PurePro™ *Caulobacter* Expression System

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PurePro™ Caulobacter Expression System

Rapid cloning of Taq polymerase-amplified PCR products into an expression vector for secretion and simplified purification of recombinant proteins in bacteria

Catalog no. K600-01

Invitrogen™
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Technical Services

Invitrogen provides Technical Services to all of our registered *Caulobacter* Expression Kit users. If you need assistance with the *Caulobacter* Expression Kit please call us at 1-800-955-6288.

Important Information

Shipping/Storage

The components in the PurePro™ *Caulobacter* Expression System are supplied in 4 boxes and are shipped as described below. For a description of the reagents in each box, see below and pages vii-viii. Upon receipt, store each box as indicated below.

Item	Contents	Shipping	Storage Temp
Box 1	pCX TOPO TA Cloning® Reagents	Dry ice	-20°C
Box 2	One Shot® TOP10F′ Chemically Competent Cells	Dry ice	-80°C
Box 3	One Shot® B5 BAC Electrocomp™ *Caulobacter* Cells	Dry ice	-80°C
Box 4	PurePro™ Accessory Kit	Room temperature	Room temperature and +4°C (see page viii for details)

pCX TOPO TA Cloning® Reagents

The pCX TOPO TA Cloning® reagents (Box 1) are listed below. **Note that the user must supply *Taq* polymerase.** Store Box 1 at -20°C.

Item	Concentration	Amount
pCX-TOPO®	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.5 1 mM EDTA 1 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM phenol red	20 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	--	1 ml

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pCX TOPO TA Cloning® Reagents, continued

Item	Concentration	Amount
M13 Reverse Sequencing Primer (for sequencing in the sense orientation)	Lyophilized in TE, pH 8	2 µg
RsaA Reverse Sequencing Primer	Lyophilized in TE, pH 8	2 µg
Control PCR Template	0.05 µg/µl in TE Buffer, pH 8	10 µl
Control PCR Primers	0.1 µg/µl each in TE Buffer, pH 8	10 µl
Expression Control Plasmid (pCX)	10 ng/µl in TE Buffer, pH 8	10 µl

One Shot® TOP10F′ Reagents

The table below describes the items included in the One Shot® TOP10F′ chemically competent cell kit (Box 2). **Store at -80°C.**

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10F′ chemically competent cells*	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-Hcl, .5 mM EDTA, pH 8.0	50 µl

*Supplied at an efficiency of 1 x 10^9 cfu/µg plasmid DNA

continued on next page

Important Information, continued

One Shot® B5 BAC Reagents

The table below describes the items included in the One Shot® B5 BAC Electrocomp™ cell kit (Box 3). **Store at -80°C.**

Item	Composition	Amount
PYE Broth (may be stored at +4°C or room temperature)	0.2% Peptone 0.1% Yeast Extract 0.02% $MgSO_4 \cdot 7H_2O$ 0.01% $CaCl_2 \cdot 2H_2O$	6 ml
B5 BAC electrocompetent cells*	--	21 x 50 µl
pCX Control DNA	10 ng/µl in TE Buffer, pH 8	10 µl

*Supplied at an efficiency of 1 x 10^6 cfu/µg plasmid DNA

PurePro™ Accessory Kit

The table below describes the items included in the PurePro™ Accessory Kit (Box 4). The amount of M11 Expression Medium concentrate provided is sufficient to prepare 5 liters of medium. Store the M11 Expression Medium at +4°C and the other accessories at room temperature.

Item	Composition	Amount
M11 Expression Medium	**Solution C, 25X stock** 7.5% glucose 8.6% sodium glutamate 125 mM imidazole, pH 7.0 50 mM K_xH_xPO4, pH 7.0 (KH_2PO_4 and K_2HPO_4 mixed to produce pH 7.0)	200 ml
	Solution M, 100X stock 105 mM Nitrilotriacetic acid (NTA)-Free Acid 260 mM KOH 221 mM $MgSO_4 \cdot 7H_2O$ 0.67% $CaCl_2 \cdot 2H_2O$ 15 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 2.963 mM $FeSO_4 \cdot 7H_2O$ 856 µM EDTA 3.809 mM $ZnSO_4 \cdot 7H_2O$ 911 µM $MnSO_4 \cdot H_2O$ 157 µM $CuSO_4 \cdot 5H_2O$ 86 µM $Co(NO_3)_2 \cdot 6H_2O$ 46 µM $Na_2B_4O_7 \cdot 10H_2O$	50 ml
Mesh	Nylon, 145 µm pore size	5 pieces
Buchner Funnel	Polypropylene	1 funnel

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Important Information, continued

Sequencing Primers

The table below lists the sequence and pmoles for the primers included in the PurePro™ *Caulobacter* Expression System. If you wish to order additional primers, see **Additional Reagents** on the next page.

Primer	Sequence	Amount
M13 Reverse	5′-CAGGAAACAGCTATGAC-3′	385 pmoles
RsaA Reverse	5′-GCCGCGCCAGCGACGCGGAGGG-3′	294 pmoles

Genotype of TOP10F′ Cells

TOP10F′ *E. coli* cells are provided for general cloning purposes. Note that this strain can be used for single-strand rescue of DNA from vectors that have an f1 origin of replication. TOP10F′ cells also contain the lacI^{q} gene encoding the lac repressor and are useful for expressing potentially toxic genes under the control of the *lac* promoter (e.g. pCX-TOPO®).

Genotype: F′ {*lac*I^{q} *Tn10*(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G

Genotype of B5 BAC Cells

B5 BAC *Caulobacter crescentus* cells are provided for expression purposes **only**. Do not use these cells for propagating or maintaining your construct. Note that the B5 BAC strain is derived from the JS4000 *Caulobacter crescentus* strain. For more information about the B5 BAC strain , see page 8.

Genotype: JS4000 (Bingle *et al.*, 1997b; Smit and Agabian, 1984) *rsaA* (Am) holdfast^{-} *rec*A(Δ*Eco*R V)::*rep*BAC

Accessory Products

Introduction

The products listed in this section are intended for use with the PurePro™ *Caulobacter* Expression System. For more information, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 47).

Additional Reagents

Many of the reagents supplied in the PurePro™ *Caulobacter* Expression System are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Amount	Catalog no.
One Shot® Kit (TOP10F′ Chemically Competent Cells)	20 reactions	C3030-03
	40 reactions	C3030-06
One Shot® Kit (B5 BAC Electrocomp™ Cells)	20 reactions	C7000-01
M13 Reverse Sequencing Primer	2 µg (385 pmoles)	N530-02
M11 Expression Medium*	5 liters	Q700-01

*Supplied as a concentrate (see page viii)

Additional Products

The table below lists additional products available from Invitrogen which you may use in conjunction with the PurePro™ *Caulobacter* Expression System.

Item	Amount	Catalog no.
pCX TOPO® TA Expression Kit	20 reactions	K6000-01
RsaA Antiserum	50 µl*	R970-25
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
S.N.A.P.™ Gel Purification Kit	25 reactions	K1999-25

*The amount of antibody supplied is sufficient for 25 western blots.

Introduction

Overview

Introduction

The PurePro™ *Caulobacter* Expression System utilizes the TOPO® Cloning technology to facilitate rapid cloning of *Taq* polymerase-amplified PCR products into a vector for high-level expression and simplified purification of recombinant proteins from *Caulobacter crescentus*. The pCX-TOPO® vector allows cloning of PCR products as N-terminal fusions with the RsaA protein. Once cloned into pCX-TOPO®, the RsaA protein directs secretion and aggregation of the fusion protein in the culture medium of *Caulobacter crescentus*. The recombinant fusion protein may then be purified to approximately 90% purity by a simple filtration step.

Caulobacter crescentus and the S-Layer

Caulobacter crescentus (hereinafter referred to as "*Caulobacter"*) is a gram-negative, non-pathogenic bacteria that is common to freshwater environments (Poindexter, 1981). *Caulobacter* possesses a dimorphic life cycle alternating between a monoflagellated swarmer cell and a nonmotile stalked cell. Because of its unusual life cycle, the bacteria has been studied for over 40 years and is well-characterized genetically and biochemically (Wheeler *et al.*, 1998).

Caulobacter is covered by a two-dimensional crystalline protein surface layer (S-layer) consisting of a hexagonal array of ring-like subunits (Smit *et al.*, 1981) composed of six copies of a single protein, RsaA (see below for more information) (Smit *et al.*, 1992). Proper crystallization and formation of the S-layer requires calcium ions (Bingle *et al.*, 1997b; Gilchrist *et al.*, 1992; Smit *et al.*, 1992; Walker *et al.*, 1992). The S-layer is anchored to the cell surface via a non-covalent interaction between the RsaA protein and a lipopolysaccharide moiety in the outer membrane (Walker *et al.*, 1994). While the function of the S-layer is not entirely understood, it is thought to act as a physical barrier to parasites and lytic enzymes. Studies have demonstrated that the S-layer can protect against predation by pathogens such as *Bdellovibrio bacteriovorus* (Koval and Hynes, 1991).

