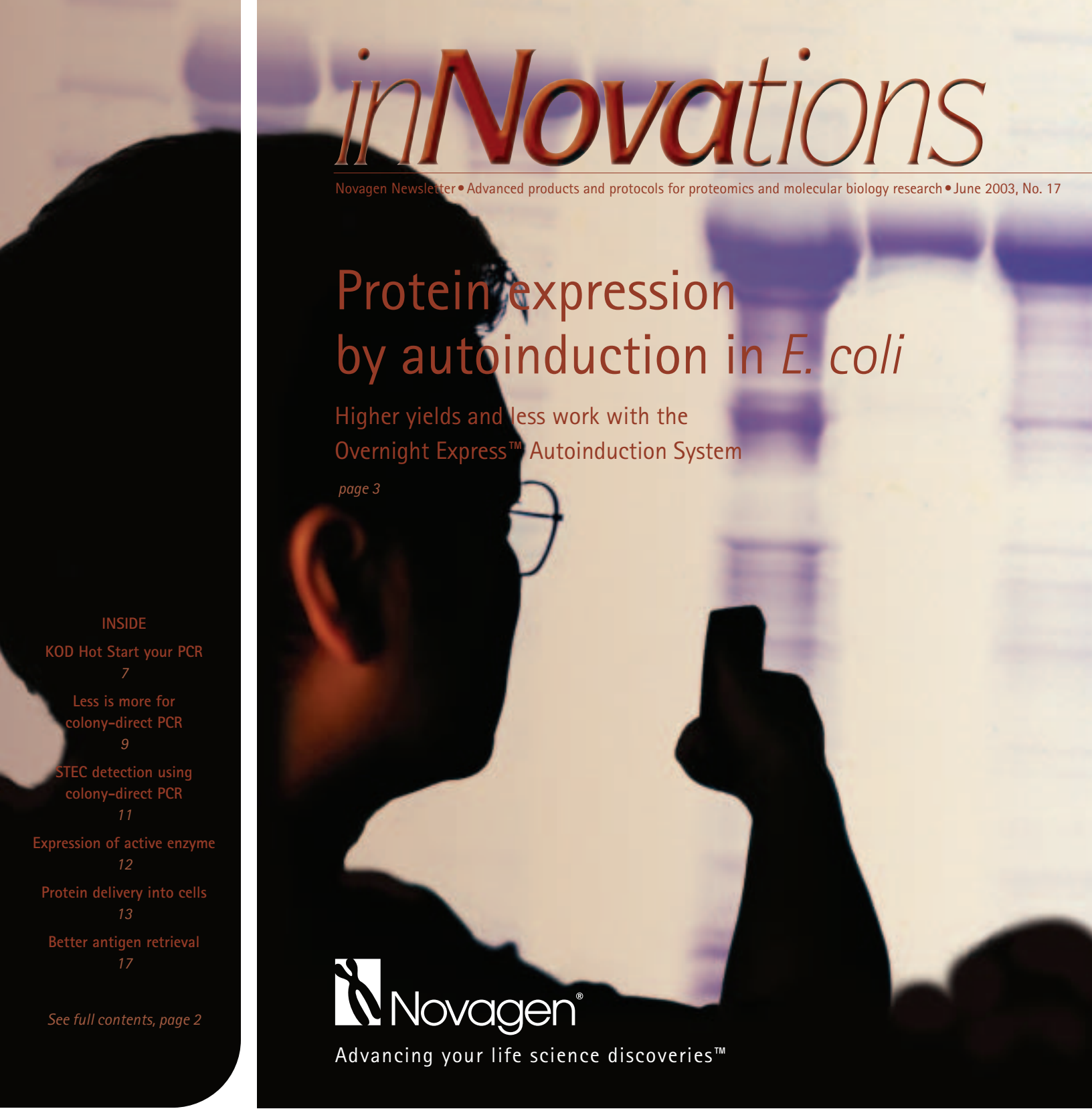

inNovations

Novagen Newsletter • Advanced products and protocols for proteomics and molecular biology research • June 2003, No. 17

Protein expression by autoinduction in *E. coli*

Higher yields and less work with the Overnight Express™ Autoinduction System

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ON THE COVER

SDS-PAGE analysis revealed greater yields of target proteins tested with the Overnight Express Autoinduction System 1 as compared to IPTG induction.

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Unattended high-density cell growth and induction of protein expression with the Overnight Express™ Autoinduction System

Anthony Grabski, Mark Mehler, and Don Drott — Novagen

A method for regulating protein expression in the pET system without the need to monitor cell growth is described. The method is based on media components that are metabolized differentially to promote growth to high density and automatically induce protein expression. The Overnight Express™ Autoinduction System 1 consists of three solutions that can be added to conventional bacterial growth media. The induction solution is a blend of carbon sources to permit high-density cell growth in the absence of expression followed by induction at high cell density. The buffering solution controls pH and provides additional nitrogen for protein synthesis. The magnesium solution provides the necessary concentration of this important cation for maximum cell density. Advantages to this system over IPTG induction include increased soluble protein yields, higher cell densities, no growth-rate monitoring, and convenience for high-throughput, parallel culturing and induction of numerous expression clones simultaneously.

Protein expression using the T7 (pET) system is highly efficient at producing a wide range of recombinant proteins in *E. coli* (1). Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost all of the metabolic resources of the cell are focused on target gene expression when fully induced, and the desired product can comprise more than 50% of the total cell protein a few hours after induction. Hosts for T7-based expression contain the λDE3 prophage. Expression of the T7 RNA polymerase in λDE3 lysogens is controlled by a modified *lac* promoter, the L8-UV5 *lac* promoter. In the absence of inducer, the binding of *lac* repressor to the *lac* operator prevents transcription of the T7 RNA polymerase gene. Addition of inducer (e.g., IPTG) relieves repressor binding, so that the cell can express T7 RNA polymerase (Figure 1). Although the *lac* and L8-UV5 promoters are well repressed in the absence of inducer, both exhibit basal activity. Basal expression of small amounts of T7 RNA polymerase can lead to expression of the target protein ("leaky expression"), and if the protein is toxic to the host, the cells may lyse, lose the expression plasmid, or accumulate mutations, resulting in low protein yield. The pET system is designed with options for more stringent regulation of basal expression of target proteins, relying on an additional *lac* operator to reduce basal transcription of the target gene by T7 RNA polymerase (T7*lac* promoter) or supplying T7 lysozyme, an inhibitor of T7 RNA polymerase. However, Grossman *et al.* report expression in the absence of added inducer if cells are grown to stationary phase in certain complex media (2). Such inadvertent expression is associated with high levels of cyclic AMP (cAMP), which are strongly influenced by the carbon sources present in the medium (2–4). In the presence of glucose, cAMP levels remain low and inadvertent expression is reduced.

Traditionally, recombinant protein production with the pET system requires culturing the cells in liquid medium until they reach early- to mid-log phase, at which time expression is induced by the manual addition of IPTG. As an alternative to IPTG, lactose has been used suc-

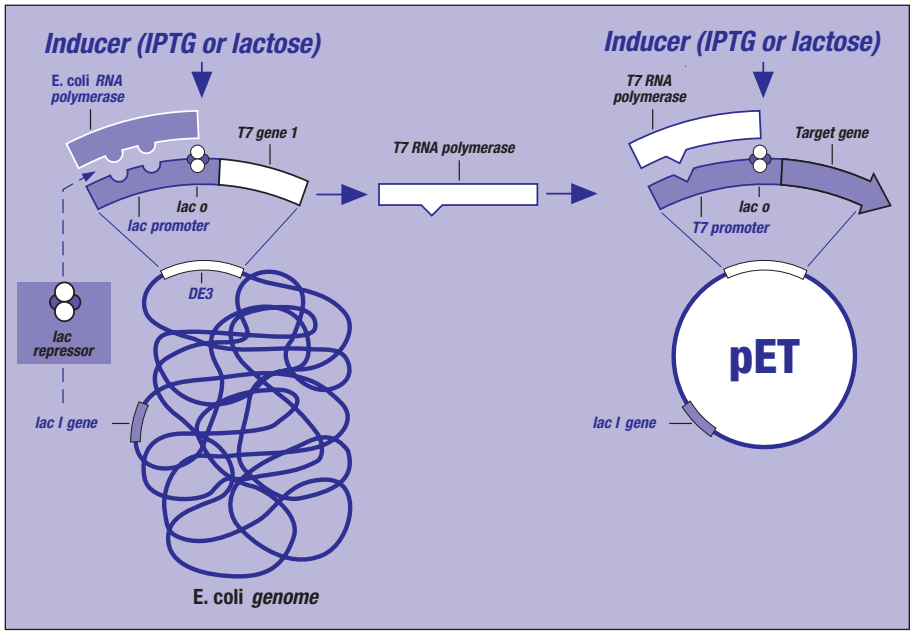

Figure 1. Induction of protein expression in the pET System

continued on page 4

continued from page 3

cessfully to induce protein expression from pET vectors in λDE3 lysogens (5). By carefully balancing the levels of glucose and lactose, yields of soluble, active protein can be maximized (6–8). Optimal recombinant protein expression requires the harmonization of a number of factors, from the choices of vector, host, and inducer to the influences of temperature and growth medium (9).

The problems associated with inadvertent expression of target proteins and attempts to understand effects of complex and defined media components on growth, catabolite repression, and induction of T7 expression strains led to the formulation of media to support growth to saturation without induction as well as fully defined and complex autoinduction media (10). In autoinduction media, cultures reliably grow uninduced to relatively high density and then spontaneously induce high levels of target protein without the need to monitor cell density or add IPTG. Key components of the autoinduction formulations are now available as the Overnight Express™ Autoinduction System 1. This article demonstrates autoinduction by the Overnight Express system (Table 1), which resulted in soluble protein yields several-fold greater than obtained by conventional induction with IPTG. Because growth and expression occur completely unattended, the Overnight Express system is convenient for high-throughput, parallel analysis of protein expression and solubility as well as purification from multiple expression clones.

The Overnight Express Autoinduction System 1 includes three components: OnEx™ Solution 1 (induction solution), OnEx Solution 2 (buffering solution), and OnEx Solution 3 (magnesium solution). OnEx Solution 1 is a blend of carbon sources optimized for tightly regulated uninduced growth to relatively high density followed by high-level induction and continued growth. Because lactose is used for induction, expression hosts should produce functional *lac* permease (encoded by the *lacY* gene) and β-galactosidase (encoded by the *lacZ* gene) for consistent results in both complex and defined media; *lacY*$^-$ strains will not efficiently transport lactose for induction and *lacZ* mutants will not convert a portion of the transported lactose into the allolactose inducer. Note that elevated levels of target gene expression in *lacY* and *lacZ* mutant strains may occur as cells approach stationary phase in some complex media, even in the absence of lactose or IPTG. However, this induction will vary depending upon medium composition, cell growth stage, and nutrient availability, all of which affect pH and the levels of cyclic AMP and acetate (2). OnEx Solution 2 is a concentrated blend of a buffer that maintains pH throughout metabolic acid production and provides additional nitrogen necessary to support increased protein synthesis. OnEx Solution 3 provides critical magnesium for maximum cell density. Addition of these components to traditional glucose-free media for culturing *E. coli*, such as LB, TB (11), or Veggie™ media (TYMV in Table 2; see page 19 for Veggie media components), results in high cell densities, autoinduction, and maximum yields of target proteins with the pET system.

Table 1. Overnight Express protocol: prepare, inoculate, incubate, and analyze

1. Prepare culture medium and add OnEx Solution 1, OnEx Solution 2, and OnEx Solution 3.
2. Inoculate culture medium with a single colony.
3. Incubate culture at appropriate expression temperature and aeration (8–24 h depending on expression host, base medium, and temperature).
4. Analyze target expression or purify protein.

Cell growth, induction, extraction, and robotic affinity purification of fusion proteins

Overnight Express autoinduction was tested using three vectors: pET-41b(+) for expression of a 35.6-kDa GST•Tag™/His•Tag®/S•Tag™ fusion protein; pET-43.1b(+) for expression of a 66.4-kDa Nus•Tag™/His•Tag/S•Tag/HSV•Tag® fusion protein; and pET-30b(+) for expression of a His•Tag/S•Tag 121-kDa β-gal fusion protein. Additionally, three eukaryotic proteins, annexin I (43.5 kDa),

Table 2. Media tested

ZY Medium	TB Medium	TYMV Medium
10 g/L tryptone	13.3 g/L tryptone	20 g/L Veggie Peptone
5 g/L yeast extract	26.7 g/L yeast extract	5 g/L Veggie Yeast Extract
	4.4 ml/L glycerol	100 mM NaCl

Table 3. Culture growth and protein expression

		Cell Density at Harvest[1]		Pure Protein Yield[2]	
Medium	Protein	OE[3]	IPTG[4]	OE	IPTG
ZY	β-gal	15.6	7.9	525	255
ZY	NusA	16.4	8.4	645	375
ZY	GST	14.2	13.1	375	300
TB	β-gal	20.7	9.7	675	225
TB	NusA	20.0	8.5	690	240
TB	GST	20.2	15.2	750	255
TYMV	β-gal	16.3	7.6	381	179
TYMV	NusA	16.5	10.0	331	208
TYMV	GST	16.1	13.3	219	239

1 OD_{600}
2 Pure protein yield expressed as µg/ml culture was determined by BCA assay (BCA Protein Assay Kit, Novagen) of purified protein fractions.
3 Overnight Express induction (OE) was accomplished by inoculating a single colony into 2 ml medium in 17 × 100-mm round-bottom, snap-cap Falcon® tubes and incubating overnight (approximately 16 h) at 30°C with shaking at 300 rpm.
4 IPTG induction (IPTG) was accomplished by inoculating a single colony into 2 ml medium containing 0.5% glucose in 17 × 100-mm round-bottom, snap-cap Falcon tubes and incubating at 30°C at 300 rpm to an average OD_{600} of 2.2 followed by addition of IPTG to 1mM final concentration and incubating an additional 5 h prior to harvest. Media for the IPTG-induced cultures included OnEx Solution 3 to eliminate $MgSO_4$ as a variable and demonstrate the influences of only OnEx Solution 1 and OnEx Solution 2 on cell harvest density and pure protein yield. However, cells cultured in these media lacking OnEx Solution 3 and induced with IPTG may not achieve similar cell densities.

annexin II (43.4 kDa), and tubulin alpha-4 (54.7 kDa) in pET-30 Ek/LIC, were produced using the Overnight Express™ system and purified to test this system with potentially toxic protein targets. The plasmids were each transformed into the BL21(DE3) expression host. All of these fusion proteins can be purified by immobilized metal chelation chromatography using His•Bind® affinity resins and detected using either the His•Tag® Monoclonal Antibody or the FRETWorks™ S•Tag™ Assay Kit. A standard induction with IPTG was performed for comparison of cell growth, protein expression level, and final purified target yield using the same vectors, host, and test proteins. Media formulations used in these experiments are shown in Table 2 (page 4). The expression and purification results are summarized in Figure 2 (at right) and Table 3 (page 4) and in Figure 3 and Table 4 (both on page 6).

