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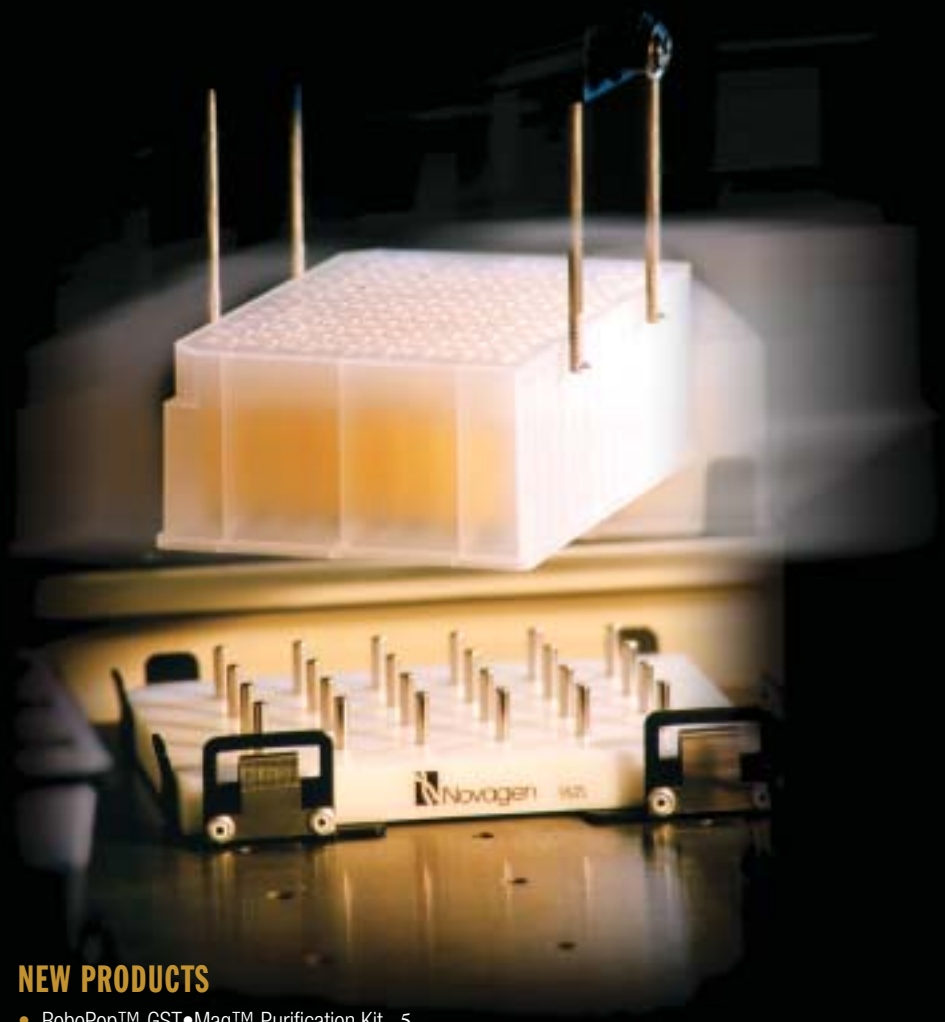

ARTICLES

NEW PRODUCTS

PRODUCT SPOTLIGHT

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Automated purification of recombinant proteins in a 96-well format with RoboPop™ Kits and robotic sample processing

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High throughput structural and functional proteomics requires development of new reagents and automated methods to streamline the process steps that convert gene sequences to purified proteins. Milligram quantities of purified proteins are required to create and utilize functional analysis platforms such as protein chips and to perform crystallization trials (1, 2). Conventional methods of cloning, protein expression, purification, and crystallography are labor intensive and throughput is only improved by increasing the number of person-hours dedicated (2). The daunting task of unlocking the proteome will not be realized through these conventional methods, but will require automation, miniaturization, and parallelization (3). Recently, robots have entered the race to assist in mapping the 3D structures of the proteome (4, 5). Although robotic liquid handling, colony picking, and sequencing technologies developed during the genome project are easily adapted to the cloning and expression steps upstream of structure determination (2), and robotic crystallographers have automated crystal mounting, data acquisition and analysis (4), the narrowest bottleneck in the structural pipeline is high throughput (HT) purification and identification of the protein variant that will crystallize to diffraction quality (2).

Many expression vectors have been developed that allow genetic attachment of purification tags to recombinant proteins. *E. coli* is the expression system of choice in HT structural proteomics due to the rapid, convenient, and inexpensive cultivation for production of recombinant proteins. Cultivation and expression of cloned proteins in *E. coli* have been adapted to 96-well deep well plates (6, 7). Until recently, existing purification technologies have been employed for isolation of individual proteins. Traditional protein isolation usually begins with culturing cells in liquid media under conditions for maximum target protein expression. Cells containing the expressed protein are harvested by centrifugation or filtration, resuspended in a buffer or lysis reagent, mechanically or chemically disrupted to prepare the cell extract, and finally the cellular components are fractionated through multiple mechanical, chemical, and biochemical processing procedures (8–11). Centrifugation and mechanical lysis steps are difficult to automate and miniaturize for the purpose of purifying small amounts of many proteins in parallel.

Table 1. Robotic processing protocol

1. Culture cells in 1.0 ml × 96 wells using a deep-well plate under conditions for target protein production.
2. Add 0.1 ml PopCulture Reagent™ containing 25 U Benzonase® Nuclease and 40 U rLysozyme™ Solution to each well, mix, and incubate 10 min at room temperature.
3. (Optional) Take a 1 µl sample from each well for screening expression levels of S•Tag™ fusion proteins using the FRETWorks™ S•Tag Assay.
4. Add equilibrated His•Mag™ or GST•Mag™ Agarose Beads, mix, and incubate 5 min at room temperature.
5. Separate the beads from the extract with the Magnetight™ HT96™ Stand and remove the supernatant.
6. Wash the beads 2 times by resuspending in 750 µl wash buffer, placing on the magnetic stand, and removing the supernatant from each well.
7. Elute the target protein by resuspending the beads in the appropriate elution buffer. Collect the beads with the magnetic stand and transfer the supernatant containing the target protein to a collection plate.

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 disruption of cells. BugBuster® Protein Extraction reagent utilizes a mixture of detergents to disrupt cell membranes and perforate bacterial cells without denaturing soluble proteins (12, 13). BugBuster Reagent combined with Benzonase Nuclease for extraction of proteins from *E. coli* results in non-viscous extracts suitable for biochemical analysis, assays, or downstream processing such as chromatography. The effectiveness of BugBuster Reagent and the need to simplify the purification process for automation led us to develop PopCulture Reagent (14). PopCulture is a concentrated mixture of specialized detergents that, when combined with lysozyme and Benzonase, enables extraction and purification of recombinant proteins from *E. coli* directly from culture media without cell harvest, mechanical disruption, or extract clarification. The following examples demonstrate an automated protein purification protocol employing RoboPop Protein Purification Kits. Each kit contains PopCulture Reagent, Novagen's stabilized recombinant lysozyme (rLysozyme Solution), Benzonase Nuclease, and magnetic His•Mag or GST•Mag affinity capture resin. The kits provide the reagents and plasticware necessary to achieve the entire process from cell culture of 96 clones to purified protein in an HT format and has been demonstrated by robotic sample processing. The general protocol we used for automation is described in Table 1.

MultiPROBE® II HT EX

The Packard-brand MultiPROBE II from PerkinElmer Life Sciences is a flexible liquid handling workstation specially designed for the efficient automation of sample preparation procedures utilized in pharmaceutical, biotech, research and clinical applications. Available in 4- and 8-tip models, MultiPROBE II Systems enable dispensing into tubes, vials and microplates using volumes as low as 100 nl. PerkinElmer's patented VersaTip™ Plus probe design enables the MultiPROBE II to switch between fixed and disposable tips in one assay. The system's user-friendly WinPREP® software can be optimized for a wide variety of applications, including

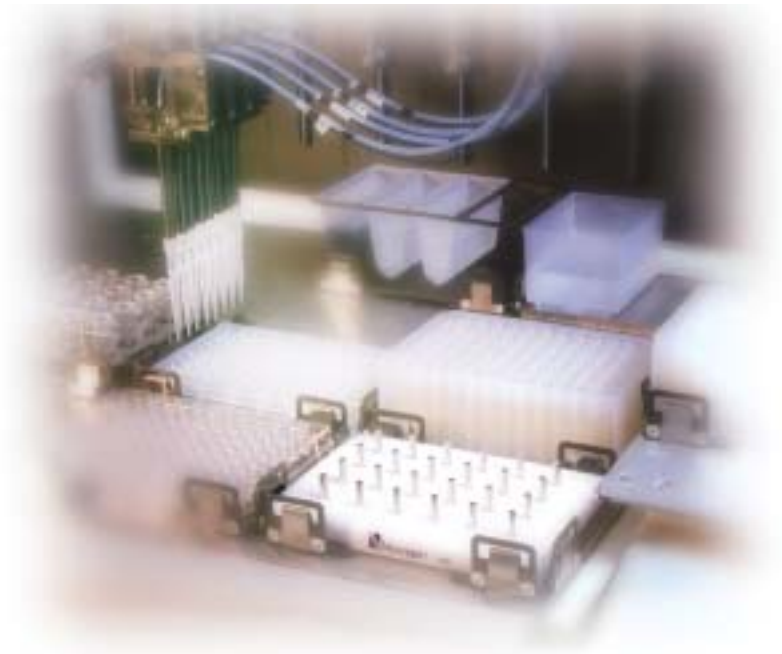


Figure 1. MultiPROBE II HT EX workstation set up for automated protein purification

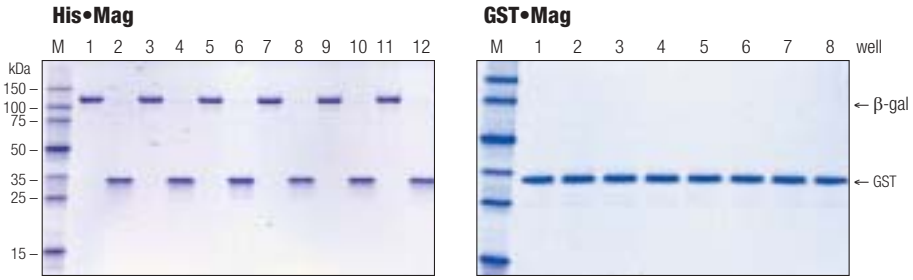
nucleic acid purification, sequencing reaction setup, PCR setup and clean up, protein purification, automated in-gel digestion, MALDI target spotting, cherry picking, dilutions, Caco-2 screening, and Solid Phase Extractions (SPE).

PerkinElmer's Packard-brand Gripper™ Integration Platform expands the capability of MultiPROBE® II EX expanded deck systems, providing an integrated gripper tool capable of "picking-and-placing" SBS-approved microplates, microplate lids, deep-well plates, extraction blocks and selected vacuum manifolds around the deck of MultiPROBE II EX systems. The Gripper also travels beyond the system's right expansion module, enabling integration with approved off-the-shelf devices, such as mixers, incubators, thermal cyclers, hotels, readers, shakers and washers. A full line of application oriented accessories such as automated temperature control of plates and reagents, automated shaker, and automated vacuum control are available to optimize the MultiPROBE II platform and enhance performance of specific applications.

Purification of fusion proteins by the RoboPop™ His•Mag™ and GST•Mag™ protocols

As test vectors for *E. coli* extraction and purification, we used pET-41b(+) for expression of a 35.6 kDa GST•Tag™/His•Tag®/S•Tag™ fusion protein and pET-28b(+) for expression of a 119 kDa His•Tag/T7•Tag® β-galactosidase fusion protein. Both fusion proteins can be purified by immobilized metal chelation chromatography (IMAC) using His•Mag Agarose Beads. The 35.6 kDa fusion protein can also be purified using GST•Mag Agarose Beads and also contains the S•Tag peptide, which enables rapid quantification of expression by the homogeneous FRETWorks™ S•Tag Assay (16).

The purification results are summarized in Figure 2. The data demonstrate the effectiveness of the RoboPop methods with an average yield of 53 µg/ml culture and purity greater than 92% when proteins were purified by the His•Mag method. Yields for purification by the GST•Mag protocol were not as high, but purity was excellent at greater than 98%. Both β-gal and GST purified by these methods were enzymatically active. The reproducibility, absence of degradation products, and lack of cross contamination is seen in the SDS-PAGE analysis of His•Tag β-gal and His•Tag GST purified simultaneously from cultures in alternate rows of the 96-well plate. Although the proteins are purified at ambient temperature and protease inhibitors were not used, no protease degradation was evident. If protease degradation of the target protein is detected, protease inhibitors such as PMSF, AEBSF, Benzamidine or Protease Inhibitor Cocktail Sets III, IV, or V may be added.



Target protein	Yield (µg/ml culture)	%Purity
His•Tag β-gal	40	95
His•Tag GST	67	92

Target protein	Yield (µg/ml culture)	%Purity
GST	30	> 98

Figure 2. Automated purification using the RoboPop His•Mag and GST•Mag Purification Kits

Separate cultures of *E. coli* strain BL21(DE3) containing pET-41b(+) and pET-28b(+) β-gal were prepared and protein expression was induced with 1 mM IPTG for approximately 3 h at 30°C. The final cultures had OD_{600} readings between 4 and 8. The cultures were dispensed (1 ml/well) into alternate rows of 2 ml 96-well plates and 100 µl PopCulture™ Reagent containing 40 units rLysozyme™ and 25 units Benzonase® was added to each well. Plates were allowed to react with mixing for 10 min at room temperature (RT). His•Mag (left panel) or GST•Mag (right panel) Agarose Beads were washed and equilibrated as a 50% slurry with 1X His•Bind® Buffer (left panel) or 1X GST•Bind™ Bind/Wash Buffer (right panel). The equilibrated beads were added to each lysis reaction, mixed, and allowed to react with mixing for 10 min at RT. The entire mixture was subjected to a magnetic field using the Magnetight™ HT96™ Stand to isolate the target-loaded beads. Spent culture media and cellular contaminants were removed with the supernatant while the beads were held by the magnetic field. The beads were washed twice with 750 µl 0.5X His•Bind Wash Buffer (left panel) or GST Bind/Wash buffer (right panel). The washes were accomplished by removing the plate from the magnetic field, resuspending the beads in wash buffer by shaking on a platform shaker, re-isolating the beads with the magnetic field, and pipetting to remove the supernatant. The purified proteins were eluted from the beads with 2 x 150 µl 0.5X His•Bind Elute Buffer (left panel) or GST•Bind Elute Buffer (right panel). The entire purification process after cell culture and induction was performed automatically by the MultiPROBE II. Samples (2 µg protein) were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining. Protein assays were performed by the Bradford method and purity determined by densitometry of the gel scan.

