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FEATURE ARTICLE

Extraction and purification of proteins from *E. coli* without harvesting cells

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To study proteins and their functions, you must produce, extract and purify them. The process usually begins with culturing cells in liquid media under conditions that maximize target protein expression. Cells containing the expressed protein are harvested by centrifugation or filtration, resuspended in a buffer or lysis reagent, and mechanically or chemically disrupted to prepare a cell extract (1–4). Following another round of centrifugation or filtration to remove residual intact cells and debris, the extract is ready for the purification phase of the process, which commonly involves column or batch chromatography. Whereas this process is satisfactory for many applications, the number and types of steps required make it unsuitable for high throughput (HT) formats, which require rapid purification of hundreds of proteins simultaneously. We describe here a novel HT-compatible method for direct affinity purification of proteins from *E. coli* that eliminates the necessity for multiple mechanical extraction and separation procedures.

The key element in this method is extraction of cells directly in their culture medium using a concentrated mixture of specialized detergents called PopCulture™ Reagent (see Figure 1). Recombinant proteins are purified from the resulting mixture by the addition of an affinity matrix, washing the matrix-target protein complex to remove spent culture medium and cellular contaminants, and elution of the purified protein from the matrix. The entire culturing, extraction, and purification process can be achieved in the original culture tube or multiwell plate. This "in-media" protein purification procedure may be adapted to high throughput robotic processing of samples for proteomics research and any application that would benefit from the increased speed and convenience it provides.

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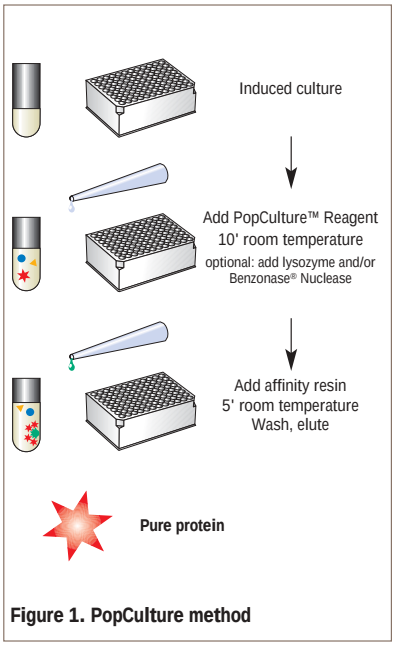

Figure 1. PopCulture method

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Background

A variety of methods exist for cellular disintegration and extraction of proteins from cells, ranging from enzymatic digestion and osmotic shock to ultrasonication, grinding, and pressure disruption (4). These "traditional" methods have inherent advantages and disadvantages. Generally, vigorous mechanical treatments reduce viscosity, but can result in heat and oxidative inactivation of labile proteins. Gentle treatments may not release the target protein from the cells, and produce extremely viscous extracts due to the high concentration of unsheared nucleic acids. Traditional methods all require an initial cell harvest step, which concentrates cell mass and removes media components. This step, as well as any mechanical lysis step, is difficult to automate and scale down for the purpose of extracting and purifying small amounts of many proteins simultaneously.

Recently, extraction and recovery of proteins produced in both prokaryotes and eukaryotes have been simplified through the application of specialized reagents that eliminate the need for mechanical or enzymatic disruption of cells. For example, Novagen's BugBuster™ Protein Extraction Reagent is a mixture of detergents capable of perforating bacterial cells without denaturing proteins (5, 6). When used with Benzonase® Nuclease, non-viscous *E. coli* cell extracts can be easily prepared and directly applied to a variety of chromatography media for target protein purification.

The "1X" ready-to-use form of BugBuster reagent effectively extracts soluble proteins from *E. coli* cells when used to resuspend cell pellets. This led us to consider the possibility that a concentrated formulation of detergents could achieve similar performance when added directly to unprocessed cultures, eliminating the need to collect the cells. Assuming that proteins could be extracted by this method, purification of intact protein may be further complicated by the presence of media components, metabolic products and cell debris in the mixture.

The following examples demonstrate that direct processing and affinity chromatography of total culture extracts can produce high yields of target proteins with purity and quality similar to conventionally purified preparations.

Table 1. General protocol for PopCulture™ extraction and purification

1. Culture cells in liquid media under conditions for target protein production.
2. Add 0.1 culture volume PopCulture™ Reagent, mix, and incubate 10 minutes at room temperature.
3. (Optional) Add lysozyme and/or Benzonase® Nuclease, mix and incubate 10–15 minutes at room temperature.
4. Add equilibrated affinity resin, mix, and incubate 5 minutes at room temperature.
5. Separate the affinity resin from the culture extract by filtration or magnetic isolation.
6. Wash the affinity resin.
7. Elute the target protein using the appropriate elution buffer.
8. Remove the affinity resin.
9. Analyze the purified protein.

Purification of a His•Tag fusion protein from *E. coli* total culture extracts

As a test vector for *E. coli* extraction and purification we used pET-41b(+), which expresses a 35.6 kDa GST•Tag™/His•Tag® fusion protein that can be purified using immobilized metal chelation chromatography (IMAC; His•Bind® Resins) or immobilized glutathione (GST•Bind™ Resins). Both affinity purification methods are compatible with the conditions of total culture extraction with the PopCulture Reagent, and magnetic formats are available that are well suited for HT applications.

For testing IMAC purification, the general protocol given in Table 1 was used with three different affinity supports: His•Bind Resin, Ni-NTA His•Bind Resin, and His•Bind Magnetic Agarose Beads. The His•Bind Resin was pre-charged with Ni^{2+} before equilibration with 1X His•Bind Buffer, and the other two supports (which are already Ni^{2+}-charged) were directly equilibrated in the same buffer at step 4 in the protocol. With His•Bind and Ni-NTA His•Bind samples, the target protein was captured in batch mode and then the resins were transferred to small columns for final washing and elution steps. Multiwell filter plates can also be used for this application. With His•Bind Magnetic Agarose Beads, the entire purification procedure was

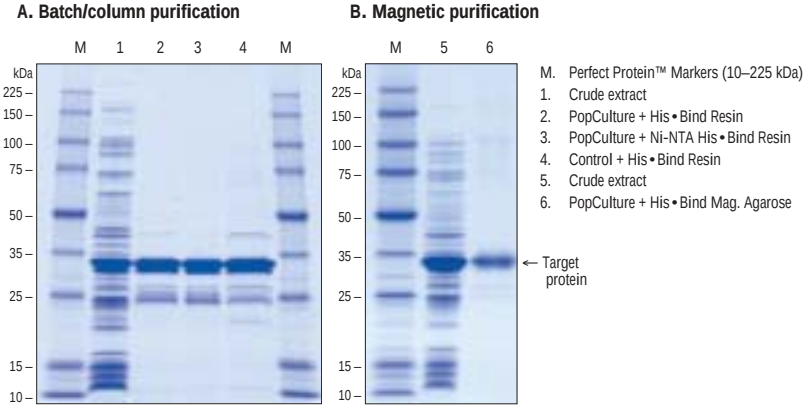

Figure 2. IMAC purification of a His•Tag fusion protein from *E. coli* total culture extracts

Panel A. *E. coli* strain BL21(DE3) containing pET-41b(+) was grown in liquid culture and protein expression induced with 1 mM IPTG for approximately 3 h (final OD_{600} = 9.0). Samples (2.7 ml) of the culture were dispensed into 15-ml tubes and 0.3 ml PopCulture Reagent was added to each tube (except for the control). The 2.7-ml control sample was centrifuged at 10,000 x g for 5 min to harvest the cells, and the supernatant removed and discarded. The cell pellet from the control was suspended in 0.3 ml BugBuster Reagent. All samples were incubated for 10 min at room temperature, treated with 2 µl Benzonase Nuclease, and processed as described in Table 1 and the text. Target proteins were eluted with 2 x 150 µl of 0.5X His • Bind Elute Buffer.

Panel B. The same recombinant used in Panel A was induced with IPTG for 3 h (final OD_{600} = 4.8). The culture was dispensed in 1.0 ml samples into a deep 96-well plate (2 ml well capacity) and 0.1 volume PopCulture Reagent was added per well. After pipetting up and down to mix, 1 µl Benzonase was added followed by another mixing step and the samples were incubated 10 min at room temperature. His • Bind Magnetic Agarose Beads (50 µl of a 50% slurry equilibrated in 1X His • Bind Binding Buffer) were added to each sample, mixed, and incubated 5 min at room temperature. The samples were subjected to a magnetic field using pin magnets to collect the beads. The beads were washed three times with 750 µl His • Bind Wash Buffer. Target protein was eluted with 200 µl 0.5X His • Bind Elute Buffer followed by 100 µl 0.5X His • Bind Elute Buffer. All samples were analyzed by SDS PAGE (4–20% gradient gels) and Coomassie blue staining.

Table 2. Purification of His•Tag® GST expressed in *E. coli*

Purification Method	Yield[1]	Purity[2]
Standard His•Bind®	74	83
PopCulture™ His•Bind	111	89
PopCulture Ni-NTA His•Bind	170	85
PopCulture His•Bind Magnetic[3]	128	94
Standard GST•Bind™	42	92
PopCulture GST•Bind	45	90
PopCulture GST•Bind Magnetic[3]	40	94

1. Yield in micrograms of target protein purified per ml of culture, as determined by BCA protein assay.
2. % purity determined by scanning densitometry of Coomassie blue stained SDS polyacrylamide gels.
3. Data represent the average of 8 separate wells processed in parallel.

performed in batch mode using 2-ml deep 96-well plates and a magnetic pin rack to collect the beads for binding, wash and elution steps. As a control, cells were harvested by centrifugation from an equal volume of culture, and protein was extracted with BugBuster™ Reagent. The control extract was clarified by centrifugation and the target protein purified using His•Bind Resin.

