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Greater yields of full-length proteins *in vitro*

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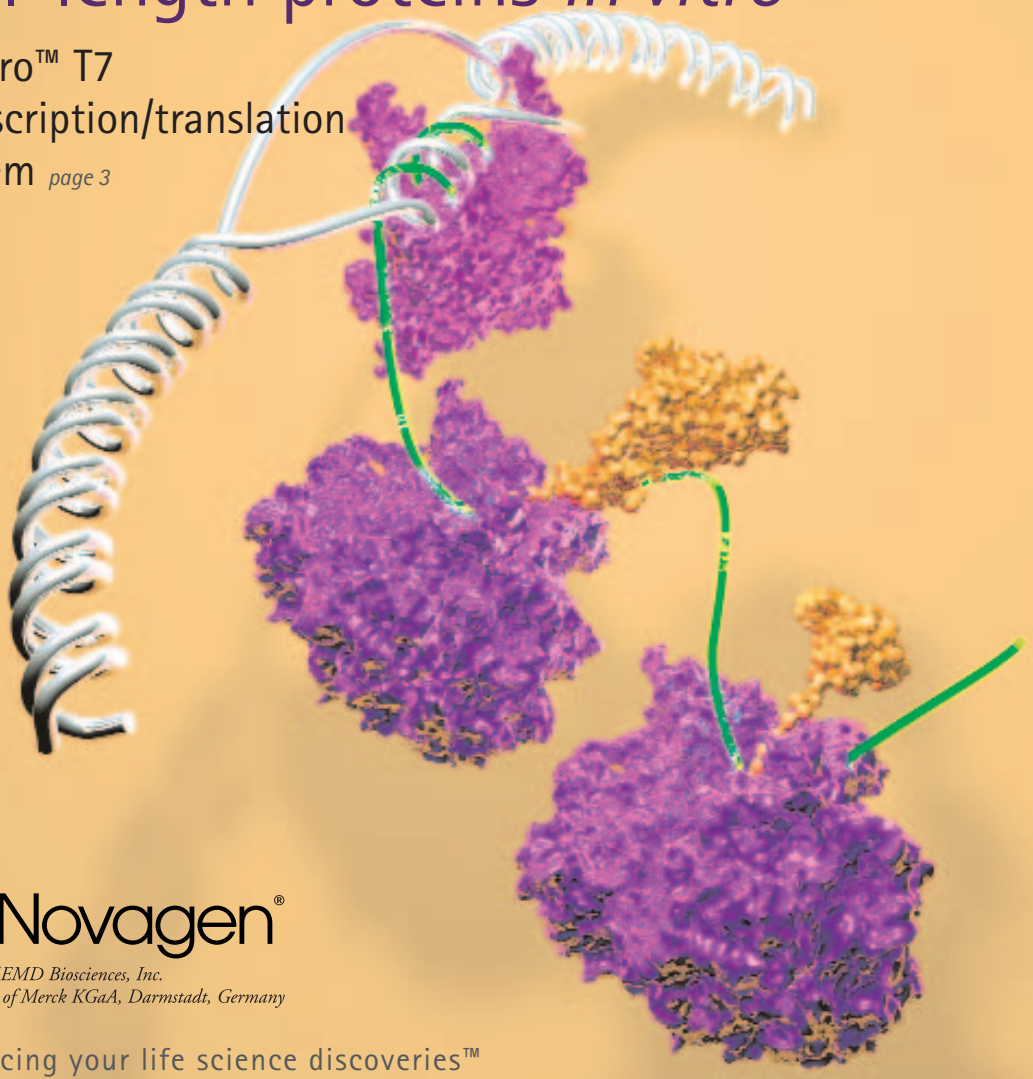

INSIDE

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

e-mail: novatech@novagen.com

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On the Cover

The cover illustration is a rendering of protein synthesis in the EcoPro T7 *in vitro* transcription/translation system. The T7 RNA polymerase is based on the Protein Database (PDB, www.rcsb.org) structure entry 1ARO, the 70S ribosomes (not to scale) are based on 1GIX and 1GIY, and the expressed protein is based on 1L10.

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Greater yields of full-length proteins using the EcoPro™ T7 transcription/translation system

Yuping Ambuel, Mark Handley, and Scott Hayes — Novagen

Since the earliest description of an *E. coli*-based *in vitro* expression system (1), researchers have sought better ways to make protein in a cell-free manner. Improvements in bacterial extract production methods and reaction conditions have led to the realization of diverse applications such as expression of toxic proteins, open reading frame confirmation, functional testing of mutations, antibiotic screening, and polysome display. The success of these techniques requires efficient synthesis of the full-length target protein with minimal production of nontarget proteins and incomplete fragments of desired species.

In this regard, calculation of protein yield based solely on incorporation of labeled amino acids can be misleading. A more meaningful analysis for most applications should quantify full-length protein. While this is generally true for all *in vitro* translation systems, it is particularly important for bacterial extracts, which are somewhat notorious for having high backgrounds relative to rabbit reticulocyte and wheat germ systems. Although protein synthetic activity is significantly higher in bacterial extracts, traditional methods of producing them often result in high levels of endogenous *E. coli* DNA (along with *E. coli* RNA polymerase) and nuclease activities that result in the accumulation of many secondary products in addition to the desired target. Even with apparently low background incorporation as determined by negative controls (no template), the full-length target may represent only a minor fraction of the total species produced during the reaction.

To overcome this disadvantage of bacterial extracts, Novagen developed the EcoPro™ T7 System. Starting with *E. coli* cells deficient in the *lon* and *ompT* proteases, Novagen prepares and fractionates an extract by a proprietary method designed to remove activities associated with the appearance of secondary products. As a result, the EcoPro T7 System retains high translational activity for maximal production of full-length proteins in coupled transcription/translation reactions (2).

Table 1. Comparison of four commercial *E. coli* transcription/translation kits for ^{35}S-methionine incorporation and synthesis of active reporter enzymes

Template	EcoPro T7	Company X	Company Y	Company Z
β-gal incorporation	22.8%	3.2%	4.6%	40%
β-gal activity	28 µg	5 µg	1 µg	0.4 µg
Fluc incorporation	24.5%	3.4%	11%	35%
Fluc activity	3.2 µg	0.8 µg	0.05 µg	2.2 µg
CAT incorporation	29.9%	10%	15.8%	36.3%
CAT activity	17.1 µg	3.6 µg	10.6 µg	7.7 µg

Genes encoding the reporter enzymes β-gal, Fluc and CAT were cloned into the pTriEx™-4 vector for use in product comparisons. Two micrograms of purified plasmids were incubated in 50-µl reactions at 37°C for 60 min. For incorporation, 40 µCi ^{35}S-methionine were added to the extracts at the time of template plasmid addition. Unlabeled reactions were used for activity assays. All reactions were performed in duplicate according to the manufacturers' recommended protocols using their respective kit components. Calculated percent incorporation reflects cpm obtained from TCA precipitation of a reaction sample (minus background) divided by total input cpm. Yields of reporter enzymes, in micrograms, were determined by comparison to standard curves using purified enzymes. The BetaRed™ β-Gal Assay Kit (Novagen) was used for β-galactosidase assays. Firefly luciferase was measured using luciferase assay reagent (Promega), and a colorimetric assay was used for calculating the yield of CAT (3). Data represent the average of duplicate reactions.

In this report, different methods were used to compare the performance of Novagen's EcoPro T7 System with *E. coli* extracts from three other companies. In addition to yield based on incorporation of ^{35}S-labeled methionine, we measured the yield of active enzymes using reporter assays and detected full-length proteins by Western blotting and fluorographic analysis of ^{35}S-labeled proteins. We also evaluated the ability of the EcoPro T7 System to produce mammalian proteins, to accept a variety of templates encoding proteins of different sizes, and to transcribe/translate multiple templates in the same reaction.

^{35}S-methionine incorporation

To begin the comparison of *E. coli* extracts, we measured protein production in 50-µl reactions by incorporation of ^{35}S-methionine and activity assays of reporter enzymes. Templates consisted of pTriEx™ vector constructs encoding chloramphenicol acetyl transferase (CAT; 25 kDa), firefly luciferase (Fluc; 61 kDa), and *E. coli* β-galactosidase (β-gal; 116 kDa) under control of the T7 promoter. All reactions were performed using the respective manufacturers' kit components and recommended reaction conditions. The results of the incorporation assay (Table 1) indicate that Company Z's reaction produced between 35 and 40 percent incorporation, which was the highest for each of the templates. The EcoPro T7 reactions produced between 23 and 30 percent, and both Company X and Company Y had relatively low levels, between 3 and 10 percent and 5 and 16 percent, respectively. Both Company X and Company Y refer to inclusion of cold methionine in their bacterial extracts, which likely explains the lower ^{35}S-methionine incorporation relative to EcoPro T7 and Company Z. For this reason, and because incorporation data alone cannot distinguish between full-length and incomplete products, it is difficult to make definitive conclusions regarding extract performance.

Reporter enzyme activity

The results of reporter assays performed in parallel unlabeled reactions are also shown in Table 1. With each template, the EcoPro T7 System produced substantially more active protein than the other extracts. As much as 28 µg full-length active protein (β-gal) was produced from a standard 50-µl EcoPro T7 reaction in 60 minutes, more than 5 times the

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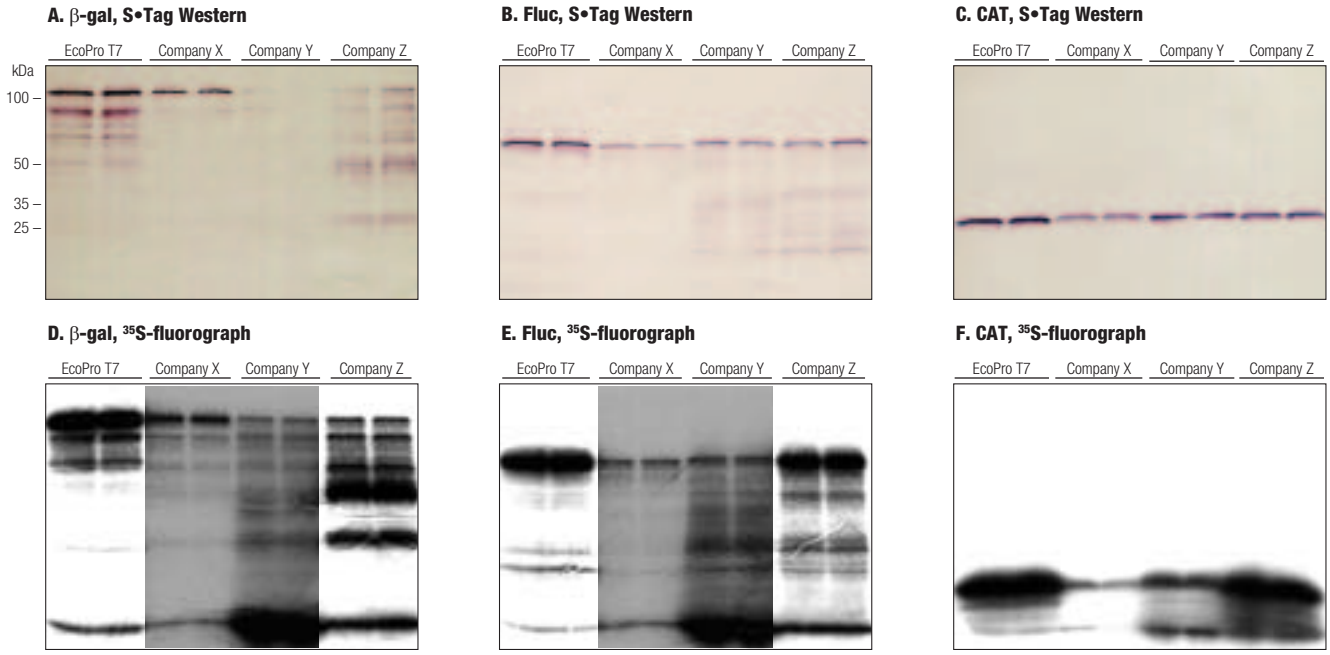


Figure 1. Gel analysis of translation products synthesized with four commercial kits

Plasmids encoding β-gal, Fluc and CAT were used in reactions according to the manufacturers' recommended protocols with their respective kit components. For fluorography of radioactive products (panels D, E, F), 40 µCi ^{35}S-methionine was added to each reaction at the time of template plasmid addition. Reactions were incubated for 60 min at 37°C. Samples from duplicate reactions were run side by side on 10–20% SDS-polyacrylamide gels followed by either transfer to nitrocellulose (Western blots, panels A, B, C) or fixation, treatment with Amplify™ (Amersham Biosciences), and exposure to x-ray film for 30 min (fluorography, panels D, E, F). The insets in panels D and E show exposures of the same gel to x-ray film for 5 h. Western blot detection was performed with the Novagen S•Tag AP Western Blot Kit.

nearest competitor. The reactions using extracts from Company X, Y, and Z produced 5, 1, and 0.4 µg of active β-gal, respectively. It should be noted that the extract from Company Z had high levels of endogenous β-gal as determined from controls lacking template DNA. These "blank" values were subtracted from samples containing template to reach the final yield of newly synthesized protein. This result clearly demonstrates the capacity of the EcoPro™ T7 System to produce full-length functional protein of large size and complex nature, especially given that the active site of β-gal resides near the C-terminus of each 116-kDa subunit, which must form tetramers for activity.

With the firefly luciferase template, the EcoPro T7 reactions again outperformed the competitor extracts, accounting for a final yield of 3.2 µg active protein. This is almost 150% higher than the nearest competitor. Particularly striking is the poor yield from Company Y's reactions, which averaged only 0.05 µg active protein.

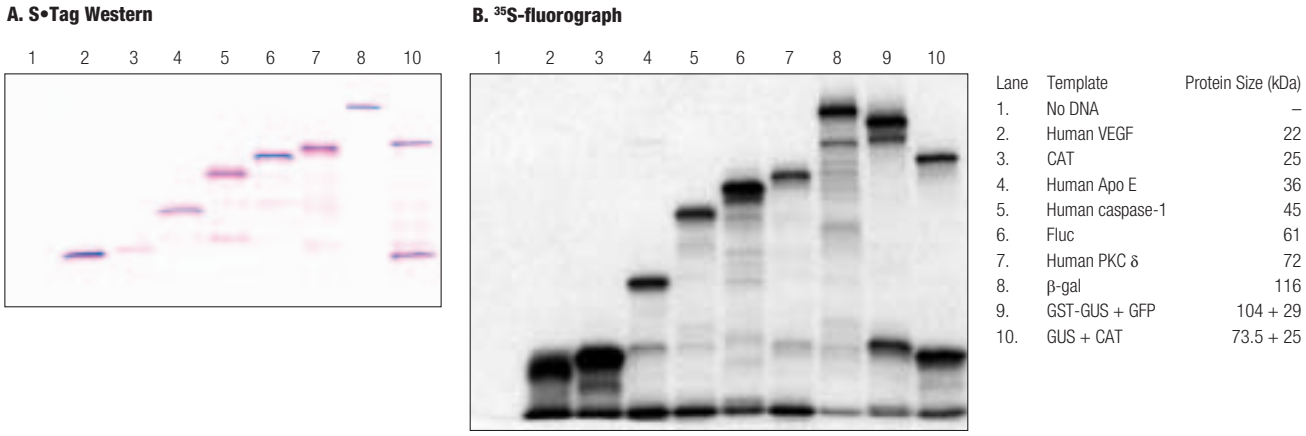
The EcoPro T7 reactions also synthesized the highest levels of CAT activity, producing 17 µg protein versus 3.6 µg, 10.6 µg, and 7.7 µg for Company X, Company Y, and Company Z, respectively.

