as chromatography (hydrophobic adsorption, ion exchange, affinity, gel filtration), dialysis and precipitation.

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Features of the Proteus DetEx Mini Spin columns:

- Rapid removal of free detergent micelles in 10 minutes.
- Generate concentrated protein free of detergent micelles
- Complete detergent exchange/removal.
- Weak anion exchanger for binding membrane proteins with pI<8.
- Universal appeal as most proteins have a pI between 4-8.
- High protein binding capacity (2 mg).
- Simple load → wash → elute procedure.
- Elution in a small volume (minimum volume 50 µl).
- Compatible with common lab instrumentation (only requires a microfuge).

Specification:

Item:	NB-45-00059-4	NB-45-00059-20
Column bed volume:	0.2 ml	0.2 ml
Membrane matrix:	Stabilized regenerated cellulose	Stabilized regenerated cellulose
Nominal pore size	3-5 µm	3-5 µm
Thickness	230-320 µm	230-320 µm
Amount of ionic groups	145-218 µEquivalents/ml for monovalent ions^{+}	145-218 µEquivalents/ml for monovalent ions^{+}
Membrane Area:	7.5 cm^2	7.5 cm^2
Working pH:	4-10	4-10
Binding capacity*:	2 mg	2 mg
Membrane chemistry:	Diethylamine Ion Exchange, $R-CH_2-NH^+-(CH_2H_5)_2$	Diethylamine Ion Exchange, $R-CH_2-NH^+-(CH_2H_5)_2$
Approximate pKa or ionic groups:	9.5	9.5
Maximum loading volume:	0.4 ml (fixed angle rotor)	0.4 ml (fixed angle rotor)
Minimum elution buffer volume:	50 µl	50 µl
Number of spin columns:	4	20
Number of collection tubes:	8	40

*NOTE: Actually yields are dependent upon the specific protein sample and selected pH and salt conditions. The estimated yields quoted were established using 1 mg/ml BSA in 25 mM Tris-HCl pH 8.0.

Chemical compatibility:

Buffer stability	
Alcohols	
Ethanol:	100%
Methanol:	60%, 100% (limited compatibility)
n-propanol:	60%
Isopropanol:	100%
Butan-2-ol:	100%
Glycerol:	100%
Acids	
HCl:	1 M
Acetic Acid:	1 M
Formic Acid:	25% (pH 1.0 solution)
Alkalis	
NaOH:	1 M
Ammonium Hydroxide:	1%
Detergents	
Octyl-β-D-Glucopyranoside (β-OG):	2%
Tween 20:	2%
Guanidine HCl:	6 M
Urea:	8 M
Other additives	
Imidazole:	500 mM
NaCl:	5 M
DTT:	100 mM
2-mercaptoethanol:	100 mM
DMSO:	100%
EDTA (Na Salt):	5%

Principles of membrane protein purification:

The solubilisation of biological membranes by excess detergent is necessary for the extraction of membrane proteins in a native state. Common non-ionic detergents such as Dodecyl β-D-maltoside (β-DDM), Decyl Maltoside (β-DM), Octylβ-D-glucoside (β-OG) mimic the lipid bilayer of the native environment facilitating solubilzation of both peripheral (extrinsic) and integral (intrinsic) membrane proteins.

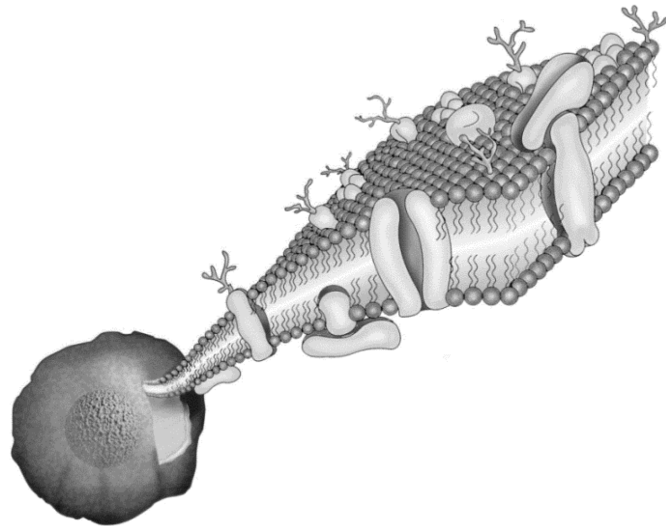


Figure 1. Schematic of a cell membrane showing the lipid bilayer, protein and sugar components.