The results are summarized in Figure 2 and Table 2. The data show that with all three types of IMAC matrix, the yield of purified target protein recovered from PopCulture total culture extracts was significantly greater than the yield from the control purification. Furthermore, the purity of the target protein was similar to that of protein purified by the standard method using centrifugation for cell harvest and extract clarification. (Several minor truncated GST products are routinely observed.) Most notably, the use of His•Bind Magnetic Agarose Beads enabled the entire procedure to be carried out in a single tube without the need for columns or centrifugation.

Purification of a GST fusion protein from *E. coli* total culture extracts

The GST•Tag/His•Tag fusion protein expressed from pET-41b(+) was also purified with GST•Bind Resin, using the affinity of the GST (glutathione-S-transferase) domain for immobilized reduced glutathione on the resin. As in the His•Bind purification experiments, two different GST•Bind formats were used. Figure 3 and Table 2 show the results of these purifications. A batch protocol was performed with the standard GST•Bind Resin (Panel A), and a magnetic protocol was used with GST•Bind Magnetic Agarose Beads (Panel B). Extraction and purification of this protein from PopCulture total culture extracts using the GST affinity produced yields and purity similar to the controls using standard harvest and extraction procedures.

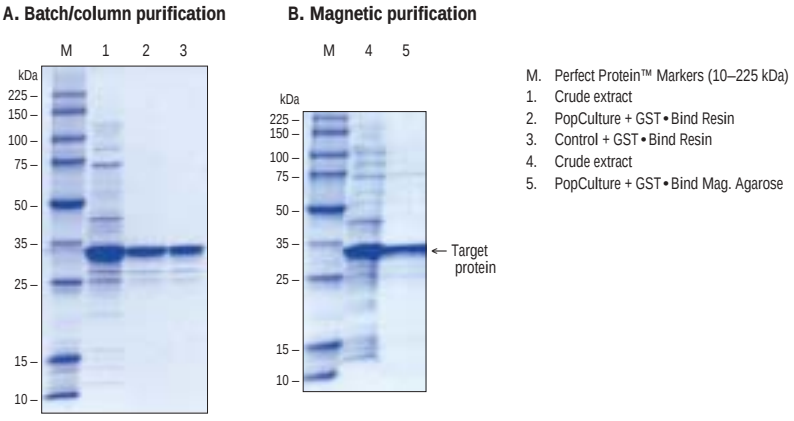

Figure 3. GST•Bind purification of a GST fusion protein from *E. coli* total culture extracts

Panel A. *E. coli* strain BL21(DE3) containing pET-41b(+) was grown in liquid culture and protein expression induced with 1 mM IPTG for approximately 3 h (final OD_{600} = 2.1). Samples (3 ml) of the culture were dispensed into 15-ml tubes and 0.3 ml PopCulture Reagent was added to each tube (except for the control). The 30-ml control sample was centrifuged at 10,000 x g for 5 min to harvest the cells, and the supernatant removed and discarded. The cell pellet from the control was suspended in 1X BugBuster Reagent at a ratio of 5 ml/g cells. All samples were incubated for 10 min at room temperature, treated with 2 µl Benzonase Nuclease, and processed as described in Table 1 and the text. Target proteins were eluted with 2 x 375 µl of GST Elute Buffer.

Panel B. The same recombinant used in Panel A was induced with IPTG for 3 h (final OD_{600} = 4.8). The culture was processed exactly as described in the legend of Figure 2B (n = 8 wells), except that GST • Bind Magnetic Agarose Beads and 1X GST Bind/Wash Buffer were used for purification. Target protein was eluted with 100 µl 1X GST Elute Buffer. All samples were analyzed by SDS PAGE (4–20% gradient gels) and Coomassie blue staining.

Effect of lysozyme

Lysozyme, which cleaves a bond in the peptidoglycan layer of the *E. coli* cell wall, is widely used to enhance cell lysis. We therefore investigated the effect of lysozyme on the efficiency of protein extraction when used in combination with the PopCulture Reagent. Table 3 and Figure 4 demonstrate that lysozyme increased the yield of proteins in PopCulture total extracts. In Table 3, BL21(DE3) and BL21(DE3)pLysS hosts were used for expression and purification of a His•Tag β-galactosidase fusion protein encoded by a pET plasmid. Parallel cultures were processed with PopCulture Reagent, either omitting or including the addition of lysozyme to the procedure. The data show that the yield of this large protein (a tetramer composed of 118 kDa subunits) was increased two- to three-fold by including a source of lysozyme in the extraction. Furthermore, extraction was equally effective using a pLysS host (which expresses low levels of T7 lysozyme), or adding purified chicken egg or recombinant lysozyme to the PopCulture extraction with a non-pLysS host.

Table 3. Effect of lysozyme on PopCulture yield of His•Tag β-gal

Host	Cell Mass[1]	Lys[2]	Yield[3]	Purity[4]
BL21(DE3)pLysS	11	–	27	94
BL21(DE3)pLysS	11	chicken	23	94
BL21(DE3)pLysS	11	recomb.	26	87
BL21(DE3)	15	–	11	84
BL21(DE3)	15	chicken	38	93
BL21(DE3)	15	recomb.	38	93

1. Wet weight, in mg/ml, as determined by harvesting cells by centrifugation and weighing the pellet.
2. Lysozyme added to PopCulture procedure as described in Table 1.
3. Yield in micrograms per ml of culture of β-gal purified using His•Bind Magnetic Agarose Beads, as determined by BCA protein assay.
4. % purity determined by scanning densitometry of Coomassie blue stained SDS polyacrylamide gels.

The gel analysis in Figure 4 further demonstrates that the overall extraction efficiency was enhanced by lysozyme for β-gal and GST fusion proteins. Again, low level expression of T7 lysozyme in the BL21(DE3)pLysS host was sufficient to improve target protein extraction efficiency to a level similar to that obtained by treating the BL21(DE3) host with either egg white or recombinant lysozyme. Therefore, when

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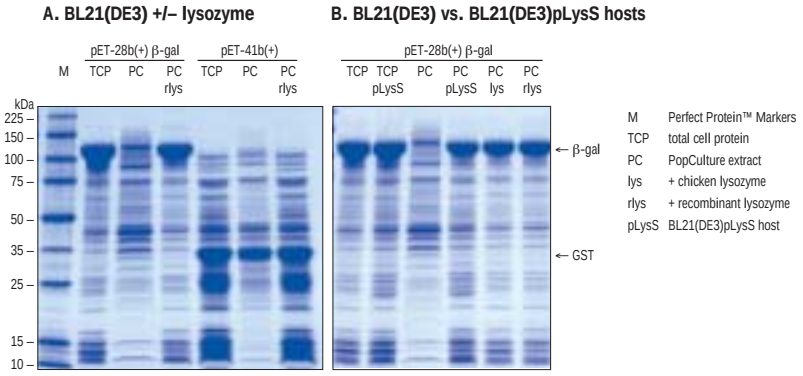

Figure 4. Effect of lysozyme on PopCulture extraction efficiency

Panel A. *E. coli* strain BL21(DE3) containing pET-28b(+) β-galactosidase or pET-41b(+) was grown in liquid culture and protein expression induced with 1 mM IPTG for approximately 3 h. To obtain sufficient protein for gel analysis, cells were concentrated prior to treatment, and were resuspended in a 1:10 dilution of PopCulture Reagent and incubated 10 min at room temperature. The indicated samples received an additional 15 min treatment with chicken egg lysozyme or recombinant lysozyme.

Panel B. BL21(DE3) and BL21(DE3)pLysS hosts containing pET β-galactosidase recombinants were grown in liquid culture and protein expression induced with 1 mM IPTG for approximately 3 h. Samples of the cultures were processed as in Panel A with the indicated lysozyme treatments. Total cell protein (TCP) samples were prepared by resuspending cell pellets in SDS sample buffer. The TCP and equal volumes of all PopCulture extracts were analyzed by SDS PAGE (4–20% gradient gels) and Coomassie blue staining.

target proteins are expressed in BL21(DE3)pLysS host strains, maximum PopCulture™ extraction efficiency may be obtained without exogenous lysozyme addition.

Effect of culture medium

As shown in Table 4, PopCulture Reagent was equally effective for extraction of proteins expressed in *E. coli* cultured in three standard media formulations. For this experiment, BL21(DE3) containing pET-41b(+) was grown in Terrific Broth (TB), 2X YT, and Luria Broth (LB, which was also used for all other experiments). The expressed His•Tag® GST fusion protein was extracted and purified using PopCulture and His•Bind® Magnetic Agarose Beads. Protein purity was similar for all media tested. However, as expected, cell mass and total protein yield were greater in the richer TB medium.

Table 4. PopCulture purification of His•Tag GST using different media

Medium	Cell Mass[1]	Yield[2]	Purity[3]
Terrific Broth	13	81	90
2X YT	11	30	95
Luria Broth (LB)	9	40	89

1. Wet weight, in mg/ml, as determined by harvesting cells by centrifugation and weighing the pellet.
2. Yield in micrograms per ml of culture of target protein purified using His•Bind Magnetic Agarose Beads, as determined by BCA protein assay.
3. Determined by scanning densitometry of Coomassie blue stained SDS polyacrylamide gels.