The crude soluble extract and purified fusion proteins were analyzed by SDS-PAGE. Figure 2 shows that the Overnight Express system produced higher amounts of total protein for a given culture volume versus IPTG-induced cells. The purified samples (2 µg loaded) showed equivalent purity between Overnight Express– and IPTG-induced cultures. These results correlate with the final cell density as well as with the purified target protein yields (Table 3, page 4). Cells cultured and induced by the Overnight Express system had an average harvest OD_{600} of 17.3 compared to 10.5 for those induced with IPTG; purified protein yields averaged, respectively, 510 and 253 µg/ml culture.

The three eukaryotic proteins, annexin I, annexin II, and tubulin alpha-4 in pET-30 Ek/LIC, were produced in BL21(DE3) cultures (5 ml culture in 10-ml × 24-well plates) according to the RoboPop™ protocol (12). Expression by either the Overnight Express System 1 or IPTG induction was compared and Ni-NTA His•Bind®-purified targets were quantified and analyzed (Figure 3 and Table 4, both on page 6). Although the very high cell densities seen in shake flasks for β-gal, NusA, and GST autoinductions were not achieved by the standard RoboPop™ cell culturing protocol, purified protein yields were equal to or greater than the yields obtained by standard IPTG induction (Table 4, page 6). Additionally, the overall protein purity of each of the annexin I and annexin II targets was greater than the purity obtained from cultures induced with IPTG.

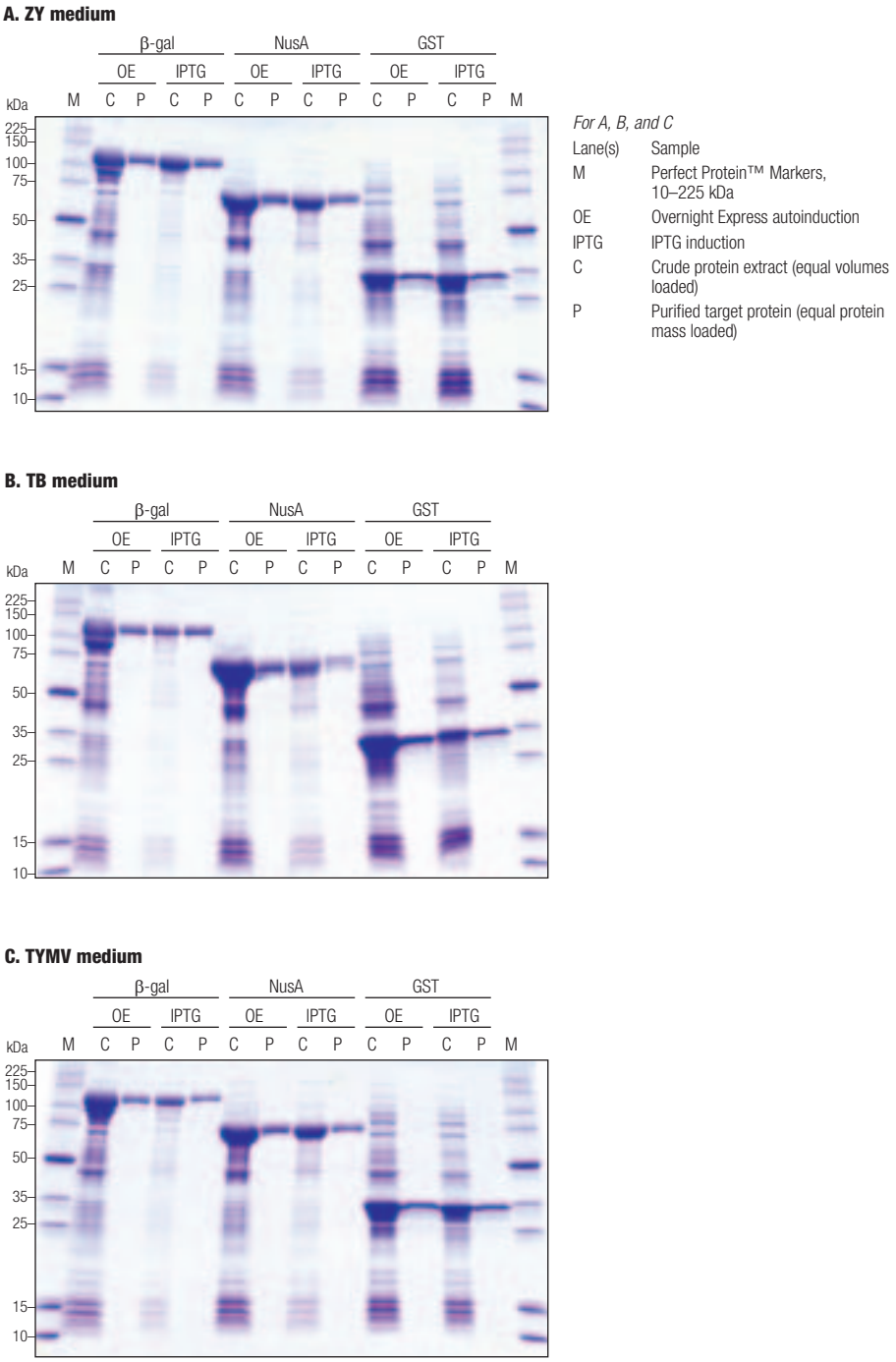

Figure 2. SDS-PAGE analysis of crude and purified proteins from cultures induced with Overnight Express versus IPTG

pET recombinants encoding β-gal, NusA, and GST His•Tag® fusion proteins were transformed into BL21(DE3). Cells were cultured and protein expression was induced in parallel cultures either by Overnight Express autoinduction or 1 mM IPTG as described in the footnotes to Table 3 (page 4). Following induction, cells were harvested by centrifugation and extracted with BugBuster® HT Protein Extraction Reagent plus rLysozyme™ Solution to produce a crude extract. Equal volumes (7 µl) of the extract were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining (C lanes). The remainder of the extract was used for robotic affinity purification using Ni-NTA His•Bind Resin. Samples (2 µg) of the purified fraction were loaded on the gels (P lanes).

Summary

We compared the Overnight Express Autoinduction System 1 with standard IPTG induction using six different pET

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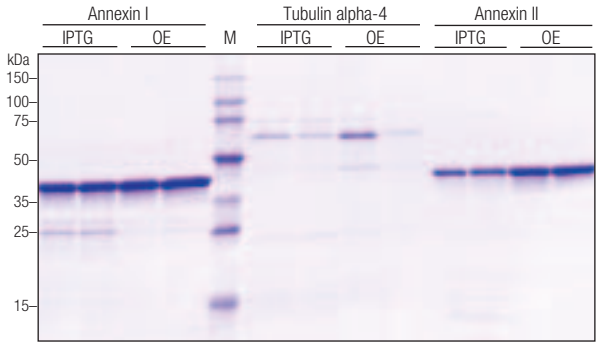

Lane(s)	Sample volume
Annexin I	4 µl
Tubulin alpha-4	4 µl
Annexin II	IPTG, 8 µl; OE, 4.5 µl

Lane(s)	Sample
IPTG	IPTG induction
OE	Overnight Express autoinduction
M	Perfect Protein™ Markers, 15–150 kDa

Figure 3. SDS-PAGE analysis of eukaryotic target proteins purified from cultures induced with Overnight Express versus IPTG

pET recombinants encoding the indicated His•Tag® fusion proteins were transformed into BL21(DE3). Cultures were grown and induced as described in the footnotes to Table 4, and target proteins were extracted and purified using the RoboPop™ Ni-NTA His•Bind® Purification Kit. The indicated sample volumes were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining.

Table 4. Culture growth and expression of eukaryotic proteins

		Cell Density at Harvest[1]		Pure Protein Yield[2]	
Medium	**Protein**	**OE[3]**	**IPTG[4]**	**OE**	**IPTG**
TB	Annexin I	6.8	5.8	240	248
TB	Annexin II	5.4	4.9	323	165
TB	Tubulin alpha-4	10	8.6	45	15

1 OD_{600}
2 Target proteins were extracted with PopCulture® Reagent and robotically purified according to protocols described in TB 346 (available at www.novagen.com). Pure protein yield was quantified as µg/ml culture (BCA Protein Assay Kit, Novagen).
3 Overnight Express induction was accomplished by inoculating a single colony into 5 ml medium in 10-ml × 24-well plates and incubating overnight (approximately 16 h) at 30°C with shaking at 250 rpm.
4 IPTG induction was accomplished by inoculating a single colony into 5 ml medium in 10-ml × 24-well plates and incubating at 16°C with shaking at 250 rpm to an average OD_{600} of 1.0 followed by addition of IPTG to 1 mM final concentration and incubating an additional 16 h prior to harvest.

recombinants and three different media formulations. Using cultures in 2-ml tubes, three different recombinants averaged 1.7-fold higher OD_{600} at cell harvest and 2-fold higher purified protein yield with the Overnight Express™ system. Using cultures in 5-ml × 24-well plates with an automated cell processing and purification protocol, SDS-PAGE analysis revealed nearly equal or greater yields of all three eukaryotic target proteins tested with the Overnight Express system as compared to IPTG induction. In addition to its superior performance, the Overnight Express Autoinduction System 1 is extremely convenient because it allows unattended growth and induction of recombinants without the need to monitor cell density or add inducer. These features greatly streamline many protein expression applications, from parallel analysis of multiple small-scale cultures to larger-scale processing for preparative uses.*

ACKNOWLEDGMENT

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Product	Size	Cat. No.
Overnight Express™ Autoinduction System 1	1 kit*	71300-3
	1 kit†	71300-4

(includes OnEx™ Solution 1, OnEx Solution 2, and OnEx Solution 3)

* 71300-3 includes enough reagents to induce 1 liter
† 71300-4 includes enough reagents to induce 5 liters

Available separately:

Product	Size	Cat. No.
Perfect Protein™ Markers, 10–225 kDa	100 lanes	69079-3
BugBuster® HT Protein Extraction Reagent	100 ml	70922-3
	500 ml	70922-4
	1 L	70922-5
rLysozyme™ Solution (30 KU/µl)	300 KU	71110-3
	1200 KU	71110-4
	6000 KU	71110-5

Note: 1 KU = 1000 units

RoboPop™ Ni-NTA His•Bind® Purification Kit	1 kit	71188-3

(includes PopCulture® Reagent, Ni-NTA His•Bind Resin, 4X Ni-NTA Bind Buffer, 4X Ni-NTA Wash Buffer, 4X Ni-NTA Elute Buffer, 2 ml 96-well Filter Plate, 1 ml 96-well Collection Plate with Sealer, rLysozyme™ Solution, rLysozyme Dilution Buffer, and Benzonase® Nuclease, Purity > 90%)

Veggie™ Peptone	500 g	71280-3
Veggie Yeast Extract	500 g	71279-3
BCA Protein Assay Kit	500 assays (2500 microplate assays)	71285-3

(includes BCA Solution; BSA Standard, 2 mg/ml; and 4% Cupric Sulfate)

* The products and technologies presented in this article were developed and are offered under the terms of a license agreement between EMD Biosciences, Inc. Novagen Brand and Brookhaven Science Associates, covering U.S. patent properties entitled "High Density Growth of T7 Expression Strains with Auto-Induction Option," filed March 14, 2003, in the name of F. William Studier and assigned to Brookhaven Science Associates, LLC., Upton, NY 11973. The Auto Induction Media Technology embodied in the Overnight Express Autoinduction System 1 is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is the subject of patent applications assigned to Brookhaven Science Associates, LLC. (BSA). This product is to be used for research purposes only. A separate license is required for any commercial manufacture or use, including the manufacture of protein products for use in the screening of compound libraries. Information about commercial licenses may be obtained from the Office of Intellectual Property and Industrial Partnerships, Brookhaven National Laboratory, Bldg. 475D, P. O. Box 5000, Upton, New York 11973-5000; Telephone (631) 344-7134.

Fidelity and reliability in PCR using KOD Hot Start

Masao Kitabayashi[1] and Alla Zilberman[2] – [1]Tsuruga BioResearch Institute, Toyobo Co. Ltd., Japan and [2]Novagen

The performance of KOD Hot Start DNA Polymerase is compared with PfuUltra *and* PfuTurbo *enzymes. The KOD enzyme exhibits higher yield with a lower mutation frequency.*

The family of recombinant *Thermococcus kodakaraensis* KOD1 DNA polymerases offers the highest elongation rates and processivity in DNA amplification, and possesses one of the highest fidelities among the thermostable DNA polymerases used for PCR. This article demonstrates the superior DNA yield in PCR amplification with KOD Hot Start DNA Polymerase* as compared to the performance of two DNA polymerases isolated from the extremophile *Pyrococcus furiosus*. In addition, KOD Hot Start DNA polymerase shows excellent fidelity compared to *Taq*, *PfuUltra*™, and *PfuTurbo*® DNA polymerases (Stratagene).

KOD Hot Start DNA Polymerase is a premixed complex of KOD HiFi DNA Polymerase, a recombinant form of *T. kodakaraensis* KOD1 DNA polymerase (1, 2), and two monoclonal antibodies that inhibit DNA polymerase and 3′–5′ exonuclease activities at ambient temperatures (3).

Table 1. DNA polymerase comparison: KOD HiFi, *Pfu*, and *Taq*

Enzyme	KOD HiFi DNA Polymerase	*Pfu* DNA Polymerase	*Taq* DNA Polymerase
Species	*Thermococcus kodakaraensis*	*Pyrococcus furiosus*	*Thermus aquaticus YT-1*
Fidelity† (mutation frequency)	0.0035	0.0039	0.013
Elongation rate (bases/second)	106–138	25	61
Processivity (nucleotide bases)	> 300	< 20	not determined

† Fidelity was measured as mutation frequency in PCR products using a sensitive blue/white phenotypic assay with a 5.2 kbp *lacZ* plasmid as template (2).

Advantages of KOD Hot Start DNA Polymerase

KOD Hot Start DNA Polymerase combines the high fidelity, rapid extension speed, and outstanding processivity of KOD HiFi DNA Polymerase (Table 1) with the high specificity that results from antibody-mediated hot start technology.

Because most of the mispriming events that can occur throughout setup at ambient temperatures and during the initial increase in temperature are avoided, nonspecific amplification is reduced. Similarly, primer degradation due to exonuclease activity at the ambient setup temperature is effectively inhibited. These features offer a distinct advantage for robotic PCR applications in which preparations may be held at ambient temperatures for varying periods before the cycling process begins.

The 3′–5′ exonuclease-dependent proofreading activity of this DNA polymerase results in a lower PCR mutation frequency than any other commercially available DNA polymerase. Elongation rates and processivities that are, respectively, 5 times and 10 to 15 times higher than *Pfu* DNA polymerase, result in a robust yield of a highly accurate product in a short reaction time.

