# inNovations

Novagen Newsletter • Advanced products & protocols for proteomics and molecular biology research • October 2003, No. 18

# Enhanced protein coexpression in *E. coli*

New Duet vectors for expanded compatibility *page 4*
Novel LIC Duet™ Adaptors for cloning two genes at once *page 7*

An EMSA alternative
*13*

PCR from blood without DNA purification
*17*

Automated protein purification
*20*

*See full contents, page 2*

Novagen®

Advancing your life science discoveries™

EMD

# TABLE OF CONTENTS

## ARTICLES


Enhanced protein coexpression
New coexpression vectors for expanded compatibilities in *E. coli* ..... 4

Enhanced protein coexpression
A rapid method for simultaneously cloning two open reading frames for coexpression in *E. coli* ..... 7

An EMSA alternative
Identification of DNA binding proteins using the NoShift™ Transcription Factor Assay Kit ..... 13

PCR from blood without DNA expression
A novel buffer system for direct PCR from whole blood ..... 17

Automated protein purification
Robotic solubility screening and purification of fusion proteins ..... 20


## NEW PRODUCTS


Vectors for expression of amino-terminal His•Tag® fusion proteins containing minimal extraneous sequences ..... 23
Overnight Express™ Autoinduction System 2 ..... 26
BugBuster® Plus Lysonase™ Kit ..... 27
Rosetta™ 2(DE3) Competent Cells for enhanced coverage of codon bias in *E. coli* ..... 28
NovaBlue T1$^R$ Singles™ Competent Cells ..... 29
Zappers™ Electrocompetent Cells ..... 29
PhosphoSafe™ Extraction Buffer ..... 30


## NOVAGEN INFORMATION


Bulk and custom packaging options ..... 31
Novagen literature ..... 31
Contact and ordering information ..... 32


## Products In This Issue!


BloodDirect™ PCR Buffer Kit, Human ..... 19
BloodDirect PCR Buffer Kit, Mouse ..... 19
BugBuster Plus Lysonase Kit ..... 27
LIC Duet™ GST•Tag™ Ek Adaptor ..... 12
LIC Duet Minimal Adaptor ..... 12
LIC Duet Nus•Tag™ Ek Adaptor ..... 12
LIC Duet T7•Tag® Ek Adaptor ..... 12
LIC Duet Trx•Tag™ Ek Adaptor ..... 12
NoShift c-Fos Reagents ..... 16
NoShift ER-α Reagents ..... 16
NoShift Sp1 Reagents ..... 16
NoShift Transcription Factor Assay Kit ..... 16
NoShift Transcription Factor Assay Kit Plus NucBuster™ ..... 16
NovaBlue T1$^R$ Singles Competent Cells ..... 29
NovaXG Zappers Electrocompetent Cells ..... 29
NovaXGF´ Zappers Electrocompetent Cells ..... 29
Overnight Express Autoinduction System 2 ..... 26
pCDF-1b DNA ..... 25
pCDF-1 Expression System ..... 25
pCDF-1 Expression System plus Competent Cells ..... 25
pCDF-2 Ek/LIC Vector Kit ..... 12, 25
pCDFDuet™-1 DNA ..... 6
pET Expression System 45b ..... 25
pET Expression System 45b plus Competent Cells ..... 25
pET-45b(+) DNA ..... 25
pET-46 Ek/LIC Vector Kit ..... 12, 25
PhosphoSafe Extraction Buffer ..... 30
pIEx™-6 DNA ..... 25
pIEx-7 Ek/LIC Vector Kit ..... 25
pRSF-1 Expression System ..... 25
pRSF-1 Expression System plus Competent Cells ..... 25
pRSF-1b DNA ..... 25
pRSF-2 Ek/LIC Vector Kit ..... 12, 25
pRSFDuet™-1 DNA ..... 6
Rosetta 2(DE3) Competent Cells ..... 29
Rosetta 2(DE3) Singles Competent Cells ..... 29
Solubility Screening 96-well Filter Plate ..... 22



*inNovations* is a free newsletter presenting research articles from Novagen scientists and external contributors. Reports present new products and techniques, novel product applications or protocols, and other information of value to the proteomics and molecular biology research communities. Your comments, including suggestions for articles, are welcome. Please direct comments to:

**EMD Biosciences, Inc.**
**Novagen Marketing Department**
**441 Charmany Dr.**
**Madison, WI 53719**

**e-mail: novatech@novagen.com**



Prices are subject to change without notice. The following are trademarks or registered trademarks of EMD Biosciences, Inc.: Advancing your life science discoveries, BloodDirect, BugBuster, Calbiochem, Clonables, CytoBuster, EcoPro, FRETWorks, GeneJuice, GigaSingles, GST•Bind, GST•Mag, GST•Tag, His•Bind, His•Mag, His•Tag, LIC Duet, Lysonase, Non-Interfering Protein Assay, NoShift, Novagen logo, Novagen name, Nova*Taq*, NucBuster, Nus•Tag, Oncogene Research Products, OnEx, Origami, Overnight Express, pACYCDuet, pBiEx, pCDFDuet, Pellet Paint, Perfect DNA, Perfect Protein, pETBlue, pETcoco, pETDuet, PhosphoSafe, pIEx, PopCulture, pRSFDuet, pTriEx, rLysozyme, RoboPop, Rosetta, RosettaBlue, Rosetta-gami, Singles, S•Tag, T7•Tag, Trail Mix, Trx•Tag, Tuner, YeastBuster, and Zappers.

AmpliTaq is a registered trademark of Applied Biosystems. Benzonase is a registered trademark of Merck KGaA, Darmstadt, Germany. Coomassie is a registered trademark of Imperial Chemical Industries, Ltd. Genesis is a registered trademark and Freedom is a trademark of Tecan Group AG. HotStarTaq is a trademark of Qiagen.

©Copyright 2003 EMD Biosciences, Inc.
All Rights Reserved.
Printed in the USA. October, 2003.


Novagen®
*A Brand of EMD Biosciences, Inc.*
*an Affiliate of Merck KGaA, Darmstadt, Germany*

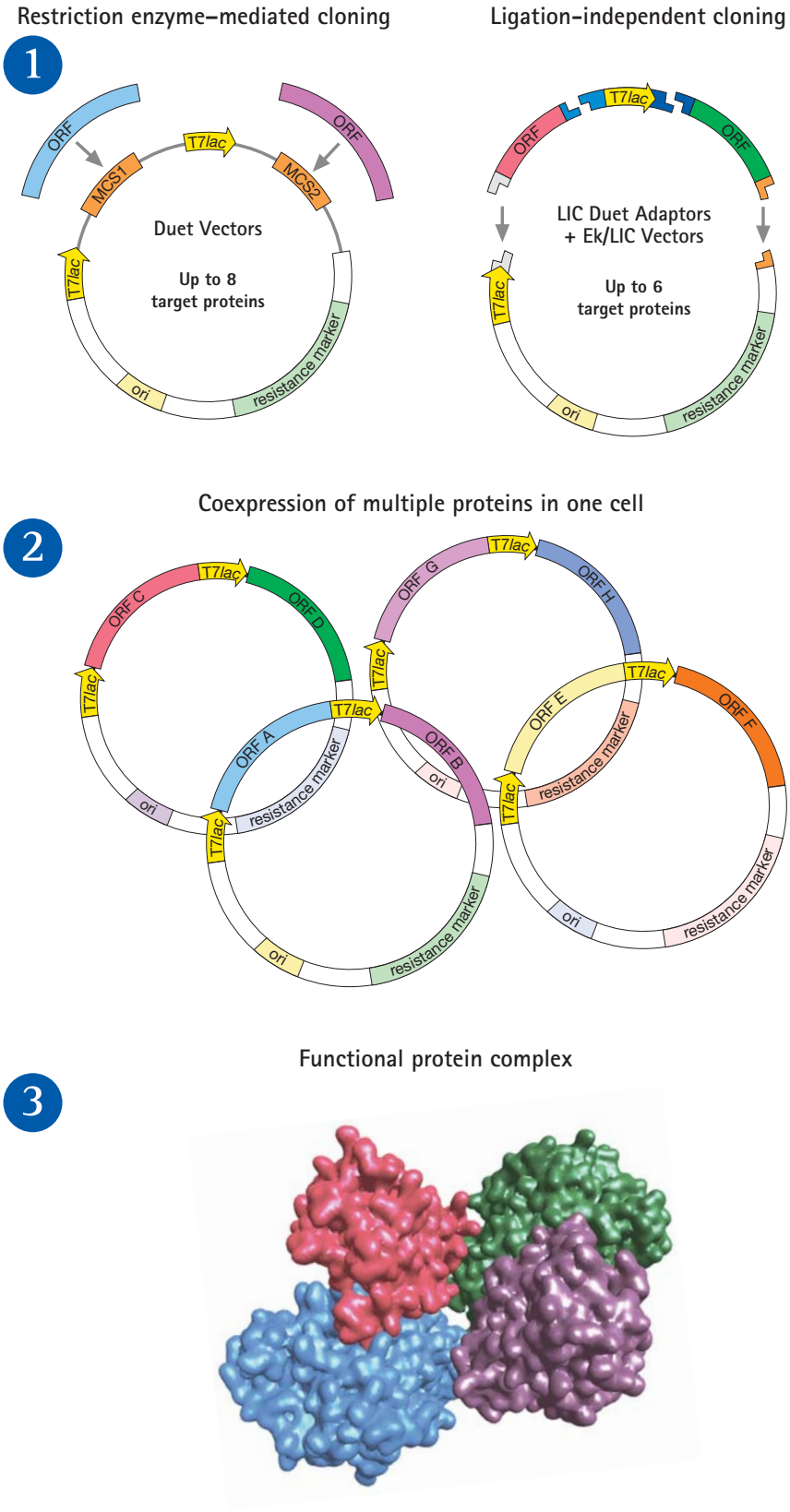
# Enhanced protein coexpression in *E. coli*

*New Duet vectors, methods, and kits for restriction enzyme–mediated and ligation-independent cloning*

An *inNovations* 15 article titled "*Coexpression of multiple target proteins in* E. coli," described the advantages of expressing multiple proteins in *E. coli* and introduced two coexpression vectors, pETDuet™-1 and pACYCDuet™-1. In this issue of *inNovations*, two articles introduce additional products to enhance cloning and protein coexpression: two new Duet vectors and five new LIC Duet™ Adaptors with new Ek/LIC vectors (see figure at right).

The first article, "*New co-expression vectors for expanded compatibilities in* E. coli," begins on page 4 and describes the new Duet vectors, pRSFDuet™-1 and pCDFDuet™-1, which have two sets of expression signals and multiple cloning site (MCS) regions. The new Duet vectors are compatible with each other and with the previously introduced Duet vectors; these vectors increase the number of host strains available for coexpression and the number of proteins that can be coexpressed.

The second article, "*A rapid method for simultaneously cloning two open reading frames for coexpression in* E. coli," begins on page 7. It describes the LIC Duet Adaptors, which allow for the simultaneous cloning of two open reading frames (ORFs) into any Novagen Ek/LIC expression vector, and introduces additional Ek/LIC vectors. When used with an appropriate combination of Ek/LIC vectors, the LIC Duet Adaptors facilitate coexpression of up to six target proteins.



# New coexpression vectors for expanded compatibilities in *E. coli*

Dustie Held, Keith Yaeger, and Robert Novy — Novagen

*We describe two new vectors for coexpression of multiple target proteins in* E. coli; *these vectors expand the set of compatible host strains available for coexpression and increase the number of target genes that can be coexpressed. The Duet family of coexpression vectors now includes four T7 expression plasmids with compatible replicons and selectable markers that enable coexpression of up to eight target proteins in the same bacterial cell.*

The previously described pETDuet™-1 and pACYCDuet™-1 vectors (1) facilitate the cloning and expression of multiple target proteins in a set of expression host strains. Many T7 expression system strains carry the pLysS plasmid and Rosetta™ strains carry the pRARE plasmid. Because the pLysS and pRARE plasmids carry the P15A replicon and encode chloramphenicol resistance ($Cm^R$), they are not compatible with the pACYCDuet-1 vector. To expand the set of compatible host strains for coexpression and to increase the number of target genes that can be coexpressed, we constructed the pCDFDuet™-1 and pRSFDuet™-1 vectors.

To create coexpression vectors compatible with the existing Duet vectors, pETDuet-1 and pACYCDuet-1, it was necessary to use different antibiotic resistance genes and plasmid replicons. The pETDuet-1 vector carries the ColE1 replicon and an ampicillin resistance marker ($Ap^R$); the pACYCDuet-1 vector carries the P15A replicon and $Cm^R$. Based on known compatibilities of various replicons (2, 3), we incorporated those from plasmids RSF1030 (also known as NTP1; 4, 5) and CloDF13 (6). The CloDF13 replicon was combined with *aadA* encoding streptomycin and spectinomycin resistance to create pCDFDuet-1. A slightly modified version of the RSF replicon was combined with kanamycin resistance to create pRSFDuet-1 (Figure 1). Table 1 summarizes the replicons of plasmids used in Novagen *E. coli* expression systems.

### Relative copy number

To compare the relative copy number of all four Duet plasmids, each was transformed separately into the NovaBlue host strain. Liquid cultures inoculated from the resulting transformants were grown in parallel. Plasmid DNA was then isolated and loaded onto an agarose gel to generate the comparative plasmid yield data shown in Figure 2 (page 5). The gel analysis shows that the relative plasmid copy numbers for the Duet plasmids are pRSF > pET > pCDF > pACYC. The differences in plasmid copy number can be used to influence relative target protein expression levels.

### Restriction enzyme–mediated cloning strategies

All Duet vectors are designed for the cloning and expression of two target genes or, more correctly, open reading frames (ORFs). These vectors each contain two expression units, each controlled by a separate T7*lac* promoter. Each T7*lac* promoter is followed by an optimal ribosome binding sequence and multiple cloning site (MCS). In each vector, MCS1 incorporates an *Nco* I site (CCATGG) at the ATG (Met) translation initiation site. The *Nco* I site can be used for expression of unfused protein (8) and is followed by a six–amino acid His•Tag® coding sequence and several restriction sites common to most Novagen pET vectors (*Bam*H I, *Eco*R I, *Sac* I, *Sal* I, *Hin*d III, *Not* I; see www.novagen.com for sequences). MCS2

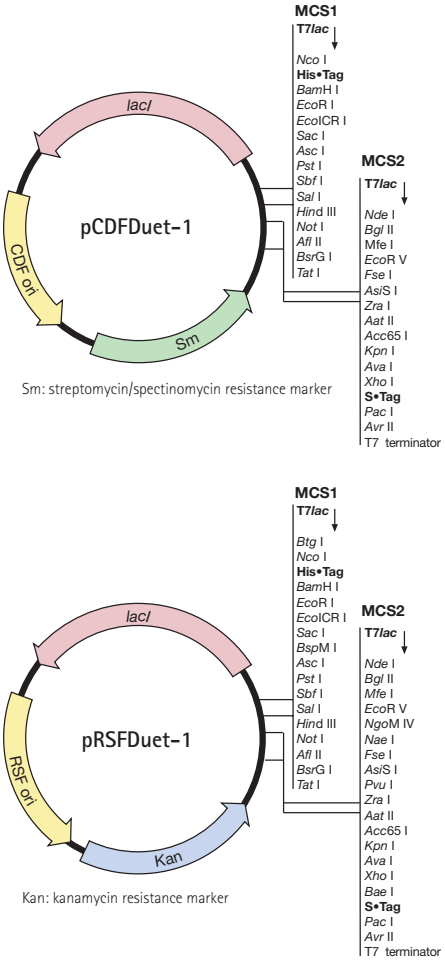


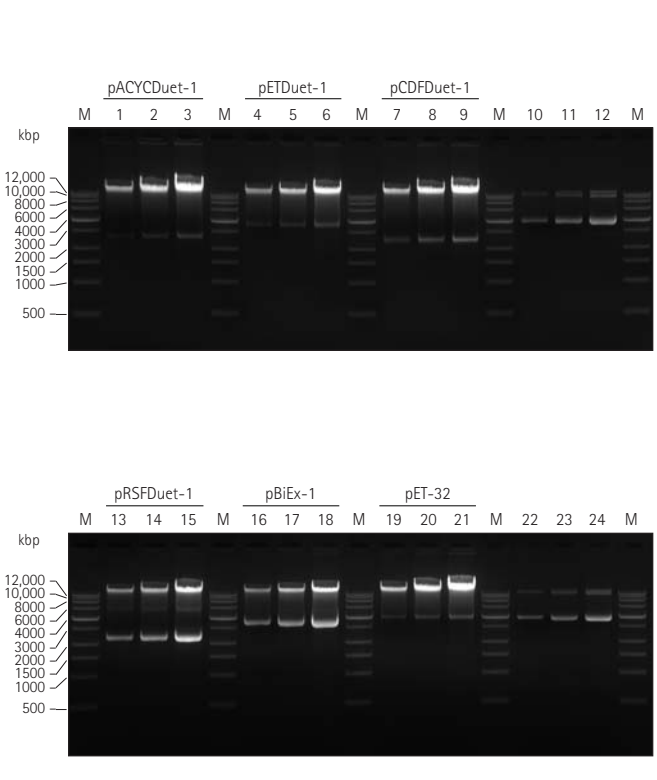

Figure 1. pCDFDuet-1 and pRSFDuet-1 vectors

Table 1: Plasmid replicons in Novagen *E. coli* expression systems

| Plasmid(s) | Replicon (source) | Copy Number |
|---|---|---|
| pET (all)<br>pETDuet-1 | ColE1 (pBR322) | ~40 |
| pACYCDuet-1<br>pLysS<br>pLysE<br>pLacI<br>pRARE | P15A (pACYC184) | 10–12 |
| pRSF (all) | RSF1030 | > 100 |
| pCDF (all) | CloDF13 | 20–40 |
| pETBlue™ (all)<br>pTriEx™ (all) | ColE1 (pUC) | > 500 |
| pETcoco™ (all) | Mini-F/RK2 (7)<br>(pBeloBAC11, RK2) | 1, amplifiable to ~40 |

begins with an *Nde* I (CATATG) site at the ATG (Met) translation initiation site, which also facilitates the generation of unfused protein. MCS2 contains *Bgl* II, *Mun* I, and *Xho* I sites for enzymes that generate overhangs compatible with *Bam*H I, *Eco*R I, and *Sal* I overhangs, respectively. Incorporating these key restriction sites into each MCS allows target genes from existing pET recombinants to be readily transferred to the Duet plasmids. The *Xho* I site in MCS2 is followed by a sequence encoding the 15–amino acid S•Tag™ peptide. The nature and positioning of the cloning sites in the Duet vectors generate several options for detection, purification, and quantification of protein complexes: unfused proteins for one or both ORFs, ORF 1–encoded fusion proteins with an amino-terminal His•Tag® sequence, and ORF 2–encoded fusion proteins with a carboxy-terminal S•Tag sequence.

When creating dual constructs, the first ORF to be inserted should lack restriction sites to be used to insert the second ORF. Both MCS regions contain a




| Lane | Sample |
|---|---|
| M | Perfect DNA™ Markers, 0.5–12 kbp |
| 1 | 1X pACYCDuet™-1 |
| 2 | 2X pACYCDuet-1 |
| 3 | 4X pACYCDuet-1 |
| 4 | 1X pETDuet™-1 |
| 5 | 2X pETDuet-1 |
| 6 | 4X pETDuet-1 |
| 7 | 1X pCDFDuet™-1 |
| 8 | 2X pCDFDuet-1 |
| 9 | 4X pCDFDuet-1 |
| 10 | 50 ng standard |
| 11 | 100 ng standard |
| 12 | 200 ng standard |

| Lane | Sample |
|---|---|
| M | Perfect DNA Markers, 0.5–12 kbp |
| 13 | 1X pRSFDuet™-1 |
| 14 | 2X pRSFDuet-1 |
| 15 | 4X pRSFDuet-1 |
| 16 | 1X pBiEx™-1 |
| 17 | 2X pBiEx-1 |
| 18 | 4X pBiEx-1 |
| 19 | 1X pET-32 |
| 20 | 2X pET-32 |
| 21 | 4X pET-32 |
| 22 | 50 ng standard |
| 23 | 100 ng standard |
| 24 | 200 ng standard |

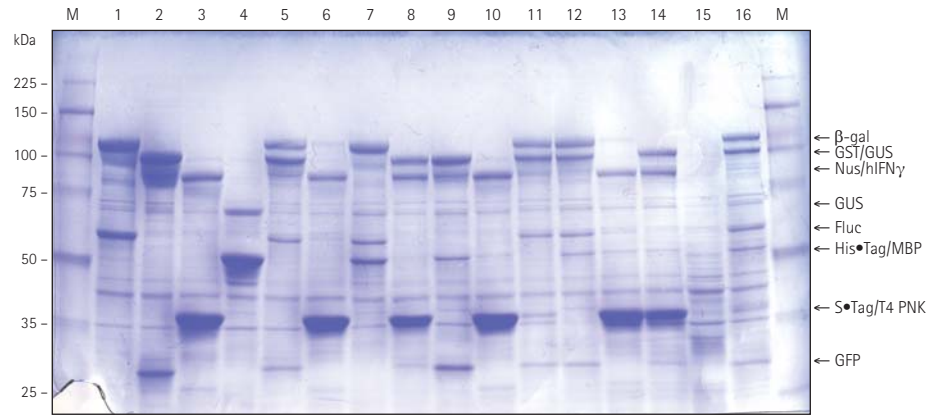
**Figure 2. Relative copy numbers of the Duet plasmids**

Plasmids were transformed into NovaBlue competent cells and grown to stationary phase in LB broth plus appropriate antibiotics at 37°C. The $OD_{600}$ of each culture was determined, and cells from 1.5 ml of each culture were collected by centrifugation (7 min, 8400 × g). Following removal of the supernatant, cell pellets were resuspended in phenol-CIAA (150 µl) and 1X TE (150 µl) and samples were mixed by vortex agitation and collected by centrifugation (10 min, 16,400 × g). The aqueous layer was then transferred to a new tube containing 1 µl RNase A Solution (Novagen). Volumes of each sample were normalized with respect to $OD_{600}$ at harvest. For each plasmid sample, three proportional load volumes (1X, 2X, and 4X) were analyzed by agarose gel electrophoresis (1.2% gel). (The intense top band in each plasmid preparation is chromosomal DNA present due to the rapid processing method used.)