Historically, most membrane protein chemists purify their protein using a poly-histidine tag with affinity for a Nickel-IMAC resin or column via the following main steps:

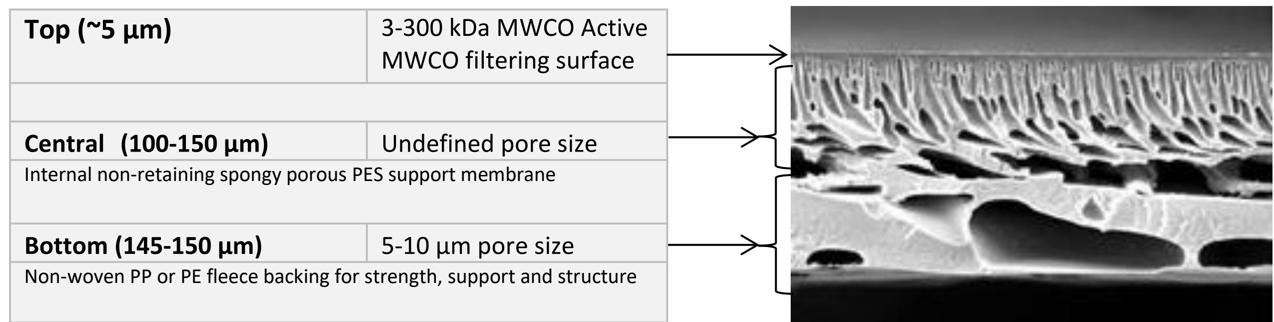
1. Cell lysis and membrane preparation and washing.
2. Solubilisation of membranes.
3. Pelleting down of un-solubilised material.
4. Incubation of the extract with the Nickel-IMAC resin (Neo-Biotech Code: NB-45-00042-25) pre-equilibrated with buffer.
5. Washing of column with equilibration buffer and elution with imidazole. *
6. Optional gel filtration or ion exchange chromatography involving elution with a salt gradient.
7. Concentration of the final sample using an Ultrafiltration (UF) device.

Purification protocols typically use buffers with detergent concentrations slightly higher than the Critical Micellar Concentration (CMC) in order to maintain protein-detergent complexes (PDC) in solution. Modest to extensive protein concentrating using Ultrafiltration (UF) or a stirred-cell is typically carried out after the final chromatography step. This process unfortunately often leads to an increase in free detergent micelles concentration to levels which, while not necessarily detrimental to protein stability and activity, can impede the crystallization process. Consequently the concentration of detergent is often unknown in the final sample leading to a lack of reproducibility in downstream processes.

Existing methods for the removal or exchange of detergent include chromatography (hydrophobic adsorption, ion exchange, affinity, gel filtration), dialysis and precipitation. Besides protein factors, the choice is dependent on the detergent properties such as its CMC, hydrophile-lipophile balance and molecular weight. Apart from the time-consuming dialysis method most are labor intensive.

Ultrafiltration

During Ultrafiltration (UF), the ability of free detergent micelles to pass through the molecular weight cutoff membrane does not always correlate well with the detergent micelle size due to the non-homogeneous and asymmetric pore structure. A UF membrane is not a mesh with defined and uniform pores. The mean pore size is smaller than the nominal (maximum) pore size. For example, a 100 kDa molecular weight cut off (MWCO) UF membrane will retain up to 80% of a 65 kDa protein (see figure 2).