RsaA

RsaA is a 1026 amino acid, 98kDa protein encoded by the *rsaA* gene (Gilchrist *et al.*, 1992). The RsaA protein comprises the sole component of the S-layer and is expressed to extremely high levels in *Caulobacter* cells. In *Caulobacter*, the RsaA protein accounts for 10-12% of the total protein in the cell. The RsaA protein exhibits the following biochemical characteristics:

- Contains many small neutral residues
- Contains few charged amino acids (with the exception of several aspartic acid groups), resulting in an acidic protein with a predicted pI of 3.46
- Contains no cysteine residues
- Possesses a high content of hydroxyl-containing residues (25% of the amino acids are serine or threonine)

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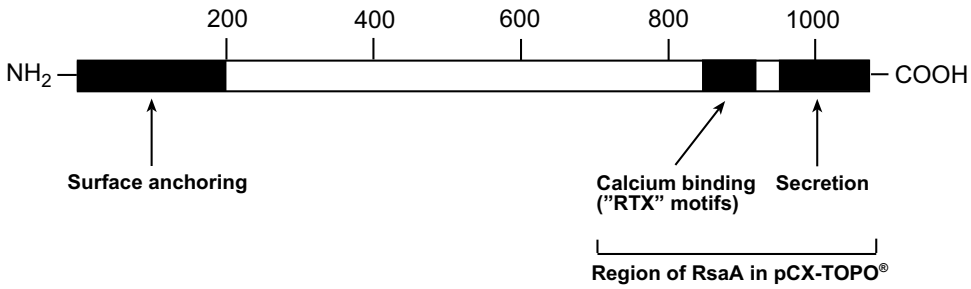
Overview, continued

Structural Features of RsaA

The RsaA protein has been well-characterized functionally and various studies have identified the following specific functional domains within the protein (see below and Figure 1):

- The N-terminus (approximately amino acids 1-200) is required for attachment of the protein to the cell surface (Bingle *et al.*, 1997a; Bingle *et al.*, 1997b)
- Glycine-rich "RTX" motifs in the C-terminal portion of the protein (amino acids 860-905) are required for calcium binding (Baumann *et al.*, 1993; Bingle *et al.*, 1997b; Bingle *et al.*, 2000)
- The extreme C-terminal portion (amino acids 944-1026) is required for secretion of the protein (Bingle *et al.*, 2000)

Figure 1. Functional regions of the RsaA Protein



Type I Secretion

Because RsaA is involved in forming the S-layer, the protein must be produced inside the cell and exported to the cell surface (see Figure 2 on the next page). Various studies have demonstrated that export of RsaA to the cell surface is mediated by a Type I, three-component, bacterial ABC transporter secretion system (Awram and Smit, 1998). S-layer proteins from other gram negative bacteria have also been shown to be secreted via a Type I secretion system (Kawai *et al.*, 1998; Thompson *et al.*, 1998). In general, Type I-secreted proteins exhibit the following characteristics:

- The secretion signal resides in the C-terminus of the protein. In RsaA, the secretion signal has been mapped to the last 82 amino acids (Bingle *et al.*, 1997a; Bingle *et al.*, 2000).
- The secretion signals are not cleaved, and remain on the exported protein.
- The C-termini of these proteins exhibit a low degree of primary structural homology to one another (Binet *et al.*, 1997).

Note

Because the Type I secretion mechanism creates a pathway to the exterior, proteins that might not be suited for secretion by other mechanisms (e.g. the general secretory pathway of bacteria involving direct insertion into membranes) might be successfully secreted by such a system.

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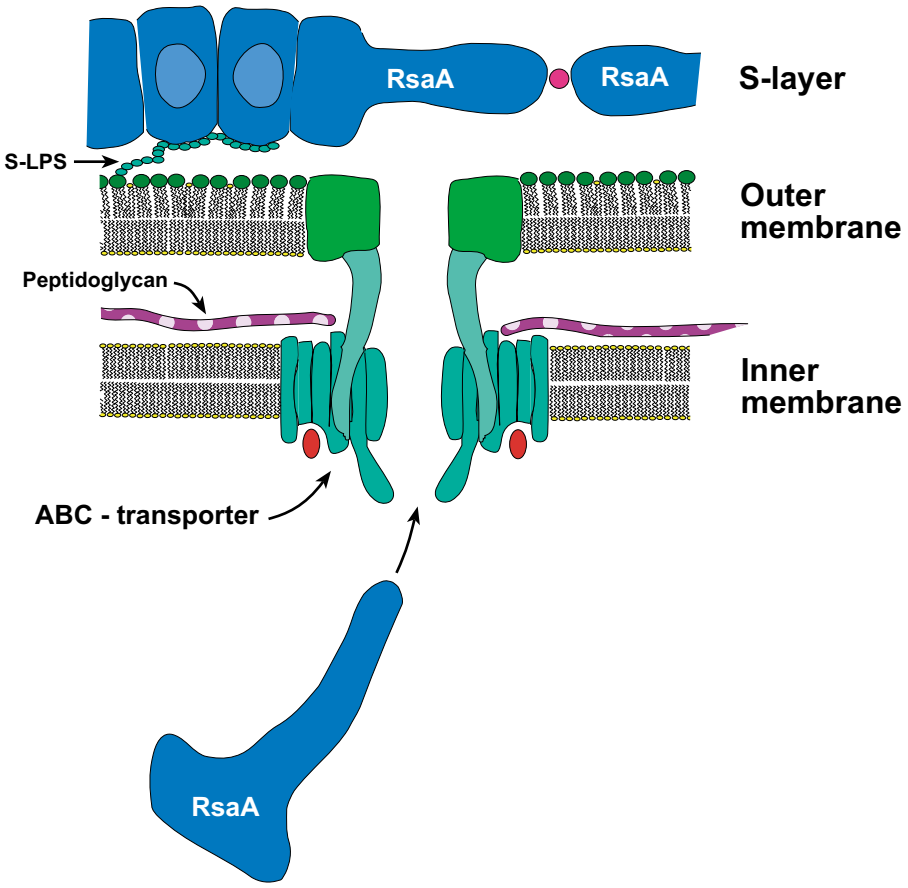
Overview, continued

Figure 2. Type I Secretion of RsaA to the Cell Surface



S-LPS = smooth lipopolysaccharide

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Figure provided courtesy of John Smit,
University of British Columbia, Canada

Overview, continued

RsaA Secretion Signal in pCX-TOPO®

As mentioned on page 2, the secretion signal resides in the C-terminal 82 amino acids of the RsaA protein. This C-terminal region has been further characterized and found to possess the following characteristics:

- It is sufficient to direct secretion and aggregation of the RsaA protein
- It is capable of autonomous secretion and is self-aggregating
- It can direct secretion and aggregation of heterologous fusion partners (Bingle *et al.*, 1997a; Bingle *et al.*, 2000)

The PurePro™ *Caulobacter* Expression System utilizes the unique characteristics of the RsaA secretion signal to allow expression and secretion of your heterologous protein of interest as a recombinant fusion protein. You will clone your gene of interest into the pCX-TOPO® expression vector as a fusion to a truncated RsaA protein (mw = 32 kDa). This truncated RsaA protein is derived from amino acids 690-1026 of the wild-type RsaA protein and includes the secretion signal and the RTX motifs. Although the secretion signal only requires the C-terminal 82 amino acids, the truncated RsaA protein in pCX-TOPO® is derived from the C-terminal 336 amino acids. In experiments at Invitrogen, we have found that use of this longer secretion signal provides the optimal length required for high-level expression of heterologous proteins in B5 BAC.

For more information about the features of pCX-TOPO®, see the next page.

Purification of RsaA Fusion Proteins

Because of the unique properties of the RsaA protein, heterologous proteins expressed as recombinant fusions can be produced to high levels in *Caulobacter* and secreted into the medium. Once secreted into the medium, the recombinant fusion proteins form a hydrated gel-like aggregate, allowing simple purification to greater than 90% purity by filtration through a coarse nylon mesh.

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The pCX-TOPO® Vector

pCX-TOPO® is a 5.4 kb expression vector designed to facilitate rapid cloning and expression of PCR products as fusions to the RsaA protein. The vector allows high-level expression of RsaA fusion proteins in *Caulobacter* cells. The vector contains the following elements:

- *lac* promoter for high-level, constitutive expression of the gene of interest in *Caulobacter* cells
- CX leader peptide for enhanced translation efficiency
- TOPO® Cloning site for rapid cloning of *Taq*-amplified PCR products (see the next page for more information)
- Truncated RsaA ORF (amino acids 690-1026 only) to direct secretion and aggregation of the recombinant RsaA fusion protein in the culture medium (see page 4)
- Chloramphenicol acetyltransferase (CAT) gene for selection of the vector in *E. coli* and *Caulobacter*
- RSF1010 origin (oriV) for high-copy replication of the plasmid in *Caulobacter* (see page 6 for more information) (Bagdasarian *et al.*, 1981)
- pUC origin for high-copy replication of the plasmid in *E. coli*

A control plasmid (pCX) is included for use as a positive control for expression and secretion (see page 41 for more information).

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How TOPO® Cloning Works

The plasmid vector, pCX-TOPO® is supplied linearized with:

- Single 3´ thymidine (T) overhangs for TA Cloning®
- Topoisomerase covalently bound to the vector (this is referred to as "activated" vector)

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products (see Figure 3 below).

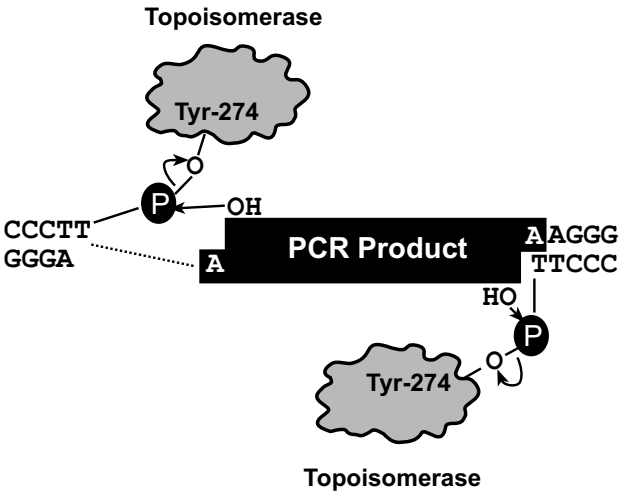

Figure 3. How TOPO® Cloning Works

oriV

The pCX-TOPO® vector contains an origin of replication (oriV) derived from the broad host range plasmid, RSF1010 (Bagdasarian *et al.*, 1981). Replication in *Caulobacter* also requires three replication factors encoded by the *repA*, *repB*, and *repC* (*repBAC*) genes. These three genes have been integrated into the genome of the JS4000 *Caulobacter* strain to generate the B5 BAC strain provided in the PurePro™ *Caulobacter* Expression System (Umelo-Njaka *et al.*, Manuscript in preparation). The presence of the *repBAC* genes in B5 BAC and the oriV from pCX-TOPO® allows high-copy replication of the plasmid in this strain. For more information about B5 BAC, see pages 8 and 20.