**Table 2. Recombinants used for coexpression analysis in Figure 3**

| Parent Vector | Target Proteins | Expected $M_r$ (kDa) |
|---|---|---|
| pRSFDuet-1 | Nus/hIFNγ | 79.1 |
| | S•Tag/T4 PNK | 39.4 |
| pCDFDuet-1 | His•Tag/MBP | 53.0 |
| | GUS | 68.5 |
| pACYCDuet-1 | Fluc | 60.7 |
| | β-gal | 115.9 |
| pETDuet-1 | GST/GUS | 99.9 |
| | GFP | 28.7 |

GFP: green fluorescent protein; GUS: β-glucuronidase; MBP: maltose binding protein; T4 PNK: T4 polynucleotide kinase




| Lane | Sample |
|---|---|
| M | Trail Mix™ Protein Markers |
| 1 | pACYCDuet-1: β-gal + Fluc |
| 2 | pETDuet-1: GST/GUS + GFP |
| 3 | pRSFDuet-1: Nus/hIFNγ + S•Tag/T4 PNK |
| 4 | pCDFDuet-1: GUS + His•Tag/MBP |
| 5 | pACYCDuet-1, pETDuet-1 combination |
| 6 | pACYCDuet-1, pRSFDuet-1 combination |
| 7 | pACYCDuet-1, pCDFDuet-1 combination |
| 8 | pETDuet-1, pRSFDuet-1 combination |
| 9 | pRSFDuet-1, pCDFDuet-1 combination |
| 10 | pETDuet-1, pCDFDuet-1 combination |
| 11 | pACYCDuet-1, pETDuet-1, pRSFDuet-1 combination |
| 12 | pACYCDuet-1, pETDuet-1, pCDFDuet-1 combination |
| 13 | pACYCDuet-1, pRSFDuet-1, pCDFDuet-1 combination |
| 14 | pETDuet-1, pRSFDuet-1, pCDFDuet-1 combination |
| 15 | Uninduced control |
| 16 | All four Duet vectors: all eight proteins |

**Figure 3. Coexpression of multiple proteins using Duet vectors**

The indicated constructs were transformed individually or together into BL21(DE3). Cultures were grown in TB plus phosphates plus glucose at 37°C to an $OD_{600}$ between 1.0 and 1.2. Target protein expression was induced by adding IPTG to a final concentration of 1 mM. Cultures were harvested by centrifugation 2.5 h after induction. Lysates were produced by sonication using equal volumes of 1% SDS and 2X SDS sample buffer. Equivalent amounts of protein (based on harvest OD) were analyzed by SDS-PAGE (4–20% gradient gel) and stained with Coomassie blue. To maximize band separation, proteins smaller than 25 kDa were allowed to migrate off the gel.

*continued on page 6*

*continued from page 5*

number of six-base recognition sequences. In addition, each includes sites for eight-base rare cutting restriction enzymes, *Sse*8387 I and *Not* I in MCS1 and *Fse* I and *Sgf* I in MCS2.

### Protein expression from Duet vectors

To test the Duet vectors for coexpression of multiple proteins, the recombinants listed in Table 2 (page 5) were transformed separately and in various combinations into the BL21(DE3) expression host. Analysis of induced cell extracts is shown in Figure 3 (page 5). Lanes 1–4 show expression of two proteins from the four different Duet vectors. Vector combinations included two plasmids coexpressing up to four proteins (lanes 5–10), three plasmids coexpressing up to six proteins (lanes 11–14), and four plasmids coexpressing eight proteins (lane 16). The various combinations of Duet recombinants yielded different expression levels that roughly correlated with relative copy number. When the higher copy number pRSFDuet™ vector was used with the pACYCDuet™ or pCDFDuet™ vector, expression from the lower copy number plasmids was reduced. Using the four-vector combination and some combinations of three vectors, expression from lower copy number plasmids was similarly reduced. Plasmids with similar copy numbers appeared to express similar levels of target proteins. Combinations of lower– and higher–copy number plasmids provide a potential means to control the relative amount of proteins expressed.

### Summary

The new pRSFDuet-1 and pCDFDuet-1 vectors represent an important addition to the Duet expression vector family because they expand the number of host strains that can be used for coexpression experiments and, by virtue of their compatibility with existing Novagen vectors, they expand the number of proteins that can be coexpressed. Table 3 outlines various vector combinations (Duet/Duet and Duet/traditional pET) that can be used for coexpression of four to eight proteins and specifies compatible expression host strains for many of these combinations. Although the table does not include every possible combination of Novagen vectors, it does include those compatible with the largest number of expression strains.

**Table 3. Vector and host strain compatibilities for coexpression of four to eight target proteins**

| Compatible Vector Combinations | | | | Target Proteins (number) | Compatible Host Strain Group |
|---|---|---|---|---|---|
| Vector 1 | Vector 2 | Vector 3 | Vector 4 | | |
| pETDuet™-1 (Ap$^R$) | pACYCDuet-1 (Cm$^R$) | pRSFDuet-1 (Kn$^R$) | pCDFDuet-1 (Sm$^R$) | 8 | A |
| pET (Ap$^R$) | pACYCDuet-1 (Cm$^R$) | pRSFDuet-1 (Kn$^R$) | pCDFDuet-1 (Sm$^R$) | 7 | A |
| pETDuet-1 (Ap$^R$) | pACYCDuet-1 (Cm$^R$) | pRSFDuet-1 (Kn$^R$) | none | 6 | A |
| pETDuet-1 (Ap$^R$) | pRSFDuet-1 (Kn$^R$) | pCDFDuet-1 (Sm$^R$) | none | 6 | B |
| pET (Ap$^R$) | pRSFDuet-1 (Kn$^R$) | pCDFDuet-1 (Sm$^R$) | none | 5 | B |
| pETDuet-1 (Ap$^R$) | pRSFDuet-1 (Kn$^R$) | none | none | 4 | B |
| pRSFDuet-1 (Kn$^R$) | pCDFDuet-1 (Sm$^R$) | none | none | 4 | B |
| pETDuet-1 (Ap$^R$) | pCDFDuet-1 (Sm$^R$) | none | none | 4 | C |

Host Strain Groups

| Group A | Group B | Group C | |
|---|---|---|---|
| B834(DE3) | B834(DE3) | B834(DE3) | RosettaBlue(DE3) |
| BL21(DE3) | B834(DE3)pLysS | B834(DE3)pLysS | RosettaBlue(DE3)pLysS |
| BLR(DE3) | BL21(DE3) | BL21(DE3) | Rosetta-gami™(DE3)* |
| HMS174(DE3) | BL21(DE3)pLysS | BL21(DE3)pLysS | Rosetta-gami(DE3)pLysS* |
| NovaBlue(DE3) | BLR(DE3) | BLR(DE3) | Rosetta-gami B(DE3) |
| Tuner™(DE3) | BLR(DE3)pLysS | BLR(DE3)pLysS | Rosetta-gami B(DE3)pLysS |
| | HMS174(DE3) | HMS174(DE3) | Tuner(DE3) |
| | HMS174(DE3)pLysS | HMS174(DE3)pLysS | Tuner(DE3)pLysS |
| | NovaBlue(DE3) | NovaBlue(DE3) | |
| | NovaBlue(DE3)pLysS | NovaBlue(DE3)pLysS | |
| | Rosetta™(DE3) | Origami™(DE3)* | |
| | Rosetta(DE3)pLysS | Origami(DE3)pLysS* | |
| | RosettaBlue™(DE3) | Origami B(DE3) | |
| | RosettaBlue(DE3)pLysS | Origami B(DE3)pLysS | |
| | Tuner(DE3) | Rosetta(DE3) | |
| | Tuner(DE3)pLysS | Rosetta(DE3)pLysS | |

* These strains carry the *rpsL* mutation that confers resistance to streptomycin; therefore, spectinomycin must be used for selection of pCDFDuet recombinants.

Resistance markers: Ap$^R$, ampicillin/carbenicillin; Kn$^R$, kanamycin; Cm$^R$, chloramphenicol; Sm$^R$, streptomycin/spectinomycin

For strain descriptions, please refer to www.novagen.com or the Novagen catalog.


## ACKNOWLEDGMENTS

The authors thank G. J. Phillips, Iowa State University, for a derivative of RSF1030 and M. K. Waldor, New England Medical Center and Tufts University Medical School, for the CloDF13 provided for these experiments.


## REFERENCES


1. Novy, R., Yaeger, K., Held, D., and Mierendorf, R. (2002) *inNovations* **15**, 2–6.
2. Selzer, G., Som, T., Itoh, T. and Tomizawa, J. (1983) *Cell* **32**, 119–129.
3. Phillips, G. J., Park, S., and Huber, D. (2000) *BioTechniques* **28**, 400–408.
4. Som, T. and Tomizawa, J. (1982) *Mol. Gen. Genet.* **187**, 375–383.
5. Cannon, P. M. and Strike, P. (1992) *Plasmid* **27**, 220–230.
6. Nijkamp, H. J. J., de Lang, R., Stuitje, A. R., van den Elzen P. J. M., Veltkamp, E., and van Putten, A. J. (1986) *Plasmid* **16**, 135–160.
7. Wild, J., Hradecna, Z., and Szybalski, W. (2002) *Genome Res.* **12**, 1443–1444.
8. Novy, R. (1999) *inNovations* **10**, 13.



| Product | Size | Cat. No. |
|---|---|---|
| pCDFDuet™-1 DNA | 10 µg | 71340-3 |
| pRSFDuet™-1 DNA | 10 µg | 71341-3 |
| pETDuet™-1 DNA | 10 µg | 71146-3 |
| pACYCDuet™-1 DNA | 10 µg | 71147-3 |
| **Product** | **Size** | **Cat. No.** |
| BL21(DE3) Competent Cells<br>guaranteed efficiency: > 2 × 10$^6$ cfu/µg | 0.4 ml<br>1 ml | 69450-3<br>69450-4 |
| BL21(DE3) Singles™ Competent Cells<br>guaranteed efficiency: > 2 × 10$^6$ cfu/µg | 11 rxn<br>22 rxn | 70235-3<br>70235-4 |
| NovaBlue Competent Cells<br>guaranteed efficiency: > 1 × 10$^8$ cfu/µg | 0.4 ml<br>1 ml | 69825-3<br>69825-4 |
| NovaBlue Singles Competent Cells<br>guaranteed efficiency: > 1.5 × 10$^8$ cfu/µg | 11 rxn<br>22 rxn | 70181-3<br>70181-4 |

# A rapid method for simultaneously cloning two open reading frames for coexpression in *E. coli*

Katie Loomis, Heather Sternard, Sue Rupp, Dustie Held, Keith Yaeger, Robert Novy, and Shou Wong — Novagen

*The LIC Duet™ Adaptor strategy enables simultaneous cloning of two open reading frames into one plasmid and can convert any Novagen Ek/LIC bacterial expression plasmid into a coexpression vector.*

Ligation-independent cloning (LIC) was developed for efficient cloning of PCR products without the need for restriction enzyme digestion or ligation reactions (1, 2). The LIC-prepared pET bacterial expression vectors place the PCR product under the control of a powerful T7*lac* promoter and optimal ribosome binding site (RBS). These expression vectors encode several different amino-terminal fusion tags to improve solubility or purification (Table 1). An additional benefit to the Novagen LIC strategy is that the insert is positioned immediately adjacent to a protease cleavage site to enable complete removal of all amino-terminal, vector-encoded amino acids.

The LIC Duet™ Adaptors were designed to complement the overall LIC strategy by enabling the cloning of two open reading frames (ORFs) into any Novagen Ek/LIC vector for coexpression of proteins in *E. coli*. The primary advantage of the LIC Duet Adaptor method is the elimination of the multiple cloning steps otherwise needed to insert two ORFs into the same expression vector. At the same time, all the advantages associated with LIC are retained in LIC Duet. For example, virtually all colonies produced contain the desired recombinants due to the highly efficient nature and directionality of LIC. Combined with the availability of numerous Ek/LIC vectors and high-quality, high-fidelity thermostable polymerases, such as KOD HiFi and KOD Hot Start, LIC and LIC Duet Adaptor methodologies offer a platform technology for shuttling PCR inserts into many different destination Ek/LIC vectors.

The LIC method exploits the 5′→ 3′ polymerase and 3′→ 5′ exonuclease activities of T4 DNA polymerase to generate long, complementary 5′-overhangs in the vectors and inserts for annealing. LIC vectors are created by treating linearized vectors with T4 DNA polymerase in the presence of only one dNTP. The 3′→ 5′ exonuclease activity of T4 DNA polymerase removes nucleotides until it encounters the first residue that corresponds to the only dNTP present in the reaction mix. At this point, the 5′→ 3′ polymerase activity counteracts the exonuclease activity of the enzyme, preventing further excision. Plasmid sequences adjacent to the site of linearization are designed to produce specific 13- and 14-base noncomplementary, single-stranded overhangs in the LIC vector.

PCR products with complementary overhangs are created by including appropriate 5′-extension sequences in the primers. The purified PCR products are treated with T4 DNA polymerase in the presence of the appropriate dNTP to generate specific LIC vector–compatible overhangs. The treated LIC vectors and PCR inserts are annealed and transformed into NovaBlue GigaSingles™ Competent Cells. Because only correctly annealed LIC vector–inserts are formed during the annealing process, cloning efficiency is extremely high with minimal nonrecombinant background. Covalent bond formation at the vector-insert junctions occurs within cells, yielding circular plasmids (3).

With the LIC Duet method, the overall procedure to generate LIC-compatible vectors and PCR inserts remains the same. However, a LIC Duet Adaptor is included in the annealing reaction with the LIC vector plus two LIC-prepared PCR fragments that represent the target ORFs (Figure 1, page 8). All LIC Duet Adaptors contain the T7*lac* promoter, a strong RBS, and an ATG start codon to drive expression of the second ORF. Four of the five adaptors allow for the expression of the second ORF fused with an amino-terminal tag plus enterokinase cleavage site (Figure 2, page 8). The LIC Duet Minimal Adaptor encodes the minimum amino-terminal fusion sequence (five amino acids) with no protease cleavage site.

### pRSF-2, pCDF-2, and pET-46 Ek/LIC vectors

The high degree of flexibility inherent in the LIC strategy is illustrated in Figure 2 (page 8), which shows all of the Ek/LIC vectors available for protein coexpression in *E. coli*. Two vectors, pRSF-2 Ek/LIC and pCDF-2 Ek/LIC, are compatible with each other and with all other pET Ek/LIC vectors, including the new pET-46 Ek/LIC vector. The pRSF-2 Ek/LIC vector was derived from pRSFDuet™-1 and the pCDF-1 Ek/LIC vector was derived from the pCDFDuet™-1 vector (Table 1; for more information about the Duet vectors, see the article beginning on page 4). By using

**Table 1. Ek/LIC vector amino-terminal fusion tags**

| | Tag(s) | | | |
|---|---|---|---|---|
| Vector | Amino | Carboxy | Replicon | Antibiotic Selection |
| pCDF-2 Ek/LIC | His•Tag®, Ek | His•Tag | CloDF13 | streptomycin/spectinomycin |
| pET-30 Ek/LIC | His•Tag, Tb, S•Tag™, Ek | His•Tag | ColE1 | kanamycin |
| pET-32 Ek/LIC | Trx•Tag™, His•Tag, Tb, S•Tag, Ek | His•Tag | ColE1 | ampicillin |
| pET-41 Ek/LIC | GST•Tag™, His•Tag, Tb, S•Tag, Ek | His•Tag | ColE1 | kanamycin |
| pET-43.1 Ek/LIC | Nus•Tag™, His•Tag, Tb, S•Tag, Ek | His•Tag | ColE1 | ampicillin |
| pET-46 Ek/LIC | His•Tag, Ek | S•Tag | ColE1 | ampicillin |
| pRSF-2 Ek/LIC | His•Tag, Ek | His•Tag | RSF1030 | kanamycin |

Recognition sites: Ek, enterokinase; Tb, thrombin

*continued on page 8*

*continued from page 7*

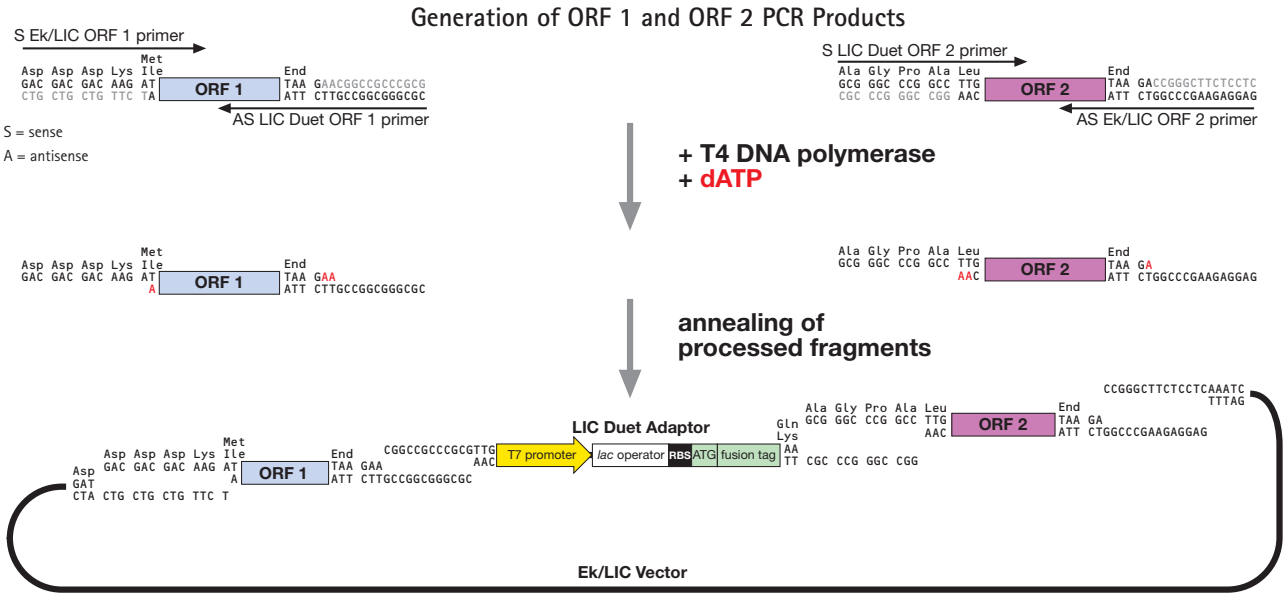
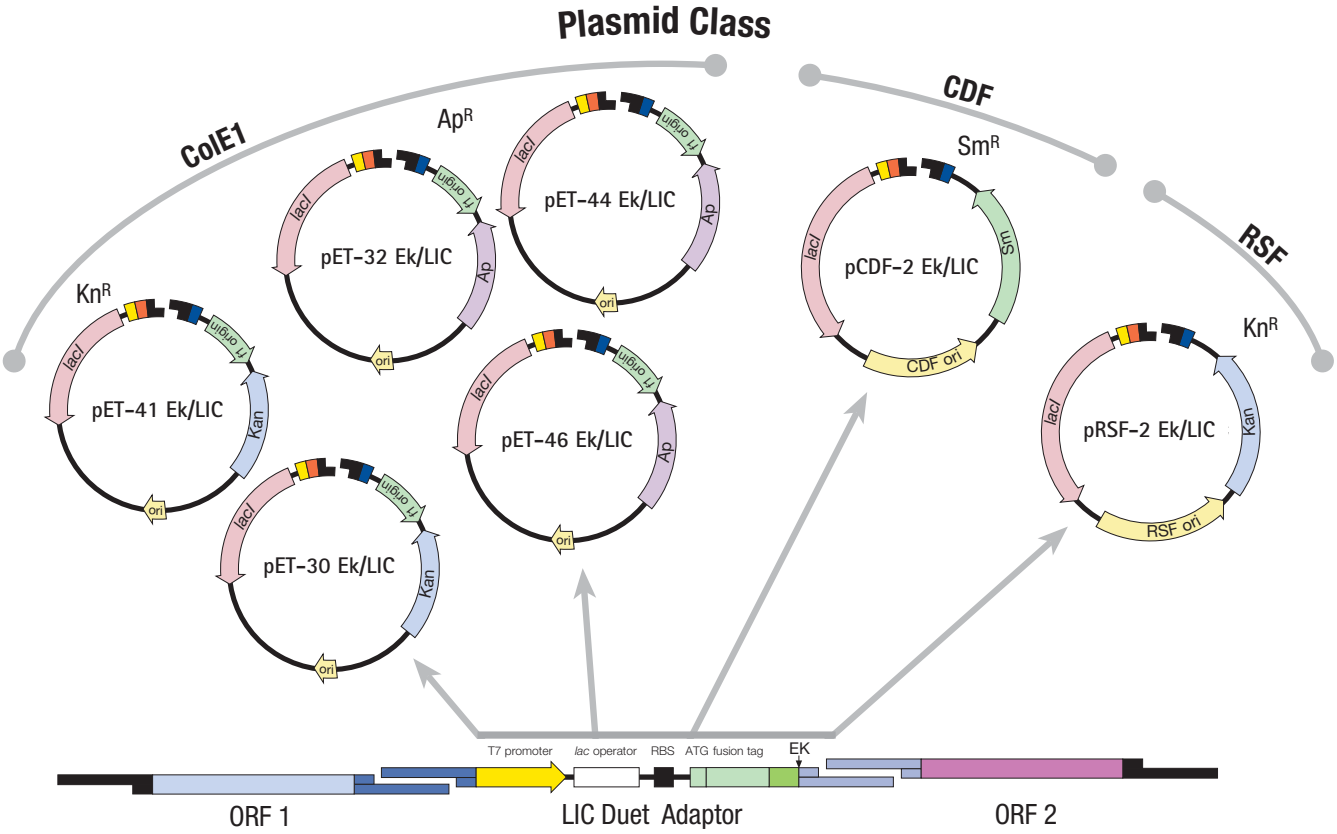



Figure 1. The LIC Duet™ Adaptor method for simultaneous cloning of two open reading frames




| Fusion Tag | Tag Size (aa) | Adaptor Size (bp) |
|---|---|---|
| none* | 5 | 138 |
| T7•Tag® | 12 | 189 |
| Trx•Tag™ | 109 | 502 |
| GST•Tag™ | 220 | 835 |
| Nus•Tag™ | 495 | 1,660 |

* LIC Duet Minimal Adaptor does not encode an EK site

Figure 2. Compatible Ek/LIC vectors for coexpression in bacteria

a two-ORFs-per-vector LIC Duet™ Adaptor cloning strategy in combination with pRSF-2 Ek/LIC, pCDF-2 Ek/LIC, and an ampicillin-based pET Ek/LIC vector, it is possible to coexpress up to six target proteins. Strains compatible with the coexpression of multiple target proteins from various Ek/LIC combinations are listed in Table 2.