Top (~5 µm)	3-300 kDa MWCO Active MWCO filtering surface
Central (100-150 µm)	Undefined pore size
Internal non-retaining spongy porous PES support membrane	
Bottom (145-150 µm)	5-10 µm pore size
Non-woven PP or PE fleece backing for strength, support and structure	

Figure 2. Asymmetric ultrafiltration membrane with a typical 300 µm thickness.

Proteus DetEx Mini Spin columns:

The Proteus DetEx Mini Spin columns offer a novel, rapid, simple and effective method for removal of free detergents micelles and complete detergent exchange. Optimized for membrane proteins with pI <8 in complex with non-ionic or zwitterionic detergents.

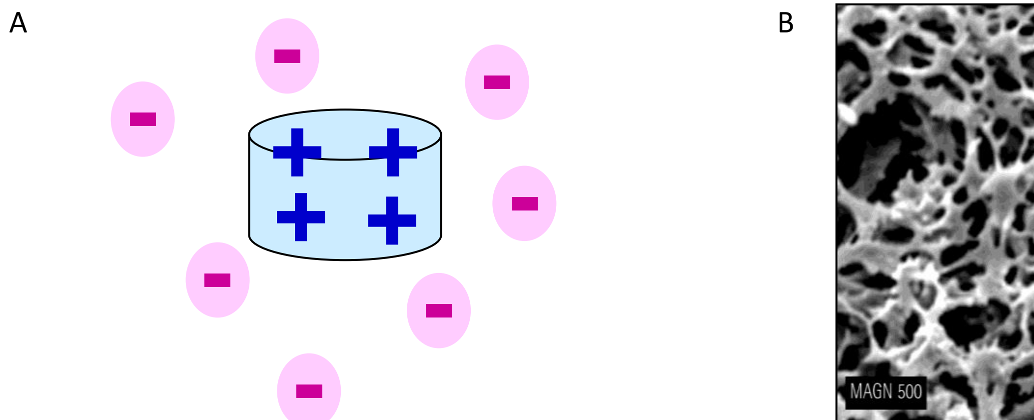


Figure 3. The Proteus DetEx Mini Spin columns are comprised of an anion exchange membrane surface which is covered with positively-charged diethylamine functional groups (A), with an homogenous and symmetrical charged microfiltration membrane (B).

Free micelle removal conditions:

This protocol describes the depletion of free detergent micelles from IMAC affinity purified recombinant His-tagged membrane proteins using a Proteus DetEx Mini Spin Column. This

requires a microfuge centrifuge that can accommodate 2.0 ml microcentrifuge tubes and is capable of spinning samples at speeds of 2,000 x g.

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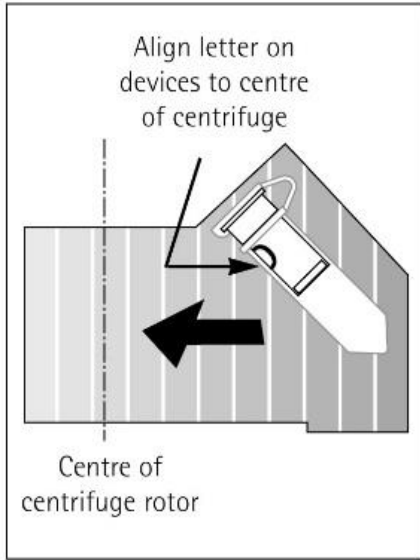

Figure 4. Orientation of Proteus DetEx Mini Spin Column for optimum performance using a fixed angle rotor.

NOTE: If using a fixed angle rotor it is important to consider the orientation of the Proteus Detergent Exchange Mini Spin Column during centrifugation. To achieve the best results, align the D letter on the Proteus column towards the centre of the rotor for all the binding, washing and elution steps. This will guarantee even liquid flow through the membrane during each chromatography step (see figure 4).

