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Phospho-specific Antibodies: New Tools to Study Protein Phosphorylation

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Protein phosphorylation is now recognized to play a critical role in the regulation of cell growth and development. As a result, it has become increasingly important to measure changes in the phosphorylation status of proteins. Key decisions in the life of a cell, such as deciding whether to divide, differentiate, or die, are all controlled by protein phosphorylation (1,2). For example, progression through the cell cycle is tightly regulated both positively and negatively by phosphorylation of the cyclin-dependent protein kinases (2). In addition, the actions of growth factors and cytokines are largely elaborated via the activation of protein kinase cascades that serve to amplify signals generated at the cell surface into complex biological responses, including the activation of transcription factors and gene expression (1-3). Multi-kinase cascades allow not only signal amplification but also signal divergence to multiple effectors that are often cell-type-specific, allowing a growth factor to stimulate mitosis of one cell and differentiation of another.

One such cascade is the MAP kinase pathway (Figure 1) that appears to mediate both mitogenic, differentiation and stress response in different cell

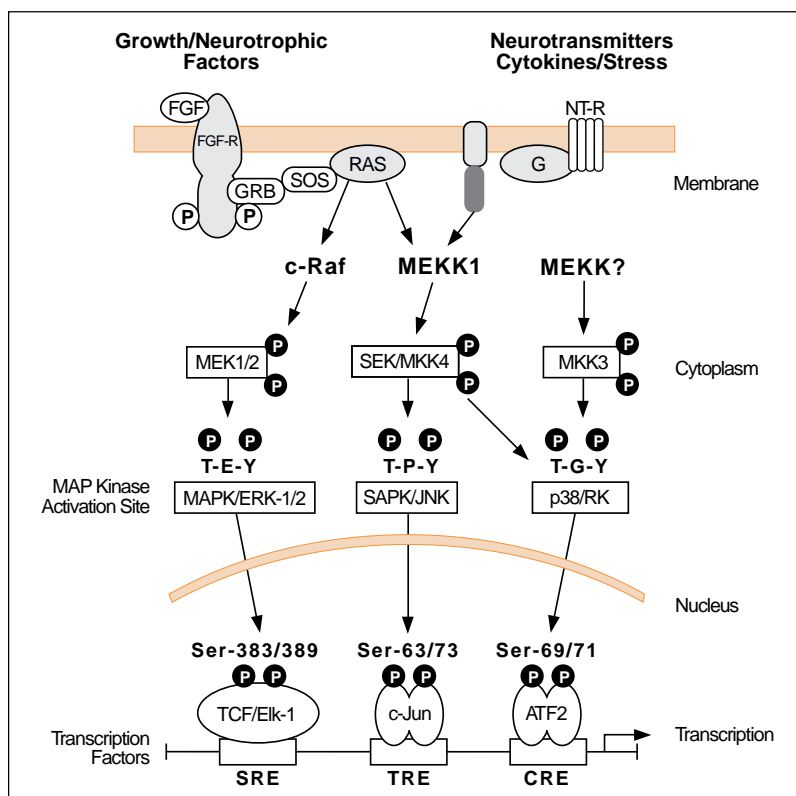


Figure 1. Growth factor and cytokine activated MAP kinase signaling pathways. The activation sites of three different MAP kinase families, ERK, SAPK and p38/RK are shown as are the activated transcription factors.

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types. Stimulation of growth factor receptors results in Ras activation followed by the sequential activation of c-Raf, MEK, and p44 and p42 MAP kinases (ERK1 and ERK2). Activated MAP kinase then phosphorylates many key regulatory proteins, including the protein kinase p90^{rsk} (4) and transcription factors such as Elk-1 (3,5,6) that are activated when MAP kinase translocates to the nucleus (7,8). Inhibition of either MEK (9) or MAP kinase (10) blocks both cell proliferation and neural differentiation and also reverses the ability of oncogenic Ras to transform cells.