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Overview, continued

Proteins Expressed in *Caulobacter*

The table below lists some of the proteins that have been expressed as RsaA fusions (under shake flask conditions) in B5 BAC *Caulobacter*. The size of the protein expressed and the yield of recombinant protein obtained after purification is also indicated. Note that except for the RsaA control, the size of the protein does not include the amino acids from the RsaA protein.

Gene	Source	Region Expressed	Size of Protein	Fusion Protein Yield (mg/L)	Reference*
rsaA	*Caulobacter crescentus*	Amino acids 690-1026 (RsaA control from pCX)	347 amino acids (includes the CX leader)	250	Invitrogen
lacZ (β-galactosidase)	*E. coli*	Amino acids 82-189	107 amino acids	118	Invitrogen
lacZ	*E. coli*	Amino acids 82-289	207 amino acids	42	Invitrogen
lacZ	*E. coli*	Amino acids 82-389	307 amino acids	14	Invitrogen
lacZ	*E. coli*	Amino acids 82-489	407 amino acids	7	Invitrogen
VP2 (viral envelope protein)	Infectious Pancreatic Necrosis Virus (Salmonid fish virus)	Amino acids 145-257	112 amino acids	140	Nomellini and Smit, unpublished results
VP2 (viral envelope protein)	Infectious Pancreatic Necrosis Virus	Amino acids 1-257	257 amino acids	138	Nomellini and Smit, unpublished results
Cex (exocellulase)	*Cellulomonas fimi*	Amino acids 3-370	368 amino acids	143	(Bingle *et al.*, 2000)
Cex (exocellulase)	*Cellulomonas fimi*	Amino acids 1-443	443 amino acids	105	Bingle, Nomellini, and Smit, unpublished results
phoA (alkaline phosphatase)	*E. coli*	Amino acids 14-246	233 amino acids	109	Nomellini and Smit, unpublished results
G peptide envelope protein	Infectious Hemato-poietic Necrosis Virus (Salmonid fish virus)	Amino acids 336-444	109 amino acids	102	Nomellini and Smit, unpublished results
G peptide envelope protein	Infectious Hemato-poietic Necrosis Virus	Amino acids 270-453	184 amino acids	62	Nomellini and Smit, unpublished results

*The results from Nomellini and Smit were obtained using a plasmid vector that is slightly different from the pCX-TOPO® vector

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Overview, continued

Important

As can be seen in the table on the previous page, the yield of recombinant fusion protein obtained varies significantly depending on the nature of the heterologous protein of interest. As a general trend with exceptions, we have found that **the yield of recombinant fusion protein decreases as the size of the heterologous protein increases** (see table on the previous page).

To date, heterologous proteins up to about 450 amino acids (in addition to the RsaA secretion signal) in size have been successfully expressed in *Caulobacter*. The upper limit of size is not established and other factors including secondary structure or "shape" of the heterologous protein during secretion may contribute to the success of an expression experiment. **In general, we recommend caution in attempting expression of heterologous proteins larger than 500 amino acids.**

Use of TOP10F′ Cells

One Shot® TOP10F′ chemically competent cells are included in the PurePro™ *Caulobacter* Expression System to provide a host for stable propagation and maintenance of your pCX-TOPO® and pCX plasmids. We have found that B5 BAC *Caulobacter* cells are not a reliable host for propagation and long-term maintenance of the pCX-TOPO® and pCX plasmids. **We recommend that you transform your TOPO® Cloning reaction into TOP10F′ cells for characterization of the construct, propagation, and maintenance.** When you are ready to perform an expression experiment, transform your construct into the B5 BAC strain (see below).

B5 BAC Strain

The B5 BAC *Caulobacter* strain is specifically included in this kit for expression and secretion of your recombinant RsaA fusion protein of interest from pCX-TOPO®. This strain should not be used for propagation and maintenance of your pCX-TOPO® expression constructs.

The B5 BAC strain is derived from the JS4000 *Caulobacter* strain and contains a mutation which eliminates its ability to express native RsaA protein. The B5 BAC strain also contains the replication genes, *rep*A, *rep*B, and *rep*C (*rep*BAC) from RSF1010 stably integrated into the genome using allelic exchange at the *rec*A locus (Umelo-Njaka *et al.*, Manuscript in preparation). The presence of the *rep*BAC genes allows high-copy replication in *Caulobacter* of plasmids containing the RSF1010 origin of replication (*ori*V).

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Overview, continued

Experimental Outline

The table below outlines the steps required to clone and express your gene of interest in pCX-TOPO®.

Step	Action	Page
1	Design PCR primers to clone your gene of interest in frame with the CX leader and the C-terminal, truncated RsaA ORF. Consult the diagram of the TOPO® Cloning site on page 11 to help you design your PCR primers.	10-11
2	Produce your PCR product with *Taq* polymerase.	12
3	TOPO® Clone your insert into pCX-TOPO® and transform into One Shot® TOP10F′ *E. coli*. Select transformants on LB agar plates containing 15 µg/ml chloramphenicol.	13-16
4	Analyze your transformants for the presence and orientation of insert by restriction enzyme digestion.	17
5	Select a transformant with the correct restriction pattern and sequence it to confirm that your gene is cloned in frame with the CX leader and the C-terminal RsaA ORF.	17
6	Prepare purified plasmid and transform into One Shot® B5 BAC Electrocomp™ *Caulobacter* cells. Select transformants on PYE agar plates containing 2 µg/ml chloramphenicol. Grow at 30°C.	20-22
7	Perform a small-scale pilot expression of the recombinant RsaA fusion protein.	23-25
8	Purify the recombinant RsaA fusion protein and assay for protein yield.	26-29
9	Scale-up expression and purify RsaA fusion protein for downstream applications.	26-29

Methods

PCR Primer Design

Introduction

The design of the PCR primers to clone your DNA sequence of interest is critical for fusion to RsaA and expression. **Remember that your PCR product will have single 3´ adenine overhangs if *Taq* is used as your polymerase.**

Note

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pCX-TOPO®.

Cloning into pCX-TOPO®

You will need to consider the following when cloning your PCR product into pCX-TOPO®:

- The CX leader contains an ATG initiation codon and surrounding sequences that allow optimal expression of heterologous fusion proteins in *Caulobacter*. Design the 5´ primer such that your PCR product will clone in frame with the CX leader.
- To fuse your gene to the N-terminus of the RsaA ORF, design your 3´ PCR primer such that the PCR product will clone in frame with the RsaA ORF. **Important:** You must clone your PCR product in frame with the RsaA ORF for proper expression and secretion of your protein of interest.

Note: Cloning efficiencies may vary depending on the 5´ nucleotide sequence of your primer (see page 33).

Use the diagram on the next page to design your PCR primers. Once you have designed your PCR primers, proceed to page 12.

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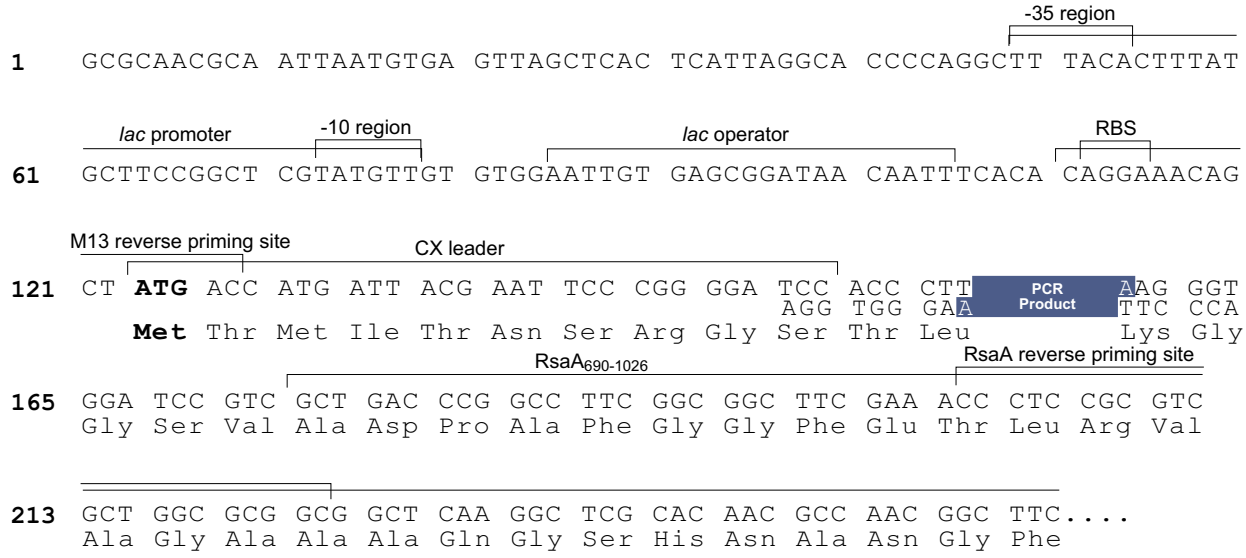
PCR Primer Design, continued

TOPO® Cloning Site of pCX-TOPO®

The diagram below is supplied to help you design appropriate PCR primers to correctly clone and express your PCR product using pCX-TOPO®. The vector is supplied linearized between base pair 158 and 159. This is the TOPO® Cloning site**. Note that the complete sequence of pCX-TOPO® is available for downloading from our World Wide Web site (www.invitrogen.com) or by calling Technical Service (see page 47).** For a map and a description of the features of pCX-TOPO®, refer to pages 39-40.



Producing PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, you are ready to produce your PCR product.

Materials Supplied by the User

You will need the following reagents and equipment.

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product (e.g. Expand™ or eLONGase™).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you can add 3′ A-overhangs using the method on page 36.

Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using a plasmid for template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full-length and 3´ adenylated.

DNA Template	10-100 ng
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Primers (0.1-0.2 µg each)	1 µM each
Sterile water	add to a final volume of 49 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single, discrete band, refer to the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before TOPO® Cloning into pCX-TOPO® (see pages 34-35). Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (Catalog no. K1220-01) from Invitrogen can help you optimize your PCR. Call Technical Service for more information (page 47).