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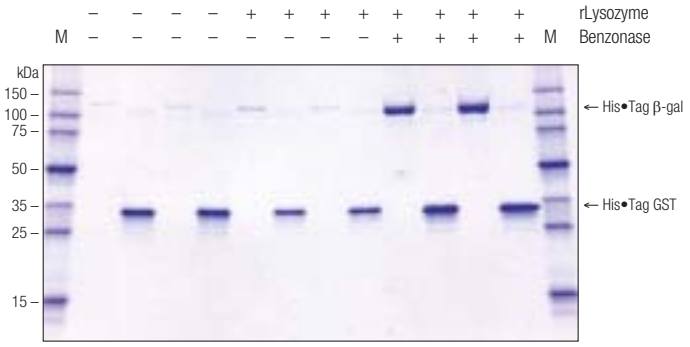


Target protein	Enzyme addition	Yield (µg/ml culture)	%Purity
His•Tag β-gal	none	2	ND
His•Tag β-gal	rLysozyme	4	ND
His•Tag β-gal	rLysozyme + Benzonase	84	97
His•Tag GST	none	97	85
His•Tag GST	rLysozyme	53	88
His•Tag GST	rLysozyme + Benzonase	144	91

Figure 3. Effect of rLysozyme and Benzonase on protein yield with the RoboPop His•Mag automated protocol

Separate cultures of *E. coli* strain BL21(DE3) containing pET-41b(+) and pET-28b(+) β-gal were prepared and protein expression was induced with 1 mM IPTG for approximately 3 h at 30°C. The final cultures had OD_{600} readings between 4 and 8. The cultures were dispensed (1 ml/well) into alternate rows of a 2 ml 96-well plate and 100 µl PopCulture™ Reagent was added to each well. Where indicated by the (+/–) sign above the lanes, the PopCulture Reagent was pre-mixed with 40 units rLysozyme and/or 25 units Benzonase prior to addition. His•Mag™ Agarose Beads were added and the plate was processed using the MultiPROBE II robot exactly as described in Figure 2. Samples (10 µl eluates) were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining. Protein yield and purity were determined by densitometry of the gel scan. M = Perfect Protein™ Markers, 15–150 kDa

Importance of rLysozyme™ Solution and Benzonase® Nuclease addition to the robotic protocol

Figure 3 shows the processing enhancements resulting from the additions of rLysozyme and Benzonase Nuclease during the extraction stage of the RoboPop™ protocol. The combined mechanism of action for these reagents is disruption of the cell membrane and perforation and exposure of the cell wall by the detergent-based PopCulture Reagent, hydrolysis of the N-acetylmuramide linkages in the cell wall by rLysozyme, and complete digestion of the released nucleic acids by Benzonase. Lanes 2–5 of the gel in Figure 3 show duplicate purifications of both β-gal and GST in the absence of rLysozyme and Benzonase. It is clear from the data that β-gal (a tetramer composed of 118 kDa subunits) was not extracted efficiently by PopCulture treatment alone, in contrast to the smaller GST fusion protein (35.6 kDa). The addition of rLysozyme during the PopCulture extraction step did not significantly increase the yield of β-gal and actually decreased the yield of GST by 45% (Figure 3, lanes 6–9 and table). Treatment with PopCulture plus rLysozyme is required for complete cell lysis, but in the absence of Benzonase, the viscosity resulting from the released nucleic acid interfered with robotic processing. The combination of PopCulture, rLysozyme, and Benzonase synergistically increased the yield of target proteins 40-fold for β-gal and 1.5-fold for GST.

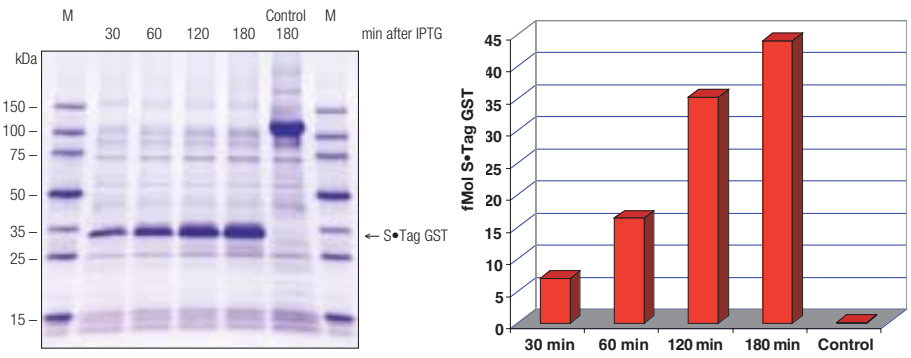


Figure 4. Time course of induction of S•Tag GST with FRETWorks S•Tag Assay

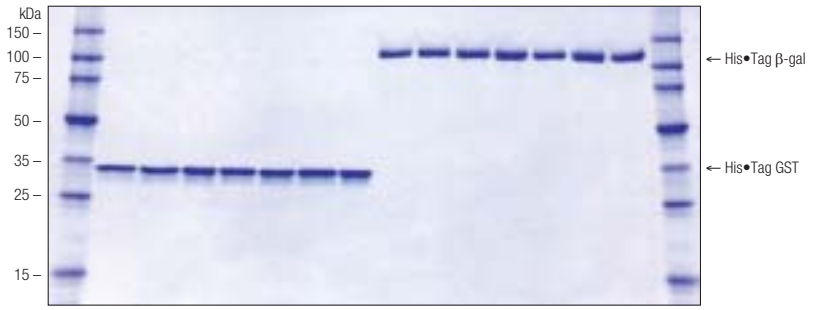
Separate cultures of *E. coli* strain BL21(DE3) containing pET-41b(+) (for expression of S•Tag GST) and pET-28b(+) β-gal (as a negative control lacking an S•Tag sequence) were grown in liquid culture and induced with 1 mM IPTG. At the indicated times, 1 ml samples were placed into sequential rows of a 96-well deep well culture plate and 100 µl of PopCulture Reagent containing 40 units of rLysozyme and 25 units of Benzonase Nuclease were added. After mixing for 10 min at room temperature, the crude extracts were used for SDS-PAGE analysis (left panel) and diluted 1:2500 for the FRETWorks S•Tag Assay according to the standard protocol (20 µl of diluted sample were used per assay). The S•Tag GST fusion protein in the crude extracts was quantified based on a standard curve with known amounts of S•Tag Standard (right panel). M = Perfect Protein Markers, 15–150 kDa.

FRETWorks™ S•Tag™ Assay screening for target protein expression levels

The 15 aa S•Tag peptide enables rapid quantification of fusion proteins by the FRETWorks S•Tag Assay. This ultrasensitive, homogeneous assay is based on the high affinity specific interaction of the S•Tag peptide with S-protein to form active ribonuclease (15), and employs a mixed ribo-deoxyribooligonucleotide FRET (fluorescent resonance energy transfer) substrate for RNase containing a fluor on the 5'-end and a quencher on the 3'-end. When cleaved by the ribonuclease S activity of the S•Tag/S-protein complex, quenching is released and a strong fluorescent signal is generated. The FRET substrate appears to be resistant to cleavage by cellular RNases and Benzonase Nuclease, which enables the assay to be used with crude extracts. Figure 4 shows the results of a time course of induction of the 36.5 kDa S•Tag GST fusion protein. The FRETWorks Assay results (Figure 4, right panel) correlated well with SDS-PAGE analysis of the crude extracts (Figure 4, left panel) prepared by PopCulture/rLysozyme/Benzonase treatment. It should be noted that this assay is routinely performed with 20 µl of a 1:2500 dilution of the crude extract and takes less than 10 minutes.

96-Well Cell Culture

In an effort to minimize variability due to culture conditions, the above experiments were performed using aliquots of cultures set up in 50 ml flasks. For true high throughput capability, the entire cell culture process must be carried out in the wells of



Target protein	Yield (µg/ml culture)	%Purity
His•Tag β-gal	32	> 98
His•Tag GST	45	88

Figure 5. Protein yield and purity from 96-well cultures

Separate cultures of *E. coli* strain BL21(DE3) containing pET-41b(+) and pET-28b(+) β-gal were grown in alternate rows of a RoboPop Culture Plate by inoculating isolated colonies from LB agar + 34 µg/ml kanamycin plates grown overnight at 37°C into 100 µl TB + phosphates + 0.5% glucose placed in the wells. The inoculated plate was incubated at 24°C with shaking at 300 rpm approximately 16 h. After adding 1.0 ml of the same media the cells were incubated at 30°C with shaking for an additional 1.5 h to an OD_{600} of 1.0 and induced with 1 mM IPTG for approximately 3 h at 30°C. The final cultures had OD_{600} readings between 3.5 and 5. For purification, the plates were processed using the RoboPop His•Mag protocol as described in Figure 2. Samples (2 µg) were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining. Protein assays were performed by the Bradford method and purity determined by densitometry of the gel scan. M = Perfect Protein™ Markers, 15–150 kDa.

automation-compatible plates. Figure 5 shows the results of RoboPop™ His•Mag™ purification using 1 ml cultures set up in a 96-well deep well culture plate. Under the conditions used (see Figure 5 legend), the induced cultures reached a final OD_{600} between 3 and 3.5, which is about 10–50% lower than obtained using LB broth in 50 ml flasks. The gel analysis and protein purity were very similar to those obtained using flask cultures; however, the yield was slightly lower (32 vs. 40 µg His•Tag® β-gal, 45 vs. 67 µg His•Tag GST), which correlates with the decrease in cell mass we observed using these conditions for 96-well culture.

Summary

Previously we demonstrated the utility of PopCulture™ Reagent and its advantages for direct processing of *E. coli* cultures and affinity purification of proteins without cell harvest or mechanical lysis (15). Here we have adapted the PopCulture purification procedures to robotic sample processing. The robotic processing protocol has been demonstrated in the purification of intact fusion proteins from total culture extracts using magnetic IMAC and GST affinity approaches and the MultiPROBE® II liquid handling workstation. We have elucidated the processing enhancements resulting from the addition of rLysozyme and Benzonase® Nuclease to the PopCulture Reagent. We have incorporated the ability to rapidly quantify expression levels in crude extracts using the FRETWorks™ S•Tag™ Assay. Finally, we have demonstrated the effectiveness of the protocol from the start of 96-well cell cultures from isolated transformants and their expression of target protein to the finish of obtaining highly purified proteins. Novagen's RoboPop Purification Kits combine PopCulture Reagent, rLysozyme™ Solution, Benzonase Nuclease, and plasticware necessary to rapidly achieve the entire process from cell culture of 96 clones to purified protein in an HT format. The increased speed and convenience of the RoboPop His•Mag and GST•Mag™ Purification Kits have opened a bottleneck in HT protein purification and will allow automated parallel processing of hundreds of proteins without the need for expensive centrifugation or cell disruption equipment.

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Product	Size	Cat. No.
RoboPop™ GST•Mag™ Purification Kit (includes PopCulture Reagent, rLysozyme Soution, Benzonase Nuclease, GST•Mag Agarose Beads, 96-well Culture and Collection Plates with Sealers, and Buffers)	1 plate	71102-3
RoboPop His•Mag™ Purification Kit (includes PopCulture Reagent, rLysozyme Soution, Benzonase Nuclease, His•Mag Agarose Beads, 96-well Culture and Collection Plates with Sealers, and Buffers)	1 plate	71103-3
PopCulture™ Reagent	15 ml 75 ml 250 ml	71092-3 71092-4 71092-5
Benzonase® Nuclease, Purity > 90%	10,000 U 2500 U	70746-3 70746-4
rLysozyme™ Solution 30 KU/µl Note: 1 KU = 1000 Units	300 KU 1200 KU 6000 KU	71110-3 71110-4 71110-5
GST•Mag™ Agarose Beads	2 ml 10 ml	71084-3 71084-4
His•Mag™ Agarose Beads	2 ml 10 ml	71002-3 71002-4
Magnetight™ HT96™ Stand		71101-3
FRETWorks™ S•Tag™ Assay Kit	100 assays 1000 assays	70724-3 70724-4

Novel single-copy pETcoco™ vector with dual controls for amplification and expression

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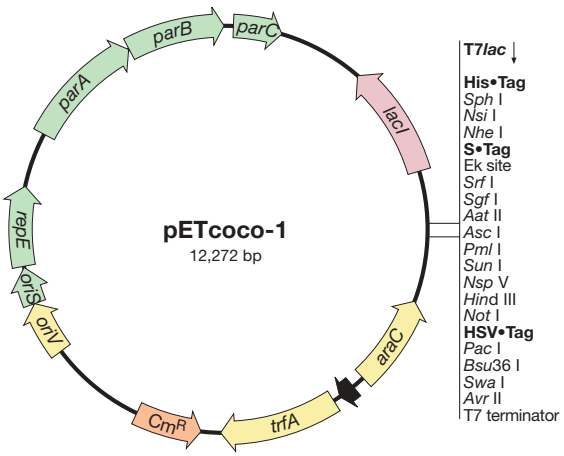
Expression vectors are designed and employed to produce large quantities of a specific product, usually a protein. However, synthesis of proteins, especially those that are toxic to the host, must be controlled, being none or at non-toxic levels in uninduced cells, and high only after induction of expression during the appropriate phase of growth. Although pET vectors (1) have been used extensively to produce high levels of heterologous proteins in *E. coli*, problems of the residual background expression reaching toxic levels and consequent plasmid instability sometimes arise. Even the uninduced levels of highly processive T7 RNA polymerase can lead to a residual expression of the cloned target gene, providing selective advantage for those cells that have lost the cloned "toxic" gene or have undergone changes that have reduced its expression and/or toxicity. For all the described expression vectors, regulation is exerted at the transcriptional level, mainly at the promoter, with constant plasmid copy number, which most often is medium to high, to assure high expression levels.