Example buffers:

Loading buffer (salt depleted):
50 mM Tris pH 7 to 8 with the detergent of choice

Wash buffer (salt and detergent depleted):
50 mM Tris pH 7 to 8 without detergent

Elution buffer:
50 mM Tris pH 7 to 8, 50-100mM salt (for example $MgSO_4$), with or without detergent

NOTE: Check that the pH of the buffer is above the iso-electric point of the protein.
e.g. a protein with a pI of 5 will be negatively charged at pH 6.5 and will bind to the Proteus DetEx Mini Spin Column. The protein can be eluted with increasing salt concentration or by lowering the elution buffer pH below pH 5.0.

NOTE: Use only for non-ionic detergents (maltosides, glucosides and polyoxyethylene glycols) and Zwitterionic detergents (Fos-Cholines, CHAPS and amine oxides).

NOTE: Use loading buffers in the pH range of 4-10; salt concentration ideally < 25 mM.

NOTE: Elute with low pH or 100 mM $MgSO_4$.

Procedure

1. **Sample preparation:**
Centrifuge your sample at 10,000-15,000 x g for 2-5 min to remove any

NOTE: Proteus Mini clarification spin columns (Neo-Biotech code: NB-45-00061).

aggregate. Alternatively, you can filter your sample using a Proteus Mini clarification spin column by pulse centrifuging for 20 seconds.

2. **Dilution:**
   Dilute a 50-100 µl sample aliquot eluted from the Nickel affinity column by 10-20x in 'loading buffer' to lower the ionic strength.

3. **Equilibration:**
   Place the column into a collection tube and equilibrate the column with 0.4 ml of 'loading buffer' by centrifuging at 2,000 x g for 1 min using a fixed-angle rotor. Repeat this step once.

4. **Binding to the column:**
   Load successive volumes (0.4 ml) of sample as required by centrifuging at 2,000 x g for 2 min.

5. **Washing:**
   Wash the bound sample with 0.4 ml of 'wash buffer' (salt and detergent-depleted) by centrifuging at 2,000 x g for 2 min. Repeat this step once.

6. **Elution:**
   Place the column in a clean collection tube. Add 50-200 µl of 'elution buffer' containing the salt of choice based upon protein stability. Centrifuge at 2,000 x g for 2 min.

7. **Collect the detergent-depleted protein sample.**

NOTE: It is advisable to test a range of salt concentrations (e.g. 50-100 mM and 100-200 mM for divalent and monovalent salts, respectively) to optimise the yield of sample recovery as well as buffer conditions (salt composition and concentration, protein concentration etc) suitable for crystallisation screening.

NOTE: Elution can also be carried out by lowering the pH provided the protein remains stable

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Performance data:

Excess detergent removal from the Photosystem II multi-subunit membrane protein complex

Photosystem II (PSII) is the light-driven Water: Plastoquinone Oxidoreductase located in the thylakoid membranes of plants, algae and cyanobacteria. Dimeric cyanobacterial PSII exists as a 700 kDa complex (Ref. 1 & 2) which can be purified using 2 consecutive anion exchange chromatography steps (Ref. 3).
Following the solubilisation of membranes from the cyanobacterium *Thermosynechococcus elongatus* WT with 0.5 % n-Dodecyl β-D-maltoside (β-DDM), the dimeric PSII protein was purified using 2 consecutive anion exchange steps (Toyo Pearl DEAE 650 S) and a final 12-40 mM $MgSO_4$ gradient. The concentration of β-DDM was kept at 0.02% (i.e. 2.4 x CMC) throughout the chromatographic steps. A post-purification concentration and excess detergent removal procedure was then carried out (detailed below):

Buffers:

Buffer 1:
40 mM MES-NaOH pH 6.0, 20 mM $CaCl_2$, 40 mM $MgSO_4$, 5 % glycerol, 0.02 % (w/v) β-DDM

Buffer 2:
40 mM MES-NaOH pH 6.0, 0.02 % (w/v) β-DDM

Buffer 3:
40 mM MES-NaOH pH 6.0

Buffer 4:
40 mM MES-NaOH pH 6.0, 50 mM $MgSO_4$

Protocol:

1. The eluted protein present in 30 ml of Buffer 1 was diluted 10 times with Buffer 2 to lower the ionic strength.

2. Using Vivaspin Turbo 15 PES centrifugal concentrators (100 KDa MWCO), 300 ml were concentrated 273 times down to 1.1 ml at 10 mg/ml protein concentration (SAMPLE 1).