Gel analysis

The extent of full-length protein production in each of the extracts was also examined by gel analysis. The reactions used for reporter enzyme analysis (Table 1) were analyzed by Western blotting as shown in Figure 1, panels A, B and C. For detection, we took advantage of the N-terminal 15-aa S•Tag™ peptide present in each of the reporter gene constructs in the pTriEx vector. This fusion tag is recognized with high specificity and affinity by Novagen's S-protein Alkaline Phosphatase (AP) Conjugate. Because the S•Tag sequence is on the N-terminus of each target protein, this method reveals any incomplete products that result from either nuclease activity or premature translation termination. As another measure of full-length protein production, ^{35}S-methionine-labeled reactions from Table 1 were used for fluorographic analysis, shown in Figure 1, panels D, E, and F.

As demonstrated in Figure 1, panels A and D, the EcoPro T7 reactions produced predominantly full-length β-gal. Extract from Company X also produced full-length protein, but considerably less than from EcoPro T7. Based only on ^{35}S-methionine incorporation (Table 1), Company Z would be expected to yield a significant amount of full-length protein. However, as seen on gels (Figure 1, panels A and D), the majority of protein consisted of incomplete translation products and full-length protein was only a small fraction of the total. The fluorographs from Company X and Company Y reactions show much less band intensity at the same exposure as EcoPro T7 and Company Z reactions, due to the presence of higher levels of endogenous methionine in the corresponding extracts. However, longer exposures revealed predominantly secondary products in the extracts from Company Y (Figure 1, panel D), in a pattern similar to that of the Company Z reactions.

Production of full-length firefly luciferase is shown in Figure 1, panels B and E. As expected from the enzyme activity data, the EcoPro T7 reactions yielded the most full-length protein. Significant secondary products were observed in both the Company Y and Company Z reactions. These products have been attributed to initiation of translation at internal methionine residues preceded by potential ribosome binding sites (4). Whereas our data cannot rule out this as a cause of some truncated products in



Lane	Template	Protein Size (kDa)
1.	No DNA	–
2.	Human VEGF	22
3.	CAT	25
4.	Human Apo E	36
5.	Human caspase-1	45
6.	Fluc	61
7.	Human PKC δ	72
8.	β-gal	116
9.	GST-GUS + GFP	104 + 29
10.	GUS + CAT	73.5 + 25

Figure 2. Expression of reporters, mammalian proteins and dual template proteins using the EcoPro T7 system

A. pTriEx recombinant plasmids encoding the indicated S•Tag fusion proteins were added to standard EcoPro T7reactions and incubated at 37°C for 60 min. Samples were precipitated with acetone and run on a 10–20% SDS-polyacrylamide gel followed by Western blotting and detection with S-protein AP Conjugate. The amounts of vector DNA used were as follows: VEGF, 4 µg; CAT, 1 µg; Apo E, 2 µg; caspase-1, 2 µg; Fluc, 1 µg; PKC δ, 4 µg; β-gal, 1 µg; GUS, 1 µg; and CAT, 1 µg.

B. Two micrograms of the indicated templates (as in A) were incubated in standard EcoPro T7 reactions containing 40 µCi ^{35}S-methionine for 60 min at 37°C. Samples were diluted in 1X SDS Sample Buffer and run on a 10–20% SDS-polyacrylamide gel followed by treatment with Amplify™ (Amersham Biosciences) and exposure to x-ray film for 30 min.

the EcoPro™ T7 and Company X reactions, the relative lack of such products in these reactions indicates that they may have another source.

Figure 1, panels C and F (page 4) show the translation products of the CAT template. CAT is a small enzyme which leads to higher turnover with less probability of producing truncated products. The Western blotting and fluorographic data support this, as none of the extracts showed appreciable secondary products. Although the differences between the competitors were less dramatic with this template, the highest yields of intact protein were again obtained with the EcoPro T7 reactions.

EcoPro T7 performance with additional templates

The results described above show that high yields of active full-length reporter enzymes can be synthesized with the EcoPro T7 System. However, for the system to be generally useful it should also perform well with templates derived from various sources, including mammalian cDNAs. Figure 2 shows both an S•Tag™ Western blot and an ^{35}S-fluorograph of the products of seven different templates encoding S•Tag fusion proteins. In addition to reporter enzymes CAT, β-gal, and β-glucuronidase (GUS), the EcoPro T7 extracts efficiently synthesized full-length human vascular endothelial growth factor (VEGF), apolipoprotein E (Apo E), caspase-1, and protein kinase C δ (PKC δ), (lanes 2, 4, 5, and 7, respectively). Another advantage of the EcoPro T7 System is that multiple proteins can be expressed in a single reaction. pTriEx™ plasmids encoding GUS and CAT were added separately to an EcoPro T7 reaction, and both proteins were produced at approximately the same levels as individual reactions (lane 10). The fluorograph in Figure 2, panel B shows the corresponding reactions in Figure 2, panel A performed in the presence of ^{35}S-methionine, plus another reaction (lane 9) that contained a pETDuet™-1 recombinant plasmid expressing both green fluorescent protein (GFP) and a glutathione S-transferase-GUS (GST-GUS) fusion protein (5). The ability to produce two proteins simultaneously in the EcoPro T7 reaction facilitates the investigation of protein-protein interactions as well as the *in vitro* synthesis of heterodimers or multimeric complexes. In applications where multiple proteins are needed, the advantage of the EcoPro T7 System in producing predominantly full-length molecules is especially critical.

EcoPro T7 Quarters™ System

The standard EcoPro T7 System includes five 350-µl vials of extract and is designed to perform 50 standard 50-µl reactions. To provide for higher-throughput sample processing, we have developed a multi-well plate format called the EcoPro T7 Quarters System. EcoPro T7 extract is predispensed in aliquots designed for 25-µl reaction volumes in a 24-well module compatible with 96-well processing formats. Figure 3, panel A (page 6) shows four 24-well modules with the HT96™ Isothermal Block, which is available separately as a means to ensure even incubation temperature.

To test the performance of the EcoPro T7 Quarters System, we used 24 individual pET and pTriEx vector recombinants expressing various human cDNAs as S•Tag fusion proteins. Purified plasmid DNA from each clone was added to the thawed EcoPro T7 extract and reactions were performed according to the standard protocol. Completed reactions were precipitated with acetone and equal portions examined by Western blotting with detection by S-protein AP Conjugate. Figure 3, panel B (page 6) shows that all 24 reactions produced proteins of the predicted size, and in 22 of 24 reactions the prominent protein band was the full-length molecule. In the other two samples, a protein of expected size was produced but other bands were more prominent (arrows indicate the predicted size).

The performance of the EcoPro T7 Quarters System in this experiment suggests that it can be used for *in vitro* synthesis of "unknown" mammalian proteins with a high success rate. The appearance of secondary bands, although detectable, was no greater than that usually observed with eukaryotic extracts, and expression levels were high enough and consistent enough between samples to allow rapid

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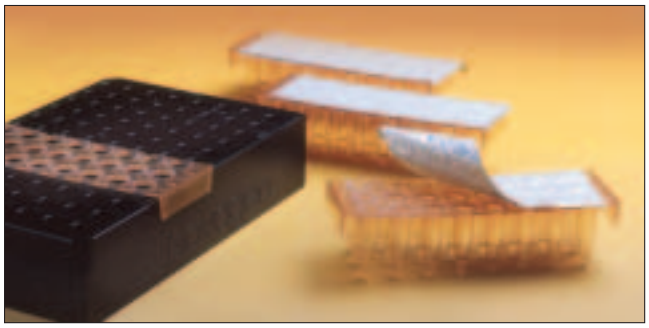
A. EcoPro T7 Quarters System



B. S•Tag Western blot of EcoPro T7 Quarters reactions

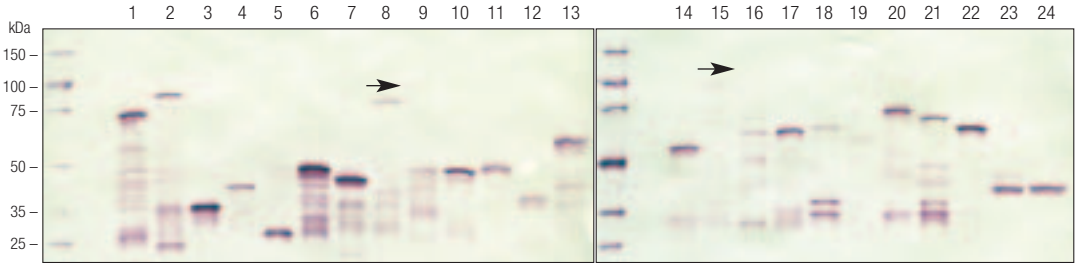


Figure 3. EcoPro T7 Quarters System for high-throughput transcription/translation

A. Photograph of EcoPro T7 Quarters modules with the HT96™ Isothermal Block. The Quarters format consists of 24-well and 4 × 24-well groups containing predispensed EcoPro T7 extracts ready for processing using multichannel pipetting devices or automated workstations.

B. Various human cDNAs were cloned into pTriEx™-4, pET-32b(+), and pET-41b(+) vectors to express different S•Tag™ fusion proteins. The recombinant plasmids were purified using the Mobius™ 200 Plasmid Kit and then tested for protein expression. One microgram of each plasmid was used in EcoPro T7 Quarters System reactions with the standard protocol. Samples were precipitated with acetone, suspended in 1X SDS loading buffer and loaded onto 10–20% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes and detected using the S•Tag AP Western Blot Kit. The Perfect Protein™ Western Markers are shown in the left-hand lane of each blot, followed by a negative control reaction without template. Lane numbers indicate the different templates.

detection by Western blotting. It also appears that any codon bias potentially caused by disparate levels of *E. coli* tRNA versus representation of mammalian codons in the target genes was not a factor in the translation efficiency of these samples. We have also observed little effect on translation efficiency of mammalian proteins known to be affected by codon bias *in vivo* using EcoPro™ T7 extracts prepared from cells containing six additional tRNAs representing codons rarely used in *E. coli* (data not shown). This apparent lack of codon bias is probably due to the supplementation of EcoPro T7 extract with exogenous tRNAs.

Summary and discussion

Our results show that the EcoPro T7 System significantly outperformed competitor systems with respect to the key feature of producing full-length proteins. The EcoPro T7 System also was capable of producing easily detectable amounts of full-length proteins from a variety of mammalian cDNA templates and could be used to coexpress multiple proteins in the same reaction. The unique properties of the EcoPro T7 System can be attributed to the fractionation process used to produce the bacterial extract, which removes factors that cause the accumulation of truncated proteins without compromising overall translation activity. The quality of the translation products with the EcoPro T7 System more closely resembles that of reticulocyte or wheat germ systems in terms of the proportion of full-length molecules, but with much greater yields. Whereas optimal yields for these eukaryotic systems are in the range of 100 ng per 50-µl batch reaction, the EcoPro T7 System can produce micrograms to tens of micrograms in the same volume and reaction time. The EcoPro T7 Quarters™ System is now available to provide a robot-friendly, high-throughput format for convenient processing of multiple samples.

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Product	Size	Cat. No.
EcoPro™ T7 System (includes EcoPro T7 Extracts, 5 mM Methionine, Nuclease-free Water, and EcoPro Control DNA)	50 rxn	70876-3
Introductory EcoPro T7 System (includes EcoPro T7 Extracts, 5 mM Methionine, Nuclease-free Water, and EcoPro Control DNA)	10 rxn	70888-3
EcoPro T7 Quarters™ System [includes EcoPro T7 Extracts, 5 mM Methionine, Nuclease-free Water, EcoPro Control DNA, Aluminum Plate Sealer(s), and 8-cap strips]	24 rxn 4 × 24 rxn	71231-3 71231-4
HT96™ Isothermal Block		71195-3

A novel high-level transient protein expression and purification system for Sf9 insect cells that facilitates automated screening

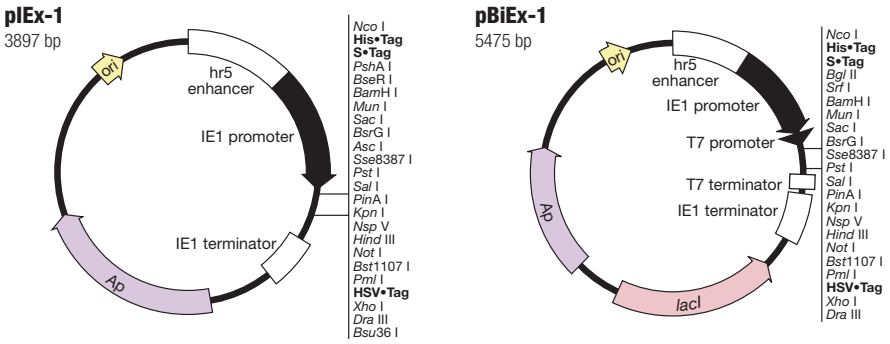
Kathryn Loomis, Robert Novy, and Keith Yaeger – Novagen

A fundamental challenge in high-throughput (HT) expression screening is to rapidly identify the appropriate expression system for hundreds or thousands of individual targets in parallel. Known or unknown open reading frames (ORFs) are typically PCR-amplified and then cloned into a variety of vectors and the recombinants used to direct target protein expression in *E. coli*, insect cells, mammalian cells or yeast. Expression in insect cells is commonly performed using a baculovirus system, which requires the construction of baculovirus recombinants. Unfortunately, baculovirus-based systems are not well suited for HT expression screening due to the time and complexities involved in creating, titering and maintaining the viral recombinants. In addition, given the trend toward miniaturization in HT protein characterization processes, less and less protein is typically required to perform downstream analyses. Therefore, baculovirus-based expression could be avoided for some applications if alternative HT-friendly insect expression systems were available.