For symbols, see references 1, 4, and 6; aa = amino acid(s)

Figure 1. pETcoco-1 vector map

The dual-control expression plasmid contains the following key features:

1. Single-copy control unit derived from BAC plasmid (*oriS/repE – parABC*)
2. Medium-copy conditional amplification elements [*oriV* and *trfA*-up, the latter controlled by the *araC*-P_{BAD} promoter system (P_{BAD} = black arrow) or alternatively by the T7 promoter]
3. Antibiotic resistance marker (Cm^R)
4. *lacI* gene
5. P_{T7}/*lacO* (T7*lac*) promoter plus T7 gene *10* ribosome binding site
6. Target gene cloning region to enable N-terminal fusion of 6 aa His•Tag®, 15 aa S•Tag™, and enterokinase cleavage sites, and optional C-terminal fusion of the 11 aa HSV•Tag® peptide to the target protein
7. T7 transcription terminator

pETcoco "copy control" vectors

For our novel expression vectors, we introduced a new concept, namely production of proteins under dual controls: (i) at transcriptional level at the T7 phage promoter (1), and (ii) at the DNA replication level by amplifying the plasmid copy number (2, 3). The main advantage of our dual-control expression plasmid is that it retains practically all of the outstanding features of the pET vectors, but in addition, when not induced, its expression background is many times lower, a feature of crucial importance for plasmids producing toxic products, which is often the case. The copy number of our dual-control expression vectors could be regulated from one copy (when about 0.2% D-glucose is present in growth medium) to about 20–50 copies upon induction. At the same time, the expression at the T7 promoter could be usually induced by as much as 2500-fold over the background. *(Ed. note: the term "medium copy" as used here refers to plasmid copy numbers of about 40–60 copies per cell, similar to pBR322 and pET vectors.)*

Structure of the pETcoco-1 vector

Dual regulation is based on two kinds of *cis*-acting elements: alternative origins of DNA replication (*oriS* for single copy and *oriV* for medium copy plasmids) and controllable promoters providing *trans* functions (Figure 1). Genes encoding *trans*-acting factors (RepE replicator and *parAB* functions acting at *oriS* and *parC* sites, ensuring the single-copy status, TrfA replicator acting at *oriV*, and activator/repressors for regulating promoters) are present on the pETcoco plasmid. As the backbone, we used pBeloBAC11, into which *oriV* was cloned at the *Xho* I site (3–6). Furthermore, segment *lacI-pT7lacO*-MCS-*tT7* [from Novagen's pET-24(+) plasmid] was cloned between the *Not* I sites. In our construct, all the *cis*-acting sites and some genes for *trans* functions were placed on the expression vector, which allows our plasmids to be used in any compatible host that provides T7 RNA polymerase in a well-regulated mode. The *oriV/trfA*-up replicon functions independently from the T7 system after induction with L-arabinose. The major advantage of our pETcoco expression system is that it is extremely tight, since any leakage from the single gene copy should be about 1/40 of the level of the corresponding pET vectors, which are maintained at about 40 copies per cell. At the same time, the level of induced expression from pETcoco becomes as high as from the pET vectors. Cloning and any DNA manipulations are very easy, since we are able to secure an ample supply of the vector by amplifying it independently of the expression function that requires T7 RNA polymerase. All constructions, cloning of target genes, and maintenance of stocks are best performed in a $RecA^-$, $EndA^-$, F^- strain such as $NovaF^-$ (available from Novagen).

Effect of glucose

It was found in our laboratory in 1998, that 0.2% glucose reduces the bacterial artificial chromosome (BAC) vector levels from two copies to one copy (3, 6). Moreover, it was observed that to keep the lowest possible level of cAMP-CAP [because of transcription stimulatory activity of CAP protein on the *lac*UV5-L8 type of promoter (6–9) as well as on the P_{BAD} and P_{cat} promoters] the maintenance medium for the lowest residual expression of *lacZ* should be LB + 0.2% glucose (Table 1). Such residual

Table 1. Expression of βGal from the pETcoco™-1.lacZ clone

	IPTG (mM)[a]	OD_{600}	Miller units[b]	plasmid copy number[c]
(1)	0.0 (host/no plasmid)	4.98	2	none
(2)	0.0	4.13	18	1–2
(3)	0.0 (+0.2% D-glucose)	4.92	9	1
(4)	0.0 (+0.01% L-arabinose)	4.09	120	20–30
(5)	0.05	4.09	11,525	3–4
(6)	0.1	4.12	18,730	4–5
(7)	0.2	3.92	37,635	4–5
(8)	0.5	3.36	48,020	3–4
(9)	1.0	3.56	44,870	2–3

a *E. coli* Tuner™(DE3) alone (line 1) or carrying a pETcoco.lacZ recombinant (lines 2–9) were inoculated (at 1:200) with overnight culture, and grown in 10 ml LB medium with 12.5 µg chloramphenicol/ml. After 1.5 h at 37°C, *lacZ* gene expression was induced for 3 h with 0.05–1.0 mM IPTG (lines 5–9). In parallel, control strains were grown in IPTG-free LB medium alone (uninduced; line 2), or supplemented with 0.2% D-glucose (uninduced, line 3) or 0.01% arabinose (induction of plasmid amplification only; line 4).

b For measurement of β-galactosidase (βGal) activity, ONPG assay was performed according to Miller (10) using 50 µl samples of each culture. We used 25 µl 0.1% SDS and 50 µl chloroform for lysis of bacteria [+ 10 sec of shaking], and 4 mg/ml ONPG stock solution in buffer Z. After color development (usually 15–30 sec for induced bacteria, and 30 min for uninduced bacteria), samples were centrifuged for 4 min, as to make OD measurement at 550 nm unnecessary, since after spinning the samples down, the values at 550 nm measurement are practically zero.

c Plasmid copy number was estimated from DNA gels (somewhat similar to those shown in Figures 2C and 3). Under certain conditions (rapid growth), the plasmid copy number in line 3 could reach two. In strains like NovaF⁻, during maintenance phase in the presence of 0.2% glucose, the plasmid copy number is one (3, 6).

lacZ expression is usually below 1–7 Miller units when the "no plasmid" background is subtracted, but could be somewhat higher under particular growth conditions. From the practical point of view, such residual expression of the target or reporter gene could often be considered as negligible, since it does not affect the stability of the uninduced plasmid or of the entire expression system.

Amplification of the vector

(a) In the absence of the target gene

For cloning or sequencing, the vector and its derivatives can be amplified in the absence of target gene expression, since in this manner one avoids any toxicity effect during the plasmid amplification. Replication from the *oriV* origin (RK2) can be initiated by adding 0.01% L-arabinose (from a 2% stock solution), which induces the P_{BAD} promoter that controls the *trfA* gene. For the uninduced control bacteria, 0.2% glucose can be added (from a 20% stock solution) to maintain tight repression of P_{lac} and P_{BAD} promoters and, consequently, the plasmid copy number. Plasmid amplification was assessed in Figures 2B, 2C, and 3 by gel electrophoresis of uncut or pre-cut plasmids using 0.65% agarose gels and appropriate dilutions of DNA adjusted according to the OD_{600} of the culture. Amplification varied from 20–50 fold depending on growth conditions and the level of the copy-up TrfA protein [Figure 2B (lane 2) and 2C (lanes 3 and 5); Figure 3 (lane 4)]. The highest plasmid levels occurred either after 0.01% arabinose induction or at 0.1 mM IPTG. Perhaps surprisingly, the TrfA levels after arabinose induction were not very high but enough to initiate replication at maximum level (Figure 2A and 2B, lanes 2). Higher IPTG levels resulted in lower plasmid amplification (Figure 2B), presumably caused by over-transcription of the *trfA* and *oriV* regions (Figure 2A, lanes 3–5). This is why there was low amplification after both arabinose and IPTG were added (Figure 3, lane 4), although in this case the target gene, *lacZ*, was present. As expected, the CAT levels reflected the levels of DNA amplification.

(b) In the presence of the target gene

Upon addition of L-arabinose, only the *trfA*-up gene is induced, the plasmid is amplified and target gene expression increased only in rough proportion to the copy number (Table 1, line 4; Figure 3, lane 3).

Upon addition of IPTG, the T7 promoter is induced, resulting in both (i) massive expression of the target gene and (ii) somewhat suppressed expression of the *trfA*-up gene, which is downstream of the target gene. There was only little amplification of DNA after IPTG induction (Figure 3, lane 5), most probably because of rather effective termination of transcription by two elements: the long *lacZ* target gene and the T7 terminator located downstream. As a confirmation, we did not observe increases in either TrfA or CAT proteins in IPTG-induced cultures (data not shown).

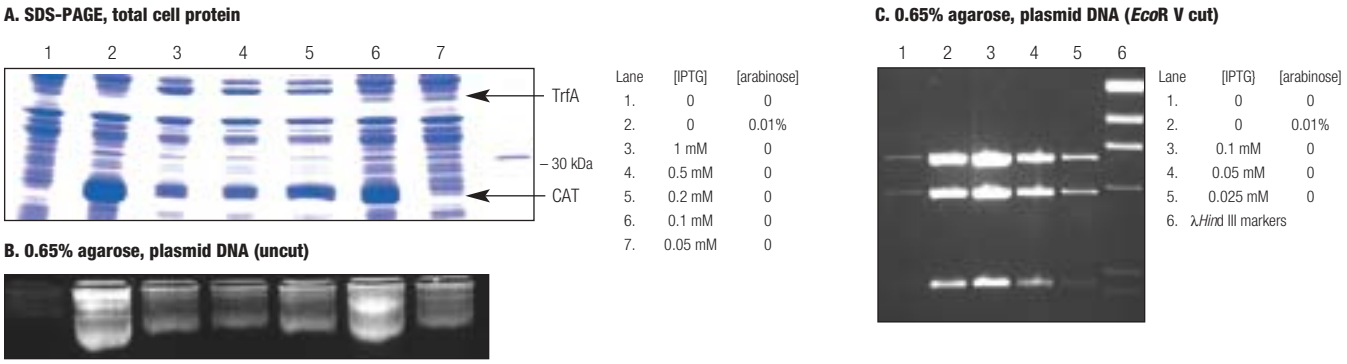


Figure 2. Relationship between protein levels and DNA amplification upon induction of the pETcoco-1 vector (not containing a target gene).

(A) Visualization of the TrfA and CAT proteins (and other plasmid and host proteins) on 0.1% SDS-10% PAGE. Cultures of *E. coli* strain Tuner(DE3) containing pETcoco-1 were started from a 1:200 inoculation of an overnight culture and grown in 10 ml LB medium (with 12.5 µg Cm/ml). After 1.5 h of cultivation at 37°C (OD_{600} = 0.2), IPTG was added at the indicated concentrations (0.01% arabinose was added only to the sample in lane 2) and incubation continued for 4 h, after which total cell protein in 50 µl samples was analyzed. The gel was stained with Coomassie brilliant blue. (B) Amplification of pETcoco-1 DNA as assessed by 0.65% agarose gel electrophoresis with aliquots of minipreps isolated from the above cultures and not pre-cut (lanes correspond to lanes in A). (C) Minipreps of the vector were prepared after 4 h of induction (like in B), and aliquots of DNA, all after thorough deproteinization (+ protease) followed by pre-cutting with *Eco*R V, were electrophoresed in 0.65% agarose gel.

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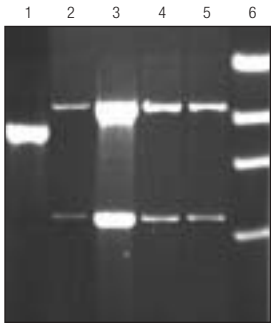


Figure 3. DNA levels for pET-24.lacZ and pETcoco-1.lacZ clones, uninduced or induced.

Plasmid DNAs were isolated from mid-late stationary phase Tuner(DE3) cultures, digested with *Bam*H I and examined on 0.65% agarose gel. Lanes: 1, pET-24.lacZ uninduced; 2, pETcoco-1.lacZ uninduced; 3, pETcoco-1.lacZ induced with 0.01% arabinose (2 h); 4, pETcoco-1.lacZ induced with 0.01% arabinose (1.5 h) and then with 2 mM IPTG for 2 h; 5, pETcoco-1.lacZ after 2 h of 2 mM IPTG induction; 6, λ*Hin*d III markers.

Basal and induced expression levels

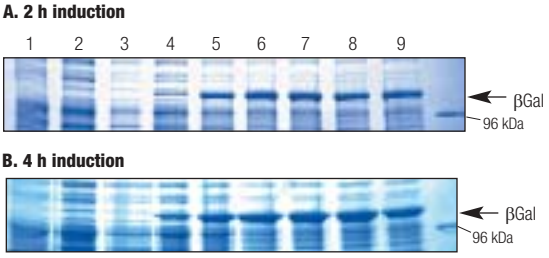
We used our pETcoco™ vector containing the full-length *lacZ* reporter gene to measure the expression controlled by the T7 promoter. Overnight cultures of *E. coli* Tuner™(DE3) carrying the pETcoco.lacZ were diluted 1:200 in LB medium and incubated at 37°C. When cultures reached OD_{600} = 0.1–0.15, we induced the T7 promoter by addition of IPTG. After 2 h (OD_{600} = 2.4–2.7), 3 h (OD_{600} = 3.3–3.6) or 4 h (OD_{600} = 4.7–4.9) of cultivation, samples were taken for the βGal assay and also for measuring of the level of plasmid amplification. Optimal induction was observed at 0.5 mM IPTG for 3 h (Table 1, line 8) and 1.0 mM IPTG for 4 h (Figure 4B, lane 8).

The results in Figure 4A and 4B corroborate the data in Table 1. However, it should be possible to obtain still higher levels of target protein by extending the induction to 5–6 h or overnight, and perhaps by further manipulation of the IPTG, and possibly the arabinose, concentrations, together with exact schedules.

Summary

A modified T7-based expression vector, pETcoco-1, was developed with the following novel and practical features:

- When not induced, recombinants are very stable, because being based on the BAC system, they contain only one copy of the expression plasmid. Only upon induction of its *oriV*/TrfA-up amplification system does it become a multi-copy plasmid.
- In the uninduced state, background expression is reduced to as little as 1/40 of the levels obtained with pET vectors (based on copy number).
- Because of such low background, the pETcoco expression system is not likely to require the use of pLysS-carrying hosts, which can reduce the induced yield and cause premature cell lysis.
- Despite their somewhat large size, the pETcoco vectors can be easily amplified to facilitate plasmid purification.
- When maintained in the uninduced state, single-copy pETcoco clones are more stable than clones in pETcoco vectors, especially if expression from the target gene is toxic to the host.
- In our tests, induced expression levels were at least as high as with the pET vectors (data not shown).
- Cell cultures of pETcoco recombinants tend to continue to grow following IPTG induction, which enables longer induction periods and potentially higher expression levels.



Lane	[IPTG]	[arabinose]
1.	0	0
2.	0	0*
3.	0	0.01%
4.	0.05 mM	0
5.	0.1 mM	0
6.	0.2 mM	0
7.	0.5 mM	0
8.	1.0 mM	0
9.	2.0 mM	0

* culture received 0.2% D-glucose

Figure. 4. Visualization of βGal synthesis in Tuner(DE3) carrying pETcoco-1.lacZ clones.

Protein extracts (from 50 µl of the bacteria pellets) were analyzed by 0.1% SDS-6.5% PAGE, after 2 h (A) and 4 h (B) of induction. Recombinants containing pETcoco-1.lacZ were prepared as described in legend to Figure 2A. After 1.5 h of cultivation at 37°C, target gene expression was induced by addition of the indicated amounts of IPTG. In parallel, control strains were grown in LB medium (uninduced; lane 1), or supplemented with 0.2% D-glucose (uninduced; lane 2) or 0.01% arabinose (induction of plasmid replication only; lane 3). Gels were stained with Coomassie brilliant blue.

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Drs. Zdenka Hradecna and Jadwiga Wild of Prof. Szybalski's laboratory have supplied us with several genetic elements and advised us about the construction of the backbone of the pBAC/oriV plasmid, including the P_{BAD}-*trfA*-up/*oriV* amplification system, similar to that present in their P_{araBAD}-based dual-control expression plasmid. Their unselfish help and advice are acknowledged with the deepest gratitude.