Summary

We have developed a detergent-based reagent, called PopCulture Reagent, that can be added directly to cultures of *E. coli* to effectively extract recombinant proteins without the need for cell harvest. Successful purification of intact fusion proteins from total culture extracts has been demonstrated using IMAC and GST affinity approaches. Based on these results, we may expect that this method would also be compatible with a number of other affinity purification strategies, including the use of immobilized antibodies and other ligands that bind to their protein targets in the presence of salts and other media components present in total culture extracts. This novel method provides a number of advantages for high-throughput purification of recombinant proteins, including:

- Elimination of the need for separating cells from culture media.
- Elimination of the need for mechanical disruption of cells.
- Elimination of the need to clarify cell extracts prior to purification.
- Direct affinity adsorption of target proteins to resin directly from the total culture extract.
- Ability to rapidly perform the entire cell growth and purification process in a single tube or well.

Our experiments have shown that the addition of lysozyme or the use of a pLysS host increases the efficiency of protein extraction with the procedure. Benzonase® Nuclease may also be added to degrade endogenous nucleic acids. While viscosity has not been a problem with these experiments, it is possible that procedures using higher cell densities, robotic manipulations, and/or specific target proteins would benefit from the addition of Benzonase.

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Product	Size	Cat. No.
PopCulture™ Reagent	15 ml	71092-3
	75 ml	71092-4
	250 ml	71092-5
PopCulture GST•Bind™ Magnetic Purification Kit (includes PopCulture Reagent, GST•Bind Magnetic Agarose Beads, Buffers and Chicken Egg Lysozyme)		71094-3
PopCulture His•Bind® Magnetic Purification Kit (includes PopCulture Reagent, His•Bind Magnetic Agarose Beads, Buffers and Chicken Egg Lysozyme)		71095-3
Benzonase® Nuclease, Purity > 90%	10,000 U	70746-3
Lysozyme, Chicken Egg White ~	1 g	4403
	5 g	
GST•Bind™ Magnetic Agarose Beads	2 x 1 ml	71084-3
	10 x 1 ml	71084-4
His•Bind® Magnetic Agarose Beads	2 x 1 ml	71002-3
	10 x 1 ml	71002-4
Magnetight™ Separation Stand (holds 4 x 1.5-ml tubes, 1 x 15-ml tube, or 1 x 50-ml tube)		69964-3
Magnetight Multitube Rack (holds 30 x 1.5-ml tubes, with 10-place removable magnet)		70747-3

~ = Calbiochem brand product

Domain mapping of nuclear transport factors

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For our studies on the mechanism of nuclear protein transport, we analyzed the interactions of one of the main factors required for transport, importin β, with other factors known to be involved in the process. Domain mapping of importin β to identify the Ran-binding domain and the nuclear pore complex binding domain was carried out using fragments of importin β expressed in Novagen's pET-30 expression vector, which produces fusion proteins containing the S•Tag™ sequence.

A family of transporters called importins/exportins, or karyopherins, mediates the transport of many proteins between the cytoplasm and nucleus (1). Each of these transporters binds to a cargo protein and carries the protein through the nuclear pore complex. The binding of Ran-GTP to the transporter regulates the binding of cargo proteins to the transporters. Exportins can only bind cargo cooperatively with Ran-GTP, whereas importins release their cargo when bound to Ran-GTP. To understand how transport is regulated, we needed to map the domains of importin β that bind to the nuclear pore and to Ran-GTP. In order to create the recombinant importin β necessary for these experiments, we cloned the human importin β cDNA into Novagen's pET-30 vector. Our choice of pET-30 for expression in bacteria was influenced by the small size of the S•Tag to minimize interference with the rest of the protein and the high affinity interaction of the S•Tag with S-protein. The S•Tag binds S-protein very specifically and under a variety of conditions allowing us some latitude in our choice of binding conditions.

Mapping of binding domains

We first mapped the Ran-binding domain on importin β (2). A number of amino and carboxyl terminal deletions of importin β were created in pET-30 and expressed in *E. coli* BL21(DE3) cells. The fusion proteins were purified binding to Novagen's Ni-NTA His•Bind® Resin using the His•Bind Buffer Kit. In some cases, the S•Tag fusion proteins were adsorbed onto S-protein Agarose from a 100,000 × g supernatant from the lysed bacteria and used directly in binding experiments. The amount of each fusion protein on a given amount of beads was adjusted so that each binding assay had an equimolar amount of importin β present. Each aliquot of beads with an importin β fusion protein was incubated with 1 µg of recombinant Ran that had been charged *in vitro* with GTP. After a 30-minute incubation at 4°C, the beads were washed and the bound proteins eluted with 1X SDS-PAGE sample buffer. The samples were resolved by SDS-PAGE and transferred to nitrocellulose. Western blotting with an antibody to Ran was used for detection. The immunoblot in Figure 1 clearly shows that the amino terminal 282 amino acids were required for efficient Ran-GTP binding. Deletion of even the first seven amino acids from the amino terminus greatly decreased binding. This domain map for Ran-binding by importin β has subsequently been confirmed by the X-ray crystal structure of the importin β/Ran-GTP complex.

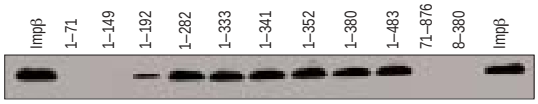

Figure 1. Domain map of Ran-GTP binding to importin β

S•Tag fusion proteins of fragments of importin β (Impβ) were expressed in pET-30, purified and bound to S-protein Agarose. Recombinant Ran-GTP was mixed with each fusion protein and bound protein was resolved by SDS-PAGE. A Western blot of the gel was probed with an antibody to Ran and the antibodies were detected by chemiluminescence. The amino acid residues of human importin β present in each construct are indicated.

In order for importin β to carry a cargo protein through the nuclear pore complex, it must bind to several components of the pore called nucleoporins. Mapping of the domain of importin β that binds to the nuclear pore complex was carried out in a permeabilized cell assay (3). Cells grown on glass coverslips are permeabilized with the glycoside digitonin in an isotonic buffer. The digitonin permeabilizes the plasma membrane but leaves all of the intracellular membranes intact. These cells are capable of carrying out nuclear protein import in the presence of added cytosol and are therefore useful to map the interactions of importin β with the nuclear pore complex. The same S•Tag importin β fusion proteins that were used to map the Ran-binding domain were used to map the pore-binding domain (4). Ni-NTA His•Bind purified proteins were dialyzed into import buffer and

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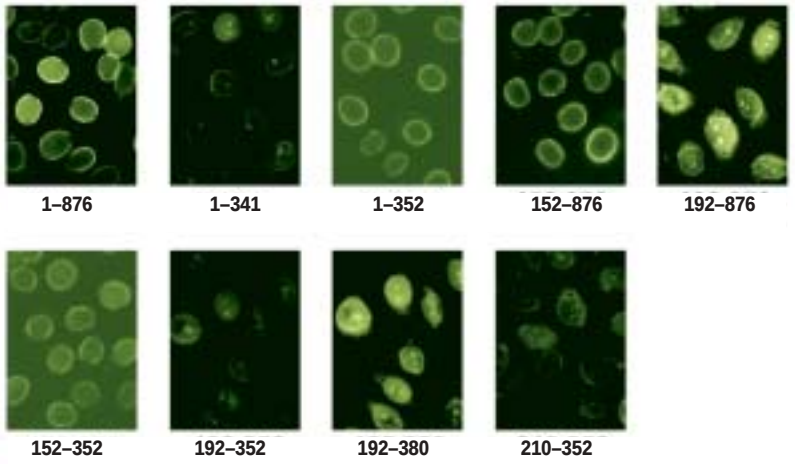

Figure 2. Binding of importin β to permeabilized cells

S•Tag fusion proteins of fragments of importin β were incubated on permeabilized MDBK cells grown on glass coverslips. The fusion proteins were detected with S-protein FITC and viewed by epi-fluorescence microscopy.

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concentrated to equalize the molar concentrations between fusion proteins. The S•Tag proteins were incubated with digitonin-permeabilized cells for 15 minutes on ice followed by washing with import buffer. The bound importin β was detected by incubating the cells with Novagen's S-protein FITC conjugate at the recommended dilution for 15 minutes on ice. After washing to remove unbound S-protein, the coverslips were mounted in import buffer and observed by epi-fluorescence microscopy. Figure 2 shows that the pore complex binding domain of importin β lies between residues 152 and 352 of the protein. This binding domain was recently confirmed by the X-ray crystal structure of importin β bound to a fragment of a pore complex protein.

Discussion

We have used the pET-30 expression system to analyze the binding domains of several other proteins involved in nuclear transport. In every case, the ability to use either the S•Tag or the His•Tag® sequences in each fusion protein has been instrumental in our success. The S•Tag is compatible with other fusion tags and is highly specific for the S-protein so proteins with different types of tags can be combined in binding assays. The high affinity interaction of the S•Tag with S-protein has enabled us to wash complexes with high stringency without eluting the fusion protein from the affinity resin. The S•Tag can be used for detection of fusion proteins on immunoblots, which can be useful in screening bacterial colonies for poorly expressed proteins. The fluoresceinated S-protein does have a low background of nuclear binding, similar to some secondary antibodies, but this background does not interfere with the detection of specific binding.