As both MEK and MAP kinase function in a kinase cascade (Figure 1), their enzymatic activities are both tightly regulated by phosphorylation at specific "activation sites". MEK is activated by phosphorylation at Ser215 and Ser219 by Raf-1 and MEK kinase (11). Similarly, MEK activates MAP kinase by dual phosphorylation at Thr187 and Tyr189 of p44 MAP kinase (12,13). Upon activation both p44 and p42 MAP kinases translocate to the nucleus (3,4,14) where they activate the transcription factor Elk-1 by phosphorylation at several sites including Ser383 and Ser389 (2,5). Since phosphorylation at these sites is required for kinase or transcriptional activity, phospho-status is an excellent indicator of both pathway and protein activity.

Measuring Phosphorylation

To facilitate the analysis of protein phosphorylation and kinase cascades, it is useful to have reagents that recognize only the active or phosphorylated form of a kinase or transcription factor. Although it is currently possible to assay activity and phosphorylation using conventional antibodies and phosphopeptide mapping, these methods are laborious, difficult to control properly and require the use of large amounts of ³²P. Analysis of phosphorylation at individual sites requires fragmentation of the protein into phosphopeptides and separation by 2-dimensional thin layer chromatography (TLC) based upon peptide size and

chromatographic properties. Furthermore, if the peptide contains multiple phosphorylation sites, mutagenesis experiments combined with mass spectrometry or other biophysical techniques may be necessary to conclusively identify the phosphorylated residue. Finally, phosphopeptides can only be generated and measured when the protein of interest is relatively abundant in the tissue homogenate, and such measurements provide only semi-quantitative estimates of phosphorylation.

The recent development of phospho-tyrosine specific antibodies has dramatically improved the analysis of protein phosphorylation. Western blots of SDS-lysed cell extracts can now be used to measure changes in phosphorylation states. The use of general phospho-tyrosine antibodies has its drawbacks; not all proteins phosphorylated on tyrosine are recognized by the antibody. In addition, to obtain information about a specific protein, the phospho-tyrosine antibody must be used in conjunction with a protein-specific antibody. Finally, phosphorylation at different sites within the same molecule cannot be distinguished.

Development

The identification of key sites of protein phosphorylation combined with the ability to synthesize or enzymatically produce phosphorylated peptides has allowed the development of highly specific immunological tools to study protein phosphoryla-

tion. A short peptide containing the phosphorylated residue is synthesized and used to immunize animals. Synthetically produced phosphopeptides of approximately 10-15 amino acids are long enough to elicit a good immune response, yet short enough to focus the immune response upon the phosphorylated residue. The phosphopeptide is first coupled to a carrier protein such as keyhole limpet hemocyanin (KLH) and injected into rabbits to prepare polyclonal antiserum. Antibodies are then purified over a protein A column and phospho-specific antibodies are isolated by affinity chromatography using a nonphosphopeptide affinity column. Antibodies that bind in a phosphorylation-independent fashion are retained on the column and removed while antibodies for which the phosphate is an essential part of the epitope will flow through the column. If necessary, a second purification step using a phosphopeptide affinity column can further purify the phospho-specific antibodies. It is also possible to immunize animals with a nonphosphorylated peptide and use a phosphopeptide column to purify antibodies that are selective for the nonphosphorylated form of the protein.

Antibodies produced in this fashion are first screened by ELISA to determine affinity and phospho-selectivity, followed by Western blotting, immunoprecipitation, and immunohistochemistry to confirm specificity against the denatured