### High cloning efficiency and success rate with LIC Duet Adaptors

The original LIC procedure exhibits high-efficiency directional cloning of one ORF at a time with minimal nonrecombinant background (3). To determine the cloning efficiency of LIC Duet for targets of different sizes, PCR products in sizes of 279, 388, 1,308, and 3,802 base pairs were grouped into four sets of pairs: set 1 represents a small/large PCR product pair (279 and 3,802 bp); set 2 represents a small/small PCR product pair (279 and 388 bp); set 3 represents a large/large PCR product pair (1,308 and 3,802 bp); and set 4 represents a large/small PCR product pair (1,308 and 388 bp). These four PCR product pairs were annealed with pET-30 Ek/LIC and the LIC Duet T7•Tag® Ek Adaptor and transformed into NovaBlue GigaSingles™ Competent Cells. Table 3 shows that pair sets containing the 388-bp insert (sets 2 and 4) exhibited approximately three- to sixfold greater cloning efficiency than the pairs containing the 3,802-bp insert (sets 1 and 3). It is important to note that LIC Duet method produced fewer transformed colonies than the standard LIC method, which allows for cloning one PCR insert at a time. A decrease in cloning efficiency using LIC Duet methodology is expected because it relies on a four-part annealing process instead of the two-part process used in the LIC method. Because only the correctly annealed products can form an intact plasmid, almost 100% of the resulting colonies contain the two PCR inserts desired.

### Cloning ORFs of interacting proteins or domains

A key application for the LIC Duet Adaptor strategy is for cloning ORFs that represent interacting proteins or domains. To test this application, we used ORFs encoding interacting domains of BRCA2 and Rad51.

The 3,418–amino acid BRCA2 protein is a tumor suppressor that contributes to genomic stability, at least in part, by playing a role in homologous recombination repair through interactions with Rad51 (4). BRCA2 interacts directly with Rad51 via eight conserved sequence motifs, or BRC repeats, composed of approximately 30 amino acids each and located between residues 990 and 2,100 in human BRCA2 (5). Appropriate regions of BRCA2 and Rad51, encoding 126 and 243 amino acids, respectively, were amplified from cDNAs in plasmid templates. The BRCA2 and Rad51 fragments were simultaneously cloned into the pET-30 Ek/LIC vector with the LIC Duet T7•Tag Ek Adaptor, generating a pET-30 BRCA2–Rad51 recombinant plasmid. In this plasmid, BRCA2 is expressed as a 19.9-kDa fusion with an amino-terminal His•Tag® sequence and Rad51 is expressed as a 28.3-kDa protein with an amino-terminal T7•Tag sequence. As controls, BRCA2 and Rad51 domains also were cloned individually into pET-30 Ek/LIC for expression of each domain alone as amino-terminal His•Tag fusions.

*Using BugBuster® Protein Extraction Reagent and Ni-NTA His•Bind® Resin, we successfully purified His•Tag/BRCA2–unfused Rad51 complexes and demonstrated that this mild, detergent-based extraction reagent did not disrupt protein–protein interactions.*

As shown in Figure 3 (page 10), when expressed as a single His•Tag fusion, most of the expressed BRCA2 domain could be purified under native conditions on Ni-NTA His•Bind® Resin (lane 4). When Rad51 was expressed as a His•Tag

**Table 3. Efficient cloning of small and large gene pairs with LIC Duet**

| Pair Set | Pair Member Sizes | Average Number of Colonies per Plate |
|---|---|---|
| 1 | Small (279 bp)/Large (3,803 bp) | 47 |
| 2 | Small (279 bp)/Small (388 bp) | 277 |
| 3 | Large (1,308 bp)/Large (3,803 bp) | 58 |
| 4 | Large (1,308 bp)/Small (388 bp) | 167 |

**Table 2. Vector and host strain compatibilities for coexpression of four to six target proteins**

| Compatible Vector Combinations | | | Number of Possible Target Proteins* | Compatible Host Strain Group |
|---|---|---|---|---|
| Vector 1 | Vector 2 | Vector 3 | | |
| pET Ek/LIC (Ap$^R$) | pRSF-2 Ek/LIC (Kn$^R$) | pCDF-2 Ek/LIC (Sm$^R$) | 6 | B |
| pET (Ap$^R$) | pRSF-2 Ek/LIC (Kn$^R$) | pCDF-2 Ek/LIC (Sm$^R$) | 5 | B |
| pET Ek/LIC (Ap$^R$) | pRSF-2 Ek/LIC (Kn$^R$) | none | 4 | B |
| pET Ek/LIC (Ap$^R$) | pCDF-2 Ek/LIC (Sm$^R$) | none | 4 | C |
| pRSF-2 Ek/LIC (Kn$^R$) | pCDF-2 Ek/LIC (Sm$^R$) | none | 4 | B |

**Host strain groups**

| Group B | | Group C | |
|---|---|---|---|
| B834(DE3) | RosettaBlue™(DE3) | B834(DE3) | Origami B(DE3) |
| B834(DE3)pLysS | RosettaBlue(DE3)pLysS | B834(DE3)pLysS | Origami B(DE3)pLysS |
| BL21(DE3) | Tuner™(DE3) | BL21(DE3) | Rosetta(DE3) |
| BL21(DE3)pLysS | Tuner(DE3)pLysS | BL21(DE3)pLysS | Rosetta(DE3)pLysS |
| BLR(DE3) | | BLR(DE3) | RosettaBlue(DE3) |
| BLR(DE3)pLysS | | BLR(DE3)pLysS | RosettaBlue(DE3)pLysS |
| HMS174(DE3) | | HMS174(DE3) | Rosetta-gami™(DE3)† |
| HMS174(DE3)pLysS | | HMS174(DE3)pLysS | Rosetta-gami(DE3)pLysS† |
| NovaBlue(DE3) | | NovaBlue(DE3) | Rosetta-gami B(DE3) |
| NovaBlue(DE3)pLysS | | NovaBlue(DE3)pLysS | Rosetta-gami B(DE3)pLysS |
| Rosetta™(DE3) | | Origami™(DE3)† | Tuner(DE3) |
| Rosetta(DE3)pLysS | | Origami(DE3)pLysS† | Tuner(DE3)pLysS |

* Assumes two target genes are cloned for each Ek/LIC vector using the LIC Duet Adaptor method

† These strains carry the *rpsL* mutation that confers resistance to streptomycin; therefore, spectinomycin must be used for selection of pCDFDuet recombinants.

Resistance markers: Ap$^R$, ampicillin/carbenicillin; Kn$^R$, kanamycin; Cm$^R$, chloramphenicol; Sm$^R$: streptomycin/spectinomycin

For strain descriptions, please refer to www.novagen.com or the Novagen catalog

*continued on page 10*

*continued from page 9*

fusion, very little of the total Rad51 was recovered under native conditions (lane 6). However, when T7•Tag® Rad 51 was coexpressed with BRCA2, excellent recovery of both domains under native conditions was exhibited (lane 2). Copurification of Rad51 and BRCA2 with Ni-NTA His•Bind® Resin was possible due to heterodimerization of these two domains. This experiment demonstrates the usefulness of LIC Duet™ Adaptors for simultaneous cloning of two ORFs into the same expression vector for copurification of interacting domains. Using BugBuster® Protein Extraction Reagent and Ni-NTA His•Bind Resin, we successfully purified His•Tag®/BRCA2–unfused Rad51 complexes and demonstrated that this mild, detergent-based extraction reagent did not disrupt protein–protein interactions.

### Fusion tag choices in LIC Duet Adaptors

The LIC Duet Adaptor strategy enables selection of the most effective fusion tag to improve solubility, enhance protein folding, and support various purification methods (Figure 2, page 8). To test the effects of different fusion tags on purification of interacting domains, we used the T7•Tag, Trx•Tag™, GST•Tag™, and Nus•Tag™ adaptors to clone ORFs that encode regions of transcription factors related to TFIID, one of the central factors that controls transcription by RNA polymerase II. The TFIID complex is composed of the TATA-binding protein (TBP) and at least 14 TBP-associated factors (TAFs; 6). Others have shown that TAF4 and TAF12 interact with each other by heterodimerization via histone-fold domains and that it is necessary to coexpress both of these factors to form soluble protein complexes in *E. coli*. When these same factors were expressed individually, TAF4 was predominantly insoluble while TAF12 was soluble (7).

We cloned and expressed PCR products that encode histone-fold domains of yTAF4 and yTAF12, which are the yeast homologs of TAF4 and TAF12. The homologs were amplified from cDNA in plasmid templates. LIC Duet T7•Tag, Trx•Tag, GST•Tag, and Nus•Tag Ek Adaptors were used with pET-30 Ek/LIC to clone, as the first insert, an ORF for the histone-fold domain of yTAF12 (amino acids 409–491) and, as the second insert, an ORF for the conserved carboxy-terminal region of yTAF4 (amino acids 186–280). The products generated were yTAF12 fused with a His•Tag sequence and yTAF4 fused with four different tag sequences (Table 4). Corresponding controls included the yTAF4 domain fused with His•Tag, Trx•Tag, GST•Tag, and Nus•Tag sequences that were generated by cloning the yTAF4 open reading frame into pET-30, pET-32, pET-41, and pET-43.1 Ek/LIC vectors, respectively.

As previously reported (7), a His•Tag/yTAF12 fusion (14.5 kDa) was expressed in the soluble fraction and purified easily by Ni-NTA His•Bind chromatography (Figure 4, lane 8, page 11). Figure 4 shows that when the yTAF4 domain was expressed alone, only a small portion of the fusion was recovered under native conditions. Conversely, when coexpressed with the yTAF12 domain, significantly more of yTAF4 domain was recovered from the soluble fractions for three out of four of the adaptors (LIC Duet GST•Tag was the exception; data not shown). It is interesting to note that a degradation pattern was exhibited for the Nus•Tag/yTAF4 fusion when expressed by itself (lane 14) but not when yTAF12 and Nus•Tag/yTAF4 domains were coexpressed, suggesting that the complex formation contributed to the stability of the complex or otherwise protected protein components from degradation. Copurification of the yTAF12–yTAF4 domain complexes by Ni-NTA His•Bind chromatography depends on the His•Tag sequence in the yTAF12 domain as well as on the formation of soluble complexes that result from heterodimerization between these two domains.

**Table 4. Fusion proteins from yeast homologs to TAF4 and TAF12**

| Homolog | Amino Acids Encoded | Fusion Tag(s) | Size of Expressed Protein (kDa) |
|---|---|---|---|
| yTAF12 | 409–491 | His•Tag | 14.5 |
| yTAF4 | 186–280 | T7•Tag | 29.4 |
| | | GST•Tag | 36.0 |
| | | Trx•Tag | 40.4 |
| | | Nus•Tag | 83.5 |

### Testing for protein or domain interactions

One advantage of the LIC Duet methodology is the ability to quickly and efficiently clone any PCR product pair

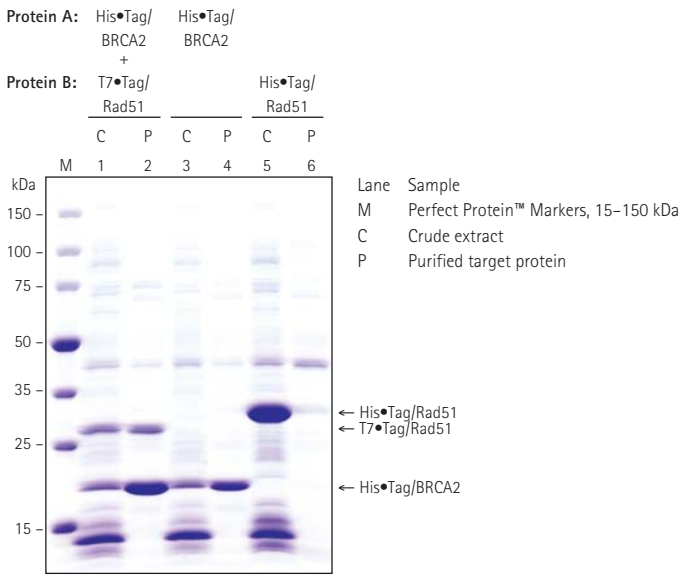



**Figure 3. Coexpression and purification of interacting domains of BRCA2 and Rad51**

Three constructs, pET-30 Ek/LIC BRCA2–Rad51, pET-30 Ek/LIC BRCA2, and pET-30 Ek/LIC Rad51, were transformed into Rosetta™(DE3) competent cells, grown in LB broth, and induced with IPTG at 26°C for 4 h. Cells were harvested by centrifugation and lysed with BugBuster Protein Extraction Reagent, rLysozyme™ Solution, and Benzonase® Nuclease. Equal volumes were purified by Ni-NTA His•Bind chromatography under native conditions. Samples representing equal cell mass were analyzed by SDS-PAGE (4–20% gradient) and stained with Coomassie blue.

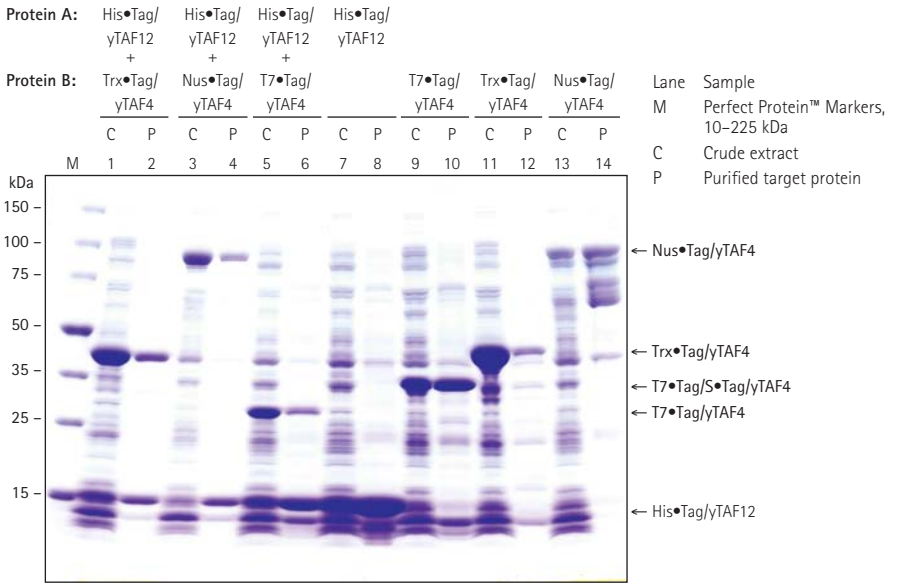



**Figure 4. Coexpression and purification of interacting domains of yTAF4 and yTAF12**

A yTAF12 fusion to the His•Tag® sequence was expressed alone in pET-30 Ek/LIC or coexpressed in the same vector with yTAF4 using the LIC Duet Trx•Tag™, Nus•Tag™, or T7•Tag® Ek Adaptors. The yTAF4 protein was also expressed alone as a His•Tag, Trx•Tag, or Nus•Tag fusion from the pET-30, pET-32, or pET-43.1 Ek/LIC vectors, respectively. The recombinant plasmids were transformed into Rosetta™(DE3), grown in LB broth, and induced with IPTG at 26°C for 4 h. Cells were harvested by centrifugation and lysed with BugBuster® Protein Extraction Reagent, rLysozyme™ Solution, and Benzonase® Nuclease. Equal volumes were purified by Ni-NTA His•Bind® chromatography under native conditions. Samples representing equal cell mass were analyzed by SDS-PAGE (4–20% gradient) and stained with Coomassie blue.

without the need to undergo a two-step cloning process. Such a capability makes LIC Duet™ an excellent choice in situations where different combinations of ORFs need to be tested to determine the best combinations of pairs to study interacting proteins or domains. To test this application, we used transcription factors E2F-1, E2F-2, and E2F-3 with heterodimeric partners DP-1 and DP-2. E2F is a cell cycle–regulated heterodimeric transcription factor that regulates the expression of a number of viral and cellular genes (8). The E2F transcription factor consists of two components, termed E2F and DP. The E2F component is encoded by a family of genes that includes at least six members (E2F-1 through E2F-6) (8, 9). E2Fs exert full transcription activity by forming heterodimers with DP partners, DP-1 and DP-2 (8). In other studies, E2Fs and DPs were expressed in the insoluble fraction when expressed individually, but formed soluble E2F–DP complexes when coexpressed in *E. coli* (10).

Regions of E2F-1, E2F-2, E2F-3, DP-1, and DP-2 that encode 181, 184, 183, 246, and 249 amino acids, respectively, were amplified from plasmid templates. Six

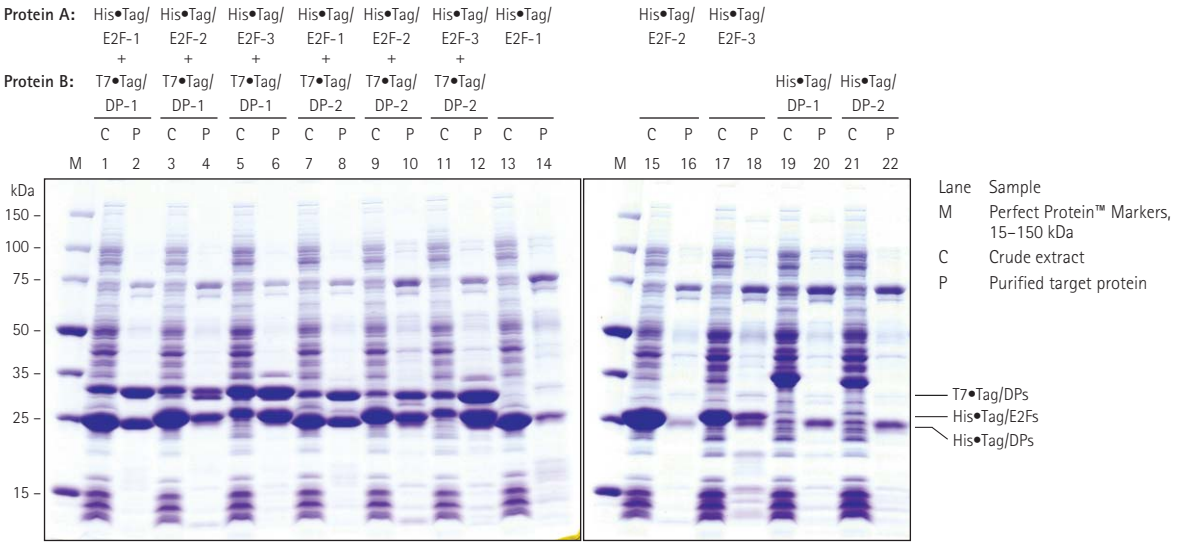



**Figure 5. Coexpression and purification of interacting domains of E2F and DP**

Combinations of E2F-1, E2F-2, or E2F-3 with DP-1 or DP-2 were coexpressed as indicated using the LIC Duet T7•Tag Ek Adaptor to simultaneously anneal both inserts into pET-30 Ek/LIC. The constructs were transformed into Rosetta(DE3) competent cells, grown in LB broth, and induced with IPTG at 26°C for 4 h. Cells were harvested by centrifugation and lysed with BugBuster Protein Extraction Reagent, rLysozyme Solution, and Benzonase Nuclease. Equal volumes were purified by Ni-NTA His•Bind chromatography under native conditions. Samples representing equal cell mass were analyzed by SDS-PAGE (4–20% gradient) and stained with Coomassie blue.

*continued on page 12*

*continued from page 11*

E2F–DP combinations were constructed using the LIC Duet™ method (Figure 5, page 11). When expressed individually, E2F-1, E2F-2, E2F-3, DP-1, and DP-2 produced low yields upon purification under native conditions (Figure 5, page 11, lanes 14, 16, 18, 20, and 22). By contrast, when E2Fs were coexpressed with DPs, soluble polypeptides formed and could be purified by Ni-NTA His•Bind® chromatography. Because the His•Tag® sequence was present only on the amino-terminus of the first protein (E2F), copurification of the E2F–DP complexes by Ni-NTA His•Bind Resin was possible due to the formation of soluble complexes as the result of heterodimerization between E2F and DP. In this analysis it appeared that all combinations of the various E2F and DP domains were expressed well when produced together in the same cell.

### Summary

LIC Duet™ Adaptor cloning is a powerful method specifically designed for simultaneous, directional cloning of two open reading frames into Novagen Ek/LIC bacterial expression vectors. The LIC Duet strategy provides a flexible and simple method to generate dual-expression constructs using various fusion tag and antibiotic resistance options. Coexpression of two to six target proteins from the same cell can be readily accomplished using this enhanced LIC Duet-based cloning method.

## ACKNOWLEDGMENTS

The authors thank Dr. Jung-Hyun Min, Dr. Bryan C. Czyzewski, and Dr. Anne-Laure Gall, from the laboratory of Dr. Nikola P. Pavletich at the Sloan-Kettering Cancer Center in New York, and Dr. Irwin Davidson, from CNRS/LGME-INSERM in Strasbourg, France, for providing DNA and plasmid templates for these experiments.