TOPO® Cloning and Transformation

Introduction

TOPO® Cloning technology allows you to ligate your PCR products into pCX-TOPO® and transform the recombinant vector into *E. coli* all in one day. It is important to have everything you need set up and ready to use to ensure you obtain the best possible results. If this is the first time you have TOPO® Cloned, we recommend performing the control reactions on pages 31-32 in parallel with your samples.

Note

Recent experiments at Invitrogen have demonstrated that inclusion of salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction results in the following:

- a 2- to 3-fold increase in the number of transformants.
- allows for longer incubation times (up to 30 minutes). Longer incubation times can result in an increase in the number of transformants obtained.

Including salt in the TOPO® Cloning reaction prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

If you do **not** include salt in the TOPO® Cloning reaction, the number of transformants obtained generally decreases as the incubation time increases beyond 5 minutes.

Important

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see below).** For this reason, two different TOPO® Cloning reaction protocols are provided to help you obtain the best possible results. Read the following information carefully.

Chemically Competent *E. coli*

For TOPO® Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies obtained. A Salt Solution (1.2 M NaCl, 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

Electrocompetent *E. coli*

For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing. The Salt Solution is diluted 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see page 15).

continued on next page

TOPO® Cloning and Transformation, continued

Materials Supplied by the User

In addition to general microbiological supplies (i.e. plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 15 µg/ml chloramphenicol (two for each transformation)
- Reagents and equipment for agarose gel electrophoresis
- 37°C shaking and non-shaking incubators

Note

There is no blue-white screening for the presence of inserts. Individual recombinant plasmids need to be analyzed by restriction analysis or sequencing for the presence and orientation of insert. Sequencing primers included in each kit can be used to sequence across an insert in the TOPO® Cloning site to confirm orientation and reading frame.

Preparation for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- For electroporation, dilute a small portion of the Salt Solution 4-fold to prepare Dilute Salt Solution (e.g. add 5 µl of the Salt Solution to 15 µl sterile water)
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® TOP10F′ cells for each transformation.

Important

Remember to use One Shot® **TOP10F′** *E. coli* to transform your TOPO® Cloning reaction. The B5 BAC *Caulobacter* strain supplied with the kit should **only** be used for expression purposes and **not** for general cloning purposes. For more information about B5 BAC cells, refer to pages 8 and 20.

continued on next page

TOPO® Cloning and Transformation, continued

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent One Shot® TOP10F′ *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 19.

Note: The red or yellow color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl

*Store all reagents at -20°C when finished. Salt solutions and water can be stored at room temperature or +4°C.

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **One Shot® Chemical Transformation** (next page) or **Transformation by Electroporation** (next page). **Note:** You may store the TOPO® Cloning reaction at -20°C overnight.

continued on next page

TOPO® Cloning and Transformation, continued

One Shot® TOP10F′ Chemical Transformation

1. Add 2 µl of the TOPO® Cloning reaction from the previous page into a vial of One Shot® TOP10F′ Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion (see above).
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature SOC medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

Transformation by Electroporation

1. Add 2 µl of the TOPO® Cloning reaction to 50 µl of electrocompetent *E. coli* in a microcentrifuge tube. Transfer the mixture to a 0.1 cm cuvette and mix gently. Do not mix by pipetting up and down. Avoid formation of bubbles.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see next page.
3. Immediately add 250 µl of room temperature SOC medium.
4. Transfer the solution to a 15 ml snap-cap tube (i.e. Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance genes.
5. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

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TOPO® Cloning and Transformation, continued

Note

Addition of the Dilute Salt Solution in the TOPO® Cloning Reaction brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analysis of Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium (3-5 ml) containing 15 µg/ml chloramphenicol.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01).
3. Analyze the plasmids by restriction enzyme analysis or by sequencing.

   The M13 Reverse and RsaA Reverse sequencing primers are included in the kit to help you sequence your insert.

   Refer to the diagram on page 11 for the sequence surrounding the TOPO® Cloning site of pCX-TOPO®. The complete sequence of the vector is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (page 47).

   Note: Resuspend each primer in 20 µl of sterile water to prepare a 0.1 µg/µl stock solution.

If you need help with setting up restriction enzyme digests or DNA sequencing, refer to general molecular biology reference texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

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TOPO® Cloning and Transformation, continued

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use a combination of one of the primers included with the kit and a primer that binds within your insert. You will have to determine the amplification conditions. If this is the first time you have used this technique, we recommend that you perform restriction enzyme analysis in parallel to confirm that PCR gives you the correct result. Artifacts may be obtained because of mispriming or contaminating template. Note that this method will allow you to check for both the presence of cloned PCR product and the orientation of the insert.

The following protocol is provided for your convenience. Other protocols are suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and *Taq* polymerase. Use a 20 µl reaction volume and multiply by the number of colonies to be analyzed (e.g. 10).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail. Don't forget to make a patch plate to preserve the colonies for further analysis.
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles using parameters previously determined (see text, above).
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 31-32. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to prepare a glycerol stock for long-term storage. We recommend that you also store the purified plasmid DNA at -20°C.

1. Streak the original colony out for single colonies on LB plates containing 15 µg/ml chloramphenicol. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 15 µg/ml chloramphenicol.
3. Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Note: Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, increase the incubation time to 30 minutes on ice.

Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

Transforming *Caulobacter*

Introduction

Once you have confirmed that your gene of interest is cloned in frame with the CX leader and the truncated RsaA ORF, you are ready to express your protein of interest in *Caulobacter*. To express your recombinant fusion product, you will transform your pCX-TOPO® construct into B5 BAC *Caulobacter* cells included with the kit. Guidelines and instructions to transform your construct into One Shot® B5 BAC cells are provided in this section.

B5 BAC

You will use One Shot® B5 BAC Electrocomp™ *Caulobacter* cells to express your recombinant fusion protein from pCX-TOPO®. The B5 BAC strain has been specifically designed to allow high-level expression of recombinant proteins from plasmids containing the RSF1010 origin of replication (oriV). In addition, the B5 BAC strain contains a mutation which eliminates its ability to express native RsaA protein.

Do not use this strain for propagation and maintenance of your plasmid. We have found that expression of recombinant proteins from pCX-TOPO® can decrease with successive subcultures when the expression construct is maintained in B5 BAC cells. We recommend that you store expression strains as frozen stocks or perform a fresh transformation of your pCX-TOPO® plasmid into B5 BAC cells before each expression experiment. **For propagation and maintenance of your plasmid, use TOP10F′.**

Note

It is possible to use other *Caulobacter* host strains for expression of your recombinant fusion protein from pCX-TOPO®; however, these strains must contain the *repBAC* genes. If you wish to prepare electrocompetent *Caulobacter* cells, a protocol is provided in the **Appendix**, pages 37-38 for your convenience.

Alternatively, One Shot® B5 BAC electrocompetent *Caulobacter* cells are available separately from Invitrogen (see page x for ordering information).

Plasmid Preparation

Purified plasmid DNA may be isolated using your method of choice. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01), or CsCl gradient centrifugation.

Note: You will need approximately 10 ng of plasmid DNA per transformation.

Positive Control

The pCX vector provided with the kit produces a fusion protein consisting of the CX leader and the truncated RsaA protein and may be used as a positive control for transformation, expression, and purification. The pCX vector will produce a recombinant RsaA fusion protein that is 33 kDa in size. For a detailed map and a description of the features of the vector, see page 41.

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Transforming *Caulobacter*, continued

Suggested Controls

We recommend including the following controls in your expression experiments.

- No DNA (mock)
- pCX vector (positive control)

Important

Caulobacter requires a different type of medium and temperature than *E. coli* for optimal growth. For general growth purposes, culture *Caulobacter* cells in PYE medium and at 30°C. To prepare PYE medium, see the recipe in the **Appendix**, page 43. Note that because of the osmolarity (salt content), *Caulobacter* cells **cannot** grow in LB medium. For the same reason, *Caulobacter* cells grows extremely poorly or not at all in tissue culture media. This is an important point to note for those users who maintain multiple gene expression systems.

Before Starting

Be sure you have the following reagents on hand.

- PYE medium (see page 43 for a recipe)
- PYE plates containing 2 µg/ml chloramphenicol (3 per transformation)
- Electroporator
- 0.1 cm cuvettes, on ice
- Microcentrifuge tubes
- 30°C incubator
- 30°C shaking incubator in a 30°C warm room

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Transforming *Caulobacter*, continued

One Shot® Electroporation Procedure

1. Add 10 ng of your purified pCX-TOPO® construct to 50 µl B5 BAC One Shot® Electrocomp™ *Caulobacter* cells in a chilled 0.1 cm cuvette and mix gently. **Do not mix by pipetting up and down.**
2. Electroporate your samples using your own protocol and your electroporator. For a Bio-Rad Gene Pulser™ unit with a Pulse Controller, use the following settings:

   Voltage: 2.5 kV

   Capacitance: 25 µF

   Resistance: 200 ohms

   Time constants of 3.6 to 4.3 msec are good. Values below 3.0 result in few transformants. **Note**: If you have a different electroporator, you may have to use different electroporation parameters. In general, conditions that work for *E. coli* are adequate for use with *Caulobacter*.
3. After electroporation, immediately add 250 µl of PYE medium and transfer to a microcentrifuge tube.
4. Incubate at 30°C with shaking (225 rpm) for 2 hours.
5. Plate 3 different volumes between 10 and 100 µl on PYE plates containing 2 µg/ml chloramphenicol.
6. Incubate at 30°C for 2 to 3 days. After day 2 you should see a haze of cells with small colonies apparent. By day 3, the colonies should be large enough to pick. You should have hundreds to thousands of colonies.

Expression and Purification of the PCR Product

Introduction

Once you have a *Caulobacter* transformant, you are ready to produce and purify your recombinant fusion protein. Be aware that *Caulobacter* grows more slowly than *E. coli*, grows at 30°C (not at 37°C), and has more specific growth requirements during protein production. We recommend that you review the material below before expressing your protein.