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Product	Size	Cat. No.
pET Expression System 30 plus Competent Cells		70782-3
Ni-NTA His•Bind® Resin	10 ml	70666-3
	25 ml	70666-4
	100 ml	70666-5
His•Bind Buffer Kit		69755-3
S-protein FITC Conjugate	200 µl	69060-3
S-protein AP Conjugate	50 µl	69598-3
S-protein Agarose	2 ml	69704-3
	5 x 2 ml	69704-4

Expression and purification of insect cadherin ectodomain using the pTriEx-2 multisystem vector in Origami and Tuner cells

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Cadherins constitute a large family of calcium-dependent single-transmembrane glycoproteins that are responsible for maintaining the integrity of cell-cell contacts in all multicellular organisms. By interacting with other cell junction proteins via their ectodomains and with the actin cytoskeleton via their cytoplasmic domains, cadherins mediate the rearrangement of cell-cell adhesions during development and growth of cells that are undergoing proliferation, differentiation and physical grouping. We have identified developmentally important cadherin molecules that are specific to the midgut epithelium of a number of insects. These midgut specific insect cadherins are crucial target receptors for the Cry toxins of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt). The toxins bind with high affinity and specificity to the cadherin receptors on the midgut epithelium surface and exert insecticidal toxic action by disrupting the structural and functional integrity of the tissue.

Characterization of insect cadherin receptors for Cry toxins is fundamentally important for continued and improved use of environmentally sound microbial insecticides based on Bt. However, membrane association of cadherin receptors and their specific expression profiles during insect development necessitate cloning and characterization of the corresponding receptor genes in different biological systems including bacteria, insect and mammalian cells. To gain insight into the biological properties of insect cadherin receptors for Cry toxins and the structural determinants involved in receptor-toxin interactions, we cloned a 265-amino acid ectodomain portion of a cadherin receptor from a lepidopteran insect (moth) and expressed the peptide with a His•Tag® sequence using the pTriEx™-2 multisystem expression vector. Expression of the recombinant pTriEx-2 plasmid in Origami™(DE3)pLacI and Tuner™(DE3)pLacI cells followed by purification of the ~35 kDa target product using His•Bind® metal chelation chromatography was accomplished flawlessly, generating high yields of the protein (Figure 3). Although expression in the bacterial hosts resulted in accumulation of the target protein in inclusion bodies, solubilization and affinity enrichment of the protein under denaturing conditions followed by purification and multistep dialysis in renaturing conditions efficiently generated refolded protein retaining complete toxin binding ability. The expressed and purified product was suitable for determining toxin-receptor interaction properties as well as for

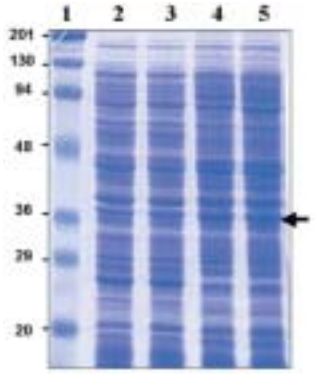

Figure 1. Expression of cadherin ectodomain fragment in LB medium supplemented with 1% glucose.

A truncated cDNA fragment encoding a 265-amino acid ectodomain portion of a lepidopteran cadherin receptor for Cry toxins was cloned into pTriEx-2 vector using NovaBlue Singles™ Competent Cells. The recombinant plasmid was later transformed into the Origami(DE3)pLacI and Tuner(DE3)pLacI bacterial hosts for expression. The cells were grown in LB medium containing 50 µg/ml carbenicillin supplemented with 1% glucose for 16 h by shaking vigorously at 37°C. To induce the expression of the target protein, IPTG was added to a final concentration of 1 mM. Protein samples from cultures of transformed Origami (lanes 2 & 3) and Tuner (lanes 4 & 5) cells, before induction (lanes 2 & 4) and 3 hours after induction (lanes 3 & 5), were analyzed by SDS gel electrophoresis. The level of target protein (band shown by arrow) expression in both hosts was not satisfactory when induction was performed in the presence of glucose.

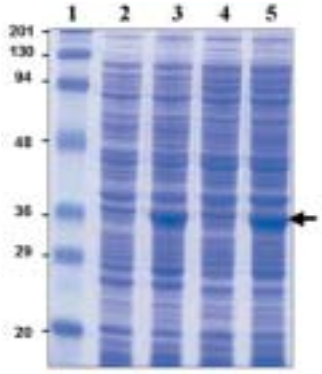

Figure 2. Expression of cadherin ectodomain fragment in LB medium without glucose.

The bacterial hosts Origami(DE3)pLacI and Tuner(DE3)pLacI were transformed with the recombinant plasmid containing an insect cadherin ectodomain cDNA fragment. The transformed cells were grown in LB medium containing 50 µg/ml carbenicillin supplemented with 1% glucose for 16 h by shaking vigorously at 37°C. Then the bacterial cells were collected by centrifugation at 2,000 x g for 20 min at 4°C. The bacteria were resuspended in LB medium without glucose. IPTG was added to the final concentration of 1 mM for induction of target protein expression. Protein samples from cultures of transformed Origami (lanes 2 & 3) and Tuner (lanes 4 & 5) cells, before induction (lanes 2 & 4) and 3 hours after induction (lanes 3 & 5), were analyzed by SDS gel electrophoresis. The expression was efficient and high yields of ~35 kDa target protein (band indicated by arrow) were achieved when both hosts were induced by IPTG in the absence of glucose.

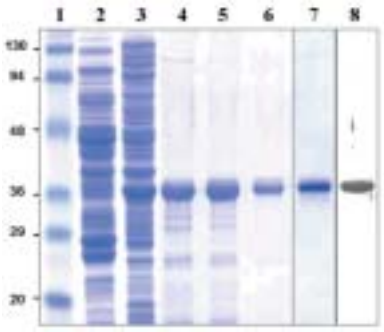

Figure 3. Purification of receptor cadherin ectodomain fragment and immuno-ligand blot analysis of toxin-receptor binding.

A 100-ml sample of bacterial culture expressing the His • Tag fusion protein was harvested after 3 h of induction by centrifugation at 10,000 x g for 10 min at 4°C. The bacterial cell pellet was weighed and resuspended in appropriate volume (5 ml/g) of BugBuster™ Reagent. The suspension was transferred into a 15-ml polypropylene tube and Benzonase® Nuclease (25 units/ml) was added. The suspension was gently mixed at room temperature for 20 min and then transferred into a 15-ml glass COREX centrifuge tube. Soluble and insoluble fractions were separated by centrifugation at 16,000 x g for 15 min at 4°C and analyzed by SDS gel electrophoresis (lanes 2 & 3, respectively). Most of the ~35 kDa target product was observed in inclusion bodies in the pellet. The pellet was solubilized in 6 ml of binding buffer (His • Bind Buffer Kit) containing 6 M urea. The solution was added to 1 ml of His • Bind Resin in a small polypropylene column and mixed gently for 30 min at room temperature. The column was washed and the target protein purified according to the protocol provided with the His • Bind Buffer Kit. The protein was eluted in two 2-ml fractions in Elute Buffer (lanes 4 & 5). The fractions were pooled, dialyzed against the binding buffer with 6 M urea and further enriched with the recharged His • Bind column. The fractions obtained at this step contained the purified target protein (lane 6). The protein was subjected to refolding by using multistep dialysis against buffer (10 mM Tris-HCl, pH 8.0) with decreasing concentrations of urea (6 M, 3 M, 1.5 M, 0 M). The final product (lane 7) efficiently bound the Cry toxin in an immuno-ligand blot experiment (lane 8).

structure analysis using CD-spectrometry and X-ray crystallography. Furthermore, efficient expression of the target receptor protein by the versatile pTriEx-2 vector facilitated studies of the receptor-toxin interactions in various heterologous cell systems without subcloning into additional vectors.

Results and discussion

Recombinant proteins containing fusion tags facilitate purification and detection of proteins and, sometimes, can simplify molecular characterization studies. There are a wide variety of expression vectors and hosts for amplification of fusion proteins. The criteria for selecting an appropriate vector and host for the amplification of a fusion construct is complicated by the nature of the target molecule and various factors that influence the expression, solubility, conformation, yield and post-translational modification of proteins. Furthermore, it is crucial that the structural and functional properties of the fusion product correlate with the actual characteristics of the target protein and that they are suitable for downstream applications. The pTriEx-2 vector was employed successfully to clone and amplify a fusion tagged cadherin ectodomain fragment from the midgut epithelial cells of a moth. The vector was very efficient in generating high yields of the target protein when expression was induced in Origami and Tuner bacterial cells. Both bacteria containing the expression vector were grown in LB medium containing 1% glucose (Figure 1), however, the expression level of the target protein was significantly higher when induction with IPTG was done in the absence of glucose (Figure 2). In addition to efficient expression in bacterial hosts, the pTriEx-2 vector also enables expression in insect and mammalian cells because it contains special promoter elements. This feature is advantageous because of the time saved by eliminating the need for cloning into different vectors. In conclusion, pTriEx-2 vector and Origami(DE3) pLacI and Tuner(DE3)pLacI bacterial cells are ideal for cloning and expression of cadherin ectodomain fragments. Furthermore, when combined with affinity purification using His•Bind Resin, the approach can provide efficient molecular tools that streamline biochemical analyses designed to examine the structure and function of recombinant proteins.

Product	Size	Cat. No.
pTriEx™ Bacterial Expression System 2 (includes pTriEx-2 vector, Tuner™(DE3)pLacI, Origami™(DE3)pLacI Competent Cells, SOC Medium, Test Plasmid)		70867-3
His • Bind® Resin	10 ml	69670-3
	50 ml	69670-4
	100 ml	69670-5
His • Bind® Buffer Kit		69755-3
BugBuster™ Protein Extraction Reagent	100 ml	70584-3
	500 ml	70584-4
Benzonase® Nuclease, Purity > 90%	10,000 U	70746-3

Use of glucose to control basal expression in the pET System

Robert Novy and Barbara Morris—Novagen, Inc.