## REFERENCES

1. Aslanidis, C. and de Jong, P. J. (1990) *Nucleic Acids Res.* **18**, 6069–6074.
2. Haun, R. S., Serventi, I. M., and Moss, J. (1992) *Biotechniques* **13**, 515–518.
3. Novy, R. E., Yaeger, K. W., and Kolb, K. M. (1996) *inNovations* **5**, 1–3.
4. Pellegrini, L., Yu, D. S., Lo, T., Anand, S., Lee, M., Blundell, T. L., and Venkitaraman, A. R. (2002) *Nature* **420**, 287–293.
5. Kowalczykowski, S. C. (2002) *Nature Struct. Biol.* **9**, 897–899.
6. Werten, S., Mitschler, A., Romier, C., Gangloff, Y., Thuault, S., Davidson, I., and Moras, D. (2002) *J. Biol. Chem.* **277**, 45502–45509.
7. Thuault, S., Gangloff, Y., Kirchner, J., Sanders, S., Werten, S., Romier, C., Weil, P. A., and Davidson, I. (2002) *J. Biol. Chem.* **277**, 45510–45517.
8. Rogers, K. T., Higgins, P. D., Milla, M. M., Phillips, R. S., and Horowitz, J. M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 7594–7599.
9. Cartwright, P., Muller, H., Wagener. C., Holm, K., and Helin, K. (1998) *Oncogene* **17**, 611–623.
10. Gall, A. L., personal communication.

| Product | Size | Cat. No. |
|---|---|---|
| LIC Duet™ Minimal Adaptor* | 20 rxn | 71362-3 |
| LIC Duet T7•Tag® Ek Adaptor* | 20 rxn | 71321-3 |
| LIC Duet Trx•Tag™ Ek Adaptor* | 20 rxn | 71322-3 |
| LIC Duet GST•Tag™ Ek Adaptor* | 20 rxn | 71323-3 |
| LIC Duet Nus•Tag™ Ek Adaptor* | 20 rxn | 71324-3 |
| * LIC Duet Adaptors include LIC Adaptor, LIC Duet Control Insert 1, and LIC Duet Control Insert 2 | | |
| pCDF-2 Ek/LIC Vector Kit† | 20 rxn | 71337-3 |
| pRSF-2 Ek/LIC Vector Kit† | 20 rxn | 71364-3 |
| pET-46 Ek/LIC Vector Kit† | 20 rxn | 71335-3 |
| pET-30 Ek/LIC Vector Kit† | 20 rxn | 69077-3 |
| pET-32 Ek/LIC Vector Kit† | 20 rxn | 69076-3 |
| pET-41 Ek/LIC Vector Kit† | 20 rxn | 71071-3 |
| pET-43.1 Ek/LIC Vector Kit† | 20 rxn | 71072-3 |
| pET-44 Ek/LIC Vector Kit† | 20 rxn | 71144-3 |
| † Ek/LIC vector kits include Ek/LIC Vector; Control Insert; T4 DNA Polymerase, LIC-qualified; 10X T4 DNA Polymerase Buffer; 100 mM DTT; 25 mM EDTA; 25 mM dATP; Nuclease-free Water; BL21(DE3) Competent Cells, BL21(DE3)pLysS Competent Cells, NovaBlue GigaSingles™ Competent Cells; SOC Medium; and Test Plasmid. | | |
| BugBuster® Protein Extraction Reagent | 100 ml<br>500 ml | 70584-3<br>70584-4 |
| rLysozyme™ Solution | 300 KU<br>1200 KU<br>6000 KU | 71110-3<br>71110-4<br>71110-5 |
| Benzonase® Nuclease, Purity > 99% | 10 KU | 70664-3 |
| Note: 1 KU = 1000 units | | |
| Ni-NTA His•Bind® Resin | 10 ml<br>25 ml<br>100 ml | 70666-3<br>70666-4<br>70666-5 |
| Perfect Protein™ Markers, 15–150 kDa | 100 lanes | 69149-3 |

| Product | Cat. No. |
|---|---|
| Ni-NTA Buffer Kit (includes 4X Ni-NTA Bind Buffer, 4X Ni-NTA Wash Buffer, and 4X Ni-NTA Elute Buffer) | 70899-3 |

| Product | Size | Cat. No. |
|---|---|---|
| Rosetta™(DE3) Competent Cells | 0.4 ml<br>1 ml | 70954-3<br>70954-4 |
| guaranteed efficiency: > 2 × $10^6$ cfu/µg | | |
| Rosetta(DE3) Singles™ Competent Cells | 11 rxn<br>22 rxn | 71099-3<br>71099-4 |
| guaranteed efficiency: > 2 × $10^6$ cfu/µg | | |

# Identification of DNA binding proteins using the NoShift™ Transcription Factor Assay Kit

Faye Bruggink and Scott Hayes – Novagen

*A microassay plate–based alternative to electrophoretic mobility shift assays identifies proteins that bind to specific DNA sequences. A biotinylated oligonucleotide is mixed with a protein sample and the protein–DNA complex is captured on streptavidin plates. The bound transcription factor is detected with a specific antibody followed by a second antibody-HRP conjugate and chromogenic substrate. This sensitive, nonradioactive assay can be completed within five hours.*

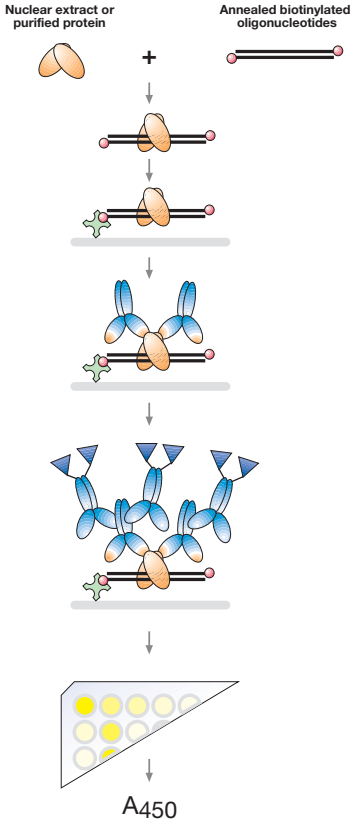
The gel shift, or electrophoretic mobility shift assay (EMSA), is a method for analyzing interactions between proteins and DNA. This technique, first published in 1981 (1, 2), is based on a shift in the electrophoretic mobility of protein–DNA complexes compared with that of DNA alone under nondenaturing conditions. Further verification of the interaction can be performed with a "supershift" assay in which an antibody against the protein further slows the migration of the complex through the gel. The traditional gel shift assay is tedious and time consuming, requires radiolabeled probe, and often requires extensive optimization.

The NoShift™ assay system is a microassay plate–based approach for analysis of DNA-binding proteins that alleviates many of the problems associated with gel shifts. The 96-well plate format is versatile and enables simultaneous analysis of multiple binding factors in less than five hours without the use of radioisotopes. Sequence-specific binding can be assessed with extracts from different growth or embryo stages, various drug treatments, and different tissues and cell lines. In this article, the performance of the NoShift assay is compared with that of conventional EMSA. We used several different transcription factors to test the specificity and flexibility of the assay.

### Comparison of NoShift and gel shift assays

Figure 1 outlines the NoShift assay procedure. To test the performance of the NoShift assay versus traditional gel shift, we induced AP-1 transcription factor activity in CHO-K1 cells by treatment with Phorbol-12-myristate-13-acetate (PMA). Nuclear extracts from cells induced with and without PMA were compared using a traditional gel shift and the NoShift assay.

As shown in Figure 2, panel A (page 14), the gel analysis revealed complex formation as evidenced by a mobility shift of the radioactively labeled DNA, but it is difficult to discern a difference in band intensity between the induced and uninduced extracts. In contrast, the NoShift assay provided a relative measurement of the PMA-induced activation, as shown in Figure 2, panel B (page 14). In the absence of PMA, the signal-to-noise ratio for CHO-K1 nuclear extracts was 2.9:1. When cells were induced with PMA, the ratio increased to 5:1, nearly two-fold higher than that seen in the uninduced extracts. This result clearly indicates PMA-induced activation of AP-1 and demonstrates the utility of the NoShift assay to quantify and assess drug




1. Anneal two oligonucleotides that define a binding site (at least one oligonucleotide should be end labeled with biotin).
2. Incubate dsDNA with the extract or protein of interest for 30 min on ice.
3. Transfer the samples to a streptavidin plate. Incubate for 1 h at 37°C.
4. Wash.
5. Add antibody specific for the DNA-binding protein of interest to the samples. Incubate for 1 h at 37°C.
6. Wash.
7. Add a secondary antibody conjugated with horseradish peroxidase (HRP). Incubate the samples for 30 min at 37°C.
8. Wash.
9. Add TMB Substrate and incubate for 10–30 min to develop colorimetric signal before quenching the reaction with 1 N HCl.
10. Read absorbance at 450 nm and record results.

**Figure 1. The NoShift procedure**

*continued on page 14*

*continued from page 13*

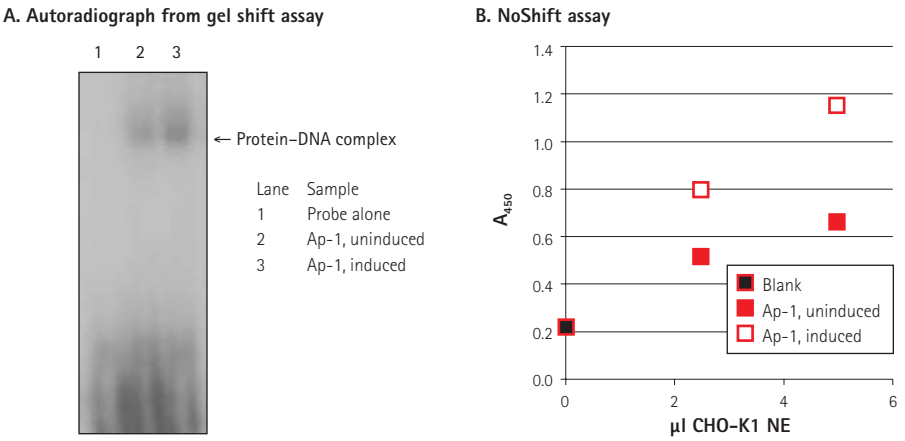



**Figure 2. Comparison of gel shift versus the NoShift assay**

To induce AP-1 expression, CHO-K1 cells were treated with and without 100 ng/ml PMA (Calbiochem) for 1 h prior to nuclear extract preparation. Nuclear extract preparations and gel shifts were performed as described (3). Total protein determination was by Non-Interfering Protein Assay™ Kit (Calbiochem). Five microliters of each of the same nuclear extracts (48 µg total protein) were used in the gel shift (panel A) and the NoShift (panel B) assays. An Ap-1 binding site (GCTAGCGCTTGATGACTCAGCCGGAATGACG) (4) was used as the target DNA for the gel shift and the probe was end labeled with $^{32}$P. Anti-c-Fos (Ab-2), Human (Rabbit) (Oncogene Research Products) was used for protein–DNA complex recognition (1:1000 dilution) followed by Anti-Rabbit IgG, H & L Chain Specific (Goat) Peroxidase Conjugate (Calbiochem) (1:1000 dilution). Well volume was maintained at 100 µl. Duplicate samples were incubated for 20 min in the presence of TMB Substrate and quenched with 1 N HCl (100 µl). Absorbance was read at 450 nm.

treatment effects on transcription factor activation.

To measure the protein concentration dependence of the NoShift™ assay, a binding study was performed with increasing amounts of nuclear extract. Figure 3 shows linearity between 0.1 and 10 µl (0.39 and 39 µg) of nuclear extract from CHO-K1 cells. Although we commonly used 5 µl of nuclear extract for each analysis, higher amounts of extract also produced clear signal. Increased amounts of extract may be required if the level of transcription factor is limiting.

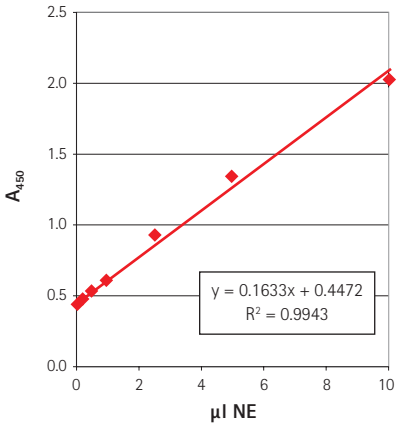



**Figure 3. Linear signal with increasing amounts of nuclear extract**

The NoShift assay was performed using a double-stranded biotinylated Sp1 recognition sequence (ATTCGATCGGGGCGGGGCGAGC) (5) and increasing amounts of CHO-K1 nuclear extract (3.9 µg/ml). Total protein determination was by Non-Interfering Protein Assay™ Kit (Calbiochem). A 1:500 dilution of Anti-Sp1, Human (Rabbit) (Oncogene Research Products) was used to detect the specific protein–DNA complex followed by incubation with Anti-Rabbit IgG, H & L Chain Specific (Goat) Peroxidase Conjugate (Calbiochem). Colorimetric reaction with TMB Substrate was performed as described in Figure 2. Absorbance was read at 450 nm for duplicate samples.

### Preparation of nuclear extracts

Just like a traditional gel shift, the NoShift assay can be performed with pure proteins or crude extracts. The protein of interest may be expressed and purified in bacteria, insect cells, or via translation *in vitro*, assuming that the activity of the recombinant protein is comparable to that of the native form. The most commonly used samples are crude eukaryotic extracts. Because activated DNA-binding proteins are located in the nucleus, it is preferable to begin with nuclear extracts. Many methods for preparation of nuclear extracts are derived from the Dignam method (6). The nuclear extracts used in this article were prepared with the NucBuster™ Protein Extraction Kit. The NucBuster procedure yields a nuclear extract suitable for DNA binding studies in less than 30 minutes (7). Two basic NoShift assay kits are available, one of which includes a complete NucBuster Protein Extraction Kit.

*Just like a traditional gel shift, the NoShift assay can be performed with pure proteins or crude extracts.*

### Sequence specificity with the NoShift assay

In classical gel shift analysis, DNA-binding specificity is tested by adding unlabeled oligonucleotide in molar excess relative to $^{32}$P-labeled probe. If the nonradioactive competitor binds to the transcription factor, less protein is bound to the $^{32}$P-labeled DNA and the intensity of the shifted band decreases. To see appreciable differences in an EMSA, a 10-fold to 100-fold molar excess of unlabeled to labeled probe is typically required. With the NoShift assay, the presence of a specific unlabeled competitor during protein–DNA complex formation means that the biotinylated probe binds less of the interacting protein and less antibody is bound to the plate.

We tested two transcription factors for specific binding in the NoShift assay. Figure 4, panel A (page 15), shows results of a competitive analysis using the transcription factor Sp1. A signal-to-noise ratio of 4.3:1 was achieved in the absence of competitor. Nonspecific, nonbiotinylated competitor did not effectively compete for Sp1 binding, resulting in a signal-to-noise ratio similar to that generated in the absence of competitor. When an equimolar amount of nonbiotinylated-specific competitor was added, the signal-to-noise ratio decreased to 2.3:1.

Figure 4, panel B (page 15), shows similar results for the transcription factor cyclic AMP–responsive element binding (CREB) protein. In this case, the signal-to-noise ratio in the absence of competitor was 5.3:1. The signal decreased as nonbiotinylated-specific competitor was added during complex formation. Using an equimolar ratio decreased the signal to background levels. In contrast, the addition of a nonbiotinylated, nonspecific probe had minimal effect; even when present with the biotinylated probe in equimolar amounts, a 4.5:1 signal-to-noise ratio was maintained.

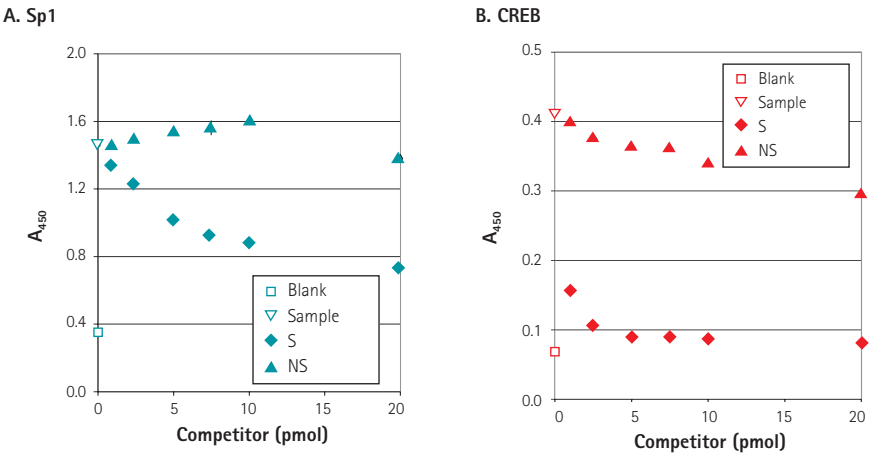



**Figure 4. NoShift–generated competition data for CREB and Sp1 binding activity**

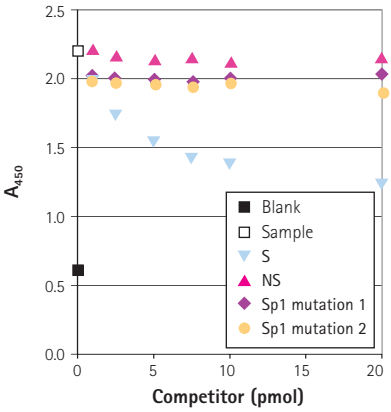
NoShift assays were performed as described in the text using 10 pmol double-stranded biotinylated consensus recognition sequence for Sp1 or CREB (8). In each case, nonbiotinylated-specific sequence (S) or nonspecific sequence (NS) was added in increasing amounts as competitor. Detection was performed with an Sp1-specific antibody (1:500 dilution) or a CREB-specific antibody (1:1000 dilution). Reactions were performed in triplicate.

*These competition studies demonstrate that the NoShift assay can effectively differentiate binding to specific transcription factor sequences from nonspecific sequences using a lower molar ratio of competitor than in traditional gel shift assays. Results also demonstrated the ability to differentiate the binding to a transcription factor recognition sequence from the binding to a similar sequence that differs by a single nucleotide.*

The two transcription factors appeared to exhibit different binding characteristics. In the assay with the CREB transcription factor, binding was extremely sensitive to a low concentration of unlabeled competitor. In contrast, the Sp1 transcription factor exhibited a more gradual signal decrease in response to increasing amounts of a specific competitor. These results, not seen in standard gel shift assays, may reflect subtle differences in the interactions of these two transcription factors with their binding sites.

The effect of single-base mutations on binding specificity was tested for Sp1 (Figure 5). The consensus sequence recognized by Sp1 is a six-nucleotide sequence, GGGCGG (5). We designed two sets of nonbiotinylated oligonucleotide competitors, each with a single nucleotide modification in the core recognition sequence: G to A in GGACGG and G to C in GGGCCG. In contrast to the results seen with specific competitor in Figure 4, panel A, none of the point mutations could effectively compete for the Sp1 transcription factor binding. The reverse experiment yielded similar information about the specificity of the DNA binding site; double-stranded biotinylated oligonucleotides of each point mutation did not produce a signal-to-noise ratio as high as 2:1 (data not shown).

These competition studies demonstrate that the NoShift™ assay can effectively differentiate binding to specific transcription factor sequences from nonspecific sequences using a lower molar ratio of competitor than traditional gel shift assays. Results also demonstrated the ability to differentiate binding to a transcription factor recognition sequence from binding to a similar sequence that differs by a single nucleotide.




**Sp1 sequences**

| | |
|---|---|
| Consensus: | ATT CGA TCG GGG CGG GGC GAG C |
| Mutation 1: | ATT CGA TCG GGA CGG GGC GAG C |
| Mutation 2: | ATT CGA TCG GGG CCG GGC GAG C |

**Figure 5. Effect of single–base mutation on Sp1 binding activity**

Assays were performed as described in Figure 4, except that single-point mutations were introduced into the Sp1 recognition sequence, as indicated, and used as nonbiotinylated competitors.

*continued on page 16*

*continued from page 15*

**Summary**

The NoShift™ assay is a microassay plate–based test to identify proteins that bind to a specific DNA sequence. One advantage of this plate-based assay over traditional gel shift assays is the remarkable specificity of the test. The shift in mobility of a DNA probe in an EMSA indicates that some protein in a crude extract binds, but the identity of the protein is unknown unless a supershift is performed with a protein-specific antibody. The NoShift assay has dual specificity: that of the protein for the DNA probe and of the antibody for the interacting protein. The convenient 96-well format of the NoShift assay permits screening for multiple DNA-binding proteins in the same plate.

The basic NoShift kit consists of a streptavidin-coated microassay plate with sealers; buffers for binding, washing, and dilution; and TMB Substrate. Also available is the NoShift Transcription Factor Assay Kit Plus NucBuster™, which includes the microassay plate and sealers, buffers, and substrate as well as a complete NucBuster Protein Extraction Kit to prepare nuclear extracts in less than 30 minutes. Three transcription factor-specific reagent kits are also offered for use with the NoShift assay kits. The c-Fos, Sp1, and ER-α reagent kits each contain biotinylated oligonucleotides that include a consensus recognition sequence, specific and non-specific competitors, a transcription factor–specific antibody, secondary antibody conjugated to HRP, and positive control nuclear extract. The NoShift assay kits and convenient reagent kits offer a fast, sensitive, nonradioactive alternative to gel shift assays.