3. A 0.2 ml aliquot of SAMPLE 1 was loaded on a Proteus Detergent Exchange Mini Spin Column pre-equilibrated with Buffer 2.

4. The spin column was washed twice with 0.4 ml of Buffer 3.

5. The collection tube was then changed (to avoid contamination with residual β-DDM) and the sample eluted with 0.2 ml of Buffer 4 containing 50 mM $MgSO_4$ (SAMPLE 2).

6. To provide comparison with an alternative method used for excess detergent removal, 0.2 ml of SAMPLE 1 was precipitated with 15 % (w/w) PEG 2000 in a 0.5 ml Eppendorf tube (Ref. 3). The pellet and tube were washed 3 times with 0.25 ml of Buffer 3 before re-suspension in 0.2 ml of Buffer 4 (SAMPLE 3).

7. The concentration of $MgSO_4$ was adjusted to 50 mM in SAMPLE 1 and the 3 samples were spun down at 15,000 x g for 5 min prior to Size Exclusion Chromatography-Multi-Angle Light Scattering (SEC-MALS) analysis.

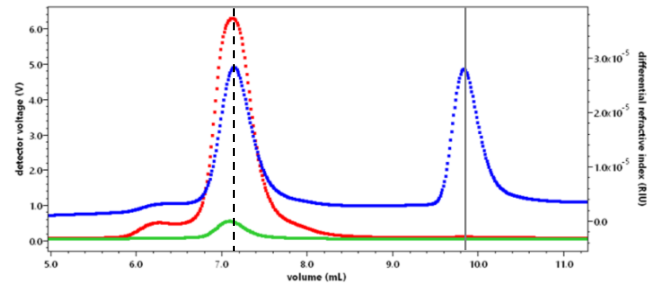
SEC-MALS analysis:

Refractive index measurements coupled with size exclusion chromatography and light scattering can be used to determine the oligomeric state of a membrane protein, the size of a PDC as well as the amount and size of free detergent micelles. The results below show the level of detergent depletion achieved by the Proteus DetEx Mini Spin Columns.

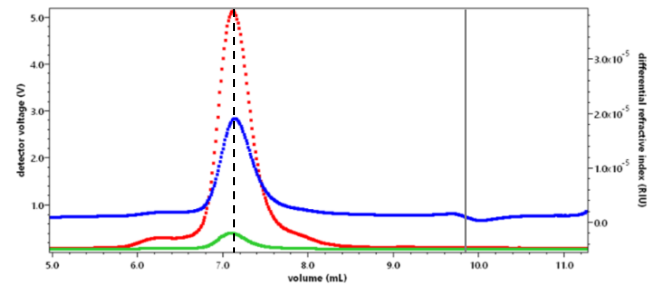
Gel filtration column:	Phenomenex Bio Sep SEC3000, 300 x 7.8 mm
Mobile phase:	40 mM MES-NaOH pH 6.0, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, 0.02 % (w/v) β-DDM
Injection volume:	20 µl at 3 mg/ml protein concentration
Flow rate:	1 ml/min
Instrument:	Agilent 1260 HPLC with Wyatt miniDAWN TREOS

Figure 5. SEC-MALS analysis of Dimeric PSII complexes prior to detergent removal (SAMPLE 1), and after detergent removal using a DetEx Mini Spin Column (SAMPLE 2) or PEG precipitation (SAMPLE 3). Light Scattering (LS) data is shown in red, Refractive Index (RI) data in blue and UV data in green. LS and RI scales are shown in the left and right-hand axes respectively.