Product	Cat. No.
pETcoco™-1 System	71131-3

Components:

- 10 µg pETcoco-1 DNA
- 0.2 ml NovaF⁻ Competent Cells
- 0.2 ml Tuner(DE3) Competent Cells
- 2 × 2 ml SOC Medium
- 10 µl Test Plasmid
- 0.2 ml Induction Control Glycerol Stock

Available separately:

Product	Size	Cat. No.
pETcoco-1 DNA	10 µg	71129-3
NovaF⁻ Competent Cells	0.4 ml	71133-3
	1 ml	71133-4
Tuner™(DE3) Competent Cells	0.4 ml	70623-3
	1 ml	70623-4

Targeted suppression of gene expression using siRNA and RiboJuice™ siRNA Transfection Reagent

Scott Hayes and Faye Bruggink — Novagen, Inc.

To selectively inhibit the expression of only one gene in a milieu of thousands is a daunting task. Recently a process called RNA interference (RNAi) seems capable of doing just that. RNAi refers to the delivery of double-stranded RNA (dsRNA) into a cell causing a sequence-dependent inhibition of gene expression. The concept of RNAi as a form of gene silencing first arose from studies in *Caenorhabditis elegans* in 1998 (1). The researchers found that introduction of dsRNA homologous to the target gene suppressed expression, much more so than introduction of either single-strand alone. Interestingly, the effect was seen with introduction of only a few molecules of dsRNA, indicating that a catalytic or amplification process was initiated. Not much was known at that time about how this process was carried out. With the discovery of a specialized form of RNAi, mediated by small interfering RNA commonly referred to as "siRNA", that would soon change.

Background

The discovery of siRNA was first made in plants in 1999 (2). This research demonstrated that post-transcriptional gene silencing (PTGS), a plant process similar to RNAi, was mediated by 25 nucleotide pieces of RNA capable of silencing homologous genes. The development of *Drosophila melanogaster* extracts, which could mimic the *in vivo* RNAi effect, led to the next breakthrough. It was found that double stranded 21–23 nucleotide RNAs direct specific target RNA cleavage (3, 4, and general reviews 5–7). Recently, some of the protein components within this pathway have been defined. A *Drosophila* protein, named Dicer, creates small interfering RNA of 21 to 25 nucleotides from longer intact dsRNA through its inherent RNase III/helicase activity (8). The siRNAs generated by Dicer target homologous mRNA for cleavage through an RNA-induced silencing complex (RISC) that contains ribonuclease activity (9). The two-step procedure leads directly to cleavage of target messenger RNA (mRNA) sharing homology with the generated siRNAs.

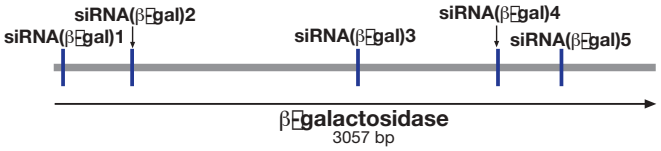

Figure 1. Locations of siRNAs relative to the β-galactosidase coding region

Five different siRNAs directed against *E. coli* β-galactosidase were synthesized for this study. siRNA(β-gal)1 (AAAACCCUGGCGUUACCCAAC) corresponds to nucleotides 35–55 relative to the translation start site, siRNA(β-gal)2 (AAGGCCAGACGCGAAUUAUUU) corresponds to nucleotides 392–412, siRNA(β-gal)3 (AAUGGUCCAUCAAAAAAUGGC) corresponds to nucleotides 1535–1555, siRNA(β-gal)4 (AAUUUAACCGCCAGUCAGGCU) corresponds to nucleotides 2255–2275, and siRNA(β-gal)5 (AAAUGGCGAUUACCGUUGAUG) corresponds to nucleotides 2573–2593. Note that the synthesis reaction adds UU to the 3'-end of the siRNAs.

Many of the guidelines for siRNA design have originated from the Thomas Tuschl lab at the Max-Planck Institute for Biophysical Chemistry. In general, an siRNA should originate at least 50 to 100 nucleotides downstream of the translation start site to avoid regulatory proteins. The sequence content should be roughly 50% G/C and the sense strand should have sequence structure of AA(N19)TT or AA(N21). Sequence design should be followed by a search of the NCBI database to ensure that only one gene is targeted by a particular siRNA (10, 11).

Novagen is now introducing a transfection reagent developed especially for synthetic siRNA delivery into the cell. RiboJuice siRNA Transfection Reagent has many of the same desirable features as Novagen's GeneJuice™ Transfection Reagent for plasmid DNA delivery. RiboJuice has been formulated to deliver siRNA into cells with high efficiency, minimal toxicity, serum compatibility, stability, and ease of use. As demonstrated in this report, RiboJuice is also compatible with GeneJuice for co-transfection of siRNA and plasmid DNA.

siRNA suppression of transfected β-galactosidase expression

To demonstrate the effectiveness of RiboJuice in siRNA delivery, a co-transfection approach was used with plasmid encoding a well-characterized reporter, β-galactosidase. A total of five different siRNA's were constructed corresponding to different regions within the β-galactosidase cDNA (see Figure 1). All siRNA's were made using the Ambion Silencer™ siRNA Construction Kit according to the manufacturer's protocol, and transfected at a final concentration of 10 nM using RiboJuice. The results shown in Figure 2 demonstrate that the selected siRNAs were differentially

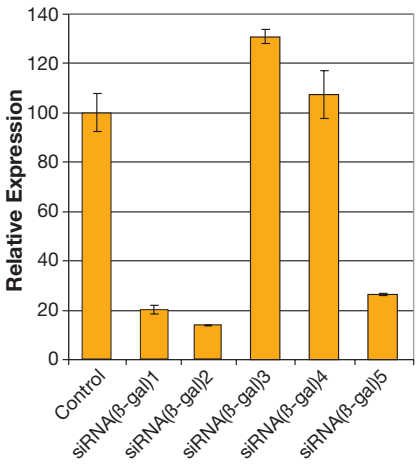

Figure 2. Effect of transfected siRNA on β-galactosidase expression in CHO-K1 cells

CHO-K1 cells were passaged the day before transfection into 24-well plates at a density of 50,000 cells per well, leading to ~70% confluency the day of transfection. Each well was transfected with two mixtures. Mixture one contained 1 µl GeneJuice Transfection Reagent and 0.25 µg pTriEx™-2(β-gal), for introducing the target gene, and 0.025 µg pTriEx-2(Fluc) for normalization. Mixture two contained 3 µl RiboJuice and 10 nM (final concentration) of siRNA(β-gal)1, 2, 3, 4, or 5. As a control, 3 µl RiboJuice was also used without any siRNA. Total volume per well was 300 µl. Twenty-four hours after transfection, cells were lysed with Reportasol™ Extraction Buffer and reporter activities measured using the BetaRed™ β-Gal Assay Kit and a standard firefly luciferase assay. All assays were performed in triplicate and variation is expressed as standard error of the mean.

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capable of suppressing β-galactosidase expression. The level of interference was as high as 86% with siRNA(β-gal)2 and was greater than 70% with both siRNA(β-gal)1 and siRNA(β-gal)5. Surprisingly, siRNA(β-gal)3 and siRNA(β-gal)4 had no suppressive effect on protein expression. The results underscore the importance of testing more than one siRNA for any given target mRNA. While RNAi has previously been demonstrated with a 300 nucleotide dsRNA targeted to LacZ (9), we believe that this is the first demonstration of suppression of β-galactosidase expression mediated by siRNA. We chose to use siRNA(β-gal)1 as a representative siRNA for further studies.

Concentration dependence and specificity

As shown in Figure 2, we determined that siRNA(β-gal)1 was capable of suppressing β-galactosidase expression at a fixed concentration of 10 nM. The next question we wanted to answer was how well this siRNA could suppress expression over a range of concentrations. Varying amounts of siRNA(β-gal)1 (0.1 nM to 25 nM final concentration), were used in a co-transfection approach following the methodology used in Figure 2. This experiment was performed in both CHO-K1 cells and HEK 293 cells. The results shown in Figure 3 demonstrated partial suppression of β-galactosidase expression at a concentration of siRNA as low as 0.1 nM (3% for HEK-293 cells and 9% for CHO-K1 cells). Expression continued to decrease with increased siRNA concentration. At the highest concentration of siRNA used, 90% and 96% inhibition of expression were observed in HEK-293 cells and CHO-K1 cells, respectively.

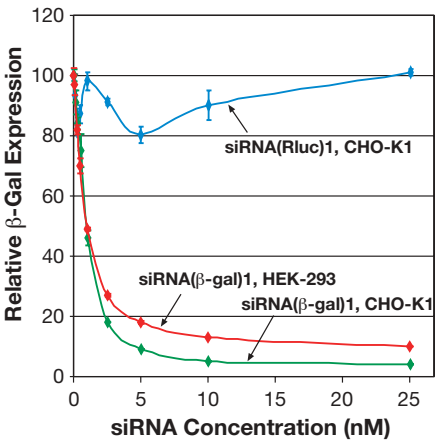

Figure 3. Concentration dependence and specificity of siRNA(β-gal)-mediated suppression

CHO-K1 and HEK-293 cells plated at 50,000 cells per well were transfected after 24 h with two mixtures. Mixture one contained 1 µl GeneJuice™ Transfection Reagent, 0.25 µg pTriEx™-2(β-gal), and 0.025 µg pTriEx-2(Fluc). Mixture two contained 3 µl RiboJuice™ and either 0.1, 0.25, 0.5, 1, 2.5, 5, 10, or 25 nM (final concentration) of the indicated siRNAs. As a control, 3 µl RiboJuice was also used without any siRNA. Total volume per well was 300 µl. Cells were lysed with Reportasol 24 h after transfection and assayed for reporter activity. All assays were performed in triplicate and variation is expressed as standard error of the mean. The sequence used for siRNA(Rluc)1 was AAACAUGCAGAAAAUGCUGUUUU.

To demonstrate the specificity of the targeted siRNA response, CHO-K1 cells were co-transfected with β-galactosidase and firefly luciferase encoding plasmids in conjunction with varying concentrations of siRNA directed against an unrelated reporter, *Renilla* luciferase [siRNA(Rluc)1]. In contrast to the result with siRNA directed against β-galactosidase, siRNA(Rluc)1 transfection had minimal effect on β-galactosidase expression (Figure 3, blue line). In addition, the expression of firefly luciferase was not affected by siRNA(Rluc)1 or siRNA(β-gal)1 introduction (data not shown).

Additional cell lines

We next wanted to determine whether siRNA introduction by RiboJuice™ was capable of suppressing protein expression in other cell lines. In addition to HEK-293 cells and CHO-K1 cells, we tested the effects of siRNA directed against β-galactosidase in the commonly used cell lines L6, BHK, Neuro-2A, HeLa, COS-7, HepG2, and NIH3T3, which represent various species and tissue types. Experiments were performed as in Figure 1 using a 10 nM final concentration of siRNA and 3 µl RiboJuice in a 24-well plate with cells that were between 50 and 70% confluent. As shown in Figure 4, siRNA(β-gal)1 suppressed β-galactosidase expression in each of the cell lines by at least 50%. Seven of the nine cell lines had siRNA mediated suppression of greater than 70%. Additional optimization of RiboJuice transfection conditions and siRNA concentration could potentially yield even higher levels of suppression. The highly efficient delivery of siRNA with minimal toxicity should make RiboJuice amenable to use with most cell lines.

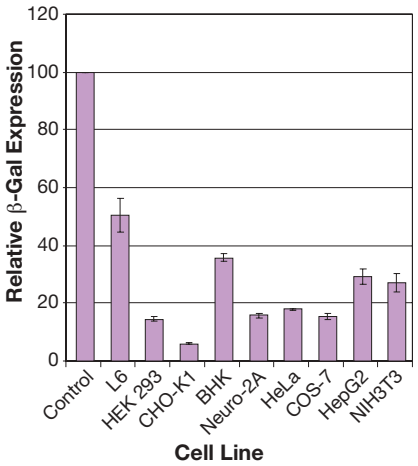

Figure 4. Suppression of β-galactosidase expression in various cell lines

The indicated cell lines were plated at 50,000 cells per well and transfected after 24 h with two mixtures. Mixture one contained 1 µl GeneJuice Transfection Reagent, 0.25 µg pTriEx-2(β-gal), and 0.025 µg pTriEx-2(Fluc). Mixture two contained 3 µl RiboJuice and 10 nM (final concentration) siRNA(β-gal)1. For each cell line, a reaction lacking siRNA was used as a control (set at 100%). Total volume per well was 300 µl. Cells were lysed with Reportasol™ 24 h after transfection and assayed for reporter activity. All assays were performed in triplicate and variation is expressed as standard error of the mean.

Suppression of firefly luciferase expression

To test the effectiveness of RiboJuice transfection with siRNA directed against another reporter gene, an siRNA was designed against firefly luciferase and tested in CHO-K1 cells. As shown in Figure 5, siRNA(Fluc)1 corresponds to nucleotides 82–102 relative to the translational start site of the codon optimized firefly luciferase coding region (Promega). The designed siRNA borders a previously characterized siRNA against firefly luciferase (GL3 siRNA; reference 10) which was shown to specifically reduce protein expression more than 80% in multiple cell lines. As the results in Figure 5 demonstrate, firefly luciferase expression was suppressed over 90% with siRNA(Fluc)1 compared to a control lacking siRNA. The suppression observed with siRNA directed against firefly luciferase was comparable to that shown with siRNA directed against β-galactosidase, verifying the utility and performance of RiboJuice siRNA transfection reagent as a vehicle for siRNA delivery into the cell.

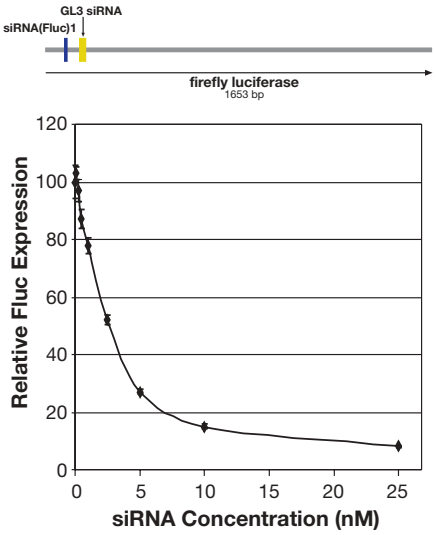

Figure 5. siRNA-mediated suppression of firefly luciferase expression

The top diagram depicts the location of siRNA(Fluc)1 (AAGGCUAUGAAGAGAUACGCCUU, corresponding to nucleotides 82–102 relative to the translation start site) and GL3 siRNA (10) relative to the firefly luciferase coding region. The bottom chart shows the results of titration with siRNA(Fluc)1. CHO-K1 cells were plated at 50,000 cells per well and transfected after 24 h with two mixtures. Mixture one contained 1 µl GeneJuice™ Transfection Reagent, 0.25 µg pTriEx™-2(Fluc), and 0.05 µg pTriEx-2(β-gal). Mixture two contained 3 µl RiboJuice and either 0.1, 0.25, 0.5, 1, 2.5, 5, 10, or 25 nM (final concentration) siRNA(Fluc)1. As a control, 3 µl RiboJuice was also used without any siRNA. Total volume per well was 300 µl. Cells were lysed with Reportasol™ 24 h after transfection and assayed for reporter activity. All assays were performed in triplicate and variation is expressed as standard error of the mean.