**REFERENCES**

1. Fried, M. and Crothers, D. M. (1981) *Nucl. Acids Res.* **9**, 6505–6525.
2. Garner, M. M. and Revzin, A. (1981) *Nucl. Acids Res.* **9**, 3047–3060.
3. Novagen (2003) *Technical Bulletin 338*, EMD Biosciences, Inc., Madison, Wisconsin.
4. Lee, W., Mitchell, P., and Tjian, R. (1987) *Cell* **49**, 741–752.
5. Briggs, M. R., Kadonaga, J. T., Bell, S. P., and Tjian, R. (1986) *Science* **234**, 47–52.
6. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucl. Acids Res.* **11**, 1475–1489.
7. Bruggink, F. and Hayes, S. (2002) *inNovations* **15**, 9–11.
8. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6682–6686.

| Product | Size | Cat. No. |
|---|---|---|
| NoShift™ Transcription Factor Assay Kit<br>[includes 4X NoShift Bind Buffer, Salmon Sperm DNA, Poly(dI-dC)•Poly(dI-dC) Solution, 10X NoShift Wash Buffer, NoShift Antibody Dilution Buffer, Streptavidin Plate, TMB Substrate, and Aluminum Plate Sealers] | 100 rxn | 71377-3 |
| NoShift Transcription Factor Assay Kit Plus NucBuster™<br>[includes NucBuster Protein Extraction Kit, 4X NoShift Bind Buffer, Salmon Sperm DNA, Poly(dI-dC)•Poly(dI-dC) Solution, 10X NoShift Wash Buffer, NoShift Antibody Dilution Buffer, Streptavidin Plate, TMB Substrate, and Aluminum Plate Sealers] | 100 rxn | 71378-3 |
| NoShift Sp1 Reagents<br>[includes CHO Positive Control Nuclear Extract 1; Anti-Sp1, Human (Rabbit); Anti-Rabbit IgG, H & L Chain Specific (Goat) Peroxidase Conjugate; Sp1 WT DNA; Sp1 Competitor DNA; Sp1 Mutant DNA] | 100 rxn | 71379-3 |
| NoShift ER-α Reagents<br>[includes MCF-7 Positive Control Nuclear Extract 1; Anti-Estrogen Receptor (Ab-1), Human (Mouse); Goat Anti-Mouse IgG HRP Conjugate; ER-α WT DNA; ER-α Competitor DNA; ER-α Mutant DNA] | 100 rxn | 71380-3 |
| NoShift c-Fos Reagents<br>[includes CHO Positive Control Nuclear Extract 1; Anti-c-Fos (Ab-2), Human (Rabbit); Anti-Rabbit IgG, H & L Chain Specific (Goat) Peroxidase Conjugate; c-Fos WT DNA; c-Fos Competitor DNA; c-Fos Mutant DNA] | 100 rxn | 71381-3 |
| Phorbol-12-myristate-13-acetate (PMA) | 1 mg<br>5 mg<br>10 mg<br>25 mg | 524400<br>524400<br>524400<br>524400 |
| Non-Interfering Protein Assay™ Kit | 1 kit | 488250 |

Available separately:

| Product | Size | Cat. No. |
|---|---|---|
| Anti-c-Fos (Ab-2), Human (Rabbit) | 100 µg | PC05L |
| Anti-Sp1, Human (Rabbit) | 100 µl | PC701 |
| Anti-Estrogen Receptor (Ab-1), Human (Mouse) | 100 µg | GR17 |
| TMB, Soluble | 100 ml | 613544 |
| Anti-Rabbit IgG, H & L Chain Specific (Goat) Peroxidase Conjugate | 2 ml | 401315 |
| Goat Anti-Mouse IgG HRP Conjugate (H + L) | 40 µl | 71045-3 |
| NucBuster™ Protein Extraction Kit<br>(includes NucBuster Extraction Reagents 1 and 2, 100 mM DTT, and Protease Inhibitor Cocktail Set I) | 100 rxn | 71183-3 |

= Calbiochem® brand product
= Oncogene Research Products™ brand product

# A novel buffer system for direct PCR from whole blood

Keith Yaeger and Alla Zilberman — Novagen

*BloodDirect™ buffers neutralize the PCR-inhibitory effects of components present in both fresh and archived blood samples. The ability to add unprocessed blood directly to the PCR mixture makes the BloodDirect PCR Buffer Kits ideal for screening multiple samples.*

Blood and other biological fluids contain a variety of substances, including polysaccharides, proteins, and lipids, that inhibit the activity of *Taq* DNA polymerase. Anticoagulants, such as heparin, are commonly added to blood samples and can also interfere with PCR amplification. Steps to remove these substances from the DNA template are usually performed prior to PCR analysis.

The most basic method for DNA isolation from biological materials requires multiple steps, including treatment with protease/detergent, extraction with phenol/chloroform, precipitation with ethanol, and resuspension in a buffer. The development of improved methods and kits for blood DNA purification have simplified the procedure; however, all still require up to 30 minutes for DNA sample preparation before PCR. The requirement of a DNA isolation procedure increases the likelihood of sample cross-contamination, especially when dealing with multiple tissue or blood samples as in the case of transgenic mouse genotyping.

To facilitate direct PCR analysis of blood samples, we used a novel buffer system that effectively neutralizes inhibitors present in blood and allows direct PCR amplification from as little as 0.5 µl blood treated with any commonly used anticoagulant.

### BloodDirect™ human and mouse blood kits

BloodDirect PCR Buffer Kits include two components: 5X BloodDirect 1 and either 5X BloodDirect Buffer A (human blood kit) or 5X BloodDirect Buffer B (mouse blood kit). These components are added to the PCR mixture in place of 10X PCR buffer. Following assembly of the PCR mixture, anticoagulant-treated human or mouse blood is added (1 µl per 50-µl mixture or 0.5 µl per 20-µl mixture) and temperature cycling is begun. A typical reaction setup and cycling conditions are shown in Table 1. BloodDirect buffers are compatible with most commercially available *Taq* DNA polymerases and antibody-mediated hot start protocols. Note that these buffers are not compatible with chemically-modified hot start DNA polymerases such as AmpliTaq® Gold DNA Polymerase (Applied Biosystems), HotStarTaq™ DNA Polymerase (Qiagen), and SureStart DNA Polymerase (Stratagene). For hot start PCR with BloodDirect kits, Novagen recommends use of an antibody-modified hot start DNA polymerase, such as Nova*Taq*™ DNA Polymerase plus *Taq* Antibody.

*...a novel buffer system that effectively neutralizes inhibitors present in blood and allows direct PCR amplification from as little as 0.5 µl blood treated with any commonly used anticoagulant.*

### Direct PCR from human blood treated with three types of anticoagulants

To evaluate the effect of anticoagulants on BloodDirect PCR, reactions were performed using four different volumes of human blood treated with sodium citrate, dipotassium EDTA, or sodium heparinate to amplify a 408-bp fragment of the β-globin gene. Fresh or frozen blood samples were added directly into 50-µl BloodDirect PCR mixtures. As shown in Figure 1 (page 18), clear amplification was achieved for each case except for the 5-µl frozen sample treated with heparin (Panel B, lane H1). The BloodDirect protocol recommends the addition of 1 µl blood per 50-µl PCR mixture. Higher amounts may affect the ability of the buffer to sequester the inhibitor.

**Table 1. Typical PCR setup and cycling conditions**

| Treatment | Final Volume 50 µl | 20 µl |
|---|---|---|
| 5X BloodDirect Buffer 1 | 10 µl | 4 µl |
| 5X BloodDirect Buffer A (*or* B) | 10 µl | 4 µl |
| dNTP mixture (2.5 mM each) | 4 µl | 1 µl |
| 5'-primer | 0.5 mM | 0.125 mM |
| 3'-primer | 0.5 mM | 0.125 mM |
| *Taq* DNA polymerase (5 U/µl) | 0.25 µl | 0.1 µl |
| PCR-grade water | to 50 µl | to 20 µl |
| Anticoagulant-treated blood | 1 µl | 0.5 µl |
| **Cycling Conditions** | | |
| 94°C, 4.5 min* | | |
| 94°C, 30 s | | |
| Annealing temperature, 1 min | | 40 cycles |
| 72°C, 1 min | | |
| 72°C, 7 min | | |

* Preheating at 80°C for 15 min is useful when fresh blood (collected on the same day as PCR amplification) is used.

### Direct PCR from dried human blood samples

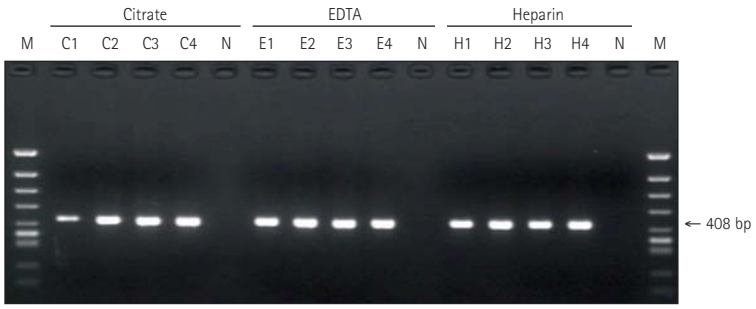
Dried blood samples archived on specimen collection cards (Guthrie Cards) are used widely in genetic screening for hereditary and metabolic disorders. Usually a drop of blood from an infant's heel is applied to the center of a filter (1–1.5 cm diameter) on the specimen collection card. The card is then stored for future testing. Amplification of DNA from dried blood samples presents significant challenges due to the presence of PCR inhibitors and the limited amounts of sample available for analysis.

We tested BloodDirect buffers in direct PCR from dried blood samples stored on Whatman Grade 3 filter paper. Six target gene sequences from four different 1-µl samples were amplified: EDTA-treated human whole blood, human blood dried in the PCR tube, human blood absorbed on Grade 3 filter paper, and purified DNA equivalent to 1 µl blood. As

*continued on page 18*

*continued from page 17*

**A. Fresh blood samples**




**B. Frozen blood samples (–20°C for four years)**

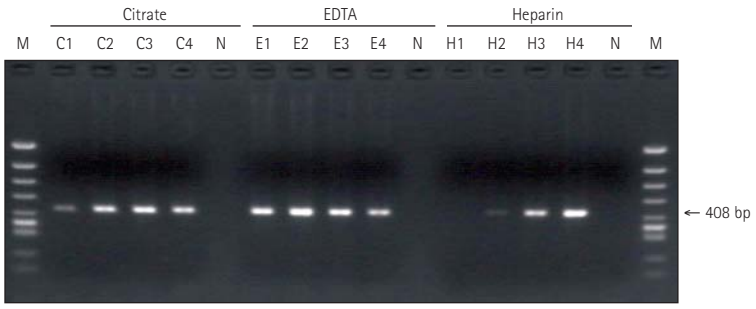
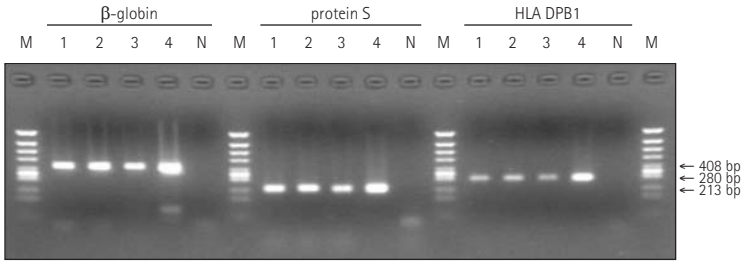



**Figure 1. Direct PCR from whole human blood**

Direct PCR was used with four different volumes of human blood treated with three different anticoagulants to amplify a 408–bp fragment of the β-globin gene. Sodium citrate (C), dipotassium EDTA (E), and sodium heparinate (H) anticoagulants were used. Fresh blood samples (panel A) or blood samples stored at –20°C for four years (panel B) were added directly to 50 µl BloodDirect mixtures. Reactions were assembled on ice using Nova*Taq* DNA Polymerase and PCR amplification was performed according to the standard cycling conditions with pretreatment (Table 1, page 17). Blood sample volumes were 5 µl (lane 1), 2 µl (lane 2), 1.3 µl (lane 3), and 0.6 µl (lane 4). One tenth (5 µl) of total reaction volume was analyzed by agarose gel electrophoresis (2.5% TAE) and stained with ethidium bromide. Lane N: negative control; Lane M: markers.

**A. Samples with β-globin, protein S, and HLA DPB1 targets**




**B. Samples with mitochondrial DNA, p53 exon 6, and p53 exon 11 targets**

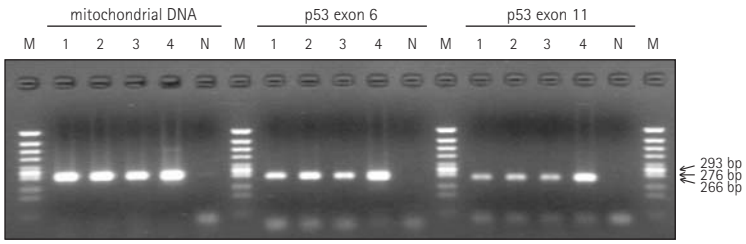



**Figure 2. Comparison of direct PCR using fresh, dried, and purified DNA samples from human blood**

PCR amplifications (50 µl) were performed with BloodDirect buffers and Nova*Taq* DNA Polymerase using the cycling conditions described in Table 1, page 17, with annealing at 55°C. Target sequences included β-globin, protein S, and HLA DPB1, shown in panel A, and mitochondrial DNA, p53 exon 6, and p53 exon 11, shown in panel B. One tenth (5 µl) of the total reaction volume was analyzed by agarose gel electrophoresis (2.5% TAE) and stained with ethidium bromide. Lane 1: 1 µl EDTA-treated human blood; Lane 2: blood dried in PCR tube; Lane 3: blood absorbed on filter paper (4-mm diameter); Lane 4: purified DNA equivalent to 1 µl blood; Lane N: negative control; Lane M: markers.

shown in Figure 2, the reactions produced PCR products of the appropriate size for all six of the target sequences from all four samples.

### BloodDirect™ buffers for direct PCR of blood from transgenic mice

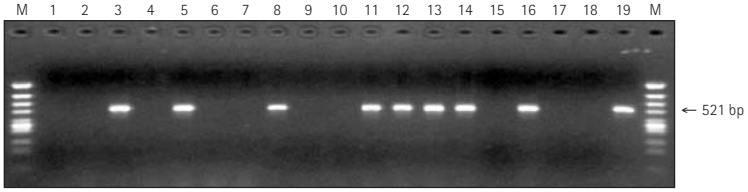
Screening for mice that carry a transgene of interest is most commonly performed by PCR analysis of DNA from blood or tail tissue samples. Genomic DNA typically is prepared by treatment of the tail tissue with Proteinase K followed by phenol/chloroform extraction or purification on silica filters. We compared the performance of the BloodDirect kit in PCR analysis using crude mouse blood and purified DNAs from the tails of 19 mice. As shown in Figure 3 (page 19), both the purified DNA and crude blood produced similar results that effectively identified the transgene-positive mice. The mouse BloodDirect PCR Buffer Kit provides a faster method to differentiate transgene-carrying mice by eliminating tedious genomic DNA preparation.

*The mouse BloodDirect PCR Buffer Kit provides a faster method to differentiate transgene-carrying mice by eliminating tedious genomic DNA preparation.*

### Amplification of longer targets with BloodDirect kits

As described above, the BloodDirect method is compatible with standard *Taq* DNA polymerases and antibody-mediated hot start PCR. We recommend standard *Taq* DNA polymerases for reliable amplification of fragments up to 1,000 bp. For the most consistent results in PCR amplification of targets longer than 1,000 bp, we recommend a hot start using *Taq* DNA polymerase/*Taq* antibody complex. To test BloodDirect for amplification of longer targets, primers were designed to amplify 437-, 742-, 1127-, 1497-, 1745-, and 2056-bp fragments of the human β-globin gene. Reactions were performed with sodium citrate-treated blood and Nova*Taq*™ DNA Polymerase/*Taq* Antibody

**A. Heparinized blood samples**




**B. Purified DNA samples**

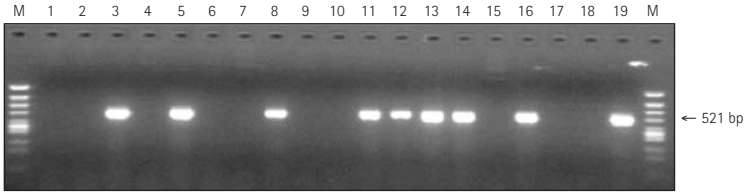



**Figure 3. Screening for transgenic mice by BloodDirect PCR analysis**

PCR analysis to detect *Lck* promoter-human D4-GD1 transgene was performed using BloodDirect buffers and Nova*Taq*™ DNA Polymerase. For heparinized blood samples, the cycling conditions described in Table 1 were used with annealing at 55°C; for purified DNA samples, the conditions were modified (30 cycles and a 30-second annealing at 55°C). Panel A shows the results from 1-µl samples of heparinized blood; Panel B shows the results from 200–800 ng of each DNA purified from the tails of the same 19 mice (C57BL/6J). A total reaction volume of 5 µl was analyzed by agarose gel electrophoresis (2.5% TAE) and stained with ethidium bromide. Lane M: markers.

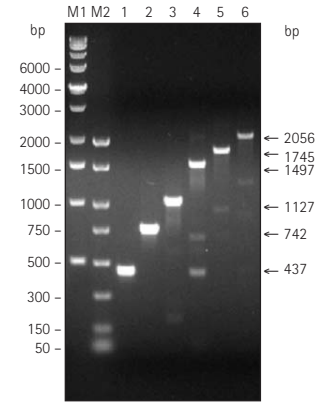



**Figure 4. BloodDirect PCR amplification of different size targets**

PCR analysis of human blood (0.5 µl per 20-µl reaction) was performed using the conditions described in Table 1 with annealing at 55°C. PCR products for six different amplicons of the β-globin gene were analyzed by agarose gel electrophoresis (1.5% TAE) and stained with ethidium bromide. Lane M1: Perfect DNA™ Markers, 0.5–12 kbp; lane M2: PCR Markers, 50–2000 bp; lanes 1–6: amplicons of the human β-globin gene ranging in size from 437 to 2056 bp.

complex. As shown in Figure 4, specific amplification of all six targets was observed.

### Summary

BloodDirect™ PCR buffer kits are ideal for direct amplification of target DNA from human and mouse blood. As little as 0.5 µl of anticoagulant-treated blood is added directly to a PCR mixture prepared with BloodDirect buffers. These PCR buffers are efficient for amplification from freshly collected blood, blood samples stored at –20°C for 4 years, or dried blood samples stored on filter paper. The BloodDirect buffers are compatible with any *Taq* DNA polymerase for standard PCR or with antibody-bound *Taq* DNA polymerase for hot start PCR. Due to the simplicity of the BloodDirect protocol, the risks of cross-contamination and sample mishandling are dramatically reduced. The system is ideal for genetic mass screening and for routine analysis of transgenic mice.

### Additional Information

Additional technical information for the BloodDirect PCR buffer kits is available in Novagen User Protocol TB404, available at www.novagen.com.

| Product | Size | Cat. No. |
|---|---|---|
| BloodDirect™ PCR Buffer Kit, Human (includes 5X BloodDirect Buffer 1 and 5X BloodDirect Buffer A) | 50 rxn<br>250 rxn | 71342-3<br>71342-4 |
| BloodDirect PCR Buffer Kit, Mouse (includes 5X BloodDirect Buffer 1 and 5X BloodDirect Buffer B) | 50 rxn<br>250 rxn | 71343-3<br>71343-4 |

Available separately:

| Product | Size | Cat. No. |
|---|---|---|
| Perfect DNA™ Markers, 0.5-12 kbp | 100 lanes | 69002-3 |
| PCR Markers, 50-2000 bp | 50 lanes | 69278-3 |
| Nova*Taq*™ DNA Polymerase | 100 U<br>500 U<br>2500 U | 71003-3<br>71003-4<br>71003-5 |

Components:

- 100, 500, *or* 5 × 500 U — Nova*Taq* DNA Polymerase (5 U/µl)
- 1, 2 *or* 7 × 1.5 ml — 10X Nova*Taq* Buffer with $MgCl_2$
- 1, 2 *or* 7 × 1.5 ml — 10X Nova*Taq* Buffer without $MgCl_2$
- 1, 2 *or* 7 × 1.5 ml — 25 mM $MgCl_2$

| | | |
|---|---|---|
| *Taq* Antibody* | 100 µl | 71088-3 |

Components:

- 100 µl — *Taq* Antibody (1 µg/µl)
- 1 ml — 10X PCR Buffer

* Manufactured by TOYOBO and distributed by Merck Biosciences. Not available from Merck Biosciences in Japan.

# Robotic solubility screening and purification of fusion proteins

Mark Mehler[1], Rick Luedke[2], and Anthony Grabski[1] – [1]Novagen and [2]Tecan-US

*Reagents for screening protein solubility and purifying recombinant protein are validated on a Tecan robotic platform.*

High throughput (HT) structural and functional proteomics requires the development of new reagents and automated methods to streamline the steps that allow for the conversion of gene sequences to purified proteins. Conventional methods of cloning, protein expression, purification, and crystallography are labor intensive and throughput is improved only by increasing the number of dedicated person-hours (1).

Parallel processing of hundreds of samples for HT proteomics research requires biological, chemical, and engineering solutions to eliminate tasks that are difficult to automate, such as sonication and centrifugation, and to minimize the number of processing steps. To meet this need, specialized lysis reagents that remove the need for mechanical disruption of cells were developed; these Novagen reagents include BugBuster®, YeastBuster™, and CytoBuster™ protein extraction reagents for *E. coli*, yeast, and insect or mammalian cells, respectively. In addition, Lysonase™ Bioprocessing Reagent, a combination of rLysozyme™ Solution and Benzonase® Nuclease, provides efficient lysis of both Gram-positive and Gram-negative bacteria and degradation of nucleic acids. The effectiveness of BugBuster reagent and the need to simplify the purification process for automation led to the development of PopCulture® Reagent (2). PopCulture Reagent is a concentrated mixture of specialized detergents that, when combined with Lysonase Bioprocessing Reagent, enables the extraction and purification of recombinant proteins from *E. coli* directly from culture media without cell harvest, mechanical disruption, or extract clarification. For insect cultures, direct cell disruption in the medium is possible with Insect PopCulture® Reagent.

Automated liquid handlers (or robotic sample processors) have become a fixture in many life science laboratories and enable researchers to perform a variety of cost-effective preparation, processing, and bioassay procedures at a throughput level previously unattainable. Because the potential for human-induced error is removed from the procedure, automated systems are inherently more robust and offer well-documented advantages of reproducibility, accuracy, and precision over manual pipetting methods. Robotic sample processors have recently been designed to facilitate the mapping of 3D structures in the proteome (3, 4). Although robotic liquid handing, colony picking, and sequencing technologies developed during the genome project are easily adapted to the cloning and expression steps upstream of structure determination (1), and many of the processes in crystallography have been automated (3), HT protein purification remains the critical bottleneck in the structure-determination pipeline.