Many popular bacterial expression systems, including the pET system, contain components of the *lac* operon. For example, in the pET system transcription of the target gene is controlled by a bacteriophage T7 promoter, and the production of the T7 RNA polymerase in expression hosts (λDE3 lysogens) is regulated by a *lac* promoter derivative, the *E. coli* L8-UV5 *lac* promoter (see Figure 1).

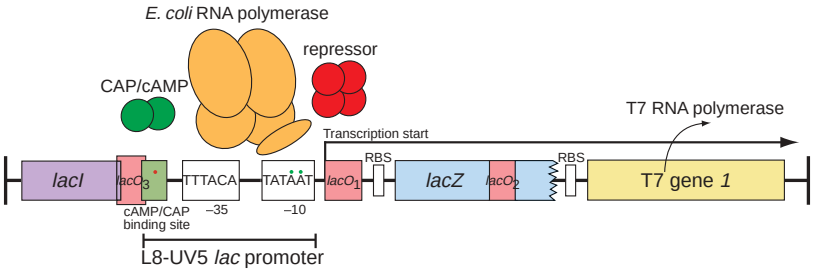

Figure 1. Transcriptional control of T7 gene *1* in λDE3 lysogens

Transcription of T7 gene *1* (encoding T7 RNA polymerase) in pET System expression hosts (λDE3 lysogens) is controlled by the L8-UV5 *lac* promoter. T7 gene *1* is transcribed as the second gene in a bicistronic mRNA (the first gene contains an N-terminal fragment of *lacZ* that includes the α-peptide coding region). Positions of the three mutations of the wild type *lac* promoter region are indicated by colored circles. The *lac* repressor (*lacI* gene product) binds to $lacO_1$, and then interacts with pseudo-operators $lacO_2$ and $lacO_3$ to prevent transcription by *E. coli* RNA polymerase. The inducer IPTG binds to the repressor, reducing its affinity for $lacO_1$ and thus enabling transcription to occur. When cAMP levels are sufficiently high (e.g., in the absence of glucose) the CAP/cAMP complex is formed and binds immediately upstream from the promoter to fully stimulate transcription. In the presence of glucose, CAP/cAMP is not formed and transcription is decreased. This is called the glucose effect, or catabolite repression.

Negative regulation by *lac* repressor

The wild type *lac* operon has two distinct mechanisms of regulation; one is negative (decreases transcription), and the other is positive (stimulates transcription). Negative regulation is mediated by the *lac* repressor. Transcription initiation begins with the binding of *E. coli* RNA polymerase to the promoter; however, the successful transition from transcription initiation to transcription elongation can be influenced by downstream elements. Between the promoter and the coding regions in the operon is the *lac* operator, which is a specific DNA sequence to which *lac* repressor binds. The binding of repressor to the operator greatly decreases the frequency of successful transcription elongation events by the RNA polymerase. Inducers of the *lac* operon (e.g., IPTG) permit transcription because they bind to the *lac* repressor and substantially decrease its binding affinity to the *lac* operator.

Positive regulation by CAP + cAMP and the glucose effect

It would seem that there should be little to no expression in cells in the absence of inducer and expression should proceed when an inducer is added. However, efficient transcription initiation also requires the presence of cyclic AMP (cAMP) and cyclic AMP receptor protein, called CRP or CAP. The CAP/cAMP complex binds just upstream of the *lac* promoter and directly stimulates transcription by RNA polymerase. Because the binding of CAP to DNA requires cAMP, induction of transcription depends on the level of cAMP in the cell. cAMP levels are strongly influenced by the carbon source present in the medium. In the presence of glucose (an easily metabolized monosaccharide), cAMP levels are low, so transcription from the *lac* promoter is low. This phenomenon is called the glucose effect or catabolite repression and is shared by a number of *E. coli* operons. When glucose is absent and the cell is forced to use an alternative carbon source, such as glycerol, cAMP levels rise. The resulting formation of the CAP/cAMP complex stimulates transcription from the *lac* promoter. Therefore, full induction of the *lac* operon is achieved only in the presence of both inducer and elevated cAMP levels.

lac elements and other transcriptional controls in the pET System

The lambda DE3 prophage encoding T7 RNA polymerase in pET expression hosts carries the L8-UV5 promoter, which has three point mutations that distinguish it from the wild type *lac* promoter (Figure 1). Two point mutations in the –10 region increase promoter strength and decrease its dependence on CAP/cAMP stimulation for full activation. The third-point mutation is located in the CAP/cAMP binding site and decreases the affinity for CAP/cAMP. This mutation reduces, but does not eliminate, sensitivity to catabolite repression. The net effect of the three-point mutations is the creation of a stronger promoter that is less sensitive to the glucose effect. This allows strong IPTG induction of T7 RNA polymerase expression even in the presence of glucose.

Although the *lac* and L8-UV5 promoters are well repressed in the absence of inducer, both exhibit detectable basal activity. In the case of λDE3 lysogens, basal expression of even a small amount of T7 RNA polymerase can lead to problems if the target gene in the pET vector produces a protein toxic to the host cell. Therefore, additional levels of control are built into the pET vectors and hosts. Vectors with a "T7*lac*" promoter have a T7 promoter followed by a *lac* operator sequence. The operator in these plasmids provides a place for *lac* repressor to bind, reducing transcription by any T7 RNA polymerase that may be expressed in the absence of inducer. Another level of control is provided in expression hosts containing the pLysS plasmid, which expresses T7 lysozyme, a protein that binds to and inhibits T7 RNA polymerase. The need for these additional sources of regulation depends on the target protein being expressed; the more damaging the protein is to bacterial cells, the more regulation is required.

As first described by Grossman et al. (1), yet another level of regulation can be employed with the pET System by exploiting the glucose effect described above, i.e.,

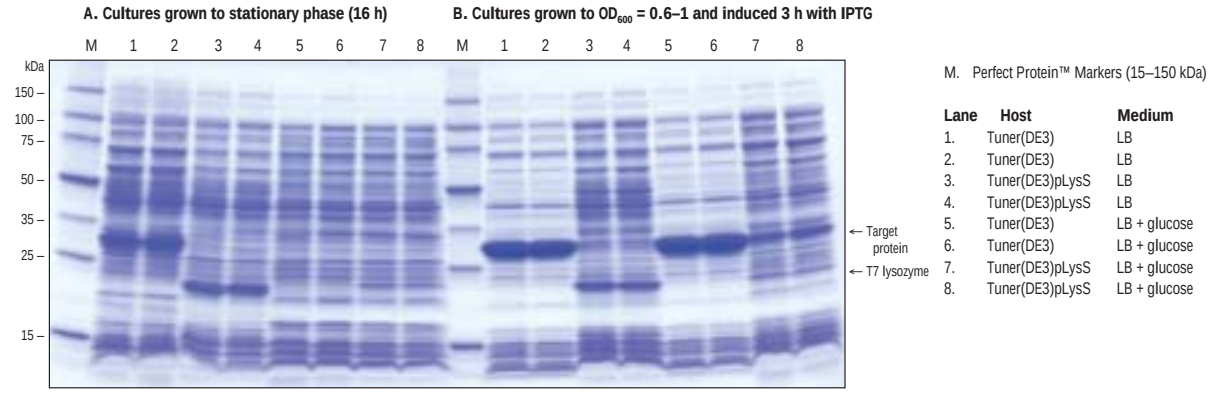

Figure 2. Expression of GFP from a pET-30 construct under different conditions

Tuner(DE3) and Tuner(DE3)pLysS hosts carrying a pET-30 Ek/LIC GFPuv recombinant were grown under various conditions and analyzed for target protein expression by SDS-PAGE of total cell extracts. In Panel A, 3-ml cultures were grown to stationary phase by overnight incubation (16 h) at 37°C with shaking at 300 rpm. In Panel B, 3-ml cultures were grown to an OD_{600} between 0.6 and 1.0 and then induced by the addition of 1 mM IPTG for 3 h at 37°C with shaking at 300 rpm. For gel analysis, cells were harvested by centrifugation and the pellets resuspended in BugBuster™ HT Protein Extraction Reagent. After the addition of 4X SDS Sample Buffer, samples corresponding to equivalent numbers of cells (based on harvest OD_{600}) were loaded on a 10–20% gradient gel. In both panels, lanes 1–4 represent cultures grown in standard LB broth and lanes 5–8 represent cultures grown in LB broth supplemented with 1% glucose. The respective hosts are indicated. Pairs of lanes represent duplicate samples derived from independent clones.

supplementing standard media such as LB with glucose to keep cAMP levels low. Apparently, although the L8-UV5 promoter is less dependent on CAP/cAMP stimulation than the wild type *lac* promoter, in practice there is still a significant reduction in basal transcription in the presence of glucose. This can be particularly important for pET vector expression when hosts that do not carry the pLysS plasmid are allowed to grow to stationary phase, where uninduced expression is maximal (1, 4). Others have also reported that supplementing LB media with glucose to a final concentration of 0.5–1.0% prevents the increased basal activity observed in cultures grown to stationary phase (1, 2).