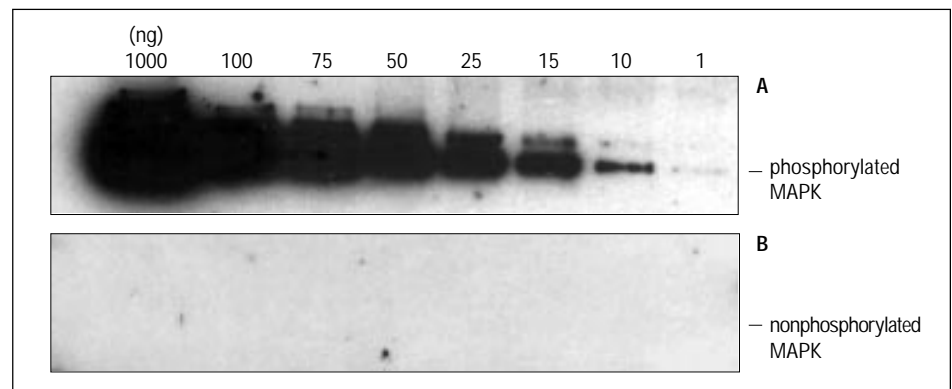


Figure 2. Specificity and selectivity of phospho-MAP kinase antibody. Western blot analysis using a polyclonal phospho-specific antibody raised against the activation site (Y189) of MAP kinase. This antibody reacts specifically with as little as 1 nanogram of phosphorylated MAP kinase (A) yet shows no reactivity with as much as 1 microgram of nonphosphorylated MAP kinase (B).

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and native protein. Good phospho-specific antibodies are highly specific for the phosphoprotein and will not react with even large amounts (μg quantities) of the nonphosphorylated protein (Figure 2).

Advantages

The great utility of phospho-specific antibodies is the ease with which one can measure changes in phosphorylation at a specific site without ^{32}P . When phospho-specific antibodies are used in conjunction with phosphorylation-independent antibodies and known phosphorylated and nonphosphorylated protein standards, it is possible to quantitate changes in phospho-

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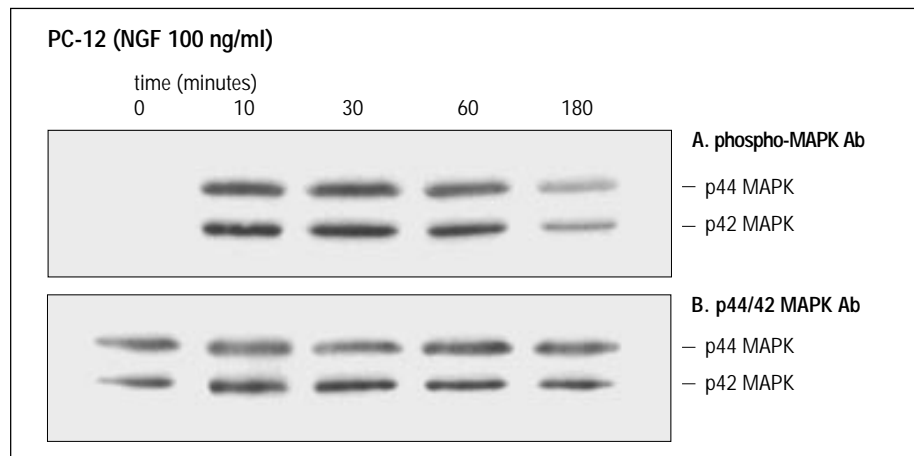


Figure 3. Time course of MAP kinase phosphorylation by NGF in PC-12 cells. Analysis of MAP kinase activation on Tyr189 using phospho-specific- (A) and control- (B) MAP kinase antibodies from SDS-lysates of total PC-12 cell extracts.

PhosphoPlus™ Kits

Phosphorylation State-Specific Antibody Kits

NEB is committed to the development of phosphorylation-state-specific antibodies useful in the analysis of growth factor and cytokine activated signaling pathways. Our phospho-specific antibody kits facilitate analysis of protein phosphorylation and include everything needed to characterize site-specific protein phosphorylation. The kits include phosphorylation-state specific and control antibodies together with control proteins that are suitable to monitor

antibody selectivity and sensitivity. The kits also include our Phototope™-Star Chemiluminescence Western Detection Kit which contains 2° antibody, anti-biotin antibody, molecular weight markers and chemiluminescent reagents (see page 7).

Our first offering of PhosphoPlus™ Antibody Kits includes phosphorylation-state specific antibodies to MAP kinase and cdc2 kinase. We are currently developing additional kits useful in the analysis of other important cell signaling

molecules, including MEK, SEK, SAPK/JNK, cdk4, and the transcription factors CREB, c-Jun, Elk-1, STAT1, STAT3 (Figure 1) and p53.

We invite your comments and suggestions as well as inquiries for more information.