Summary

In conclusion, we have demonstrated the effectiveness of RiboJuice for siRNA delivery in a variety of cell lines with multiple siRNAs. In most cases, homologous siRNA was able to reduce target gene expression by at least eighty percent. The ability to target specific genes for gene silencing is a powerful tool that researchers can now perform more easily, using RiboJuice™ siRNA Transfection Reagent.

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Product	Size	Cat. No.
RiboJuice™ siRNA Transfection Reagent	0.3 ml 1 ml	71115-3 71115-4
GeneJuice™ Transfection Reagent	1 ml 10 × 1 ml 0.3 ml	70967-3 70967-4 70967-5
pTriEx™-2 DNA	20 µg	70826-3
Reportasol™ Extraction Buffer	25 ml 125 ml	70909-3 70909-4
BetaRed™ β-Gal Assay Kit	500 assays 2500 assays	70978-3 70978-4

pET-44 series for Nus•Tag-enhanced protein solubility and improved purification with His•Mag Agarose Beads

Robert Novy and Don Drott — Novagen, Inc.

Nus•Tag™ vectors enable target proteins to be expressed as fusions to the *E. coli* NusA protein. This protein was identified by modeling as having a very high probability (> 90%) for being soluble when expressed in *E. coli* (1, 2), and has proven successful in increasing the solubility of many target proteins when fused with them. Novagen's pET-43.1a–c(+) vectors encode an N-terminal Nus•Tag/His•Tag®/S•Tag™ fusion sequence, followed by protease cleavage sites, multiple cloning site region, and optional C-terminal HSV•Tag® and His•Tag sequences. The additional small peptide tags on Nus•Tag fusion proteins provide versatile options for detection, assay, and purification. In particular, the His•Tag sequence facilitates simple purification by metal chelation chromatography (IMAC) using Novagen's His•Bind® matrices.

The recently introduced His•Mag™ Agarose Beads employ the same IDA-based chemistry for purification as the His•Bind Resins, but offer batchwise magnetic processing instead of filtration or centrifugation-based protocols. When the His•Mag method is combined with Novagen's BugBuster® or PopCulture™ Reagents for cell lysis, the entire procedure for protein purification is greatly simplified and becomes amenable to high-throughput automation. (See article on page 2.)

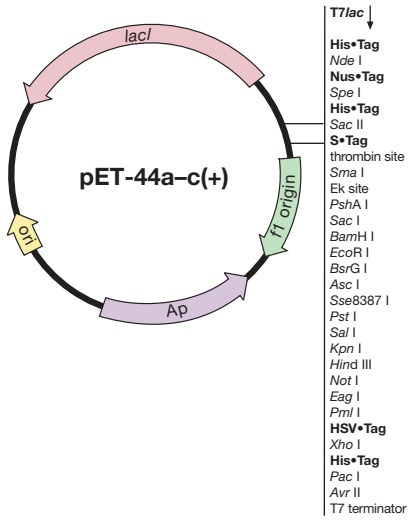

Figure 1. pET-44a–c(+) vector map

During the testing of a pET-43.1 construct with the His•Mag purification scheme, we discovered that the recovery of target protein was lower than expected based on analysis of the total soluble protein fraction. One possible explanation was that the single internal His•Tag in this construct was not sufficiently exposed to the affinity matrix under the conditions employed to enable efficient binding.

To address this possibility, we constructed and tested the pET-44 series, which are derived from the pET-43.1 series and express an additional His•Tag peptide at the N-terminus (see Figure 1). The vectors listed in Figure 2 were transformed into expression host Tuner™(DE3) for expression and purification experiments. Figure 2 also shows the configuration of 6-mer His•Tag peptides encoded by these vectors. In the case of the "a" reading frame vectors, the C-terminal His•Tag is not expressed by the empty vector. Therefore, the fusion protein generated by pET-43.1a(+) encodes only the internal His•Tag, whereas the fusion protein expressed from pET-44a(+) encodes both the internal and N-terminal His•Tag peptides. Both "b" reading frame vectors produce proteins containing an additional C-terminal His•Tag. Fusion proteins generated from these vectors were purified using His•Mag Agarose Beads and His•Bind Resin column chromatography in separate experiments.

His•Mag Agarose Bead purification

The gel in Figure 2 shows the results of purification using His•Mag Agarose Beads. All four constructs expressed similar levels of target protein, as estimated from the relative intensities of the appropriate bands in

Sample	Vector	Expressed protein	Protein structure
1.	pET-43.1a(+)	589 aa, 64.9 kDa	Nus•Tag · His•Tag · S•Tag
2.	pET-44a(+)	601 aa, 66.2 kDa	His•Tag · Nus•Tag · His•Tag · S•Tag
3.	pET-43.1b(+)	602 aa, 66.4 kDa	Nus•Tag · His•Tag · S•Tag · HSV•Tag · His•Tag
4.	pET-44b(+)	614 aa, 67.8 kDa	His•Tag · Nus•Tag · His•Tag · S•Tag · HSV•Tag · His•Tag

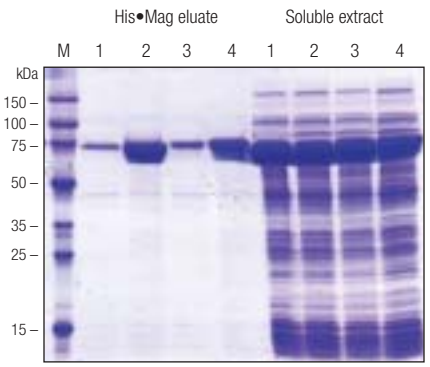

Figure 2. Expression and His•Mag purification of proteins from pET-43.1 and pET-44 vectors

Tuner(DE3) cultures carrying the indicated vectors were grown to an OD_{600} of approximately 1.0 in LB broth containing 0.5% glucose and 50 µg/ml carbenicillin. Expression was then induced with 1 mM IPTG for 2.5 h at 37°C. A 1 ml aliquot of each induced culture was harvested by centrifugation. The cell pellets were resuspended in 200 µl BugBuster HT Protein Extraction Reagent. Egg white lysozyme was added to a final concentration of 10 µg/ml and incubated for 5 minutes at room temperature. The lysate was centrifuged at 14,000 x g for 10 minutes to separate the soluble and insoluble fractions. An aliquot of the soluble fraction was removed and added to SDS Sample buffer for gel analysis. For purification, 140 µl of the soluble fraction was transferred to a new tube, mixed with 50 µl His•Mag Agarose Beads (50% slurry) and then incubated for 5 minutes at room temperature. The beads were captured by placing the tube in Novagen's Magnetight™ Separation Stand, and then washed 3 times with 400 µl 1X His•Bind Wash Buffer. The fusion protein was eluted with 50 µl 0.5X His•Bind Elute Buffer. Sample buffer was added and the equivalent of 25 µl eluate was analyzed by SDS-PAGE. Samples are indicated.

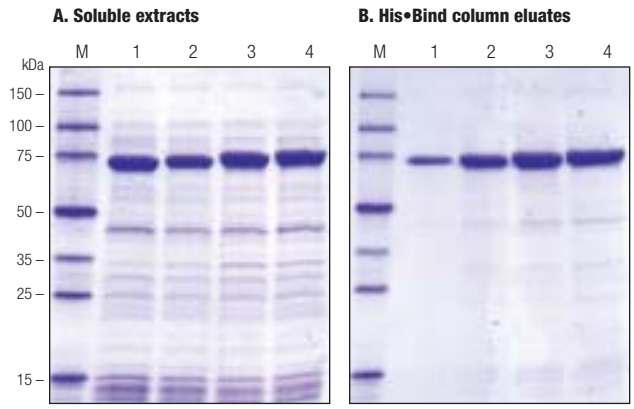

Figure 3. His•Bind column purification of proteins expressed from pET-43.1 and pET-44 vectors

Tuner™(DE3) cultures (50 ml) carrying the indicated vectors (see Figure 1 legend) were grown to an OD_{600} = 0.4 in LB broth + 0.5% glucose + 50 µg/ml carbenicillin and then induced with 0.5 mM IPTG for 3 h at 30°C. The cells were harvested by centrifugation and pellets resuspended in 5 ml BugBuster HT Protein Extraction Reagent. Chicken lysozyme (10 µg/ml) was added and the mixture was incubated for 5 min at room temperature. The lysate was centrifuged at 15,000 x g for 20 min and the supernatant (soluble fraction) transferred to a new tube. An aliquot of the soluble fraction was removed and added to SDS Sample buffer for gel analysis (Panel A). A 0.5 ml sample of each soluble fraction (representing 10 ml original culture) was then applied to separate 1 ml bed volume His•Bind™ Resin columns. The columns were washed with 1X His•Bind Buffer followed by 1X His•Bind Wash Buffer. Proteins were eluted with 2 ml 0.5X His•Bind Elute Buffer. Sample buffer was added and the equivalent of 25 µl eluate was analyzed by SDS-PAGE (Panel B).

the soluble extracts. However, the yield of purified protein (His•Mag eluate in Figure 2) was several fold greater with the pET-44a(+) and pET-44b(+) constucts than with the corresponding pET-43.1a(+) and pET-43.1b(+) constructs (compare lanes 2 and 4 vs. 1 and 3). The additional N-terminal His•Tag™ sequence in the pET-44 constructs dramatically increased the efficiency of purification under the conditions tested for His•Mag™ Agarose Beads. The N-terminal location of the His•Tag appears to be important for this effect, since the presence of an additional C-terminal His•Tag sequence in either pET-43b(+) or pET-44b(+) construct had little effect on yield (compare lanes 1 vs. 3 and 2 vs. 4 in Figure 2).

His•Bind® Resin column purification

Figure 3 shows the results of a similar experiment in which IMAC purification was carried out using column chromatography rather than the batchwise magnetic protocol. As in the previous experiment, the gel in Panel A indicates that the expression level of all of the constructs was similar. The His•Bind column eluates are shown in Panel B. In this case, no noticeable benefit of the additional N-terminal His•Tag was observed with the "b" frame clones (lanes 3 and 4), which already express an additional C-terminal His•Tag relative to the "a" frame clones. However, the yield of purified fusion protein from pET-43.1a(+) (which contains a single internal His•Tag immediately downstream from the Nus•Tag™ polypeptide) was noticeably less than that obtained from the other constructs.

Discussion and summary

These results demonstrate that the addition of a His•Tag to the N-terminus of the Nus•Tag sequence can dramatically enhance purification yields when purifying a target protein with His•Mag Agarose Beads from lysates generated with the BugBuster® HT Reagent. Similar enhancements have been observed when utilizing these magnetic beads to purify target proteins from PopCulture™ lysates (data not shown). Although the benefit was not as dramatic, an enhanced yield was also observed for the pET-44a(+) protein using His•Bind Resin column chromatography with a BugBuster lysate. Under the conditions tested here, the "b" frame clones, which encode an additional C-terminal His•Tag relative to the "a" frame clones, benefitted from the presence of an additional N-terminal His•Tag only in the case of the His•Mag batch purification.

The differences in binding efficiency observed here may be partly due to differences in exposure of the His•Tag ligands to the solid phase between batch and column based protocols. In the His•Mag protocol, exposure to the magnetic beads is relatively brief, while many more opportunities for binding are available with the column format. This is unlikely to be the only reason for our observations, because other fusion proteins do not display such large differences in yield between column and batch modes of purification. The additional N-terminal His•Tag peptide encoded by the pET-44 series appears to alleviate any potential interference by the Nus•Tag polypeptide with IMAC purification. The pET-44 series of vectors should enhance target protein recovery with His•Mag Agarose Bead purifications and for constructs that do not express the C-terminal His•Tag sequence.

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Product		Cat. No.
pET NusA Fusion System 44		71125-3
System plus Competent Cells		71126-3
Components:		
• 10 µg	pET-44a(+), pET-44b(+), and pET-44c(+) DNA (each)	
• 0.2 ml	BL21 Glycerol Stock	
• 0.2 ml	BL21(DE3) Glycerol Stock	
• 0.2 ml	BL21(DE3)pLysS Glycerol Stock	
• 0.2 ml	Induction Control Glycerol Stock	
System plus Competent Cells also includes:		
• 0.2 ml	NovaBlue Competent Cells	
• 0.2 ml	BL21(DE3) Competent Cells	
• 0.2 ml	BL21(DE3)pLysS Competent Cells	
• 2 × 2 ml	SOC Medium	
• 10 µl	Test Plasmid	

Available separately:

Product	Size	Cat. No.
pET-44a(+) DNA	10 µg	71122-3
pET-44b(+) DNA	10 µg	71123-3
pET-44c(+) DNA	10 µg	71124-3
BugBuster® HT Protein Extraction Reagent	100 ml 500 ml 1 L	70922-3 70922-4 70922-5
rLysozyme™ Solution 30 KU/µl	300 KU 1200 KU 6000 KU	71110-3 71110-4 71110-5
Note: 1 KU = 1000 Units		
Benzonase® Nuclease, Purity > 90%	10,000 U 2500 U	70746-3 70746-4
4X SDS Sample Buffer	2 ml	70607-3
Perfect Protein™ Markers, 15–150 kDa	100 lanes	69149-3

Protocols to detect Cre recombinase expression in transgenic mouse tissues by immunostaining and Western blotting

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Because embryonic lethality is often encountered in the mouse using traditional gene knockout methods, conditional gene deletion strategies, including the Cre/*lox* method (1), are becoming more widespread. To fully understand the phenotypes observed following conditional gene deletion, it is essential to properly define the temporal and spatial expression of Cre in each line of transgenic mice. Our laboratory has investigated the effects of tissue-specific deletion of transcription factors implicated in the stress response using several independent lines of transgenic mice generated to target Cre expression to the either the mammary gland, ovary or epidermis of skin. Over the course of these experiments, we have routinely used Novagen's polyclonal Anti-Cre Antibody (Cat. No. 69050-3) to characterize which cell types within each tissue express Cre, the relative percentage of cells in each tissue that express Cre and the relative levels of Cre protein expression between independent mice.