Example of the glucose effect on pET expression

Figure 2 demonstrates the dramatic difference that glucose can make when cultures are grown to stationary phase by overnight incubation at 37°C. pET-30 recombinants expressing green fluorescent protein (GFP) in Tuner™(DE3) and Tuner(DE3)pLysS hosts were tested under different growth conditions. Cultures in LB medium lacking or containing 1% glucose were grown to stationary phase (16 hours) or to log phase (OD_{600} = 0.6 and 1.0). The log phase cultures were then induced with IPTG for 3 hours at 37°C. Each condition was carried out in duplicate with two independent recombinants. Cells were harvested at the end of the culture period and total cell protein (TCP) samples were analyzed by SDS-polyacrylamide gel electrophoresis.

Figure 2, panel A shows the gel profiles of the overnight cultures grown without added inducer. The overnight Tuner(DE3) cultures lacking glucose (lanes 1–2) exhibited easily detectable levels of target protein production, whereas target protein was undetectable in the same cultures supplemented with 1% glucose (lanes 5–6). In contrast, the Tuner(DE3)pLysS cultures grown to stationary phase did not require glucose to prevent uninduced expression (lanes 3–4 without glucose vs. lanes 7–8 with glucose).

Supplementing LB medium with 1% glucose prevents increased basal expression in λDE3 lysogens grown to stationary phase.

Figure 2, panel B demonstrates that glucose addition did not interfere with IPTG induction of the target protein. In fact, IPTG induction from the pLysS host appeared to be enhanced in the presence of glucose. High induction was observed from the Tuner(DE3) cultures regardless of glucose addition (panel B, lanes 1–2 vs. lanes 5–6). Much lower expression of the target protein was observed from the pLysS-based host grown in glucose (lanes 7–8), but it was barely detectable in the host grown without glucose (lanes 3–4).

One possible explanation for the low IPTG induction results observed in the pLysS host is that in the absence of glucose the expression of T7 lysozyme from pLysS may be substantially elevated. In uninduced pLysS host cultures, some transcription of the LysS gene is probably achieved via read-through transcription from the upstream chloramphenicol acetyltransferase (CAT) promoter. In the absence of glucose, cAMP levels would be expected to rise during the later stages of the growth cycle. Because the CAT promoter is also stimulated by the CAP/cAMP complex (3), elevated transcription of the lysozyme gene from the CAT promoter would occur. In Figure 2, a unique protein band was observed between the 15 and 25 kDa protein markers in pLysS cultures, which corresponds to the predicted size of T7 lysozyme (17 kDa). This band was significantly more intense in pLysS cultures grown without glucose relative to those grown in the presence of glucose. A sufficiently high level of T7 lysozyme may saturate all of the available T7 RNA polymerase and thereby block target gene transcription. This may account for the variability that is sometimes observed when attempting to induce target proteins in pLysS hosts.

Summary

In conclusion, supplementing culture media with glucose provides a simple, inex-

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pensive method to maintain very low basal expression levels of T7 RNA polymerase in the λDE3 lysogenic expression hosts used in the pET System. This is especially true when λDE3 hosts carrying pET plasmids are grown to stationary phase. A disadvantage with glucose addition is that after an initial rapid growth phase the metabolic breakdown products of glucose will lead to acidic culture conditions and lower cell density at stationary phase. The data presented in Figure 2 demonstrate that strong induction can be achieved from λDE3 lysogens in the presence of glucose for some target proteins. Note, however, that theoretically the strongest induction of T7 RNA polymerase would be expected when glucose is absent and cAMP levels are elevated. Accordingly, in some cases (see preceding article), higher target protein expression may be observed in the absence of glucose. Overall, the optimal combination of stringent uninduced repression and high induced expression may be achieved by initial growth in the presence of glucose, followed by switching to medium without glucose for induction.

Novagen's recommendations for growth and induction of pET constructs in expression hosts are based on the information presented above. For innocuous proteins, any pET vector and λDE3 lysogen are suitable in a variety of media. But for proteins that are potentially toxic to the bacterial cell, we recommend using either a pET vector with a T7*lac* promoter or expression hosts that carry the pLysS plasmid. In addition, our general advice is to avoid growing a λDE3 lysogen carrying a pET plasmid to stationary phase. If the cells must be grown to stationary phase, we recommend the addition of 0.5 to 1.0% glucose to the medium, so that the glucose effect can be exploited to reduce basal expression.

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Preparation of protein samples for SDS-polyacrylamide gel electrophoresis: procedures and tips

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Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used analytical method to resolve separate components of a protein mixture. It is almost obligatory to assess the purity of a protein through an electrophoretic method. SDS-PAGE simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix (1). The technique is also a powerful tool for estimating the molecular weights of proteins (2, 3). The success of SDS-PAGE as an indispensable tool in protein analysis has been attributed to three innovations that permitted the correlation of electrophoretic mobility with a protein's molecular mass (4). First was the introduction of discontinuous buffer systems where the sample and gel running buffers differ in both composition, Tris-HCl/Tris-glycine, and pH, 6.8/8.3, respectively (5, 6). Discontinuous buffer systems allow larger sample volumes to be loaded while maintaining good resolution of sample components because the proteins are focused, or "stacked," as thin bands prior to entering the resolving gel. Second was the use of the detergent sodium dodecyl sulfate (SDS) and reducing agents to denature proteins (7). SDS binds strongly to proteins at an approximate ratio of 1 dodecyl sulfate molecule per 2 amino acid residues (8). Therefore, the negative charge/unit mass ratio when SDS is bound to the polypeptide chain is similar for all proteins. Third was the combination of the first two discoveries employing a simple Tris-glycine buffer system (9). More recently, buffer combinations such as Tris-borate (10) and Tris-tricine (11) have improved the resolving power of the original methods. Modern SDS-PAGE has evolved to use microslab precast gels (12). Precast and packaged gels in a wide variety of gel formulations, acrylamide percentages, thicknesses, well formats, and buffer systems are now commercially available from several manufacturers. Therefore, successful SDS-PAGE analysis of protein samples no longer depends on tedious gel casting, buffer preparation and apparatus set-up, but on careful sample preparation and treatment prior to loading the gel. This article describes techniques and procedures as a guide for preparation of protein samples for SDS-PAGE analysis.

Sample buffer preparation

To ensure consistent and successful PAGE analysis, the highest purity reagents should be used to prepare sample buffer stock solutions. After a reliable source of electrophoresis reagents has been identified, the vendor and buffer component chemicals should be maintained. High purity electrophoresis, Ultrol® grade, and molecular biology grade reagents are available through Novagen's partner brand, Calbiochem. Solutions must be carefully and safely prepared, dated, and chemical lot numbers recorded. Concentrated stock solutions should not be stored for long periods of time. Tris base, rather than Tris-Cl, should be used for buffer preparation and pH adjustment made with HCl. Use of Tris-Cl

will result in a higher ionic strength, poor migration and diffuse protein bands (13). A respirator or dust mask should be worn when handling powdered SDS. Sulfhydryl reagents, dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) can be unstable in solution and are toxic. These chemicals should be measured in a fume hood while wearing gloves and safety glasses. Although 2-ME is historically the chemical of choice for reduction of protein disulfide bonds in SDS-PAGE (7, 9), DTT is also a very effective alternative (14). Glycerol is added to increase the sample density, facilitating gel loading and preventing convective migration out of the sample wells. A small amount of bromphenol blue is added as a visual aid during sample loading and as a tracking dye, allowing easy monitoring of electrophoretic progress.

The sample buffer recipes listed in Table 1 are commonly used for Tris-glycine SDS-PAGE analysis of protein samples under denaturing, reduced conditions (7, 9, 13). When preparing these buffers, wear gloves to avoid keratin (skin protein) contamination. A heterogeneous cluster of bands around 55 kDa can be seen when a keratin-contaminated sample or sample buffer is used. This is particularly obvious with high sensitivity silver staining methods. The sample buffer should be divided into 1-ml aliquots and can be stored frozen (–70°C) for several months. Prior to use, warm (37°C) and mix the solution briefly to completely dissolve the SDS.

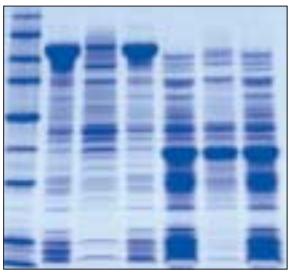
Table 1. SDS-PAGE sample buffer recipes

Component	Concentration	
	2X	4X
Tris-HCl, pH 6.8[1]	0.125 M	0.25 M
SDS	4%	8%
2-ME[2]	5%	10%
DTT[3]	0.15 M	0.3 M
Glycerol	20%	30%
Bromphenol blue	.01%	.02%

1. Prepared using Tris base, pH adjusted with HCl.
2. If 2-ME is used, omit DTT.
3. If DTT is used, omit 2-ME.

Protein sample preparation

Sample preparation is critical for clear and accurate resolution of protein bands. Photographic quality results are routinely possible if samples are carefully prepared. Common mistakes during sample preparation include using an incorrect protein-to-sample buffer ratio, delayed heating, overheating, failure to remove insoluble material, and overloading and underloading of protein. To prevent inadequate sample buffer-to-protein ratios, overloading, and underloading of samples, the protein concentration of the sample should be determined using a standard protein assay such as the CB-Protein Assay™, Non-Interfering Protein Assay™, or bicinchoninic acid (BCA) assay prior to sample buffer addition. Loading too much protein will result in distorted, poorly resolved bands in the overloaded lane and distorted electrophoretic patterns in adjacent lanes. Underloading simply prevents detection of minor components while even major bands will be too faint for photographic reproduction of the gel. Depending on the well size and gel thickness, the amount of protein loaded should range from 0.5–4.0 µg for purified samples and from 40–60 µg for crude samples if a Coomassie blue stain (e.g., RAPIDstain™) is used. Silver staining methods (such as the FASTsilver™ Kit) are approximately 100-fold more sensitive, and therefore require less protein per sample.