M11 Expression Medium

Expression of your recombinant fusion protein in *Caulobacter* requires a specialized medium, M11 Expression Medium. M11 medium is used because it can support high density growth of *Caulobacter* cells without high osmolarity. When using M11 medium, cell densities can reach as high as 5 OD_{600} units.

M11 Expression Medium concentrate is provided with the kit to allow you to prepare 5 liters of medium. The medium is supplied as two stock solutions which are diluted into sterile, deionized water prior to use (see page 24 to prepare the medium). Additional M11 Expression Medium may be obtained separately from Invitrogen (see page x for ordering information).

Pilot Expression

If you are expressing your recombinant fusion protein in *Caulobacter* for the first time, we recommend that you perform a small scale pilot study to determine the optimal conditions for expression.

Before Starting

Be sure to have the following reagents and equipment on hand for each expression experiment.

- 5 ml PYE Medium containing 2 µg/ml chloramphenicol
- 50 ml M11 Expression Medium containing 2 µg/ml chloramphenicol in a 250 ml Erlenmeyer culture flask (if performing pilot-scale expression)
- 850 ml M11 Expression Medium containing 2 µg/ml chloramphenicol in a 2 L Erlenmeyer culture flask (if performing large scale expression)
- 30°C shaking incubator or a tube roller in a 30°C warm room
- 1 piece of mesh (145 µm pore size; included in the kit or may be obtained separately from VWR Scientific, Catalog no. 25000-014)
- Buchner funnel (included in the kit or may be obtained separately from VWR Scientific, Catalog no. 30305-040)
- 1-3 liters distilled, deionized water
- Distilled, deionized water in a squirt bottle
- 30 ml glass or polypropylene centrifuge tubes

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Expression and Purification of the PCR Product, continued

Preparation of M11 Medium

Follow the instructions below to prepare 850 ml of M11 Expression Medium from the stocks provided with the kit. If you wish to prepare more or less M11 Expression Medium, scale the volumes up or down as appropriate. If you wish to prepare your own M11 Expression Medium, a recipe is provided in the **Appendix**, pages 43-45.

1. Autoclave 807.5 ml of deionized water on liquid cycle and allow it to cool overnight to ensure that the water is reoxygenated.
2. Prior to use, add the following components to the water using sterile technique.

Solution C	34 ml
Solution M	8.5 ml

3. Store at +4°C until use. **Note:** Use media within one week for optimal results.

Positive Control

We strongly recommend that you include pCX as the positive control for expression of the control RsaA protein.

Expression Procedure

1. Inoculate a single colony (from Step 6, page 22) into 5 ml of PYE medium containing 2 µg/ml chloramphenicol.
2. Incubate at 30°C on a tube roller or with shaking at 150-200 rpm for 16-18 hours (overnight). **Note:** Overnight growth should yield a culture with visible turbidity. OD_{600} values will usually range from 0.1 to 1. The rate of growth may vary because of the nature of the inoculum.
3. Inoculate the following amount of overnight culture into M11 Expression Medium. Do not use a baffled flask.
   - If you are performing pilot scale expression, inoculate 2 ml of overnight culture into 50 ml of M11 Expression Medium containing 2 µg/ml chloramphenicol.
   - If you are performing large scale expression, inoculate all of the overnight culture into 850 ml of M11 Expression Medium containing 2 µg/ml chloramphenicol.
4. Incubate for 2 days at 30°C with slow, gentle shaking (80-100 rpm). **Tips:** Do not shake the flask faster than 100 rpm. Vigorous shaking will disrupt the protein aggregates or prevent their formation. Adjust the shaking so that no foaming or bubbles occur.

   Important Note: This step is a critical determinant in the success of your expression experiment. Some empirical experimentation may be necessary to determine the optimal shaking speed for aggregate formation.

   Adequate expression of your construct should yield aggregated protein after 2 days of incubation. The aggregate is often pink to red in color because of a small amount of hydrophobic pigment that *Caulobacter* produces. Compare protein aggregate production from your construct with the positive control. You may continue to incubate the culture for up to 4 days to see if protein aggregate production increases.
5. Once you are ready to harvest the protein aggregate, let the culture sit for a minute or two to allow the aggregate to settle. Proceed to **Purification of Recombinant Fusion Protein**, next page.

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Expression and Purification of the PCR Product, continued

Nylon Mesh

You may use any nylon mesh with the appropriate pore size (80-150 µm) to filter your protein aggregate. Five pieces of reusable nylon mesh are supplied in the kit for your convenience. The pore size of the supplied nylon mesh is approximately 145 µm. We have found that at this pore size, the mesh will retain the hydrated protein aggregate and allow the cells to flow through.

For a protocol to wash the nylon mesh for reuse, see page 27. Additional mesh may be obtained from VWR Scientific (Catalog no. 25000-014) or Fisher Scientific (Catalog no. NC9524681).

Note that although nylon mesh with smaller pore sizes (80-110 µm) may be used to filter the protein aggregate, you may find it more difficult to filter the aggregate efficiently as the mesh may become clogged with aggregate. We recommend using nylon mesh with larger pore sizes (110-150 µm) and using nylon mesh with smaller pore sizes only if necessary. An example of the latter may be cases when only a small amount of finely divided aggregate is produced.

When using glass centrifuge tubes, we have found that the protein aggregates may sometimes adhere to the sides of the tube. You may want to pre-treat your glass centrifuge tubes with γ-methacryloxypropyltrimethoxysilane before use to avoid this problem. A protocol for treatment of glass with γ-methacryloxypropyltri-methoxysilane may be found in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

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Expression and Purification of the PCR Product, continued

Purification of Recombinant Fusion Protein

1. Take the Buchner funnel and disconnect the funnel part from the stem.
2. Place the mesh in between the funnel part and the stem in a single layer and push the two pieces together. Alternatively, you may hold the mesh over the top of the funnel.
3. Place the funnel over a 1 liter waste container and pour the culture slowly through the funnel.
4. The culture medium will drain through, leaving the protein aggregate behind. Large chunks will be retained in the funnel while the mesh retains smaller chunks. **Note:** If you are holding the mesh over the top of the funnel, all of the protein aggregates will be retained on the mesh.
5. After pouring all of the culture through the funnel, rinse the flask with a volume of distilled, deionized water equal to the culture volume. Pour the solution through the funnel. See the next page for disposal of the flow-through.
6. Pour or scrape the gel-like aggregate into a clean 30 ml thick-wall, glass or polypropylene centrifuge tube (see **Recommendation** on the previous page). Use a squirt bottle filled with distilled water to back-flush the funnel and/or the mesh. Note that there may be a few cells present.
7. To remove residual cells, centrifuge the aggregate as follows. Bring the rotor with your sample up to 3000 to 5000 x g and immediately decrease the speed to zero. Remove the supernatant carefully. Using these conditions the cells are not pelleted, but the aggregates will compact significantly.
8. Wash the protein aggregate with a volume of distilled, deionized water equal to the aggregate volume and centrifuge as described in Step 7. Repeat twice. Store as described below.

What to Expect

The yield of recombinant fusion protein may vary significantly depending on the nature of the cloned DNA. As with any expression system, expression levels are protein-dependent. We generally obtain the following amounts of hydrated protein aggregate when expressing the control protein from the pCX plasmid:

- Approximately 0.5-1 g wet weight of protein aggregate from 50 ml of culture
- Approximately 10 g wet weight of protein aggregate from 850 ml of culture

Note: Typically, 95-98% of the wet weight is water, yielding about 150-250 mg of protein/liter of culture.

Storage of Protein Aggregate

You may store the protein aggregate at +4°C, -20°C, or -80°C. Storage at +4°C may result in some proteolysis after 1-2 weeks, as a few residual cells may be present. Storage at -20°C or -80°C is recommended for long-term storage although some dehydration of aggregates occurs which may increase the difficulty of solubilizing some recombinant aggregates with urea (see page 29).

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Expression and Purification of the PCR Product, continued

Discarding the Flow-Through

No special precautions are required when disposing of the flow-through (see Steps 3-5 on the previous page). As a standard precaution, we recommend that you treat the flow-through with bleach or autoclave to kill the bacteria before disposal.

Reusing the Mesh

Once you have filtered your protein aggregate, you may wash and reuse the mesh and funnel. The mesh may be reused at least 5 times. We recommend washing the mesh as soon as you have finished filtering your protein aggregate. The mesh is more difficult to wash if the residual cells have dried and hardened. Follow the procedure below to wash the mesh and funnel.

1. Use detergent and warm water to wash the mesh and funnel.
2. Rinse in warm water and allow the mesh and funnel to dry.
3. Store at room temperature.

Analyzing Your Fusion Protein

Introduction

Once you have completed the purification procedure, you should have hydrated protein aggregate containing recombinant fusion protein that is approximately 90% pure. You may now analyze your recombinant fusion protein using SDS-PAGE. Because it is difficult to accurately sample the aggregate, special procedures are needed to solubilize the protein aggregate for analysis by SDS-PAGE. Guidelines are provided below to help you solubilize the protein aggregate and quantitate the yield of recombinant fusion protein.

Before Starting

Be sure to have the following reagents and equipment on hand.

- 8 M urea in 100 mM Tris-HCl, pH 8.5 (see page 46 for a recipe)
- SDS-PAGE sample buffer (any are suitable)
- SDS-PAGE gel and apparatus (see below)

Polyacrylamide Gel Electrophoresis

To facilitate separation of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The patented NuPAGE® Novex Gel System prevents the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits for visualization of proteins. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 47).

Detection of Recombinant Fusion Protein

The yield of purified recombinant fusion protein obtained is generally high enough to be readily visualized on a Coomassie™ stained gel. If desired, you may also perform western blot analysis to detect your recombinant fusion protein. You may use the RsaA Antiserum available separately from Invitrogen (see page x for ordering information) or an antibody to your protein of interest to detect your recombinant fusion protein.

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Solubilizing the Protein Aggregate

Follow the procedure below to solubilize the protein aggregate. Note that you will use a urea solution to solubilize the protein aggregate. The urea solution will denature your recombinant protein.

1. Dissolve a portion of the protein aggregate from Step 8, page 26 in one volume of 8 M urea in 100 mM Tris-HCl, pH 8.5. To increase the rate at which pellets solubilize, disperse the aggregate by brief pulses of sonication.