Because Cre transgenes are driven by specific gene regulatory sequences, often only certain populations of cells within an individual tissue are capable of expressing the recombinase. For this reason, Southern blotting and PCR methods of detection for Cre may underestimate expression levels if DNA is prepared from whole tissues. In contrast, immunostaining allows for simultaneous analysis of both cell type expression and percentage of expressing cells per total cells. In initial experiments, we attempted to detect Cre protein in formalin-fixed, paraffin-embedded sections via fluorescent, directly-conjugated, mouse monoclonal primary antibodies. Two clones, available from two independent vendors, were used; however, neither resulted in positive staining, and the antibodies were too expensive to warrant further testing. The Novagen product was subsequently chosen for several reasons, including a modest cost compared to other vendors' products, and previous reports of its use in the murine mammary gland (2). Another advantage included that it is produced as a purified IgG fraction of rabbit polyclonal serum, eliminating mouse-on-mouse cross-reactivity concerns. Finally, because the antibody is not conjugated, more flexible staining protocols could be designed in order to amplify the signal during immunostaining, immunofluorescence or Western blotting.

Figure 1. Cre immunostaining and Western blotting protocols

Immunofluorescence

1. Fix samples 2 h to overnight in either buffered formalin or chilled 4% paraformaldehyde/PBS.
2. Paraffin-embed, cut sections (5–7 microns).
3. Dewax and dehydrate according to standard protocols.
4. Perform antigen retrieval as follows. Place slides in a glass slide tray submerged in a 2 liter beaker filled to 600 ml with 1X (10 mM) citrate buffer, pH 6.0. Microwave slides at highest power setting for 5 min, repeat three more times for a total of four rounds. After each interval, replace evaporated volume with distilled water.
5. Let slides cool to room temperature (RT) while submerged (~20 min).
6. Wash 4 × 5 min in distilled water, then 1 × 2 min in 1X PBS.
7. Block in 10% normal goat serum/PBS+ 0.1% Tween (PBST) for 1 h at RT in humid chamber, drain.
8. Incubate with Anti-Cre Antibody diluted in 10% NGS/PBST at 1:1000 at RT several hours to overnight in humid chamber. (NGS = normal goat serum)
9. Wash 3 × 10 min in PBST.
10. Incubate with a biotinylated anti-rabbit secondary in 10% NGS/PBS, 1:1000 1 h RT.
11. Wash 3 × 10 min in PBST.
12. Incubate with a 1:750 dilution of streptavidin-Texas Red in 10% NGS/PBST for 30 min RT.
13. Wash 3 × 10 min in PBST.
14. To visualize nuclei, stain with 0.5 µg/ml DAPI in PBS for 5 min, wash 1 × 5 min.
15. Mount in aqueous medium, seal edges.

Colorimetric detection

1. Dewax slides, then dehydrate to 100% ethanol.
2. Block endogenous peroxidase activity in 3% H_20_2/methanol 10 min RT (if alkaline phosphatase based detection, skip this step).
3. Complete dehydration to PBS.
4. Perform antigen retrieval, blocking and primary incubations as in steps 4–8 above.
5. Proceed with standard colorimetric protocols beginning with secondary antibody incubation. Staining should develop in under two minutes.

Western blotting

1. Isolate whole cell extracts.
2. Perform SDS-PAGE with 60 µg input/lane and transfer to PVDF membrane.
3. Block membrane in 5% nonfat dry milk in PBST for 1 h at RT.
4. Add the Anti-Cre Antibody diluted in blocking buffer at 1:10000.
5. Incubate with agitation for 1 h at RT or overnight at 4°C.
6. Wash membrane with PBST 3 × 5 min.
7. Add peroxidase-labeled anti-rabbit IgG secondary antibody diluted to 1:5000 in PBST.
8. Incubate with agitation for 1 h RT.
9. Wash the membrane with PBST 3 × 5 min.
10. Detect with ECL™ reagent.

Immunostaining

To analyze Cre expression patterns, colorimetric and fluorescent protocols were optimized using formalin or paraformaldehyde-fixed, paraffin-embedded sections. Protocols for Cre immunostaining were modified based on a protocol taken from Selbert et al. (2). Minor modifications were made, including a switch to microwaving in citrate buffer in lieu of protease digestion to retrieve the antigen from formalin-fixed sections. Protocols were first optimized for both the mammary gland and ovary; both of these tissues can withstand microwave retrieval without negatively affecting morphology. Using the protocol presented in Figure 1, we were able to confirm that transgene expression was restricted to the mammary epithelium and that a majority of the mammary epithelial cells (at least 75%) expressed the transgene at lactation, as previously estimated by Southern blotting and crosses to reporter gene mice (3). As can be seen in Figure 2, dilution of the Anti-Cre Antibody to 1:1000 resulted in intense staining for Cre detected by either standard colorimetric (3, 3'-diaminobenzidine, DAB) or immunofluorescence staining. Importantly, at this dilution of primary antibody, there was little to no background staining in Cre-positive sections, and no signal was observed in non-transgenic samples. For our purposes, immunofluorescent detection was preferred over colorimetric because of the higher contrast between red (Cre; Texas Red®) and blue staining (4', 6 diamidino-2-phenylindole; DAPI) compared to the brown and blue staining observed with colorimetric detection, thereby facilitating cell counts. Although developed

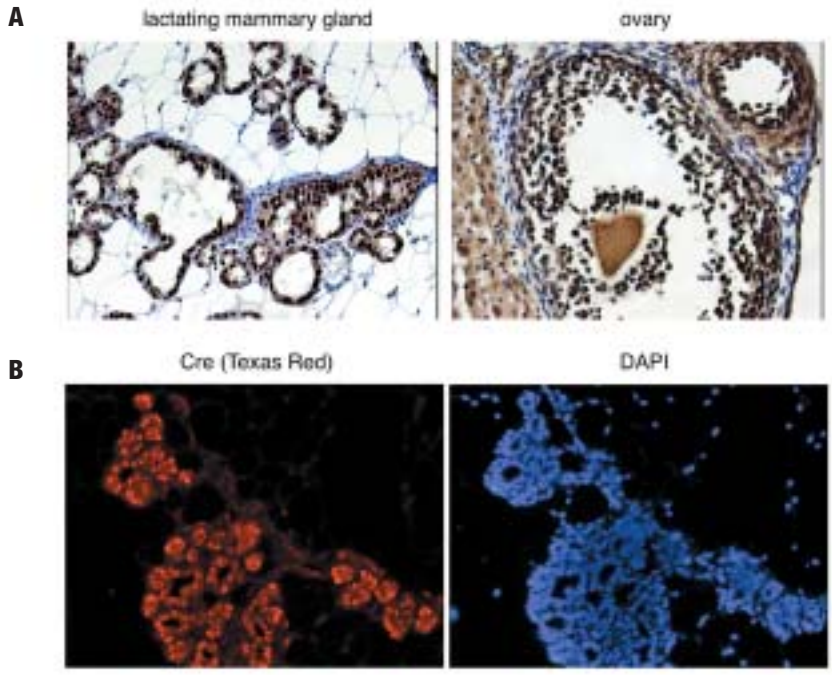

Figure 2. Immunohistological detection of Cre recombinase in the mammary gland and ovary.

(A) Cre was detected with standard DAB peroxidase enzyme substrate following by counterstaining with hematoxylin. (B) Cre staining (red) was visualized using a biotinylated secondary antibody, followed by incubation with streptavidin-Texas Red® and counterstaining with DAPI. Comparisons to DAPI indicate that a majority, but not all, of the mammary epithelial cells express Cre at this stage of development.

for the mammary gland, this immunostaining protocol was successful for the ovary as well, as indicated in Figure 1. However, the morphology of some tissues, such as the epidermis, cannot withstand microwave retrieval, therefore in these cases antigen retrieval was performed by heating the slides at 95°C in 1X citrate buffers for 45 minutes. A representative example of Cre staining in the epidermis detected by alkaline phosphate-based colorimetric detection (blue signal) is presented in Figure 3. Since the Novagen Anti-Cre Antibody produces very clean staining with a high signal to noise ratio, it may be possible to further decrease primary antibody concentrations.

Western blotting

Western blotting was also performed to determine level of Cre expression from whole cell extracts prepared from the epidermis. Briefly, sixty micrograms of extract were resolved on a polyacrylamide gel and staining was performed using a 1:10000 dilution of primary antibody, followed by incubation in a secondary antibody and development using enhanced chemiluminescent (ECL) substrate. As indicated in Figure 4, there were no detectable background bands in whole cell extracts of the epidermis using this technique and no detectable expression of Cre in the non-transgenic control. Western blotting was also used to confirm that the gene of interest, *c-Jun*, was deleted following Cre activation in the epidermis, and that loading of the protein was approximately equivalent.

Summary

In summary, in contrast to anti-Cre antibodies offered by other manufacturers, the Novagen product offered the most benefits.

Figure 3. Immunohistological analysis of Cre-positive, c-jun+^{flox}/+^{flox} mice.

Paraffin-embedded sections of skin prepared from 8-week old Cre-positive, c-jun +^{flox}/+^{flox} mice were stained with a 1:1000 dilution of Anti-Cre Antibody, visualized by alkaline phosphatase (blue) staining, 400X. The expression of Cre in this particular line of transgenic mice is restricted to the epidermis and is relatively uniformly distributed.

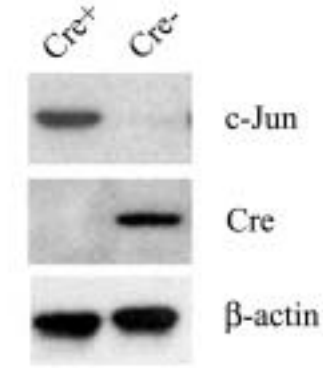

Figure 4. Western blot analysis of skin samples prepared from transgenic and nontransgenic c-jun+^{flox}/+^{flox} mice.

Protein extracts from the epidermis of Cre-negative and Cre-positive mice were immunoblotted with c-Jun, Cre and b-actin antibodies. As expected, the majority of c-Jun expression is eliminated in the transgenic mice.

Not only was the reagent easy to use, but it produced positive staining with no background, even from our first trial runs of immunostaining or Western blotting. Furthermore, the immunostaining protocol required very little optimization in order to detect Cre in the epidermis of skin samples, while preserving morphology. Finally, because Cre recombinase is commonly used to create conditional gene deletion mouse models, it is especially convenient that the antibody is a rabbit polyclonal, preventing cross-reactivity problems. These features are further enhanced by the economical costs of product. Overall, the Anti-Cre Antibody is highly recommended for detection of Cre in murine tissues.

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Product	Size	Cat. No.
Anti-Cre Antibody	50 µl	69050-3
Cre Recombinase	250 U	69247-3

Rosetta™(DE3) and Rosetta(DE3)pLysS Singles™ Competent Cells

High efficiency transformation of E. coli in less than 8 minutes

Novagen's Singles Competent Cells are designed for ultimate convenience and reliability in plasmid transformation. The cells are grown and made competent* by an optimized procedure. Cells are provided in 50 µl aliquots that eliminate the need to subaliquot, freeze/thaw or waste partially used vials, thus saving time and increasing performance. For use, simply thaw, add DNA, incubate 5 minutes on ice, heat shock for 30 seconds, place on ice for 2 minutes, and plate directly (when selecting for ampicillin resistance) or after incubation at 37°C for 30 minutes (when selecting for kanamycin resistance).

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1. Novy, R., Drott, D., Yaeger, K., and Mierendorf, R. (2001) *in**Nova**tions* **12**, 1–3.

* not intended for electroporation

Product	Size	Cat. No.
Rosetta™(DE3) Singles™ Competent Cells guaranteed efficiency: > 2 × 10^6 cfu/µg	11 rxn 22 rxn	71099-3 71099-4
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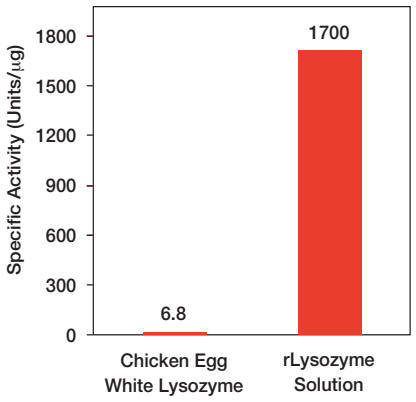
Unit Definition: one unit of rLysozyme is defined as the amount of enzyme necessary to cause a decrease of 0.025 A_{450} units per minute at 25°C in a 1.0 ml suspension (1 mg/ml) of Tuner™(DE3) cells in 0.5 × BugBuster diluted with 50 mM Tris-HCl, pH 7.5.



Comparison of chicken egg white lysozyme and rLysozyme activities

Activities were measured in a standard activity assay.

Product	Size	Cat. No.
rLysozyme™ Solution (30 KU/µl)	300 KU 1200 KU 6000 KU	71110-3 71110-4 71110-5

Note: 1 KU = 1000 Units

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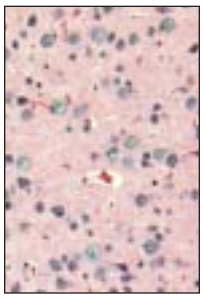
Mouse Tissue Macroarray 1	1 set (5 slides)	71109-3
Human Normal Prostate Tissue	1 set (5 slides)	71098-3

Mouse Tissue Macroarray 1

Haematoxylin and Eosin stained tissue sections from Mouse Tissue Macroarray 1. An entire slide is shown above.



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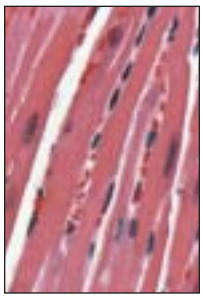

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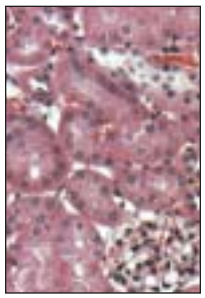
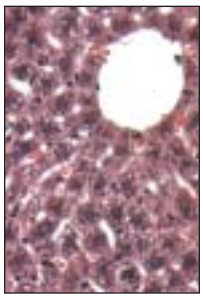
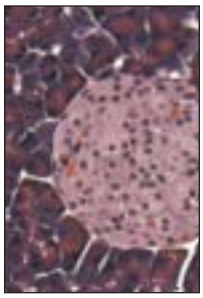


kidney

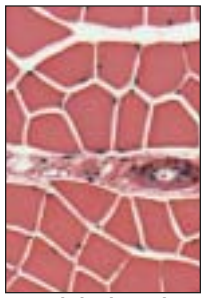


liver

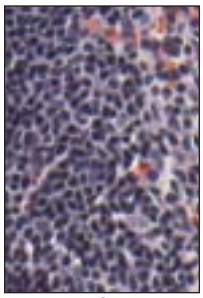
lung



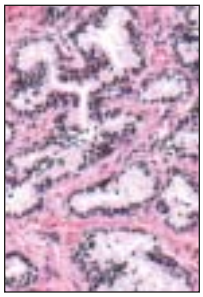
pancreas

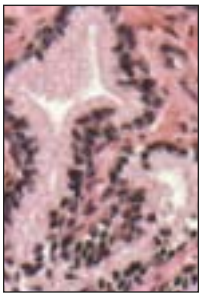


skeletal muscle



spleen





Normal Human Prostate Tissue

Haematoxylin and Eosin stained tissue sections at 10X (left) and 20X (right) magnification.