Ordering Information

PhosphoPlus MAPK Antibody Kit
#9100 10 Western mini-blot

Phospho-specific MAPK Antibody
#9101S 100 μl
#9101L 300 μl

p44/42 MAPK (control) Antibody
#9102 200 μl

MAPK Control Proteins
#9103 10 Western mini-blot

PhosphoPlus cdc2 (Tyr15) Antibody Kit
#9110 10 Western mini-blot

Phospho-specific cdc2 (Tyr15) Antibody
#9111S 100 μl
#9111L 300 μl

Control cdc2 (Tyr15) Antibody
#9112 100 μl

cdc2 Control Proteins
#9113 10 Western mini-blot

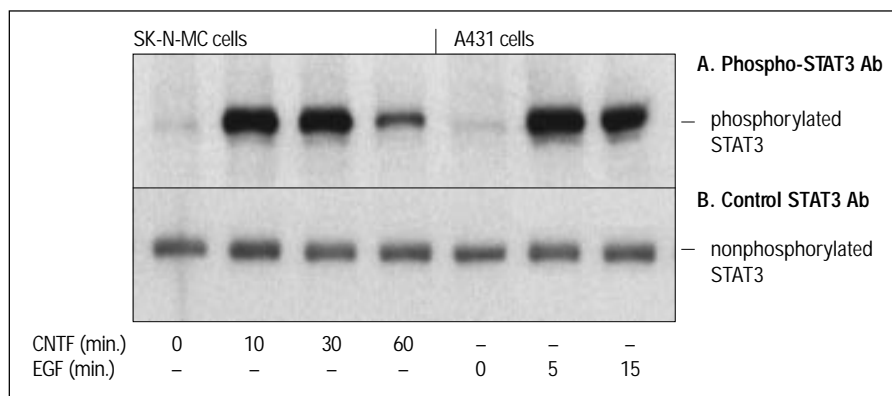


Figure 1. Time course of STAT3 phosphorylation by CNTF in SK-N-MC cells and by EGF in A431 cells. Analysis of STAT3 activation on Tyr705 using phospho-specific- (A) and control- (B) STAT3 antibodies from SDS-lysates of total cell extracts.

continued from page 3

rylation that have occurred *in vivo*. These reagents allow one to treat cells with a cytokine, growth factor, or cell cycle inhibitor, prepare total cell extracts using SDS sample buffer and rapidly measure changes in phosphorylation by Western blotting (Figure 3). This simple method not only provides quantitative information regarding phosphorylation at a specific site within a protein, but also reduces artifactual changes in phosphorylation due to the liberation or activation of phosphatases, since samples are rapidly incubated with SDS.

Since these antibodies detect only phosphorylated proteins, they can be used to selectively visualize an active protein and provide insight into its function. For example, immunoprecipitation using phospho-specific antibodies can detect protein:protein complexes selectively formed with the phosphoprotein as well as changes in protein complex formation induced by phosphorylation. Most

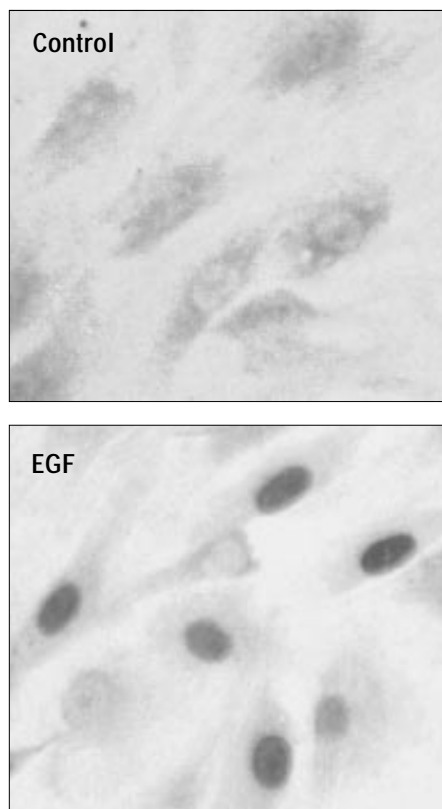


Figure 4. Phospho-specific antibody allows *in situ* detection and subcellular resolution of epidermal growth factor (EGF)-induced MAP kinase phosphorylation and nuclear translocation.

importantly, phosphorylation-induced changes in cellular distribution can be easily visualized. For example, although it has been suggested that MAP kinase translocates to the nucleus upon activation, this has been difficult to prove immunohistochemically because of the high background of inactive cytoplasmic MAP kinase. However, using a phospho-specific antibody to the activation site of MAP kinase, nuclear translocation upon growth factor treatment can be easily visualized as only the active (phosphorylated) molecule is visualized (Figure 4) (data unpublished).