   Note: For applications where activity of the fusion protein may be examined, we have found that 0.5 – 1 M urea will often solubilize a significant fraction of the aggregates. In some instances, it is possible that such conditions may not inactivate or denature the target protein.

2. Incubate the protein solution at room temperature for 2 hours. Vortex the protein solution every 15 minutes and examine the degree of solubilization.

   Note: After two hours, the aggregates should be completely solubilized. If aggregates persist, you may add more 8 M urea in 100 mM Tris-HCl, pH 8.5 and sonicate as needed. Alternatively, you may simply remove residual aggregates by centrifugation.

3. Mix an aliquot of the solubilized protein aggregate with 2X SDS-PAGE sample buffer. It is not necessary to boil the solution.
4. Load and run the SDS-PAGE gel using established protocols. The recombinant fusion protein should be visible on a Coomassie™ blue-stained gel. Use the RsaA protein expressed from pCX as a positive control.
5. Store the solubilized protein at +4°C. The solubilized protein may be stored for several months in this manner.

Note

Fusion of your PCR product with the CX leader and the RsaA protein will increase the size of your protein by approximately 33 kDa.

Dialysis of Solubilized Protein

To use the solubilized recombinant fusion protein in downstream applications, we recommend that you dialyze the recombinant protein to remove the urea. While any typical method is suitable, we generally use the following conditions to dialyze the solubilized recombinant fusion protein:

- The total volume of solubilized protein is generally 2 to 3 ml for small-scale expression.
- Use a 12 ml Slide-A-Lyzer® dialysis cassette (Pierce, Rockford, IL) to dialyze the recombinant protein against one liter of phosphate-buffered saline (PBS; see page 46 for a recipe).
- Dialyze overnight at +4°C without any change in buffer.

Once you have dialyzed your recombinant fusion protein, use any method of choice to quantitate the yield of recombinant fusion protein. We typically use the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) and BSA as a standard to quantitate the yield of recombinant fusion protein obtained. Most fusion proteins should be maintained at a concentration of 2 mg/ml or less to avoid the possibility of precipitation.

Troubleshooting Expression

Introduction

The section below lists some potential problems and possible solutions that you may use to help you troubleshoot your expression experiment.

No Expression

- Sequence your construct to make sure that your PCR product is cloned in frame with the CX leader and the truncated RsaA ORF.
- If the positive control is expressed, but you don't see any expression from your construct on a Coomassie™ blue-stained gel, re-run your samples on an SDS-PAGE gel and perform a western blot. Use the RsaA Antiserum available from Invitrogen (see page x for ordering information) or an antibody to your protein to detect your recombinant fusion protein.

  Note: In our experience, if a recombinant fusion protein is not successfully secreted from *Caulobacter*, it is rapidly degraded. We seldom see significant amounts of recombinant fusion protein accumulating within *Caulobacter* cells.

Low Expression

A number of reasons may be attributed to observing low expression of your protein of interest.

Reason	Solution
Poor aggregation	• Adjust the shaker speed. • Add extra $CaCl_2$ (5 ml/L of 100 mM $CaCl_2$ stock) to the M11 Expression Medium to promote aggregation.
Poor secretion	The factors controlling whether a recombinant fusion protein can be successfully secreted are not well understood. Some possible reasons for poor secretion may be: • Codon bias problems. To date, proteins containing as much as 34% rare codons for *Caulobacter* have been successfully expressed. If your gene contains more than this level or if rare codons are clustered, you may want to synthesize the gene of interest and optimize the codon usage. A codon usage table for *Caulobacter* may be found at www.kazusa.or.jp/codon • High cysteine content. Proteins with extremely high cysteine content do not appear to be secreted. Some expression difficulties may be experienced for proteins containing moderate cysteine content. • Specific secondary structure or shape issues. We have had little success expressing proteins containing transmembrane domains.

Appendix

pCX TOPO TA Cloning® Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions using the reagents included in the kit involves producing a control PCR product containing the *lac* promoter/operator and the LacZα protein. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing chloramphenicol, X-gal, and IPTG.

Before Starting

Prepare the following reagents before performing the control reaction:

- 40 mg/ml X-gal in dimethylformamide (see page 42 for recipe)
- 100 mM IPTG (see page 42 for recipe)
- LB plates containing 15 µg/ml chloramphenicol, X-gal, and IPTG (two per transformation)
- To add X-gal and IPTG to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml X-gal stock solution and 40 µl of 100 mM IPTG onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

Producing Control PCR Product

1. To produce the 500 bp control PCR product containing the *lac* promoter/operator and LacZα, set up the following 50 µl PCR:

Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	60°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Analyze 10 µl of the reaction by agarose gel electrophoresis. A discrete 500 bp band should be visible. Proceed to the **TOPO® Cloning Reactions**, next page.

continued on next page

pCX TOPO TA Cloning® Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Control PCR Product	--	1 µl
Salt Solution or Dilute Salt Solution	1 µl	1 µl
Sterile Water	4 µl	3 µl
TOPO® vector	1 µl	1 µl

2. Incubate at 25°C (room temperature) for 5 minutes and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® TOP10F′ cells (page 16).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 15 µg/ml chloramphenicol, X-gal, and IPTG. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 85% of these will be blue and contain the 500 bp insert.

The 'vector only' plate should contain only a few colonies (<15% of the vector + PCR insert plate).

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10F′ competent cells. Transform with 1 µl pg of pUC19 per 50 µl of cells using the protocol on page 16. Plate 10 µl of the transformation mixture plus 20 µl SOC on LB plates containing 50 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

continued on next page

pCX TOPO TA Cloning® Control Reactions, continued

Factors Affecting Cloning Efficiency

Note that lower transformation and/or cloning efficiencies will result from the following variables.

Variable	Solution
pH>9	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product. **Note:** You may use up to 4 µl of your PCR product in the TOPO® Cloning reaction.
Cloning blunt-ended fragments	Add 3´ A-overhangs by incubating with Taq polymerase (page 36).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (pages 34-35) or optimize your PCR.
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	Taq polymerase is less efficient at adding a nontemplate 3´ A next to another A. Taq is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996).

Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>1 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below for your convenience.

Note

Note that cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 12).

Using the S.N.A.P.™ Gel Purification or S.N.A.P.™ MiniPrep Kit

The S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25) or the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) are available from Invitrogen to facilitate rapid purification of PCR products from regular agarose gels. If you are using the S.N.A.P.™ MiniPrep Kit, a protocol is provided below. Before beginning, you will need to prepare 6 M sodium iodide in sterile water. Add sodium sulfite to a final concentration of 10 mM to the NaI solution to prevent oxidation.

1. Electrophorese amplification reaction on a 0.8% to 5% regular TAE agarose gel. **Note**: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2.5 volumes of the 6 M NaI, 10 mM sodium sulfite solution.
3. Add 1.5 volumes Binding Buffer (provided in the S.N.A.P.™ MiniPrep Kit).
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer (provided in the S.N.A.P.™ MiniPrep Kit).
7. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
8. Centrifuge again at maximum speed for 1 minute to fully dry the resin.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on pages 15-16.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 15). Be sure to make the gel slice as small as possible for best results.

continued on next page

Purifying PCR Products, continued

Low-Melt Agarose Method

Note that gel purification from low-melt agarose will result in a dilution of your PCR product. Use only chemically competent cells for transformation.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 15.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes.** This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into TOP10F′ One Shot® cells using the method on page 16.

Note

Note that the cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by *Vent*® or *Pfu* polymerases into TOPO TA Cloning® vectors is often difficult because of very low cloning efficiencies. These low efficiencies are caused by the lack of the terminal transferase activity associated with proofreading polymerases which adds the 3´ A-overhangs necessary for TA Cloning®. A simple method is provided below to clone these blunt-ended fragments.

Before Starting

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

1. After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for ligation into the TOPO® vector.

Note: If you plan to store your sample(s) overnight before proceeding with TOPO® Cloning, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.

Note

You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu* (see pages 34-35). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10-15 minutes at 72°C. Use 4 µl in the TOPO® Cloning reaction.

Preparing Electrocompetent *Caulobacter*

Introduction

The procedure below describes how to prepare electrocompetent *Caulobacter* for transformation.

Before Starting

You will need the following reagents and equipment.

- 30°C shaking incubator
- 250 to 500 ml PYE medium in a 1 liter or 2 liter flask
- Sterile 250-300 ml centrifuge bottles
- Sterile 30 ml centrifuge tubes
- Low speed floor centrifuge set at +4°C
- Appropriate rotors for centrifuge bottles and tubes
- Ice-cold, sterile, distilled water
- Ice-cold, sterile 10% glycerol in distilled water
- 1.5 ml microcentrifuge tubes

Preparation of Cells

The following procedure will yield enough competent cells for 15 transformations. You may scale up production if desired.

1. Inoculate a single colony into 5 ml PYE medium. Grow at 30°C with shaking until the OD_{600} is 0.5-1.0.
2. Record the OD_{600} of the above culture and calculate the amount you would add to 250 ml or 500 ml of fresh PYE medium to achieve an OD_{600} of 0.4-0.6 in 18 hours (assuming the doubling time of *Caulobacter* is about 3 hours). **Tip**: Set up two cultures (250 ml each) and inoculate the second culture with 2 to 4 times as much as you originally calculated. After overnight growth, one of the cultures should have an OD_{600} between 0.4 and 0.6.
3. Harvest the cells by centrifuging at 10,000 x g for 10 minutes at +4°C.
4. Carefully remove the supernatant by aspiration and suspend the cells in 1 volume (250 ml) of ice-cold sterile, distilled water. Resuspend the cells by pipetting. **Do not vortex.**
5. Centrifuge again as described in Step 3.
6. Remove the supernatant and resuspend the cells in one half volume (125 ml) of ice-cold sterile, distilled water.
7. Centrifuge as in Step 3.
8. Remove the supernatant and resuspend the cells in 1/20 the original volume (12.5 ml) of ice-cold, sterile 10% glycerol. Transfer to 30 ml centrifuge tubes if desired.

continued on next page

Preparing Electrocompetent *Caulobacter*, continued

Preparation of Cells, continued

9. Centrifuge as in Step 3.
10. Remove the supernatant and resuspend the cells in 750 µl of ice-cold, sterile 10% glycerol (3 ml per liter of original culture).
11. Dispense cells into 50 µl aliquots in 1.5 ml microcentrifuge tubes.
12. Store at -80°C. **Note**: There's no need to flash-freeze the cells. When ready to transform, thaw cells on ice and follow the procedure below.

