February 2009

QIAGEN® Ni-NTA Membrane Protein Kit Handbook

For purification of recombinant His-tagged membrane proteins from *E. coli* cultures and insect cells

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Contents

Kit Contents

Ni-NTA Membrane Protein Kit	**(5)**
Catalog no.	**30610**
Number of preps	**5**
Buffer NTI-10-G	160 ml
Buffer NTI-25-G	12.5 ml
Buffer NTI-500-G	3 ml
Buffer TS	20 ml
Ni-NTA Superflow	2.5 ml
Benzonase® Nuclease (25,000 units/ml)	400 µl
Lysozyme	120 mg
Penta·His Antibody, BSA-free	3 µg
n-Octyl-ß-D-glucopyranoside (OG)	350 mg
n-Decyl-ß-D-maltopyranoside (DM)	144 mg
N,*N*-Dimethyldodecylamine-*N*-oxide (LDAO)	102 mg
n-Dodecyl-ß-D-maltoside (DDM)	129 mg
Cymal 6 (Cy6)	134 mg
n-Nonyl-ß-D-glucopyranoside (NG)	169 mg
FOS-choline-16 (FOS)	7 mg
Disposable Columns	5
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Storage

Buffers NTI-10-G, NTI-25-G, NTI-500-G, and TS should be stored at room temperature (15–25°C). Ni-NTA Superflow and Lysozyme should be stored at 4°C. All other kit components should be stored at –20°C.

Penta·His Antibodies should be stored lyophilized until they are to be used. They can be stored lyophilized for one year at 2–8°C. In solution, they can be stored for three months at 2–8°C or for up to 6 months in aliquots at –20°C.

Avoid repeated freezing and thawing. Dissolve Penta·His Antibody (3 µg) in 15 µl water per tube (final concentration, 0.2 mg/ml).

When stored under the recommended conditions, all other kit components should be stable for one year.

Product Use Limitations

The QIAGEN Ni-NTA Membrane Protein Kit is intended for molecular biology applications. This product is neither intended for the diagnosis, prevention, or treatment of a disease, nor has it been validated for such use either alone or in combination with other products. Therefore, the performance characteristics of the product for clinical use (ie., diagnostic, prognostic, therapeutic, or blood banking) are unknown.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the QIAGEN Ni-NTA Membrane Protein Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

The following risk and safety phrases apply to components of the QIAGEN Ni-NTA Membrane Protein Kit.

Detergent FOS–choline–16

Contains miltefosin: Toxic, irritant. Risk and safety phrases:* T, R22-36-48/25, S13-22-26-36/37/39-45

Detergent LDAO

Contains *N*,*N*-dimethyldodecylamine-*N*-oxide: Corrosive. Risk and safety phrases:* C, R34, S26-36/37/39-45

Detergent NG

Contains *n*-nonyl-ß-D-glycopyranoside: Irritant. Risk and safety phrases:* Xi, R36/37/38, S26-36/37/39-45

Ni-NTA Superflow

Contains nickel-nitrilotriacetic acid, ethanol: Harmful, sensitizer, flammable. Risk and safety phrases:* Xn, R10-22-40-42/43, S13-26-36-46

Penta · His Antibody

Contains anti-His antibody: Sensitizer. Risk and safety phrases:* Xn, R42/43, S24-26-36/37

* R10: Flammable; R22: Harmful if swallowed; R34: Causes burns; R36: Irritating to eyes; R37: Irritating to respiratory system; R38: Irritating to skin; R36/37/38: Irritating to eyes, respiratory system and skin; R40: Limited evidence of a carcinogenic effect; R42/43: May cause sensitization by inhalation and skin contact; R48/25: Toxic: danger of serious damage to health by prolonged exposure if swallowed; S13: Keep away from food, drink and animal feeding stuffs; S22: Do not breathe dust; S24: Avoid contact with the skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S36/37: Wear suitable protective clothing and gloves; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection; S45: In case of accident or if you feel unwell, seek medical advice immediately; S46: If swallowed, seek medical advice immediately and show container or label.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAGEN Ni-NTA Membrane Protein Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Membrane proteins

Proteins can be separated into two general classes according to their location: proteins that are associated with cellular membranes (membrane proteins) and proteins that are not associated with cellular membranes (soluble proteins).

With respect to their localization, membrane proteins can be found in cellular membranous structures (e.g., the endoplasmic reticulum, mitochondria and other vesicles), and the plasma membrane. However, their functions within these respective biomembranes can be very different. The class of transmembrane proteins — which forms the largest group of membrane proteins — is characterized by the presence of a variable number of transmembrane helices. Such proteins include receptors and channels.

Another class of membrane proteins is not embedded in the membrane but instead is covalently attached to the lipid bilayer. Other membrane proteins are attached to the membrane via noncovalent interactions with other proteins.

Approximately 30% of a genome encodes for membrane proteins. They are one of the most important classes of proteins in that they receive, differentiate, and in some cases, transmit intra- and intercellular signals. Some examples of membrane proteins include cell-adhesion molecules, translocases, and receptors in signaling pathways. Moreover, defects in membrane proteins cause a number of serious disorders such as neurodegenerative diseases (e.g., Alzheimer's) and diabetes. Thus, membrane proteins constitute approximately 50% of known and novel drug targets.

For functional assays (e.g., ligand binding assays) and structural studies (e.g., via crystallization or NMR) membrane proteins must be purified after expression. Recombinant membrane proteins can be expressed in vivo using prokaryotic (e.g., *E. coli* and *Lactococcus lactis*) or eukaryotic (yeast, insect, and mammalian) cells or synthesized in vitro using cell-free expression systems (e.g., EasyXpress® Kits).

Once expressed, the isolation of membrane proteins becomes the main challenge. In contrast to soluble proteins, the hydrophobic part of membrane proteins makes it difficult to solubilize them. Detergents are polar molecules (with hydrophobic and hydrophilic parts) and adhere to the hydrophobic part of a membrane protein, thus resolubilizing it from the membrane. The resolubilization results in a water soluble protein/detergent complex that can be isolated and subsequently purified. The selection of an effective detergent can be crucial for effective solubilization and purification of membrane proteins.

Principle and procedure

The Ni-NTA Membrane Protein Kit enables the solubilization and purification of His-tagged membrane proteins from prokaryotic or eukaryotic cell cultures or cell-free expression systems. An initial screening procedure determines the detergent best suited for solubilization of a particular membrane protein. Aliquots of the cell lysate containing the expressed protein are solubilized using each detergent in the kit (see Detergent Screening Procedure, page 11) and the optimal detergent is determined by SDS-PAGE separation of the soluble fractions. The Penta·His Antibody is used to detect His-tagged proteins after Western blotting.

Once the optimal detergent has been identified, larger scale amounts of the protein are purified on a Ni-NTA matrix using buffers containing the chosen most suitable detergent (see Membrane Protein Purification Procedure, page 12).

Typical results using the Ni-NTA Membrane Protein Kit in *E.coli* and Sf9 insect cells are shown in Figures 1–3. In Figures 1 and 2, His-tagged NhaA, a Na^+/H^+ antiporter, was overexpressed in *E.coli*. Solubilization of the protein was screened in each of the seven detergents provided in the kit (Figure 1). DDM was chosen as the most suitable resolubilizing detergent. DM, LDAO, Cy6, and FOS also worked well for resolubilization while OG and NG showed poor resolubilzation efficiency for this protein (see Figure 1B). NhaA was subsequently purified in the presence of DDM and eluted from Ni-NTA Superflow resin in a monomeric (35 kDa) and dimeric form (70 kDa) (see Figure 2, lane E).

Similarly, His-tagged OGCP was expressed in insect cells (see Figure 3), and the detergent screening procedure determined that DDM was the most suitable detergent for resolubilization (see Figure 3A). Purification of OGCP in the presence of DDM is shown in Figure 3B (detection of eluted protein in lanes E1 and E2).

Once the best-suited detergent has been selected for a specific protein, the complete procedure described in this handbook can be scaled up reproducibly for larger sized pellets. Bulk amounts of the detergents and Ni-NTA Superflow are available for this purpose. For more information, go to www. qiagen.com.

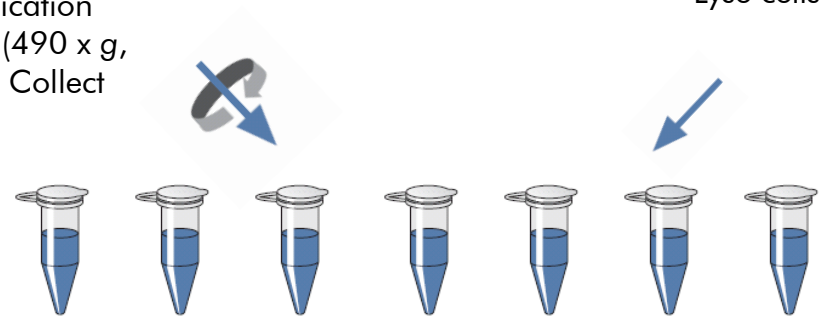
Detergent Screening Procedure

***E. coli* cell pellet**

Lyse cells by sonication and clear lysate (490 x *g*, 15 min at 4°C). Collect supernatant.

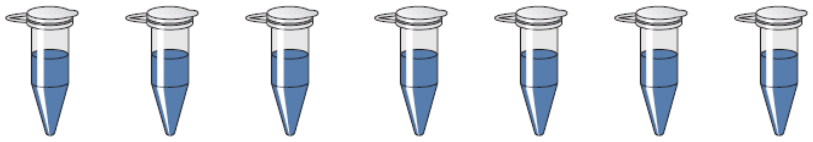
Insect cell pellet

Lyse cells



Distribute lysate between 7 tubes

20,000 x *g*, 1 h at 4°C

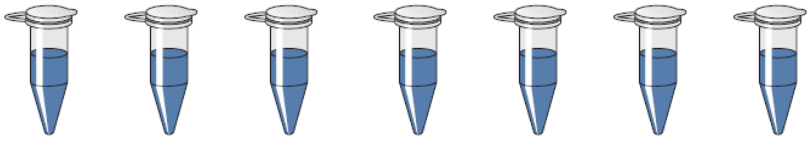


OG DM LDAO DDM Cy6 NG FOS

Resuspend pellets using different detergents

End-over-end shaker, 1 h at RT → 7 x 10 µl aliquots = Total protein in detergent

20,000 x *g*, 30 min at 4°C



OG DM LDAO DDM Cy6 NG FOS

Collect supernatants

7 x 10 µl aliquots from supernatant = Solubilized membrane protein fraction

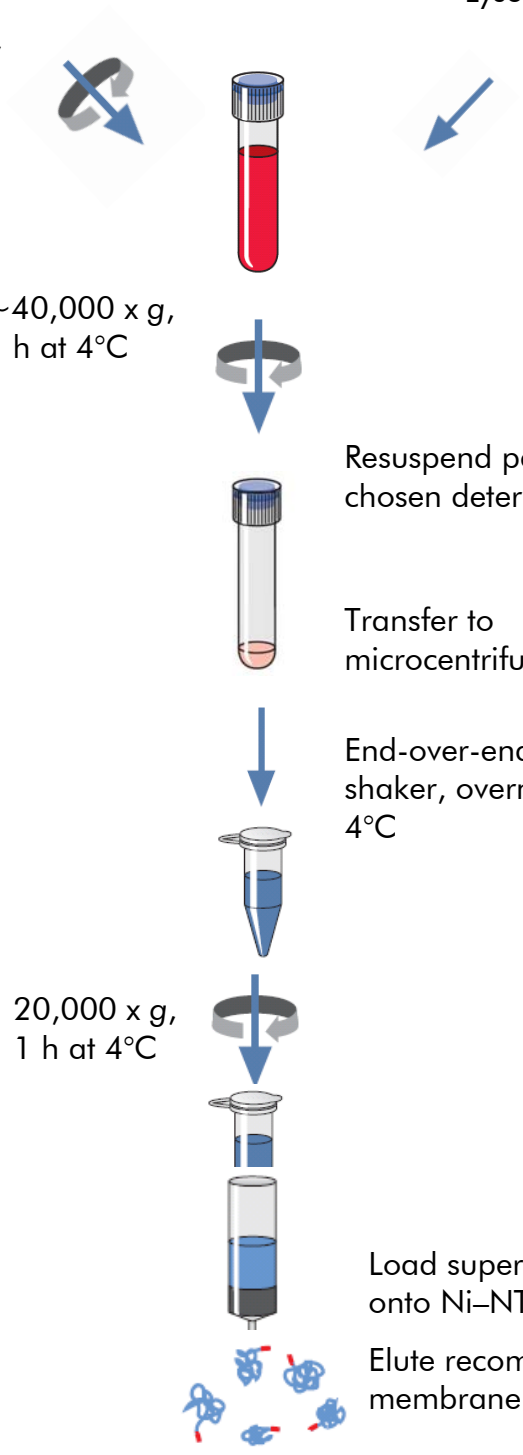
Membrane Protein Purification Procedure

***E. coli* cell pellet**

Lyse cells by sonication and clear lysate (490 x *g*, 15 min at 4°C). Collect supernatants.

Insect cell pellet

Lyse cells



Purifying a bacterial membrane protein from bacterial cultures

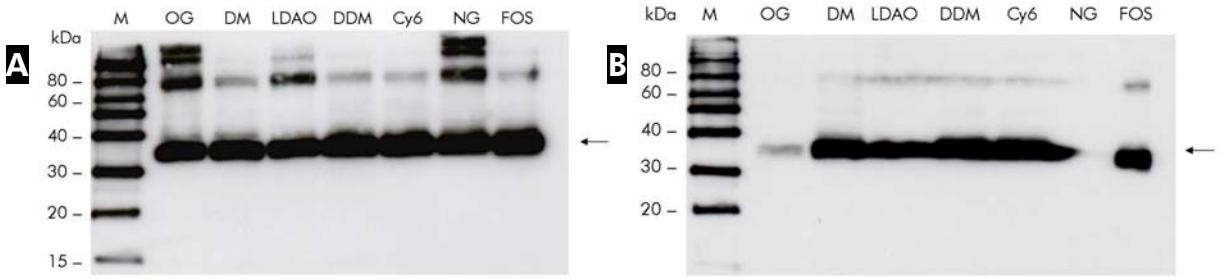


Figure 1. Screening for the optimal detergent. *E. coli* cells overexpressing the 35 kDa membrane protein NhaA were pelleted and lysed. The cell membranes were resuspended in the indicated detergents (included in the Ni-NTA Membrane Protein Kit). Aliquots of the total protein fraction were taken for SDS-PAGE and Western analysis. After centrifugation of the mixture, aliquots of the soluble membrane fraction were taken for SDS-PAGE and Western analysis. **A** Western blot of total protein fraction, and **B** Western blot of the soluble membrane fraction. NhaA protein is visualized by immunodetection of the His tag (arrows). As shown in **B**, detergents OG and NG are poor solubilizers of NhaA. **M**: His-tagged marker proteins.

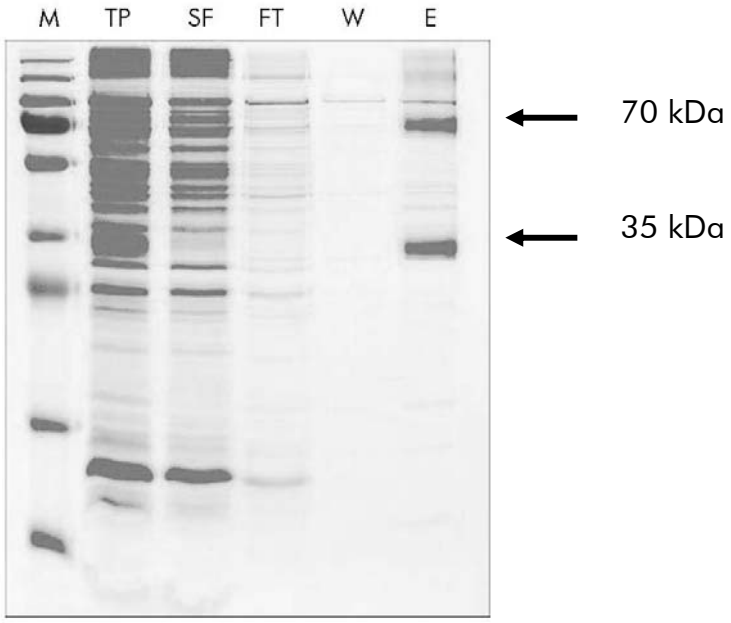


Figure 2. Purification of a membrane protein using Ni-NTA. NhaA was purified on Ni-NTA Superflow using buffers containing DDM. Fractions were separated on an SDS-PAGE gel and proteins visualized by Coomassie® staining. NhaA can be visualized in the stained gel in both its monomeric and dimeric forms (arrows). **TP**: total protein; **SF**: soluble fraction; **FT**: flow-through fraction; **W**: wash fraction; **E**: eluate; **M**: markers.

Purifying a mammalian membrane protein from insect cell cultures

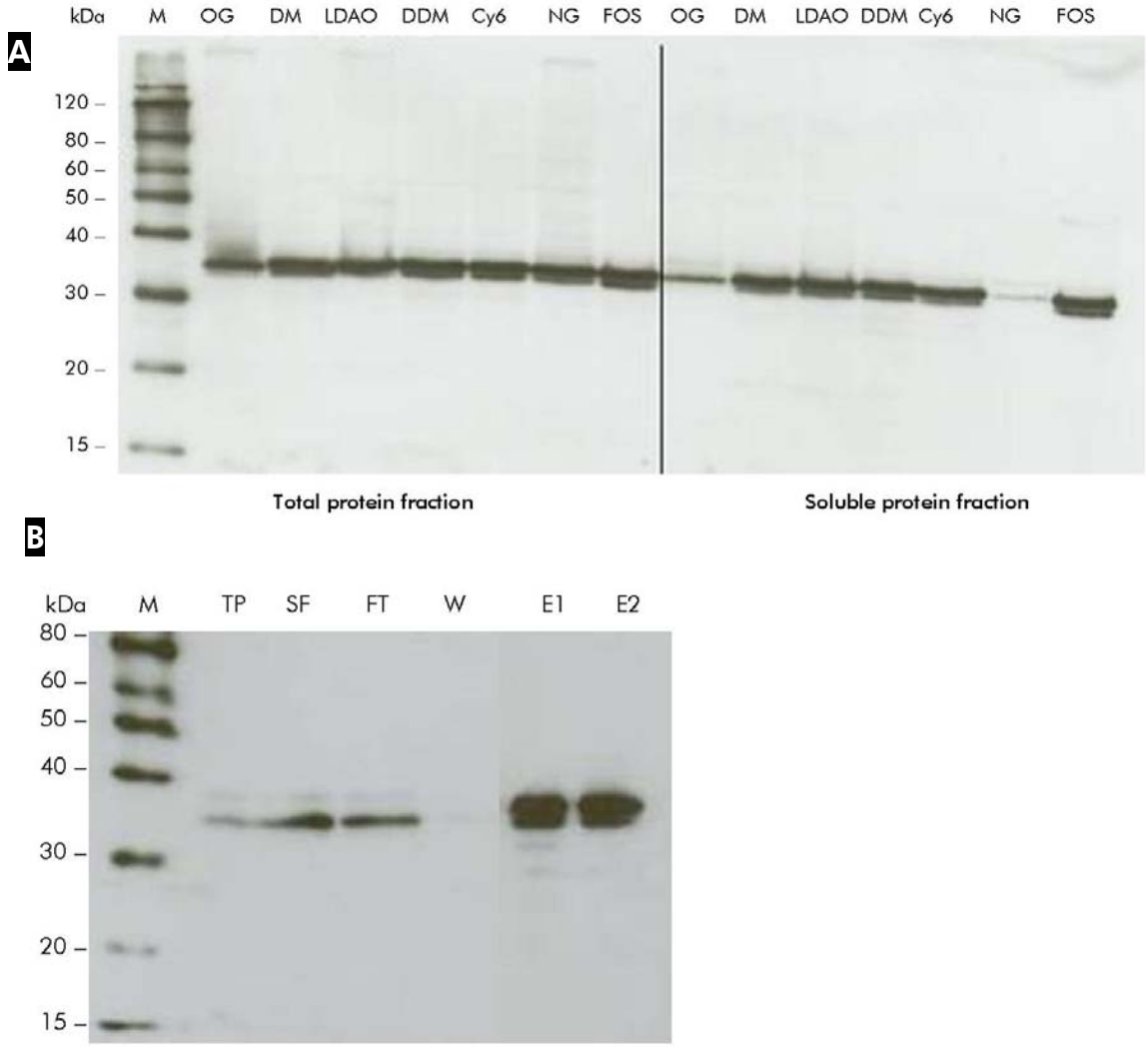


Figure 3. Screening for the optimal detergent and purification from insect cells. **A** Insect cells overexpressing the 34 kDa rat membrane protein mitochondrial-2-oxoglutarate/malate carrier protein (OGCP) were pelleted and lysed. The cell membranes were resuspended in the indicated detergents (supplied in the Ni-NTA Membrane Protein Kit). Aliquots of the total protein fraction were taken for SDS-PAGE and Western analysis. After centrifugation of the mixture, aliquots of the soluble membrane fraction were taken for SDS-PAGE and Western analysis. OGCP was visualized by immunodetection of the His tag. Note that OG and NG are poor solubilizers of OGCP. **B** OGCP expressed in insect cells was purified using Ni-NTA Superflow and buffers containing DDM. **TP**: total protein; **SF**: soluble fraction; **FT**: flow-through fraction; **W**: wash fraction; **E**: eluate; **M**: His-tagged marker proteins.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Ultrasonic homogenizer
- Refrigerated table-top centrifuge
- Refrigerated centrifuge capable of generating ~40,000 x *g* (e.g., Beckman Avanti® J Series or J2 Series centrifuge with a JA-17 rotor)
- End-over-end shaker

Protocol: Screening Detergents for Resolubilization of Membrane Proteins

This protocol is used to screen the seven detergents supplied in the kit to determine which one is best suited to solubilize a particular protein.