The recently developed phospho-CREB antibody specifically recognizes the transcription factor CREB only when phosphorylated at its key regulatory site, Ser133 (15,16). This antibody has greatly facilitated analysis of CREB phosphorylation and the regulatory pathways that activate CREB (15,16,17). It has also been used to map activity, as measured by changes in CREB phosphorylation, in neural pathways as well as to detect cells in the brain and other tissues that have been activated after different drug treatments or as a result of behavioral paradigms. It can be expected that development of phospho-specific antibodies to other important components of intracellular signaling pathways will similarly facilitate and stimulate analysis of the functional changes associated with protein phosphorylation.

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The Modern Art

of Protein Phosphorylation



The state of phosphorylation is a prime regulator of the activity of proteins in eukaryotic cells. New England Biolabs is committed to supplying a full range of reagents to study protein phosphorylation. Already available are numerous kinases, phosphatases and selective inhibitors that feature exceptional purity, rigorous quality assessment and remarkably low cost. All are purified from clones in *E. coli* to ensure the absence of trace levels of other eukaryotic kinases and phosphatases, with the exception of the recombinants p34^{cdc2}/cyclin B and CaM Kinase II, which are purified from a baculovirus system.

These reagents are complemented by peptide substrates and protein molecular markers available unstained, prestained or biotinylated, and a new line of phospho-specific antibodies targeted against key signal transducing molecules.

Protein Kinases and Inhibitors

Recent additions, MAP Kinase (ERK2) and Abl Tyrosine Kinase are produced in *E. coli* in fully active form. Others, such as Phosphorylase Kinase and Cdk2/cyclin A or E are under development.

Abl Tyrosine Kinase

#6050S	2,000 units
#6050L	10,000 units

cAMP Dependent Protein Kinase (PKA)

#6000S	250 units
#6000L	1,250 units

Heat Stable PKA Inhibitor (PKI)

#6005S	250 units
#6005L	1,250 units

CaM Kinase II

#6060S	1,000 units
#6060L	5,000 units

Casein Kinase I

#6030S	20,000 units
#6030L	100,000 units

Casein Kinase II

#6010S	10,000 units
#6010L	50,000 units

Glycogen Synthase Kinase 3 (GSK-3)

#6040S	100 units
#6040L	500 units

p34^{cdc2}/cyclin B Kinase (cdc2)

#6020S	100 units
#6020L	500 units

MAP Kinase (ERK2)

#6080S	100 units
#6080L	500 units

Kinase Peptide Substrates

Kemptide (PKA Peptide Substrate)

#6001S	0.5 mg
#6001L	2.5 mg

CKI Phosphopeptide Substrate

#6031S	0.5 mg
#6031L	2.5 mg

CKII Peptide Substrate

#6011S	0.5 mg
#6011L	2.5 mg

GSK-3 Phosphopeptide Substrate

#6041S	0.5 mg
#6041L	2.5 mg

p34^{cdc2}/cyclin B Peptide Substrate

#6021S	0.25 mg
#6021L	1.25 mg

Phospho-specific Antibody Kits

A new line of research tools for growth factor signaling and cell cycle research.