As a major step toward this goal, Novagen has developed the pIEx™ and pBiEx™ series of transient expression vectors. The pIEx series is designed for optimal expression in *Spodoptera* cell lines and is available in an HT-compatible Ligation Independent Cloning format, Ek/LIC. The pBiEx series is designed for expression of the same target gene in both *E. coli* and insect cells, which enables screening in both systems with the same set of constructs. In addition, Novagen has developed a high-efficiency insect cell transfection reagent and automation-compatible His•Tag® fusion protein purification system for insect cells to facilitate expression screening.

pIEx and pBiEx vectors for high-yield, nonlytic expression in insect cells

Novagen's new pIEx and pBiEx vectors feature an optimal combination of AcNPV baculovirus-derived transcription elements, the hr5 enhancer and the IE1 (immediate early) promoter, to direct expression in insect cells (1–5). This promoter/enhancer combination recruits endogenous insect cell transcription machinery, thereby avoiding baculovirus infection and its associated cytopathic effects. Although hr5/IE1-based plasmids, including the pIE1 vectors offered by Novagen, have been primarily used to create stable Sf9 insect cell lines that continuously express low levels of target proteins, we have discovered that this enhancer/promoter combination produces dramatically higher expression levels in transient transfection applications. The features of the pIEx and pBiEx vectors are shown in Figure 1.



Vector	Promoter(s)	Signal sequence	Fusion tags		Protease cleavage sites	Ek/LIC vector
			N-terminal	C-terminal		
pIEx-1	hr5/IE1	No	His•Tag/S•Tag™	HSV•Tag®	Tb/Ek	Yes
pIEx-2	hr5/IE1	No	GST•Tag™/His•Tag/S•Tag	HSV•Tag	Tb/Ek	Yes
pIEx-3	hr5/IE1	Yes	GST•Tag/His•Tag/S•Tag	HSV•Tag	Tb/Ek	Yes
pIEx-4	hr5/IE1	No	None	S•Tag/His•Tag	None	No
pIEx-5	hr5/IE1	Yes	None	S•Tag/His•Tag	None	No
pBiEx-1	hr5/IE1, T7*lac*	No	His•Tag/S•Tag	HSV•Tag	Tb/Ek	No
pBiEx-2	hr5/IE1, T7*lac*	No	GST•Tag/His•Tag/S•Tag	HSV•Tag	Tb/Ek	No
pBiEx-3	hr5/IE1, T7*lac*	No	None	S•Tag/His•Tag	None	No

Figure 1. pIEx and pBiEx vectors

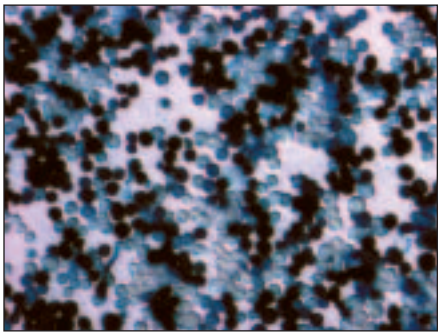


Figure 2. Sf9 cells transfected with pIEx-1/β-gal using Insect GeneJuice Transfection Reagent

Cells were washed 3X with HBSS (Hanks Buffered Saline Solution), fixed with 0.5% glutaraldehyde for 5 min at room temperature, and washed again 3X with HBSS. The cells were then stained for β-gal activity using Novagen's BetaBlue™ Staining Kit and incubated at 37°C for 8 h.

Efficient transfection using Insect GeneJuice™ Transfection Reagent

In addition to the promoter strength, expression levels in transient transfection experiments depend on the successful introduction of the target plasmid DNA into cells and their survival following treatment. To address this need we have developed Insect GeneJuice Transfection Reagent, a liposome-based formulation

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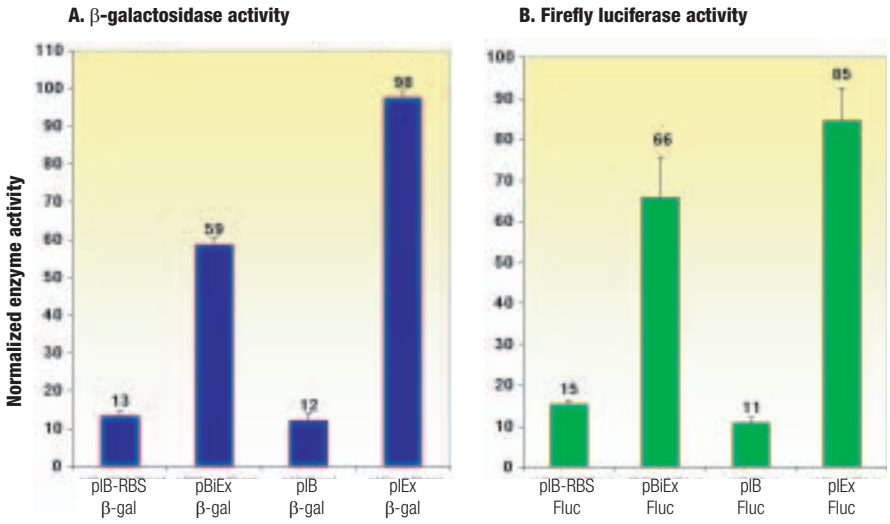


Figure 3. Reporter enzyme activity in Sf9 cells transfected with pIEx, pBiEx and pIB recombinants

A. β-galactosidase assays. Purified plasmid DNA (0.4 µg) from pIEx-1/β-gal and pIB/β-gal constructs was mixed with Insect GeneJuice™ Transfection Reagent using a 4:1 ratio of transfection reagent to plasmid. Both components were diluted in serum-free BacVector® Insect Cell Medium prior to mixing and complex formation. The plasmid–transfection reagent–media mixture was applied to Sf9 cells for 4 h followed by removal and replacement with fresh medium supplemented with 5% FBS. Forty-eight h post-transfection, cells were lysed with Reportasol™ Extraction Buffer. The concentration of total cell protein in the lysate was determined by a BCA assay and β-gal activity measured using Novagen's BetaRed™ β-Gal Assay Kit. Readings were normalized based on the total protein concentration of each sample.

B. Firefly luciferase assays. Transfection, extract preparation, and protein determinations were performed using pIEx-1/Fluc and pIB/Fluc constructs as described in panel A. Fluc activity was assayed using a luciferin substrate (Promega) and Fluc readings normalized based on the total protein concentration of each sample.

optimized for maximal transfection efficiency of *Spodoptera* insect cells with minimal toxicity. The reagent can be used for both transient and stable transfections in serum-containing or serum-free media . Figure 2 (page 7) shows the high transfection efficiencies obtained with this reagent for the transfection of pIEx™-1 reporter plasmids into Sf9 cells.

Cloning with the pIEx and pBiEx™ vectors

An extensive region of multiple cloning sites is available in both pIEx and pBiEx series for cloning target genes by a variety of restriction enzyme approaches. Two reporter enzymes, β-galactosidase (β-gal) and firefly luciferase (Fluc), were chosen to test the performance of Novagen's new vectors and reagents. β-gal and Fluc were subcloned using traditional restriction enzyme methods. *Nco* I/*Not* I fragments of these genes including N-terminal His•Tag®/S•Tag™ fusions were inserted into the *Nco* I/*Not* I sites of pIEx-1 and pBiEx-1 to create the corresponding recombinants. For comparative purposes, the same fragments were also cloned into an insect expression vector based on the OpNPV (*Orgyia pseudotsugata*) OpIE2 promoter, pIB/His B (Invitrogen). To ensure a valid comparison, we created the same translation initiation sequence in the pIB constructs as in the pIEx and pBiEx recombinants. Although the pIB vector contains an *Nco* I site overlapping the translation initiation ATG start site, the site is not unique. Therefore, it was necessary to cut the vector with *Nsp* V (slightly upstream of *Nco* I) and *Not* I and insert the reporter genes with the aid of *Nsp* V–*Nco* I adaptors. To create comparable constructs to the pIEx vectors, the adaptor recreated the original pIB vector sequence upstream of the ATG start site. The resulting pIB clones were designated pIB/β-gal and pIB/Fluc. To create comparable constructs to the pBiEx vectors, the adaptor was designed to include the same *E. coli* ribosome binding site (RBS; 5′-untranslated region from –13 to –1) as that found in the pBiEx vectors. The resulting pIB clones were designated pIB-RBS/β-gal and pIB-RBS/Fluc, respectively. All vector/insert ligation reactions were performed using the Clonables™ 2X Ligation Premix.

The pIEx-1 and pIEx-2 vectors are also available as Ek/LIC vectors, which are linearized and treated with T4 DNA polymerase in the presence of dTTP to create long, single-stranded overhangs. Inserts are amplified with special primers and similarly treated to create corresponding overhangs that anneal with the vector. This strategy circumvents the use of restriction enzymes and features very low background. In addition, the Ek/LIC cloning strategy includes a recognition site for enterokinase as part of the LIC overhang, which allows the removal of all N-terminal vector-encoded tags from fusion proteins. Two kinase cDNAs, a mouse MAP kinase and human cdc2 kinase, were amplified from Novagen's cDNA libraries and cloned into the pIEx-1 and pIEx-2 Ek/LIC Vectors.

All recombinants were established in NovaBlue Singles™ Competent Cells. Transfection-quality plasmid DNA was prepared using the UltraMobius™ 200 Plasmid Kit. The vector-insert junctions of

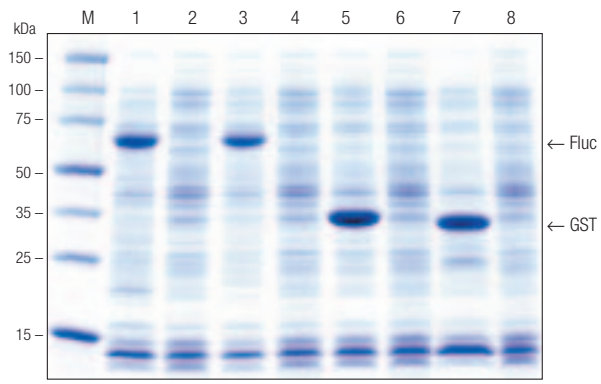


Figure 4. pBiEx expression results in *E. coli*

Duplicate Tuner™(DE3) cultures of the indicated recombinants were grown at 37°C in LB broth + 0.5% glucose to an OD_{600} between 0.6 and 1.0. One set of cultures received 1 mM IPTG and incubation continued for another 3 h at 37°C. Total cell protein samples (representing equivalent numbers of cells) from induced and uninduced cultures were loaded in adjacent lanes of a 10–20% SDS-polyacrylamide gel. Bands were visualized with Coomassie blue staining.

all recombinants were verified by sequence analysis.

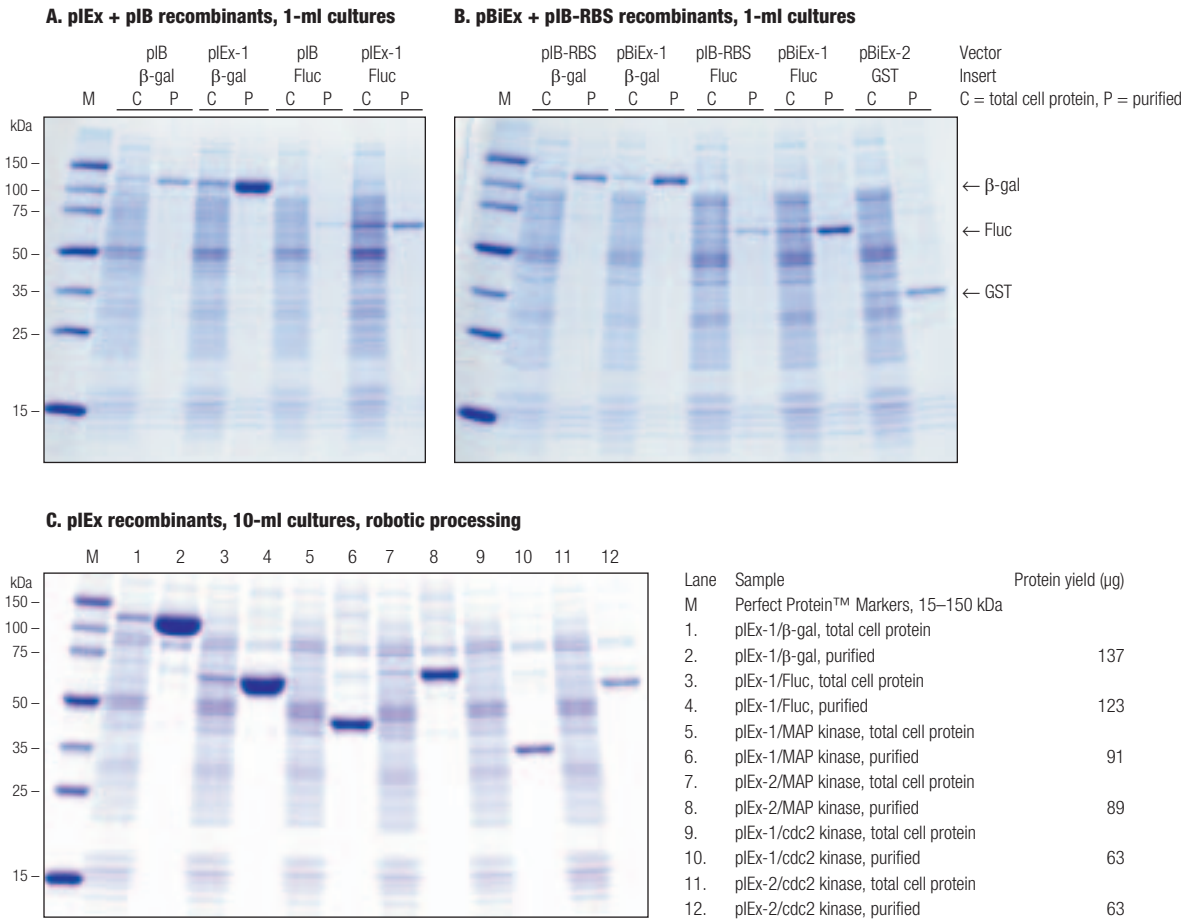
Expression in Sf9 insect cells

To compare transient expression levels, Insect GeneJuice™ Transfection Reagent was used to transfect the pIEx™, pBiEx™ and pIB β-gal and Fluc constructs into Sf9 cells grown in 24-well plates (2×10^5 cells/well). Forty-eight hours post-transfection the cells were lysed by the addition of Reportasol™ Extraction Buffer, which is optimized for gentle extraction and preservation of reporter enzyme activity. Results of β-gal and Fluc assays of the extracts are shown in Figure 3 (page 8). With both reporter genes, cells transfected with the pIEx constructs yielded activity 8-fold higher than the pIB constructs. Although the pBiEx constructs produced about 60% of the activity observed with the corresponding pIEx vectors, they were still 4.5-fold higher than the comparable pIB-RBS constructs. The difference in performance cannot be attributed to significant differences in transfection efficiencies because the relevant pIEx and pIB recombinants do not differ greatly in size and all plasmids were prepared in parallel using the same procedure. As noted above, the pIB controls contained the same immediate 5′-UTR and translation initiation sequence as the pBiEx vectors to ensure that differences in expression could not be due to sequence discrepancies.