Electroporation Procedure

1. Thaw cells on ice and aliquot 50 µl into a chilled 0.1 cm cuvette.
2. Add 10 ng DNA and mix.
3. Electroporate the cells/DNA mixture. For a Bio-Rad Gene Pulser™ unit with a Pulse Controller, use the following settings:

   Voltage: 2.5 kV

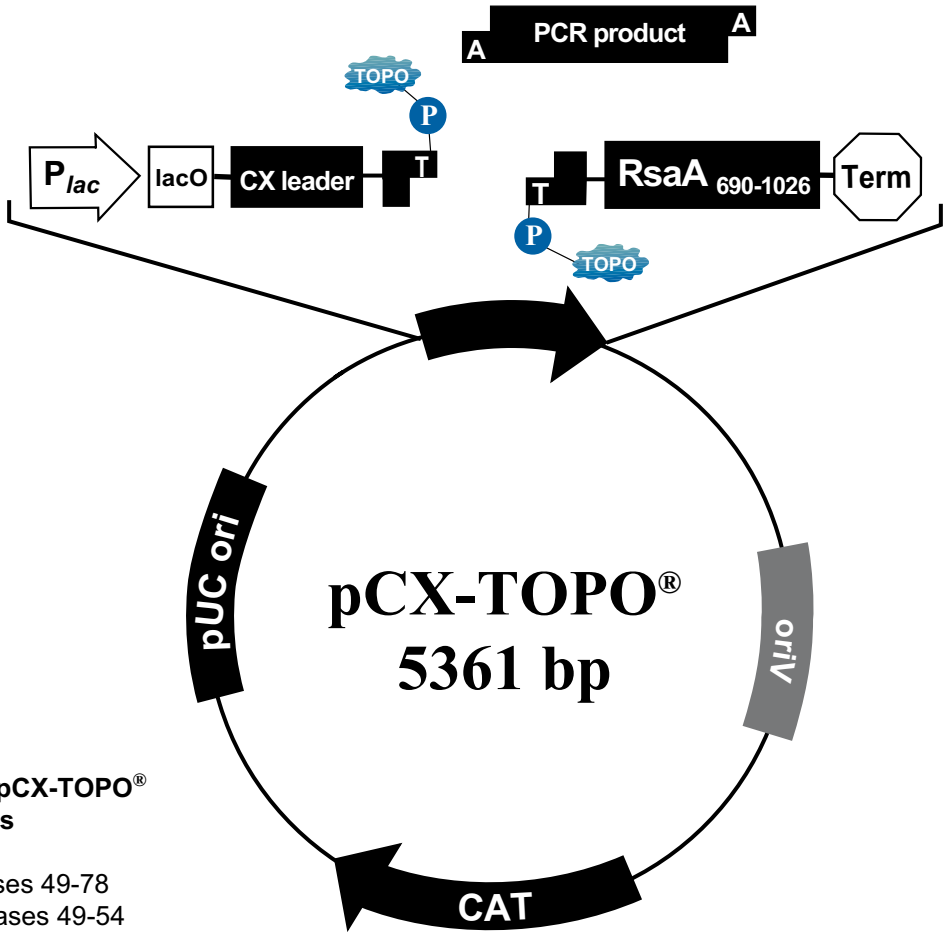
   Capacitance: 25 µF

   Resistance: 200 ohms
4. Time constants of 3.6 to 4.3 msec are good. Values below 3.0 result in few transformants. **Note**: If you have a different electroporator, you may have to use different electroporation parameters. In general, conditions that work for *E. coli* are adequate for use with *Caulobacter.*
5. After electroporation, immediately add 250 µl of PYE medium and transfer to a microcentrifuge tube.
6. Incubate at 30°C with shaking (150-200 rpm) for 2 hours.
7. Plate 3 different volumes between 10 and 100 µl on PYE plates containing 2 µg/ml chloramphenicol.
8. Incubate at 30°C for 2 to 3 days. After day 2 you should see a haze of cells with small colonies apparent. By day 3, the colonies should be large enough to pick. You should have hundreds to thousands of colonies.

pCX-TOPO® Vector

Map

The figure below summarizes the features of the pCX-TOPO® vector. The vector is supplied linearized between base pairs 158 and 159. This is the TOPO® Cloning site. **The complete nucleotide sequence for pCX-TOPO® is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 47).**



Comments for pCX-TOPO®
5361 nucleotides

lac promoter: bases 49-78
-35 region: bases 49-54
-10 region: bases 73-78
lac operator: bases 85-105
Ribosome binding site: bases 112-115
M13 reverse priming site: bases 111-127
CX leader: bases 123-152
TOPO® Cloning site: bases 158-159
Truncated RsaA ORF (amino acids 690-1026 only): bases 174-1181
RsaA reverse priming site: bases 202-223
oriV: bases 1640-2085
Kanamycin promoter: bases 2940-3046
Chloramphenicol acetyltransferase (CAT) ORF: bases 3082-3741
pUC origin: bases 4472-5146

continued on next page

pCX-TOPO® Vector, continued

Features

The pCX-TOPO® vector contains the following elements. All features have been functionally tested and the vector fully sequenced.

Feature	Benefit
lac promoter	Permits high-level, constitutive expression of the gene of interest
lac operator (lacO)	Serves as the binding site for the Lac repressor to reduce basal expression of the gene of interest from the *lac* promoter in *E. coli* (Gilbert and Maxam, 1973)
CX leader sequence	Provides an ATG initiation codon for proper initiation of translation and serves to enhance translation efficiency
M13 Reverse priming site	Permits sequencing of your insert in the sense orientation
TOPO® Cloning site	Allows insertion of your PCR product in frame with the CX leader and the C-terminal, RsaA secretion signal
RsaA ORF (amino acids 690-1026 only)	Truncated *rsaA* gene which serves to direct secretion and aggregation of a heterologous fusion partner
RSF1010 origin (oriV)	Permits high-copy replication of the plasmid in *Caulobacter* (Bagdasarian *et al.*, 1981; Scholz *et al.*, 1989)
Kanamycin promoter	Allows expression of the CAT ORF in *E. coli* and *Caulobacter*
Chloramphenicol acetyltransferase (CAT) ORF	Allows selection of the vector in *E. coli* and *Caulobacter crescentus*
pUC origin	Permits high-copy replication and maintenance of the plasmid in *E. coli*

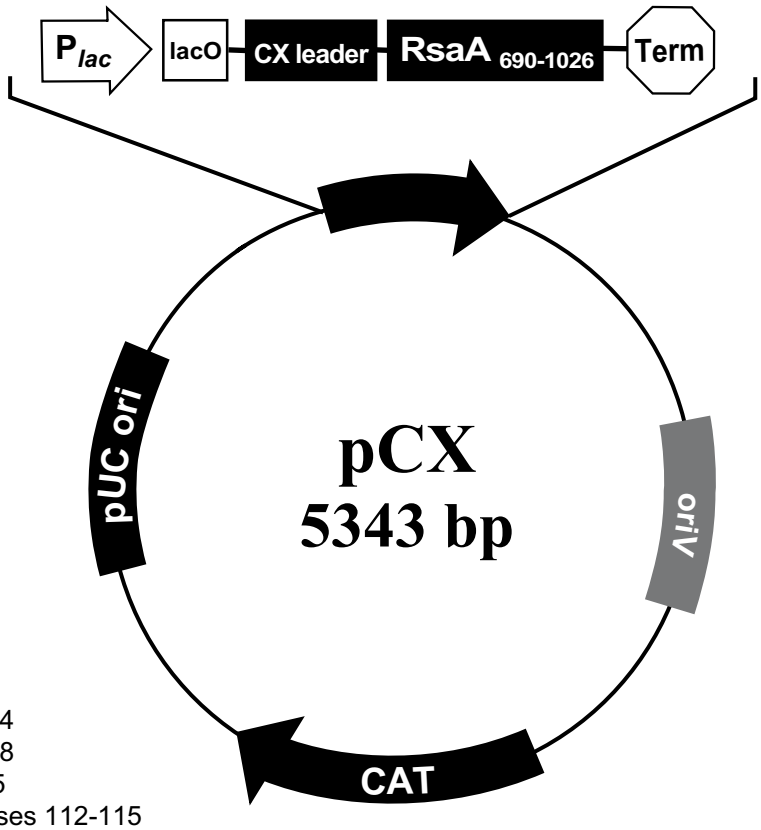
pCX Vector

Description

pCX is a 5343 bp control vector which expresses a truncated RsaA protein (amino acids 690-1026). pCX is the parent vector of pCX-TOPO® and may be used as a positive expression control. The recombinant RsaA protein produced from pCX is 33 kDa in size.

Map of Control Vector

The figure below summarizes the features of the pCX vector. **The complete nucleotide sequence for pCX is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (see page 47).**



Comments for pCX 5343 nucleotides

lac promoter: bases 49-78
-35 region: bases 49-54
-10 region: bases 73-78
lac operator: bases 85-105
Ribosome binding site: bases 112-115
M13 reverse priming site: bases 111-127
CX leader: bases 123-152
Truncated RsaA ORF (amino acids 690-1026 only): bases 156-1163
RsaA reverse priming site: bases 184-205
oriV: bases 1622-2067
Kanamycin promoter: bases 2922-3028
Chloramphenicol acetyltransferase (CAT) ORF: bases 3064-3723
pUC origin: bases 4454-5128

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add 15 µg/ml chloramphenicol, if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add 15 µg/ml chloramphenicol, and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

X-Gal Stock Solution

1. To make a 40 mg/ml stock solution, dissolve 400 mg X-Gal in 10 ml dimethylformamide.
2. Protect from light by storing in a brown bottle at -20°C.
3. To add to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

100 mM IPTG

1. To prepare a 100 mM stock solution, dissolve 0.238 g of IPTG in 10 ml of deionized water.
2. Filter-sterilize and store in 1 ml aliquots at -20°C.

continued on next page

Recipes, continued

PYE Medium and Plates

This is a general purpose medium for *Caulobacter* and is used to maintain cultures.