*PopCulture Reagent is a concentrated mixture of specialized detergents that, when combined with Lysonase™ Bioprocessing Reagent, enables the extraction and purification of recombinant proteins from* E. coli *directly from culture media without cell harvest, mechanical disruption, or extract clarification. For insect cultures, direct cell disruption in the medium is possible with Insect PopCulture Reagent.*

One automated solution for performing HT purification is the Freedom™ robotic workstation (Tecan). A successor to Tecan's Genesis® platform, the Freedom is an open-architecture robotic workstation that may be configured by the end-user for a specific application or set of applications. For microplate-based solubility screening and recombinant protein purification, the Freedom is configured with two independent processing arms. One is an 8-channel, high precision liquid handling or pipetting tool. The other is a robotic manipulator, or gripping tool, for transporting labware and accessing integrated devices throughout the workspace. Modules utilized for the purification protocols include the TeShake, an on-board microplate shaker, the TeVacS vacuum manifold, and a high performance six-slot microplate incubator. Combining the Tecan Freedom technology with the Novagen chemistry enables complete, unattended automation of both magnetic and filtration-based purification procedures. An integrated SpectraFluor Plus (GENios Plus may be substituted) multidetection microplate reader automates the solubility screening protocol.

The general protocol for PopCulture extraction and RoboPop® purification uses a microplate culture of *E. coli* cells in liquid media under conditions optimal for target protein production. A premixed solution of PopCulture Reagent and Lysonase Bioprocessing Reagent representing 10% of the total culture volume is added to each well, mixed, and incubated for 10 minutes at room temperature. At this point, a sample may be removed from each well for Western blotting or solubility screening (as described below). Equilibrated His•Mag™, GST•Mag™, Ni-NTA His•Bind®, or GST•Bind™ affinity resin is added to the extract, mixed, and incubated for 5 minutes at room temperature. After incubation, the affinity resin is separated from the culture extract by filtration or magnetic isolation, followed by a wash of the affinity resin. The pure

target protein is eluted using the appropriate buffer. Results of automated purifications of eukaryotic fusion proteins (Table 1) using His•Mag™ and Ni-NTA His•Bind® resins are shown in Figure 1.

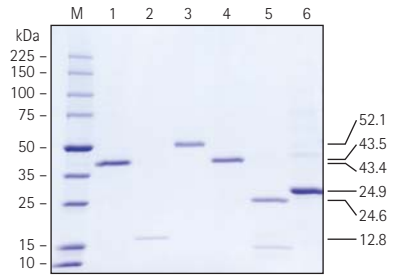
RoboPop™ solubility screening can be performed before or as part of the purification procedure. The solubility screening begins with the same *E. coli* lysates resulting from PopCulture® Reagent plus Lysonase™ treatment. Using a non-fouling filtration plate and 96-well collection plate seated within the TeVacS manifold, a 200-µl sample from each extract is transferred to the 96-well filter plate and a vacuum is applied. The soluble proteins in the flow-through are recovered in the collection plate and the plate is removed and replaced with a second collection plate. Next, a 200-µl aliquot of 4% SDS denaturing solution is added to each well of the filter plate and incubated for 10 minutes at ambient temperature. This step solubilizes the insoluble inclusion body fraction. Vacuum is applied, and the solubilized proteins are collected in the second plate. Analysis of target proteins present in the soluble and insoluble fractions can then be performed (Figure 2, page 22). His•Tag® fusion proteins can be detected by Western blotting or ELISA using the His•Tag Monoclonal Antibody or His•Tag Antibody Plates, respectively. As an alternative, S•Tag™ fusion proteins

*RoboPop Solubility Screening can be performed before or as part of the purification procedure...Analysis of target proteins present in the soluble and insoluble fractions can then be performed.*

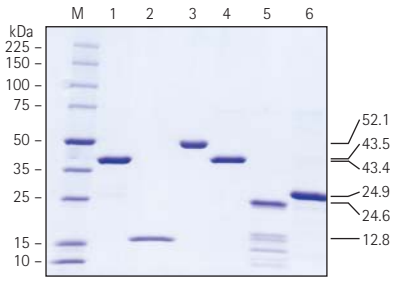
**Table 1. pET-30 Ek/LIC vector constructs used for analysis reported in Figure 1 and Figure 2**

| Fusion Protein | Expected Size |
|---|---|
| His•Tag/S•Tag Lipocortin I | 43.5 kDa |
| His•Tag/S•Tag Protein kinase inhibitor alpha | 12.8 kDa |
| His•Tag/S•Tag Enolase | 52.1 kDa |
| His•Tag/S•Tag Lipocortin II | 43.4 kDa |
| His•Tag/S•Tag Myosin regulatory light chain 2 | 24.6 kDa |
| His•Tag/S•Tag Casein alpha | 24.9 kDa |

**A. Magnetic-based affinity purification**




**B. Filtration-based affinity purification**




| Lane | Target protein | Magnetic (His•Mag Agarose Beads) Yield (µg/ml culture) | Magnetic (His•Mag Agarose Beads) % Purity | Filtration (Ni-NTA His•Bind Resin) Yield (µg/ml culture) | Filtration (Ni-NTA His•Bind Resin) % Purity |
|---|---|---|---|---|---|
| 1 | Lipocortin I | 61 | > 98 | 61 | > 98 |
| 2 | Protein kinase inhibitor alpha | 26 | > 98 | 20 | > 98 |
| 3 | Enolase | 40 | > 98 | 146 | > 98 |
| 4 | Lipocortin II | 47 | > 98 | 36 | > 98 |
| 5 | Myosin regulatory light chain 2 | 56 | 90 | 35 | 72 |
| 6 | Casein alpha | 122 | > 98 | 54 | > 98 |

**Figure 1. Automated purification of His•Tag fusion proteins using Novagen magnetic- and filtration-based affinity purification kits and Tecan workstation**

Cultures of *E. coli* strain BL21(DE3) containing the vector constructs described in Table 1 were grown at 30°C and target protein expression was induced by Overnight Express™ Autoinduction System 1 (5). Following incubation for approximately 16 h at 30°C, the cultures were processed robotically according to the RoboPop procedures described in the text. Panel A (approximately 1 µg protein load) and panel B (approximately 2 µg protein load) show purified protein samples analyzed by SDS-PAGE (10–20% gradient gel with Coomassie blue staining). The entire purification process after cell culture and induction was performed automatically by the Tecan Genesis® 200. Protein assays were performed by the Bradford method and purity was determined by densitometry of the scanned gel. Lane M: Perfect Protein™ Markers, 10–225 kDa.

*continued on page 22*

*continued from page 21*

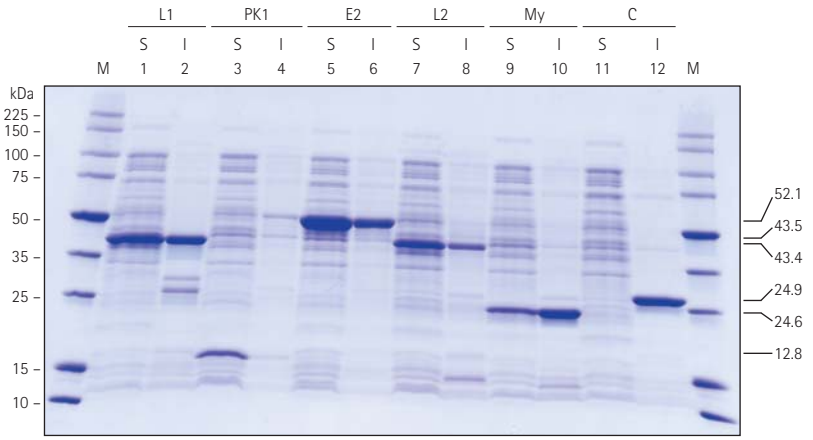



**Figure 2. SDS-PAGE analysis of soluble and insoluble fractions**

L1: lipocortin 1; PK1: protein kinase inhibitor alpha; E2: enolase 2; L2: lipocortin II; My: myosin regulatory light chain 2; C: casein alpha; S: soluble; I: insoluble; Lane M: Perfect Protein™ Markers, 10–225 kDa.

can be rapidly quantified directly using the FRETWorks™ S•Tag™ assay (Table 2; 6). The activity of native proteins can be assayed directly from the soluble fraction.

### Summary

Here we have validated methods for automated HT cell lysis, affinity tag-based protein expression screening and fusion protein purification employing the Tecan Genesis® Freedom™ 200 liquid handling workstation and the RoboPop™ kits. The combination of advanced engineering and novel biochemistry provide solutions to address common procedural bottlenecks encountered during the transition from manual processing of individual samples to HT parallel processing of hundreds of samples. Tedious cell harvest, lysis, extract fractionation, and sample analysis protocols have been automated by employing efficient liquid handling, potent chemical and biological activities, and highly selective ligand–affinity tag interactions. These comprehensive proteomics solutions allow for the rapid identification and selection of ideal host-vector combinations, expression conditions, and robotic purification parameters to facilitate HT production of proteins for structural or functional analysis.

### REFERENCES


1. Stevens, R. C. and Wilson, I. A. (2001) *Science* **293**, 519–520.
2. Grabski, A. C., Drott, D., Handley, M., Mehler, M., and Novy, R. (2001) *inNovations* **13**, 1–4.
3. Service, R. F. (2001) *Science* **292**, 187–188.
4. Brush, M. (2002) *Scientist* **16**, 38–41.
5. Grabski, A. C., Mehler, M., and Drott, D., (2003) *inNovations* **17**, 3–6.
6. Keleman, B. R., Klink, T. A., Behlke, M. A., Eubanks, S. R., Leland, P. A., and Raines, R. T. (1999) *Nucl. Acids Res.* **27**, 3696–3701.


Adapted from Grabski, A. C., Mehler, M., and Luedke, R. (2003) *Am. Biotechnol. Lab.*, in press.

**Table 2. FRETWorks S•Tag assays of soluble and insoluble fractions**

| pET-30 Ek/LIC Construct | % in Fraction: Soluble | Insoluble |
|---|---|---|
| Lipocortin I | 51 | 49 |
| PK1 alpha | 74 | 26 |
| Enolase 2 | 58 | 42 |
| Lipocortin II | 54 | 46 |
| Myosin regulatory light chain 2 | 45 | 55 |
| Casein alpha | 34 | 66 |


| Product | Cat. No. |
|---|---|
| RoboPop™ Solubility Screening Kit<br>[includes PopCulture® Reagent, Lysonase™ Bioprocessing Reagent, Solubility Screening 96-well Filter Plate, 4% SDS, Collection Plate with Sealers (2)] | 71255-3 |
| RoboPop GST•Mag™ Purification Kit<br>[includes PopCulture Reagent; Sterile 96-well Deep Well Culture Plate with Sealers (3); Collection Plate with Sealer; GST•Mag Agarose Beads; 10X GST Bind/Wash Buffer; 10X Glutathione Reconstitution Buffer; Reduced Glutathione; rLysozyme™ Solution; rLysozyme Dilution Buffer; Benzonase® Nuclease, Purity > 90%] | 71102-3 |
| RoboPop His•Mag™ Purification Kit<br>[includes PopCulture Reagent; Sterile 96-well Deep Well Culture Plate with Sealers (3); Collection Plate with Sealer; His•Mag Agarose Beads; 8X Binding Buffer; 8X Wash Buffer; 4X Elute Buffer; rLysozyme Solution; rLysozyme Dilution Buffer; Benzonase Nuclease, Purity > 90%] | 71103-3 |
| RoboPop GST•Bind™ Purification Kit<br>(includes PopCulture Reagent; GST•Bind Resin; 10X GST•Bind/Wash Buffer; Reduced Glutathione; 10X Glutathione Reconstitution Buffer; 2-ml 96-well Filter Plate; 1-ml 96-well Collection Plate with Sealer; rLysozyme Solution; rLysozyme Dilution Buffer; Benzonase Nuclease, Purity > 90%) | 71189-3 |
| RoboPop Ni-NTA His•Bind® Purification Kit<br>(includes PopCulture Reagent; Ni-NTA His•Bind Resin; 4X Ni-NTA Bind Buffer; 4X Ni-NTA Wash Buffer; 4X Ni-NTA Elute Buffer; 2-ml 96-well Filter Plate; 1-ml 96-well Collection Plate with Sealer; rLysozyme Solution; rLysozyme Dilution Buffer; Benzonase Nuclease, Purity > 90%) | 71188-3 |

All RoboPop kit reagents are also available separately. Please inquire.

| Product | Size | Cat. No. |
|---|---|---|
| FRETWorks™ S•Tag™ Assay Kit<br>(includes S•Tag Grade S-protein, FRET ArUAA Substrate, 10X FRET Assay Buffer, 10X FRET Stop Solution, and S•Tag Standard) | 100 assays<br>1000 assays | 70724-3<br>70724-4 |
| Overnight Express™ Autoinduction System 1<br>(includes OnEx™ Solution 1, OnEx Solution 2, and OnEx Solution 3)<br>* includes enough reagents to induce 1 liter<br>† includes enough reagents to induce 5 liters | 1 kit*<br>1 kit† | 71300-3<br>71300-3 |
| His•Tag® Monoclonal Antibody<br>(Anti His•Tag Monoclonal Antibody) | 100 µg<br>3 µg | 70796-3<br>70796-4 |
| His•Tag Antibody Plate | 1 plate<br>5 plates | 71184-3<br>71184-4 |
| Solubility Screening 96-well Filter Plate | 1 plate | 71258-3 |
| Perfect Protein™ Markers, 10–225 kDa | 100 lanes | 69079-3 |

# Vectors for expression of amino-terminal His•Tag® fusion proteins containing minimal extraneous sequences

*Eight new expression vectors*

A series of new vectors is designed specifically to minimize the number of extraneous amino acids encoded when expressing amino-terminal His•Tag® fusion proteins. These His•Tag vectors are available 1) as uncut plasmids with an extensive multiple cloning site (MCS) region for restriction enzyme cloning methods or 2) as linearized versions prepared for ligation-independent cloning (LIC). The specific characteristics of these eight new His•Tag vectors are compared in Table 1.

## Features

- Amino-terminal fusion tag (His•Tag) coding sequence, with optional protease cleavage
- Minimal number of extraneous amino acids encoded, depending on cloning strategy
- Optional carboxy-terminal S•Tag™ sequence
- New replicons and antibiotic resistance marker
- Compatibility with other vectors for coexpression
- Expression characteristics similar to pET vectors (with the exception of the pIEx™ vectors; see below)

## Compatible vectors for cloning and expression in *E. coli*

The pET-45b(+), pCDF-1b, and pRSF-1b plasmids rely on the strong T7*lac* promoter for high-level, tightly regulated protein expression. These three vectors are compatible with each other for coexpression of up to three proteins in one cell from separate plasmids (Table 1). The relative plasmid copy numbers for these vectors are pRSF > pET-45b(+) > pCDF. Figure 1 shows vector maps.

The MCS region is designed to allow the generation of amino-terminal His•Tag fusion proteins with a minimum of vector-encoded extraneous amino acids.

**Table 1. Vector characteristics**

| Vector | | | | | | |
|---|---|---|---|---|---|---|
| Standard Cloning* | LIC | Promoter | Replicon | Resistance Marker† | Copy Number‡ | Expression Host |
| pET-45b(+) | pET-46 Ek/LIC | T7*lac* | ColE1 | Ap | ~40 | *E. coli* |
| pCDF-1b | pCDF-2 Ek/LIC | T7*lac* | CloDF13 (1, 2) | Sm | 20–40 | *E. coli* |
| pRSF-1b | pRSF-2 Ek/LIC | T7*lac* | RSF1030 (3, 4) | Kan | > 100 | *E. coli* |
| pIEx-6 | pIEx-7 Ek/LIC | hr5/IE1 | ColE1 (pUC) | Ap | > 500 | insect cells |

* Standard cloning methods rely on restriction enzyme sites for insertion of target DNA.

† Resistance markers: Ap, ampicillin; Sm, streptomycin/spectinomycin; Kan, kanamycin

‡ Copy number was estimated based on gel analysis.

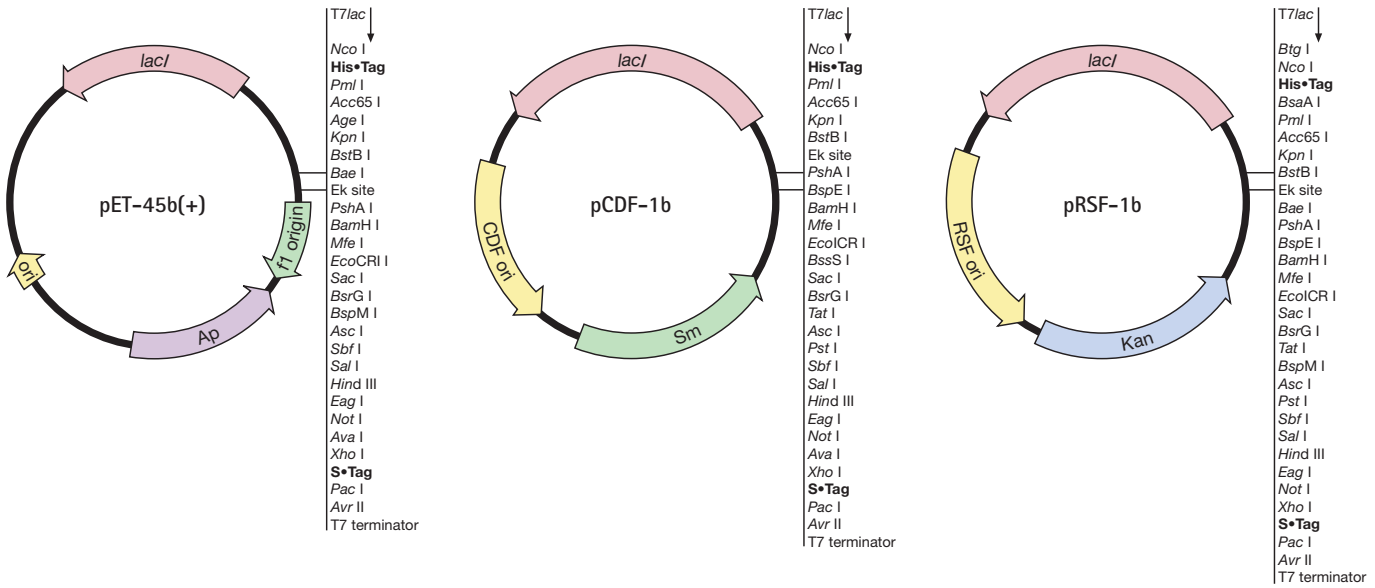



**Figure 1. pET-45b(+), pCDF-1b, and pRSF-1b vector maps**

*continued on page 24*

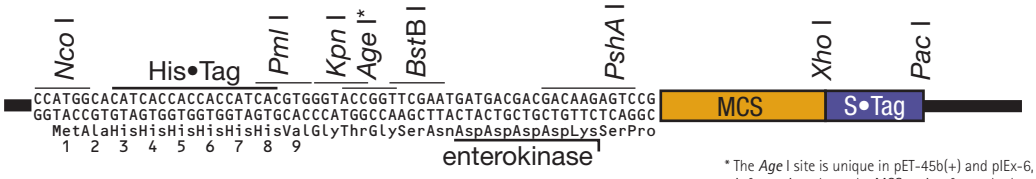
*continued from page 23*

The CAC triplet that encodes the sixth histidine in the His•Tag® coding sequence is also part of the recognition site for *Pml* I (CAC↓GTG). For blunt inserts in the appropriate reading frame, this cloning site allows direct fusion to the His•Tag sequence such that the target ORF begins at the ninth codon (Figure 2). If an application requires the ability to remove the His•Tag sequence, inserts can be cloned into the MCS downstream from the enterokinase (EK) site. Overlapping the coding region for the EK site is the *PshA* I site, can be used to re-create a full EK site to enable removal of all amino-terminal amino acids encoded by the vector (Figure 2). The remainder of the MCS facilitates insert transfer with restriction sites similar to those found in other Novagen vectors and offers an S•Tag™ sequence to generate a carboxy-terminal tag compatible with purification, detection, and quantification methods (5).

### Compatible Ek/LIC vectors for expression in *E. coli*

As with the vectors described above, the pET-46 Ek/LIC, pCDF-2 Ek/LIC, and pRSF-2 Ek/LIC plasmids carry a strong T7*lac* promoter, but lack the extensive MCS (Figure 2, panel A, below, and Table 1, page 23). These plasmids are supplied as Ek/LIC-prepared linearized DNA ready to anneal to appropriately prepared inserts. The Ek/LIC strategy places PCR products in a defined orientation immediately adjacent to the EK cleavage site and optional carboxy-terminal S•Tag sequence. When any of these three Ek/LIC vectors is used with Novagen LIC Duet™ Adaptors, two PCR products can be cloned simultaneously into one plasmid for expression of two ORFs from the same vector. Recombinants derived from these vectors can be used for coexpression of up to six target proteins in one cell from appropriate host strains (see the article beginning on page 7 for more information about LIC Duet Adaptors).

**A. Cloning region for pET-45b(+), pCDF-1b, pRSF-1b, and pIEx-6 vectors**




* The *Age* I site is unique in pET-45b(+) and pIEx-6, but not in pCDF-1b or pRSF-1b. For more information about the MCS region for each plasmid, refer to the vector maps in Figure 1 (page 23) and Figure 3 (page 25). Detailed vector maps are also available at ww.novagen.com.

**B. Cloning site for pET-46 Ek/LIC, pCDF-2 Ek/LIC, pRSF-2 Ek/LIC, and pIEx-7 Ek/LIC vectors**


**Figure 2. Common regions of multiple cloning site and Ek/LIC site**

Panel A shows the cloning region shared by pET-45b(+), pCDF-1b, pRSF-1b, and pIEx™-6 vectors. Panel B shows the analogous Ek/LIC cloning site shared by pET-46 Ek/LIC, pCDF-2 Ek/LIC, pRSF-2 Ek/LIC, and pIEx-7 Ek/LIC vectors.

**Vectors for direct expression in insect cells**

pIEx™ vectors are designed for direct expression in *Spodoptera* cell lines by transient transfection (6). Stable cell lines also can be constructed by cotransfection of the pIE1-neo plasmid and selection on G418. Using Insect GeneJuice® Transfection Reagent, the recombinant plasmid can be transfected efficiently into insect cells. Target gene transcription is driven by the baculovirus hr5 enhancer and immediate early promoter (IE1), which are recognized by the cellular RNA polymerase. The pIEx-6 vector offers the same MCS as pET-45b(+), which is convenient for restriction enzyme cloning, whereas the pIEx-7 Ek/LIC vector lacks the extensive MCS and is supplied as an Ek/LIC-prepared linearized plasmid (Figure 2, page 24, and Figure 3, below). For additional characteristics of these two insect expression vectors, refer to Table 1 (page 23).