Expression in *E. coli* using pBiEx

To test the performance of the pBiEx vectors in *E. coli*, pBiEx-1/Fluc, pBiEx-2 (which encodes a GST•Tag™/His•Tag®/S•Tag™/HSV•Tag® fusion) and two comparable pET vector controls, pET-28b(+)/Fluc and pET-41b(+), were transformed into the *E. coli* expression host Tuner™(DE3). Total cell protein fractions of both induced and uninduced cultures were analyzed by SDS-PAGE. Figure 4 (page 8) shows that, based on the Coomassie blue-stained target band intensities, the expression level obtained from the pBiEx constructs was equivalent to that obtained from the pET constructs. These results verify that pBiEx constructs can also be used for high-yield expression in *E. coli*.

It should be noted that like the pIEx vectors, the pBiEx vectors are high-copy number plasmids, which facilitates purification of sufficient quantities of DNA for transfection studies. The pBiEx vectors also contain the *lacI* gene to provide suf-



Lane	Sample	Protein yield (µg)
M	Perfect Protein™ Markers, 15–150 kDa	
1.	pIEx-1/β-gal, total cell protein	
2.	pIEx-1/β-gal, purified	137
3.	pIEx-1/Fluc, total cell protein	
4.	pIEx-1/Fluc, purified	123
5.	pIEx-1/MAP kinase, total cell protein	
6.	pIEx-1/MAP kinase, purified	91
7.	pIEx-2/MAP kinase, total cell protein	
8.	pIEx-2/MAP kinase, purified	89
9.	pIEx-1/cdc2 kinase, total cell protein	
10.	pIEx-1/cdc2 kinase, purified	63
11.	pIEx-2/cdc2 kinase, total cell protein	
12.	pIEx-2/cdc2 kinase, purified	63

Figure 5. Target protein expression levels and purification from transfected Sf9 cells

Sf9 cultures in 6-well plates (1×10^6 cells in 1 ml/well; panels A and B) or 10-ml suspension cultures (1×10^6 cells/ml; panel C) were transfected with 2 µg and 20 µg, respectively, of the indicated plasmids using Insect GeneJuice Transfection Reagent. Total culture extracts were prepared 48 h later by the addition of Insect PopCulture™ Reagent (50 µl for 1-ml cultures and 500 µl for 10-ml cultures) followed by the addition of Benzonase® Nuclease (0.5 µl for 1-ml cultures and 5 µl for 10-ml cultures). Samples were taken at this point to represent the total cell protein. Ni-NTA His•Bind® Resin (50 µl per culture) was then added to the extracts. The 1-ml cultures were processed manually and the 10-ml cultures were processed robotically using a MultiPROBE® II HT EX Liquid Handling Station (PerkinElmer). Target protein purified from the 1- and 10-ml cultures was eluted in a volume of 100 µl and 150 µl, respectively. Ten microliters of the crude and purified fractions from each transfection were loaded in adjacent lanes of a 10–20% SDS polyacrylamide gel, which was stained with Coomassie blue. Purified protein yields were determined by BCA assay.

continued on page 10

continued from page 9

ficient *lac* repressor for low basal expression in λDE3 lysogenic hosts.

Processing Sf9 cell cultures with the Insect RoboPop Ni-NTA Purification Kit

A complete system for automated expression screening must also provide for compatible cell lysis and target protein purification. Novagen has recently developed the Insect RoboPop™ Ni-NTA His•Bind® Purification Kit to fill this need. This kit includes Novagen's Insect PopCulture™ Reagent to extract proteins from total transfected cultures without centrifugation, Benzonase® Nuclease to reduce viscosity arising from liberated nucleic acids, Ni-NTA His•Bind Resin and buffers to purify the target protein, and the required 96-well filtration and collection plates. The improved method provided by Insect PopCulture–based extraction increases processing efficiency and target protein yields primarily due to the inclusion of both medium and cell fractions in the total culture extract (6). Insect PopCulture Reagent can be used for protein extraction from insect cells in suspension or adherent cells on tissue culture plates. The kit is configured for robotic or manual processing of transfected 10-ml suspension cultures. Insect PopCulture Reagent and Benzonase Nuclease are added directly to the suspension cell culture. The lysate is transferred to an appropriate-size tube or deep-well plate for robotic processing. Ni-NTA His•Bind Resin is added to the lysate to bind His•Tag® fusion proteins. After mixing, the lysate-resin mixture is transferred to a filter plate to capture the affinity resin and bound target protein while the unbound contaminants are removed by filtration. Following wash steps, the purified His•Tag fusion protein is eluted from the resin and captured in a collection plate for further analysis.

Protein purification

To ascertain the yield and purity of target protein that can be obtained using a transient expression approach, various recombinant plasmids were transfected into Sf9 cells grown in either 6-well plates or in 10-ml suspension cultures. In separate experiments, β-gal and Fluc recombinants were used for the 6-well plates and pIEx™-1/β-gal, Fluc and kinase recombinants used for the 10-ml suspension cultures. Total culture extracts were prepared 48 hours post-transfection by the addition of Insect PopCulture Reagent followed by Benzonase Nuclease. The 10-ml cultures were processed robotically using a MultiProbe® II HT EX Liquid Handling Station (PerkinElmer) and the 6-well cultures were processed manually. Figure 5 (page 9) shows the Coomassie blue–stained SDS-polyacrylamide gel and protein yields. Substantially higher yields were obtained from the pIEx constructs than from the pIB constructs, which correlated with the enzyme assay data. The pBiEx constructs also yielded more protein than the pIB-RBS control constructs but less than the pIEx clones. The robotically processed 10-ml pIEx transfections expressing β-gal, Fluc, MAP kinase and cdc2 kinase also gave good yields of highly purified protein. Protein yields ranging from 6 to 14 µg/10^6 cells were obtained for these targets (60–140 µg per 10 ml culture using 50 µl resin).

Summary

The goal in developing the vectors, transfection reagent, and purification products described in this article was to create a system that facilitates HT expression screening in insect cells. The pIEx and pBiEx vectors are a key element in this system because they enable the expression of high levels of target protein in small-scale insect cell cultures using a transient transfection protocol. Direct comparisons of the performance of pIEx, pBiEx, and pIB vectors showed that pIEx generated 8-fold and pBiEx 4.5-fold higher reporter activity relative to pIB, and these results correlated with gel analysis. Expression levels in *E. coli* using pBiEx constructs were comparable to analogous pET vector constructs. Finally, we were able to purify 60–140 µg target protein from 10 ml transfected Sf9 cell cultures using several test proteins, including mammalian kinases, with an automated process. Greater yields can be obtained by increasing the amount of resin per sample to 100 µl (200 µl slurry); this amount is provided in the Insect RoboPop Ni-NTA His•Bind Purification Kit.

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6. Loomis, K., Grabski, A., and Wong, S. C. (2002) *inNovations* **15**, 16–17.

Product	Size	Cat. No.
pIEx™-1 DNA	20 µg	71241-3
pIEx-2 DNA	20 µg	71238-3
pIEx-3 DNA	20 µg	71243-3
pIEx-4 DNA	20 µg	71235-3
pIEx-5 DNA	20 µg	71242-3
pBiEx™-1 DNA	20 µg	71234-3
pBiEx-2 DNA	20 µg	71233-3
pBiEx-3 DNA	20 µg	71232-3
pIEx-1 Ek/LIC Vector Kit*	20 rxn	71237-3
pIEx-2 Ek/LIC Vector Kit*	20 rxn	71240-3
pIEx-3 Ek/LIC Vector Kit*	20 rxn	71245-3
pIEx-1 Ek/LIC Vector (linearized vector)	1 µg	71236-3
pIEx-2 Ek/LIC Vector (linearized vector)	1 µg	71239-3
pIEx-3 Ek/LIC Vector (linearized vector)	1 µg	71244-3
AS HSV•Tag® Primer	500 pmol	71246-3
AS S•Tag™ 18mer Primer	500 pmol	71262-3
IE1 Terminator Primer	500 pmol	71247-3
Insect GeneJuice™ Transfection Reagent	0.3 ml 1 ml 10 × 1 ml	71259-3 71259-4 71259-5

* Kits include Ek/LIC Vector, Ek/LIC β-Gal Control Insert, LIC-qualified T4 DNA Polymerase, T4 DNA Polymerase Buffer, 100 mM DTT, 25 mM EDTA, 25 mM dATP, Nuclease-free Water, SOC Medium, Test Plasmid, and NovaBlue GigaSingles™ Competent Cells.

Product	Cat. No.
Insect RoboPop™ Ni-NTA His•Bind® Purification Kit	71257-3

Components:

- 50 ml Insect PopCulture™ Reagent
- 10 KU Benzonase® Nuclease, Purity > 90%
- 10 ml Ni-NTA His•Bind Resin
- 125 ml 4X Ni-NTA Bind Buffer
- 2 × 125 ml 4X Ni-NTA Wash Buffer
- 50 ml 4X Ni-NTA Elute Buffer
- 1 2-ml 96-well Filter Plate
- 1 Collection Plate with Sealer

Note: 1 KU = 1000 units

Automated solubility screening of recombinant proteins in a 96-well format

Anthony Grabski, Don Drott, and Mark Mehler — Novagen

Bacterial expression systems are frequently employed to produce large quantities of heterologous protein for structural and functional analysis irrespective of the source, sequence, or abundance of the protein in its natural host. Unfortunately, the ease and efficiency of bacterial expression systems for recombinant protein production do not always correlate with high yield of soluble, correctly folded, active protein. Instead, insoluble inclusion bodies are formed due to rapid, high expression of the protein, inadequate or low concentrations of chaperone helper proteins, complexities of folding, and limited solubility of folded domains (1). The yield of soluble, correctly folded protein can often be increased by optimizing the primary sequence of the target protein, the genetic background of the host strain, and growth conditions, such as temperature and induction methods (2). Conventional methods for screening the effectiveness of these solubility optimization experiments are tedious and inefficient. These methods typically involve cell harvest by centrifugation, mechanical disruption by sonication or French press, and separation of soluble proteins from insoluble proteins, debris and residual intact cells by a second centrifugation step. The soluble supernatant fraction is subsequently analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and/or activity assay to detect and quantify the presence and quality of the target protein.

We describe here a novel high throughput–compatible method for protein solubility screening. The procedure incorporates a nonfouling filtration plate capable of retaining insoluble inclusion bodies while allowing soluble proteins to be collected for rapid quantification and analysis.

Parallel processing of hundreds of samples for high-throughput (HT) proteomics research requires biological, chemical, and engineering solutions to eliminate tasks that are difficult to automate, such as sonication and centrifugation, while minimizing multiple processing steps. We have developed specialized lysis reagents that eliminate the need for mechanical disruption of cells. These reagents include BugBuster®, YeastBuster™, and CytoBuster™ Protein Extraction Reagent, for *E. coli*, yeast, and insect or mammalian cells, respectively. The effectiveness of BugBuster Reagent and the need to simplify the purification process for automation led us to the development of PopCulture™ Reagent. PopCulture is a concentrated mixture of specialized detergents that when combined with rLysozyme™ Solution and Benzonase® Nuclease enables extraction and purification of recombinant proteins from *E. coli* directly from the culture media without cell harvest, mechanical disruption, or extract clarification. The combined activities of rLysozyme and Benzonase Nuclease are now available as Lysonase™ Bioprocessing Reagent (see page 20). Lysonase significantly increases protein extraction efficiency and reduces sample viscosity, thereby facilitating downstream processing and robotic pipetting. PopCulture, rLysozyme, Benzonase, affinity resins, and plasticware are conveniently formatted into the RoboPop™ Protein Purification Kits for automated protein purification from *E. coli* or insect cells.

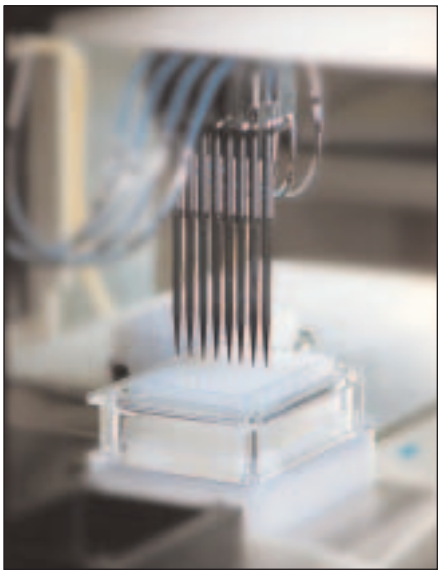


Figure 1. MultiPROBE II HT EX workstation set up for automated solubility screening

We have now expanded the applications of PopCulture and Lysonase for use in parallel or automated expression-level solubility screening. We describe here a novel high throughput–compatible method for protein solubility screening.

Table 1. RoboPop solubility screening protocol

1. Culture *E. coli* cells in liquid medium under conditions for target protein production.
2. Add 0.1 culture volume premixed PopCulture Reagent + Lysonase Bioprocessing Reagent to each culture, mix, and incubate 10 min at room temperature.
3. Place the 96-well Collection Plate and Filter Plate into the vacuum manifold.
4. Transfer 200 µl extract from each culture to the 96-well Filter Plate.
5. Apply vacuum*, collecting the flow-through containing soluble proteins in the Collection Plate.
6. Remove the Collection Plate containing the soluble fraction and replace it with a new Collection Plate.
7. Add 200 µl 4% SDS denaturing solution to each well of the Filter Plate and incubate 10 min at room temperature. This step solubilizes the inclusion body fraction.
8. Apply vacuum*, collecting the solubilized proteins in a Collection Plate.
9. Quantify target proteins present in soluble and insoluble fractions (step 5 and step 9, respectively). His•Tag® fusion proteins may be detected by Western blotting or ELISA using the His•Tag Monoclonal Antibody or His•Tag Antibody Plates, respectively. S•Tag™ fusion proteins may be quantified directly using the FRETWorks™ S•Tag assay. Activity of native proteins may be assayed directly from the soluble fraction. SDS-PAGE followed by staining may also be used for highly expressed proteins.

* Samples may be collected by substituting centrifugation of the Filter Plate over the Collection Plate at 2000 × g for 5 min.

continued on page 12

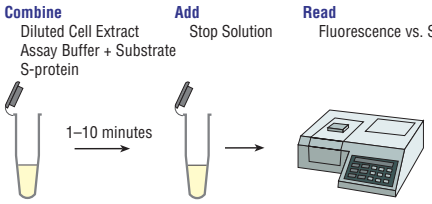
The procedure incorporates a nonfouling filtration plate capable of retaining insoluble inclusion bodies while allowing soluble proteins to be collected for rapid quantification and analysis. Insoluble proteins retained by the filtration plate are solubilized with 4% SDS denaturing solution, collected, and quantified separately. The protocol has been automated on the Packard-brand MultiPROBE® II from PerkinElmer Life Sciences (Figure 1, page 11). The general protocol we used for automation is described in Table 1 (page 11).