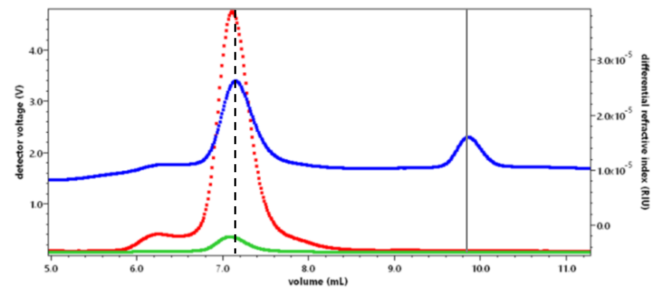
SAMPLE 1. Dimeric PSII protein prior to detergent removal.



Sample 2. Dimeric PSII protein after micelle removal using a Proteus Detergent Exchange Mini Spin Column.



SAMPLE 3. PSII protein after PEG 2000 precipitation and re-suspension.



Dimeric PSII complexes (700 KDa) elute after 7.15 ml (dashed line). Free β-DDM micelles (50-70kDa, Ref 4) elute after 9.85 ml (solid grey line).

Results demonstrate that the Proteus DetEx Mini Spin Columns efficiently removes excess β-DDM micelles from the concentrated PSII sample while maintaining the protein's oligomeric state and maximizing protein stability.

Storage conditions:

Item:	NB-45-00059-4	NB-45-00059-20
Shipping:	Room temperature	Room temperature
Long-term storage:	Room temperature	Room temperature

Questions and answers:

1. *Can the Proteus Detergent Exchange Mini Spin Column be used with Zwitterionic or positively charged detergents?*
   Yes. Excess Zwitterionic detergents are routinely removed by standard ion-exchange chromatography. Although not tested in our laboratories, positively charged detergent can in principle be used.

2. *Why should proteins have a pI below 8?*
   The reason is to maximise sample recovery as proteins with a pI below 8 will bind to the Proteus membrane.

3. *Do detergent molecules fall off the detergent belt when the sample is diluted many times in detergent-depleted buffer, leading to precipitation?*
   This is unlikely and depends on factors such as the hydrophobicity of the detergent bound to the protein and buffer conditions. The susceptibility of a protein to aggregate or precipitate can be measured by diluting it many times in detergent-depleted buffer in a 2 ml microfuge tube and spinning it at 10-15,000g for 5 min. The absence or presence of a pellet indicates whether the protein D is stable or has precipitated/aggregated.

4. *Are detergents mass spectrometry compatible?*
   Most *detergents* are not *compatible* with downstream *mass spectrometry* analysis. The Proteus Detergent Exchange Mini Spin Column allows fast and efficient excess detergent removal to enhance the output from mass spectrometry analysis.

5. *Can detergent exchange be performed into a higher CMC (e.g. less hydrophobic) detergent?*
   The routine protocol requires that a detergent exchange should normally be carried out into a more hydrophobic detergent. E.g. β-OG (n-octyl-β-D-glucopyranoside) <β-NG (n-nonyl-β-D-glucopyranoside) < DM (n-decyl-β-D-maltopyranoside) < β-DDM (n-dodecyl-β-D-maltopyranoside). It is, however, possible to exchange into a less hydrophobic destination detergent provided the PDC is washed with a buffer containing a much higher concentration of destination detergent. Detergent micelles are dynamic structures; detergent monomers within the micelle are in constant, rapid exchange with free detergent monomers in solution.

6. *Will the Proteus spin columns work well with all protein sizes?*
   Yes, provided the proteins can be separated by anion exchange chromatography. There are no upper or lower protein size limits.

7. *How do you use the Proteus spin columns for detergent exchange?*

For exchange of detergents, wash the column several times (0.4 ml times per wash) with a buffer containing the destination detergent.