PhosphoPlus™ MAPK Antibody Kit

#9100	10 Western mini-blot
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PhosphoPlus™ cdc2 (Tyr15) Antibody Kit

#9110	10 Western mini-blot
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Protein Molecular Weight Markers

Protein Marker, Broad Range

#7701S	100 mini-gel lanes
#7701L	500 mini-gel lanes

Prestained Protein Marker, Broad Range

#7707S	125 mini-blot lanes
#7707L	625 mini-blot lanes

Biotinylated Protein Marker, Broad Range

#7710BTS	125 mini-blot lanes
#7710BTL	625 mini-blot lanes

Protein Phosphatases and Inhibitors

Tyrosine Specific

LAR Phosphatase

#750S	200 units
#750L	1,000 units

T-Cell Phosphatase

#752S	200 units
#752L	1,000 units

YOP Phosphatase

#751S	2,000 units
#751L	10,000 units

Serine/Threonine Specific

Protein Phosphatase 1 (PP1)

#754S	40 units
#754L	200 units

Protein Phosphatase Inhibitor 2 (I-2)

#755S	20 µg
#755L	100 µg

Dual Specificity

Lambda Phosphatase

#753S	20,000 units
#753L	100,000 units

CDP-Star™

The Next Generation of Chemiluminescent Detection

Chemiluminescent detection of nucleic acids and proteins is becoming a popular alternative to radioactive detection due to sensitivity, convenience, safety and cost. CDP-Star™, the next generation of substituted dioxetane chemiluminescent substrates, is now incorporated into our Phototope™ Detection Kits.

NEB's Phototope Detection for nucleic acids uses biotin associated with the target DNA to provide the handle for the chemiluminescent detection. Biotin can be incorporated directly by enzymatic polymerization of DNA with a biotinylated primer (DNA sequencing) or by polymerization in the presence of biotinylated nucleotide triphosphates (NEBlot™ Phototope™ System, NEB #7550).

Biotinylated DNA is detected on a membrane support by first exposing the membrane to streptavidin which binds to the biotinylated DNA. Next, biotinylated alkaline phosphatase, which binds to the streptavidin, is added. This results in a conjugate between the alkaline phosphatase and the DNA on the membrane. Finally, the chemiluminescent reagent (Lumigen-PPD® or CDP-Star™) is added. Alkaline phosphatase catalyzes the removal of a phosphate from the phenylphosphate-substituted 1,2 dioxetane to yield a moderately stable intermediate which then spontaneously decays and emits light (Figure 1). The

emitted light is detected by exposing the membrane to X-ray film for 2 to 10 minutes.

CDP-Star can be used for the same applications as Lumigen-PPD, but the kinetics of light emission are significantly different. CDP-Star decays rapidly, giving rise to strong signals after a short "ramp" time (Figure 2). The signal persists for hours to days allowing multiple exposures and precise signal control.

Ordering Information

Phototope Detection Kit
(detection for 6,000 cm² of membrane)
#7006 with Lumigen-PPD

Phototope-Star Detection Kit
(detection for 20,000 cm² of membrane)
#7020 with CDP-Star

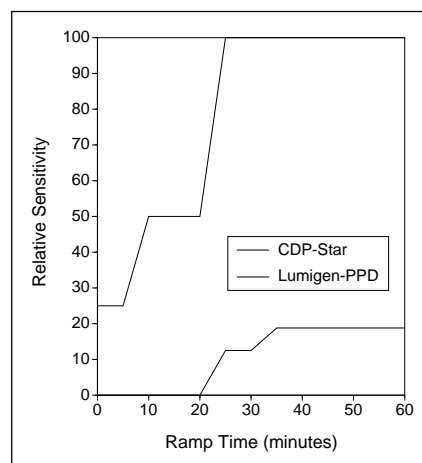


Figure 2. Comparison of signal strength of CDP-Star and Lumigen-PPD

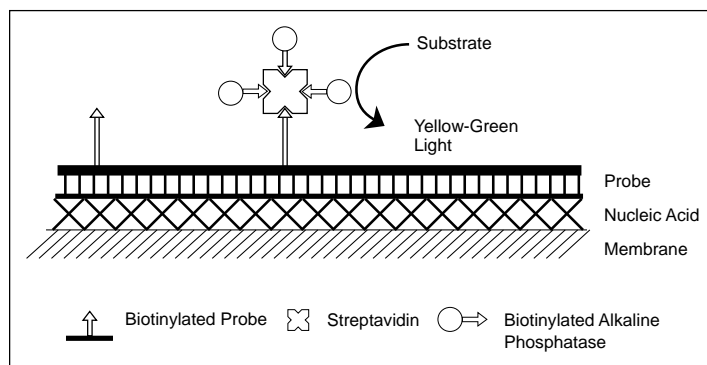


Figure 1. Overview of chemiluminescent detection.