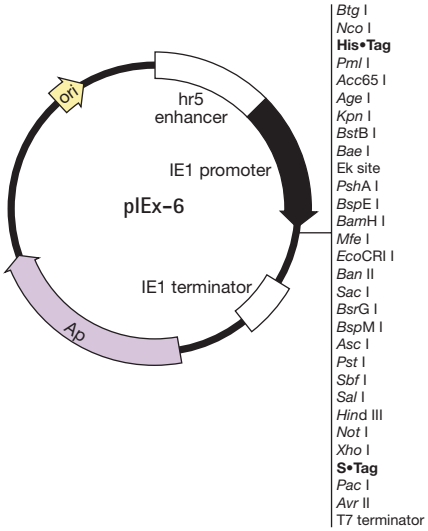



Figure 3. pIEx-6 vector map

## REFERENCES


1. Cannon, P. M. and Strike, P. (1992) *Plasmid* **27**, 220–230.
2. Nijkamp, H. J. J., de Lang, R., Stuitje, A. R., van den Elzen P. J. M., Veltkamp, E., and van Putten, A. J. (1986) *Plasmid* **16**, 135–160.
3. Som, T. and Tomizawa, J. (1982) *Mol. Gen. Genet.* **187**, 375–383.
4. Phillips, G. J., Park, S., and Huber, D. (2000) *BioTechniques* **28**, 400–408.
5. Kim, J. S. and R. T. Raines (1993) *Protein Sci.* **2**, 348–356.
6. Loomis, K., Novy, R., and Yaeger, K. (2003) *inNovations* **16**, 7–10.


| Product | Size | Cat. No. |
|---|---|---|
| pET-45b(+) DNA | 10 µg | 71327-3 |
| pCDF-1b DNA | 10 µg | 71330-3 |
| pRSF-1b DNA | 10 µg | 71363-3 |
| pIEx™-6 DNA | 20 µg | 71333-3 |

| Product | Size | Cat. No. |
|---|---|---|
| pET-46 Ek/LIC Vector Kit | 20 rxn | 71335-3 |
| pCDF-2 Ek/LIC Vector Kit | 20 rxn | 71337-3 |
| pRSF-2 Ek/LIC Vector Kit | 20 rxn | 71364-3 |
| pIEx-7 Ek/LIC Vector Kit | 20 rxn | 71339-3 |

Ek/LIC Vector Kit Components:

- 1 µg Ek/LIC Vector
- 25 units T4 DNA Polymerase, LIC-qualified
- 50 µl 25 mM EDTA
- 40 µl 25 mM dATP
- 8 µl Ek/LIC β-gal Control Insert
- 100 µl 100 mM DTT
- 50 µl 10X T4 DNA Polymerase Buffer
- 1.5 ml Nuclease-free Water
- 5 × 2 ml SOC Medium
- 10 µl Test Plasmid
- 0.2 ml BL21(DE3)pLysS Competent Cells*
- 0.2 ml BL21(DE3) Competent Cells*
- 22 × 50 µl NovaBlue GigaSingles™ Competent Cells

* not included with pIEx-7 Ek/LIC Vector Kit

| Product | Cat. No. |
|---|---|
| pRSF-1 Expression System | 71375-3 |
| System plus Competent Cells | 71376-3 |
| pCDF-1 Expression System | 71331-3 |
| System plus Competent Cells | 71332-3 |
| pET Expression System 45b | 71328-3 |
| System plus Competent Cells | 71329-3 |

Expression System Components:

- 10 µg pRSF-1b, pCDF-1b, or pET-45b(+) DNA
- 0.2 ml Induction Control D†
- 0.2 ml BL21 Glycerol Stock
- 0.2 ml BL21(DE3) Glycerol Stock
- 0.2 ml BL21(DE3)pLysS Glycerol Stock

† included with pET Expression System 45b only

System plus Competent Cells Components:
*(in addition to Expression System Components)*

- 0.2 ml NovaBlue Competent Cells
- 0.2 ml BL21(DE3) Competent Cells
- 0.2 ml BL21(DE3)pLysS Competent Cells
- 10 µl Test Plasmid
- 2 × 2 ml SOC Medium

Available separately:

| Product | Size | Cat. No. |
|---|---|---|
| Insect GeneJuice® Transfection Reagent | 0.3 ml | 71259-3 |
| | 1 ml | 71259-4 |
| | 10 × 1 ml | 71259-5 |

# Overnight Express™ Autoinduction System 2

*Combines System 1 plus three new components to create a complete, defined medium*

Overnight Express™ Autoinduction System 2 provides a complete chemically defined medium for high-level protein expression with the pET System without the need to monitor cell growth. System 2 combines the basic features of Overnight Express Autoinduction System 1 (1) with three additional components. In Overnight Express media, cultures reliably grow uninduced to relatively high density and then automatically induce high levels of target protein expression—without the need to monitor cell density or add IPTG. Additionally, Overnight Express Autoinduction System 2 can be used for selenomethionine (Se-Met) labeling of proteins to be crystallized for x-ray diffraction studies. System 2 contains sufficient methionine (Met) to support growth of the Met auxotroph B834 while providing the ability to reduce the level of unlabeled Met for Se-Met incorporation by Met auxotrophs.

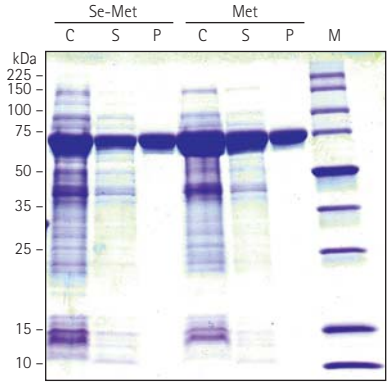



**Figure 1. SDS-PAGE analysis of crude, soluble, and purified proteins from cultures grown in Overnight Express Autoinduction System 2 medium**

pET-44b(+) recombinants were transformed into B834(DE3) cells and grown for 16 h at 37°C in 500-ml baffled flasks in Overnight Express System 2 medium containing Se-Met (125 µg/ml) or methionine (Met; OnEx Solution 6). Cells were collected by centrifugation and resuspended in Ni-NTA Bind Buffer containing AEBSF, Hydrochloride (Calbiochem), Benzamidine, Hydrochloride (Calbiochem), and Lysonase™ Bioprocessing Reagent. The suspension was sonicated and centrifuged at 12,000 × g for 10 min. The supernatant, representing soluble protein, was processed by Ni-NTA His•Bind® chromatography. Two micrograms of purified protein (P), 5 µl of soluble extract (S), and an aliquot of crude extract (C; standardized to harvest $OD_{600}$) were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining. The predicted molecular mass for the pET-44b(+) protein is 67.8 kDa. Protein was assayed using the BCA Protein Assay Kit (Novagen). Lane M: Perfect Protein™ Markers, 10–225 kDa.

The system includes six concentrated sterile solutions: OnEx™ solutions 1 through 6. Addition of these six components to sterile water results in a defined medium promoting high cell densities, autoinduction of expression, and maximum soluble protein yields. Optional addition of Se-Met allows efficient labeling of target proteins.

OnEx Solution 1 is a blend of carbon sources optimized for tightly regulated uninduced growth to relatively high cell density followed by high-level induction. OnEx Solution 2 is a concentrated buffer and nitrogen blend that mediates metabolic acid production and provides additional nitrogen for increased protein synthesis. OnEx Solution 3 provides magnesium for maximal cell density. OnEx Solution 4 provides trace metals to minimize growth limitations associated with mineral deficiencies and satisfy the metal requirements of metal-containing target proteins even at high expression levels. OnEx Solution 5 is a mixture of amino acids lacking methionine. OnEx Solution 6 is a separate methionine solution.

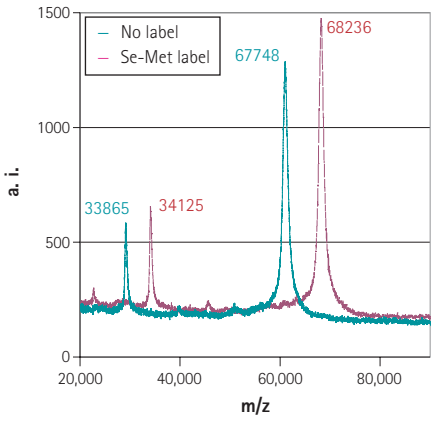



**Figure 2. Mass spectra**

Mass spectroscopy analysis shows Se-Met incorporation into target proteins with Overnight Express System 2 autoinduction as described in Figure 1. (Spectra provided by the Mass Spectrometry/Bioanalytical Facility at the University of Wisconsin Biotechnology Center.)

The Overnight Express Autoinduction System 2 is extremely convenient for routine expression of proteins in multiple cultures and is ideal for high-throughput parallel analysis of protein expression, solubility screening, and purification from multiple expression clones. The tedium of preparing a defined medium from dozens of components has been simplified with the ready-to-use sterile solutions included in the Overnight Express Autoinduction System 2.

## REFERENCES

1. Grabski, A., Mehler, M., and Drott, D. (2003) *inNovations* **17**, 3–6.

| Product | Size | Cat. No. |
|---|---|---|
| Overnight Express™ Autoinduction System 2 | 1 kit* | 71366-3 |
| | 1 kit† | 71366-4 |
| (includes OnEx™ Solution 1, OnEx Solution 2, OnEx Solution 3, OnEx Solution 4, OnEx Solution 5, and OnEx Solution 6) | | |
| Available separately: | | |
| **Product** | **Size** | **Cat. No.** |
| Overnight Express Autoinduction System 1 | 1 kit* | 71300-3 |
| | 1 kit† | 71300-4 |
| (includes OnEx Solution 1, OnEx Solution 2, and OnEx Solution 3) | | |
| * includes enough reagents to induce 1 liter | | |
| † includes enough reagents to induce 5 liters | | |
| L-Selenomethionine | 250 mg | 561505 |
| | 1 g | 561505 |
| AEBSF, Hydrochloride | 50 mg | 101500 |
| | 100 mg | 101500 |
| | 500 mg | 101500 |
| | 1 g | 101500 |
| Benzamidine, Hydrochloride | 5 g | 199001 |
| | 25 g | 199001 |
| Ni-NTA His•Bind® Resin (resin pre-charged with $Ni^{2+}$) | 10 ml | 70666-3 |
| | 25 ml | 70666-4 |
| | 100 ml | 70666-5 |
| Ni-NTA Buffer Kit | | 70899-3 |
| BCA Protein Assay Kit | 500 assays (2500 microplate assays) | 71285-3 |
| (includes BCA Solution, BSA Protein Standard, and 4% Cupric Sulfate) | | |
| Perfect Protein™ Markers, 10–225 kDa | 100 lanes | 69079-3 |

= Calbiochem® brand product

* The products and technologies presented in this article were developed and are offered under the terms of a license agreement between EMD Biosciences, Inc. Novagen Brand and Brookhaven Science Associates, covering U.S. patent properties entitled "High Density Growth of T7 Expression Strains with Auto-Induction Option," filed March 14, 2003, in the name of F. William Studier and assigned to Brookhaven Science Associates, LLC., Upton, NY 11973. The Auto Induction Media Technology embodied in the Overnight Express Autoinduction Systems is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is the subject of patent applications assigned to Brookhaven Science Associates, LLC. (BSA). This product is to be used for research purposes only. A separate license is required for any commercial manufacture or use, including the manufacture of protein products for use in the screening of compound libraries. Information about commercial licenses may be obtained from the Office of Intellectual Property and Industrial Partnerships, Brookhaven National Laboratory, Bldg. 475D, P. O. Box 5000, Upton, New York 11973-5000; Telephone (631) 344-7134.

# BugBuster® Plus Lysonase™ Kit

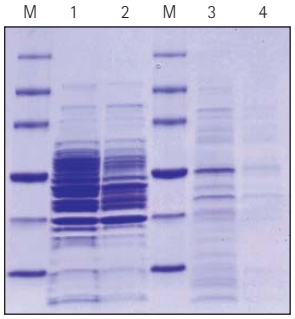
*Cell lysis and nucleic acid removal for Gram-negative and Gram-positive bacteria*

BugBuster® Protein Extraction Reagent has become an indispensable tool for efficient extraction of active soluble proteins from *E. coli* without the need for sonication or any other method of mechanical disruption. Lysonase™ Bioprocessing Reagent is a blend of rLysozyme™, a highly purified and stabilized recombinant lysozyme, and Benzonase® Nuclease, a recombinant nonspecific endonuclease that degrades all forms of DNA and RNA. The new BugBuster Plus Lysonase Kit combines the activities of both reagents to significantly increase protein extraction efficiency and facilitate downstream processing of protein extracts, thereby enabling maximum recovery of active soluble protein from both Gram-negative and Gram-positive bacteria. Use 5 ml BugBuster and 10 µl Lysonase per gram of cell paste. Two kit sizes provides sufficient reagents for protein extraction from either 20 or 100 g cell paste.

| Product | Size | Cat. No. |
|---|---|---|
| BugBuster® Plus Lysonase™ Kit | 1 kit* | 71370-3 |
| | 1 kit† | 71370-4 |

* includes 100 ml BugBuster Protein Extraction Reagent and 0.2 ml Lysonase Bioprocessing Reagent, sufficient for protein extraction from 20 g cell paste

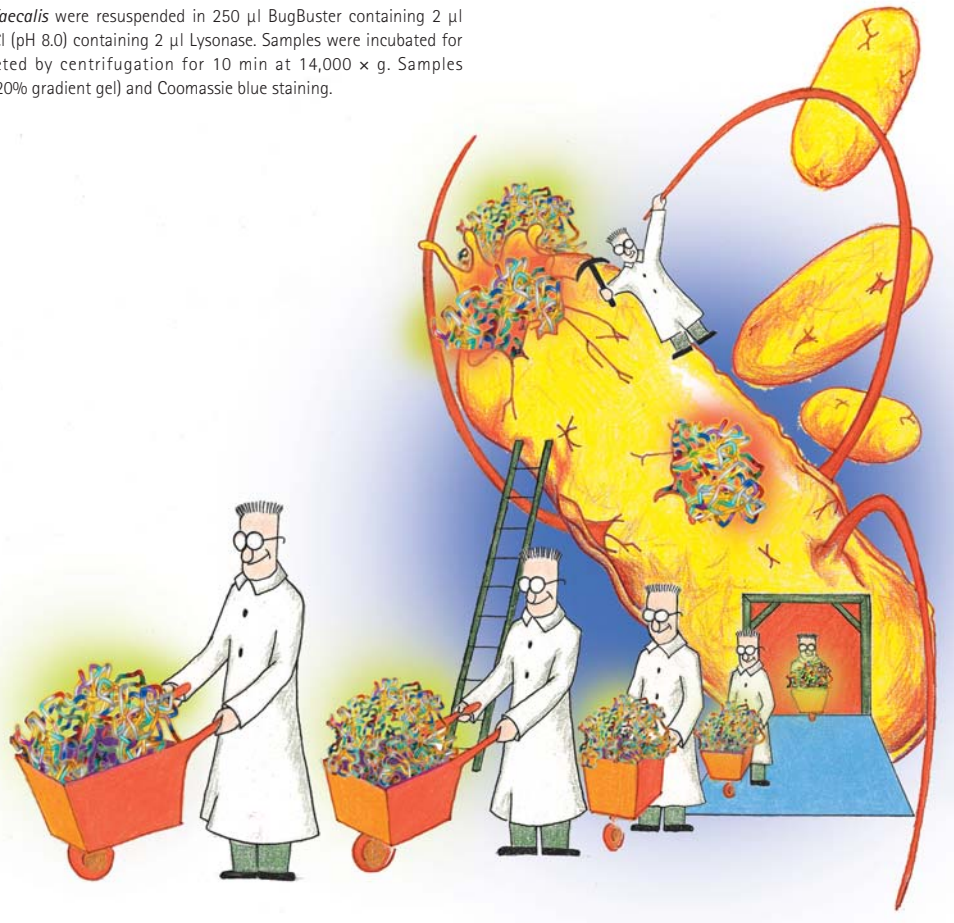
† includes 500 ml BugBuster Protein Extraction Reagent and 1 ml Lysonase Bioprocessing Reagent, sufficient for protein extraction from 100 g cell paste




| Lane | Sample |
|---|---|
| M | Perfect Protein™ Markers, 15–150 kDa |
| 1 | 10 µl *B. subtilis* BugBuster plus Lysonase |
| 2 | 10 µl *B. subtilis* Tris plus Lysonase |
| 3 | 10 µl *E. faecalis* BugBuster plus Lysonase |
| 4 | 10 µl *E. faecalis* Tris plus Lysonase |

**Figure 1. Protein extraction from Gram-positive bacteria**

Cell pellets (25 mg) of *Bacillus subtilis* and *Enterococcus faecalis* were resuspended in 250 µl BugBuster containing 2 µl Lysonase Bioprocessing Reagent or in 250 µl 50 mM Tris-HCl (pH 8.0) containing 2 µl Lysonase. Samples were incubated for 10 min at room temperature and cell debris was pelleted by centrifugation for 10 min at 14,000 × g. Samples (15 µl) of the supernatants were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining.



# Rosetta™ 2(DE3) Competent Cells for enhanced coverage of codon bias in *E. coli*

*Enhancing heterologous protein expression in* E. coli

The new Rosetta™ 2(DE3) strain is designed to alleviate codon bias when expressing heterologous proteins in *E. coli*. When the mRNA of heterologous genes is overexpressed in *E. coli*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population (1–4; Table 1). It has been well established that insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frameshifting, and amino acid misincorporation (2).

*...the pRARE plasmid was modified to include the* argX *gene and, thereby, an enhanced version, pRARE2 (Figure 1) was created. This new derivative was transformed into BL21(DE3) to create Rosetta 2(DE3).*

**Table 1. Rare codons in *E. coli***

| Amino acid | Codon | Fraction in all genes | Fraction in Class II |
|---|---|---|---|
| Arg | AGG | 0.022 | 0.003 |
| Arg | AGA | 0.039 | 0.006 |
| Arg | CGG | 0.098 | 0.008 |
| Arg | CGA | 0.065 | 0.011 |
| Arg | CGU | 0.378 | 0.643 |
| Arg | CGC | 0.398 | 0.330 |
| Gly | GGG | 0.151 | 0.044 |
| Gly | GGA | 0.109 | 0.020 |
| Gly | GGU | 0.337 | 0.508 |
| Gly | GGC | 0.403 | 0.428 |
| Ile | AUA | 0.073 | 0.006 |
| Ile | AUU | 0.507 | 0.335 |
| Ile | AUC | 0.420 | 0.659 |
| Leu | UUG | 0.129 | 0.034 |
| Leu | UUA | 0.131 | 0.055 |
| Leu | CUG | 0.496 | 0.767 |
| Leu | CUA | 0.037 | 0.008 |
| Leu | CUU | 0.104 | 0.056 |
| Leu | CUC | 0.104 | 0.080 |
| Pro | CCG | 0.525 | 0.719 |
| Pro | CCA | 0.191 | 0.153 |
| Pro | CCU | 0.159 | 0.112 |
| Pro | CCC | 0.124 | 0.016 |

Codon usage is expressed as the fraction of all possible codons for a given amino acid. "All genes" is the fraction represented in all 4,290 coding sequences in the *E. coli* genome (5). "Class II" is the fraction represented in 195 genes highly and continuously expressed during exponential growth (6).

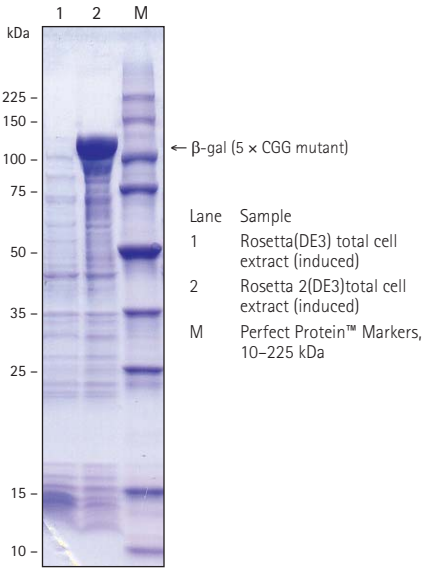
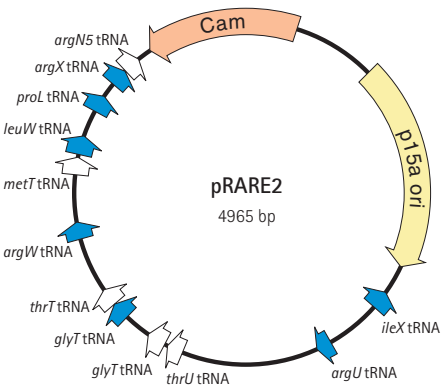



**Figure 2. Effect of consecutive CGG rare codons on target protein expression**

A pET-15b recombinant plasmid containing five consecutive CGG codons near the 5′-end of the β-gal coding region was transformed into Rosetta(DE3) and Rosetta 2(DE3). Cells were grown in LB broth with carbenicillin and chloramphenicol to an $OD_{600}$ between 1.0 and 1.2, induced with 1 mM IPTG (3 h at 37°C), and harvested by centrifugation. Cells were resuspended and lysed in SDS sample buffer, followed by sonication to reduce sample viscosity. Proteins were separated on a 4–20% SDS polyacrylamide gel and stained with Coomassie blue.

### Rosetta increases the level of rare tRNAs

The original Novagen Rosetta strain was designed to increase the level of rare tRNAs by carrying the pRARE plasmid (7). This multicopy plasmid increases rare tRNA levels by increasing the dosage of the respective tRNA genes. The original pRARE plasmid carries tRNA genes that decode AGG and AGA (*argU*), GGA (*argU*), AUA (*argU*), CUA (*argU*), and CCC (*argU*). Research since the introduction of the original Rosetta strain has revealed that the rarely used *E. coli* CGG (Arg) codon also can cause problems for target protein expression. This research also revealed that enhanced yields of the specific target protein can be obtained by the inclusion of a plasmid that carries the cognate *argX* tRNA gene (8). Using this information, the pRARE plasmid was modified to include the *argX* gene and, thereby, an enhanced version, pRARE2 (Figure 1) was created. This new derivative was transformed into BL21(DE3) to create Rosetta 2(DE3).




### Testing Rosetta 2(DE3)

Functional testing of Rosetta 2(DE3) was accomplished by creating a mutant β-gal gene encoding five consecutive rare CGG (Arg) codons near the amino-terminus. A pET-15b derivative carrying the mutant 5x-CGG β-gal mutant was transformed into both Rosetta (DE3) and Rosetta 2(DE3) for expression testing. Figure 2 demonstrates the dramatic effect that multiple consecutive rare codons can have on target protein expression. Very little full-length β-gal was expressed in Rosetta (DE3), however, a major β-gal band was observed in Rosetta 2(DE3).

### REFERENCES

1. Kane, J. F. (1995) *Curr. Opin. Biotechnol.* **6**, 494–500.
2. Kurland, C. and Gallant, J. (1996) *Curr. Opin. Biotechnol.* **7**, 489–493.
3. Goldman, E. E., Rosenberg, A. H., Zubay, G., and Studier, F. W. (1995) *J. Mol. Biol.* **245**, 467–473.
4. Baca, A. M. and Hol, W. G. J. (2000) *Int. J. Parasitology* **30**, 113–118.

5. Nakamura, Y., Gojobori, T., and Ikemura, T. (2000) *Nucl. Acids Res.* **29**, 292.
6. Henaut, A. and Danchin, A. (1996) in *Escherichia coli and Salmonella typhimurium cellular and molecular biology*, vol. 2, (Neidhardt, F., Curtiss III, R., Ingraham, J., Lin, E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M., and Umbarger, H., eds), pp. 2047–2066. American Society for Microbiology, Washington, DC.
7. Novy, R., Drott, D., Yaeger, K., and Mierendorf, R. (2001) *inNovations* **12**, 1–3.
8. McNulty, D. E., Claffee, B. A., Huddleston, M. J., and Kane, J. F. (2003) *Protein Expr. Purif.* **27**,

| Product | Size | Cat. No. |
|---|---|---|
| Rosetta™ 2(DE3) Competent Cells | 0.4 ml<br>1 ml | 71397-3<br>71397-4 |
| guaranteed efficiency: > 2 × $10^6$ cfu/µg | | |
| Rosetta 2(DE3) Singles™ Competent Cells | 11 rxn<br>22 rxn | 71400-3<br>71400-4 |
| guaranteed efficiency: > 2 × $10^6$ cfu/µg | | |

## NovaBlue T1$^R$ Singles™ Competent Cells

*Featuring T1 and T5 phage resistance*

NovaBlue T1$^R$ Singles™ Competent Cells have all the same features of NovaBlue Singles, with the added benefit of being resistant to T1 and T5 phage. NovaBlue is a K-12 strain ideally suited as an initial cloning host due to its high transformation efficiency, blue-white screening capability (with appropriate plasmids), and the high yields and excellent quality plasmid DNA that results from *recA endA* mutations. Cells are provided as 50-µl aliquots that eliminate the need to subaliquot, freeze/thaw, or waste partially used vials. All kits include SOC Medium and Test Plasmid.

### Features

- T1 and T5 phage resistant
- Chemically competent
- High quality plasmid DNA preparation
- Easy-to-use Singles format
- Includes Test Plasmid, SOC Medium, and protocol

### Genotype

*endA1 hsdR17* ($r_{K12}^-m_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proA*$^+$*B*$^+$ *lacI*$^q$*ZΔM15*::Tn*10* (Tc$^R$)] *tonA*.

| Product | Size | Cat. No. |
|---|---|---|
| NovaBlue T1$^R$ Singles™ Competent Cells | 11 rxn<br>22 rxn | 71318-3<br>71318-4 |
| guaranteed efficiency: > 1.5 × $10^8$ cfu/µg | | |

## Zappers™ Electrocompetent Cells

*Extremely high cloning efficiency in two versatile strains*

NovaXG and NovaXGF′ Zappers™ Electrocompetent Cells combine favorable genotype with high transformation efficiency for the most demanding cloning applications. NovaXG features deletions of genes involved in restriction (*mrr-hsd*RMS-*mcr*BC), which allow for the cloning of methylated DNA and library construction without deletions or rearrangements, and *recA endA* mutations, which facilitate high yields of excellent quality plasmid DNA. NovaXGF′ cells have the same genotype as NovaXG, but harbor an F′ plasmid which confers tetracycline resistance and allows for infection by M13 for ssDNA production. Because the F′ plasmid carries the *lacI*$^q$ repressor gene, addition of IPTG is required for blue/white screening of inserts. In the absence of IPTG, transcription of insert DNA from the *lacZ* promoter is kept to a minimum. Both strains are manufactured for high transformation efficiency (> 1 × $10^{10}$ cfu/µg) by electroporation to deliver a maximum number of transformants, which is especially important when working with limited amounts of DNA or when constructing large or complex libraries. The cells are packaged in a convenient two-transformation-per-tube format to minimize thawing of excess cells.

### Features

- Convenient packaging format
- Guaranteed transformation efficiency of > 1 × $10^{10}$ cfu/µg
- Methylation restriction minus
- Recombination and endonuclease minus
- T1 and T5 phage resistant
- Includes Test Plasmid and protocol

### Genotypes

NovaXG:
F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *endA1 recA1* ϕ80d*lacZΔM15* Δ*lacX74 araD139* Δ(*ara-leu*)7697 *galU galK rpsL nupG*λ$^-$ *tonA*

NovaXGF′:
*mcrA* Δ(*mrr-hsdRMS-mcrBC*) *endA1 recA1* ϕ80d*lacZΔM15* Δ*lacX74 araD139* Δ(*ara-leu*)7697 *galU galK rpsL nupG*λ$^-$ *tonA* F′[*lacI*$^q$Tn*10*(Tc$^R$)]

### Additional Information

Additional technical information for NovaXG and NovaXGF′ Zappers™ Electrocompetent Cells is available in Novagen User Protocol TB400, available at www.novagen.com.

| Product | Size | Cat. No. |
|---|---|---|
| NovaXG Zappers™ Electrocompetent Cells | 10 rxn<br>20 rxn | 71315-3<br>71315-4 |
| guaranteed efficiency: > 1 × $10^{10}$ cfu/µg | | |
| NovaXGF′ Zappers Electrocompetent Cells | 10 rxn<br>20 rxn | 71317-3<br>71317-4 |
| guaranteed efficiency: > 1 × $10^{10}$ cfu/µg | | |

Note: NovaXG Zappers and NovaXGF′ Zappers Electrocompetent Cells do not include SOC Medium. Novagen recommends using TB medium as described in the user protocol.

Available separately:

| Product | Size | Cat. No. |
|---|---|---|
| 100 mM IPTG Solution (10 × 1.5 ml) | 15 ml | 70527-3 |
| X-Gal Solution | 3 × 1 ml | 71077-3 |

# PhosphoSafe™ Extraction Buffer

*When phosphorylation state matters*

PhosphoSafe™ Extraction Buffer efficiently extracts cytosolic proteins from mammalian and insect cells while preserving their phosphorylation state. This reagent contains the same formula as CytoBuster™ Protein Extraction Reagent (1), but also includes four phosphatase inhibitors: sodium fluoride, sodium vanadate, β-glycerophosphate, and sodium pyrophosphate. PhosphoSafe is compatible with kinase assays, protein interaction analysis, and other applications.

To test the preservation of phosphorylation state by PhosphoSafe, protein extracts were prepared from L6 myoblasts using PhosphoSafe and CytoBuster reagents. An antibody against myosin light chain 2 (anti-phospho-MLC2) that specifically recognizes the combination of phosphorylated threonine and serine at amino acid positions 18 and 19, respectively, was used in a Western blot analysis of the protein extracts. A much stronger detection signal was seen when extracts were prepared with PhosphoSafe buffer than in extracts prepared without it (Figure 1).

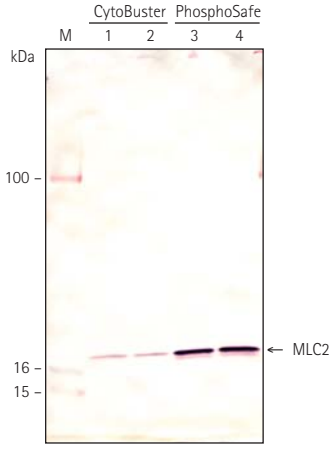



**Figure 1. Detection of phosphorylated MLC2**

Monolayers of subconfluent L6 myoblasts were extracted with CytoBuster reagent or PhosphoSafe buffer for 10 min at room temperature. Extracts were centrifuged and assayed for protein concentration using the BCA Protein Assay Kit (Novagen). Duplicates of each extract (10 µg) were analyzed by SDS-PAGE (4–20% gradient gel). After proteins were transferred to a nitrocellulose membrane, protein phosphorylation state was assessed with anti-phospho-MLC2 as the primary antibody. Goat Anti-Rabbit IgG AP Conjugate (Novagen) was added and detected by staining with the AP Detection Reagent Kit (Novagen).

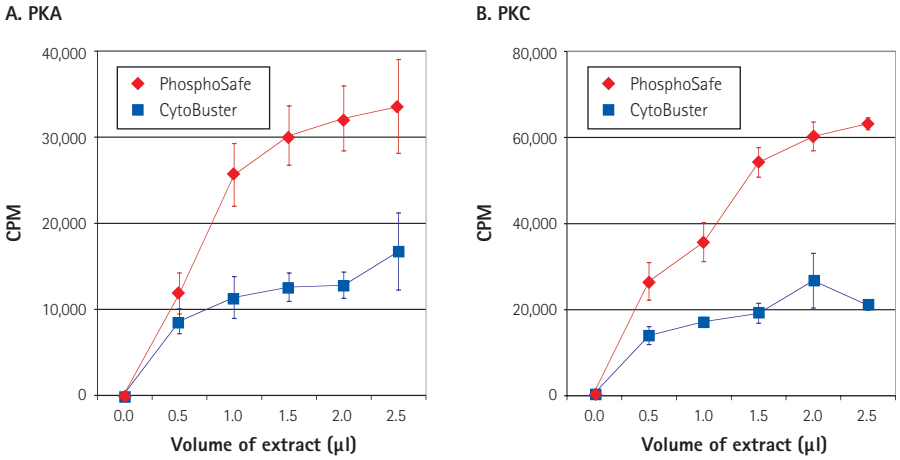



**Figure 2. Kinase assays for PKA and PKC**

Monolayers of subconfluent CHO-K1 cells were extracted with CytoBuster reagent or PhosphoSafe buffer for 10 min at room temperature. Biotinylated peptides corresponding to the PKA phosphorylation site of HNF-6 and the pseudosubstrate region of PKC were incubated with increasing amounts of extract (0.6 mg/ml) in the presence of $\gamma$-$^{32}$P ATP using the Protein Kinase Assay Kit, Universal (Calbiochem). Phosphate transfer mediated by PKA (panel A) and PKC (panel B) was detected by scintillation counting. Protein concentration was determined using the BCA Protein Assay Kit (Novagen).

Figure 2 shows kinase assay data from CHO-K1 extracts prepared using PhosphoSafe buffer and CytoBuster reagent. Panel A shows phosphorylation of a peptide corresponding to the hepatocyte nuclear factor-6 (HNF-6) by protein kinase A (PKA) (2). Panel B shows phosphorylation of a peptide from the pseudosubstrate region of protein kinase C (PKC) (3). For both assays, the level of $\gamma$-$^{32}$P transfer to the peptide during the reaction was on average 2.5-fold higher with PhosphoSafe buffer than with CytoBuster reagent. This enhanced signal transfer is especially important when working with low levels of protein kinase.

### Additional Information

Additional technical information for PhosphoSafe Extraction Buffer is available in Novagen User Protocol TB402, available a www.novagen.com.

### REFERENCES

1. Trim, J. E. and Sawyer, D. L. (2001) *inNovations* **12**, 5–6.
2. Streeper, R. S., Hornbuckle, L. A., Svitek, C. A., Goldman, J. K., Oeser, J. K., and O'Brien, R. M. (2001) *J. Biol. Chem.* **276**, 19111–19118.
3. Lee, C. L., Linton, J. M., Soughaver, J. S., Sims, C. E., and Allbritton, N. L. (1999) *Nature Biotechnol.* **17**, 759–762.

| Product | Size | Cat. No. |
|---|---|---|
| PhosphoSafe™ Extraction Buffer | 25 ml | 71296-3 |
| | 5 × 25 ml | 71296-4 |

Available separately:

| Product | Size | Cat. No. |
|---|---|---|
| AP Detection Reagent Kit (NBT, BCIP, 20X AP Buffer) | 1 ea | 69264-3 |
| | 5 ea | 69264-4 |
| Protein Kinase Assay Kit, Universal | 1 kit | 539551 |
| Trail Mix™ Western Markers | 25 lanes | 70982-3 |
| BCA Protein Assay Kit (includes BCA Solution, BSA Protein Standard, and 4% Cupric Sulfate) | 500 assays (2500 microplate assays) | 71285-3 |
| Goat Anti-Rabbit IgG AP Conjugate ($F_c$ specific) | 40 µl | 69265-3 |

= Calbiochem® brand product

## Need larger quantities, alternative packaging or different concentrations? Ask about bulk quantities or custom configurations.

Merck Biosciences welcomes your inquiries for planning long-term or large-scale projects with any of our Novagen products. Examples of products that can be formatted in larger quantities or in alternative packaging include:

**PCR Amplification**

- Nova*Taq*™ DNA Polymerase
- Pellet Paint® Co-Precipitants
- PCR Markers, 50–2000 bp

**Transformation**

- Clonables™ 2X Ligation Premix
- Competent Cells

**Phage Display**

- T7 Packaging Extracts

***in vitro* Transcription/Translation**

- EcoPro™ T7 Extracts

**Protein Extraction**

- rLysozyme™ Solution
- Benzonase® Nuclease, Purity > 99%
- Benzonase Nuclease, Purity > 90%
- BugBuster® Protein Extraction Reagents
- Lysonase™ Bioprocessing Reagent
- PopCulture® Reagent
- Insect PopCulture Reagent

**Protein Purification**

- His•Bind® Resin
- His•Mag™ Agarose Beads
- GST•Bind™ Resin
- GST•Mag™ Agarose Beads

**Protease Cleavage**

- Thrombin, Restriction Grade
- Biotinylated Thrombin
- Recombinant Enterokinase
- Factor Xa, Restriction Grade

**Contact the Novagen Bulk Team**

For help meeting your large-quantity or alternate-packaging needs, contact any member of the Novagen Bulk Team:

**USA and Canada**
Tel: 1 800 207 0144
Email: bulk@novagen.com

**Germany**
Tel: 0800 69 31 000
Email: customer.service@merckbiosciences.de

**United Kingdom, Republic of Ireland**
UK Freephone: 0800 622 935
ROI Toll-free: 1800 409 445
Email: customer.service@merckbiosciences.co.uk

**Or your local sales office.**

Request Novagen literature by calling your local sales representative or by visiting our website at www.novagen.com.


**HIS•TAG® AND GST•TAG™ PURIFICATION AND DETECTION TOOLS**

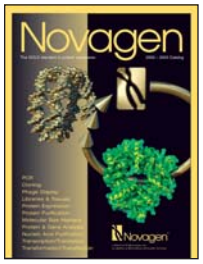
Popular tools arranged in one convenient brochure to help simplify your protein purification and detection:

- Protein extraction reagents
- Protein purification and detection tools
- Site-specific proteases and cleavage capture kits
- Accessory products

**HOST STRAIN COMPETENT CELL SELECTION GUIDE**

Featuring exclusive strains and convenient packaging options designed to help solve your most challenging protein expression problems:

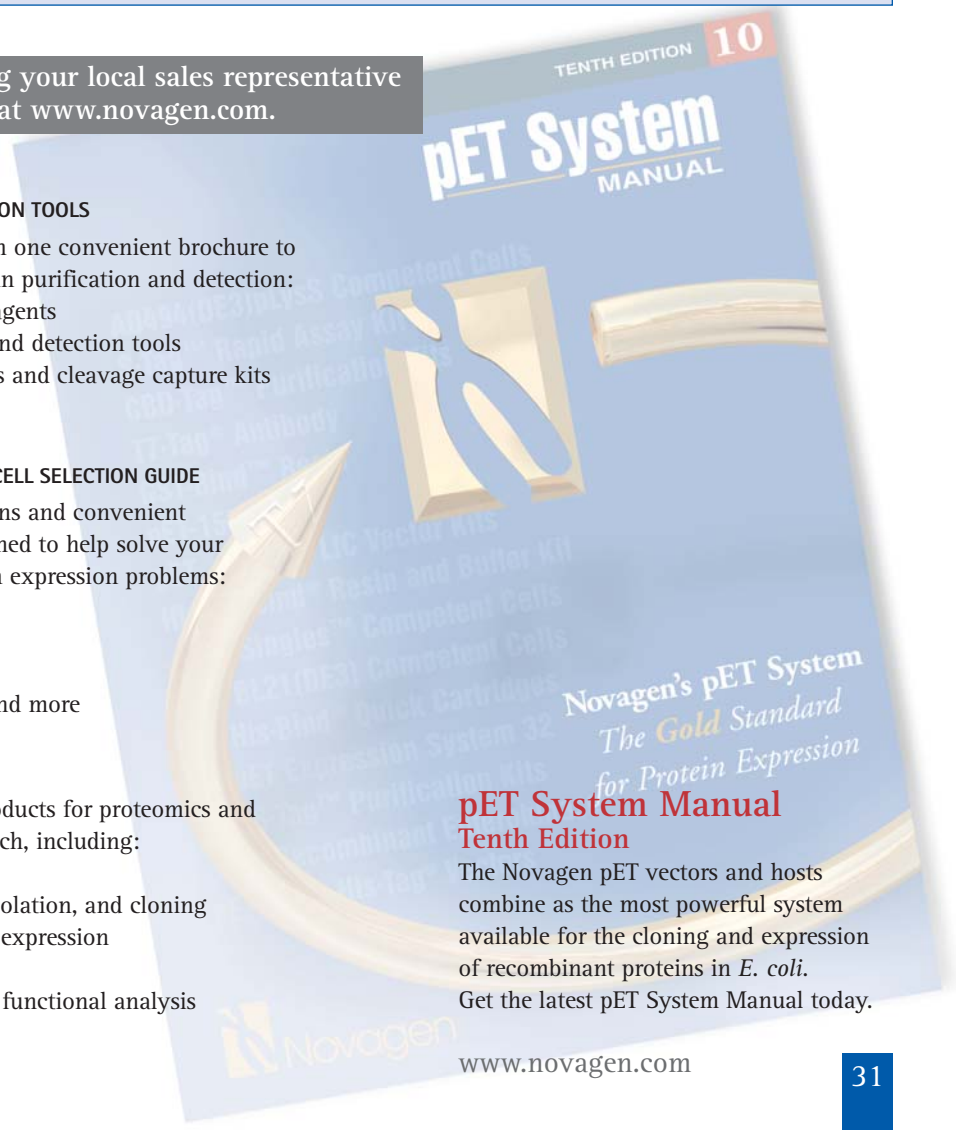
- Codon bias
- Insoluble proteins
- Inactive proteins
- Truncated proteins, and more




**NOVAGEN CATALOG**

Featuring innovative products for proteomics and molecular biology research, including:

- Gene analysis
- DNA amplification, isolation, and cloning
- Recombinant protein expression
- Protein purification
- Protein structure and functional analysis




**pET System Manual**
**Tenth Edition**

The Novagen pET vectors and hosts combine as the most powerful system available for the cloning and expression of recombinant proteins in *E. coli*. Get the latest pET System Manual today.

### United States and Canada

EMD Biosciences, Inc.
441 Charmany Drive
Madison, WI 53719
U.S.A.

*Main Desk:*
Tel: 800 526 7319

*Ordering:*
Tel: 800 854 3417
Tel: 608 238 6110
Fax: 608 238 1388
e-mail: customerservice@novagen.com
web: www.novagen.com

*Technical Service:*
Tel: 800 207 0144
e-mail: novatech@novagen.com

VWR International

*Ordering:*
Tel: 800 932 5000
web: www.vwr.com

### Germany

Merck Biosciences GmbH
Postfach 1167, 65796 Bad Soden
Ober der Röth 4, 65824 Schwalbach/Ts.

*Ordering:*
Freecall: 0800 69 31 000
Freefax: 0800 62 36 100
e-mail: customer.service@merckbiosciences.de
web: www.merckbiosciences.de

*Technical Service:*
Freecall: 0800 100 34 96
e-mail: techservice@merckbiosciences.de

VWR International GmbH
Hilpertstraße 20a
64295 Darmstadt

*Ordering:*
Tel: 06151 3972 0
Fax: 06151 3972 450
e-mail: biotech@de.vwr.com
web: www.vwr.com

### United Kingdom

Merck Biosciences Ltd.
Boulevard Industrial Park
Padge Road, Beeston
Nottingham NG9 2JR

*Ordering and Technical Service:*
Freephone: 0800 622935
Tel: 0115 943 0840
Fax: 0115 943 0951
e-mail: customer.service@merckbiosciences.co.uk
web: www.merckbiosciences.co.uk

VWR International Ltd.
Hunter Boulevard
Magna Park
Lutterworth LE17 4XN

*Ordering:*
Freephone: 0800 22 33 44
Fax: 01455 55 85 86
e-mail: uk.sales@uk.vwr.com
web: www.vwr.com

### Republic of Ireland

Merck Biosciences Ltd.
Boulevard Industrial Park
Padge Road, Beeston
Nottingham NG9 2JR

*Ordering and Technical Service:*
Toll-free: 1800 409445
Fax: +44 115 943 0951
e-mail: customer.service@merckbiosciences.co.uk
web: www.merckbiosciences.co.uk

Novagen is a Brand of EMD Biosciences, Inc.
An Affiliate of Merck KGaA, Darmstadt, Germany