Solubility screening of fusion proteins from *E. coli* total culture extracts

We used four different plasmid recombinants for expression-level solubility screening, as listed in Table 2. All of these fusion proteins can be purified by immobilized metal chelation chromatography using His•Bind® affinity resins and detected using either the His•Tag® Monoclonal Antibody or the FRETWorks™ S•Tag™ Assay (Figure 2). Figure 3 shows the results of a solubility screening experiment. The data demonstrate the effectiveness of the RoboPop™ Solubility Screening Kit for fractionation and quantification of the expressed soluble and insoluble target protein from each construct. The SDS-PAGE analysis (Figure 3, panel A) shows that under the expression conditions tested, GUS was entirely insoluble, β-gal was approximately 50% soluble, NusA was approximately 90% soluble, and GST was approximately 75% soluble. Although the fractions obtained with the kit may be analyzed by conventional SDS-PAGE to estimate the degree of soluble target protein expression, sensitivity and throughput are low and quantification is difficult by this method. A highly sensitive, robot-friendly protocol has been developed to quantify S•Tag fusion proteins in crude extracts using the homogeneous FRETWorks S•Tag Assay (3). This assay is based on the affinity of the 15–amino acid S•Tag peptide in the fusion proteins for the 104–amino acid S-protein in the assay buffer. The S•Tag–S-protein interaction reconstitutes ribonuclease activity, cleaving the FRET substrate and producing a fluorescent signal as the quencher is released from the fluorescent molecule. Results of the FRETWorks assay for quantification of

Table 2. Vector constructs used for analysis in Figure 3

Vector/construct	Fusion protein – "name"	Expected size
pET-41b(+)	GST•Tag™/His•Tag/S•Tag – "GST"	35.6 kDa
pET-30b(+)/β-gal	His•Tag/S•Tag/β-gal – "β-gal"	121 kDa
pET-43.1b(+)	Nus•Tag™/His•Tag/S•Tag/HSV•Tag® – "NusA"	66.4 kDa
pTriEx™-4/GUS	His•Tag/S•Tag/GUS/HSV•Tag – "GUS"	73.5 kDa

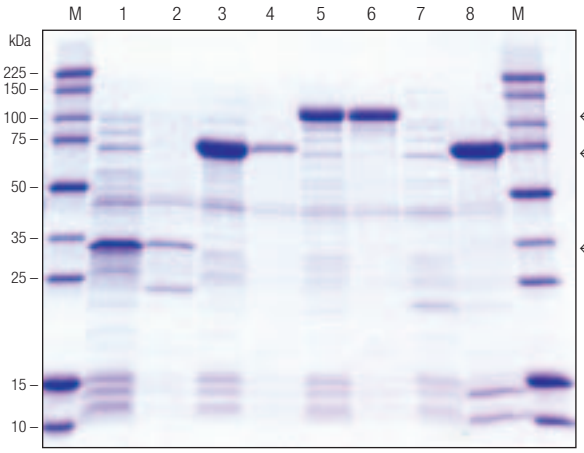


FRETWorks S•Tag Assay

Substrate	FRET ArUAA
Endpoint	fluorescence (520 nm)
Elapsed time	< 10 min
Detection limit	< 1 fmol

Figure 2. FRETWorks S•Tag Assay

A. SDS-PAGE analysis of soluble and insoluble fractions



Lane	Sample
M	Perfect Protein™ Markers, 10–225 kDa
1.	GST, soluble
2.	GST, insoluble
3.	NusA, soluble
4.	NusA, insoluble
5.	β-gal, soluble
6.	β-gal, insoluble
7.	GUS, soluble
8.	GUS, insoluble

B. FRETWorks S•Tag Assay of soluble and insoluble fractions

Construct	Fraction	pmol/ml	µg/ml	% in fraction
GST	soluble	116	209	65.1
GST	insoluble	62.2	112	34.9
NusA	soluble	33.2	121	74.7
NusA	insoluble	11.2	41	25.3
β-gal	soluble	33.7	202	44.7
β-gal	insoluble	41.7	250	55.3
GUS	soluble	1.0	3	0.7
GUS	insoluble	140	477	99.3

Figure 3. Solubility screening using four different vector constructs

Cultures of *E. coli* strain BL21(DE3) containing the vector constructs described in Table 2 were grown at 37°C to an OD_{600} = 1.5 and target protein expression was induced by adding IPTG to a final concentration of 1 mM. Following incubation for approximately 3 h at 30°C (final OD_{600} = 5–6), the cultures were dispensed (1 ml/well) into sequential rows of 2-ml 96-well plates, and 100 µl PopCulture™ Reagent containing 2 µl Lysonase™ Reagent was added to each well. The procedure described in Table 1 was used to generate soluble and insoluble protein fractions. Panel A shows SDS-PAGE analysis (Coomassie blue staining) of the indicated samples (15 µl extract). Panel B shows the results of the FRETWorks S•Tag Assay performed with the same fractions. For the assay, samples were serially diluted 1:25 to 1:2500, and the dilutions analyzed according to the standard protocol (20 µl diluted sample was used per assay). The S•Tag fusion proteins were quantified with a standard curve based on known amounts of S•Tag Standard.

GUS, β-gal, NusA and GST (Figure 3, panel B, page 12) correlated well with the SDS-PAGE analysis of the same samples. The RoboPop™ Solubility Screening Kit may also be used without the aid of a robotic platform by employing simple multichannel pipetting for the robotic liquid handling steps, and vacuum filtration may be replaced by centrifugation in the robotic or manual protocol.

Summary

The RoboPop Solubility Screening Kit increases the efficiency of solubility optimization by streamlining tedious cell lysis, extract fractionation, and sample analysis procedures. The screening kit provides a robot-friendly alternative to conventional labor-intensive, expression-level protein solubility screening through PopCulture™ cell lysis and HT 96-well filtration-based fractionation. We have combined the chemistry of PopCulture, the biological activities of Lysonase™ Solution, and the engineering of an innovative Filtration Plate to eliminate common bioprocessing bottlenecks. Together, Novagen's RoboPop Solubility Screening Kit and RoboPop purification kits allow rapid identification and selection of ideal host-vector combinations, expression conditions, and purification parameters toward production of proteins for structural or functional analysis.

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Product		Cat. No.
RoboPop™ Solubility Screening Kit [includes PopCulture™ Reagent, Lysonase™ Bioprocessing Reagent, 350-µl 96-well Filter Plate, 4% SDS, Collection Plate with Sealer (2)]		71255-3

Product	Size	Cat. No.
PopCulture Reagent	15 ml	71092-3
	75 ml	71092-4
	250 ml	71092-5
Lysonase Bioprocessing Reagent	0.2 ml	71230-3
	1 ml	71230-4
	5 ml	71230-5
FRETWorks™ S•Tag™ Assay Kit (Includes S•Tag Grade S-protein, FRET ArUAA Substrate, 10X FRET Assay Buffer, 10X FRET Stop Solution, and S•Tag Standard)	100 assays	70724-3
	1000 assays	70724-4

Available separately:

Product	Size	Cat. No.
S•Tag Grade S-protein	250 µg	69215-3
FRET ArUAA Substrate	0.2 ml	70714-3
	2 ml	70714-4
S•Tag Standard	500 µl	69217-3

Simple, powerful promoter and enhancer analysis with the MightyLight™ System

Keith Fourrier and Scott Hayes — Novagen

Determination of eukaryotic promoter and enhancer activity is useful for analysis of the factors that impact gene expression at the transcriptional level. These studies benefit from sensitive reporter systems with simple assay endpoints and robust activities with low background in mammalian cells. To address this need, Novagen is pleased to introduce the MightyLight™ System for *Renilla* luciferase expression and detection. This assay system is based on a luciferase enzyme derived from the sea pansy, *Renilla reniformis*. A 36-kDa monomeric protein, the *Renilla* luciferase enzyme (Rluc) catalyzes the oxidative decarboxylation of coelenterazine (a luciferin) to form blue light (λ_{max} = 480 nm), CO_2 and oxyluciferin (1). The bioluminescent properties of *Renilla* luciferase are well suited for use as a reporter enzyme because light emission can be quantitatively captured in a photometer and light output directly correlated with the amount of enzyme present. Rluc assays take a matter of seconds as compared to other reporter systems that may require lengthy incubations and tedious reagent additions. The protein is functionally active immediately after translation and is not dependent on divalent cations or ATP, so that one needs only to add extract to substrate in an appropriately buffered solution to measure activity (1).

The gene encoding *Renilla* luciferase was cloned and sequenced in 1991 by Lorenz et al. (2). Availability of the sequence for *Renilla* luciferase has allowed construction of plasmids designed to quantitatively assess transcriptional activation through promoter and enhancer evaluation. In this report we describe optimization of the luciferase cDNA for enhanced reporter performance, addition of a signal peptide to enable noninvasive assays from growth medium, easy-to-use plasmid configuration, and an assay kit designed to maximize the reporter signal while maintaining low background.

Codon optimization and transcription factor binding sites

For reporter utilization, a high signal-to-noise ratio is of utmost importance. The enzyme expression should be very robust when initiated by a promoter but silent when no promoter is present. One method to maximize expression and activity is to alter codon usage. This is most effective in bacterial systems, where usage of particular codons found more frequently in eukaryotes may be extremely rare (3), but altering codon usage has also been shown to benefit mammalian systems. Because the *Renilla* luciferase gene

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is derived from a coelenterate, changes in codon sequence to mammalian preference may also be advantageous. In addition, a reporter sensitive to transcription factor binding and promoter activation should contain minimal sequence corresponding to potential transcription factor binding sites. We achieved both aims by creating a synthetic version of the *Renilla* luciferase gene. To maximize codon usage in mammalian cells and exclude putative binding sites, 258 of 933 (28%) nucleotides were changed to provide an open reading frame composed entirely of codon-optimized sequence. Except for exchange of an alanine for a threonine at the second amino acid position, all mutations were silent. The change in codon 2 is advantageous in that it places a guanine residue at position +4, and forms the optimal consensus sequence for mammalian expression (4). In comparison to transcription factor recognition sequences, at least 135 sequence elements could possibly be recognized as binding sites for transcription factors within the native *Renilla* luciferase cDNA sequence. To prevent promoter-independent transcription of the reporter, 98% of the putative binding sites were altered to a nonconsensus sequence that maintained optimal codon usage.

Promoter analysis using pMLuc-1

For promoter analysis, the pMLuc-1 vector (Figure 1) contains a multiple cloning site upstream of the *Renilla* luciferase cDNA, followed by the rabbit β-globin polyadenylation sequence for transcription termination. A second synthetic terminator sequence upstream of the cloning site was introduced to decrease read-through transcription. For increased convenience, the pMLuc-1 vector is also available as an AccepTor™ Vector format. AccepTor vectors are designed for simplified cloning of PCR products generated using nonproofreading thermostable DNA polymerases, such as Nova*Taq*™ DNA Polymerase, that leave single 3′-dA overhangs on their reaction products (5).

To test the performance of the pMLuc-1 vector, separate recombinants containing two promoters of the chicken glutamine synthetase (GS) gene were constructed (6). The promoters extend 122 and 436 base pairs upstream of the transcriptional start site and share a common 3′-end at +34. The promoters were tested in conjunction with empty pMLuc-1 vector and a positive control vector (pMLuc-1 CMV Positive Control) comprised of the strong, constitutive CMV promoter driving expression of *Renilla* luciferase. All plasmids were tested for promoter performance in three separate cell lines: COS-1, 293 and BHK. As demonstrated in Figure 1, panel B, the pMLuc-1 vector alone had very low activity in each cell line. When the pMLuc-1 GS(-122) plasmid was introduced into cells, reporter expression driven by the promoter increased by a minimum of 14.7-fold (COS-1) to as much as 90.3-fold (BHK). The pMLuc-1 GS(-436) plasmid yielded similar results, producing an increase of 18-fold (COS-1) to 79-fold (BHK) above background. Reporter activity in 293 cells fell midway between that of COS-1 and BHK cells, which likely reflects cell type–specific differences for this promoter. Reporter activity of the positive control was well above the activity generated with the two glutamine synthetase promoters and was as much as 1,667-fold above background from the promoterless vector (negative control).

Enhancer analysis with pMLuc-2

When analyzing promoter activity, one often wants to test the contribution of putative transcription factor binding sites. A sensitive assay is required to differentiate true enhancer activity from background. The new pMLuc-2 vector (Figure 2, page 15) provides this capability. The vector contains a minimal thymidine kinase (TK) promoter driving expression of the optimized *Renilla* luciferase reporter. To assess enhancer activity, the cloning site has been placed downstream of the rabbit β-globin polyadenylation signal. Placement at this distal position separates the enhancer from the minimal promoter and provides a more accurate measure of enhancer function since activity should be independent of orientation and location relative to the promoter. Like pMLuc-1, the pMLuc-2 vector contains an artificial polyadenylation site upstream of the minimal TK promoter to suppress read-through transcription (and is available as an AccepTor™ Vector format). The minimal TK promoter, derived from herpes simplex virus, retains low activity in the vast majority of cell lines used. As demonstrated in Figure 2 (page 15), reporter activity driven by the minimal TK promoter is sufficiently above background to easily distinguish activity from noise, but is low enough that inclusion of an enhancer can augment activity several fold. Background, in this case, refers to activity generated by transfection of cells with pMLuc-1 and varied, depending on the cell line tested, between 3.3-fold and

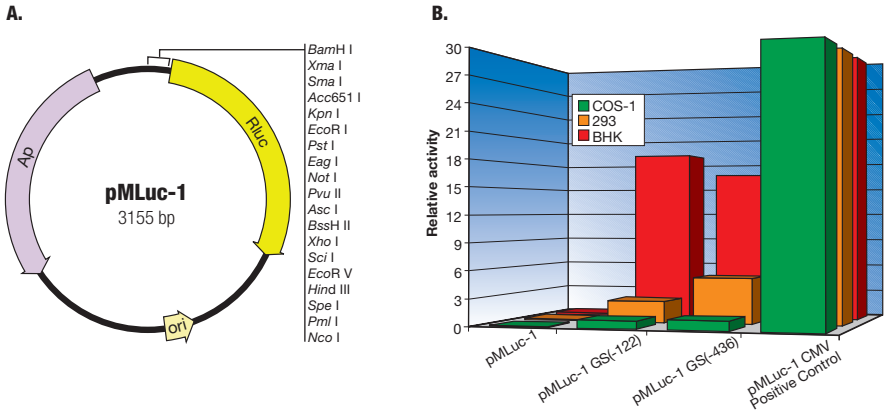

Figure 1. Promoter activity driving *Renilla* luciferase expression from the pMLuc-1 vector

A. The pMLuc-1 vector. Putative promoter sequences are cloned upstream of the *Renilla* luciferase sequence.

B. *Renilla* luciferase assays of promoter activity in COS-1, 293 cells and BHK cells. Two separate glutamine synthetase promoters extending from –122 to +34 and –436 to +34 relative to the transcriptional start site were cloned into the *Kpn* I and *Pml* I sites of pMLuc-1 and tested against the empty vector and the pMLuc-1 CMV Positive Control. All plasmids were purified using the UltraMobius™ 1000 Plasmid Kit. The day prior to transfection, 2.5×10^4 cells of each cell line were plated into wells of a 24-well plate. For transfection, 0.25 µg plasmid DNA was complexed with GeneJuice™ Transfection Reagent and transferred into wells. Twenty-four h post-transfection, cells were harvested using Reportasol™ Extraction Buffer and extracts were assayed for reporter activity. Extract was added to 100 µl reconstituted MightyLight™ Assay Buffer and photon emission measured in a Dynex MLX Microtiter® Plate Luminometer. All values reflect an average of three replicate cultures. Data are normalized to protein concentration and activity is expressed as a percent of Positive Control expression.