GST•Tag™ Monoclonal Antibody

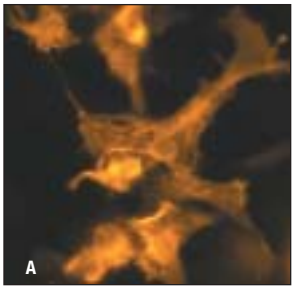
For sensitive detection of GST•Tag fusion proteins

The GST•Tag Monoclonal Antibody is a mouse monoclonal antibody (IgG_1) with high affinity to the 26 kDa glutathione-S-transferase (GST) domain from *Schistosoma japonicum*. This purified antibody is suitable for detecting fusion proteins containing the GST•Tag sequence expressed in *E. coli*, yeast, mammalian, and *in vitro* transcription/translation systems by Western blotting, immunofluorescence or immunoprecipitation. GST•Tag fusion proteins can be efficiently expressed using Novagen's pET-41 or pET-42 vector series.

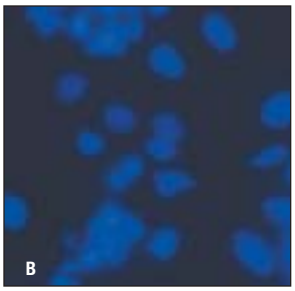
The 50 µg package size provides enough purified antibody to perform 50 Western blots (10 cm × 10 cm).

1. Smith, D. B., and Johnson, K. S. (1988) *Gene* **67**, 31–40.
2. Toye, B., Zhong, G. M., Peeling, R., and Brunham (1990) *Infect. Immunol.* **58**, 3909–3913.
3. Fikrig, E. Barthold, S. W., Kantor, F. S., and Flavell, R. A.(1990) *Science* **250**, 553–556.
4. Beekman, J. M., Cooney, A. J., Elliston, J. F., Tsai, S. Y., and Tsai, M. J. (1994) Gene **146**, 285–289.
5. Poon, R. Y., and Hunt, T. (1994) *Anal. Biochem.* **218**, 26–33.

GST detection



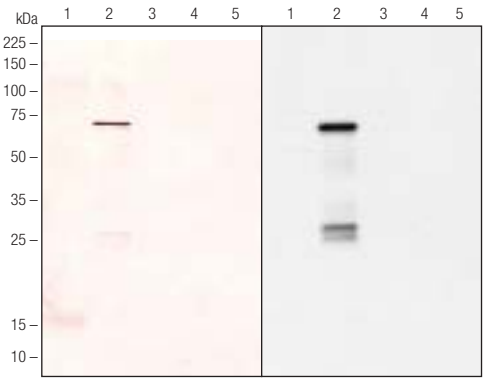
Nuclear staining



Immunofluorescent detection of GST expressed in transfected COS-1 cells

A pTriEx™ vector expressing GST was transiently transfected into COS-1 cells with GeneJuice™ Transfection Reagent. Twenty-four hours after transfection, cells were fixed, blocked with BSA and horse serum, and then exposed to GST•Tag Monoclonal Antibody (1:10,000 dilution) followed by a Cy3 conjugated Goat Anti-Mouse IgG. Hoechst 33258 was used for visualization of cell nuclei. A, Fluorescent staining of GST; B, Hoechst staining of the same field as in A showing both transfected and non-transfected cells. Magnification for both images was 32,000X.

Specificity	220 aa GST protein; precise epitope not determined
Species/Isotype	Mouse monoclonal IgG_1
Cross-reactivity	Negligible with bacterial, yeast, insect, or mammalian cell lysates
Sensitivity	2.5–5 ng (Western blot developed with chromogenic substrates) < 1 ng (AP or HRP conjugate developed with chemiluminescent substrates)
Applications	Western blot, immunoprecipitation, and immunolocalization
Form	Stabilized solution (1 mg/ml) in 50% glycerol
Working dilution	1:10,000 for Western blotting and immunofluorescence



1. Trail Mix™ Protein Markers
2. pET-41a(+) GFP, induced extract, 100 ng
3. *E. coli* BL21(DE3) extract, 100 ng
4. Sf9 insect cell control extract, 5 µg
5. CHO-K1 mammalian cell extract, 5 µg

Western blot detection of a GST•Tag GFP fusion protein

Two parallel blots were incubated with GST•Tag Monoclonal Antibody, then incubated with Anti-Mouse IgG AP or HRP Conjugate and processed by colorimetric (left panel) or chemiluminescent (right panel) detection.

Product	Size	Cat. No.
GST•Tag™ Monoclonal Antibody	50 µg	71097-3
	250 µg	71097-4

Nus•Tag™ Monoclonal Antibody

For convenient detection of Nus•Tag fusion proteins

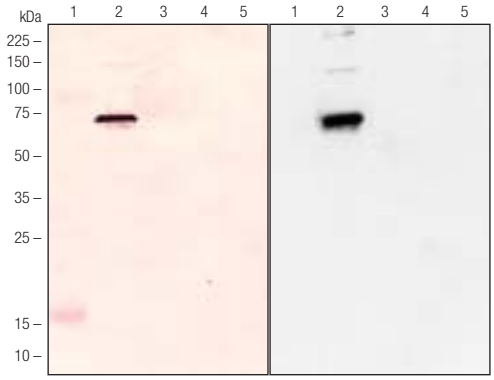
The Nus•Tag Monoclonal Antibody is a mouse monoclonal antibody (IgG_1) with high affinity for the 54.8 kDa NusA protein from *E. coli* (1, 2). This purified antibody specifically detects fusion proteins containing the Nus•Tag sequence expressed with the pET-43.1 and pET-44 vector series. Expression of polypeptides fused with the 495 aa NusA (Nus•Tag) can markedly enhance the solubility of recombinant proteins expressed in *E. coli*.

The 50 µg package size provides enough purified antibody to perform 50 Western blots (10 cm × 10 cm).

1. Harrison, R. G. (2000) *inNovations* **11**, 4–7.
2. Davis, G. D., Elisee, C., Newham, D. M., and Harrison, R. G. (1998) *Biotechnol. Bioeng.* **65**, 382–388.

Specificity	495 aa NusA protein; precise epitope not determined
Species/Isotype	Mouse monoclonal IgG_1
Cross-reactivity	Negligible with bacterial, yeast, insect, or mammalian cell lysates
Sensitivity	2–5 ng (Western blot developed with chromogenic substrates) < 1 ng (AP or HRP conjugate developed with chemiluminescent substrates)
Applications	Western blot
Form	Stabilized solution (1 mg/ml) in 50% glycerol
Working dilution	1:10,000 for Western blotting

Product	Size	Cat. No.
Nus•Tag™ Monoclonal Antibody	50 µg	71127-3
	250 µg	71127-4



1. Trail Mix™ Protein Markers
2. pET-41a(+) GFP, induced extract, 100 ng
3. *E. coli* BL21(DE3) extract, 100 ng
4. Sf9 insect cell control extract, 5 µg
5. CHO-K1 mammalian cell extract, 5 µg

Western blot detection of a Nus•Tag fusion protein

Two parallel blots were incubated with Nus•Tag Monoclonal Antibody, then incubated with Anti-Mouse IgG AP or HRP Conjugate and processed by colorimetric (left panel) or chemiluminescent (right panel) detection. Lanes are indicated.

Other Fusion Tag Antibodies and Conjugates

For superior detection of other fusion proteins

Tag	Product	Specificity	Isotype
His•Tag	His•Tag® Monoclonal Antibody	within the His•Tag sequence: HHHHH; N-terminal, C-terminal or internal	IgG_1
GST•Tag	GST•Tag™ Monoclonal Antibody	220 aa GST protein	IgG_1
T7•Tag	T7•Tag® Monoclonal Antibody	T7•Tag peptide: MASMTGGQQMG; N-terminal, C-terminal or internal	IgG_{2b}
	Biotinylated T7•Tag Monoclonal Antibody		
	T7•Tag Antibody AP Conjugate		
	T7•Tag Antibody HRP Conjugate		
S•Tag	Biotinylated S-protein	S•Tag peptide: KETAAAKFERQHMDS; N-terminal, C-terminal or internal	--
	S-protein AP Conjugate		
	S-protein HRP Conjugate		
	S-protein FITC Conjugate		
HSV•Tag	HSV•Tag® Monoclonal Antibody	HSV•Tag peptide: QPELAPEDPED; C-terminal or internal	IgG_1

Product	Size	Cat. No.
His•Tag® Monoclonal Antibody	100 µg	70796-3
	3 µg	70796-4
GST•Tag™ Monoclonal Antibody	50 µg	71097-3
	250 µg	71097-4
T7•Tag® Monoclonal Antibody	50 µg	69522-3
	250 µg	69522-4
Biotinylated T7•Tag Monoclonal Antibody	125 µl	69968-3
T7•Tag Antibody AP Conjugate	50 µl	69999-3
T7•Tag Antibody HRP Conjugate	100 µl	69048-3
Biotinylated S-protein	250 µl	69218-3
S-protein AP Conjugate	50 µl	69598-3
S-protein HRP Conjugate	50 µl	69047-3
S-protein FITC Conjugate	200 µl	69060-3
HSV•Tag® Monoclonal Antibody	40 µg	69171-3
	200 µg	69171-4

Restriction Grade Thrombin and Biotinylated Thrombin

Thrombin, Restriction Grade

Restriction Grade Thrombin is qualified to specifically cleave target proteins containing the recognition sequence LeuValProArg↓GlySer. The preparation is functionally tested for activity with fusion proteins and is free of detectable contaminating proteases. Thrombin is supplied with 10X Thrombin Cleavage Buffer and a Cleavage Control Protein.

Unit definition: one unit is defined as the amount of enzyme needed to cleave 1 mg of fusion protein in 16 hours at 20°C in a 200 µl reaction containing 20 mM Tris-HCl pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 50 µg fusion protein and enzyme.

Biotinylated Thrombin

Biotinylated Thrombin is similar to Restriction Grade Thrombin, but has covalently attached biotin for easy removal of the enzyme from cleavage reactions using immobilized streptavidin. Novagen's preparation is tested for activity using the same assay as for unmodified thrombin, and for greater than 99% binding to Streptavidin Agarose (Cat. No. 69203-3).

Thrombin Cleavage Capture Kit

The Thrombin Cleavage Capture Kit is designed for cleavage of fusion proteins followed by convenient and quantitative removal of thrombin protease. The method is based on the use of Biotinylated Thrombin for digestion and its subsequent removal with Streptavidin Agarose. The kit is suitable for use with any fusion protein that contains a thrombin recognition sequence. A Cleavage Control Protein is included in the kit to monitor performance of cleavage conditions. The 48 kDa control protein is cleaved by thrombin into two proteolytic fragments of 35 kDa and 13 kDa, which are easily visualized by SDS-PAGE.

Product	Size	Cat. No.
Thrombin, Restriction Grade	50 U	69671-3
Biotinylated Thrombin	50 U	69672-3

Components:

- 50 U Thrombin *or* Biotinylated Thrombin
- 1 ml 10X Thrombin Cleavage Buffer
- 2 ml 1X Thrombin Dilution/Storage Buffer
- 10 µg Cleavage Control Protein

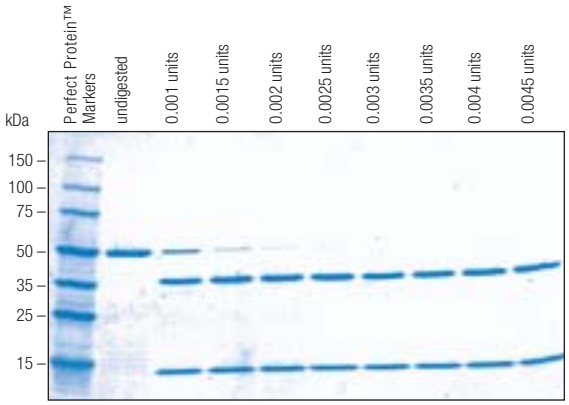
Product	Cat. No.
Thrombin Cleavage Capture Kit	69022-3

Components:

- 50 U Biotinylated Thrombin
- 5 × 1 ml 10X Thrombin Cleavage Buffer
- 2 ml 1X Thrombin Dilution/Storage Buffer
- 2 × 0.4 ml Streptavidin Agarose
- 10 µg Cleavage Control Protein
- pkg/10 Spin Filters, 2 ml capacity

Available separately:

Product	Size	Cat. No.
Streptavidin Agarose	5 ml	69203-3
Cleavage Control Protein	10 µg	69069-3
Spin Filter, 2 ml	pkg/10	69072-3



Biotinylated Thrombin cleavage

The indicated amounts of Biotinylated Thrombin were used to cleave 2 µg of Cleavage Control Protein in an overnight digestion. Samples were analyzed by SDS-PAGE (4–20% gradient gel) followed by staining with Coomassie blue. The 0.0045 units lane represents a 2.25-fold overdigestion.

Recombinant Enterokinase

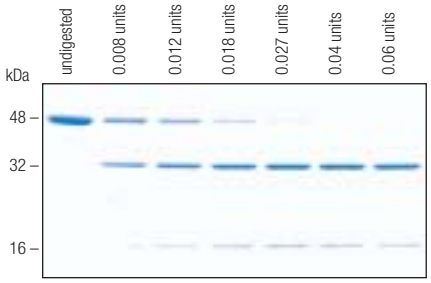
Recombinant Enterokinase

Recombinant Enterokinase (rEK) is a highly purified preparation of the catalytic subunit of bovine enterokinase, which recognizes the identical cleavage site as the native enzyme (i.e., AspAspAspAspLys↓) and has similar enzymatic activity. rEK exhibits superior rates of cleavage of fusion proteins containing the recognition sequence when compared to the native enzyme (1). Novagen's rEK is purified to near homogeneity and, unlike some preparations of native bovine enterokinase, exhibits no secondary cleavage arising from contaminating proteases. The preparation is also functionally tested for activity with fusion proteins.

Unit definition: one unit is defined as the amount of enzyme needed to cleave 50 µg of fusion protein in 16 hours at 23°C in a buffer containing 20 mM Tris-HCl pH 7.4, 50 mM NaCl, and 2 mM $CaCl_2$.

Enterokinase Cleavage Capture Kit

The Enterokinase Cleavage Capture Kit is designed for highly specific cleavage of fusion proteins followed by rapid, affinity-based capture and removal of enterokinase.



Recombinant Enterokinase (rEK) cleavage

The Cleavage Control Protein (3 µg) was digested with increasing amounts of rEK in separate reactions under standard assay conditions. Samples were analyzed by SDS-PAGE (4–20% gradient gel) followed by staining with Coomassie blue. The 0.06 units lane corresponds to 1 enzyme unit per 50 µg target protein, which exhibits > 95% cleavage.