Sample preparation is critical for clear and accurate resolution of protein bands.

SDS-PAGE sample buffer treatment is designed to completely dissociate all proteins into their subunit polypeptides. Proteins heated in the presence of SDS are denatured and imparted with a strong negative charge. Thiol reagents in the sample buffer reduce disulfide bonds. It is important to use enough sample buffer in order to maintain an excess of SDS. Most polypeptides bind SDS in a constant mass ratio of 1.4 µg SDS per 1.0 µg polypeptide, but a ratio of 3:1 is recommended (15). The 2X sample buffer prepared as shown in Table 1 contains 40 µg/µl SDS. Maintained reduction of protein sulfhydryls is essential in order to prevent intramolecular disulfide bond formation through oxidized cysteines. If artifactual band heterogeneity or unusual doublets are noted in SDS-PAGE results from samples containing sulfhydryls, insufficient reducing agent was present during sample treatment or the oxidation of cysteines may have occurred during the stacking phase of electrophoresis. These artifacts may be prevented if samples are treated with iodoacetamide (IAA) after heating in the appropriate concentration of sample buffer. The IAA treatment irreversibly blocks sulfhydryls and destroys excess reducing agent (16). Therefore, the sample buffer recipes in Table 1 do not necessarily indicate functional dilution factors, but rather they are convenient stock concentrations permitting correct addition of reagents to samples of high and low protein concentration.

Delayed heating of samples after sample buffer addition or excessive heating can cause electrophoretic artifacts due to protein degradation and peptide bond cleavage, respectively. Upon addition of SDS sample buffer, samples should be immediately mixed and heated to 85°C for three minutes. This treatment is usually sufficient to reduce disulfides, solubilize and dissociate proteins without peptide bond cleavage. Addition of SDS sample buffer will begin to denature most proteins. However, proteases are known to be resistant to SDS denaturation alone (15, 17). Partially denatured samples (particularly crude extracts) are therefore extremely sensitive to proteolytic degradation as protease active sites within the polypeptides become exposed by SDS treatment. Immediate heating limits degradation by completely denaturing all proteins including resistant proteases through the combination of heat, SDS, and reductant. Protease inhibitors may also be used during sample preparation to limit proteolysis. Excessive heating, e.g., 100°C for prolonged periods, may break peptide bonds or cause selective aggregation and band smearing (18). Asp-Pro bonds have been demonstrated to be sensitive to thermal cleavage.

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In some cases more extreme heating may be necessary to completely denature the protein (19, 20). Therefore, if prolonged heating at 100°C is necessary for complete dissociation of a thermally stable protein, the effects of such treatment upon peptide bond cleavage must be considered (21). Some proteins such as histones and membrane proteins may not completely dissolve by heating in SDS sample buffer alone and may require addition of 6–8 M urea or a nonionic detergent such as Triton X-100 (22, 23). After heat treatment in SDS sample buffer, insoluble material must be removed by brief centrifugation. This is easily accomplished by a two-minute spin in a microcentrifuge at 17,000 × g. Failure to remove precipitated insoluble material from the sample will cause streaking within the gel. The supernatant of the treated sample is now ready to load. The sample may be stored at 4°C overnight or frozen at –20°C for longer periods. Warm stored samples briefly at 37°C to redissolve the SDS and recentrifuge to remove insoluble material prior to loading.

Preparation of difficult samples

Samples that are dilute, acidic, very viscous, or that contain interfering compounds pose unique challenges to the SDS-PAGE analysis method. However, these difficult samples can be analyzed by SDS-PAGE through the application of one or more of the following pre-treatment techniques. Samples too dilute for analysis can be concentrated by several methods including lyophilization, spin concentrators, dialysis against concentrated polyethylene glycol (PEG), and absorption of excess solvent by exposure of the dialysis bag containing sample to dry PEG, Aquacide or gel filtration media such as Sephadex®. Samples concentrated through these methods may be dialyzed against 50 mM Tris-HCl, pH 6.8 to remove low molecular weight impurities prior to addition of SDS sample buffer. Dilute samples, acidic samples and samples containing interfering compounds such as potassium, guanidine hydrochloride, or ionic detergents can be precipitated by trichloroacetic acid or acetone to concentrate the proteins and remove contaminants. Protocols for TCA, acetone/methanol, and ethanol precipitation are described in references 4, 15, 17 and 24. A modified acetone precipitation method is also described in Novagen Technical Bulletin 012, available at www.novagen.com. Crude cell extracts are often extremely viscous due to the high concentration of unsheared nucleic acids. The high viscosity is problematic during gel loading, because samples are difficult to pipet and will not be evenly distributed in the sample well. Viscosity can be eliminated by treatment of samples with Benzonase® Nuclease prior to addition of sample buffer. This recombinant endonuclease completely degrades all forms of DNA and RNA and is free from proteolytic activity. Viscosity can also be reduced by physical shearing of the nucleic acids through sonication or by vigorous vortex mixing of the heated sample. Employing these pre-treatment protocols will allow successful SDS-PAGE analysis of samples that are very dilute, viscous or contaminated with interfering compounds.

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pET-41 & pET-43.1 Ek/LIC Vector Kits for high efficiency, directional PCR cloning

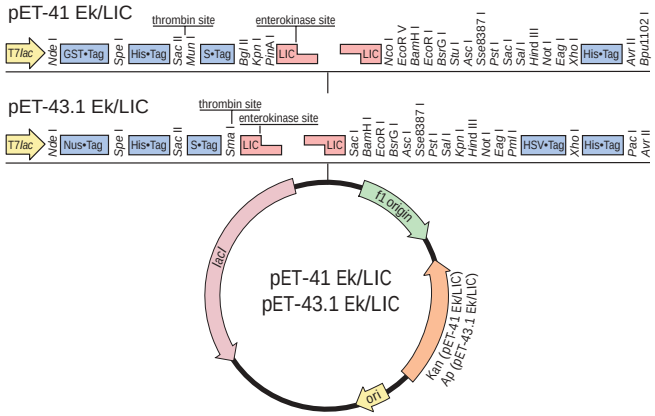
Novagen's popular pET-41 and pET-43.1 expression vectors are now available as prepared Ek/LIC vectors for convenient cloning. LIC Vector Kits offer rapid, directional cloning of PCR products without restriction enzyme digestion or ligation reactions. Special primer extensions enable the generation of long single-stranded overhangs by treatment with T4 DNA Polymerase included in the kits. The extension sequences are designed so that the exonuclease activity of the polymerase digests one strand from the 3'-end until it encounters a dTTP residue, which corresponds to the dATP present in the reaction mix. The polymerase then fills in the dATP, leaving the defined overhang. The insert anneals with the vector in the correct orientation, and no ligation is necessary prior to transformation.

The Ek/LIC site in Ek/LIC vectors (see map below) has a 13-base, single-stranded overhang on the left side and a 14-base, single-stranded overhang on the right side. The left side is designed to encode the recognition site for enterokinase (DDDDK↓). This feature enables removal of all of the vector encoded fusion sequences from expressed proteins by cleavage with enterokinase. The following sequences must be added to the 5'-end of the target gene PCR primers to generate vector-compatible overhangs:

Sense primer: 5'-GAC GAC GAC AAG ATX*

Antisense primer: 5'-GAG GAG AAG CCC GGT

* The first nucleotide of the insert-specific sequence must complete the codon ATX to give Ile (X = A, C or T) or Met (X = G).



The antisense primer may encode a stop codon or allow read-through to the vector-encoded stop codon present after the C-terminal His•Tag® sequence.

Kits contain prepared Ek/LIC vector, LIC-qualified T4 DNA Polymerase, buffers, NovaBlue Singles™ Competent Cells, BL21(DE3) Competent Cells, BL21(DE3)pLysS Competent Cells, SOC Medium and Test Plasmid.

Advantages

- Directional cloning into powerful expression vectors: pET-41 for GST fusion and pET-43.1 for NusA fusion
- No restriction digestion or ligation
- Ultra-low background: > 95% recombinants
- Complete removal of N-terminal fusion sequences with enterokinase
- Same insert preparation is compatible with all Novagen Ek/LIC vectors

Product	Size	Cat. No.
pET-41 Ek/LIC Vector Kit	20 rxn	71071-3
pET-43.1 Ek/LIC Vector Kit	20 rxn	71072-3

UltraMobius™ 200 Plasmid Kit for midi-scale isolation of ultra-high quality plasmid DNA

The UltraMobius™ 200 Plasmid Kit is designed for fast, convenient and economical isolation of up to 200 µg ultrapure, endotoxin-free plasmid DNA from 35-ml overnight bacterial cultures, using high copy number plasmids (up to 25 µg for low copy number plasmids). The kit features the same high capacity anion exchange tentacle resin as the other Mobius™ kits and incorporates a unique filter basket that minimizes the centrifugation steps required for clarification of bacterial lysates. The UltraMobius 200 Plasmid Kit differs from the Mobius 200 Plasmid Kit by including an extra step in the procedure that further reduces already low endotoxin levels reached with the Mobius Kit. Purified plasmid DNA is suitable for the most demanding applications such as DNA microinjection and transfection of sensitive cell lines.