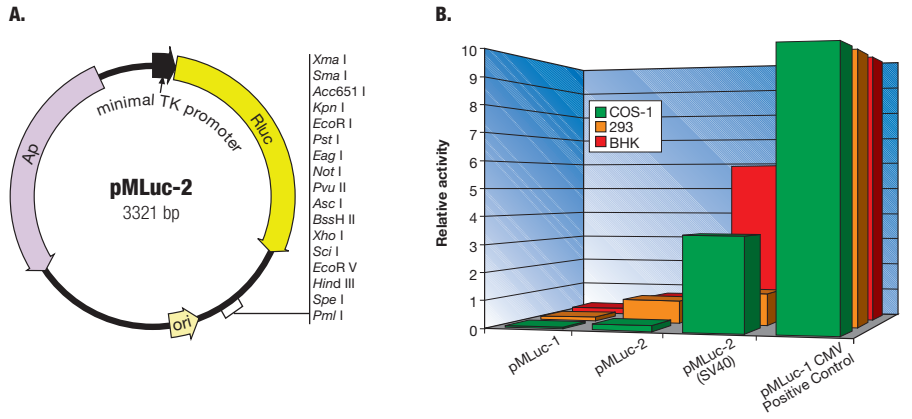

Figure 2. Enhancer activity demonstrated in the pMLuc-2 vector

A. The pMLuc-2 vector. Putative enhancer sequences are cloned downstream of the minimal TK promoter-driven *Renilla* luciferase cDNA and transcription termination sequence.

B. *Renilla* luciferase assays of enhancer activity in COS-1, 293 cells, and BHK cells. A 243-bp SV40 enhancer was cloned into the *Kpn* I and *Pml* I sites of pMLuc-2 and tested against the empty pMLuc-1 and pMLuc-2 vectors as well as the pMLuc-1 CMV Positive Control. Transfection and MightyLight reporter assays were performed as described in Figure 1 (page 14).

6-fold above the control background (Figure 2). Introduction of a single enhancer, such as the SV40 enhancer (derived from the SV40 early promoter), into the multiple cloning site significantly impacted the reporter expression level. Interestingly, there was a differential effect depending on which cell line is transfected. As shown in Figure 2, COS-1 cells and BHK cells demonstrated an enhancer-driven increase in activity that was 8.4-fold and 16-fold, respectively, over the background activity exhibited by pMLuc-2 alone. In contrast, 293 cells showed less than a two-fold increase in reporter activity. This may indicate that the SV40 enhancer is not utilized as efficiently in 293 cells as in the other cell lines.

Promoter analysis using pMLuc-3 for detection of secreted *Renilla* luciferase

Novagen is the first company to offer a promoterless vector encoding a secreted luciferase reporter. Only two secreted reporters have been used to any degree: the secreted alkaline phosphatase (SEAP) and the human growth hormone assay (hGH) (7, 8). These assays offer many advantages over traditional intracellular-based assays, but can be time consuming. A commercial version of the SEAP assay takes a minimum of 30 minutes to perform and the hGH assay can take more than 4 hours. In contrast, the MightyLight™ assay can be performed in a matter of minutes. Aside from decreased assay time, secreted luciferase allows sampling of time points without disruption of cells. This is particularly useful if a cell treatment agent is valuable or in short supply. Secretion of *Renilla* luciferase is made possible by a fusion of DNA encoding the signal peptide of human interleukin-2 (IL-2) to the DNA encoding the normally cytoplasmic *Renilla* luciferase (9). The encoded protein is 339 amino acids long and is processed through signal peptide cleavage to 318 amino acids (cleavage between amino acids 21 and 22). We made three notable changes in the secreted *Renilla* luciferase sequence. The second amino acid of the IL-2 sequence was changed from tyrosine to glutamate to obtain an optimal translation initiation sequence (4). Substitution of leucine for methionine at amino acid number four was found to increase utilization of the initiating methionine (data not shown). An additional change that differentiated the secreted Rluc reporter from the nonsecreted pMLuc-1 and pMLuc-2 versions was a change of cysteine at position 152 of the preprotein to alanine. Alteration at this amino acid can improve assay sensitivity as much as 100-fold as compared to the native version (10). The pMLuc-3 vector is also available as an AccepTor™ Vector format.

To test the performance of secreted luciferase, the –436 to +34 and –122 to +34 promoters of the chicken glutamine synthetase gene were cloned into the pMLuc-3 vector. As shown in Figure 3 (page 16), both promoters had similar activity to that obtained in the pMLuc-1 cytoplasmic *Renilla* vectors (Figure 1, page 14). In COS-1 cells, pMLuc-3 GS(-122) and pMLuc-3 GS(-436) had activity that was 18.5- and 19.8-fold, respectively, above the background obtained with an empty pMLuc-3 vector. The CMV-driven positive control vector (pMLuc-3 CMV Positive Control) had activity 449-fold above background. In 293 and BHK cells, the pMLuc–3 GS(-122) construct produced 10.0- and 35.7-fold increases, respectively. This increased to 18.8- and 44.8-fold above background for assays in which the pMLuc-3 GS(-436) vector was used to assess reporter activity. These results reflect cell-specific expression caused by differential activation through the glutamine synthetase promoter. Results were obtained at 24 hours in the presence of DMEM containing 10% fetal bovine serum (FBS). It has been our experience, and documented by others (10), that decreasing the amount of FBS in the medium may enhance assay performance (data not shown).

The MightyLight Rluc Assay Kit

A common problem encountered with previous *Renilla* luciferase assay systems is auto-fluorescence due to oxidation of the coelentrazine substrate. The MightyLight Rluc Assay Kit has been specifically designed to maximize reporter activity with maintenance of very low background. Novagen provides the substrate as a stabilized 100X concentrate to be used in conjunction with MightyLight Assay Buffer. This combination leads to a substantial increase in assay performance as compared to published methods (1). Sufficient reagent is provided for 100 assays of 100 µl each (equivalent to a 96-well plate). Reportasol™ Extraction Buffer is provided in the assay kit for preparation of the cellular extracts and is composed of a buffered mixture of non-ionic and zwitterionic detergents designed for optimal preservation of *Renilla* luciferase activity. The pMLuc-1 CMV Positive Control, which can be used to test assay performance and serve as a positive transfection control, the pMLuc-3 CMV Positive Control, and pMLuc-specific primers for sequencing and PCR are available separately.

Summary

We have demonstrated that the MightyLight assay system provides a

continued on page 16

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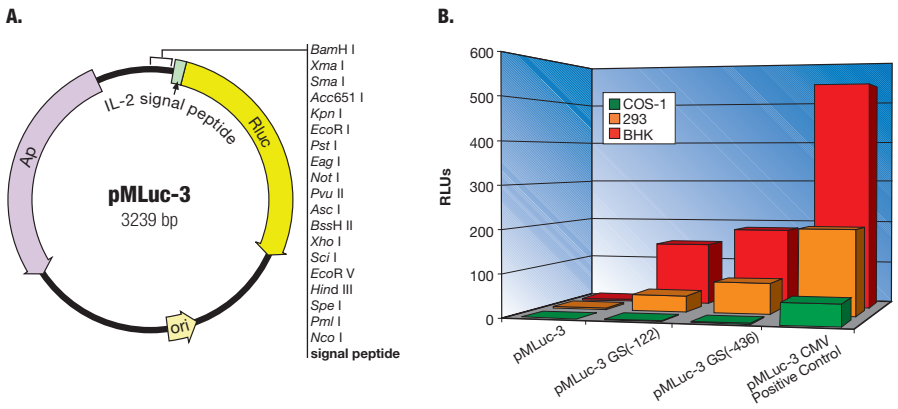

Figure 3. Promoter activity driving expression of secreted *Renilla* luciferase from the pMLuc-3 vector

A. The pMLuc-3 vector. Putative promoters are cloned upstream of the secreted form of *Renilla* luciferase. **B. *Renilla* luciferase assays in culture media from transfected COS-1, 293 cells, and BHK cells.** Two separate glutamine synthetase promoters extending from –122 to +34 and –436 to +34 relative to the transcriptional start site were independently cloned into the *Kpn* I and *Pml* I sites of pMLuc-3 and tested against the empty vector and pMLuc-3 CMV Positive Control. Assays were performed essentially as described in Figure 1 except that cells were not extracted with Reportasol. Instead, 20 µl samples culture media were assayed with reconstituted MightyLight Assay Buffer 24 h after transfection. All values reflect an average of three replicate cultures. Activity is expressed as a percent of secreted *Renilla* luciferase Positive Control expression.

comprehensive tool for promoter and enhancer analysis. Two features unique to the Novagen system offer significant advantages over available luciferase reporter systems. First, the AccepTor™ Vector versions of pMLuc-1, pMLuc-2, and pMLuc-3 offer simpler cloning procedures for PCR-based promoter analysis and are not dependent on restriction enzymes or specialized primers. Amplified target sequence can be directly cloned into the recipient vector without intermediate steps. Second, the format provides for easy determination of bi-directional promoter activity or enhancer activity because inserts can be cloned into the pMLuc plasmids in either direction. This is especially relevant for new techniques such as chromatin immunoprecipitation (ChIP; for review see 11), in which the orientation of the inserted target DNA is unknown. This procedure utilizes a process by which promoter-bound transcription factors are cross-linked to chromatin DNA followed by a shearing or sonication step to break the DNA up into manageable sizes. The transcription factors are then immunoprecipitated and the copurified chromatin DNA is released through a decrosslinking step. Ideally, the isolated DNA is then cloned into a vector expressing a reporter gene, and promoter or enhancer activity is assessed. The AccepTor Vector cloning approach simplifies this procedure. Ends from sonicated or sheared DNA can be treated with a non-proofreading thermostable DNA polymerase to add single 3′-dA overhangs on the reaction products. Novagen offers the Single dA™ Tailing Kit to facilitate this procedure. The treated DNA can then be simply ligated into the relevant AccepTor Vector without the need for complicated linker addition, restriction enzyme treatment and fractionation steps.

The secreted version of *Renilla* luciferase also offers capabilities and experimental design flexibility not found in other luciferase assay systems. Samples can be taken at various times to create a time course of promoter activation or suppression without disruption of cells. This capability increases the ease with which researchers can test drug or chemical impact on cell signaling pathways. The noninvasive measurement of luciferase secreted into the medium also provides for downstream utilization of the cells for techniques such as RNA isolation. *Renilla* luciferase activity produced by the pMLuc vectors is easily assessed with the MightyLight™ Rluc Assay Kit. Samples can be measured rapidly and conveniently in 96-well plates or as individual assays. Inclusion of Reportasol™ Extraction Buffer provides for efficient recovery of reporter protein in a buffered, passive lysis reagent specifically designed to maintain optimal *Renilla* luciferase activity.

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11. Johnson, K. D. and Bresnick, E. H. (2002) *Methods, A Companion to Methods in Enzymology* **26**, 27–36.

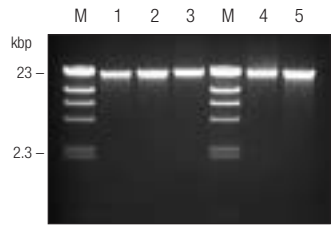
Product	Size	Cat. No.
pMLuc-1 DNA	20 µg	71116-3
pMLuc-2 DNA	20 µg	71118-3
pMLuc-3 DNA	20 µg	71120-3
pMLuc-1 AccepTor™ Vector Kit	20 rxn	71214-3
pMLuc-2 AccepTor Vector Kit	20 rxn	71216-3
pMLuc-3 AccepTor Vector Kit	20 rxn	71218-3
pMLuc-1 AccepTor Vector	20 rxn	71117-3
pMLuc-2 AccepTor Vector	20 rxn	71119-3
pMLuc-3 AccepTor Vector	20 rxn	71121-3
MightyLight™ Rluc Assay Kit (includes Rluc Assay Buffer, Rluc Substrate, and Reportasol™ Extraction Buffer)	100 assays 1000 assays	71220-3 71220-4
pMLuc-1 CMV Positive Control	20 µg	71249-3
pMLuc-3 CMV Positive Control	20 µg	71219-3
MLuc1/3UP Primer	500 pmol	71221-3
MLuc1DOWN Primer	500 pmol	71222-3
MLuc2UP Primer	500 pmol	71223-3
MLuc2DOWN Primer	500 pmol	71224-3
MLuc3DOWN Primer	500 pmol	71225-3
Single dA™ Tailing Kit	20 rxn	69282-3

MagPrep® Bacterial Genomic DNA Kit: Efficient isolation of genomic DNA from Gram-positive and Gram-negative bacterial species

Don Drott[1] and Karl Holschuh[2] – [1]Novagen and [2]Merck KGaA, Darmstadt, Germany

Preparation of genomic DNA from bacteria often relies on the use of hazardous organic solvents such as phenol and chloroform. Novagen's new MagPrep® Bacterial Genomic DNA Kit is designed for rapid purification of genomic DNA from both Gram-positive and Gram-negative bacteria without the use of organic solvents. This kit combines the advantages of a silica-based resin with magnetic-based separation. The method is based on the lysis of bacterial cells in proximity to magnetic silica particles, and the immobilization of large DNA molecules to the surface of the particles (1, 2). The resulting DNA:magnetic particle complex is then washed to remove impurities and purified genomic DNA is eluted. The procedure for Gram-negative bacteria can be performed in as little as 30 minutes, and Gram-positive genomic DNA can be purified in as little as 75 minutes with much of the procedure being hands-off time. Up to 30 µg genomic DNA can be purified from 1 ml of culture. Genomic DNA prepared with the MagPrep Bacterial Genomic DNA Kit is suitable for a variety of downstream applications, including restriction endonuclease digestion, RFLP analysis, Southern blotting, library construction, and many PCR-based applications.