KOD Hot Start amplifies problematic GC-rich sequences, genomic DNA templates up to 12 kbp long, and plasmid and λ DNA templates up to 21 kbp long. It produces blunt-ended PCR fragments suitable for cloning with Novagen's Perfectly Blunt® Cloning Kits or other blunt-end cloning methods.

PCR Yield

We compared the DNA yield in PCR reactions performed with three DNA polymerases: KOD Hot Start, *PfuTurbo*, and *PfuUltra* high-fidelity. PCR reactions using 100 ng human genomic DNA templates were performed according to the following protocol: initial denaturation at 95°C for 2 minutes, 35 amplification cycles (30 seconds at 95°C, 30 seconds at 60°C, 2 minutes at 72°C), and final extension at 72°C for 2 minutes. (Note: 72°C is an optimal extension temperature for both *Pfu* enzymes but is suboptimal for KOD Hot Start genomic DNA amplification.) The primers were designed to amplify DNA fragments of 0.6, 0.8, 1.3, and 2.0 kbp. As shown in Figure 1, the reaction performed with KOD Hot Start DNA Polymerase resulted in the highest yield of all four target PCR fragments. Both *PfuTurbo* and *PfuUltra* high-fidelity DNA polymerases generated lower yields of products under the same conditions.

PCR Fidelity

The fidelity of replication was measured as the mutation frequency in PCR products using a modified *rpsL*⁺ fidelity

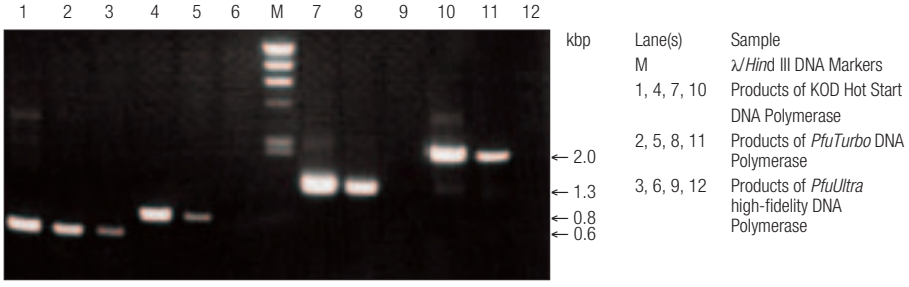

Figure 1. Performance of KOD Hot Start, *PfuTurbo*, and *PfuUltra* high-fidelity DNA polymerases in amplification of human genomic DNA

The indicated DNA fragments were amplified using sets of specific primers and 100 ng human genomic DNA. Reactions contained 0.3 µM each primer, the appropriate PCR buffer, 0.2 mM each dNTP, and 1 mM $MgSO_4$ (KOD Hot Start reactions) or approximately 2 mM $MgCl_2$ (*PfuTurbo* and *PfuUltra* reactions, included in 1X PCR buffer). Cycling parameters are described in the text. Samples (equal volumes) were analyzed by agarose gel electrophoresis (1.2% TAE) and stained with ethidium bromide.

* Manufactured by Toyobo and distributed by EMD Biosciences Inc. Novagen Brand. Not available through Novagen in Japan.

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Table 2: Mutation frequency comparison: KOD Hot Start, *PfuTurbo*, *PfuUltra*, and *Taq*

DNA Polymerase	Number of Colonies		Percentage of Mutants
	Total	Mutant	Mutation Frequency (%)
KOD Hot Start	51200	51	
PfuUltra	49900	53	
PfuTurbo	65900	164	
Taq	7000	354	
Mutation Frequency = (Number of mutant colonies/Number of total colonies) × 100%			

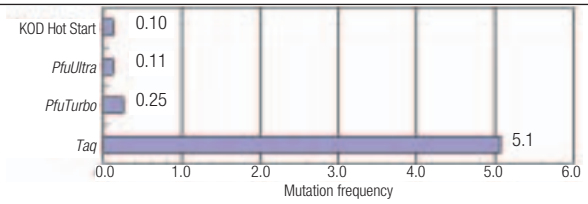

Table 3. PCR cycling parameters

		DNA Polymerase			
		PfuTurbo	***PfuUltra* high-fidelity**	**KOD Hot Start**	
PCR Segment	**Number of cycles**	genomic, λ, or plasmid DNA	genomic, λ, or plasmid DNA	λ or plasmid DNA	genomic DNA
Denaturation	1	95°C, 2 min	95°C, 2 min	94°C, 2 min	94°C, 2 min
Amplification	25–40	95°C, 30 s Primer (T_m – 5)°C, 30 s 72°C, 1 min per kbp	95°C, 30 s Primer (T_m – 5)°C, 30 s 72°C, 1 min (targets < 10 kbp) or 68°C, 2 min per kbp (targets > 10 kbp)	94°C, 15 s Primer [T_m – (5–10)]°C, 30 s 72°C, 20 s per kbp[1] (targets < 21 kbp)	94°C, 15 s Primer [T_m – (5–10)]°C, 30 s 68°C, 30 s per kbp[1] (targets < 12 kbp)
Final extension	1	72°C, 10 min	72°C, 10 min	Omit	Omit

1 Length of the extension step is 10 s per kbp for KOD HiFi. Set up PCR reactions on ice (Novagen TB 320, available at www.novagen.com).

Table 4. Typical PCR reaction setup

Component[1]	DNA Polymerase		
	PfuTurbo	***PfuUltra* high-fidelity**	**KOD Hot Start**
Specific 10X PCR buffer	5 µl	5 µl	5 µl
dNTPs	0.2 mM each	0.2 mM each	0.2 mM each
$MgSO_4$	none	none	1 mM
$MgCl_2$	about 2 mM[2]	about 2 mM[2]	none
5' primer	0.2–0.5 µM	0.2–0.5 µM	0.3 µM
3' primer	0.2–0.5 µM	0.2–0.5 µM	0.3 µM
Template DNA			
Reverse transcriptase reaction mixture	n/a[3]	n/a[3]	2 µl
Genomic DNA	50–100 ng	50–100 ng	200 ng
λ DNA/plasmid DNA	10 pg–100 ng	0.1–30 ng	1–50 ng
DNA polymerase	2.5 U	2.5 U	1 U
PCR grade water	X µl	X µl	X µl
Total volume	50 µl	50 µl	50 µl

1 Final reaction concentrations of the components shown are for typical PCR reactions with the indicated DNA polymerases.
2 $MgCl_2$ is included as a component of the 10X PCR buffer for *PfuTurbo* and *PfuUltra* high-fidelity DNA polymerases.
3 Data not available.

assay (4, 5). The 4-kbp pMOL21 plasmid, which includes the *bla* gene for ampicillin resistance and the *rpsL*$^+$ gene for the streptomycin-sensitive phenotype, was used as a template. This study compared mutant-colony production from different DNA polymerases: KOD1 (KOD Hot Start), *Pfu* (*PfuTurbo*® and *PfuUltra*™), and *Taq*.

The *rpsL* gene of *E. coli* encodes ribosomal protein S12, which binds streptomycin and causes a streptomycin-sensitive phenotype. Mutations in this gene arising from errors during plasmid amplification can result in proteins incapable of binding streptomycin, which leads to streptomycin resistance. Thus, following transformation, the mutation rate can be measured by comparing the number of colonies that grow on amp plus strep plates with the number that grow on amp plates. The number of mutant colonies resulting from each of the four DNA polymerases examined (Table 2) demonstrates comparable mutation frequencies between KOD Hot Start and *PfuUltra*, two-fold higher mutation frequency using *PfuTurbo*, and 500-fold greater mutation frequency using *Taq*.

Summary

We have demonstrated that KOD Hot Start DNA Polymerase is a superior proofreading DNA polymerase capable of generating high yields of PCR amplicons and exhibiting very high fidelity, comparable to that of *PfuUltra* DNA Polymerase. By using KOD Hot Start DNA Polymerase in place of *Pfu* or *Taq* DNA polymerase in a standard PCR protocol, better results can be achieved in shorter times (Tables 3 and 4).

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Rapid microbial gene detection and amplification techniques using colony-direct PCR

Naofumi Tsuchizaki[1] and Kunimoto Hotta[2] – [1]Technology Unit, Japan Microbiological Clinic, Ltd., Atsugi, Japan and [2]Department of Bioactive Molecules, National Infectious Disease Research Institute, Tokyo, Japan

We demonstrate a method for rapid amplification of DNA directly from microbial colonies. KOD Hot Start DNA Polymerase permits multiplex PCR using primers for multiple targets. A critical factor for successful colony-direct PCR is limiting the amount of bacteria in the template.

PCR has become an effective means for detecting infectious disease organisms, especially bacteria, fungi, and viruses that are difficult to handle or impossible to culture. PCR can also detect variations and mutations in genes. In some cases, PCR techniques offer a viable option for direct analysis of materials as opposed to indirect techniques based on antibody detection or differential culture.

In colony-direct PCR (CD-PCR), the polymerase chain reaction is performed by adding a template of intact bacteria directly from a colony into the reaction mixture. CD-PCR has been used successfully as a detection method for Gram-negative (Gram–) bacterial strains such as *E. coli*. Because reproducibility has been an issue with *Staphylococcus aureus* and other Gram-positive (Gram+) bacteria, CD-PCR had been considered inadequate for this type of detection analysis (1).

Here we present CD-PCR as a practical method for quick analysis of multiple antibiotic resistance in methicillin-resistant *S. aureus* (MRSA) and for detection of other Gram+ and Gram– bacterial strains. (For a description of multiplex CD-PCR for identification of Shiga toxin–producing *E. coli*, see page 11.) Severely limiting the amount of bacteria added to the reaction mixture and use of KOD Hot Start DNA Polymerase* are key factors for successful and reproducible CD-PCR using MRSA or other Gram+ bacteria.

Detect MRSA with multiplex CD-PCR

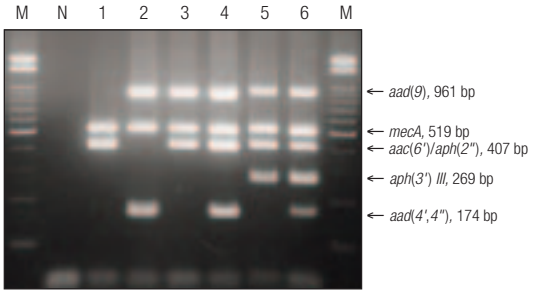
To perform multiplex CD-PCR, we identified five *S. aureus* drug-resistance genes as targets. Primers were designed to produce amplification products of a unique size for each target gene. KOD Hot Start DNA Polymerase was used in all PCR reactions. To collect a small amount of bacteria, the tip of a sterilized toothpick was lightly touched to the surface of the test colony grown on agar medium. No sample was visible on the toothpick tip, which was immersed into the PCR reaction mixture and quickly withdrawn; stirring was carefully avoided. PCR reactions were performed using the following cycling conditions: initial denaturation at 95°C for 3 minutes; 30 cycles at 95°C for 30 seconds, 50°C for 30 seconds, 68°C for 1 minute; and final extension at 68°C for 3 minutes.

Table 1. MRSA PCR reaction setup

Component	Concentration
PCR buffer for KOD Hot Start	1X
dNTP Mix	0.2 mM each
$MgSO_4$	1 mM
aac(6′)/aph(2″) primers	0.5 µM each
aad(9) primers	0.4 µM each
mecA primers	0.2 µM each
aph(3′) III primers	0.2 µM each
aad(4′, 4″) primers	0.2 µM each
KOD Hot Start DNA Polymerase	0.4 U

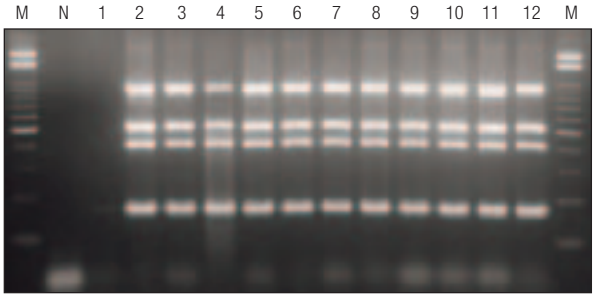
Figure 1 shows the results of the multiplex CD-PCR performed using the above primers for five target genes with a colony from a clinical MRSA isolate. Clear amplification was achieved for each of the target genes.

Next, contributing factors that could affect the CD-PCR were investigated. Results were not affected by the type of culture medium or by extended post-cultivation colony storage at 4°C (for up to six months, data not shown); the only observable effect was attributed to the amount of bacteria added (Figure 2). To study the effect of the amount of bacteria used for the PCR template, a dilution series was made. Each of 12 sample tubes was



Lane	Sample
M	Marker (100-bp ladder)
N	Negative control (without added bacteria)
1	*mecA, aac(6′)/aph(2″)*
2	*aad(9), mecA, aad(4′,4″)*
3	*aad(9), mecA, aac(6′)/aph(2″)*
4	*aad(9), mecA, aac(6′)/aph(2″), aad(4′,4″)*
5	*aad(9), mecA, aac(6′)/aph(2″), aph(3′) III*
6	*aad(9), mecA, aac(6′)/aph(2″), aph(3′) III, aad(4′,4″)*

Figure 1. MRSA drug-resistant DNA detection using multiplex CD-PCR



Lane(s)	Sample
M	Marker (100-bp ladder)
N	Negative control (without added bacteria)
1	Visible amount of bacteria on toothpick
2–12	Successive light touches with same toothpick

Figure 2. Amplification using visible versus minute quantities of MRSA

* Manufactured by Toyobo and distributed by EMD Biosciences Inc. Novagen Brand. Not available through Novagen in Japan.

continued on page 10

continued from page 9

Table 2. Strains used for CD-PCR

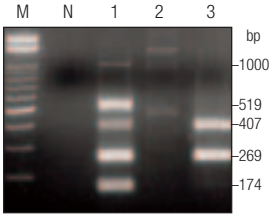
Organisms	Target Genes	PCR Products	Primers
E. faecalis	*aad(9)*	961 bp	
E. faecium	*mecA*	519 bp	
S. aureus	*aac(6′)/aph(2″)*	407 bp	multiplex
S. aureus	*aph(3′) III*	269 bp	
S. aureus	*aad(4′,4″)*	174 bp	
S. griseus	*aac(3)*	930 bp	
S. kasugaensis	*aac(2″)*	810 bp	multiplex
Streptomyces strain 8	*aac(6′)*	622 bp	
L. pneumophila	*mip*	168 bp	multiplex
L. pneumophila	5S rDNA	108 bp	
S. marcescens	*aac(6′)-Ib*	395 bp	
C. albicans	SAP6	265 bp	

Table 3. PCR conditions for other strains

Organisms	PCR*	Primer		Product
	Anneal	size (mer)	%GG	%GC
MSRA-Ent.	50°C	19–20	33–52	29–47
Actinomycetes	65°C	20	55–60	67–73
Serratia	55°C	17, 19	59, 37	54
Legionella	60°C	18–24	38–56	42–49
Candida	58°C	10, 22	50, 36	39