Following cleavage of the target protein, rEK is removed with > 99% efficiency from the reaction by affinity capture on EKapture™ Agarose. Following capture of rEK, the EKapture Agarose is removed by spin filtration. Because the same buffer conditions are used for both cleavage and capture, no buffer changes are necessary.

The kit also includes a Cleavage Control Protein for conducting control digests in parallel with experimental samples, or to test cleavage under customized buffer conditions. The 48 kDa Cleavage Control Protein is cleaved by rEK into two proteolytic fragments of 32 kDa and 16 kDa, which are easily visualized by standard SDS-PAGE followed by Coomassie blue staining (see figure below). The Cleavage Control Protein also features an amino terminal S•Tag™ sequence enabling sensitive detection of the 16 kDa proteolytic product with Western blot reagents.

1. Collins-Racie, L. A., McColgan, J. M., Grant, K. L., DiBlasio-Smith, E. A., McCoy, J. M., and LaVallie, E. R. (1995) *Bio/Technology* **13**, 982–987.

Product	Size	Cat. No.
Recombinant Enterokinase	50 U	69066-3

Components:

- 50 U Recombinant Enterokinase
- 2 ml 1X rEK Dilution/Storage Buffer
- 1 ml 10X rEK Cleavage Buffer
- 10 µg Cleavage Control Protein

Product	Cat. No.
Enterokinase Cleavage Capture Kit	69067-3

Components:

- 50 U Recombinant Enterokinase
- 2 ml 1X rEK Dilution/Storage Buffer
- 5 ml 10X rEK Cleavage Buffer
- 1.5 ml EKapture Agarose
- 10 µg Cleavage Control Protein
- pkg/10 Spin Filters, 2 ml capacity

Available separately:

Product	Size	Cat. No.
EKapture™ Agarose	1.5 ml	69068-3
	10 ml	69068-4
Cleavage Control Protein	10 µg	69069-3
Spin Filter, 2 ml	pkg/10	69072-3

Restriction Grade Factor Xa

Factor Xa, Restriction Grade

Restriction Grade Factor Xa is a highly purified enzyme isolated from bovine plasma and activated with Russell's viper venom. Novagen's preparation is purified to near homogeneity and shows no secondary cleavage from contaminating proteases. The preparation is also functionally tested for activity with fusion proteins.

Like enterokinase, Factor Xa cleaves at the C-terminal side of its recognition sequence (IleGluGlyArg↓) and can therefore be used for removing all vector-encoded sequences from appropriately designed constructs.

Unit definition: one unit of Restriction Grade Factor Xa cleaves 50 µg Xa Cleavage Control Protein to > 95% completion in 16 hours at 21°C in a buffer containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl, and 5 mM $CaCl_2$.

Factor Xa Cleavage Capture Kit

The Factor Xa Cleavage Capture Kit is designed for highly specific cleavage of fusion proteins followed by convenient affinity-based capture and removal of Factor Xa. After cleavage of the target protein, Factor Xa is removed with greater than 95% efficiency from the reaction by affinity capture on Xarrest™ Agarose. Following capture of Factor Xa, the agarose is removed by spin-filtration. No buffer changes are necessary because the same buffer conditions are used for both cleavage and capture. The kit also includes a Cleavage Control Protein for conducting control digests in parallel with experimental samples, or to test cleavage under customized buffer conditions. The 49 kDa Xa Cleavage Control Protein is cleaved into two proteolytic fragments of 32 kDa and 17 kDa, which are easily visualized by standard SDS-PAGE followed by Coomassie blue staining (see figure below). The Xa Cleavage Control Protein also features an amino terminal S•Tag sequence enabling sensitive detection of the 17 kDa proteolytic product with Western blot reagents.

Product	Size	Cat. No.
Factor Xa, Restriction Grade	400 U	69036-3

Components:

- 400 U Restriction Grade Factor Xa
- 2 ml Factor Xa Dilution/Storage Buffer
- 1 ml 10X Factor Xa Cleavage Buffer
- 10 µg Cleavage Control Protein

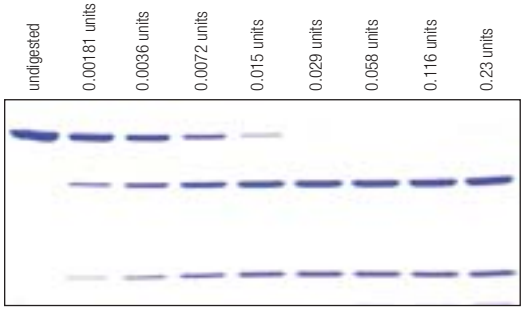
Product	Cat. No.
Factor Xa Cleavage Capture Kit	69037-3

Components:

- 400 U Restriction Grade Factor Xa
- 2 ml Factor Xa Dilution/Storage Buffer
- 5 ml 10X Factor Xa Cleavage Buffer
- 2 × 2.5 ml Xarrest Agarose
- 10 µg Xa Cleavage Control Protein
- pkg/10 Spin Filters, 2 ml capacity

Available separately:

Product	Size	Cat. No.
Xarrest™ Agarose	5 ml	69038-3
Xa Cleavage Control Protein	10 µg	69051-3
Spin Filter, 2 ml	pkg/10	69072-3



Factor Xa cleavage

The Xa Cleavage Control Protein (3 µg) was digested with increasing amounts of Factor Xa in separate reactions under standard assay conditions. Samples were analyzed by SDS-PAGE (4–20% gradient gel) followed by staining with Coomassie blue. The 0.015 units lane corresponds to 0.25 units enzyme per 50 µg target protein, which exhibits > 95% cleavage.

Pellet Paint® Co-Precipitant

Rapid, quantitative precipitation of DNA and RNA, including PCR clean-up

Pellet Paint Co-Precipitant* is a visible dye-labeled carrier formulated specifically for use in alcohol precipitation of nucleic acids (1, 2). The five minute precipitation protocol requires no low temperature incubations or prolonged centrifugation. Both RNA and DNA are efficiently precipitated from even the most dilute solutions (2 ng/ml) and the pellet is easily located by its vivid pink color. The pellet can be easily followed during washing steps and prevents losses during handling.

Most applications of PCR benefit from a clean-up step in which primers and other reactants are removed and the target DNA is concentrated (see reference 3 for an example). Pellet Paint Co-Precipitant is ideal for this purpose because the procedure is rapid, primers < 50 nt in length are efficiently removed, and the DNA is quantitatively recovered. Furthermore, it is easy to tell when the DNA has been completely resuspended following the precipitation step.

Pellet Paint is compatible with most molecular biology procedures and is free of contaminating nucleic acids and nucleolytic enzymes. Although Pellet Paint absorbs in the UV range, accurate spectrophotometric measurements of DNA or RNA samples are possible; the absorbance ratio (provided with each package of Pellet Paint) can be used as a correction factor when determining nucleic acid concentration (2). Pellet Paint is compatible with Cy5-based automated sequencers. Pellet Paint NF is recommended for use with PE Applied Biosystems automated sequencers.

1. McCormick, M. (1995) *in**Nova**tions* **4**, 10–11.
2. McCormick, M. (1996) *in**Nova**tions* **5**, 10.
3. Taggart et al. (1998) *J. Clin. Microbiol.* **36**, 3408–3409.

* patent pending

Pellet Paint Procedure

1. Add 2 µl Pellet Paint or Pellet Paint NF Co-Precipitant + 0.1 volume 3 M Na Acetate to sample and mix briefly
2. Add 2 volumes ethanol (or 1 volume isopropanol) and briefly vortex
3. Incubate at room temperature for 2 minutes
4. Spin sample for 5 minutes
5. Discard supernatant, wash and resuspend pellet

Product	Size	Cat. No.
Pellet Paint® Co-Precipitant	125 rxn	69049-3
	1000 rxn	69049-4
	5000 rxn	69049-5

Components:

- 250 µl *or* 2 ml *or* 10 ml Pellet Paint Co-Precipitant
- 1 ml *or* 8 ml *or* 40 ml 3 M Sodium Acetate, pH 5.2

Pellet Paint NF Co-Precipitant

Non-fluorescent visible DNA co-precipitant for automated sequencing and other applications

Pellet Paint NF Co-Precipitant* is a non-fluorescent dye labeled carrier compatible with fluorescent sequencing. It facilitates rapid removal of BigDye™ Terminators (PE Corp.) during the alcohol precipitation of cycle sequencing reaction products. Cycle sequencing reactions can be precipitated rapidly with centrifugation times of 10 minutes. The easily visualized carrier provides a simple confirmation that precipitation has occurred. Sequencing reaction products are efficiently pelleted and dye-labeled terminators remain in the supernatant during alcohol precipitation using the standard Applied Biosystems precipitation protocols. Resuspension of pelleted sequencing reaction products in deionized formamide can be confirmed by checking for dissolution of the carrier pellet. Pellet Paint NF Co-Precipitant is fully compatible with the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction. To avoid extra sample handling, Pellet Paint NF Co-Precipitant can be added directly to the reaction mix, template DNA, crude PCR samples, or dilution buffer prior to the cycle sequencing reaction. Pellet Paint NF Co-Precipitant has no detectable effect on the sequencing reaction or sequence accuracy. Pellet Paint NF is also an effective substitute for the original Pellet Paint in applications where fluorescent detection is used.

Features

- Efficient and rapid precipitation of BigDye cycle sequencing products
- Efficient removal of dye terminators
- Direct visualization and tracking of precipitated material
- No effect on sequencing reaction
- Substitute for original Pellet Paint Co-Precipitant for fluorescent detection applications

* patent pending

Product	Size	Cat. No.
Pellet Paint® NF Co-Precipitant	125 rxn	70748-3
	1000 rxn	70748-4
	5000 rxn	70748-5

Components:

- 125 µl *or* 1 ml *or* 5 ml Pellet Paint NF Co-Precipitant
- 1 ml *or* 8 ml *or* 40 ml 3 M Sodium Acetate, pH 5.2

GeneJuice™ Transfection Reagent

High efficiency transfection of mammalian cells

Product	Size	Cat. No.
GeneJuice™ Transfection Reagent	1 ml 10 × 1 ml 0.3 ml	70967-3 70967-4 70967-5

GeneJuice Transfection Reagent is a proprietary formulation optimized for maximal transfection efficiency, ease of use, and minimal cytotoxicity. This transfection reagent is a superior alternative to a wide variety of other techniques including calcium phosphate coprecipitation, electroporation, microinjection, biolistic particle delivery, and complex formation with DEAE-dextran.

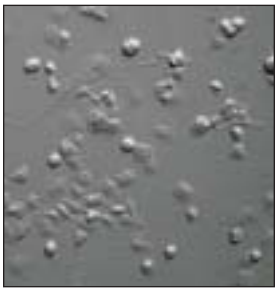
Whereas many available transfection reagents are based on cationic lipid formulations, GeneJuice is composed of a nontoxic cellular protein and a small amount of a novel polyamine. The unique chemistry provides several advantages over lipid-based transfection, including:

- Highly efficient DNA transfer for both stable and transient transfections
- Minimal cellular toxicity
- Compatibility with both serum-containing and serum-free media
- Simple protocol—no need for media changes
- Ideal for high-throughput (HT) transfection in a multi-well plate format

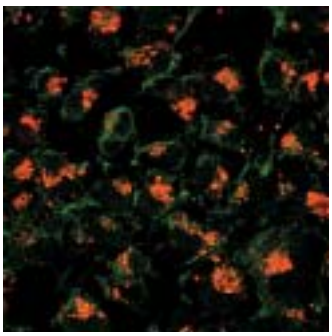
GeneJuice Transfection Reagent has been demonstrated to provide excellent performance in both stable and transient transfection of eukaryotic cells and is ideal for use with Novagen's pTriEx™ transient and stable expression vectors.

The 1 ml size provides enough reagent to perform up to 500 transfections in standard 35 mm plates. The reagent is also available in an introductory 0.3 ml size. GeneJuice is supplied as a ready-to-use sterile solution.

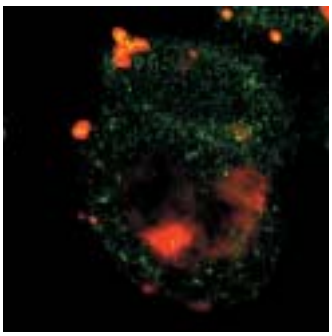
No transfection



Cationic lipid based reagent



COS-7



HeLa

GeneJuice transfection of COS-7 and HeLa cells with rhodamine-labeled DNA

Cells grown on polylysine-coated coverslips to 50% confluency were transfected with rhodamine-labeled pTriEx-2 DNA using GeneJuice. Labeled plasmid DNA was complexed with GeneJuice in serum-free medium, and the complexes were added directly to the cells in complete medium. Twenty-four hours after transfection, the cells were washed in PBS, fixed in 4% formalin for 10 minutes, and washed again in PBS. Coverslips were mounted on glass slides and sealed for confocal microscopy. The transfected DNA is seen in red. Unfiltered reflected light from the 533 laser was collected to image the cell boundaries.

GeneJuice

Toxicity comparison

Three replicate COS-7 cultures were left untreated, transfected with a popular cationic lipid based transfection reagent, and transfected with GeneJuice according to recommended protocols. Cellular damage is visualized by rounding up and detachment from the plate surface. The photographs, taken 48 h post transfection, show that GeneJuice caused much less cytotoxicity than the other reagent.

Cell types successfully transfected with GeneJuice Transfection Reagent

10T1/2	BC3	CFPAC-1	HCT-116	IEC-6	Melanocyte	PS-1	SW-837
3T3-L1	BCBL	Chang Liver	HEK293	JEG-3	MG-63	R2C	T3M4
A204	BHK-21	CHO	HeLa	Jurkat	Neuroblastoma	RAW 264.7	TM4
A431	C3H/10T1/2	COS-1	Hep 3B2.1-7	KB	NPK	RBL-2H3	U937
A549	C6	COS-7	HepG2	L57-3-11	NT2/D1	RMP-41	UCD
alpha TC1-6	C2C12	DDTI MF-2	Hepa 1-6	L-6	OV-1063	SC-1	Vero
AR 42J	Caco-2	DT40	Ht-29	L-929	OVCAR3	Schneider line2	WE-38
As4.1	Caki-1	ECV304	HTB-37	MA-10	P4	SK-N-MC	Primary aortic smooth muscle cells
AtT-20	Calpan-1	EL4	HTB-45	McA-RH7777	P19	SK-N-SH	
B50	Calu-1	ES-E14TG2a	Huh-7	MCF-7	PC12	SKOV3	Primary keratinocytes
BC-1	Calu-6	EVSCC17M	HUVEC	MCF-10-2A	PA317	STO	
BC-2	CCL-131	H9c2	IC21	MDCK	PAM212	SW-480	

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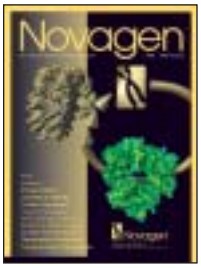

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