Product	Size	Cat. No.
UltraMobius™ 200 Plasmid Kit	25 rxn	71090-3

Enzymes and Kits for PCR

Novagen now offers a complete selection of enzymes and kits for PCR, featuring KOD HiFi DNA Polymerase. This unique proofreading enzyme, isolated from the extreme thermophile *Thermococcus kodakaraensis* KOD1, possesses superior processivity and fidelity that enable faster, more accurate PCR amplification than with conventional enzymes, including *Pfu* DNA polymerase (1). KOD HiFi DNA Polymerase is also available in a Hot Start version for high specificity and increased read length (2), and as a blend (KOD XL DNA Polymerase) recommended for very long templates (3).

For increased specificity and convenience with standard PCR, we now offer Nova*Taq*™ Hot Start DNA Polymerase and the *Taq* Antibody. Nova*Taq* Hot Start DNA Polymerase is a chemically modified form of *Taq* DNA polymerase that becomes active when heated at 95°C for 7–10 minutes. The *Taq* Antibody is available as an alternative means to provide hot start capability to Nova*Taq* DNA Polymerase as well as other sources of *Taq* DNA polymerase. Please refer to the following table as a guide to select the appropriate enzyme combination for your application.

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Enzyme	PCR Product Size	Elongation Rate	Specificity	Fidelity	Yield	GC-rich Templates	PCR Product Ends
Nova*Taq* DNA Polymerase	< 5 kbp	60 bases/s	•	•	•	nr	3'-dA
Nova*Taq* Hot Start DNA Polymerase	< 5 kbp	60 bases/s	•••	•	••	nr	3'-dA
Nova*Taq* DNA Polymerase + *Taq* Antibody	< 5 kbp	60 bases/s	•••	•	••	nr	3'-dA
KOD HiFi DNA Polymerase	< 6 kbp	120 bases/s	•	•••	•••	••	blunt
KOD Hot Start DNA Polymerase	< 20 kbp	120 bases/s	•••	•••	•••	•••	blunt
KOD XL DNA Polymerase	< 30 kbp	120 bases/s	•	•••	•••	•••	blunt + 3'-dA

• = satisfactory •• = good ••• = excellent nr = not recommended

Nova*Taq*™ DNA Polymerase

Nova*Taq* DNA Polymerase* is a premium quality recombinant form of *Thermus aquaticus* DNA polymerase. This thermostable enzyme is suitable for a wide range of PCR applications. The enzyme leaves single 3'-dA overhangs that make the products suitable for cloning by T-vector or **A**ccep**T**or™ Vector methods, as well as Novagen's Perfectly Blunt® Vector Kits. The enzyme is available separately or in Nova*Taq* PCR Kits and PCR Master Mix.

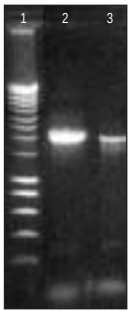
Product	Size	Cat. No.
Nova*Taq*™ DNA Polymerase	100 U	71003-3
	500 U	71003-4
	2,500 U	71003-5
Nova*Taq* PCR Kit	250 U	71005-3
Nova*Taq* PCR Kit PLUS	250 U	71006-3
Nova*Taq* PCR Master Mix	250 U	71007-3

Nova*Taq* Hot Start DNA Polymerase

Nova*Taq* Hot Start DNA Polymerase* is a heat-activatable, chemically modified form of Nova*Taq* DNA Polymerase, which is inactive at room temperature. Nova*Taq* Hot Start DNA Polymerase provides improved specificity and yield when compared to standard *Taq* DNA polymerase and can eliminate the presence of non-specific amplification products such as primer-dimers and misprimed products.

Advantages

- Higher PCR specificity and yield
- Improved low-copy target amplification
- Automated room temperature set up
- Target amplification of up to 5 kbp
- Ideal for quantitative PCR applications



1. Markers
2. 1.0 kbp fragment amplified using Nova*Taq* Hot Start DNA Polymerase
3. 1.0 kbp fragment amplified using Competitor A chemically modified *Taq* DNA polymerase

Product	Size	Cat. No.
Nova*Taq*™ Hot Start DNA Polymerase	250 U	71091-3
	5 x 250 U	71091-4

Taq Antibody

Taq Antibody is a monoclonal antibody for automated hot start PCR. It provides an antibody-mediated hot start that enhances the specificity and sensitivity of PCR. The antibody inhibits both native and recombinant *Taq* DNA polymerase activities, and is effective with Nova*Taq* DNA Polymerase. One microgram (1 µl) of antibody inhibits > 95% of 5 units of *Taq* DNA polymerase.

Product	Size	Cat. No.
Taq Antibody	100 µg	71088-3

KOD HiFi DNA Polymerase

KOD HiFi DNA Polymerase*† is a recombinant form of *Thermococcus kodakaraensis* KOD1 DNA polymerase. It is the most efficient thermostable DNA polymerase, exhibiting higher accuracy, elongation rate and processivity than any other commercially available DNA polymerase. KOD is simply the best proofreading DNA polymerase available on the market!

Advantages

- Higher fidelity than *Pfu* DNA polymerase—excellent for cloning
- Greater yield—extension speed is 2X faster than *Taq* DNA polymerase and 5X faster than *Pfu* DNA polymerase
- More accurate PCR in a shorter time
- No truncated amplification products in PCR reaction
- Free 2 mM dNTP Mix included

Product	Size	Cat. No.
KOD HiFi DNA Polymerase	250 U	71085-3

KOD Hot Start DNA Polymerase

KOD Hot Start DNA Polymerase*† is a heat-activatable form of KOD HiFi DNA Polymerase for automated PCR set up. KOD Hot Start DNA Polymerase combines the high fidelity, fast extension speed and outstanding processivity of KOD HiFi DNA Polymerase with the high yield and specificity of the antibody-based Hot Start technology.

Advantages

- Highest accuracy, yield and processivity among commercially available proofreading DNA polymerases
- Amplifies genomic DNA templates up to 12 kbp
- Amplifies plasmid DNA templates up to 20 kbp
- Successfully amplifies GC-rich sequences
- Eliminates mispriming and primer-dimer formation
- Convenient room temperature set up
- Optimal KOD Hot Start Buffer for PCR performance over a wide range of targets
- Free 2 mM dNTP Mix included

1. Markers
2. 8.4 kbp myosin heavy chain gene fragment amplified from human genomic DNA using KOD Hot Start DNA Polymerase
3. 12.3 kbp β-globin gene fragment amplified from human genomic DNA using KOD Hot Start DNA polymerase

Product	Size	Cat. No.
KOD Hot Start DNA Polymerase	200 U	71086-3
	5 x 200 U	71086-4

KOD XL DNA Polymerase

KOD XL DNA Polymerase*†‡ is a high performance enzyme mix for long and accurate PCR. The enzyme mixture is designed for reliable amplification of long, complex targets with robust yield and high accuracy.

Advantages

- Ideal for amplification of large DNA fragments from purified DNA or crude samples
- Amplifies DNA templates up to 30 kbp
- Successfully amplifies GC-rich sequences
- Free 2 mM dNTP Mix included

Product	Size	Cat. No.
KOD XL DNA Polymerase	250 U	71087-3
	5 x 250 U	71087-4

One Step RT-PCR Kit

The One Step RT-PCR Kit† is a convenient one-enzyme, single-buffer system for RT-PCR. It is specifically designed for rapid, easy RNA PCR screening using the thermostable r*Tth* DNA Polymerase.

r*Tth* DNA Polymerase acts as both a reverse transcriptase and a DNA polymerase in a unique single buffer system. The kit eliminates cumbersome northern blotting techniques to identify specific RNA species. The speed and sensitivity of the One Step RT-PCR Kit make it ideally suited for routine screening of multiple samples where the goal is to detect gene expression.

Each kit provides sufficient reagents to perform 50 RT-PCR reactions. Positive control RNA and primers set are also included.

Advantages

- Robust one-step, one-enzyme system for easy sample screening
- Minimizes carryover contamination
- High temperature reverse transcription for RNA targets with secondary structures or high GC content
- Ideal for gene expression studies

Product	Size	Cat. No.
One Step RT-PCR Kit	50 rxn	71089-3

* Sold under licensing arrangements with F. Hoffmann-La Roche Ltd., Roche Molecular Systems, Inc. and PE Corporation.
† Manufactured by TOYOBO and distributed by Novagen.
‡ Licensed under US Patent Number 5,436,149 owned by Takara Shuzo Co., Ltd.

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Proteomics I represents the dedicated effort of Novagen and Calbiochem to develop and bring together unique products specifically for the emerging field of proteomics. We are pleased to offer an extensive range of quality reagents for Protein Extraction, Purification and Analysis. Request your copy of Proteomics I by calling your local Novagen office or by visiting our web site at www.novagen.com.

Novagen, the Gold Standard in protein expression…now sets the standard in quality reagents for proteomics research.

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For your added convenience, Novagen products can also be obtained through VWR in the United States and Canada. Contact VWR at 800-932-5000 for more information or to place an order.

UPCOMING MEETINGS

Novagen representatives will be attending the **American Society for Cell Biology, 41st Annual Meeting** December 8–12, 2001 in Washington, D.C., and the **Nature Biotechnology Winter Symposia, "The Genome and Beyond—Genomics and Structural Biology for Medicine," ** February 2–6, 2002 in Miami Beach, FL. At the February meeting we will be presenting a workshop entitled, "Accelerated High-Efficiency Cloning and Multisystem Protein Expression."

Novagen

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ADDRESS CORRECTION REQUESTED

Comments and suggestions regarding the current issue of *in**Nova**tions* or ideas for articles in future issues are welcome and should be directed to: Marketing Dept., Novagen, Inc., 601 Science Dr., Madison, WI 53711.

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