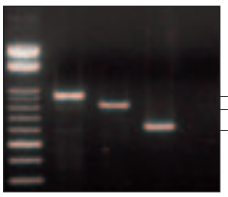
* PCR Conditions: KOD Hot Start DNA Polymerase; extension at 68°C; denaturation at 95°C (except for *Actinomycetes*, at 98°C)

A. *Enterococci*



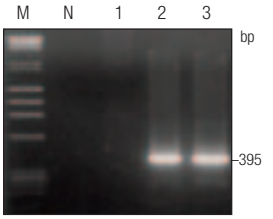
Lane	Sample
M	Marker (100-bp ladder)
N	Negative control (without added bacteria)
1	*S. aureus*
2	*E. faecium* BG-0019
3	*E. faecalis* BG-0009

B. *Actinomycetes*



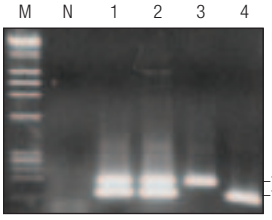
Lane	Sample
M	Marker (100-bp ladder)
1	*S. griseus* SS-1198PR
2	*S. kasugaensis* MB273
3	*Streptomyces* strain 8
N	Negative control (without added bacteria)

C. *Serratia*



Lane	Sample
M	Marker (100-bp ladder)
N	Negative control (without added bacteria)
1	*S. aureus*
2	*S. marcescens* MT1M2 (colony)
3	*S. marcescens* MT1M2 (total DNA)

D. *Legionella*



Lane	Sample
M	Marker (100-bp ladder)
N	Negative control (without added bacteria)
1	*L. pneumophila* ATCC33152 (multiplex)
2	*L. pneumophila* ATCC33215 (multiplex)
3	ATCC33215 *mip* only
4	ATCC33215 rDNA only

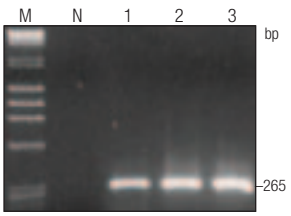
Figure 3. CD-PCR for *Enterococci, Actinomycetes, Serratia*, and *Legionella*

filled with reaction mixture, then a sterilized toothpick with a visible amount of bacteria on its tip was rinsed off into the first sample tube (Figure 2, lane 1, page 9). For samples 2–12 (Figure 2, lanes 2–12, page 9), this same toothpick was lightly touched to the solution in each successive tube. The inhibition of PCR amplification (Figure 2, lane 1, page 9) indicated that the addition of a visible amount of bacteria comparable to the amount of bacteria necessary for successful CD-PCR from *E. coli* did not produce favorable CD-PCR results with MRSA. Conversely, the positive results observed for less-concentrated samples confirmed that stable PCR amplification was possible when extremely small amounts of bacteria are used.

The amount of DNA template needed to yield PCR product sufficient for visualization through agarose gel analysis after a 30-cycle PCR reaction (approximately 10 ng) was calculated as several hundred femtograms. To meet this condition, theoretically between 10^2 and 10^4 colony-forming units (cfu) per 20-µl reaction volume were required. The number of cells added to the PCR reaction is a key factor in achieving successful CD-PCR for MRSA (1). Furthermore, using KOD Hot Start DNA Polymerase is important for CD-PCR with reproducible results; this same order of consistency was not possible with *Taq* DNA polymerase (1).

CD-PCR with other bacteria and with fungi

Aside from MRSA, we also confirmed positive results using CD-PCR for Gram+ and Gram– bacteria and for fungi. Gram+ bacteria used were *Enterococcus faecalis*, *E. faecium*, *S. aureus* (three strains), *Streptomyces griseus*, *S. kasugaensis*, and a rare *Streptomyces* strain (strain 8).



Lane	Sample
M	Marker (100-bp ladder)
N	Negative control (without added bacteria)
1	Visible amount of cells
2	Relatively smaller amount of cells
3	Microscopic amount of cells

Figure 4. CD-PCR for *Candida*

Gram– bacteria used were two strains of clinically-derived *Legionella pneumophila* and one of *Serratia marcescens*. For fungi (yeast), one strain of *Candida albicans* was used (Table 2).

As shown in Table 2, primer pairs designed for the detection of multiple target genes were used in the multiplex CD-PCR with *E. faecalis*, *Streptomyces*, and *Legionella*. PCR cycling conditions are described in Table 3. Because the DNA in *Streptomyces* has a high GC content (roughly 70%), the denaturation temperature for *Actinomycetes* was raised to 98°C.

As shown in Figure 3, the targeted genes for each organism were amplified using CD-PCR. In each case, as with *S. aureus*, a favorable amplification was obtained by adding a microscopic amount of sample to the reaction. Adding a macroscopic (visible) amount of cells inhibited amplification in each case except *Candida* (Figure 4).

When a multiplex CD-PCR was performed (*E. faecalis*, *Streptomyces* and *Legionella*), a known gene profile and corresponding gene amplification pattern were confirmed, verifying that these PCR conditions offered high selectivity. The data from the target gene of *E. faecalis*, also common in *S. aureus*, further corroborated these results (data not shown).

For PCR using *Candida*, DNA obtained from a zymolase-treated (5 min at 37°C) colony can be used as a template (2). As described here, sufficient DNA amplification is possible by the CD-PCR method. In contrast to CD-PCR from bacterial strains, using a visible amount of yeast did not adversely affect amplification.

Summary

In many procedures for colony PCR, template DNA is extracted by incubation of a bacterial colony in water for 3 minutes at 95°C. When colony-direct PCR (especially multiplex CD-PCR) is used, this

extraction step is no longer necessary, and the detection of multiple strains of bacteria becomes more efficient. A common concern with CD-PCR has been reproducibility. In these studies, KOD Hot Start DNA Polymerase and microscopic amounts of cells generated reproducible colony-direct PCR results.

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

[includes KOD Hot Start DNA Polymerase (1.0 U/µl), 10X PCR Buffer for KOD Hot Start DNA Polymerase, 25 mM $MgSO_4$, and dNTP Mix (2 mM each)]

Detection of Shiga toxin-producing *E. coli* using multiplex colony-direct PCR with KOD XL DNA Polymerase

Okitsu, T., Suzuki, R., Yamai, H. — Bacterial Pathology Department, Kanagawa Prefecture Health Research Institute, Japan

Certain strains of *E. coli* are known to produce a family of related toxins, referred to as Shiga toxin 1 (Stx1, encoded by *stx1*) and Shiga toxin 2 (Stx2, encoded by *stx2*). Shiga toxin-producing *E. coli* (STEC), represented by serotype O157:H7, has a strong infectious capacity and pathogenicity. In recent years, this bacterium has been affecting an increasing number of victims, resulting in life-threatening illness such as hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura (1). The morbidity and mortality associated with STEC disease highlight the threat these organisms pose to public health. For this reason, there is an increasing demand for fast and efficient methods for the detection of virulent strains of STEC in fecal samples and in meat and dairy products.

Table 1. STEC PCR reaction setup[1]

Component	Concentration
PCR buffer for KOD XL	1X
dNTP mix	0.2 mM
stx1 primers (*stx1*-F + *stx1*-R)	0.2 µM each
stx2 primers (*stx2*-F + *stx2*-R)	0.2 µM each
eaeA primers (*eaeA*-F + *eaeA*-R)	0.2 µM each
hlyA primers (*hlyA*-F + *hlyA*-R)	0.2 µM each
IS1203v primers (1203v-F + 1203v-R)	0.1 µM each
Bacterial colony	approximately 10^4 cfu
KOD XL DNA Polymerase	2.5 U

[1] When using KOD XL DNA Polymerase, set up the PCR reaction on ice.

PCR is generally considered the most sensitive means for determining if a food or fecal sample contains STEC. A multiplex PCR method developed by Paton and Paton (2) enables simultaneous determination of *stx1*, *stx2*, and correlated genes that encode accessory STEC virulence factors, such as *eaeA* and *hlyA*, in crude DNA extracts from primary fecal cultures.

In this study, we developed a rapid typing system for STEC that improves upon the original multiplex PCR assay. With our method, a bacterial colony from a food or fecal culture was used directly as the template. In addition, four target genes were examined for the presence of the IS1203v insertion sequence discovered in *stx2* genes (3, 4) with IS1203v-specific primers. To reduce the time needed for the PCR, *Taq* DNA polymerase was replaced with the faster KOD XL DNA Polymerase*. Multiplex CD-PCR analysis for eleven STEC strains and one control K-12 strain isolated at Kanagawa Prefecture, Japan, between 1996 and 1999 was performed (Figure 1). Table 1 identifies the final concentrations of the PCR reaction components. PCR reactions were performed using the following conditions: initial denaturation at 94° for 5 minutes, 30 cycles of 98° for 15 seconds, 60° for 5 seconds, and 74° for 30 seconds. After the PCR, one-tenth of the reaction solution was analyzed by agarose gel electrophoresis (2% TAE gel, Figure 1). The results of the multiplex-PCR showed a clear amplification for each target: 180 bp for *stx1*, 255 bp for *stx2*, 384 bp for *eaeA*, 534 bp for *hlyA*, and 910 bp for IS1203v, in 11 STEC strains (Figure 1). Amplification of the target genes from the control K-12 strain was negative. The results clearly demonstrated that this system is effective for STEC typing.

By using KOD XL DNA Polymerase, PCR was completed in less than 1.5 hours, a significant time savings compared to the nearly 3.5 hours of the original method, and a great advantage when multiple specimens require quick processing.

Lane(s)	Sample
M	Markers (100-bp ladder)
1–11	STEC strains
12	K-12 strain

Figure 1. STEC identification by CD-PCR

Eleven STEC strains and one K-12 strain were used for CD-PCR. Reaction products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

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4. Okitsu, T., Suzuki, R, and Yamai, S. (2001) *Upload* **63** (Toyobo Co., Ltd., Japan newsletter).

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

[includes KOD XL DNA Polymerase (2.5 U/µl), 10X PCR Buffer for KOD XL DNA Polymerase, dNTP Mix (2 mM each)]

* Manufactured by Toyobo and distributed by EMD Biosciences Inc. Novagen Brand. Not available through Novagen in Japan.

Efficient production of recombinant plant chitinase with Novagen's *E. coli* Origami™(DE3) strain

Takayuki Ohnuma[1], Mikado Yagi[2], Toki Taira[2], Takeshi Yamagami[2], and Masatsune Ishiguro[2] — [1]University of Illinois at Urbana-Champaign, USA and [2]Kyushu University, Japan

The bacterial production of an active plant chitinase is possible using a host strain that permits disulfide bond formation in the cytoplasm at a reduced temperature. The purified enzyme expressed in Origami™(DE3) exhibits similar activity and CD spectrum as the native protein.

Chitin, a β-1,4-linked polymer of *N*-acetyl-D-glucosamine, is one of the most abundant contributors to biomass in nature and an important structural component of many organisms, comprising the cuticles of insects, shells of crustaceans, and cell walls of many fungi. Chitinases (EC 3.2.1.14), known to degrade internal glycosidic linkages of chitin, are widely distributed in a variety of organisms, including higher plant species. Plant chitinases have been postulated to safeguard plants against fungal pathogens by degrading the chitin that constitutes the cell walls of pathogenic fungi. Experimental evidence from both *in vitro* and *in vivo* studies supports the theory that these enzymes play an important role in plant defense. Because of their high antifungal activity, plant chitinases have been receiving attention from plant biotechnologists.

Yamagami and others have reported the purification, characterization, and amino acid sequence of basic chitinase (RSC-c) from rye (*Secale cereale*) seeds. The RSC-c molecule consists of 243 amino acid residues and three disulfide bonds (C23/C85, C97/C105, and C223/C236) (1). To understand the structure/function relationship for RSC-c, we cloned a cDNA encoding RSC-c from rye seeds and attempted to develop a bacterial production system for functional recombinant RSC-c using Novagen's *E. coli* strains BL21(DE3) and Origami(DE3).

Here, we report the construction of an efficient production system for functional rRSC-c using *E. coli* Origami(DE3).

Results and Discussion

To understand the structure/function relationship for the desired protein, a relatively large amount of protein should be available for detailed biochemical and structural analysis. On first attempt to produce the desired recombinant protein, *E. coli* cells often are used as host. However, many eukaryotic proteins fail to fold into their native state if they are produced in *E. coli* cells and, instead, form insoluble aggregates (inclusion bodies) that are not useful for structural studies. Thus, large-scale production of the desired protein in soluble form in *E. coli* cells is the first step for the study of their structure.

The cDNA that encodes mature RSC-c was amplified by PCR and ligated into the expression vector pET-22b(+) (*Nde* I and *Bam*H I sites; note that this cloning strategy removes the signal peptide coding sequence). The resulting plasmid, pET-22b/RSC-c, was introduced into *E. coli* BL21(DE3) and Origami(DE3). When *E. coli* BL21(DE3) was used as a host for production of rRSC-c under the standard induction condition (37°C for 4 h), the expressed protein was produced completely as an insoluble aggregate (Figure 1, panel A). Because RSC-c has three disulfide bonds in the molecule, rRSC-c may form insoluble aggregates due to insufficient disulfide bond formation in the cytoplasm of the BL21(DE3) host. Therefore, *E. coli* Origami(DE3) was tested as an alternative host for production of rRSC-c. Mutations in the thioredoxin reductase and glutathione reductase genes of *E. coli* greatly enhance the formation of disulfide bonds in proteins produced in the cytoplasm (2).

Under the standard induction condition with the Origami(DE3) host, only a very small increase in the solubility of rRSC-c was observed (Figure 1, panel C). However, the yield of soluble rRSC-c increased dramatically when induction of expression was conducted at a lower temperature and for a longer time (20°C for 24 h) (Figure 1, panel D). When BL21(DE3) carrying pET-22b/RSC-c was induced under this condition, the increase was not seen (Figure 1, panel B).

The Origami(DE3) isolate was grown and induced under the modified conditions, and rRSC-c was purified from the soluble fraction in one step using S-Sepharose column chromatography. Purified rRSC-c exhibited chitinase activity similar to that of the native enzyme (Table 1), inhibited the growth of *Trichoderma* sp. *in vitro*, and reacted with anti-RSC-c antiserum (data not shown). In addition, the short-wavelength CD spectra of the native and recombinant enzymes

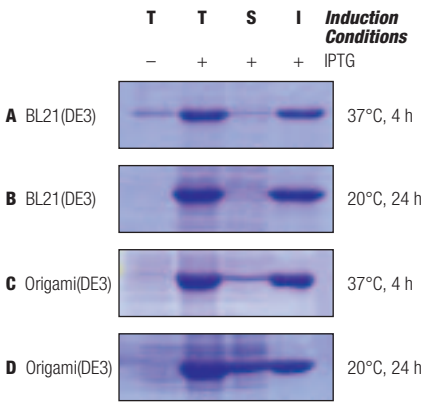

Figure 1. Expression of rRSC-c from a pET-22b construct under different conditions

BL21(DE3) and Origami(DE3) hosts carrying a pET-22b/RSC-c were grown to OD_{600} 0.6–1.0 before induction with 1 mM IPTG. After addition of IPTG to the cultures, growth was continued either for 4 h at 37°C or for 24 h at 20°C. Samples of the total (T), soluble (S), and insoluble (I) protein fractions from cells containing a pET-22b/RSC-c were prepared and analyzed on 15% SDS-PAGE followed by staining with Coomassie blue.