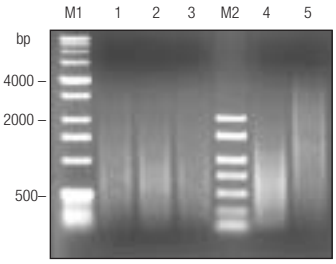
To test the performance of MagPrep Bacterial Genomic DNA Kit, five different strains of bacteria (three Gram-negative and two Gram-positive) were cultured and processed using the standard protocol. Agarose gel analysis of the purified genomic DNA (Figure 1) shows that all cultures produced similar yields of DNA with sizes in the range of 20–25 kbp. Restriction endonuclease digestion of the same genomic DNAs is shown in Figure 2. *E. coli* genomic DNA samples were also used as template to successfully amplify a 805-bp region using *nusA*-specific primers (data not shown). These experiments illustrate the ability of MagPrep Bacterial Genomic DNA Kit to purify high molecular weight genomic DNA from Gram-negative and Gram-positive bacteria. To date, the kit has been tested on nearly 40 species and subspecies of pathogenic and non-pathogenic Gram-positive and Gram-negative bacteria (Table 1).



Lane	Sample
M	λ *Hind* III Markers
1.	*Escherichia coli* K-12 (NovaBlue)
2.	*Escherichia coli* B (BL21)
3.	*Salmonella enterica*
4.	*Bacillus subtilis*
5.	*Enterococcus faecalis*

Figure 1. Agarose gel analysis of genomic DNA isolated from Gram-negative and Gram-positive bacteria

Bacterial cultures were incubated overnight in LB broth at 30°C, standardized to the equivalent of 1 ml of 1.0 OD_{600} bacteria, and processed with the MagPrep Bacterial Genomic DNA Kit according to the protocol. Agarose gel analysis of genomic DNA isolated from 5 different bacteria is shown. Equal volumes (15 µl) of DNA were loaded on a 0.8% 1X TAE agarose gel. Total elution volume was 100 µl.



Lane	Sample
M1	Perfect DNA™ Markers, 0.1–12 kbp
1.	*Escherichia coli* K-12 (NovaBlue)
2.	*Escherichia coli* B (BL21)
3.	*Salmonella enterica*
M2	PCR Markers, 50–2000 bp
4.	*Bacillus subtilis*
5.	*Enterococcus faecalis*

Figure 2. Agarose gel analysis of *Sau*3A I-digested genomic DNA isolated from Gram-negative and Gram-positive bacteria

Equal volumes (15 µl) of the same DNAs in Figure 1 were digested with 2 units *Sau*3A I in a 25-µl reaction at 37°C for 1 h. Digested DNAs were resolved on a 1.0% 1X TAE agarose gel.

Bacillus cereus
Bacillus subtilis
Enterococcus faecalis
Salmonella enterica
Escherichia coli K-12
Escherichia coli B
Enterococcus faecium
Leuconostoc mesenteroides
Lactococcus lactis
Micrococcus luteus
Erysipelothrix rhusiopathiae
Aerococcus viridans
Staphylococcus simulans
Staphylococcus aureus
Lactobacillus casei
Alcaligenes faecalis
Citrobacter freundii
Enterobacter cloacae
Klebsiella pneumoniae
Proteus vulgaris
Shigella sonnei
Serratia marcescens
Agrobacterium radiobacter
Yersinia enterocolitica
Vibrio parahämolyticus
Listeria innocua + 12 subspecies

Table 1. Strains processed successfully with MagPrep Bacterial Genomic DNA Kit

REFERENCES

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Product	Size	Cat. No.
MagPrep® Bacterial Genomic DNA Kit	100 rxn	71256-3
Components:		
• 2 × 1 ml	MagPrep Silica Particles	
• 10 ml	Bacterial Resuspension Buffer	
• 0.1 ml	RNase A Solution	
• 20 mg	Lysozyme, Chicken Egg White	
• 0.5 ml	Proteinase K Solution	
• 10 ml	MagPrep BG Lysis Buffer	
• 2 × 75 ml	MagPrep BG Wash Buffer	
• 10 ml	MagPrep BG Elute Buffer	

Available separately:

Product	Size	Cat. No.
MagPrep Silica Particles	1 ml	70912-3
RNase A Solution	0.1 ml	70856-3
Proteinase K Solution	2 ml	71049-3
600 mAU/ml	10 ml	71049-4

NovaBlue GigaSingles™ Competent Cells

10^9 efficiency in chemically competent cells

NovaBlue GigaSingles™ Competent Cells produce greater than 1×10^9 colonies/µg plasmid for cloning applications requiring the highest efficiency transformations. NovaBlue is a K-12 strain ideally suited as an initial cloning host due to its blue-white screening capability (with appropriate plasmids), and *recA endA* mutations, which facilitate high yields of excellent quality plasmid DNA. 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

- Chemically competent
- Greater than 1×10^9 cfu/µg
- Ideal for high efficiency cloning
- High quality plasmid DNA preparation
- Easy-to-use Singles™ format

Introductory pricing

Introductory pricing is available until May 31, 2003. Please inquire.

Product	Size	Cat. No.
NovaBlue GigaSingles™ Competent Cells guaranteed efficiency: > 1×10^9 cfu/µg	11 rxn 22 rxn	71227-3 71227-4

PCR cloning kits and LIC vector kits featuring higher-efficiency competent cells

PCR Cloning Kits

Novagen has packaged the super-efficient NovaBlue GigaSingles Competent Cells with several PCR cloning kits. The pSTBlue-1 **A**ccep**T**or™ Vector Giga Kits and pSTBlue-1 Perfectly Blunt® Giga Cloning Kits feature the new NovaBlue GigaSingles Competent Cells for increased transformation efficiency (> 1×10^9 cfu/µg). **A**ccep**T**or Vector Kits are designed for cloning of PCR products generated using thermostable DNA polymerases that leave single 3′-dA nucleotide overhangs. The vector enables direct ligation by virtue of single 3′-dU overhangs that anneal with 3′-dA extensions on PCR products. The dU residues are converted to dT residues *in vivo*. Perfectly Blunt Cloning Kits are designed for cloning of DNA generated by PCR using any type of DNA polymerase. This allows for the use of high fidelity proofreading enzymes for amplification, thereby decreasing the probability of generating mutations in the target sequence.

LIC Vector Kits

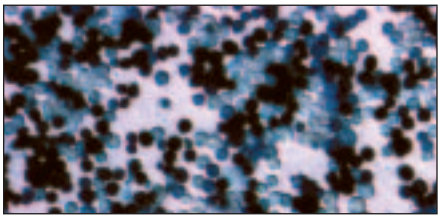
All Ek/LIC and Xa/LIC vector kits now include NovaBlue GigaSingles Competent Cells for even higher efficiency cloning. Ligation-independent cloning (LIC) was developed for directional cloning without restriction enzyme digestion or ligation reactions. LIC vectors are prepared to have very specific, noncomplementary 12-to-15 nucleotide, single-stranded overhangs. PCR products with overhangs complementary to the vector are easily created by building 5′ extensions into the primers followed by treatment with LIC-qualified T4 DNA Polymerase in the presence of the appropriate dNTP. Directional cloning of the insert is achieved with minimal nonrecombinant background, and cloning is so efficient that virtually all of the resulting colonies contain the desired recombinant. In addition to these features, all vector-encoded sequences can be removed with either Recombinant Enterokinase or Factor Xa digestion following purification.

Product	Size	Cat. No.
pSTBlue-1 **A**ccep**T**or™ Vector Giga Kit (includes **A**ccep**T**or Vector, Positive Control Insert, Clonables 2X Ligation Premix, Nuclease-free Water, NovaBlue GigaSingles™ Competent Cells, and Test Plasmid)	20 rxn 40 rxn	71228-3 71228-4
pSTBlue-1 Perfectly Blunt® Giga Cloning Kit (includes Blunt Vector, Positive Control Insert, End Conversion Mix, T4 DNA Ligase, Nuclease-free Water, NovaBlue GigaSingles Competent Cells, and Test Plasmid)	20 rxn 40 rxn	71229-3 71229-4

Insect GeneJuice™ Transfection Reagent

High-efficiency insect cell transfection

Insect GeneJuice™ Transfection Reagent is a proprietary liposome formulation optimized for maximal transfection efficiency of Sf9 insect cells. The reagent features extremely low toxicity to the cells and can be used for both transient and stable transfections in serum-containing or serum-free media, and for co-transfection of transfer plasmids with linearized virus DNA for the production of recombinant baculoviruses. Insect GeneJuice Transfection Reagent is ideal for large-scale protein expression when using pIEx™ and pBiEx™ vectors in suspension-culture transfections of Sf9 insect cells.



Sf9 cells transfected with pIEx-1/β-gal using Insect GeneJuice Transfection Reagent

Insect GeneJuice Transfection Reagent is provided as a 2 mg/ml suspension in 20 mM MES, 150 mM NaCl, pH 6.2 buffer. One milliliter is sufficient for 10 or 100 transfections in 10-ml suspension-culture flasks or a 35-mm plates, respectively.

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

Insect RoboPop™ Ni-NTA His•Bind® Purification Kit

High-throughput processing and purification

The Insect RoboPop™ Ni-NTA His•Bind® Purification Kit is designed for filtration-based 96-well format purification of His•Tag® fusion proteins directly from cultures of insect cells without cell harvest. This kit includes Insect PopCulture™ Reagent for protein extraction from total cultures without centrifugation, Benzonase® Nuclease for viscosity reduction, Ni-NTA His•Bind Resin and buffers, 2-ml 96-well Filter Plate and Collection Plate with Sealer.

The improved Insect PopCulture method recovers both protein released into the medium and intracellular protein, increasing processing efficiency and protein yields (1). The kit can be used with the insect cells grown in suspension or adherent cells grown on tissue culture plates.

The kit is configured for robotic processing of transfected 10-ml suspension cultures and purifies up to 400 µg His•Tag fusion protein per culture based on binding capacity of the resin. Protein yields ranging from 60 µg to 140 µg per 10 ml culture were obtained with β-gal, Fluc, MAP kinase and cdc2 kinase expressed in the pIEx™ transient protein expression system. The Filter Plate is compatible with standard vacuum manifolds for either automated or manual processing. The reagents and protocol have been validated for robotic sample processing with the MultiPROBE® II liquid handling workstation from PerkinElmer Life Sciences.

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Product	Cat. No.
Insect RoboPop™ Ni-NTA His•Bind® Purification Kit (includes Insect PopCulture™ Reagent, Benzonase® Nuclease, Ni-NTA His•Bind Resin, Buffers, 96-well Filter Plate, Collection Plate with Sealer)	71257-3

Veggie™ Singles™ Competent Cells for cloning and protein expression

Certified animal-free competent cells

Veggie™ Singles™ Competent Cells are the newest addition to Novagen's extensive competent cell offering. All Veggie Singles cells are maintained and manufactured with media and reagents derived from nonanimal sources, making these cells ideally suited for applications in which animal-free materials are desired. Cells are provided as 50-µl aliquots that eliminate the need to subaliquot, freeze/thaw, or waste partially used vials. Included with each kit is Veggie SOC Medium and Test Plasmid.

Veggie NovaBlue Competent Cells are available for routine cloning applications. 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 *recA endA* mutations, which result in high yields of excellent-quality plasmid DNA.

Veggie BL21(DE3) Singles Competent Cells and Veggie BL21(DE3)pLysS Singles Competent Cells are available for protein expression using vectors containing the T7 promoter. BL21 has the advantage of being deficient in both *lon* and *ompT* proteases for increased protein stability. These hosts carry a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter. The pLysS plasmid carries the gene for T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase and serves to suppress basal expression of T7 RNA polymerase prior to induction, thus stabilizing recombinants encoding target proteins that affect cell growth and viability.

Features

- Manufactured free of animal-derived media and components
- Chemically competent
- Protease-deficient hosts for protein expression (BL21)
- High-efficiency cloning and high-quality plasmid preparation (NovaBlue)
- Easy-to-use Singles format

Product	Size	Cat. No.
Veggie™ NovaBlue Singles™ Competent Cells (guaranteed efficiency: > 1.5×10^8 cfu/µg)	11 rxn	71251-3
	22 rxn	71251-4
Veggie BL21(DE3) Singles Competent Cells (guaranteed efficiency: > 2×10^6 cfu/µg)	11 rxn	71252-3
	22 rxn	71252-4
Veggie BL21(DE3)pLysS Singles Competent Cells (guaranteed efficiency: > 2×10^6 cfu/µg)	11 rxn	71253-3
	22 rxn	71253-4

0.5 M THP Solution

His•Bind®-compatible reducing agent

THP [Tris(hydroxypropyl)phosphine] is a water-soluble, odorless, sulfhydryl reducing agent that is more stable and effective than dithiothreitol (DTT). The reducing capacity of THP against oxidized glutathione is greater than that of TCEP (0.232 meq/g for THP versus 0.182 meq/g for TCEP). THP is also more resistant to air oxidation than DTT and is compatible with immobilized metal affinity chromatography with His•Bind and Ni-NTA His•Bind resins at concentrations up to 1.0 mM. The common working concentration of THP for many applications is 1–5 mM. THP is provided as a ready-to-use 0.5 M solution in water. Purity > 90% by NMR. CAS 4706-17-6. Molecular formula $C_9H_{21}O_3P$. M.W. 208.2.

Product	Size	Cat. No.
0.5 M THP Solution	1 ml	71194-3
	5 × 1 ml	71194-4

Lysonase™ Bioprocessing Reagent

Convenient blend of rLysozyme Solution and Benzonase Nuclease

Lysonase™ Bioprocessing Reagent is an optimized, ready-to-use blend of rLysozyme™ Solution and Benzonase® Nuclease. rLysozyme Solution contains a highly purified and stabilized recombinant lysozyme with specific activity 250 times greater than that of chicken egg white lysozyme. Benzonase Nuclease is a genetically engineered nonspecific endonuclease that degrades all forms of DNA and RNA (single stranded, double stranded, circular, linear), reducing extract viscosity, and increasing protein yield. The combined activities of rLysozyme and Benzonase Nuclease significantly increase protein extraction efficiency and facilitate downstream processing of protein extracts.

For efficient protein extraction with BugBuster® Protein Extraction Reagent, use 10 µl Lysonase per 1 g cell paste. For efficient protein extraction with PopCulture™ Reagent, add 2 µl Lysonase per 1 ml culture. Store at –20°C.