Things to do before starting

- Obtain a pellet from 175 ml *E. coli* culture or 3.6 x 10^7 insect cells (approximately six T-75 flasks) expressing a His-tagged membrane protein.

Procedure

***E. coli* cultures**

1a. Suspend the bacterial pellet in 14 ml Buffer NTI-10-G.

2a. Dissolve 14 mg Lysozyme in 500 µl distilled water and add to the bacterial pellet together with 21 µl (525 units) of Benzonase Nuclease. Incubate for 30 min at room temperature, then for 30 min on ice.

3a. Disrupt the cells using an ultrasonic homogenizer. Sonicate the cell lysate for 3 min.

Keep the cell lysate on ice during sonication to prevent warming.

4a. Centrifuge the lysate at 490 x *g* for 15 min at 4°C.

5a. Transfer the supernatant into a fresh tube, mix briefly, and distribute between seven clean 2 ml tubes. Centrifuge tubes at 20,000 x *g* for 1 h at 4°C. Proceed to step 6.

Insect cell cultures

1b. Suspend the insect cell pellet in 1.75 ml Buffer TS.

2b. Add 12 µl (300 Units) Benzonase Nuclease.

3b. Incubate for 30 min on ice.

4b. Pipet 200 µl of the lysate into seven clean 2 ml tubes

5b. Centrifuge the tubes at 20,000 x *g* for 1 h at 4°C. Proceed to step 6.

6. During the centrifugation, prepare the detergent stock solutions. Add 2 ml of Buffer NTI-10-G to each detergent tube and mix by gently vortexing.

Detergent stock solutions can be stored at –20 °C for up to six months.

7. Discard the supernatants from step 5 and label each tube with the name of one of the seven detergents.

8. **Resuspend the pellets in the relevant volume of Buffer NTI-10-G as indicated in Table 1.**

   For example, add 421 µl Buffer NTI-10-G to the tube labeled DDM and resuspend the pellet.

9. **Add the relevant volume of detergent stock solution to the resuspended pellets (see Table 1).**

   For example, add 79 µl DDM stock solution to the tube labeled DDM.

10. **Incubate the dissolved pellets on an end-over-end shaker for 1 h at room temperature (15–25°C).**

11. **Remove a 10 µl aliquot (= total protein in detergent) from each tube for Western blot analysis.**

    Label each sample carefully, e.g., DDM-TPD, FOS-TPD, etc.

12. **Centrifuge the remaining 490 µl at 20,000 x *g* for 30 min at 4°C.**

Table 1. Buffer and detergent stock solution volumes for screening

Detergent	Vol. Buffer NTI-10-G added in step 8	Vol. detergent stock solution added in step 9	Final detergent conc.
n-Octyl-β-D-glucopyranoside (OG)	457 µl	43 µl	51 mM
n-Decyl-β-D-maltopyranoside (DM)	430 µl	70 µl	21 mM
N,N-Dimethyldodecylamine-*N*-oxide (LDAO)	432 µl	68 µl	30 mM
n-Dodecyl-β-D-maltoside (DDM)	421 µl	79 µl	20 mM
Cymal 6 (Cy6)	424 µl	76 µl	20 mM
n-Nonyl-β-D-glucopyranoside (NG)	441 µl	59 µl	33 mM
FOS-choline-16 (FOS)	421 µl	79 µl	1.3 mM

13. **Remove a 10 µl aliquot of the supernatant (= soluble membrane fraction) from each tube for analysis.**

    Label each sample carefully, e.g., DDM-SMF, FOS-SMF, etc.

14. **Add 10 µl 2x SDS-PAGE sample buffer to the samples collected in steps 11 and 13 and heat for 30 min at 46°C.**

15. **Analyze the samples by SDS-PAGE and Western blotting (see Appendices A–D).**

    If analysis is not performed directly, samples can be stored at –20°C.

16. **Choose a detergent that demonstrates suitability for resolubilization for the subsequent protein purification procedure.**

Protocol: Purification of Membrane Proteins Using Ni-NTA Superflow

This protocol is suitable for purification of 6xHis-tagged proteins using Ni-NTA Superflow and one of the best-suited detergents determined in the solubilization screening protocol.

Things to do before starting

- Obtain a pellet from 300 ml *E. coli* culture or 4.8 x 10^7 insect cells (approximately eight T-75 flasks) expressing a His-tagged membrane protein.

Procedure

E. coli cultures

1a. Suspend the bacterial pellet in 10 ml Buffer NTI-10-G.

2a. Dissolve 10 mg Lysozyme in 500 µl distilled water and add to the bacterial pellet together with 36 µl (900 Units) Benzonase Nuclease. Incubate for 1 h on ice.

3a. Disrupt the cells using an ultrasonic homogenizer. Sonicate the cell lysate for 3 min.

Keep the cell lysate on ice during sonication to prevent warming.

4a. Centrifuge the lysate at 490 x *g* for 15 min at 4°C.

5a. Carefully transfer the supernatant to a centrifuge tube and centrifuge at ~40,000 x *g* for 1 h at 4°C. Proceed to step 6.

Insect cell cultures

1b. Suspend the insect cell pellet in 2.25 ml Buffer TS.

2b. Add 16 µl (400 Units) Benzonase Nuclease.

3b. Incubate for 1 h on ice.

4b. Transfer the lysate to a centrifuge tube.

5b. Centrifuge the tube at ~40,000 x *g* for 1 h at 4°C. Proceed to step 6.

6. During the centrifugation, thaw the stock solution of the chosen detergent (determined in the previous protocol).

7. Discard the supernatant from step 5 and, according to the detergent to be used, resuspend the pellet in the volume of Buffer NTI-10-G indicated in Table 2.

For example, when using *n*-Decyl-β-D-maltopyranoside (DM), resuspend the pellet in 1289 µl Buffer NTI-10-G.

8. **Add the relevant volume of detergent stock solution to the resuspended pellet (see Table 2).**

   For example, when using *n*-Decyl-β-D-maltopyranoside (DM) add 211 µl DM stock solution to the resuspended pellet.

Table 2. Buffer and detergent stock solution volumes for purification

Detergent	Vol. Buffer NTI-10-G added in step 7	Vol. detergent stock solution added in step 8	Final detergent conc.
n-Octyl-β-D-glucopyranoside (OG)	1373 µl	127 µl	51 mM
n-Decyl-β-D-maltopyranoside (DM)	1289 µl	211 µl	21 mM
N,*N*-Dimethyldodecylamine-*N*-oxide (LDAO)	1297 µl	203 µl	30 mM
n-Dodecyl-β-D-maltoside (DDM)	1263 µl	237 µl	20 mM
Cymal 6 (Cy6)	1272 µl	228 µl	20 mM
n-Nonyl-β-D-glucopyranoside (NG)	1324 µl	176 µl	33 mM
FOS-choline-16 (FOS)	1264 µl	236 µl	1.3 mM

9. **Transfer the dissolved pellet into a clean 2 ml tube and incubate on and end-over-end shaker overnight at 4°C.**
10. **Remove a 10 µl aliquot (= total protein fraction) from the tube for SDS-PAGE analysis. Centrifuge the remainder at 20,000 x *g* for 1 h at 4°C.**
11. **Transfer the supernatant to a fresh tube. This is the soluble membrane fraction that will be purified in the next section of this protocol. Remove a 10 µl aliquot for SDS-PAGE analysis (= soluble fraction).**
12. **Pipet 0.5 ml (for *E. coli* lysate purification) or 0.25 ml (for insect cell lysate purification) Ni-NTA Superflow suspension into a 1 ml disposable column and rinse with 2 x 5 ml distilled water.**
13. **Add the appropriate amount of detergent stock solution to the volumes of purification buffer given in Table 3.**

For example, when using *n*-Decyl-β-D-maltopyranoside (DM), add 70 µl DM stock solution to 430 µl Buffer NTI-10-G, 40 µl DM stock solution to 246 µl Buffer NTI-25-G, and 8 µl DM stock solution to 492 µl Buffer NTI-500-G.

14. **Equilibrate the column with 0.5 ml Buffer NTI-10-G containing the relevant detergent.**
15. **Pipet the soluble membrane protein fraction from step 11 onto the column and retain the flow-through fraction. Reapply the flow-through fraction to the column twice.**
16. **Wash the column with 2 x 1.25 ml volumes of Buffer NTI-25-G containing the relevant detergent.**
17. **Elute the protein in 0.5 ml Buffer NTI-500-G containing the relevant detergent. Remove a 10 µl aliquot for SDS-PAGE analysis.**
18. **Add 10 µl 2x SDS-PAGE sample buffer to each sample and heat for 30 min at 46 °C.**
19. **Analyze the samples by SDS-PAGE (see Appendices A–D).**
    If analysis is not performed directly, samples can be stored at –20°C

Table 3. Volumes of detergent stock solution added to protein purification buffers

Detergent	Buffer NTI-10-G	Buffer NTI-25-G	Buffer NTI-500-G
n-Octyl-β-D-glucopyranoside (OG)	43 µl + 457 µl buffer	156 µl + 2344 µl buffer	31 µl + 469 µl buffer
n-Decyl-β-D-maltopyranoside (DM)	70 µl + 430 µl buffer	40 µl + 2460 µl buffer	8 µl + 492 µl buffer
N,*N*-Dimethyldodecylamine-*N*-oxide (LDAO)	68 µl + 432 µl buffer	51 µl + 2449 µl buffer	10 µl + 490 µl buffer
n-Dodecyl-β-D-maltoside (DDM)	79 µl + 421 µl buffer	4 µl + 2496 µl buffer	1 µl + 499 µl buffer
Cymal 6 (Cy6)	76 µl + 424 µl buffer	16 µl + 2484 µl buffer	3 µl + 497 µl buffer
n-Nonyl-β-D-glucopyranoside (NG)	59 µl + 441 µl buffer	88 µl + 2412 µl buffer	18 µl + 482 µl buffer
FOS-choline-16 (FOS)	79 µl + 421 µl buffer	6 µl + 2494 µl buffer	2 µl + 498 µl buffer
Final volume of detergent solutions	0.5 ml	2.5 ml	0.5 ml

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Screening Detergents for Resolubilization of Membrane Proteins:	
Protein bands are faint or not visible upon Western blotting of the total protein fraction	
a) *E. coli* expression level is too low	Increase the culture volume. Change the expression conditions (e.g., temperature, time, medium, IPTG-concentration, bacterial strain). See also the *QIAexpressionist*, 5th edition, at www.qiagen.com.
b) Insect cell expression level is too low	Adjust the viral titer. Increase the time of infection and/or time of expression. Increase or decrease the cell density.
Protein bands are faint or not visible upon Western blotting of the soluble protein fraction	
Resolubilization is incomplete	Increase the resolubilization time. Increase the resolubilization temperature.
Purification of Membrane Proteins using Ni-NTA Superflow:	
Purified protein is not detectable on Coomassie gel	
a) *E. coli* expression level is too low	Increase the culture volume. Change the expression conditions (e.g., temperature, time, medium, IPTG-concentration, bacterial strain)

	Comments and suggestions
b) Insect cell expression level is too low	Adjust the viral titer. Increase the time of infection and/or time of expression. Increase or decrease the cell density.
c) Resolubilization is incomplete	Increase the resolubilization time. Increase the resolubilization temperature.
d) Coomassie stain not sensitive enough	Western blot the protein gel
e) Not enough Ni-NTA Superflow added to the Ni-NTA column	Increase amount of Ni-NTA Superflow added

Protein does not enter the separating gel

Sample pretreated incorrectly	Be sure to treat sample as follows prior to loading onto gel: 1x SDS-PAGE buffer, heat 30 minutes at 46°C

Immunodetection of His-tagged Proteins with Penta·His Antibody (Chemiluminescent method):

Protein bands appear weak or diffuse (by Ponceau staining) after transfer from gel to membrane

Transfer of proteins was incomplete or inefficient	Increase transfer time or increase power on the transfer apparatus. Put a weight on top of the lid of the blotting chamber. Ensure that all air bubbles have been removed from filter paper layers by gently rolling a Pasteur pipette over each layer in the sandwich.

Background signal is too high

a) Membrane was not washed enough	Increase the wash time, the number of washes, or amount of detergent in the wash.
b) Nonspecific binding of the antibody	Optimize the antibody dilution
c) Membrane blocked insufficiently	Increase blocking time or concentration of blocking agents

Bands on Western blot are diffuse	
Excessive protein loaded into the gel	Dilute the sample and try again

Appendix A: Separation of Proteins by SDS-PAGE

Materials and equipment to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Gel apparatus and electrophoresis equipment
- 30% acrylamide/0.8% bis-acrylamide stock solution[†1] — this can also be conveniently purchased as a ready-to-use solution from several companies, e.g., Rotiphorese Gel 30 (Roth, Cat. No. 3029.1)
- 2.5x separating gel buffer
- 5x stacking gel buffer
- TEMED (*N*,*N*,*N*',*N*'-tetramethylethylenediamine)
- 10% ammonium persulfate
- Isopropanol
- 5x electrophoresis buffer
- Protein samples
- 2x SDS-PAGE sample buffer

Note: Use only high-quality reagents and water for SDS-PAGE.

For buffer and reagent compositions, see Appendix E.

Procedure

A1. Assemble gel plates with spacers according to the manufacturer's instructions.

A2. Mark the level to which the separating gel should be poured — a few millimeters below the levels where the wells will be formed by the comb.

The size of the gel apparatus used will determine the volumes of gel solutions necessary. The following are used for a 12% acrylamide 8 x 8 or 8 x 10 cm, 1mm thick, minigel.

[1] Acrylamide is a potent neurotoxin and is absorbed through the skin. Take appropriate safety measures particularly when weighing solid acrylamide/bisacrylamide, but also when working with the solutions and gels.

A3. For a 12% acrylamide gel, mix the following in a beaker or similar vessel.

2.2 ml 30% acrylamide/0.8% bis-acrylamide stock solution

2.2 ml separating gel buffer

1.1 ml distilled water

5 µl TEMED

The volume of acrylamide solution and water should be adjusted according to the percentage acrylamide required (dependent on the size of 6xHis-tagged protein to be separated).

A4. Add 50 µl 10% ammonium persulfate, and mix well. Immediately pour the acrylamide solution between the assembled gel plates to the level marked in step 2. Overlay with butanol.

As soon as ammonium persulfate is added, pour the gel quickly before the acrylamide polymerizes.

Water can be used instead of butanol when using apparatus that may be damaged by the use of isopropanol — see the manufacturer's instructions.

A5. After polymerization is complete, pour off isopropanol, rinse with water, and dry.

Water remaining on the plates can be removed using pieces of filter paper.

A6. For the stacking gel, mix the following:

0.28 ml 30% acrylamide stock solution

0.33 ml stacking gel buffer

1 ml distilled water

2 µl TEMED

A7. Add 15 µl 10% ammonium persulfate, and mix well. Immediately pour the stacking gel solution on top of the separating gel and insert the comb, avoiding introduction of air bubbles.

As soon as ammonium persulfate is added, the stacking gel should be poured quickly before the acrylamide polymerizes.

A8. Prepare samples for loading: See step 14 (page 17) and step 18 (page 20) in Protocols.

A9. After the stacking gel polymerizes, the gel can be placed in the electrophoresis chamber. Fill the chamber with 1x electrophoresis buffer, remove comb, load samples, and run the gel. For electrophoresis conditions refer to the recommendations provided by the manufacturer of the apparatus.

Appendix B: Western Transfer of Proteins

Materials and equipment to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Transfer apparatus
- Filter paper
- PVDF (polyvinylidene difluoride) membrane (e.g., Immun-Blot® PVDF Membrane, Bio-Rad cat. no. 162-0177)
- SDS polyacrylamide gel containing separated proteins
- Semi-dry transfer buffer for PVDF membranes
- 100% methanol

For buffer and reagent compositions, see Appendix E.

Semi-dry–transfer procedure

B1. Cut 8 pieces of filter paper and a piece of membrane to the same size as the gel.

To avoid contamination, always handle the filter paper, gel, and membrane with gloves.

B2. Immerse membrane briefly in 100% methanol.

B3. Soak filter paper in semi-dry–transfer buffer.

B4. Avoiding air bubbles, place 4 sheets of filter paper on the cathode (negative, usually black), followed by the gel, the membrane, 4 sheets of filter paper, and finally the anode (positive, usually red).

Air bubbles may cause localized nontransfer of proteins. They can be removed by gently rolling a Pasteur pipette over each layer in the sandwich.

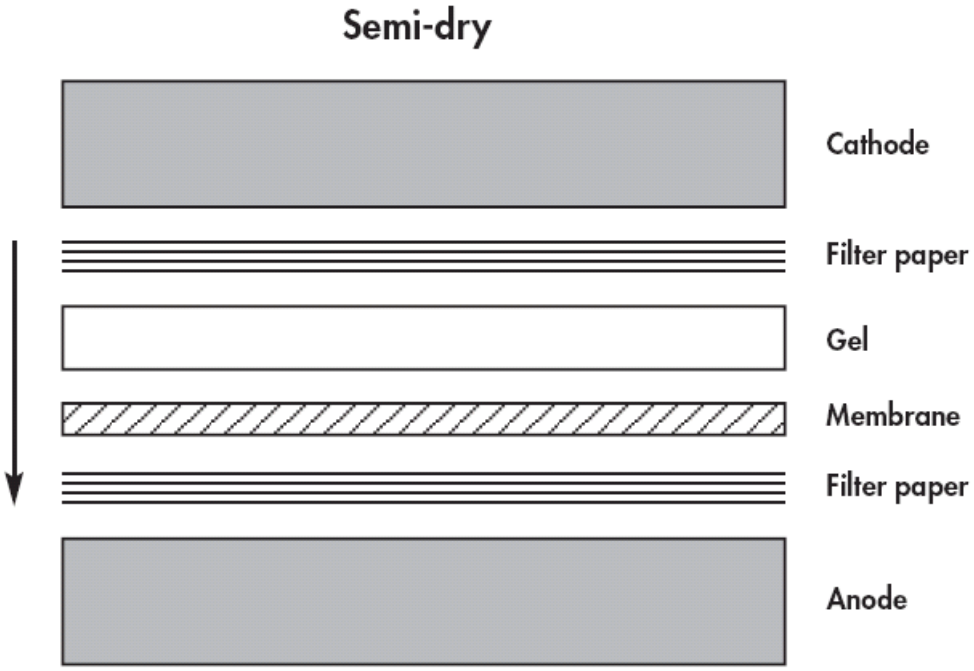


Figure 4. Assembly of the layers for semi-dry Western transfer of proteins. The arrow indicates the direction of protein transfer.

B5. For current, voltage, and transfer times consult the manufacturer's instructions.

Time of transfer is dependent on the size of the proteins, percentage acrylamide, and gel thickness. Transfer efficiency should be monitored by staining with Ponceau S (see Appendix C).

The field strength required is determined by the surface area and thickness of the gel: 0.8 mA/cm^2 is a useful guide (1 h transfer).

B6. After transfer, mark the orientation of the gel on the membrane using a soft pencil.

Appendix C: Staining Proteins after Western Transfer

Materials and equipment to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Staining solution: 0.5% Ponceau S, 1% acetic acid
- Western blot

Procedure

C1. Incubate membrane in staining solution (0.5% Ponceau S, 1% acetic acid) with gentle agitation for 2 min.

C2. Destain in distilled water until bands are visible.

If the 6xHis Protein Ladder has been used as a positive control, the bands should be weakly visible after this staining procedure. Check that the proteins of different sizes have been transferred uniformly to the membrane.

C3. Mark membrane or cut as desired.

C4. The orientation of the gel on the membrane should be marked with a soft pencil. It is not necessary to mark the positions of bands of the 6xHis Protein Ladder. They will become visible after the His-tag detection procedure.

C5. Proceed with the detection protocol (Appendix D).

The blot will be destained in the washing or blocking solution at the beginning of the immunological detection protocol. If the membrane is to be stored at this stage it should be blocked and washed (steps D1–D2 of the protocol on page 30), dried, and then stored at 4 °C. The length of time that the blot can be stored is dependent on the samples on the blot.

Appendix D: Immunodetection of His-tagged Proteins with Penta·His Antibody (Chemiluminescent Method)

Of the two most commonly used immunodetection methods (chemiluminescent and chromogenic detection), chemiluminescence is the more sensitive. A protocol for chromogenic detection of His-tagged proteins and a comprehensive Troubleshooting Guide can be found in the *QIA*express *Detection and Assay Handbook*.

Materials and equipment to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Western blot
- TBS Buffer
- TBS-Tween/Triton Buffer
- Blocking buffer

- Anti-mouse secondary antibody conjugate (e.g., goat-anti-mouse IgG/HRP-conjugate from Dianova [Cat. No. 115-035-003])

Chemiluminescent substrates

Please refer to manufacturer's recommendations. CDP-Star™ (e.g., from Applied Biosystems) can be used with AP-conjugated secondary antibodies, and the ECL™ system from GE Heathcare can be used in combination with HRP-conjugated secondary antibodies. The blocking reagents supplied with the CDP-Star system are compatible with Penta·His Antibodies and can be used according to the manufacturer's instructions, instead of the blocking buffers and secondary antibody dilution buffers described in the following protocol.

Procedure

D1. Wash membrane twice for 7 min each time with TBS buffer at room temperature (15–25°C).

D2. Incubate for 1 h in blocking buffer at room temperature or overnight at 4°C.

D3. Discard blocking buffer and incubate membrane in Penta·His Antibody solution (1/1000–1/2000 dilution of antibody or conjugate stock solution in blocking buffer) at room temperature (15–25°C) for 1 h.

Membrane can be sealed in plastic bags.

D4. Wash twice for 7 min each time in TBS-Tween/Triton buffer at room temperature (15–25°C).

D5. Wash for 7 min in TBS buffer at room temperature (15–25°C).

D6. Incubate with secondary antibody solution for 1 h at room temperature (15–25°C).

Dilute in blocking buffer according to the manufacturer's recommendations. Use the lowest recommended concentration to avoid false signals.

D7. Wash 4 times for 15 min each time in TBS-Tween/Triton buffer at room temperature (15–25°C).

D8. Perform chemiluminescent detection reaction and expose to X-ray film according to the manufacturer's recommendations.

Appendix E: Buffer Compositions

TBS (1 liter)

10 mM Tris·Cl	1.21 g Tris base (MW 121.14 g/mol)
150 mM NaCl	8.77 g NaCl (MW 58.44 g/mol)

Adjust pH to 7.5 using HCl and sterile filter (0.2 or 0.45 µm).

SDS-PAGE sample buffers

2x SDS-PAGE sample buffer	0.09 M Tris·Cl pH 6.8; 20% glycerol; 2% SDS; 0.02% bromophenol blue; 0.1 M DTT

Solutions for SDS-PAGE

30% acrylamide/ 0.8% bis-acrylamide stock	30% acrylamide/0.8% bis-acrylamide (*N*,*N*'-methylene-bis-acrylamide) solution (e.g., Roth, Cat. No. 3029.1)
2.5x separating gel buffer	1.875 M Tris·Cl pH 8.9; 0.25% (w/v) SDS
5x stacking gel buffer	0.3 M Tris-phosphate pH 6.7; 0.5% (w/v) SDS
5x electrophoresis buffer	0.5 M Tris base; 1.92 M glycine; 0.5% (w/v) SDS. Should be pH 8.8. Do not adjust.

Solutions for Western transfer

Semi-dry transfer buffer for PVDF membranes	25 mM Tris base; 150 mM glycine; 5% methanol. Adjust to pH 9.

Solutions for detection procedures

TBS buffer	10 mM Tris·Cl, pH 7.5; 150 mM NaCl
TBS-Tween/Triton buffer	20 mM Tris·Cl, pH 7.5; 500 mM NaCl; 0.05% (v/v) Tween 20 (Sigma, cat. no. P1379); 0.2% (v/v) Triton X-100 (Sigma, cat. no. X-100)
Blocking buffer	5% skim milk powder (Fluka, cat. no. 70166) in TBS buffer; 0.05% (v/v) Tween 20

Coomassie staining solutions

Coomassie staining solution	0.05% (w/v) Coomassie Brilliant Blue R-250*; 40% (v/v) ethanol; 10% (v/v) glacial acetic acid. For 1 liter, dissolve 500 mg Coomassie Brilliant Blue R-250 in 400 ml 100% ethanol. Add 100 ml glacial acetic acid and water to 1 liter. Filter before use.
Destaining solution	40% (v/v) ethanol; 10% (v/v) glacial acetic acid

* e.g., Sigma, cat. no. B 0149

Buffers for protein purification

Binding Buffer = NTI-10-G

Dissolve 2.5 Tris base, 29.2 g NaCl and 0.7 g imidazole in approximately 700 ml water. Adjust pH to 7.5 with 25% HCl. Add 0.4 g sodium azide, 126 g glycerol, and adjust volume to 1 L with water. Mix for 20 minutes, adjust pH as necessary to maintain pH 7.5 and filter with a 0.45 µm filter. Store at room temperature.

Washing Buffer = NTI-25-G

Dissolve 2.5 g Tris base, 29.2 g NaCl and 1.7 g imidazole in approximately 700 ml water. Adjust pH to 7.5 with 25% HCl. Add 0.4 g sodium azide, 126 g glycerol and adjust volume to 1 L with water. Mix for 20 minutes, adjust pH as necessary to maintain pH 7.5 and filter with a 0.45 µm filter. Store at room temperature.

Elution Buffer = NTI-500-G

Dissolve 2.5 g Tris base, 29.2 g NaCl and 34 g imidazole in approximately 700 ml water. Adjust pH to 7.5 with 25% HCl. Add 0.4 sodium azide, 126 g glycerol, and adjust volume to 1 L with water. Mix for 20 minutes, adjust pH as necessary to maintain pH 7.5 and filter with a 0.45 µm filter. Store at room temperature.

Insect Cell Lysis Buffer = TS

Dissolve 0.6 g Tris base with approximately 800 ml water. Adjust pH to 7.5 with 25% HCl. Add 85.6 g sucrose and 0.4 g sodium azide, and adjust volume to 1L with water. Mix for 20 minutes, adust pH as necessary to maintain pH 7.5 and filter with a 0.45 µm filter. Store at room temperature.

Appendix F: Detergent Information

Table 4. Critical micellar concentration (CMC)* of detergents

Detergent	CMC in mM
n-Octyl-β-D-glucopyranoside (OG)	20-25
n-Decyl-β-D-maltopyranoside (DM)	1.6
N,*N*-Dimethyldodecylamine-*N*-oxide (LDAO)	3
n-Dodecyl-β-D-maltoside (DDM)	0.15
Cymal 6 (Cy6)	0.56
n-Nonyl-β-D-glucopyranoside (NG)	6.5
FOS-choline-16 (FOS)	0.013

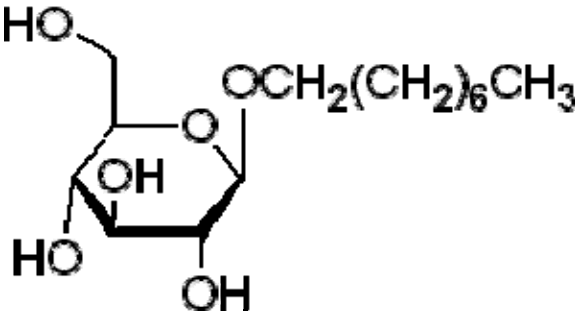
* Please note that CMC refers to a concentration in water. CMC in buffers containing salts results in significantly changes with respect to the concentrations.

Molecular Structures

n-Octyl-ß-D-glucopyranoside (OG): nonionic detergent

Molecular formula: $C_{14}H_{28}O_6$;