0.2% Peptone
0.1% Yeast Extract
0.02% $MgSO_4 \cdot 7H_2O$
0.01% $CaCl_2 \cdot 2H_2O$

Note: Calcium ions are required for optimal growth of *Caulobacter*.

1. For 1 liter, combine 2 g peptone, 1 g yeast extract, 1 ml of 20% sterile $MgSO_4 \cdot 7H_2O$, and 1 ml of sterile 10% $CaCl_2 \cdot 2H_2O$ in 1 liter of deionized water. **Note:** If you are preparing plates, add 15 g agar per liter.
2. Autoclave on liquid cycle for 20 minutes.
3. Cool the medium to ~55°C and add 2 µg/ml chloramphenicol, if desired (or see next step).
4. Store the medium at room temperature. Medium without chloramphenicol is stable indefinitely if it is not contaminated. Chloramphenicol may be added to the liquid medium before use. Plates are stable for about 2 weeks at +4°C.

M11 Expression Medium

M11 Expression Medium is used to express RsaA fusion proteins from pCX-TOPO® and pCX and is complex and somewhat laborious to prepare. Because of the complexity involved in preparation, this medium can easily become contaminated. We recommend obtaining the premade M11 Expression Medium from Invitrogen (see page x for ordering information). For your convenience, stock solutions of M11 Expression Medium sufficient to prepare 5 liters of medium are provided with the kit.

If you wish to prepare the medium, you will need to prepare several stock solutions. Recipes and procedures are provided on pages 44-45. **Important:** Note that the stock solutions that you will prepare below are different from those provided with the kit.

Note: If, during the course of use, you suspect the medium concentrate has become contaminated, you may filter-sterilize the medium using aseptic technique. **Do not autoclave the concentrate; it will yield a precipitate, making it unusable.**

Before Starting

You will need to have the following solutions on hand before preparing M11 Expression Medium. Recipes are provided on pages 44-45.

- 1 M imidazole, pH 7.0
- 2 M potassium phosphate, pH 6.8
- 10% glucose
- 10% glutamate
- 100 mM $CaCl_2$
- Metals 44
- Hutner's mineral base concentrate

continued on next page

Recipes, continued

M11 Expression Medium

1. To prepare 1 liter of M11 Expression medium, add the following to 925 ml of deionized water:

1 M imidazole, pH 7.0	5 ml
2 M potassium phosphate buffer, pH 6.8	1 ml

2. Autoclave for 30 minutes on liquid cycle and allow the solution to cool to ~55°C.
3. Add the following to the cooled solution:

10% glucose	15 ml
10% glutamate	15 ml
100 mM $CaCl_2$	1.25 ml
Hutner's Mineral Base	2.5 ml

4. Medium is now ready for use. Store at +4°C. **Note:** Because of the manipulation involved, this medium is more likely to become contaminated. Be sure to practice sterile technique.

   Note: Solid medium can be prepared (add agar to a concentration of 12-15 g/liter), if desired.

Hutner's Mineral Base Concentrate

Nitrilotriacetic acid (NTA), free acid	20 g
$MgSO_4 \cdot 7H_2O$	54.5 g
10% $CaCl_2 \cdot 2H_2O$ solution	66.7 ml
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (ammonium molybdate)	18.5 mg
$FeSO_4 \cdot H_2O$	198 mg (323.7 mg if it is $7H_2O$)
Metals 44	100 ml

1. For 1 liter, place NTA in 750 ml deionized water (1 liter beaker) and slowly add 14.6 g of KOH pellets to dissolve the NTA.
2. Add the other components in the order given, dissolving each one before adding the next component.
3. Adjust the pH to 6.8 with 5 N KOH (~18 ml). **Note:** This is the most difficult part of making this solution. Care must be taken to avoid overshooting the pH. In addition, a precipitate may form as base is added, but it will disappear with stirring. The color will also darken as base is added. **Do not autoclave until ALL the precipitate is dissolved.**
4. Bring the volume to 1 liter, transfer to a 2 liter Erlenmeyer flask and add a stir bar.
5. Autoclave 20 minutes on liquid cycle. The yellow color darkens even more after autoclaving. If a precipitate forms during autoclaving, stir the hot solution overnight as it cools. Store at +4°C. This solution should be stable for up to one year if stored properly.

continued on next page

Recipes, continued

Metals 44

EDTA (free acid)	2.5 g
$ZnSO_4 \cdot 7H_2O$	10.95 g
$FeSO_4 \cdot 7H_2O$	5 g
$MnSO_4 \cdot H_2O$	1.54 g
$CuSO_4 \cdot 5H_2O$	392 mg
$Co(NO_3)_2 \cdot 6H_2O$	250 mg
$Na_2B_4O_7 \cdot 10H_2O$	177 mg (200 mg if it is $8H_2O$)

1. For 1 liter, add EDTA to 800 ml deionized water. Heat and stir for 30 to 45 minutes to dissolve as much as possible. **Note**: Solution will remain cloudy until $ZnSO_4$ is added.
2. Dissolve other ingredients in the order given above. **Do not add the next component until the previous component has completely dissolved.** A few drops of H_2SO_4 may be added to retard precipitation.
3. Bring the volume to 1 liter. You should have a clear, lime-green solution. **Do not autoclave**. Store at +4°C. This solution should be stable for up to one year if stored properly.

1 M Imidazole, pH 7.0

1. Add 6.8 g imidazole to 90 ml deionized water.
2. Adjust pH to 7 with concentrated HCl.
3. Bring the volume to 100 ml and autoclave on liquid cycle.
4. Store at room temperature.

2 M Potassium Phosphate, pH 6.8

1. Prepare 2 M stock solutions (100 ml each) of KH_2PO_4 and K_2HPO_4.
2. Combine 50.3 ml of 2 M KH_2PO_4 and 49.7 ml of K_2HPO_4.
3. Adjust the pH if necessary with one or the other stock solutions.
4. Autoclave on liquid cycle and store at room temperature.

10% Glucose

1. Dissolve 100 g glucose in 1 liter of deionized water.
2. Sterilize by autoclaving on liquid cycle.
3. Store at room temperature.

10% Glutamate

1. Dissolve 100 g glutamic acid in 900 ml of deionized water.
2. Adjust the pH to 7 with NaOH and bring the volume to 1 liter.
3. Sterilize by autoclaving on liquid cycle.
4. Store at room temperature.

Note: You may also use monosodium glutamate at a concentration of 11.5%.

100 mM $CaCl_2$

1. Dissolve 1.47 g in 100 ml deionized water.
2. Sterilize by autoclaving on liquid cycle.
3. Store at room temperature.

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Recipes, continued

8 M Urea in 100 mM Tris-HCl, pH 8.5

1. For 100 ml, add 10 ml 1 M Tris-HCl, pH 8.5 buffer to 60 ml of water and then dissolve 48.05 g urea.
2. Adjust the pH with concentrated HCl and bring the volume up to 100 ml with water.

2X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol (100%)	2.0 ml
β-mercaptoethanol	0.4 ml
Bromophenol blue	0.02 g
SDS	0.4 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at –20°C until needed.

Phosphate-Buffered Saline (PBS)

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

1. Dissolve: 8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   in 800 ml deionized water.
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter. You may wish to filter-sterilize or autoclave the solution to increase shelf life.

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Technical Service, continued

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Product Qualification

Introduction

This section describes the criteria used to qualify the components in the PurePro™ Caulobacter Expression System.

Vector

The pCX plasmid (parental vector of pCX-TOPO®) is qualified by restriction digest prior to adaptation with topoisomerase I. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel. The table below lists the restriction enzymes used to digest the vector and the expected fragments. Note that restriction sites used to qualify the parental vector may no longer be present in the topoisomerase I-adapted vector.

Restriction Enzyme	Expected fragments (bp)
*Bam*H I	5343
Hpa I	711, 4632
Nae I	280, 1149, 3914
Nco I	2093, 3250

TOPO® Cloning Efficiency

Once the supercoiled vector has been adapted with topoisomerase I, it is lot-qualified using the control reagents included in the kit. Under conditions described on pages 31-32, a 500 bp control PCR product is TOPO®-Cloned into pCX-TOPO® and subsequently transformed into the One Shot® TOP10F′ competent *E. coli* included with the kit.

Each lot of vector should yield greater than 85% cloning efficiency.

Sequencing Primers

The sequencing primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® Competent *E. coli*

All competent cells are qualified as follows:

- Cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells.
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

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Product Qualification, continued

One Shot® B5 BAC Electrocomp™ *Caulobacter*

50 µl of electrocompetent cells are transformed with 10 ng of supercoiled pCX plasmid. Transformed cultures are plated on PYE plates containing 2 µg/ml chloramphenicol and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1 x 10^6 cfu/µg DNA.

Untransformed cells are plated on:

- PYE plates containing 2 µg/ml chloramphenicol to verify the absence of chloramphenicol resistant contamination.
- PYE plates as a lawn to verify the absence of phage contamination.

M11 Expression Medium

To assess the performance of each lot of M11 *Caulobacter* Expression Medium, medium is prepared as described on page 24 and used to test the expression and aggregation of the RsaA control protein from pCX in *Caulobacter crescentus* cells. Briefly, 1 ml of frozen B5 BAC cells transformed with the pCX plasmid is inoculated into 400 ml of 1X M11 Expression Medium containing 2 µg/ml chloramphenicol. Cells are grown at 30°C with slow shaking for 3-4 days. A substantial amount of reddish protein precipitate should be readily visible. The protein aggregates are harvested and purified using the protocol described on page 26. The amount of hydrated protein aggregate obtained should yield a wet weight of greater than 6.5 g.

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