8. *Can the Proteus spin columns be used for preparing samples for NMR analyses?*
Excess detergent can lead to phase separation problems or viscosity increases in NMR experiments. The Proteus DetEx Spin Columns can efficiently remove excess free micelles from protein-detergent complexes (PDC) and exchange to deuterated non-ionic or zwitterionic detergent.

Troubleshooting assistance:

Protein sample doesn't bind:

- The ionic strength may still be too high and the sample should be diluted further in salt depleted buffer. Consideration must be given to the fact that some proteins require a certain concentration of salt to remain soluble. In such case increasing the detergent concentration further while lowering the ionic strength may help find conditions for binding to the resin. The absorbance of the flow through at 280 nm should be measured to confirm binding.

Protein sample doesn't elute:

- The ionic strength of the elution buffer is insufficient and needs to be increased. Try optimising he conditions by testing different salts.
- It is possible that the protein has precipitated and stuck to the resin during the detergent depleted buffer washes. The ionic strength and/or the detergent concentration may require increasing.

Glossary:

affinity chromatography - chromatographic separation based on a specific interaction between an immobilized ligand and a binding site on a macromolecule.

β-DDM - Dodecyl-β-D-Maltopyranoside. Commonly used detergent for membrane protein solubilisation.

β-DM - Decyl-β-D-Maltopyranoside. Commonly used detergent for membrane protein solubilisation.

*β-OG - Octyl-*β-D-*Glucopyranoside.* Commonly used detergent for membrane protein solubilisation.

CHAPS - 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate. Zwitterionic detergent that is especially well suited for protecting the native state of proteins.

CMC - critical micellar concentration. Concentration of detergent above which micelles form.

column bed volume(CV) - the total volume occupied by the chromatographic packed bed. It is also referred to as the column volume or CV.

his - a 3 letter symbol for L-histidine

his-tag – a permanent affinity tag engineered into the expression vector upstream or downstream of the gene of interest to facilitate the purification of the recombinant protein. The His-tag doesn't normally have any effect upon the protein structure or function, it comprises 6-8x Histidine residues (polyhistidine) and has a molecular weight of 0.7-0.9 kDa

immobilized metal ion affinity chromatography (IMAC) – method of protein affinity purification using immobilized metal ions.

ion exchange chromatography - chromatographic separation based on different charge properties of macromolecules.

isoelectric point - the pH at which the protein has no net charge.

PDC - protein-detergent complexes.

recombinant protein – a protein coded for by a cloned gene which has often been modified *to increase the expression of that protein or to alter the properties of the protein.*

SEC-MALS - size-exclusion chromatography with multi-angle light scattering. Method for accurate size determination of macromolecules (including proteins and micelles) whereby a liquid sample is passed down a size-exclusion chromatography column before passing through a multi-angle light scattering detector. As the macromolecules pass through the detector they scatter the light from multiple light sources at different angles which is then used to estimate the molecular mass.

Literature:

1. Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J. and Iwata, S. (2004). Architecture of the photosynthetic oxygen-evolving center. S*cience* **303**:1831-1838.

2. Umena, Y., Kawakami, K., Shen, J.R. and Kamiya, N. (2011) Crystal tructure of oxygen-evolving photosystem II at a resolution of 1.9 Å. *Nature* **473** (7345): 55-80.

3. Kern, J., Loll, B., Luneberg, C., DiFiore, D., Biesiadka, J., Irrgang, K. D. and Zouni, A. (2005). Purification, characterisation and crystallisation of photosystem II from *Thermosynechococcuselongatus*cultivated in a new type of photobioreactor. *Biochim. Biophys. Acta***1706**:147-157.

4. Anatrace Inc., Available from: www.anatrace.com

Ordering information:

Product	Spin Columns	Order Code
Proteus Detergent Anion Exchange Mini Kit	20	NB-45-00059-20
Proteus Detergent Anion Exchange Mini Trial Pack	4	NB-45-00059-4

Technical support:

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- This product is for research use only and is not intended for use in clinical diagnosis. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed.