USER GUIDE

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by life technologies™

MembraneMax™ Protein Expression Kits

(MembraneMax™ and MembraneMax™ *HM*)

For expression of recombinant membrane proteins in a cell-free protein synthesis system

Catalog numbers A10632, A10633, A10634, A10635

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For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

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Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Amount*	Cat. no.
MembraneMax™ Protein Expression Kit	20 reactions	A10632
	100 reactions	A10633
MembraneMax™ *HN* Protein Expression Kit	20 reactions	A10634
	100 reactions	A10635

*Amount based on a standard 100 µl reaction size.

Kit Components

The MembraneMax™ Protein Expression Kits include the following components.

Component	Cat. no.			
	A10632	A10633	A10634	A10635
MembraneMax™ Expression Module (20 reactions)	√			
MembraneMax™ Expression Module (100 reactions)		√		
MembraneMax™ *HN* Expression Module (20 reactions)			√	
MembraneMax™ *HN* Expression Module (100 reactions)				√
Expressway™ Mini Expression Module	√		√	
Expressway™ Maxi Expression Module		√		√
Expressway™ Mini Amino Acid Module	√		√	
Expressway™ Maxi Amino Acid Module		√		√
Expressway™ Control Vector Kit	√	√	√	√
MembraneMax™ Protein Expression Kits Manual	√	√	√	√
MembraneMax™ Protein Expression Kits Quick Reference Card	√	√	√	√

For a detailed description of the contents of each module described above, as well as their shipping and storage conditions, see the next page.

Shipping/Storage

The MembraneMax™ Protein Expression Kits are shipped as described below. All reagents are guaranteed for six months if stored properly. Upon receipt, store boxes as detailed below.

Box	Component	Shipping	Storage
1	MembraneMax™ or MembraneMax™ *HN* Expression Module (20 or 100 reactions)	Dry ice	–20°C
2	Expressway™ Mini or Maxi Expression Module	Dry ice	–80ºC
3	Expressway™ Mini or Maxi Amino Acid Module	Dry ice	–80°C
4	Expressway™ Control Vector	Dry ice	–20ºC

Note

For convenience, you may store the entire contents of the MembraneMax™ and MembraneMax™ *HN* Protein Expression Kits at **–80°C**.

MembraneMax™ Expression Modules

The MembraneMax™ or MembraneMax™ *HN* Expression Modules contain the following reagents. **Store the entire box at –20°C or –80°C.**

Item		Amount
MembraneMax™ or MembraneMax™ *HN* Reagent	(20 reactions)	40 µL
	(100 reactions)	5 × 40 µL
pEXP5-CT/bR Control Vector (0.5 µg/µL in TE buffer, pH 8.0)*		20 µL
10 mM All-trans retinal in dimethyl sulfoxide (DMSO)		25 µL

***TE buffer, pH 8.0:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Important

All-trans retinal is photolabile and must be stored in the dark.

Product Use

For research use only. Not intended for any human or animal diagnostic or therapeutic uses.

Continued on next page

Expressway™ Mini and Maxi Expression Modules

The following reagents are included in the Expressway™ Expression Modules. The Expressway™ Mini and Maxi Expression Modules supply enough reagents for 20 or 100 cell-free membrane protein synthesis reactions, respectively. **Store the entire box at –80°C.**

Item	Module Size	Amount
E. coli slyD⁻ Extract	Mini	400 µL
	Maxi	5 × 400 µL
2.5X IVPS Reaction Buffer (–amino acids)	Mini	400 µL
	Maxi	5 × 400 µL
2X IVPS Feed Buffer	Mini	500 µL
	Maxi	5 × 500 µL
T7 Enzyme Mix	Mini	20 µL
	Maxi	100 µL
DNase/RNase-Free Water	Mini	2 mL
	Maxi	5 × 2 mL

Expressway™ Mini and Maxi Amino Acids Modules

The following reagents are included in the Expressway™ Amino Acids Modules. The Expressway™ Mini and Maxi Amino Acids Modules supply enough amino acids for 20 or 100 cell-free membrane protein synthesis reactions (Mini and Maxi Amino Acids Modules, respectively). **Store at –80°C.**

Item	Composition	Module Size	Amount
50 mM Amino Acids (–Methionine)	All amino acids (50 mM) except for methionine in 50 mM HEPES, pH 11	Mini	160 µL
		Maxi	800 µL
75 mM Methionine	75 mM in 50 mM HEPES, pH 7.5, 4 mM DTT	Mini	120 µL
		Maxi	600 µL

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Expressway™ Control Vector pEXP5-NT/CALML3

The pEXP5-NT/CALML3 control vector (10 µg) is included in the kit for use as a positive control for Expressway™ Expression Modules and allows expression of an N-terminally-tagged human calmodulin-like 3 (CALML3) protein (GenBank accession number NM_005185) from pEXP5-NT/TOPO®.

The vector is supplied as 20 µL at 0.5 µg/µL in TE buffer, pH 8.0.
Store at –20°C or –80°C.

MembraneMax™ Control Vector pEXP5-CT/bR

The pEXP5-CT/bR control vector (10 µg) is included in the kit for use as a positive control for the MembraneMax™ Expression and MembraneMax™ *HN* Expression Modules, and allows the expression of the bacteriorhodopsin protein (bR; GenBank accession no. J02755). Together with its cofactor, all-trans retinal, bR can be used as a positive control for membrane protein expression in a rapid colorimetric control assay.

The vector is supplied as 20 µL at 0.5 µg/µL in TE buffer, pH 8.0.
Store at –20°C or –80°C.

Additional Products

Accessory Products

Some of the reagents supplied in the MembraneMax™ Protein Expression Kits as well as other products suitable for use with the kits are available separately from Life Technologies. Ordering information is provided below. For detailed instructions on how to use any of the accessory products, refer to the manual provided with each product. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (page 34).

Products for Generating DNA Template

The following products are recommended for cloning your gene of interest and generating your DNA template (i.e., expression construct) to be used in the MembraneMax™ Protein Expression Kits.

Product	Quantity	Cat. no.
pEXP5-NT/TOPO® TA Expression Kit	10 reactions	V960-05
pEXP5-CT/TOPO® TA Expression Kit	10 reactions	V960-06
pEXP1-DEST Vector Kit	1 kit	V960-01
pEXP3-DEST Vector Kit	1 kit	V960-03
pEXP4-DEST Vector Kit	1 kit	V960-04
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions 20 reactions	C4040-10 C4040-03
Gateway® LR Clonase® II Enzyme Mix	20 reactions	11791-020
UltraPure™ DNase/RNase-Free Water	500 mL	10977-015
RNase *AWAY*® Reagent	250 mL	10328-011
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
PureLink® HiPure Plasmid Midiprep Kit	25 preps 50 preps	K2100-04 K2100-05
PureLink® HiPure Plasmid Maxiprep Kit	10 preps 25 preps	K2100-06 K2100-07
PureLink® Quick Plasmid Miniprep Kit	250 reactions	K2100-11
PureLink® PCR Purification Kit	50 reactions	K3100-01

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Additional Products, continued

Products to Detect Recombinant Fusion Protein

If you have expressed your recombinant membrane protein using MembraneMax™ Protein Expression Kit (but **not** MembraneMax™ *HN*) you may detect expression of your recombinant fusion membrane protein using an antibody to the appropriate epitope. The amount of antibody supplied is sufficient for 25 western blots.

For use with	Product	Epitope	Cat. no.
pEXP1-DEST	Anti-Xpress™ Antibody	Detects the 8 amino acid Xpress™ epitope: DLYDDDDK	R910-25
	Anti-Xpress™-HRP Antibody		R911-25
pEXP1-DEST, pEXP3-DEST, pEXP5-NT/TOPO®	Anti-HisG Antibody	Detects the N-terminal polyhistidine (6xHis) tag followed by glycine: HHHHHHG	R940-25
	Anti-HisG-HRP Antibody		R941-25
	Anti-HisG-AP Antibody		R942-25
pEXP4-DEST, pEXP5-CT/TOPO®	Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag: HHHHHH-COOH (requires the free carboxyl group for detection (Lindner *et al.*, 1997)	R930-25
	Anti-His (C-term)-HRP Antibody		R931-25
	Anti-His (C-term)-AP Antibody		R932-25
pEXP1-DEST, pEXP3-DEST, pEXP4-DEST, pEXP5-NT/TOPO®, pEXP5-CT/TOPO®	Penta-His mouse IgG1 monoclonal Antibody	Detects both N- and C-terminal polyhistidine (6xHis) tag	P21315

Important

If you are using the MembraneMax™ *HN* Protein Expression Kit and want to express your protein as a recombinant fusion membrane protein for detection and purification purposes, you must utilize a tag other than polyhistidine and choose the corresponding antibody appropriate for the tag. For a list of antibodies available from Life Technologies, visit **www.lifetechnologies.com/antibodies**.

Lumio™ Technology

If you have used the pEXP3-DEST and pEXP4-DEST vectors to clone your protein of interest in frame with the N- or C-terminal Lumio™ tag for expression in a MembraneMax™ or MembraneMax™ *HN* kit, you may detect expression of your recombinant fusion protein using the Lumio™ Detection Reagents available separately from Life Technologies. For more information on the Lumio™ Technology, refer to **www.lifetechnologies.com** or contact Technical Support (page 34).

Product	Quantity	Cat. no.
Lumio™ Green Detection Kit	100 in-gel detections	LC6090
BenchMark™ Fluorescent Protein Standard	125 µL	LC5928

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Products to Purify Recombinant Fusion Membrane Protein

Life Technologies offers a wide range of protein purification products. If you have used the MembraneMax™ *HN* Kit (with the His-tagged MembraneMax™ Reagent) or expressed your protein of interest in frame with an N- or C-terminal affinity tag, you may use affinity chromatography appropriate for your tag for protein purification. Many expression vectors available from Life Technologies also contain specific recognition sites for proteases to facilitate the easy removal of affinity tags upon purification. See the table below for ordering information. For more information on these and other protein purification products, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 34).

Product	Quantity	Cat. no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-chelating Resin	50 mL 150 mL	R801-01 R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 mL 25 mL	R901-01 R901-15
Glutathione Transferase Fusion Protein Purification Kit	5 purifications	G-21801
Glutathione agarose, linked through sulfur (sedimented bead suspension)	10 mL 100 mL	G-2879 G-21800
Purification Columns (10 mL polypropylene columns)	50 each	R640-50
AcTEV™ Protease (pEXP3-DEST and pEXP5-NT/TOPO® only)	1,000 units	12575-015

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Recommended Electrophoresis Products

Life Technologies offers a wide range of pre-cast polyacrylamide gel systems such as the NuPAGE®, Novex® Tris-Glycine, and E-PAGE™ electrophoresis systems that are ideally suited for analyzing your purified protein samples. For analyzing your protein-MembraneMax™ Reagent complexes, you may employ non-denaturing gel systems such as the NativePAGE™ gel system. Each gel system has its own advantages and comes in a ready-to-use, pre-cast format. For more information on the pre-cast polyacrylamide gel systems available from Life Technologies, and which gel system to use for your analysis, refer to **www.lifetechnologies.com** or contact Technical Support (page 34).

Recommended Electrophoresis Accessory Products

In addition to the pre-cast polyacrylamide gel systems, Life Technologies offers a wide range of pre-mixed buffers, protein standards, and stains, each with its own advantages. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (page 34).

Product	Quantity	Cat. no.
NuPAGE® LDS Sample Buffer (4X)	10 mL 250 mL	NP0007 NP0008
Novex® Tris-Glycine SDS Sample Buffer (2X)	20 mL	LC2676
NativePAGE™ Sample Buffer (4X)	10 mL	BN2003
SimplyBlue™ SafeStain	1 L	LC6060
SilverQuest™ Silver Staining Kit	1 kit	LC6070
SilverXpress® Silver Staining Kit	1 kit	LC6100
Colloidal Blue Staining Kit	1 kit	LC6025
Novex® Sharp Protein Standard Pre-stained Unstained	2 × 250 µL 2 × 250 µL	LC5800 LC5801
SeeBlue® Plus2 Pre-Stained Standard	500 µL	LC5925
NativeMark™ Unstained Protein Standard	5 × 50 µL	LC0725
MagicMark™ XP Western Protein Standard	250 µL	LC5602
iBlot® Gel Transfer Device	1 unit (self-contained)	IB1001
UltraPure™ Sodium Dodecyl Sulfate (SDS)	500 g	15525-017

Introduction

Overview

Introduction

The MembraneMax™ Protein Expression Kits are designed for *in vitro* expression of soluble membrane proteins from template DNA in a single, scalable reaction that is amenable to high-throughput. This versatile system contains all the components for the production of recombinant membrane protein of interest from an expression construct in less than 4 hours with microgram to milligram yields. Once purified, the resulting recombinant membrane protein is suitable for use in other downstream applications including biochemical, biophysical, and structural characterization.

The MembraneMax™ Protein Expression Kits are offered in four configurations: MembraneMax™ Protein Expression Kit and MembraneMax™ *HN* Protein Expression Kit, each available in two sizes (20 reactions and scalable from 20-100 reactions).

Advantages of MembraneMax™ Protein Expression

- Production of soluble and monodispersed membrane protein population
- Microgram to milligram quantities of membrane protein in a homogeneous population achieved by cell-free expression
- Convenient purification/enrichment scheme for both native and tagged membrane proteins
- Expression of toxic membrane proteins that otherwise show poor expression *in vivo* and compromised yield in cell based systems
- Easy, scalable membrane protein synthesis reactions that are amenable to high-throughput for a wide range of expression needs
- Convenience of a complete kit with all the reagents supplied

Possible Applications

Recombinant membrane proteins that are expressed using the MembraneMax™ Protein Expression Kits can be used in a variety of downstream applications upon purification, such as structural and functional studies, ligand binding, antibody production, and X-Ray crystallography, and studies with mutant proteins.

Note: There are many factors that affect protein yield, solubility, and function. Therefore, your purified protein might **not** be suitable for all the downstream applications listed above.

Continued on next page

Components of the System

The major components of the MembraneMax™ Protein Expression Kits include:

- MembraneMax™ Reagent containing native (MembraneMax™) or polyhistidine-tagged (MembraneMax™ *HN*) nanolipoprotein particles that are optimized for high-yield production, increased stability, and enhanced solubility of the membrane protein of interest
- An optimized *E. coli* extract (Zubay, 1973) for increased stability of DNA constructs during transcription and translation and increased production of soluble membrane protein
- An optimized reaction buffer composed of an ATP regenerating system (Kim *et al.*, 1996; Lesley *et al.*, 1991; Pratt, 1984) to provide an energy source for protein synthesis
- An optimized feed buffer containing salts and other substrates (Kim and Swartz, 1999) to replenish components depleted or degraded during protein synthesis, thus enhancing recombinant membrane protein yield
- Amino acids (–Met) required for protein synthesis to occur
- Methionine provided separately to allow production of labeled recombinant protein, if desired
- Proprietary T7 Enzyme Mix containing T7 RNA polymerase and other components optimized for T7-based expression from DNA templates (Studier *et al.*, 1990)
- The pEXP5-CT/bR control plasmid (expressing bacteriorhodopsin) and all-trans retinal (cofactor for bacteriorhodopsin) for use as a positive control for membrane protein expression in a rapid colorimetric activity assay
- The pEXP5-NT/CALML3 control vector for use as a positive control for protein expression with Expressway™ Expression Modules

Continued on next page

Overview, continued

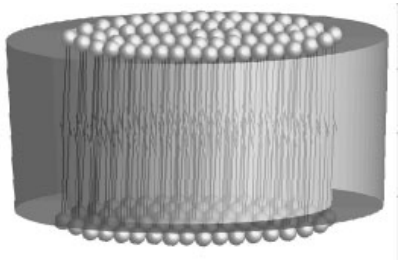
MembraneMax™ Reagent

The MembraneMax™ Protein Expression Kits deliver high yields of soluble membrane proteins using the proprietary MembraneMax™ Reagent, an optimized lipid-protein formulation with cellular membrane-like properties containing nanolipoprotein particles (NLPs) available in native (MembraneMax™) or polyhistidine-tagged (MembraneMax™ *HN*; *HN*: His-tagged NLP) formulations.

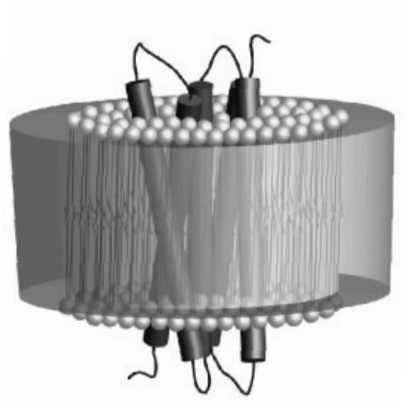
NLPs are discoidal particles of approximately 10 nm in diameter consisting of a proprietary formulation of a scaffold protein ring that encloses a planar DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) lipid bilayer. When combined with the highly efficient Expressway™ Cell-free *E. coli* Expression System from Life Technologies, the MembraneMax™ Reagent allows the production of microgram to milligram quantities of soluble membrane proteins (Katzen *et al.*, 2008).

MembraneMax™ Reagent works conveniently without the need of any detergent or tedious reconstitution into membrane vesicles, because the membrane protein of interest is synthesized into an environment that mimics its cellular surroundings. Furthermore, with its high uniformity and consistent structure, MembraneMax™ Reagent minimizes the formation of membrane protein aggregates or clumping often caused by inconsistent size, shape and structure typically observed with microsomes or micelles. Upon completion of its synthesis, the membrane protein of interest is accessible from either side of the lipid bilayer.

The schematic below depicts the native (i.e., untagged) MembraneMax™ Reagent in an "empty" state (i.e., before the initiation of MembraneMax™ protein synthesis reaction) and after the completion of the membrane protein expression with a hypothetical membrane protein embedded in the lipid bilayer.



MembraneMax™ Reagent



MembraneMax™ Reagent with embedded membrane protein

MembraneMax™ Protein Expression

How the System Works

The MembraneMax™ Protein Expression Kits combine the convenience of the Expressway™ Cell-free *E. coli* Expression System with the cellular membrane-like environment provided by the MembraneMax™ Reagent.

The addition of the DNA template into the reaction mixture initiates the MembraneMax™ Protein Synthesis reaction (see page 6 for generating the DNA template). While the MembraneMax™ Reagent ensures proper folding and stability of the membrane protein, the Expressway™ Cell-free *E. coli* Expression System (included in the kit) allows high levels of protein synthesis with a proprietary mix of an ATP regenerating reaction buffer, amino acids, and an optimized feed buffer that replenishes the salts, amino acids, and other substrates that are depleted during protein synthesis.

E. coli slyD⁻ Extract

All Life Technologies Expressway™ Expression Modules supplied with MembraneMax™ and MembraneMax™ *HN* Protein Expression Kits contain an optimized *E. coli slyD⁻* extract. The *slyD⁻* extract promotes the high yield expression of full-length, active protein from DNA constructs under the reaction conditions specified in this manual.

Important

If you have used other Life Technologies Expressway™ Cell-free *E. coli* Expression Systems, note that some of the components including the Expressway™ IVPS *E. coli* Extract and the Expressway™ 2.5X IVPS Reaction Buffer supplied with older Expressway™ kits contain different formulations and **may not** be compatible with the Expressway™ Expression Modules included in MembraneMax™ and MembraneMax™ *HN* Protein Expression Kits.

For optimal results, use the components supplied in this kit to perform the protein synthesis reaction.