Product	Size	Cat. No.
Lysonase™ Bioprocessing Reagent	0.2 ml	71230-3
	1 ml	71230-4
	5 × 1 ml	71230-5

PopCulture™ Reagent: new, lower prices

Protein extraction from E. coli *without harvesting cells*

PopCulture™ Reagent is a 10X-concentrated mixture of specialized detergents and buffer that enables extraction and purification of recombinant proteins from *E. coli* directly from the culture media without cell harvest, mechanical disruption, or extract clarification. Recombinant proteins can be directly screened in the crude extract, or purified by adding an affinity matrix, washing the matrix–target protein complex to remove spent medium and cellular contaminants, and eluting the purified protein from the matrix. The ability to perform the entire procedure in the original culture tube or multi-well plate leads to increased convenience and speed when processing multiple samples. Now you can "pop cultures" at a lower cost per sample. We have optimized the production protocol for the reagent and would like to pass our cost savings on to you! Please inquire.

Product	Size	Cat. No.
PopCulture™ Reagent	15 ml	71092-3
	75 ml	71092-4
	250 ml	71092-5

RingMaster™ Nuclease

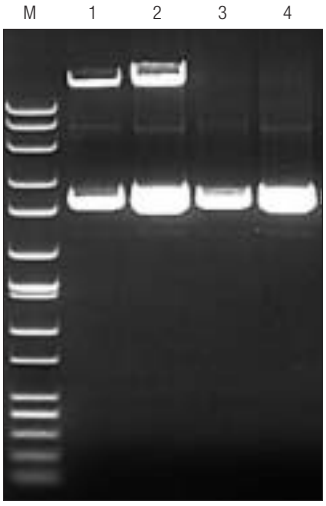
Eliminates linear DNA from plasmid preparations

RingMaster™ Nuclease is a recombinant exonuclease that hydrolyses linear double stranded DNA from a 5′-phosphorylated end, releasing 5′-phosphomononucleotides. The enzyme does not affect circular supercoiled DNA.

Bacterial genomic DNA often contaminates standard plasmid preparations, especially in the case of low-copy number plasmids. Using RingMaster Nuclease to eliminate linear DNA as the final treatment step in your plasmid purification protocol improves plasmid DNA quality. The enzyme can be completely inactivated by heating treated plasmid DNA for 10 minutes at 75°C. One unit of the enzyme is sufficient for removal of > 95% bacterial genomic DNA contamination from 1 µg plasmid DNA in 1 hour at 37°C.

RingMaster Nuclease is available in sizes of 1 KU, 5 KU, and 25 KU at a concentration 100 U/µl (1 KU = 1000 units).

Unit Definition: one unit is defined as the amount of enzyme that digests 200 ng of lambda DNA in 30 minutes at 37°C.



Lane	Sample
M	Perfect DNA™ Markers, 0.05–10 kbp
1.	0.5 µg plasmid DNA, untreated
2.	1 µg plasmid DNA, untreated
3.	0.5 µg plasmid DNA + RingMaster
4.	1 µg plasmid DNA + RingMaster

Removal of genomic DNA contamination from a plasmid preparation by RingMaster Nuclease

E. coli strain NovaBlue carrying pET-15b (5708 bp) was incubated overnight in LB medium plus 50 µg/ml carbenicillin, and plasmid DNA isolated according to a standard protocol. The preparation contained a significant amount of bacterial genomic DNA as indicated by the upper band observed in lanes 1 and 2 on this 0.8% agarose gel stained with ethidium bromide. Treatment of the preparation with RingMaster Nuclease (1 U/µg DNA) for 1 h at 37°C removed greater than 95% of the genomic contaminant without affecting circular plasmid, as shown in lanes 3 and 4.

Product	Size	Cat. No.
RingMaster™ Nuclease	1 KU	71250-3
	5 KU	71250-4
	25 KU	71250-5

Components:

- 1, 5 *or* 25 KU — RingMaster Nuclease (100 U/µl)
- 1.5 ml — 10X RingMaster Nuclease Buffer
- 1.5, 3 × 1.5 *or* 6 × 1.5 ml — Nuclease-free Water

Note: 1 KU = 1000 units

New T7Select® phage display host strains: Origami™ B 5615, Rosetta™ 5615, and Rosetta-gami™ B 5615

Novagen's T7Select® Phage Display System is a novel display system for identifying peptides and proteins that bind to ligands such as proteins, RNA, and DNA. Target proteins or peptides are expressed as fusions to the C-terminus of the T7 capsid protein and displayed on the viron surface where they are accessible for interaction. Simple panning procedures provide for rapid and simultaneous isolation of open reading frames and their encoded proteins because the target protein is physically associated with the corresponding DNA sequence as a phage particle.

As a surface display platform, the T7Select System is unique because the vector is a lytic phage. The cytoplasmic assembly and release of the phage by cell lysis avoids the requirement for export through the bacterial inner membrane during phage protein synthesis. The secretory pathway required by filamentous phage vectors prevents the recovery of phage displaying amino acids that interfere with secretion. The T7Select System offers high-, mid- and low-copy vectors and can display proteins as large as 1200 amino acids on the virus surface. Although the high-copy number peptide display vector can complete its life cycle in any T7-compatible host, the mid- and low-copy number vectors (with the larger protein display potential) require specific hosts that provide another source of the native capsid protein. The new T7Select hosts have features that facilitate folding and expression of the displayed protein, while providing the necessary capsid protein.

Our new T7Select host strains are closely related to the Rosetta™ and Origami™ host strains (see descriptions below). These new strains, however, carry an ampicillin-resistant, medium-copy number plasmid, pAR5615, that expresses the native T7 capsid protein under the control of the *lacUV5* promoter.

T7Select host strain genotypes and descriptions

Strain*	
Origami B 5615	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lacY1 ahpC gor522*::Tn*10* (Tc^R) *trxB*::kan pAR5615 (Ap^R) Greatly facilitates the expression of active, soluble proteins in *E. coli*. Mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes create a cytoplasmic environment compatible with the formation of disulfide bonds (1, 2).
Rosetta 5615	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lacY1* pRARE2^2 (Cm^R) pAR5615 (Ap^R) Designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs under the control of their native promoters for the codons AUA, AGG, AGA, CGG, CUA, CCC and GGA on a compatible chloramphenicol-resistant plasmid, pRARE-2.
Rosetta-gami™ B 5615	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lacY1 ahpC gor522*::Tn*10* (Tc^R) *trxB*::kan pRARE2 (Cm^R) pAR5615 (Ap^R) Combines the *trxB*/*gor* mutations of Origami for disulfide bond formation with the supply of rare tRNAs of Rosetta.

* All of the new T7Select host strains are derived from BL21, an *E. coli* B strain that lacks the *lon* and *ompT* proteases. The cells also contain the Ap^R plasmid, pAR5615, which expresses the T7 gene 10A capsid protein under the control of the *lacUV5* promoter, making them suitable as hosts for the mid- and low-copy number display vectors T7Select1-1, 1-2, and 10-3.

REFERENCES

1. Bessette, P. H., Aslund, F., Beckwith, J. and Georgiou, G. (1999) *Proc. Natl. Acad. Sci.* **96**, 13703–13708.
2. Ritz, D., Lim, J., Reynolds, C. M., Poole, L. B., and Beckwith, J. (2001) *Science* **294**, 158–160.

Product	Size	Cat. No.
Origami™ B 5615 Glycerol Stock	0.2 ml	71263-3
Rosetta™ 5615 Glycerol Stock	0.2 ml	71266-3
Rosetta-gami™ B 5615 Glycerol Stock	0.2 ml	71264-3

Use of BugBuster® and Lysonase™ Reagents for efficient protein extraction from Gram-positive bacteria

BugBuster® Protein Extraction Reagent has become a valuable tool for extraction of proteins from *E. coli* without the need for mechanical disruption. It is also known that supplementing BugBuster (as well as its derivative, PopCulture™ Reagent) with a small amount of lysozyme enhances the efficiency of extraction from *E. coli*, especially for larger proteins (1). Furthermore, the addition of Benzonase® Nuclease to the extraction mixture degrades the liberated nucleic

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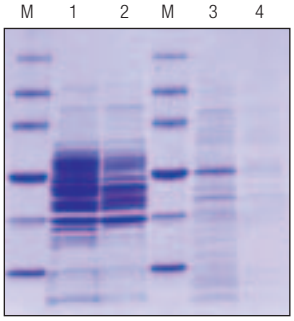

Lane	Sample
M	Perfect Protein™ Markers, 15–150 kDa
1.	10 µl *B. subtilis* BugBuster + Lysonase
2.	10 µl *B. subtilis* Tris + Lysonase
3.	10 µl *E. faecalis* BugBuster + Lysonase
4.	10 µl *E. faecalis* Tris + Lysonase

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 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. Equal-volume samples (15 µl) of the supernatants were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining.

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acids, reducing viscosity and potential interference of DNA and/or RNA with purification procedures (2). With this issue of *inNovations*, we introduce Lysonase™ Bioprocessing Reagent, which is an optimized blend of rLysozyme™ Solution and Benzonase® Nuclease that can be used alone or in conjunction with BugBuster® or PopCulture™ Reagent for any of their applications.

Figure 1 (page 21) shows that the combination of BugBuster and Lysonase greatly enhanced the release of soluble proteins from two different species of Gram-positive bacteria. Extraction is accomplished by resuspending cells in BugBuster containing 1/125 volume of Lysonase followed by a 20-minute incubation at room temperature. Insoluble material is simply removed by centrifugation and the supernatant can be used directly for purification or activity assays.

REFERENCES

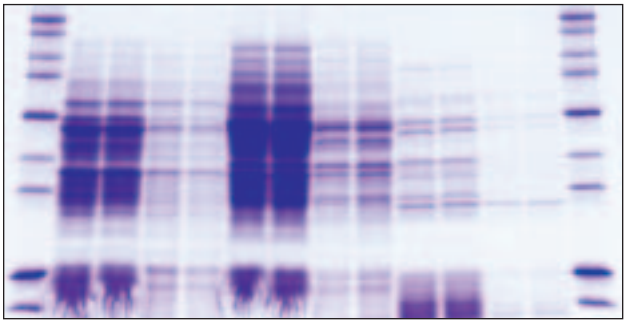
1. Grabski, A., Drott, D., Handley, M., Mehler, M., and Novy, R. (2001) *inNovations* **13**, 1–4.
2. Grabski, A., McCormick, M., and Mierendorf, R. (1999) *inNovations* **10**, 17–19.

Product	Size	Cat. No.
Lysonase™ Bioprocessing Reagent	0.2 ml	71230-3
	1 ml	71230-4
	5 × 1 ml	71230-5
BugBuster® Protein Extraction Reagent	100 ml	70584-3
	500 ml	70584-4
BugBuster 10X Protein Extraction Reagent	10 ml	70921-3
	50 ml	70921-4
	100 ml	70921-5
BugBuster (primary amine-free) Protein Extraction Reagent	100 ml	70923-3
	500 ml	70923-4
PopCulture™ Reagent	15 ml	71092-3
	75 ml	71092-4
	250 ml	71092-5

Extraction of proteins from three different yeast species using YeastBuster™ Protein Extraction Reagent

YeastBuster™ Protein Extraction Reagent allows simple and efficient extraction of proteins from yeast cells without harsh mechanical disruption or inconsistent enzymatic treatments. We have previously shown that YeastBuster works well for extraction of recombinant proteins from *Saccharomyces cerevisiae* and that the extracts are compatible with GST•Bind™ and Ni-NTA His•Bind® affinity chromatography (1). Here we demonstrate that YeastBuster is also effective for extraction of proteins from *Pichia stipidis* and *Schizosaccharomyces pombe*. Analysis of protein samples extracted from these species plus *S. cerevisiae* is shown in Figure 1.

For all three species, duplicate cell samples were extracted with YeastBuster and a glass bead method. Whereas the extractions were performed at room temperature for *S. cerevisiae* and *P. stipidis*, optimal protein yields were obtained from *S. pombe* by performing both extraction procedures at 45°C. The results demonstrate that YeastBuster extracted more protein over the entire size range than the glass bead method with all three species.



Lane	Sample
M	Perfect™ Protein Markers, 10–225 kDa
1.	*S. cerevisiae* YB
2.	*S. cerevisiae* YB
3.	*S. cerevisiae* GB
4.	*S. cerevisiae* GB
5.	*P. stipidis* YB
6.	*P. stipidis* YB
7.	*P. stipidis* GB
8.	*P. stipidis* GB
9.	*S. pombe* YB
10.	*S. pombe* YB
11.	*S. pombe* GB
12.	*S. pombe* GB

YB = YeastBuster
GB = glass bead lysis buffer

Protein yield (mg)

	S. cerevisiae	*P. stipidis*	*S. pombe*
YeastBuster	2.65	3.3	0.9
Glass Bead Lysis Buffer	0.8	1.0	0.2

Figure 1. SDS-PAGE analysis and Coomassie blue staining of extracted soluble proteins

Duplicate samples of pelleted yeast cells (100 mg wet weight) in 1.5-ml microcentrifuge tubes were resuspended in 500 µl YeastBuster plus 5 µl 100X THP Solution, or 500 µl of glass bead lysis buffer containing 50 mM Tris-HCl, 250 mM LiCl, 100 mM $(NH)_2SO_4$, 1 mM DTT, and 2% glycerol, plus 100 µl acid-washed glass beads (100–150 µm diameter). All samples contained protease inhibitors: 1 mM EDTA; 0.5 µM AEBSF; and 15 µg/ml benzamidine. After resuspension of pellets by pipetting, *S. cerevisiae* and *P. stipidis* suspensions in YeastBuster were agitated gently at room temperature for 20 min. *S. pombe* suspensions in YeastBuster were incubated at 45°C for 20 min with intermittent agitation. *S. cerevisiae* and *P. stipidis* suspensions in glass bead lysis buffer were vortexed on high for 4 min with intermittent chilling on ice. *S. pombe* suspensions in glass bead lysis buffer were incubated at 45°C for 20 min with intermittent vortexing. All samples were centrifuged at 16,000 × g for 5 min prior to SDS-PAGE analysis. Equal-volume samples (7.5 µl) of the extracts were loaded on the gel. Protein yields based on Non-Interfering Protein Assay™ are also shown.

REFERENCES

1. Drott, D., Bahairi, S., and Grabski, A. C. (2002) *inNovations* **15**, 14–16.

Product	Size	Cat. No.
YeastBuster™ Protein Extraction Reagent (includes 100X THP Solution)	100 ml	71186-3
	500 ml	71186-4

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- PopCulture™ Reagent
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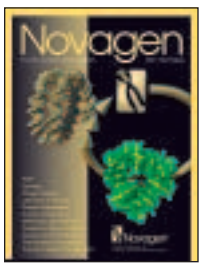

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