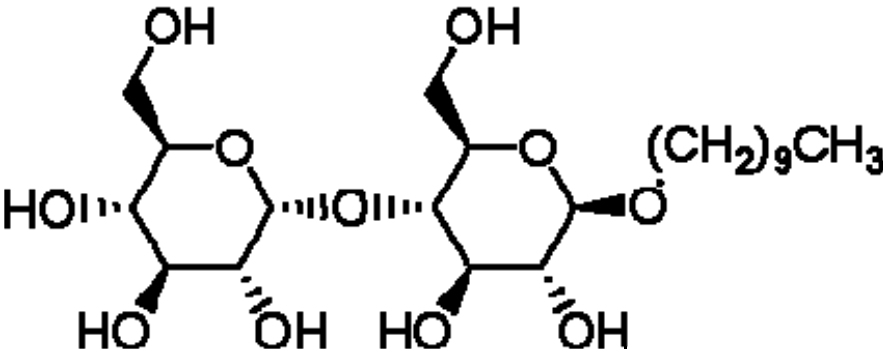
Molecular weight: 292.37



n-Decyl-ß-D-maltopyranoside (DM): nonionic detergent

Molecular formula: $C_{22}H_{42}O_{11}$

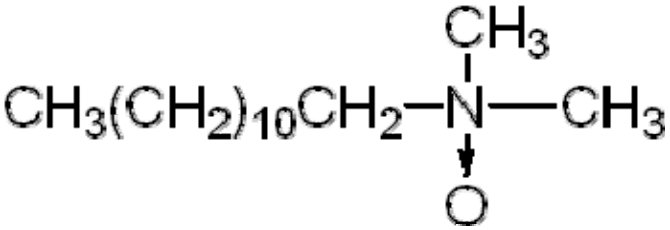
Molecular weight: 482.56



N, *N*-Dimethyldodecylamine-*N*-oxide (LDAO): zwitterionic detergent

Molecular formula: $CH_3(CH_2)_{11}N(O)(CH_3)_2$

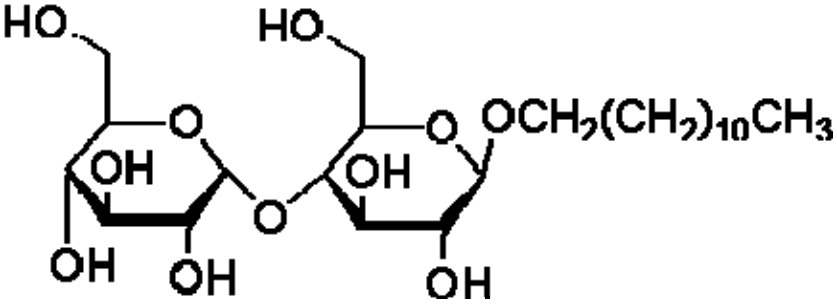
Molecular weight: 229.40



n-Dodecyl-β-D-maltoside (DDM): nonionic detergent

Molecular formula: $C_{24}H_{46}O_{11}$

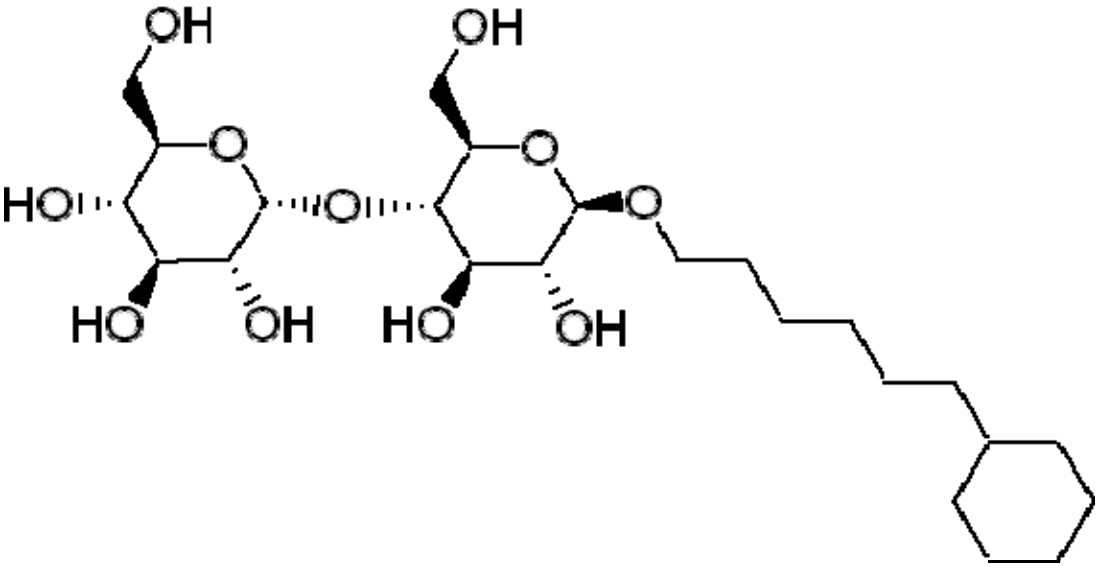
Molecular weight: 510.62



Cymal 6 (Cy6): nonionic detergent

Molecular formula: $C_{24}H_{44}O_{11}$

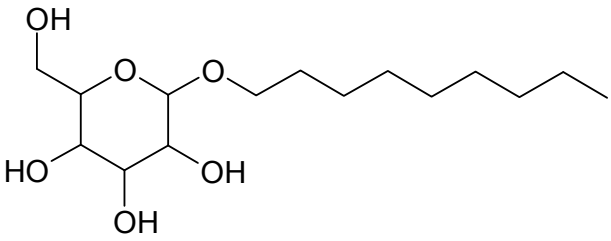
Molecular weight: 508.60



n-Nonyl-ß-D-glucopyranoside (NG): nonionic detergent

Molecular formula: $C_{15}H_{30}O_6$

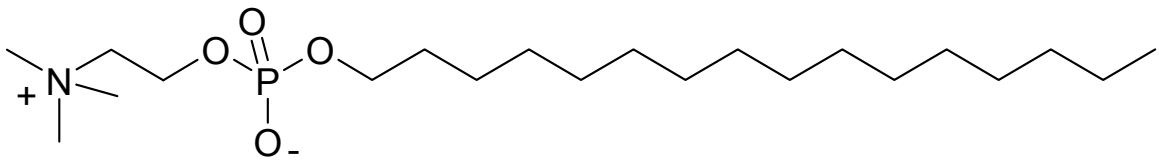
Molecular weight: 306.40



FOS-choline-16: zwitterionic detergent

Molecular formula: $C_{21}H_{46}NO_4P$

Molecular weight: 407.50



References

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Product	Contents	Cat. no.
Ni-NTA Agarose (25 ml)	25 ml nickel-charged resin (max. pressure: 2.8 psi)	30210
Ni-NTA Superflow (25 ml)	25 ml nickel-charged resin (max. pressure: 140 psi)	30410
Ni-NTA Superflow Cartridges (5 x 1 ml)	5 cartridges are prefilled with 1 ml Ni-NTA Superflow: for automated purification of His-tagged proteins using liquid chromatography systems	30721
EasyXpress Protein Synthesis Kit (5)	For 5 x 50 µl reactions: *E. coli* extract, reaction buffer, RNase-Free water, and positive control DNA	32501
EasyXpress Insect Kit II (5)	For 5 x 50 µl reactions; *Spodoptera frugiperda* insect cell extract, reaction buffers, in vitro transcription reaction components, RNase-Free water, gel-filtration columns, and positive control DNA	32561
QIAgenes Expression Kit *E. coli*	QIAgenes Expression Construct *E. coli*, TNFα positive control, Penta·His Antibody, 4 Ni-NTA Spin Columns	Varies
QIAgenes Expression Kit Insect/Mammalia	QIAgenes Expression Construct Insect/Mammalia, QIAgenes Insect/Mammalia Positive Control, Penta·His Antibody (BSA-free), Ni-NTA Magnetic Agarose Beads	Varies
Penta·His Antibody, BSA-free (100 µg)	100 µl mouse anti-$(H)_5$ (lyophilized, BSA-free, for 1000 ml working solution)	34660
Penta·His HRP Conjugate Kit	125 µl Penta·His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer (10x concentrate)	34460
Detergent Cymal 6*	2 g Cymal 6 (Cy6)	34174
Detergent DM*	2 g *n*-Decyl-ß-D-maltopyranoside (DM)	34114

* Coming soon.

Product	Contents	Cat. no.
Detergent DDM*	2 g *n*-Dodecyl-ß-maltoside (DDM)	34124
Detergent OG*	5 g *n*-Octyl-ß-D-glucopyranoside (OG)	34134
Detergent LDAO*	2 g *N,N*-Dimethyldodecylamine-*N*-oxide (LDAO)	34144
Detergent NG*	2 g *n*-Nonyl-ß-D-glucopyranoside (NG)	34154
Detergent FOS-choline-16*	1 g FOS-choline-16 (FOS)	34164

* Coming soon.

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