Bacteriorhodopsin protein

The *Halobacterium salinarum* bacteriorhodopsin (bR) (GenBank acc # J02755) protein is light driven proton pump with 7 α-helical transmembrane domains. Although its mechanism does not involve the activation of G proteins, bR has been used as a structural model for rhodopsin and other GPCR family members When retinal is covalently bound to the Lys-216 residue in the cofactor binding pocket of bR via Schiff base formation, a purple color and characteristic absorption peaks at 558 and 568 nm (trimer) or at 546 and 553 nm (monomer) indicate a correctly folded, functional bR, thus rendering the utilization of the bR-retinal complex an ideal positive control for the expression of membrane proteins using the MembraneMax™ Reagent.

Experimental Overview

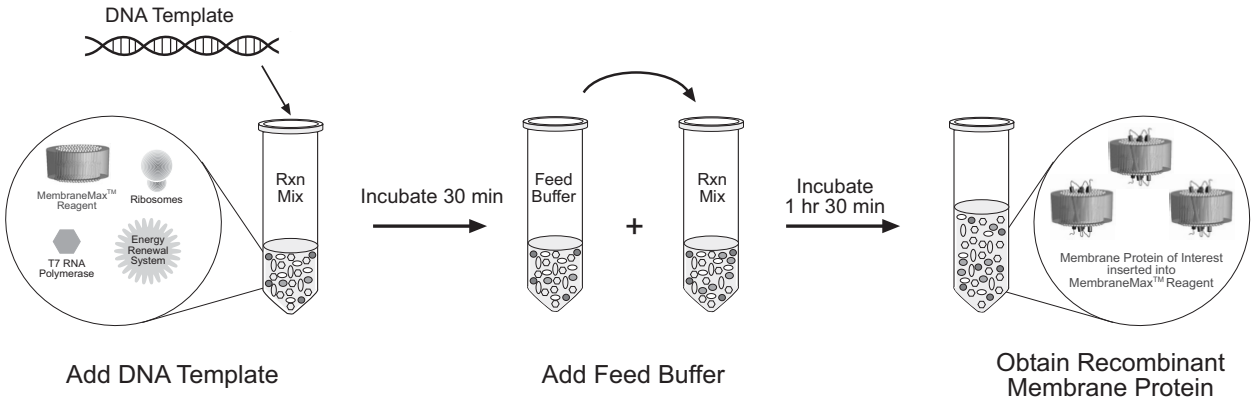
Experimental Outline

The table below describes the major steps required to synthesize your recombinant membrane protein of interest using MembraneMax™ or MembraneMax™ *HN* Protein Expression Kits. Refer to the specified pages for details to perform each step.

Step	Action	Pages
1	Generate the DNA template.	6–10
2	Purify your DNA template.	12
3	Perform the membrane protein synthesis reaction.	13–17
4	Analyze recombinant protein by polyacrylamide gel electrophoresis or other method of choice.	19–24
5	Purify your recombinant protein, if desired.	25

MembraneMax™ Protein Synthesis Reaction

The schematic below depicts the major steps of the standard protein synthesis reaction using the MembraneMax™ or MembraneMax™ *HN* Protein Expression Kit after generating and purifying the expression construct.



Methods

Choice of MembraneMax™ Reagents

Correct MembraneMax™ Reagent

The choice of the correct MembraneMax™ reagent is crucial for successful expression and purification of your membrane protein of interest. Follow the guidelines below to choose the correct MembraneMax™ reagent for your expression and purification needs.

- **MembraneMax™ Protein Expression Kit:** Use this kit if you wish to express your membrane protein as a fusion protein containing an N- or C-terminal polyhistidine tag (6xHis) or any other tag.
- **MembraneMax™ *HN* Protein Expression Kit:** Use this kit if you wish to express your membrane protein in its native state (i.e., untagged) or if you wish to use a tag other than 6xHis.

Note: MembraneMax™ *HN* Reagent contains a 6xHis tag, therefore it cannot be used to express fusion proteins tagged with 6xHis.

Note

You can utilize tandem purification methods to approach 100% membrane protein-NLP complex purity if:

- your fusion membrane protein of interest contains a tag other than 6xHis, and
- you have used MembraneMax™ *HN* Protein Expression Kit to synthesize your protein.

MembraneMax™ Reagent Choice Scenarios

The table below provides several hypothetical scenarios to guide you in your choice of the MembraneMax™ Reagent. This table **does not** contain an exhaustive list of affinity tags or purification systems that can be used with the MembraneMax™ Protein Expression Kits. For more information on expression vectors and protein purifications systems available from Life Technologies, refer to **www.lifetechnologies.com** or contact Technical Support (page 34).

If your expression construct has…	Then use…	And purify by…
N-terminal polyhistidine tag	MembraneMax™ Reagent **only**	Nickel-chelation chromatography (e.g., ProBond™ or NiNTA purification systems; see page xi)
C-terminal polyhistidine tag	MembraneMax™ Reagent	Nickel-chelation chromatography
	MembraneMax™ *HN* Reagent **only if** you add a stop codon upstream of the polyhistidine tag.	Nickel-chelation chromatography
GST tag	MembraneMax™ Reagent	GST affinity chromatography (e.g., Glutathione Transferase Fusion Protein Purification Kit; see page xi)
	MembraneMax™ *HN* Reagent	Nickel-chelation or GST affinity chromatographies, or both methods for tandem purification

Generating the DNA Template

Introduction

The Expressway™ Expression Module supplied with the MembraneMax™ Protein Expression Kits is used to synthesize the recombinant membrane protein of interest from an expression construct. Successful use of this module requires the addition of a DNA template containing the gene of interest placed within the proper context of transcription and translation regulatory elements including the bacteriophage T7 RNA polymerase promoter ("T7 promoter"), the prokaryotic Shine-Dalgarno ribosome binding site (RBS), the ATG initiation codon, the stop codon, and the T7 terminator. However, protein yield can be significantly enhanced if the DNA template is optimally configured.

If you wish to design your own expression construct, general guidelines are provided in this section.

Factors Affecting Protein Yield

The yield of protein produced in cell-free systems, including MembraneMax™ Protein Expression Kits, is generally dependent on many factors, such as:

- Size of the protein
- Sequence of the gene of interest
- Positioning of the gene of interest relative to the T7 promoter and the Shine-Dalgarno ribosome binding site in the DNA template
- Expression of protein as a fusion with an N- or C-terminal tag (typically added to facilitate detection and purification of recombinant protein)
- Optimized codon usage
- Quality of the DNA template
- Stability of mRNA

Recommendations and guidelines to generate a DNA template with the optimal configuration and to purify the DNA template are provided in this section. The size of the protein and its sequence will vary depending on your gene of interest. Any variability in protein yield due to these two factors will require empiric experimentation to optimize expression conditions.

Continued on next page

Generating the DNA Template, continued

DNA Templates

The following DNA templates may be used in MembraneMax™ Protein Expression Kits:

- Supercoiled plasmid DNA (recommended to obtain the highest yields)
- Linear DNA
- PCR product

Many expression vectors or DNA templates may be used. For proper expression, all templates must contain the T7 promoter, an initiation codon, and a prokaryotic Shine-Dalgarno ribosome binding site (RBS) upstream of the gene of interest. See below for a discussion of template optimization.

Designing Your Own Template

If you are designing your own expression construct, we recommend generating a DNA template that contains the following elements (see the figure below for reference).

- Gene of interest placed downstream of a T7 promoter and a ribosome binding site (RBS). The gene of interest **must** contain an ATG initiation codon and a stop codon.
- Sequence upstream of the T7 promoter containing a minimum of 6–10 nucleotides (nt) for efficient promoter binding (**required** for linear PCR products). This sequence need not be specific.
- Sequence following the T7 promoter containing a minimum of 15–20 nt which forms a potential stem-and-loop structure as described by Studier *et al*., 1990 (see **MembraneMax™ Compatible Vectors**, next page, for more information).
- Sequence of 7–9 nt between the RBS and the ATG initiation codon for optimal translation efficiency of the protein of interest. This sequence need not be specific.
- A T7 terminator located 4–100 nt downstream of the gene of interest for efficient transcription termination and message stability.

MembraneMax™ Compatible Vectors

Minimum Requirements

Many T7-based expression vectors are suitable for use as templates for the Expressway™ Expression Module in MembraneMax™ Kits. At a minimum, these vectors must contain the T7 promoter, RBS, and T7 terminator with the suitable spacing and sequence configuration for optimal expression of protein.

The following section includes important information on T7-based Gateway® and TOPO® Cloning vectors that are recommended for use with the Expressway™ Module in MembraneMax™ Kits.

Important

The pEXP1-DEST, pEXP3-DEST, pEXP4-DEST, pEXP5-NT/TOPO®, and pEXP5-CT/TOPO® vectors, as well as any other vector system that would allow the expression of polyhistidine-tagged proteins, must be used with the MembraneMax™ Protein Expression Kit, and **not** the MembraneMax™ *HN* Protein Expression Kit. See page 6 for details.

Any expression vectors containing a sequence for C-terminal polyhistidine tag, including pEXP4-DEST and pEXP5-CT/TOPO®, can be used with the MembraneMax™ *HN* Protein Expression Kit, **only if**:

- the polyhistidine tag is not used for the purification of the membrane protein, or
- the gene for the membrane protein of interest and the sequence for the polyhistidine tag are separated by a stop codon in the expression construct.

Note

We recommend using N-terminal 6xHis tags for generating fusion membrane proteins because of higher expression levels in the MembraneMax™ protein synthesis reactions as compared to the C-terminally tagged fusion proteins.

We **do not** recommend the pEXP2-DEST vector for use with the MembraneMax™ Protein Expression Kits because of low expression levels observed.

MembraneMax™ Compatible Vectors, continued

Recommended Expression Vectors

The following expression vectors, available separately from Life Technologies, are ideally suited for use with the MembraneMax™ Protein Expression Kits (see page ix for ordering information). For detailed instructions on cloning your gene of interest into an expression vector, refer to the manual for the specific vector you are using. For more information on T7-based vectors, refer to our website at **www.lifetechnologies.com** or contact Technical Support (page 34).

Note: See previous page for important information on using vector systems that allow the expression of polyhistidine-tagged recombinant fusion membrane proteins with MembraneMax™ *HN* Protein Expression Kits.

pEXP5-NT/TOPO® and pEXP5-CT/TOPO® Vectors

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors available from Life Technologies are ideally suited for use with MembraneMax™ Protein Expression Kits. Both vectors include the following features:

- T7 promoter, RBS, and T7 terminator with spacing and sequence configuration optimized to allow the high levels of protein expression in MembraneMax™ Protein Expression Kits
- Adapted with topoisomerase I to allow highly efficient, 5-minute, TOPO® Cloning of *Taq* polymerase-amplified PCR products for rapid generation of expression constructs
- An N-terminal peptide containing the 6xHis tag and a Tobacco Etch Virus (TEV) recognition site to allow synthesis of a recombinant fusion membrane protein that may be easily detected and purified (pEXP5-NT/TOPO® only). The TEV recognition site allows the AcTEV™ Protease-mediated removal of the N-terminal tag to generate nearly native recombinant protein with only 2 amino acids added to N-terminus)
- A C-terminal peptide containing the 6xHis tag to allow synthesis of a recombinant fusion membrane protein that may be easily detected and purified (pEXP5-CT/TOPO® only)

pEXP1-DEST Vector

The pEXP1-DEST vector allows T7-based, high-level expression of recombinant fusion proteins in the Expressway™ Cell-free *E. coli* Expression System and can be used with the MembraneMax™ Kits. The vector contains the following elements:

- Bacteriophage T7 promoter for high-level, inducible expression of the recombinant membrane protein of interest in the MembraneMax™ Protein Expression Kit
- N-terminal Xpress™ and 6xHis fusion tags for detection and purification of recombinant fusion proteins
- Two recombination sites, *att*R1 and *att*R2 downstream of the T7 promoter for Gateway® cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- *ccd*B gene located between the attR sites for negative selection

Note: We **do not** recommend the pEXP2-DEST vector for use with the MembraneMax™ Protein Expression Kits because of the low membrane protein expression levels observed.

MembraneMax™ Compatible Vectors, continued

pEXP3-DEST and pEXP4 Vectors

The pEXP3-DEST and pEXP4-DEST vectors are designed to allow T7-based, high-level expression of N- and C-terminal tagged recombinant fusion proteins using the Expressway™ Lumio™ Cell-free Expression and Detection System, and can be used with the MembraneMax™ Kits. The vectors contain the following elements:

- Bacteriophage T7 promoter for high-level, inducible expression of the recombinant membrane protein of interest in the MembraneMax™ Protein Expression Kit
- N- or C-terminal 6xHis fusion and Lumio™ tags for detection and purification of recombinant fusion proteins (pEXP3-DEST and pEXP4-DEST, respectively)
- An N-terminal peptide containing TEV recognition site to allow the easy removal of the N-terminal tags from your recombinant fusion protein using the recombinant AcTEV™ Protease (pEXP3-DEST only)
- Two recombination sites, *att*R1 and *att*R2 downstream of the T7 promoter for Gateway® cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- *ccd*B gene located between the attR sites for negative selection

Important

Any vector system that would allow the expression of polyhistidine-tagged proteins must be used with the MembraneMax™ Protein Expression Kit, and **not** the MembraneMax™ *HN* Protein Expression Kit.

Any expression vectors containing a sequence for C-terminal polyhistidine tag can be used with the MembraneMax™ *HN* Protein Expression Kit, **only if**:

- the polyhistidine tag is not used for the purification of the membrane protein,

  or
- the gene for the membrane protein of interest and the sequence for the polyhistidine tag are separated by a stop codon in the expression construct.

Purifying the DNA Template

Sequencing

Once you have generated your DNA template, we recommend that you sequence the expression construct to confirm the presence and orientation of the gene of interest. If you have generated a fusion construct verify that your gene of interest is in frame with the appropriate N- or C-terminal tag.

Purifying the DNA Template

After you have generated the DNA template, you must purify the DNA before proceeding to the protein synthesis reaction. You may use a variety of methods to purify your DNA template including commercial DNA purification kits (see below) or CsCl gradient centrifugation. When purifying your DNA template, keep the following in mind:

- **Do not gel-purify** your DNA template. Purified DNA solution obtained from agarose gels significantly inhibits the protein synthesis reaction.
- For rapid isolation of high quality purified plasmid DNA, we recommend using the PureLink® HiPure Plasmid Midi- or Maxiprep Kits available from Life Technologies (see page ix for ordering information). Other commercial DNA purification kits are also suitable.
- For rapid isolation of high quality DNA from PCR reactions, we recommend using the PureLink® PCR Purification Kit available from Life Technologies (see page ix for ordering information).
- Ammonium acetate is not recommended for use in DNA precipitation as any residual contamination may inhibit translation. Use sodium acetate.
- Make sure that the purified DNA is free of RNase contamination. Wear gloves and use RNase-free reagents when preparing DNA.
- Make sure that purified DNA is free of excess ethanol or salt as both can inhibit translation.

  Note: Carefully wash ethanol precipitated DNA with 70% ethanol to remove excess salt and dry.
- Resuspend purified DNA in 1X TE Buffer or water to a final concentration of at least 500 ng/µL.

MembraneMax™ Reaction Conditions

Introduction

After you have obtained purified template DNA, you are ready to synthesize recombinant membrane protein using the MembraneMax™ or the MembraneMax™ *HN* Protein Expression Kit. This section provides guidelines and the standard protocol to synthesize your protein.

Important

RNase contamination may affect protein yield. To reduce the chances of RNase contamination, wear gloves and use RNase-free reagents when performing the protein synthesis reaction. To eliminate RNase from surfaces, use RNase *AWAY*® available separately from Life Technologies (see page ix).

Note

Membrane protein yields may vary depending on the nature of the protein expressed and the template utilized, as well as the reaction conditions used during synthesis. **For highest membrane protein yields, the reaction conditions must be optimized.** See page 18 for suggestions to optimize your MembraneMax™ protein synthesis reaction.

Reaction Volumes

The volume of the protein synthesis reaction may be scaled, based on your needs. For screening reactions, the standard volume is 100 µL (50 µL initial reaction + 50 µL Feed Buffer), but this can be decreased to 25 µL reaction volume and increased up to 10 mL reaction volume. For scaling the reaction, adjust the volumes of the reagents accordingly. You can also use 96-well plates (or any other plate configurations) for high-throughput membrane protein synthesis with the reaction volumes adjusted appropriately for the size of the wells.

Amount of DNA Template

For a 100 µL protein synthesis reaction, use 1 µg of template DNA (plasmid or linear DNA). For a 2 mL reaction, use 10–15 µg of template DNA. For optimal results, purify DNA template before use (see page 12).

Reaction Vessel

Use a reaction vessel that contains a large enough surface area to allow moderate mixing to occur. We recommend performing the 100 µL membrane protein synthesis reaction in a sterile, RNase-free 1.5-mL microcentrifuge tube. If you require larger reaction volumes, you may use sterile, RNase-free 50-mL conical tubes. Other reaction vessels including 96-well, 6-well, or 12-well untreated culture plates are suitable.

Continued on next page

MembraneMax™ Reaction Conditions, continued

Incubation Conditions

- To obtain optimal protein yield, **it is critical to mix the reaction thoroughly throughout the incubation period.** We recommend using a thermomixer set to 1,200 rpm or a shaking incubator set to 300 rpm. **Do not** use stationary incubators such as incubator ovens or water baths as protein yields may be reduced by up to 30–50%.
- Incubate the protein synthesis reaction at a temperature ranging from 30°C to 37°C. The optimal temperature should be determined empirically. Higher protein yields are generally obtained with incubation at higher temperatures (i.e., 37°C). The MembraneMax™ Reagent solubilizes the membrane protein at both 30°C and 37°C; however, lower temperatures decrease the rate of translation.
- You may obtain your protein of interest in 1.5 hours of incubation after feeding (2 hours total). **Many reactions yield 75% of total protein within the first hour**. Although longer incubation times up to more than 4 hours will increase total protein yield, the buildup of lipids in the reaction mix that are displaced from the NLP bilayer by membrane proteins during synthesis could trigger the formation of polydispersed micelles.

Amino Acid Concentration

Use an amino acid concentration ranging from 1 mM to 4 mM in the protein synthesis reaction. The recommended amino acid concentration is 1.25 mM each, but may be adjusted according to the protein being synthesized and your application (see **Using Unnatural Amino Acids**, below).

Feed Buffer

Add 1 volume of Feed Buffer (containing Expressway™ 2X IVPS Feed Buffer and amino acids) to the protein synthesis reaction after the initial 30-minute incubation. Higher protein yields may be obtained by adding one half-volume of Feed Buffer at 30 minutes and one half-volume of Feed Buffer again at 1 hour after initiating the protein synthesis reaction. If you choose longer incubation times, you may add the Feed Buffer at empirically determined intervals.

Using Unnatural Amino Acids

Methionine is supplied separately in the kit to allow you to incorporate unnatural amino acids into your recombinant protein and adjust the amino acid concentration in the protein synthesis reaction. Depending on your application, you may use the following unnatural amino acids:

- **Radiolabeled methionine:** Use [^{35}S]-Methionine to produce radiolabeled protein for use in expression and purification studies.
- **Heavy metal-labeled methionine:** Use selenomethionine (Budisa *et al.*, 1995; Doublie, 1997; Hendrickson *et al.*, 1990) to produce labeled protein for use in X-ray crystallographic studies.

Note: See **Performing the MembraneMax™ Protein Synthesis Reaction**, page 17, for the recommended amounts of labeled and unlabeled methionine.
When using selenomethionine, **do not** use any unlabeled methionine in the protein synthesis reaction.

Continued on next page

MembraneMax™ Reaction Conditions, continued

Handling Reagents

- **Do not** store the *E. coli slyD*⁻ Extract, 2.5X IVPS Reaction Buffer (–amino acids), or 2X IVPS Feed Buffer at –20°C or room temperature as this may result in loss of activity.
- Freezing and thawing the *E. coli slyD*⁻ Extract, 2.5X IVPS Reaction Buffer (–amino acids), and 2X IVPS Feed Buffer once or twice is acceptable. However, you should avoid multiple freeze/thaw cycles as this may result in loss of activity. You may also aliquot the reagents and freeze them to minimize multiple freeze/thaw cycles.
- Although multiple freeze/thaw cycles do not affect the performance of the MembraneMax™ Reagent, keep the freeze/thaw cycles to a minimum.