Table 1. Chitinase activity of the original and recombinant chitinases toward the polymer substrate, glycolchitin

Enzyme	Specific activity (U/mg)
RSC-c	205.0
rRSC-c	233.7

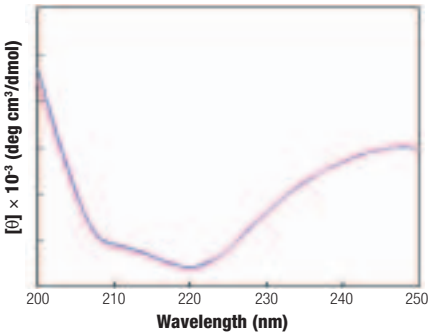

Figure 2. CD spectra of RSC-c and rRSC-c

The spectra in the far-UV range, 200–250 nm, were recorded at room temperature on a Jasco J-720 spectropolarimeter. Proteins were dissolved to a final concentration of 100 µg/ml in distilled water. Signal averaging during accumulation of 4 scans was performed automatically. Blue line, RSC-c; Pink line, rRSC-c.

were virtually identical (Figure 2). These results suggest that the soluble rRSC-c obtained from the Origami™(DE3) host was similar in structure and function to the native enzyme. The yield of rRSC-c was approximately 20 mg/liter of induced culture. Subsequently, we successfully constructed a number of rRSC-c mutants for further characterization using the same expression and purification conditions (3).

Overall, our studies indicate that *E. coli* Origami(DE3) is a useful system for the production of relatively high levels of rRSC-c, which are sufficient for structural analysis using site-directed mutagenesis and crystallization techniques.

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Product	Size	Cat. No.
Origami™(DE3) Singles™ Competent Cells guaranteed efficiency > 2 × 10⁶ cfu/µg	11 rxn 22 rxn	70630-3 70630-4
Origami(DE3)pLysS Singles Competent Cells guaranteed efficiency > 2 × 10⁶ cfu/µg	11 rxn 22 rxn	70631-3 70631-4

Protein delivery into mammalian cells using ProteoJuice™ Protein Transfection Reagent

Scott Hayes – Novagen

Novagen's new transfection reagent permits the rapid delivery of intact active proteins into a variety of mammalian cell lines. Data are presented to demonstrate the activity and subcellular localization of proteins transported with ProteoJuice™ Protein Transfection Reagent.

Introduction of a target protein into cells permits functional analysis in the living cellular environment. The most rapid method for protein introduction is to transfect a specific protein directly into cells. An effective protein delivery system should work with a wide variety of cells and maintain both cell viability and protein functionality. Here, we demonstrate these and other characteristics of ProteoJuice Protein Transfection Reagent that make it an excellent choice for protein transfections.

Standard plasmid transfections offer the ability to introduce a DNA sequence into a cell, observe the effects of encoded protein and reporter expression, and test putative promoters and enhancers (1, 2). The amount of protein expressed in a transfected cell depends on a variety of factors, including the cell type, transfection efficiency, and strength of the relevant promoter in the cells. Typically, expression is monitored for 24 to 72 hours after transfection with plasmid DNA. While this method has proven useful for many proteins and cell types, it is not always successful. Some cells are transfected poorly or not at all by plasmid DNA, and, for various reasons, some proteins are not suited to study by means of plasmid introduction.

Another method for determining the function of a protein within a cell is to perform gene silencing using small interfering RNA (siRNA) and observe the resultant changes. For siRNA transfections, the principal challenge has been the design of an appropriate RNA sequence for maximum suppression of expression in the cell. After the siRNA molecule is transfected into the cell, it mediates cleavage of the target mRNA (3). Gene expression is usually monitored for 24 to 72 hours after siRNA transfection. The applications of this exciting new technology are currently under investigation by many laboratories.

To complement our GeneJuice® Transfection Reagent (for DNA) and RiboJuice™ siRNA Transfection Reagent (for siRNA), we introduce ProteoJuice Protein Transfection Reagent for the transfection of intact functional protein into mammalian cells. The chemistry of the ProteoJuice method differs substantially from protein delivery systems based on lipids or mediated by protein transduction domains (PTDs). In contrast, ProteoJuice forms a non-covalent complex with the protein that protects against degradation by endosomal proteases to ensure delivery of intact protein inside the cell.

Unlike microinjection or electroporation techniques, ProteoJuice reagent is easy to use, maintains cell viability, and enables the detection of the target protein within 3 to 5 hours. The ProteoJuice reagent has successfully delivered protein to A549, BHK-21, CHO-K1, COS-7, CV-1,

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HEK 293, HeLa, Hep G2, L6, MCF-7, Neuro2A, NIH 3T3, PC12, and Raw 264.7 cell lines. Proteins used successfully with ProteoJuice reagent range in size from 11 kDa to approximately 465 kDa (the β-galactosidase tetramer).

Delivery, localization, and functionality of transfected proteins

In the figures on this and the following pages, we demonstrate proper subcellular localization and function of a variety of proteins delivered to several cell lines with ProteoJuice™ Protein Transfection Reagent. In the general protocol, cells were plated at a density of 5×10^4 cells/well in a 4-well chambered coverslip one day before protein transfection. When ProteoJuice was used, transfection mixtures consisted of 1.25 µl ProteoJuice reagent, the protein of interest, and serum-free medium in a total volume of 25 µl. Protein complex formation was allowed to proceed for 25 minutes at room temperature, after which an additional 225 µl serum-free medium was added. During complex formation, culture medium was removed from the chamber slide and the slide was washed three times with 500 µl serum-free medium. Following complex formation, all medium was aspirated from the chamber slide and the entire 250 µl protein/ProteoJuice mixture was added directly to the cells. The cells were incubated with the mixture for 3.5 to 5 hours (37°C, 5% CO_2). The mixture was then removed with a pipet, cells were washed four times with 500 µl serum-free medium, and transfected protein was detected by appropriate assay or microscopic examination.

As a rapid test for delivery into cells and retention of functionality of the protein, green fluorescent protein (GFP) was complexed with ProteoJuice and transfected into monolayers of L6 and Neuro2A cells. After 3.5 hours, cells were washed and observed. The fluorescent signal arising from the intact chromophore was distributed throughout the cytoplasm of both L6 cells and Neuro2A cells (Figure 1).

The delivery of a large protein was demonstrated by transfecting an FITC-conjugated goat anti-mouse antibody into L6 and Neuro2A cells. As demonstrated in panels A and B of Figure 2, fluorescence could be observed throughout the cytoplasm of recipient cells.

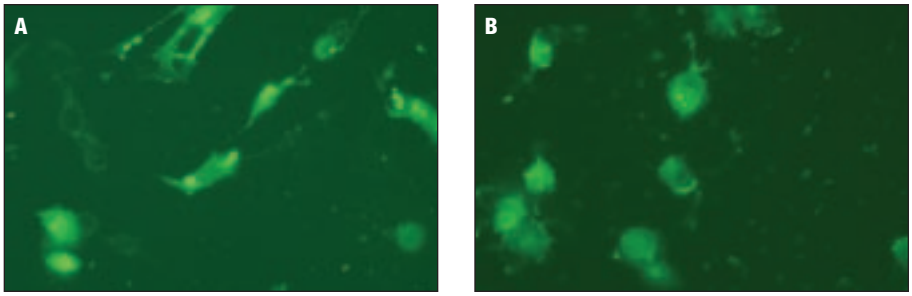

Figure 1. Green fluorescent protein (GFP) transfection into Neuro2A and L6 cells

Cells were transfected as described in the text using 0.625 µg GFP for 3.5 h. Images depict GFP fluorescence in L6 (panel A) and Neuro2A cells (panel B).

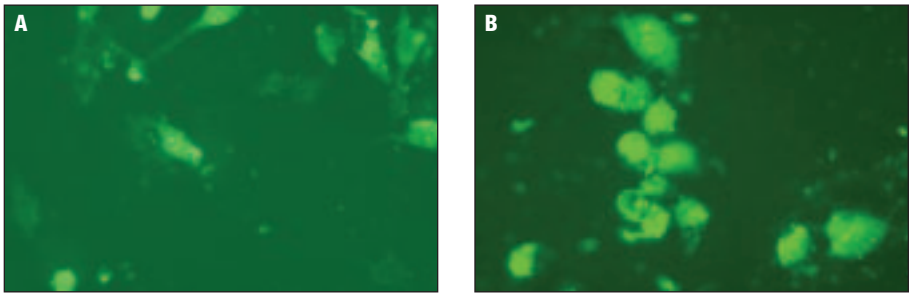

Figure 2. FITC-conjugated antibody transfection into Neuro2A and L6 cells

Cells were transfected as described in the text using FITC-conjugated antibody (0.625 µg per transfection) for 5 h. Images depict FITC-conjugated antibody fluorescence in L6 (panel A) and Neuro2A cells (panel B).

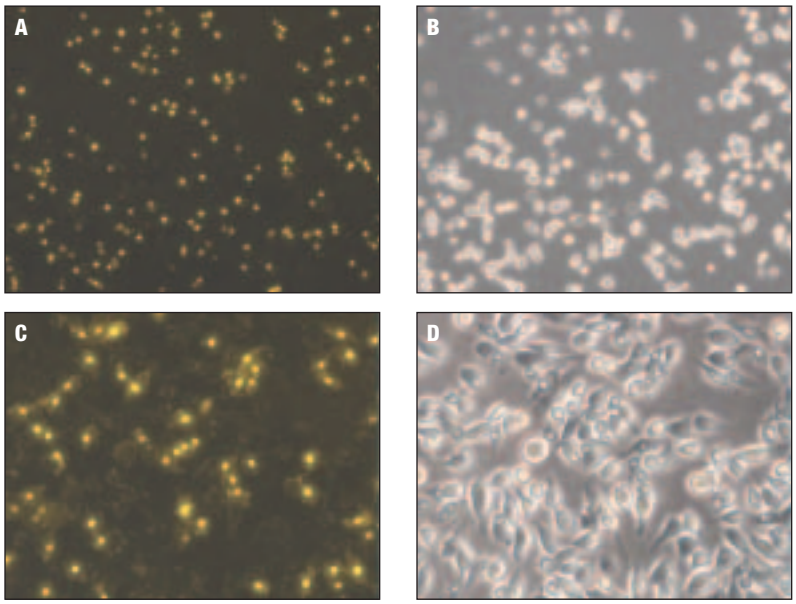

Figure 3. Transfected histone localization in PC12 and CHO-K1 cells

Cells were transfected as described in the text using 1.25 µg Cy™ 3-conjugated histone H2A for 3.5 h. Panels A and B: PC12 cells; panels C and D: CHO-K1 cells. Images depict fluorescent (panels A and C) and brightfield (panels B and D) views of the same cells.

In Figure 3, Cy™3 fluorescent dye (Amersham) was conjugated to purified histone H2A and transfected into PC12 and CHO-K1 cells using ProteoJuice reagent. Panels A (PC12 cells) and C (CHO-K1 cells) show delivery of fluorescent histone to the nuclei following transfection. Brightfield images of the same field of view are shown in panels B and D. ProteoJuice enabled protein delivery to the expected subcellular location in a rapid and efficient manner.

Though the localization of the alpha form of estrogen receptor (ERα) has been controversial, it is now generally accepted that endogenous ERα, as part of a multiprotein complex, is localized to the nucleus in the presence of estradiol (4, 5). To visualize the localization of purified recombinant estrogen receptor, transfections of FITC-labeled ERα were performed with HepG2 cells in the presence and absence of 17β-estradiol. Brightfield microscopy revealed all cells within the field of view (Figure 4, panels A and D) and diaminophenylindole (DAPI) staining was used to define the cell nucleus (Figure 4, panels C and F). In the absence of hormone, (Figure 4, panel B), the transfected estrogen receptor exhibited cytoplasmic localization. In contrast, in the presence of estradiol, the labeled receptor appeared to translocate and become concentrated in the nuclei of recipient cells (Figure 4, panel E). Because ProteoJuice™ reagent allows exogenous receptor to be placed directly into cells, its use makes it possible to study agonist or antagonist effects on cell-signaling events. ProteoJuice Protein Transfection Reagent can offer insight into protein localization and the mechanism of action for a receptor.

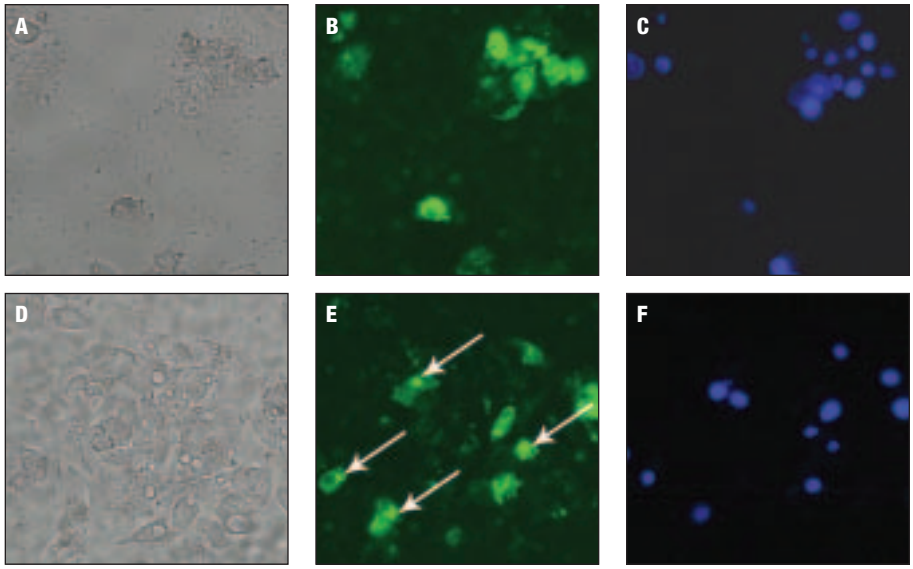

Figure 4. Transfected estrogen receptor localization in HepG2 cells

HepG2 cells were transfected with FITC-labeled estrogen receptor as described in the text. After incubation, washed cells were exposed to DAPI nuclear stain for 2 min, followed by four additional washes in PBS before microscopy. Panels A, B, and C: no estradiol treatment; panels D, E, and F: estradiol (Calbiochem, 17β-estradiol) treatment. Images depict brightfield (panels A and D), FITC fluorescence (panels B and E), and DAPI fluorescence (panels C and F) views, with the same cells in panels A, B and C and in panels D, E, and F. Arrows indicate estrogen receptor localization in nuclei.

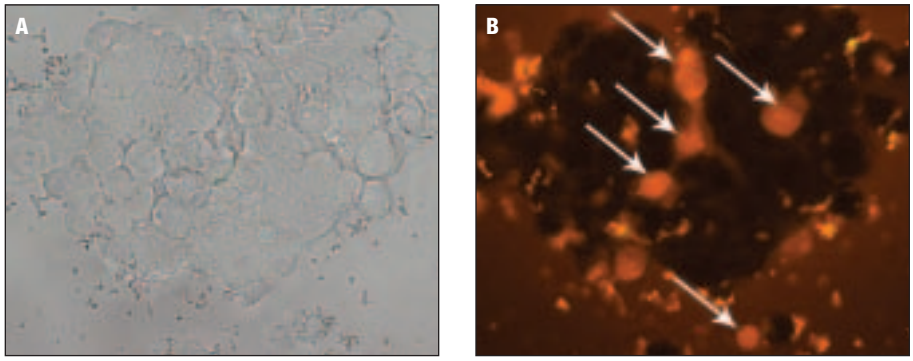

Figure 5. Transfected caspase-initiated apoptosis in MCF-7 cells

Cells were transfected as described in the text with the following modifications. Caspases (6.25 U each of Caspase-2, Caspase-3, and Caspase-7; Calbiochem, Caspase Enzymes Set, Group II) were transfected with 1.25 µg β-gal. Cells exposed to the protein/ProteoJuice mixture for 3.5 h were washed three times in serum-free medium. Medium was completely removed and 250 µl PhiPhiLux® G_2D_2 substrate solution (Calbiochem, Caspase-3 Intracellular Activity Assay Kit II) was added to the chamber. Cells were incubated for 45 min (37°C, 5% CO_2) and then washed four times with PBS before microscopy. Images depict brightfield (panel A) and fluorescence (panel B) views of the same cells. Arrows indicate apoptotic cells.

Handling difficult proteins and large molecules

For experiments that may be difficult to accomplish through standard plasmid transfection techniques, ProteoJuice reagent is an excellent alternative. For example, in studies of apoptosis, it is impractical to transfect cells with a mammalian expression plasmid encoding a caspase gene because of variability in peak expression times and expression levels of caspase cDNAs. Because of these variations in expression, cells tend to undergo apoptosis at widely variant times. When toxic proteins are delivered by plasmids, the maximal effects of protein expression normally seen between 24 and 72 hours may be masked by insufficient expression (cells incorporating small amount of plasmid) or cell death (in cells expressing a high level of toxic protein or incorporating large quantities of plasmid). In contrast, meaningful analysis of the effects of these toxic agents is possible within the narrower time frame needed for protein transfection. Here we demonstrate the direct transfection of caspases into human breast cancer cells and the resulting apoptotic response.

To visualize cells undergoing apoptosis, we used a FRET assay with a fluorescent-labeled peptide substrate susceptible to cleavage by caspase-3. In the presence of caspase activity, the quencher and fluorophore separate, unmasking the fluorescent signal and enabling the identification of apoptotic cells by fluorescent emission. An active mixture of caspase-2, caspase-3, and caspase-7 was introduced into the human breast cancer line MCF-7. Because only a small amount of the toxic protein could be used, a protein carrier, β-gal, was included. Cells were exposed to the caspase mixture for 3.5 hours followed by removal of the transfection mixture and washes. Figure 5 shows a brightfield image of the cells (panel A) and the caspase-mediated red fluorescence exhibited by apoptotic cells (arrows in panel B).

Comparison to lipid- and PTD-based transfection reagents

The efficacy of ProteoJuice was compared with two commercially available transfection reagents: a lipid formulation

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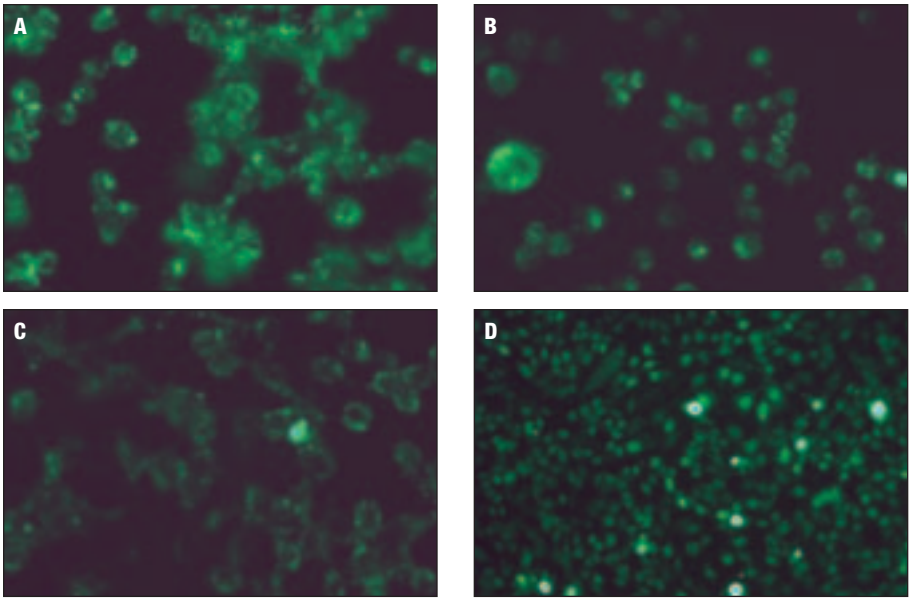

Figure 6. Histone transfection in CHO-K1 cells using commercially available reagents

Cells were transfected as described in the text. Histone H1 (1.25 µg) conjugated with Alexa Fluor 488 protein labeling kit was complexed with each of three protein transfection reagents according to the manufacturers' specifications. Nuclear staining indicates histone delivery. Panel A, no transfection reagent added; panel B, PTD-based transfection reagent; panel C, lipid-based transfection reagent; panel D, ProteoJuice Protein Transfection Reagent.

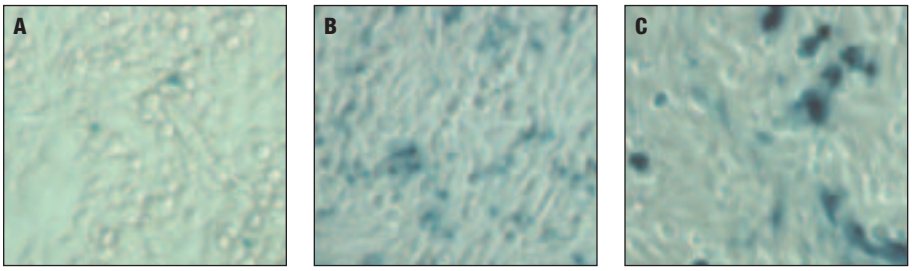

Figure 7. β-gal transfection in CHO-K1 cells using commercially available reagents

β-gal (1.25 µg) was complexed with each of three protein transfection reagents according to the manufacturers' specifications. After incubation (5 h), cells were rinsed four times with PBS, fixed (4% paraformaldehyde, 15 min), and stained with Novagen's BetaBlue™ Staining Kit. Staining indicates β-gal delivery. Panel A, PTD-based transfection reagent; panel B, lipid-based transfection reagent; panel C, ProteoJuice Protein Transfection Reagent.

and a PTD-delivery system. Histone labeled with Alexa Fluor® 488 Protein Labeling Kit (Molecular Probes) was used to test efficiency of protein transfer. As demonstrated in Figure 6, delivery of histone into the cells, and specifically into the nucleus, was not very efficient with either comparative reagent. After protein transfection, images from the other products (panels B and C) showed no significant difference in cell appearance from the negative control (panel A). The peripheral staining seen in these cells may be due to interaction of the basic histone with the plasma membrane. In contrast, protein delivery mediated by ProteoJuice™ reagent led to specific incorporation of histone into the nucleus without noticeable peripheral fluorescence (panel D).

We next assessed the capabilities of each reagent to transfer large proteins into cells. For this purpose, β-gal (M_r = 465 kDa) was complexed with each reagent and exposed to CHO-K1 cells. Color development with the Novagen BetaBlue™ Staining Kit revealed that the degree of protein transfer varied greatly among the three reagents. Cells shown in Figure 7, panel A, transfected with the PTD-based reagent, exhibited very little transfer of the protein into cells. Use of the lipid-based reagent increased β-gal staining, but the signal localized near the periphery of the cells (Figure 7, panel B), indicating inefficient protein delivery. In contrast, the cells transfected with ProteoJuice showed effective internalization of the β-gal protein and widespread distribution throughout the cytoplasm (Figure 7, panel C).

Summary

ProteoJuice Protein Transfection Reagent efficiently delivers a wide range of intact proteins to a variety of cell types in five hours or less. Our results have shown that functional transfected proteins became appropriately localized within cells and were delivered with greater efficacy than proteins from lipid- and PTD-protein delivery systems.

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Product	Size	Cat. No.
ProteoJuice™ Protein Transfection Reagent	0.125 ml	71281-3
	4 × 0.125 ml	71281-4
Available separately:		
Product	**Size**	**Cat. No.**
GeneJuice® Transfection Reagent	1 ml	70967-3
	10 × 1 ml	70967-4
	0.3 ml	70967-5
RiboJuice™ siRNA Transfection Reagent	0.3 ml	71115-3
	1 ml	71115-4
BetaBlue™ Staining Kit	100 ml	71074-3
Caspase Enzymes Set, Group II	1 set	218817
Caspase-3 Intracellular Activity Assay Kit II (PhiPhiLux® G_2D_2)	1 kit	235432
17β-Estradiol	1g	3301

Improved immunohistochemical staining of fixed tissues

Alla Subbotin and Mark A. Handley – Novagen

For histological studies, chemically fixed tissue sections provide a significant advantage because they can be collected when the biological material is available and saved for later analysis. The process of formalin or formaldehyde fixation is important for preserving the structural integrity of the tissue and protecting proteins and nucleic acids from degradation. However, during preservation and throughout subsequent storage, the proteins within the embedded tissues form methylene bridges between free amino groups. These cross-linked bridges can significantly mask certain epitopes such that subsequent antibody reactions can be weak or undetectable.

Because paraformaldehyde- or formalin-fixed tissues occasionally show low reactivity to antibodies used for immunohistochemistry (IHC), it is advantageous to identify a set of conditions under which the protein structure and reactivity to antibodies can be recovered. This process is called antigen retrieval (AR). Although the specific mechanism of action for AR is unclear, it is possible that some modification of the cross linkages (loosening or breakage) or renaturation of the protein epitopes occurs (1–3). The conditions for effective AR are usually determined somewhat empirically and vary depending on the tissue source and antigen-antibody combination.

The first described method of antigen retrieval was a simple high-temperature technique that involved boiling formalin-fixed, paraffin-embedded tissue sections in water. This method was developed for IHC of archival tissue sections and delivered an increased intensity of staining for a variety of antibodies tested (4). Since the first descriptions of antigen retrieval, the application of the technique for tissue IHC has become a routine procedure in many laboratories. Today, the majority of publications dealing with IHC for formalin-fixed, paraffin-embedded tissues adopt some variant of the AR technique as a necessary pretreatment to achieve optimal immunostaining.

Determining if antigen retrieval is necessary

Whenever a new antibody reagent for immunohistochemistry is characterized, it must first be established whether the antibody requires antigen retrieval. Second, the most favorable conditions for the antigen-antibody reaction must be determined. If the antigen must be retrieved (i.e., if signal for the desired IHC target is low), it is necessary to determine which procedure and retrieval solution combination will produce the best results. For example, although some antigens require heating in buffer at a specific pH to achieve the strongest staining intensity (5), antigen retrieval is also possible at physiological pH without the more traditional heating processes. This is a benefit for some of the more fragile tissues (e.g., Figure 2). Conditions for proper controls and optimal dilution of primary and secondary antibodies for each tissue will depend on the epitope retrieval procedure selected. After the optimal AR procedure

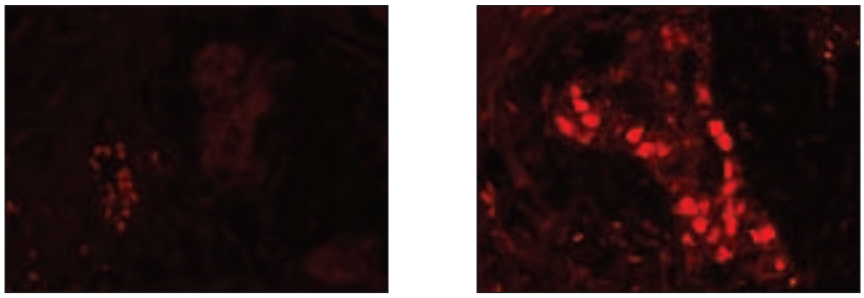

Figure 1. Human colon adenocarcinoma stained with anti-p53 antibody

Human colon adenocarcinoma sections (Novagen) were either untreated or heat treated using 5X AntigenPlus™ Buffer (pH 10), diluted 1:5, as described in the text. The processed slides were incubated for 18 h at 4°C with anti-p53 antibody [p53 (Ab-5) (Wild type) Monoclonal Antibody, Oncogene Research Products] diluted 1:20. Slides were washed three times with PBS and incubated for 1 h at room temperature with Goat anti-Mouse IgG, TEXAS RED® Conjugate (Oncogene Research Products) diluted 1:100 in dilution buffer. Slides were examined using an Axioplan® 2 fluorescent microscope (Carl Zeiss, Göttingen, Germany) at 200X magnification and photographed using a rhodamine/Texas Red filter.

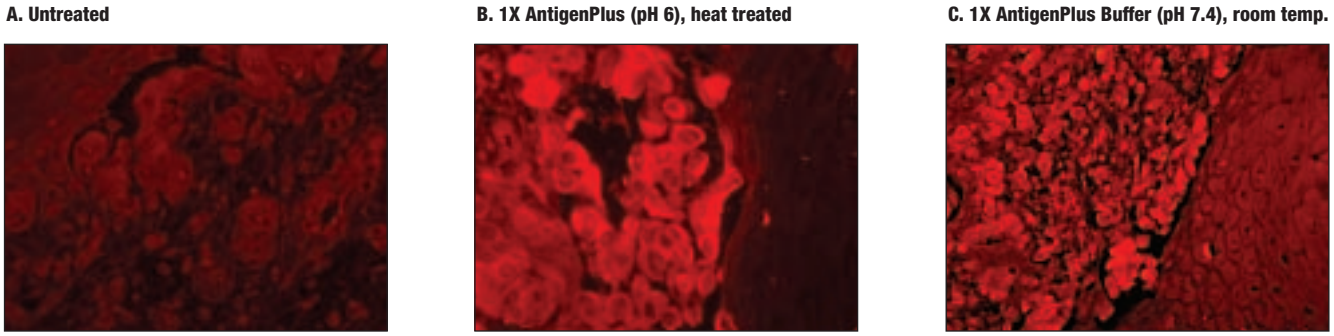

Figure 2. Human skin melanoma stained with anti-vimentin antibody

Human skin malignant melanoma sections (Novagen) were either untreated, heat treated in 5X AntigenPlus Buffer (pH 6), diluted 1:5, or treated at room temperature with 5X AntigenPlus Buffer (pH 7.4), diluted 1:5, as described in the text. Anti-vimentin antibody [Vimentin/LN6 (Ab-1) Monoclonal Antibody, Oncogene Research Products], diluted 1:100, was incubated with the processed slides for 18 h at 4°C. Slides were washed three times with PBS, and incubated for 1 h at room temperature with Goat anti-Mouse IgG, TEXAS RED Conjugate diluted 1:100 in dilution buffer. Slides were examined as described in Figure 1, except that panels A and B were examined at a higher magnification (630X).

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is determined, the antibody dilution can be fine-tuned.

Using 5X AntigenPlus™ Buffers to optimize antigen retrieval

For tissues and antibodies that require antigen retrieval, it is usually worthwhile to test several different buffers and reaction conditions to achieve optimal staining. Novagen is pleased to introduce the 5X AntigenPlus Buffers, designed for convenient optimization of antigen retrieval from fixed, paraffin-embedded tissues. As demonstrated in Figures 1–4, dramatic signal enhancement was achieved using various antibodies and tissues with 5X AntigenPlus Buffers at pH 6, 7.4, and 10.

The tissues shown were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned into 5-micron slices, and mounted on glass slides. Prior to IHC, each pair of slides was deparaffinized by soaking for a total of 20 minutes in xylene (2 × 10 min each) and then treated for a total of 4 minutes with 100% ethanol (2 × 2 min each). The slides were washed three times with water prior to AR treatment. One slide of each pair received no further treatment (each panel A in Figures 1 and 2, page 17, and Figures 3 and 4) and the other slide was treated with the indicated 5X AntigenPlus Buffer (diluted 1:5 to 1X working concentration with deionized distilled water) either with heat (immersed in 300 ml buffer and heated 3 × 5 min in a 600-W microwave) or at room temperature (5 min). Slides were then washed with PBS (3 × 5 min), treated with blocking buffer (1 h at room temperature), washed again with PBS (3 × 5 min), and then these processed slides were probed with the indicated antibodies. Incubations with fluorescent secondary antibody conjugates are described in the figure legends. The blocking buffer was PBS containing 2% bovine serum albumin (BSA) and 1% normal goat serum; the antibody dilution buffer was PBS containing 0.1% Triton X-100 and 0.2% BSA.

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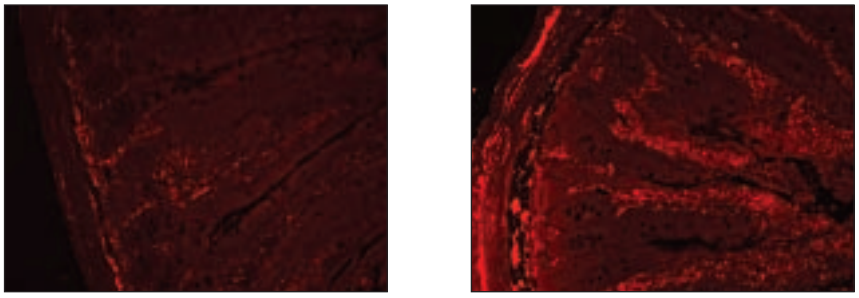


Figure 3. Normal rat intestine (smooth muscle) stained with anti-vimentin antibody

Rat intestine smooth muscle sections (Novagen) were either untreated or heat treated using 5X AntigenPlus Buffer (pH 6), diluted 1:5, as described in the text. Anti-vimentin antibody, diluted 1:100, was incubated with the processed slides for 18 h at 4°C. Slides were washed three times with PBS and incubated for 1 h at room temperature with Goat anti-Mouse IgG, TEXAS RED® Conjugate diluted 1:100 in dilution buffer. Slides were examined as described in Figure 1.

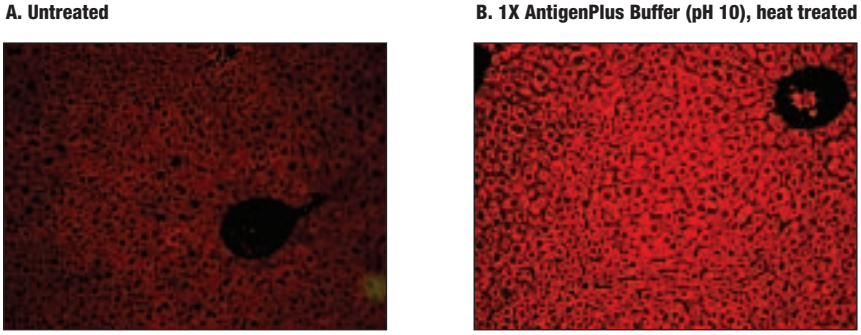


Figure 4. Normal mouse liver stained with anti-insulin receptor antibody

Mouse liver sections (Novagen) were either untreated or heat treated using 5X AntigenPlus Buffer (pH 10), diluted 1:5, as described in the text. Slides were washed 3 × 5 min in PBS and incubated for 1 h at room temperature with blocking buffer. Anti-insulin receptor antibody [Insulin Receptor (β-Subunit) Monoclonal Antibody, Oncogene Research Products] labeled with Alexa Fluor® 594 Mouse IgG_1 Labeling Kit (Molecular Probes) was diluted 1:5 in labeling reagent and incubated for 2 h at room temperature with the processed slides. Slides were examined as described in Figure 1.

Product	Size	Cat. No.
5X AntigenPlus™ Buffer (pH 6)	100 ml	71289-3
5X AntigenPlus Buffer (pH 7.4)	100 ml	71291-3
5X AntigenPlus Buffer (pH 10)	100 ml	71290-3
AntigenPlus Buffer Set (includes pH 6, pH 7.4, and pH 10 buffers)	1 set	71292-3
Available separately:		
Mouse Adult Tissue	**No. Slides**	**Cat. No.**
Liver	10	69568-3
Rat Adult Tissue	**No. Slides**	**Cat. No.**
Smooth Muscle (intestine)	10	69694-3
Human Diseased Tissue	**No. Slides**	**Cat. No.**
Colon Tumor:		
Adenocarcinoma	5	70337-3
Skin Tumor:		
Malignant Melanoma	5	70417-3
Product	**Size**	**Cat. No.**
p53 (Ab-5) (Wild type) Monoclonal Antibody	20 µg	OP33
Vimentin/LN6 (Ab-1) Monoclonal Antibody	100 µg	IF01
Insulin Receptor (β-Subunit) Monoclonal Antibody	100 µg	GR36
Goat anti-Mouse IgG, TEXAS RED® Conjugate	100 µg	DC18L

= Oncogene Research Products™

Animal-free reagents

Certified animal-free Veggie™ media components and competent cells; Veggie-grade lysis reagent

Novagen is committed to expanding its selection of animal-free products to help you reliably meet safety needs and regulatory requirements. We have developed products specifically to meet animal-free certification requirements. These products carry the Veggie™ product-line name. Novagen products identified by the Veggie Grade name are manufactured under the same strict guidelines as animal-free products, but include use of reagents derived from certified disease-free animal origin. Contact Novagen for information on raw material certification for any Veggie or Veggie Grade product.

Recently we introduced Veggie Singles™ Competent Cells for cloning and protein expression applications. The latest additions to Novagen's Veggie family include Veggie Peptone and Veggie Yeast Extract media components and a recombinant replacement for egg white lysozyme—rLysozyme™ Solution, Veggie Grade. Use our certified animal-free Veggie products for applications that require the absence of animal-derived materials or Veggie Grade products when certified absence of detectable contaminants and adventitious agents is required.

Veggie Media Components

Use Veggie Peptone and Veggie Yeast Extract as direct replacements for tryptone and yeast extract, respectively, in growth and expression media. Veggie Peptone, derived from papain-digested soymeal, is supplied as a dry powder while Veggie Yeast Extract is granulated.

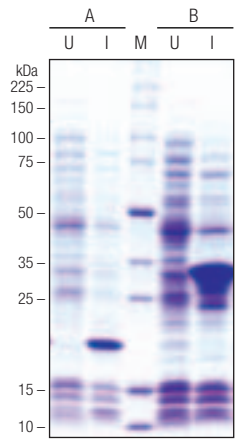
Veggie Singles Competent Cells

Veggie NovaBlue Singles Competent Cells (K-12 strain) are available for routine and high-efficiency cloning applications and, when appropriate plasmids are used, offer blue-white colony screening capabilities. Veggie BL21(DE3) Singles Competent Cells and Veggie BL21(DE3)pLysS Singles Competent Cells are available for protein expression using vectors containing the T7 promoter.

rLysozyme Solution, Veggie Grade

rLysozyme Solution, Veggie Grade is a special grade of rLysozyme prepared using certified animal-free or disease-free reagents. All of the steps in the preparation of the recombinant enzyme are carried out using reagents of nonanimal origin, with the exception of the IPTG used to induce protein expression. IPTG is chemically synthesized by a stringent process from D-galactose isolated from lactose, a milk sugar. The lactose is derived from certified disease-free cows. rLysozyme Solution, Veggie Grade has the same stability and specific activity as the rLysozyme Solution and requires no change to the protocol.

With its high specific activity (1,700 KU/mg, Note: 1 KU = 1000 units), only a small amount of rLysozyme Solution, Veggie Grade is required to break the cell wall of *E. coli*. Use the enzyme at 3–5 KU/g cells to enhance protein recovery from cells treated with BugBuster® or BugBuster HT protein extraction reagents or with PopCulture® Reagent. Or, treat thawed cell pellets with only the enzyme using 45–60 KU/g cells. Optimal protein purification is achieved by treating the cell lysate with Benzonase® Nuclease, which efficiently reduces extract viscosity by degrading the nucleic acids liberated as cells lyse. rLysozyme Solution, Veggie Grade is supplied as a ready-to-use solution at a concentration of 30 KU/µl in 50% glycerol containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Triton X-100. The solution is stable at –20˚C.



Lane	Sample
A	pET-32b(+)(Thioredoxin)
B	pET-41b(+)(GST)
U	Uninduced total cell protein
I	Induced total cell protein
M	Perfect Protein™ Markers, 10–225 kDa

Protein expression using Veggie Singles competent cells and Veggie media components

Veggie BL21(DE3) was transformed with two constructs, grown in TB medium made with Veggie Peptone and Veggie Yeast Extract, and induced for 3 h with 1 mM IPTG. Samples of total cell protein were analyzed by SDS-PAGE and Coomassie blue staining.

Product	Size	Cat. No.
Veggie™ Peptone	500 g	71280-3
Veggie Yeast Extract	500 g	71279-3
rLysozyme™ Solution, Veggie Grade	1200 KU 6000 KU	71297-4 71297-5
Veggie NovaBlue Singles™ Competent Cells guaranteed efficiency: > 1.5×10^8 cfu/µg	11 rxn 22 rxn	71251-3 71251-4
Veggie BL21(DE3) Singles Competent Cells guaranteed efficiency: > 2×10^6 cfu/µg	11 rxn 22 rxn	71252-3 71252-4
Veggie BL21(DE3)pLysS Singles Competent Cells guaranteed efficiency: > 2×10^6 cfu/µg	11 rxn 22 rxn	71253-3 71253-4

Available separately:

Product	Size	Cat. No.
BugBuster® Protein Extraction Reagent	100 ml 500 ml	70584-3 70584-4
BugBuster HT Protein Extraction Reagent	100 ml 500 ml 1 L	70922-3 70922-4 70922-5
Benzonase® Nuclease, Purity > 90%	10 KU 2.5 KU	70746-3 70746-4
Benzonase Nuclease HC, Purity > 90%	25 KU	71205-3
Benzonase Nuclease, Purity > 99%	10 KU	70664-3
Benzonase Nuclease HC, Purity > 99%	25 KU	71206-3

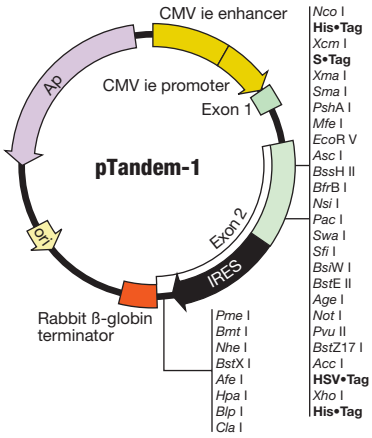
Note: 1 KU = 1000 units

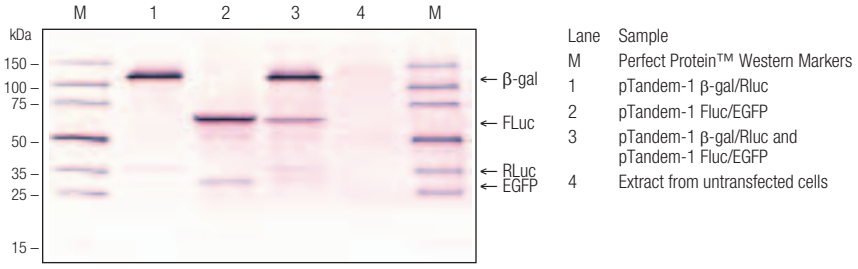
pTandem™-1 mammalian dual expression vector

Coexpression of two genes from one vector

The pTandem™-1 mammalian dual expression vector is designed for coexpression of two genes in mammalian cells from a bicistronic RNA. This vector contains separate multiple cloning sites (MCS) located on both sides of an internal ribosome binding site (IRES). These features provide for convenient insertion of two open reading frames and efficient translation of both encoded proteins from a single bicistronic mRNA.

Transcription is controlled by the cytomegalovirus (CMV) promoter. The rabbit β-globin terminator and polyadenylation signal direct correct processing of the 3′ end of the message. pTandem-1 recombinants can be used in transient transfections or for stable cell line selection by cotransfecting with a stable cell line selection vector, such as our new pTK-neo DNA (see below).





Lane	Sample
M	Perfect Protein™ Western Markers
1	pTandem-1 β-gal/Rluc
2	pTandem-1 Fluc/EGFP
3	pTandem-1 β-gal/Rluc and pTandem-1 Fluc/EGFP
4	Extract from untransfected cells

Transfection with pTandem-1 constructs

HEK 293 cells were plated at 54,000 cells/well in 24-well plates and later transfected with 0.25 µg pTandem-1 constructs containing β-gal/Rluc coding sequence (lane 1), Fluc/EGFP coding sequence (lane 2), or both constructs (lane 3). All of the target proteins contained an N-terminal S•Tag™ fusion sequence, which enabled their simultaneous detection using S-protein AP Conjugate. After 24 h, cells were harvested using CytoBuster™ Protein Extraction Reagent. Samples (10 µg total protein) were loaded onto an SDS-polyacrylamide gel for S•Tag Western blot analysis.

Product	Size	Cat. No.
pTandem™-1 DNA	20 µg	71283-3
TandemDOWN1 Primer	500 pmol	71301-3
TandemUP2 Primer	500 pmol	71302-3
TandemDOWN2 Primer	500 pmol	71303-3

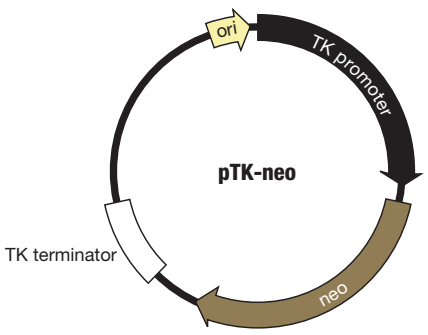
Available separately:

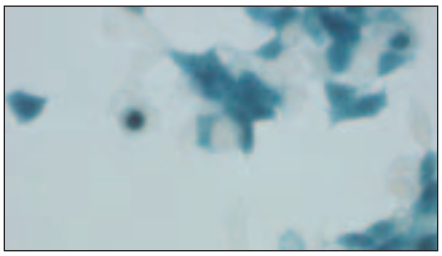
Product	Size	Cat. No.
pTK-neo DNA	20 µg	71284-3
GeneJuice® Transfection Reagent	1 ml 10 × 1 ml 0.3 ml	70967-3 70967-4 70967-5
CytoBuster™ Protein Extraction Reagent	50 ml 250 ml	71009-3 71009-4
Perfect Protein™ Western Markers	25 lanes	69959-3
S-protein AP Conjugate	50 µl	69598-3

pTK-neo DNA

For selection of stably transfected mammalian cell lines

The pTK-neo vector can select stably transformed mammalian cell lines using G 418. A minimal thymidine kinase (TK) promoter controls expression of the neomycin resistance gene. This promoter facilitates selection for stable integration of both the selection plasmid and a cotransfected expression plasmid into transcriptionally active sites in the genome. Try pTK-neo DNA as a cotransfection plasmid with pTandem-1 mammalian dual expression vector (see above), pTriEx™ vectors, pMLuc, or any mammalian expression vector.





Transfected cell selection with pTK-neo DNA

CHO-K1 cells plated at 1 × 10^6 in T-75 flasks were cotransfected with 15 µg β-gal expression plasmid (pTandem-1) and 1.5 µg pTK-neo using GeneJuice® Transfection Reagent. Cells were maintained for two weeks under G 418 selection (1 mg/ml). β-galactosidase expression was visualized using the BetaBlue™ Staining Kit.

Product	Size	Cat. No.
pTK-neo DNA	20 µg	71284-3

Available separately:

Product	Size	Cat. No.
G 418 Sulfate, Sterile-Filtered Aqueous Solution, Cell Culture Tested (50 mg/ml)	10 ml 20 ml 50 ml	345812
pTriEx™-1.1 DNA	20 µg	70840-3
pMLuc-1 DNA	20 µg	71116-3
pTandem™-1 Mammalian Dual Expression Vector	20 µg	71283-3
GeneJuice® Transfection Reagent	1 ml 10 × 1 ml 0.3 ml	70967-3 70967-4 70967-5
BetaBlue™ Staining Kit	100 ml	71074-3

EMSA Accessory Kit

Qualified reagents for DNA-binding reactions

An electrophoretic mobility shift assay (EMSA) determines whether a specific sequence of DNA binds to a protein. A discernible band shift between the free DNA fragments (faster migration) and stable protein-DNA complexes (slower migration) is visible when analyzed by electrophoresis under non-denaturing conditions.

Novagen's EMSA Accessory Kit contains three key reagents needed to determine whether labeled DNA probes bind specifically to proteins. Ready-to-use components include Poly(dI-dC)•Poly(dI-dC) Solution, 4X EMSA Buffer, sonicated Salmon Sperm DNA, and 100 mM DTT. Designed as a companion product to our NucBuster™ Protein Extraction Kit, the Novagen EMSA Accessory Kit is compatible with traditional methods of nuclear extract isolation and provides sufficient reagents to complete 100 reactions (17-µl reaction volume).

Product	Size	Cat. No.
EMSA Accessory Kit [includes Poly(dI-dC)•Poly(dI-dC) Solution, 4X EMSA Buffer, Salmon Sperm DNA, and 100 mM DTT]	100 rxn	71282-3

Available separately:

Product	Size	Cat. No.
NucBuster™ Protein Extraction Kit	100 rxn	71183-3

BCA Protein Assay Kit

Rapid, sensitive determination of total protein concentration

The BCA protein assay* is based on a biuret reaction, which is the reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline solution with concentration-dependent detection of the monovalent copper ions. Bicinchoninic acid is a chromogenic reagent that chelates the reduced copper, producing a purple complex with strong absorbance at 562 nm (1, 2). This assay can be used to quantify protein concentration with a wide variety of samples and can be performed in minutes.

Novagen's BCA Protein Assay Kit can be used to determine protein concentration in the range of 20–2,000 µg/ml in either a standard assay or microassay configuration. Kit components are sufficient to complete 500 standard-size reactions (50 µl protein sample plus 1 ml reagent) or 2,500 micro-scale reactions (25 µl protein sample plus 200 µl reagent) (3). A BSA standard (3 × 1 ml at 2 mg/ml) is provided for convenient preparation of standard curves.

This assay is robust and can be performed in the presence of many chemical compounds. Some reagents, including chelating agents, strong acids or bases, and reducing agents, interfere with the reduction and chelating reactions on which this assay depends (4). The BCA assay is compatible with the following Novagen protein extraction and lysis reagents: BugBuster® Protein Extraction Reagent, PopCulture® Reagent, CytoBuster™ Protein Extraction Reagent, Reportasol™ Extraction Buffer, and Insect PopCulture Reagent. Options for the removal or dilution of interfering substances are described in the kit literature.

REFERENCES

1. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85.
2. Wiechelman, K., Braun, R., and Fitzpatrick, J. (1988) *Anal. Biochem.* **175**, 231–237.
3. Hinson, D. L. and Webber, R. J. (1988) *BioTechniques* **12**, 496–499.
4. Brown, R., Jarvis, K. , Hyland, K. (1989) *Anal. Biochem.* **180**, 136–139.

Product	Size	Cat. No.
BCA Protein Assay Kit (includes BCA Solution; BSA Standard, 2 mg/ml; and 4% Cupric Sulfate)	500 assays (2500 microplate assays)	71285-3

Available separately:

Product	Size	Cat. No.
BugBuster® Protein Extraction Reagent	100 ml 500 ml	70584-3 70584-4
PopCulture® Reagent	15 ml 75 ml 250 ml	71092-3 71092-4 71092-5
CytoBuster™ Protein Extraction Reagent	50 ml 250 ml	71009-3 71009-4
Reportasol™ Extraction Buffer	25 ml 5 × 25 ml	70909-3 70909-4
Insect PopCulture Reagent	50 ml 250 ml	71187-3 71187-4

* BCA protein assay is covered by U.S. Patent #4,839,295 and its use is permitted for research purposes only.

ProteoExtract™ Kits from CALBIOCHEM® *(a Brand of EMD Biosciences, Inc.)*

Rapid extraction of proteins suitable for differential display analysis

ProteoExtract™ Kits offer fast and reproducible extraction of proteins from a wide range of biological sources. Kits are tailored with optimized protocols and components to produce samples compatible with isoelectric focusing, functional assays, gel electrophoresis, or liquid chromatography. Successful differential display analysis depends on standardized and reliable sample preparation. These kits solve the problems associated with reproducible solubilization of all protein types, prevent protein degradation, and efficiently remove contaminating nucleic acids.

Three families of ProteoExtract Proteome Extraction Kits (PEK) are available: Subcellular Proteome Extract Kit (S-PEK), Complete Proteome Extract Kits (C-PEK), and Partial Proteome Extract Kits (P-PEK). (See page 23.) Each kit is sufficient for preparation of up to 20 samples. All kits contain proteomics-grade, ultra-pure chemicals and buffers to deliver reproducible results and optimal resolution of the protein patterns visualized by 2D-gel electrophoresis (2DGE). Kits also include Benzonase® Nuclease, a nonspecific nuclease effective at eliminating nucleic acids, reducing sample viscosity, and improving spot resolution. With the exception of S-PEK, all kits are designed to examine denatured proteins.

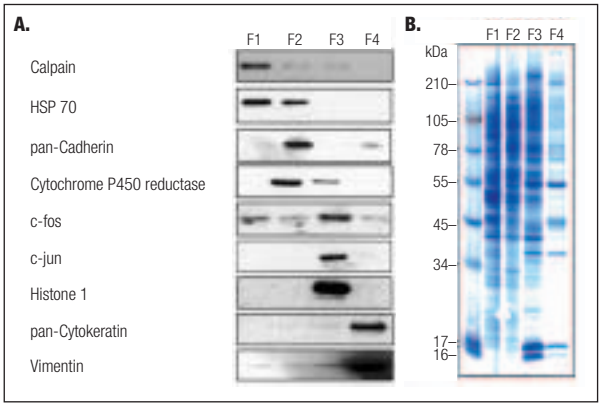


Figure 1. Stepwise extraction with S-PEK demonstrates distinct protein patterns for each isolated subcellular fraction

Panel A: Immunoblots of selected marker proteins demonstrate separation efficiency greater than 80% for subcellular compartments of mammalian tissue culture cells. For detection of c-fos, the protein was immunoprecipitated before electrophoresis. **Panel B:** Polyacrylamide gel analysis of subcellular fractions after S-PEK extraction of adherent tissue culture cells demonstrates clearly distinct protein patterns for each fraction. Fractions are as follows: F1, cytosolic fraction; F2, membrane/organelle fraction; F3, soluble nuclear fraction; F4, cytoskeletal/nuclear matrix fraction.

Prepare native protein fractions from mammalian subcellular compartments

S-PEK includes an innovative sequential extraction buffer system to yield four native proteome fractions suitable for analysis in activity assays or microarrays. Use S-PEK to:

- Prepare subcellular protein fractions from adherent cells or suspension-cultured cells
- Obtain four distinct protein fractions: cytosolic, plasma membrane plus organelle, soluble nuclear, and cytoskeletal plus nuclear matrix
- Extract native, functional proteins
- Perform the entire procedure in less than three hours without ultracentrifugation

Partition proteomes by hydrophobicity

Select P-PEK to extract bacterial, yeast, or mammalian proteomes into four fractions based on solubility characteristics. These kits:

- Offer an all-in-one solution for fractionating complex protein mixtures using buffers with increasing solubilization strength
- Provide an optimal chaotrope/detergent mixture for each subset of cellular proteins
- Partition the proteome into four fractions (three for yeast) based on hydrophobicity, increasing the probability of visualizing low-abundance proteins
- Produce samples ready for 2DGE

Isolate total protein

Isolate the complete proteome in just two steps using a single microcentrifuge tube and one extraction reagent. C-PEK provides the components necessary to:

- Extract total proteins in one fraction from bacterial, yeast, or mammalian cell sources (depending on kit selected)
- Improve cellular protein solubilization
- Produce samples ready for 2DGE

ProteoExtract™ Subcellular Proteome Extract Kit (S-PEK)

Product	Size	Cat. No.
ProteoExtract Subcellular Proteome Extraction Kit (includes Wash Buffer, extraction buffers, Protease Inhibitor Cocktail, and Benzonase® Nuclease. Components are sufficient for approximately 20 extractions based on T-25 flask of adherent cells or 1×10^6 suspended cells per sample.)	20 rxn	539790

ProteoExtract Partial Proteome Extract Kits (P-PEK)

Product	Size	Cat. No.
ProteoExtract Partial Bacterial Proteome Extraction Kit (includes Wash Buffer, extraction reagents, SDS buffers, Protease Inhibitor Cocktail, Benzonase Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 2×10^{11} cells per sample.)	20 rxn	539780
ProteoExtract Partial Yeast Proteome Extraction Kit (includes Wash Buffer, extraction reagents, SDS buffers, Protease Inhibitor Cocktail, Benzonase Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 2×10^7 cells per sample.)	20 rxn	539785
ProteoExtract Partial Mammalian Proteome Extraction Kit (includes Wash Buffer, extraction reagents, SDS buffers, Protease Inhibitor Cocktail, Benzonase Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on $1–2 \times 10^8$ cells per sample.)	20 rxn	539789

ProteoExtract Complete Proteome Extract Kits (C-PEK)

Product	Size	Cat. No.
ProteoExtract Complete Bacterial Proteome Extraction Kit (includes Wash Buffer, Resuspension Buffer, Extraction Reagent, Reducing Reagent, Benzonase Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 2×10^{11} cells per sample.)	20 rxn	539770
ProteoExtract Complete Yeast Proteome Extraction Kit (includes Wash Buffer, Extraction Reagent, Benzonase Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on 2×10^7 cells per sample.)	20 rxn	539775
ProteoExtract Complete Mammalian Proteome Extraction Kit (includes Wash Buffer, Resuspension Buffer, Extraction Reagent, Reducing Reagent, Benzonase Nuclease, and Glass Beads. Components are sufficient for approximately 20 extractions based on $1–2 \times 10^8$ cells per sample.)	20 rxn	539779

Available separately:

Product	Size	Cat. No.
Benzonase® Nuclease, Purity > 90%	10 KU 2.5 KU	70746-3 70746-4
Benzonase Nuclease, Purity > 99% Note: 1 KU = 1000 units	10 KU	70664-3

ProteoExtract™ Kits Application Guide

Cell Type	Starting Material	Fractionation and Enrichment	Applications	ProteoExtract Kit
bacterial		Total proteome extracted into one fraction	IEF/2DGE[1] PAGE Western Blot	Bacterial C-PEK
		Produces 4 protein fractions based on solubility: • Most soluble • Moderately soluble • Highly insoluble (membrane proteins) • Most insoluble (SDS[1] buffer)		Bacterial P-PEK
yeast		Total proteome extracted into one fraction	IEF/2DGE[1] PAGE Western Blot	Yeast C-PEK
		Produces 3 protein fractions based on solubility: • Most soluble • Highly insoluble (membrane proteins) • Most insoluble (SDS[1] buffer)		Yeast P-PEK
mammalian		Total proteome extracted into one fraction	IEF/2DGE[1] PAGE Western Blot	Mammalian C-PEK
		Produces 4 protein fractions based on solubility: • Most soluble • Moderately soluble • Highly insoluble (membrane proteins) • Most insoluble (SDS[1] buffer)		Mammalian P-PEK
		Produces 4 *native* protein fractions based on subcellular localization: • Cytosol • Plasma membrane and organelles • Soluble nuclear • Cytoskeleton and nuclear matrix ideal for functional assays		S-PEK

Key:

E. coli · Mammalian · Yeast · Adherent cells · Cell pellet · Homogenized tissue

C-PEK = ProteoExtract Complete Proteome Extract Kit
P-PEK = ProteoExtract Partial Proteome Extract Kit
S-PEK = ProteoExtract Subcellular Proteome Extract Kit

IEF = isoelectric focusing
2DGE = 2-dimensional gel electrophoresis
[1]SDS must be removed before IEF

United States and Canada

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

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Ordering:
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e-mail: biotech@de.vwr.com
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