Important

All-trans retinal is light-sensitive. Although it is provided in an amber bottle, you should keep its exposure to light at a minimum and store it in the dark after use.

Positive Control Vectors

There are two positive control vectors included in the MembraneMax™ Protein Expression Kits.

- pEXP5-NT/CALML3 is for use with the Expressway™ Expression Module, and expresses the N-terminally-tagged human CALML3 protein.
- pEXP5-CT/bR is for use with the MembraneMax™ and MembraneMax™ *HN* Expression Modules in the rapid colorimetric control assay, and expresses bacteriorhodopsin.

For details about the control vectors, refer to pages 32 and 33. To propagate and maintain the control plasmids:

1. Use 10 ng of plasmid to transform a *rec*A, *end*A *E. coli* strain such as TOP10, DH5α™, or equivalent using your method of choice.
2. Select transformants on LB agar plates containing 100 µg/mL ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

MembraneMax™ Protein Synthesis Reaction

Materials Needed

- Expression construct or other suitable DNA template (purified; resuspended in TE or water at a concentration greater than 500 ng/µL)
- For each reaction, sterile, RNase-free 1.5-mL microcentrifuge tubes or other suitable reaction vessels (see page 13)
- RNase-free pipette tips and microcentrifuge tubes
- Thermomixer (set to 30°C or 37°C and 1,200 rpm)

  Optional: Standard shaking incubator (set to 300 rpm) instead of thermomixer

  Optional: Labeled methionine (if producing labeled recombinant protein, see page 14)

  Supplied with the kit:
- MembraneMax™ or MembraneMax™ *HN* Reagent (thaw at room temperature)
- Expressway™ *E. coli slyD*⁻ Extract (thaw on ice)
- Expressway™ 2.5X IVPS Reaction Buffer (–amino acids) (thaw on ice)
- Expressway™ 2X IVPS Feed Buffer (thaw on ice)
- T7 Enzyme Mix (keep on ice; store at –20°C after initial use)
- 50 mM Amino Acids (–Met)

  Note: When thawing the 50 mM Amino Acids (–Met), the solution may have a brown or yellowish tint. This is normal and does not affect the activity of the amino acids.
- 75 mM Methionine
- DNase/RNase-free Water (supplied with the kit)

  Optional: pEXP5-CT/bR control plasmid (0.5 µg/µL in TE Buffer, pH 8.0) and all-trans retinal substrate (10 mM in DMSO) (if performing the rapid colorimetric control reaction)

Scaling the MembraneMax™ Protein Synthesis Reaction

The MembraneMax™ Protein Expression Kits in their 100 reaction configuration are supplied with five tubes of the appropriate MembraneMax™ Reagent, as well as five tubes each of Expressway™ *E. coli slyD*⁻ Extract (400 µL per tube), Expressway™ 2.5X IVPS Reaction Buffer (–amino acids) (400 µL per tube), and Expressway™ 2X IVPS Feed Buffer (500 µL per tube).

It is possible to synthesize recombinant membrane protein in various reaction volumes, such as for screening experiments (i.e., 100 µL reaction) or to obtain larger amounts of protein (i.e., up to 10 mL per reaction). For small reaction volumes, we recommend the following:

1. **Thaw on ice** the MembraneMax™ or MembraneMax™ *HN* Reagent, *E. coli slyD*⁻ Extract, 2.5X IVPS Reaction Buffer (–amino acids), and 2X IVPS Feed Buffer.
2. Remove the amount of MembraneMax™ or MembraneMax™ *HN* Reagent, *E. coli slyD*⁻ Extract, 2.5X IVPS Reaction Buffer (–amino acids), and 2X Feed Buffer needed for the protein synthesis reaction and return tubes to a –80°C freezer.

Continued on next page

Performing the MembraneMax™ Protein Synthesis Reaction

Use the protocol below to synthesize your recombinant membrane protein from the DNA template. The amounts given are for a standard 100 µL reaction; if you are scaling up the reaction, adjust the volume of reagents accordingly.

1. **For each sample**, add the following reagents to the appropriate reaction vessel (see page 13):

Reagent	Protein Synthesis Rxn	Positive Control Rxn	Negative Control Rxn
E. coli slyD⁻ Extract	20 µL	20 µL	20 µL
2.5X IVPS Reaction Buffer (–amino acids)	20 µL	20 µL	20 µL
50 mM Amino Acids (–Met)	1.25 µL	1.25 µL	1.25 µL
75 mM Methionine*	1 µL	1 µL	1 µL
MembraneMax™ or MembraneMax™ *HN* Reagent	2 µL	2 µL	–
T7 Enzyme Mix	1 µL	1 µL	1 µL
DNA Template	1 µg	–	–
pEXP5-CT/bR	–	2 µL	2 µL
DNase/RNase-Free Water	To a final volume of 50 µL		

2. Close the tube and incubate sample in the thermomixer (1,200 rpm) at 30°C–37°C for 30 minutes. During incubation, prepare the Feed Buffer by adding the following reagents to a sterile, RNase-free microcentrifuge tube **for each sample**. For multiple samples, you may scale up the volume of reagents used accordingly and prepare one master mix.

Reagent	Protein Synthesis Rxn	Positive Control Rxn	Negative Control Rxn
2X IVPS Feed Buffer	25 µL	25 µL	25 µL
50 mM Amino Acids (–Met)	1.25 µL	1.25 µL	1.25 µL
75 mM Methionine*	1 µL	1 µL	1 µL
10 mM all-trans retinal	–	0.5 µL	0.5 µL
DNase/RNase-Free Water	To a final volume of 50 µL		

***Note:** To generate radiolabeled membrane protein using [^{35}S]-methionine, add 1 µL each of [^{35}S]-methionine and unlabeled 75 mM methionine when initiating the reaction, then again in the feed buffer. To generate labeled protein using selenomethionine, use 2 µL of selenomethionine only, **do not** add unlabeled methionine.

3. After 30 minutes of incubation (from Step 2 above), add 50 µL of the Feed Buffer to the sample (total volume = 100 µL).
4. Cap the tube and return the sample to thermomixer (1,200 rpm). Incubate for 1.5–2 hours at 30°C –37°C as appropriate (see page 14).
5. Place the reaction on ice and proceed to **Analyzing Samples**, pages 19–21 or store the sample at –20°C for future processing or analysis.

Continued on next page

Optimizing the MembraneMax™ Protein Synthesis Reaction

Depending on your protein and based on your initial results, you may want to optimize your MembraneMax™ Protein Synthesis reaction. The guidelines presented below **do not** constitute an exhaustive list, but are provided as a starting point in optimization, which must be approached empirically.

- You may incubate the reaction longer than the recommended 2 hours (up to 4 hours) to increase protein yield, although this may result in the formation of polydispersed micelles (see page 14, **Incubation Conditions**). Since 2 hours of incubation typically results in more than 75% expression for most proteins, longer incubation is usually unnecessary.
- You may increase the incubation temperature to boost the rate of translation or decrease the temperature to allow the protein more time to fold properly. **Do not** decrease the incubation temperature below 24°C, which is the transition temperature of the DMPC lipid bilayer of the MembraneMax™ Reagent.
- Changing the oxygenation of the reaction will also alter its kinetics. You may achieve this by increasing or decreasing the speed of the thermomixer or shaking incubator.
- You may modify the feeding schedule of the synthesis reaction. For example, you may initiate the reaction with 50% reaction volume, and then add 25% reaction volume of feed buffer twice at 30 minute intervals (e.g., 50 µL initial volume + 25 µL feed buffer twice for a total reaction volume of 100 µL). In the standard MembraneMax™ Protein Synthesis protocol, the reaction is initiated with 50% of the final reaction volume, and after 30 minutes, is supplemented with feed buffer at 50% of the total reaction volume (e.g., 50 µL initial volume + 50 µL feed buffer for a total reaction volume of 100 µL).
- Protein yield is reaction volume dependent. You may scale up the reaction to obtain larger quantities of membrane protein.
- Increasing the concentration of the MembraneMax™ Reagent in the reaction mix will not improve protein yield; however, you may titrate down the amount of the reagent used.

Analyzing Samples

Introduction

Once you have performed the membrane protein synthesis reaction, you may use any method of choice to analyze your sample. Generally, the amount of membrane protein produced in a MembraneMax™ Protein Synthesis reaction is sufficient to allow detection on a Coomassie-stained protein gel or by western blot analysis. If you have trace-labeled your membrane protein with [^{35}S]-methionine during synthesis, you can analyze your protein by autoradiogram. Alternatively, you can use enzymatic activity assays or affinity purification for more robust biochemical analysis.

- The rapid colorimetric control assay performed in parallel to the membrane protein synthesis reaction (see below) indicates whether the components of the MembraneMax™ Protein Expression Kit are functioning as intended.
- If you plan to analyze your sample using polyacrylamide gel electrophoresis, first precipitate the proteins with acetone to remove background smearing. A protocol to perform the acetone precipitation and other general guidelines for gel electrophoresis are provided on the next page.
- If you have performed trace labeling using [^{35}S]-methionine, you may use TCA precipitation to determine the amount of radiolabeled methionine incorporated and to calculate the yield of protein (see **Determining Protein Yield**, page 23).

Interpreting the MembraneMax™ Colorimetric Control Assay

Successful expression of the correctly-folded, functional bacteriorhodopsin (bR) in the presence of its cofactor all-trans retinal causes the reaction mixture to change from a pale yellow to a characteristic pink color in as little as 15 minutes. Longer incubation times (up to the recommended 2 hours or more) results in higher bR yields and stronger color change. See page 4 for more information on the bacteriorhodopsin protein.

If the MembraneMax™ Protein Synthesis reaction proceeds correctly, you will observe the emergence of the characteristic pink color in your positive control reaction mixture, while the negative control mixture remains pale yellow.

The control sample may also be analyzed by any one of the methods described on the following pages.

Note

The expression levels of membrane proteins may vary from protein to protein depending on the nature of the protein, the configuration of the DNA template, and the conditions of the MembraneMax™ Protein Synthesis reaction. If you have used small reaction volumes for preliminary experiments or for screening purposes, you may not be able to visualize your membrane protein on a Coomassie-stained protein gel. In such cases, we recommend silver staining the protein gel, or using western blot analysis or autoradiography to visualize your membrane protein.

Continued on next page

SDS-PAGE Analysis

To facilitate separation and visualization of your recombinant membrane protein by SDS polyacrylamide gel electrophoresis, Life Technologies offers a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus, as well as a large selection of staining kits and molecular weight protein standards, including Novex® Sharp™, BenchMark™, and HiMark™. For ordering information, see page xii. For more information on the various electrophoresis products available from Life Technologies, visit **www.lifetechnologies.com** or contact Technical Support (page 34).

Before SDS-PAGE analysis, you must first precipitate the protein with acetone to remove background smearing. A protocol to perform acetone precipitation and other general guidelines for gel electrophoresis are provided below.

Materials Needed for SDS-PAGE Analysis

Acetone (room temperature)

1X NuPAGE® LDS or Novex® Tris-Glycine SDS Sample Buffer (or any other compatible sample buffer appropriate to the gel used; see page xii)

Appropriate polyacrylamide gel to resolve your protein of interest

Appropriate protein standard and stain (e.g., Novex® Sharp™ Protein Standard, SimplyBlue™ SafeStain; see page xii)

Note: You may also use standard SDS-PAGE gels and sample buffers.

Acetone Precipitation

Use the following protocol to precipitate your proteins prior to loading on the polyacrylamide gel for analysis. Before starting this procedure, make sure that you have an appropriate gel for your protein size, or use one of the pre-cast polyacrylamide gels available from Life Technologies (see page xii).

1. Add 5 µL of the protein reaction product from Step 5, page 17, to 20 µL of acetone. Mix well.
2. Centrifuge for 5 minutes at room temperature in a microcentrifuge at 10,000 × *g*.
3. Carefully remove the supernatant, taking care not to disturb the protein pellet.
4. Resuspend pellet in 20 µL of 1X NuPAGE® LDS Sample Buffer.
5. Heat at 70–80°C for 10–15 minutes and centrifuge briefly. Proceed to **Polyacrylamide Gel Electrophoresis**, next page.

   Note: Alternatively, samples may be stored at –20°C until needed.

Continued on next page

Polyacrylamide Gel Electrophoresis

1. Load 5–10 µL of the sample from Step 5, previous page, on an SDS-PAGE gel and electrophorese following manufacturer's recommendations. You may also save your sample by storing at –20°C, if desired.
2. Depending on your assay of choice, perform the following:

If you are…	Then…
Visualizing your protein using an appropriate protein stain	Stain gel by following the manufacturer's instructions.
Visualizing your protein using the Lumio™ Green Detection Kit (if your protein contains the Lumio™ tag)	Follow the instructions in the manual provided with the Lumio™ Green Detection Kit (see below).
Analyzing your protein by western blot	Transfer proteins electrophoretically to a suitable membrane and use an appropriate antibody to detect the protein of choice (see below).

Note: For radiolabeled proteins, the signal may be enhanced by placing the gel in a commercially available reagent that enhances the signal. Dry the gel and expose to x-ray film for 1–4 hours.

Detecting Fusion Proteins

If you have expressed your recombinant membrane protein with a fusion tag from pEXP1-DEST, pEXP3-DEST, pEXP4-DEST, pEXP5-NT/TOPO®, or pEXP5-CT/TOPO®, you may perform western blot analysis and detect protein expression utilizing an antibody appropriate to the epitope tag.

For selecting and ordering the appropriate antibody for your expression vector, see page x. For more information on antibodies, refer to **www.lifetechnologies.com**, or contact Technical Support (page 34).

Detection of Lumio™-tagged Proteins

If you are using pEXP3-DEST or pEXP4-DEST expression vectors, you can choose to express your recombinant membrane protein fused to the Lumio™-tag, a tetracysteine sequence tag that is specifically recognized and bound by the Lumio™ Detection Reagent, thus allowing both real-time and in-gel protein detection with high sensitivity. For more information on the Lumio™ Technology, see the Lumio™ Green Detection Kit manual or the Expressway™ Lumio™ Cell-free Expression and Detection System manual, both available at **www.lifetechnologies.com**, or by contacting Technical Support (page 34).

Continued on next page

Detecting CALML3 Control Protein

If you use pEXP5-NT/CALML3 as a positive control for protein expression with the Expressway™ Expression Module, you may be able to detect the CALML3 fusion protein on a Coomassie-stained gel using 5 µL of the reaction. A typical 100 µL protein synthesis reaction should yield at least 75 µg of the 19.5 kDa fusion protein.

Note

The CALML3 fusion protein expressed from pEXP5-NT/CALML3 is **not** a membrane protein. It is provided as a positive expression control for the Expressway™ Expression Module. If the rapid colorimetric control assay provides a positive result (i.e., the reaction mixture turns pink), you do not need to use the pEXP5-NT/CALML3 plasmid. However, if the positive control reaction mixture remains a pale yellow, you may try expressing CALML3 using the Expressway™ Expression Module in order to pinpoint the source of the glitch in your reaction.

A negative colorimetric assay followed by positive expression of the CALML3 fusion protein would implicate a degraded MembraneMax™ Reagent, while the lack of CALML3 protein expression would point to the degradation of the reagents in the Expressway™ Expression Module as a potential source.

Note: All MembraneMax™ Protein Expression Kit reagents are guaranteed for six months if stored properly. Upon receipt, store boxes as detailed on page vi.

What to Do Next

Once you have verified expression, you may use the recombinant membrane protein in any downstream application of your choice. If you plan to use the recombinant protein for structural analyses including x-ray crystallography, note that you must purify the recombinant protein before use. Use any method of choice to purify your recombinant protein.

- If you have cloned your gene in frame with the N-terminal or C-terminal polyhistidine tag in your expression vector, you may purify your recombinant fusion protein using a metal-chelating resin such as Ni-NTA or ProBond™. For guidelines to purify recombinant membrane protein using Ni-NTA or ProBond™, see page 25.
- Similarly, affinity purification with a metal-chelating resin can be employed to purify a native recombinant membrane protein or a recombinant protein containing a tag other than polyhistidine if it is synthesized using the MembraneMax™ *HN* Kit.
- A recombinant membrane protein containing an affinity tag other than a polyhistidine tag (e.g., GST or MBP) can be purified using the appropriate affinity chromatography method in tandem with a metal-chelating resin (such as Ni-NTA or ProBond™) provided that the membrane protein is expressed using the MembraneMax™ *HN* Kit.

  Note: Other metal-chelating resins are also suitable.

Determining Protein Yield

Introduction

If you have included radiolabeled methionine in the membrane protein synthesis reaction, you may use TCA precipitation to determine the amount of radiolabeled methionine incorporated and to calculate the yield of protein.

Determining Total Counts

1. Mix and spot 5 µL of each radiolabeled reaction from Step 5, page 17 on a glass microfiber filter (Type GF/C; Whatman, Cat. no. 1822-021).
2. Set aside and let dry. **Do not** wash or TCA precipitate these filters.

Performing TCA Precipitation

Two protocols are provided below for performing TCA precipitation; one to perform standard TCA precipitation and one to perform TCA precipitation using a vacuum filtration device (*e.g.* Millipore 1225 Sampling Manifold). Choose the protocol that best fits your needs.

Performing Standard TCA Precipitation

1. Mix and spot 5 µL of each radiolabeled reaction from Step 5, page 17 on a separate set of individual glass fiber (GF/C) filters and allow to air dry for approximately 5–10 seconds.
2. Place filter in a beaker and wash once with cold 10% TCA for 10 minutes at room temperature while shaking gently (use approximately 10–20 mL per filter).
3. Wash with 5% TCA for 5 minutes at room temperature while shaking gently. Repeat wash.
4. Rinse filters with methanol to facilitate drying.
5. Allow filters to dry, place in scintillation vials, and add scintillation fluid. Count samples in a scintillation counter.
6. Proceed to **Calculating Protein Yield**, next page.

Performing TCA Precipitation Using a Vacuum Filtration Device

1. Aliquot 5 µL of each radiolabeled reaction from Step 5, page 17 into separate glass tubes.
2. Add 100 µL of 1 N NaOH to each reaction and incubate for 5 minutes at room temperature.
3. Add 3 mL of 10% TCA to each glass tube and incubate tubes at +4ºC for 20 minutes.
4. Wet individual glass fiber (GF/C) filters with 10% TCA and place onto the vacuum filtration device.
5. Turn the vacuum on and pour the TCA solution from each glass tube into a sample well.
6. Wash filters twice with 5% TCA.
7. Wash filters once with 100% ethanol. Leave the vacuum on for 1 minute to allow the filters to dry.
8. Turn the vacuum off and remove the filters. Place the filters in scintillation vials, and add scintillation fluid. Count samples in a scintillation counter.
9. Proceed to **Calculating Protein Yield**, next page.

Continued on next page

Determining Protein Yield, continued

Calculating Protein Yield

Use the equations below to calculate the yield of protein obtained. You need to determine the pmoles of methionine present in your specific reaction. Remember to account for both radiolabeled and unlabeled methionine. You also need to determine the total counts incorporated using TCA precipitation (see previous page).

Background counts = Precipitable counts obtained in a mock reaction in the absence of DNA

$$\text{Total counts} = \text{total cpm per 5 } \mu\text{l spotted} \times \frac{\text{total reaction volume}}{5}$$

$$\text{Specific activity} = \frac{\text{total counts}}{\text{pmoles of methionine}}$$

$$\text{pmoles methionine incorporated} = \frac{\left[(\text{TCA precipitable counts} - \text{background counts}) \times \frac{\text{total reaction volume}}{5}\right]}{\text{specific activity}}$$

$$\text{pmoles of protein} = \frac{\text{pmoles of methionine incorporated into protein}}{\text{number of methionines in protein}}$$

$$\text{Yield of protein (in } \mu\text{g)} = \frac{\text{moles of protein} \times \text{molecular weight of protein}}{10^6}$$

Purifying the Recombinant Membrane Protein

Introduction

The presence of the N-terminal or C-terminal 6xHis tag in the pEXP1-DEST, pEXP3-DEST, pEXP4-DEST, pEXP5-NT/TOPO®, and pEXP5-CT/TOPO® vectors allows purification of your recombinant protein with a metal-chelating resin such as ProBond™ and Ni-NTA (see page xi for ordering information). This section provides guidelines for purification.

Note

pEXP3-DEST and pEXP5-NT/TOPO® vectors contain a Tobacco Etch Virus (TEV) recognition site that allows the removal of the N-terminal tag from your recombinant fusion protein using the AcTEV™ Protease (see page xi for ordering information).

ProBond™ and Ni-NTA resins

ProBond™ and Ni-NTA are nickel-charged agarose resins that can be used for affinity purification of:

- Fusion membrane proteins containing the 6xHis tag
- Native membrane proteins synthesized using the MembraneMax™ *HN*
- Membrane proteins containing an affinity tag other than polyhistidine if they are expressed using MembraneMax™ *HN* (tandem affinity purification)

To purify your fusion protein using another metal-chelating resin, refer to the manufacturer's instructions.

Guidelines for Purification

Use these guidelines for purifying your polyhistidine-tagged membrane protein (expressed using the MembraneMax™ Protein Expression Kit) or if you have expressed your protein using the MembraneMax™ *HN* Protein Expression Kits (either native or as a fusion containing a tag other than polyhistidine). Remember to use criteria appropriate for purification under native conditions. For details, refer to the ProBond™ or the Ni-NTA manual, as appropriate.

1. Prepare the purification column containing ProBond™ or Ni-NTA agarose resin. After applying the resin to the column, wash with 4 volumes of water followed by 8 volumes of Binding Buffer (supplied with the kit; 50 mM $NaPO_4$, pH 8.0, 500 mM NaCl) to equilibrate.
2. Centrifuge the protein synthesis reaction at 15,000 × *g* for 10 minutes at room temperature to remove insoluble material.
3. Load the supernatant containing soluble protein onto the equilibrated resin and incubate (i.e., batch binding) for 30 minutes at the desired temperature.
4. Wash the column twice with 2 volumes of Binding Buffer each time.
5. Wash the column twice with 2 volumes of Binding Buffer containing 20 mM imidazole.
6. Elute the protein using an Elution buffer containing an appropriate amount of imidazole (e.g., 250 mM imidazole).
7. Analyze the fractions using SDS-PAGE.
8. Pool the desired fractions and dialyze, if necessary.

Sample Membrane Protein Synthesis Experiment

Introduction

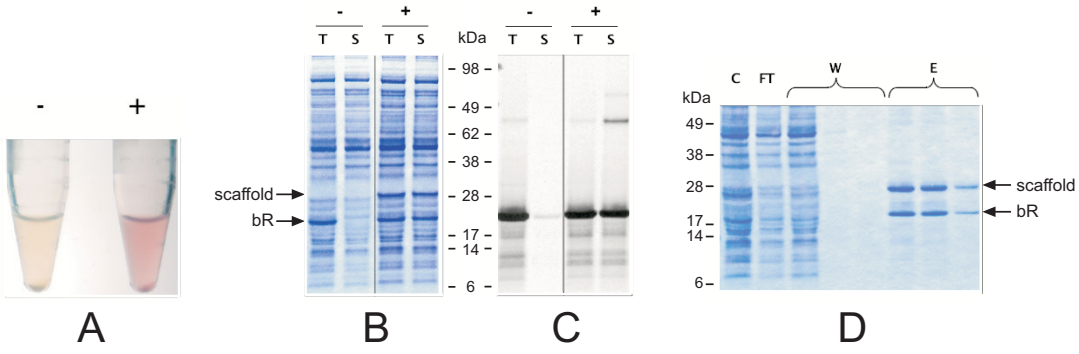
This section provides an example of a typical protein synthesis experiment performed using the MembraneMax™ Protein Expression Kit and results obtained.

Experimental Conditions

In this experiment, *Halobacterium salinarum* bacteriorhodopsin gene (bR; GenBank accession no. J02755) was PCR amplified and TOPO® cloned into pEXP5-CT/TOPO® to generate the 3435 bp pEXP5-CT/bR expression vector.

Upon purification, 1 µg of pEXP5-CT/bR was used in a 100 µl protein synthesis reaction with the components supplied in the MembraneMax™ *HN* Protein Expression Kit (containing polyhistidine-tagged MembraneMax™ *HN* Reagent) according to the protocol on page 17. The reaction mixtures contained 0.2 µCi/µL [^{35}S]-methionine for trace labeling and 0.5 µL 10mM all-trans retinal for the colorimetric control assay. As a negative control, pEXP5-CT/bR plasmid from the same preparation was used to express bR in an *in vitro* protein synthesis reaction that lacked the MembraneMax™ *HN* Reagent.

5 µL aliquot from each reaction was electrophoresed on a NuPAGE® Novex® 4–12% Bis-Tris Gel and stained (B, Coomassie Stained) or exposed to x-ray film (C, Autoradiogram), while the remainder of the synthesis reactions was purified using the Ni-NTA Purification System, separated by SDS electrophoresis and Coomassie-stained (D). Panel A depicts the results of the colorimetric assay in the absence (–) or the presence (+) of the MembraneMax™ *HN* Reagent.



A. bR expression in the presence (+) or absence (–) of MembraneMax™ *HN* Reagent and All-trans retinal with the characteristic pink color indicating correctly folded bR.

B. Coomassie-stained gel showing total crude extract (T) and soluble fractions (S) of bR synthesis reactions in the presence (+) or absence (–) of MembraneMax™ *HN* Reagent.

C. Autoradiogram of the gel depicted in panel B. Note the substantial increase in the soluble protein yield.

D. Coomassie-stained SDS gel depicting bR expressed in the presence of the MembraneMax™ *HN* Reagent and subsequently purified over a nickel column. C, FT, W, and E represent crude extract, flow-through, wash, and imidazole eluted fractions, respectively.

Scaffold protein is the polyhistidine-tagged protein ring enclosing the lipid bilayer as a part of the MembraneMax™ *HN* Reagent.

Although eluted from the nickel column intact, the bR-MembraneMax™ *HN* complex is denatured on the SDS gel, resulting in two distinct bands of correct sizes.

Troubleshooting

Introduction

Review the information in this section to troubleshoot your cell-free membrane protein expression experiment.

Problem	Reason	Solution
Low or no yield of protein (but control reaction produces protein)	DNA template not optimally configured	• Use one of the recommended pEXP1-DEST, pEXP3-DEST, pEXP4-DEST, pEXP5-NT/TOPO®, or pEXP5-CT/TOPO® expression vectors or follow the guidelines on page 8 to generate a DNA template with the optimal configuration. • Make sure that the ATG initiation codon is in the proper context for expression (i.e., check spacing and placement after the RBS). • Fusion of your protein to an N- or C-terminal tag may affect RNA structure and lower translation levels. Try moving the fusion tag to the other terminus.
	Gene of interest not cloned in frame with the N- or C-terminal tag	Generate a new expression construct, making sure that your gene of interest is cloned in frame with the N- or C-terminal tag; confirm by sequencing.
	DNA template not pure • Contaminated with ethanol, sodium salt, or ammonium acetate • Contaminated with RNases	• Prepare new DNA template taking care to remove excess ethanol and/or salt after precipitation. • Do not use ammonium acetate to precipitate DNA. Use sodium acetate. • Wear gloves and use RNase-free reagents when preparing DNA.
	DNA template purified from agarose gel	Do not purify your DNA from a gel. See the purification guidelines on page 12.
	Insufficient amount of DNA template used	• Use 10–15 µg of template DNA in a 2 mL protein synthesis reaction. • If you are expressing a large protein, increase the amount of DNA template used in the protein synthesis reaction to 20 µg.

Continued on next page

Problem	Reason	Solution
Low or no yield of protein (but control reaction produces protein), continued	Sample incubated in a water bath or non-shaking incubator	Incubate sample in a thermomixer or incubator with shaking (see page 14).
	Insufficient amount of MembraneMax™ reagent used	Increase the amount of MembraneMax™ reagent in reaction mixture.
	Incorrect MembraneMax™ Reagent used.	See page 6 to determine which MembraneMax™ Reagent is appropriate for your expression construct.
	Insufficient feeding	• Add one volume of Feed Buffer to the sample (i.e., 1 mL Feed Buffer to 1 mL sample) 30 minutes after initiating protein synthesis. • Add one-half volume of Feed Buffer to the sample (i.e., 25 µL Feed Buffer to 50 µL sample) 30 minutes and 1 hour after initiating protein synthesis.
	Large protein being expressed	• Protein yield may decrease as the size of the protein increase; optimize reaction conditions. • Reduce incubation temperature to 25°C–30°C during protein synthesis.
	Sample not mixed before spotting on filter for TCA precipitation (radiolabeled samples only)	Mix sample before spotting on filter for TCA precipitation.
	Degraded protein	• Limit incubation to <2 hours. • Minimize handling in between reaction, analysis, and purification steps. • Upon completion of expression, store reaction products immediately at –20°C.
	Expression vector contains the *lac* operator sequence and the *lacI* gene encoding the lac repressor	Add IPTG to induce protein expression.

Continued on next page

Problem	Reason	Solution
Control reactions produce no protein	Reagents have lost activity	• Store Expressway™ Expression Module reagents at –80°C. • Store the T7 Enzyme Mix at –20°C after initial use. • Use care when freezing and thawing the Expressway™ *E. coli slyD*⁻Extract, Expressway™ 2.5X IVPS *E. coli* Reaction Buffer, and Expressway™ 2X IVPS Feed Buffer. Follow handling guidelines on page 15. One or two freeze/thaw cycles are acceptable. Avoid multiple freeze/thaw cycles. • Our long-term stability studies reveal MembraneMax™ Reagent to be very stable at a wide range of temperatures.
	Reagent(s) contaminated with RNases	• Wear gloves and use RNase-free supplies when handling the reagents supplied in the kit. • Use RNase *AWAY*® available from Life Technologies (see page ix) to eliminate RNase from surfaces.
Colorimetric control assay does not produce pink color, but protein is expressed	All-trans retinal is bleached	All-trans retinal is a photolabile substrate; store in the dark.
Protein has low biological activity	Improper protein folding	• Reduce incubation temperature to as low as 25°C during protein synthesis. • Membrane lipid composition is crucial for proper membrane protein folding. MembraneMax™ reagent may not contain the optimal lipid composition for the protein of interest.
	Post-translational modifications required	The Expressway™ *E. coli slyD*⁻ Extract will not introduce post-translational modifications such as phosphorylation or glycosylation to the recombinant protein.
	Synthesized protein requires co-factors for complete activity	Add required co-factors to the protein synthesis reaction.

Continued on next page

Problem	Reason	Solution
Multiple bands observed on the polyacrylamide gel	Scaffold protein is detected	The scaffold protein from the MembraneMax™ Reagent will migrate as a 28 kDa band on an SDS-PAGE gel.
	Proteins denatured for too long	Add 1X SDS-PAGE sample buffer to the sample and incubate at 70°C–80°C for 10–15 minutes before loading on the gel.
	Large eukaryotic membrane protein (>100 kDa) is expressed	Expression of particularly large eukaryotic membrane proteins (>100 kDa) may produce a small portion of truncated products.
	Old [^{35}S]-methionine used (radiolabeled samples only)	Use fresh [^{35}S]-methionine.
	Not enough SDS in the 1X SDS-PAGE sample buffer	• Use a pre-mixed sample buffer available from Life Technologies (see page xii) • Prepare a fresh solution of SDS-PAGE sample buffer with correct enough SDS.
	Internal ATG codons in the context of RBS-like sequences	• Check the sequence of your gene and search for potential RBSs with the proper spacing from internal methionines. • Replace the methionine or change RBS sequence(s) using point mutation(s). • Clone your gene of interest into pEXP5-NT/TOPO® or pEXP5-CT/TOPO®.
	There is codon usage bias	Optimize codon usage.

Continued on next page

Smearing on the gel	Samples not precipitated with acetone	Precipitate the proteins with acetone to remove background smearing. Follow the protocol provided on page 20.
	Too much protein loaded	Reduce the amount of protein loaded on the gel.
	Gel not clean	• Rinse the gel briefly before exposing to film. • If you have stained the gel with Coomassie blue, destain the gel in water or 50% methanol, 7.5% glacial acetic acid for 15–30 minutes before drying. • If you have already destained the gel, repeat destaining procedure.
	Ethanol present in the protein synthesis reaction	Make sure that any residual ethanol is removed during DNA purification.
	Expired pre-cast gels	Do not use pre-cast gels after the expiration date.

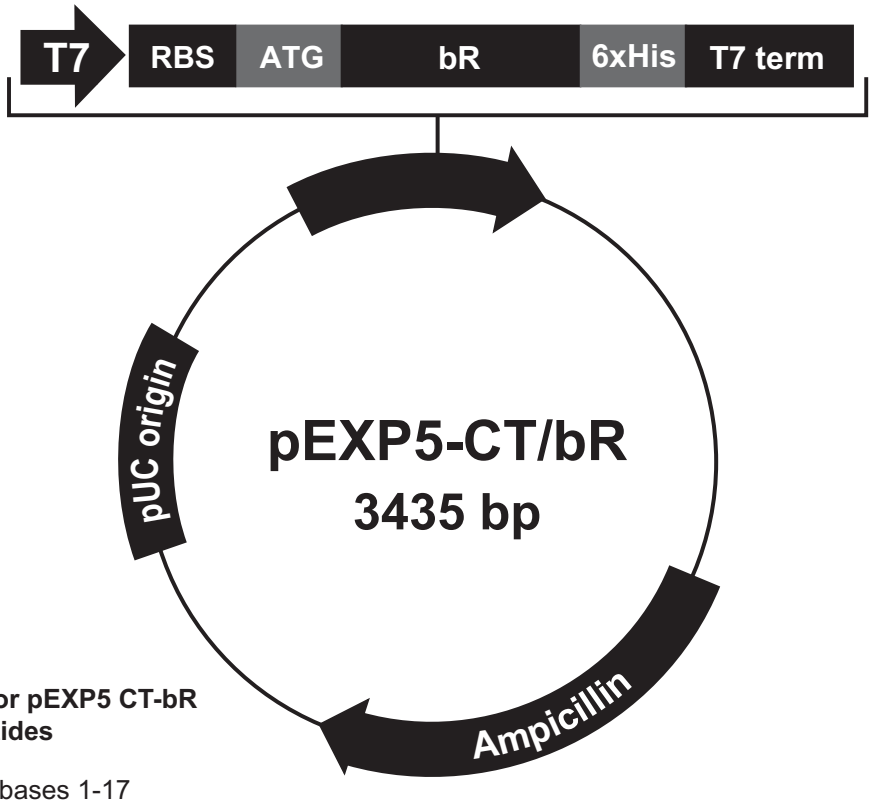
Appendix

Map and Features of pEXP5 CT-bR

pEXP5-CT/bR Map

The pEXP5-CT/bR vector (3435 bp) contains the *Halobacterium salinarum* bacteriorhodopsin gene (bR; GenBank accession number J02755) that has been TOPO® Cloned into pEXP5-CT/TOPO® in frame with the C-terminal polyhistidine tag.

The complete sequence of pEXP5-CT/bR is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (page 34).



Comments for pEXP5 CT-bR 3435 nucleotides

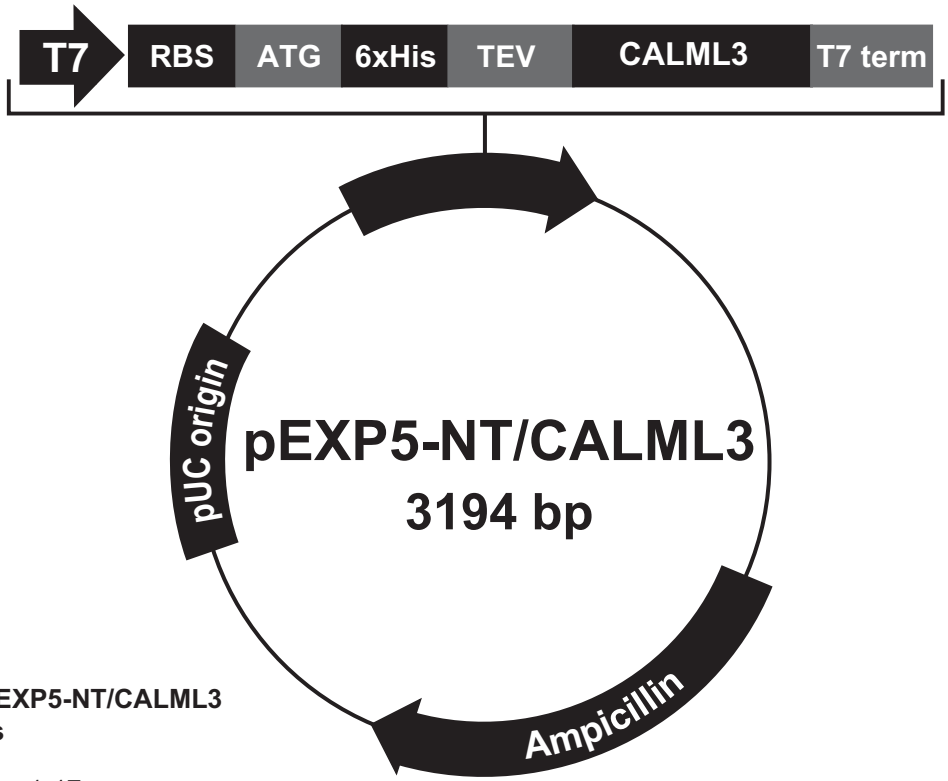
T7 promoter: bases 1-17
T7 forward priming site: bases 1-20
Ribosome binding site (RBS): bases 69-74
Initiation ATG: bases 82-84
bR: bases 82-828
Polyhistidine (6xHis) region: bases 838-855
T7 reverse priming site: bases 955-977
T7 transcription terminator: bases 893-977
bla promoter: bases 1089-1187
Ampicillin resistance gene: bases 1118-2048
pUC origin: 2193-2866

Map and Features of pEXP5-NT/CALML3

pEXP5-NT/CALML3 Map

The pEXP5-NT/CALML3 vector (3194 bp) contains a human calmodulin-like 3 gene (CALML3; GenBank accession number NM_005185) that has been TOPO® cloned into pEXP5-NT/TOPO® in frame with the N-terminal polyhistidine tag. The size of the CALML3 fusion protein is approximately 19.5 kDa.

The complete sequence of pEXP5-NT/CALML3 is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (page 34).



Comments for pEXP5-NT/CALML3
3194 nucleotides

T7 promoter: bases 1-17
T7 forward priming site: bases 1-20
Ribosome binding site (RBS): bases 68-73
Initiation ATG: bases 80-82
Polyhistidine (6xHis) region: bases 92-109
HisG epitope: bases 92-112
TEV recognition site: bases 122-142
CALML3: bases 146-595
T7 reverse priming site: bases 647-666
T7 transcription terminator: bases 608-736
bla promoter: bases 848-946
Ampicillin resistance gene: bases 947-1807
pUC origin: 1952-2625

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Purchaser Notification

Introduction

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Notes

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