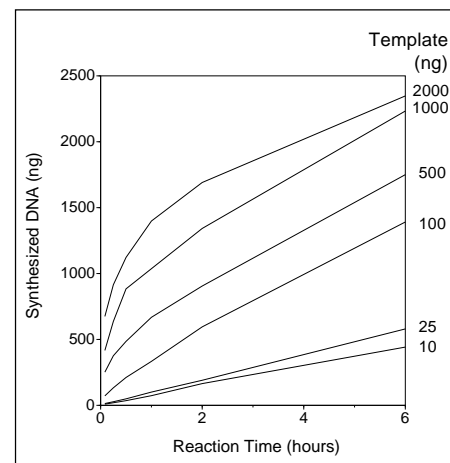
Improved Random Primer Labeling with Klenow (exo⁻)

The NEBlot™ Phototope™ Kit for random primer biotin labeling generates biotinylated probes. Random biotinylated octamers are used *in vitro* to prime DNA synthesis from denatured double-stranded template DNA. We have recently found that incorporation of biotinylated dATP, particularly into low abundance templates, can be enhanced by performing extension reactions with Klenow Fragment (3' → 5' exo⁻) (NEB #212). The resulting probes are highly biotinylated and provide superior sensitivity in nucleic acid detection.

The enhanced biotin incorporation of Klenow Fragment (3' → 5' exo⁻) in the NEBlot Phototope Kit in combination with the increased sensitivity of Phototope™-Star Detection allow experiments which were previously impractical using nonisotopic methods. For example, it is now possible to perform Southern blotting from single copy human genes with µg amounts of genomic DNA and Northern blotting for low abundance message. Screening thousands of colony hybridizations or plaque lifts can now be performed at significantly reduced cost.

Ordering Information

NEBlot Phototope Kit
#7550 25 reactions



Biotin incorporation into Hind III fragments of Lambda DNA using NEBlot Phototope Kit protocols. Template concentrations varied from 10 to 2,000 ng. Reaction times varied from 15 minutes to 6 hours. Incorporation measured by colabeling with biotin and ³H.

Western Detection

Phototope™-Star Chemiluminescent Detection for Western Blotting

Experience greater sensitivity with the Phototope™-Star Western Blot Detection Kit designed for the chemiluminescent detection of proteins in standard Western blot applications. Protein samples and biotinylated molecular weight markers (included) are separated by SDS-PAGE and transferred onto PVDF membrane. Following incubation with your primary antiserum, alkaline phosphatase-linked (AP-linked) secondary antibody is bound and then reacted with CDP™-Star reagent. The light emitted by dephosphorylated CDP-Star reagent is subsequently captured on X-ray film.

Method Overview

The six basic steps of the Phototope-Star Western Blot Detection Kits.

- 1. Electrophoresis of Proteins**
Separate the protein samples and molecular weight standards by polyacrylamide gel electrophoresis.
- 2. Transfer**
Transfer the protein to PVDF membrane by standard electroblotting.
- 3. Block Membrane**
Block to saturate nonspecific binding sites on the membrane.
- 4. 1° antibody**
Incubate the membrane with your primary antibody.
- 5. 2° antibody**
Incubate the membrane with AP-linked 2° antibody (anti-rabbit, mouse or human IgG) and AP-linked anti-biotin antibody.
- 6. Chemiluminescent Detection**
Add CDP-Star substrate and capture the emitted light on X-ray film.

Ordering Information

Phototope-Star Western Blot Detection Kit (detection for 5,000 cm² of membrane)

#7051	anti-Rabbit IgG
#7052	anti-Mouse IgG
#7053	anti-Human IgG

Biotinylated Protein Marker, Broad Range

#7710BTS	125 mini-blot lanes
#7710BTL	625 mini-blot lanes

Anti-Rabbit IgG, AP-linked

#7051-1	1.0 ml
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Anti-Mouse IgG, AP-linked

#7052-1	1.0 ml
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Anti-Human IgG, AP-linked

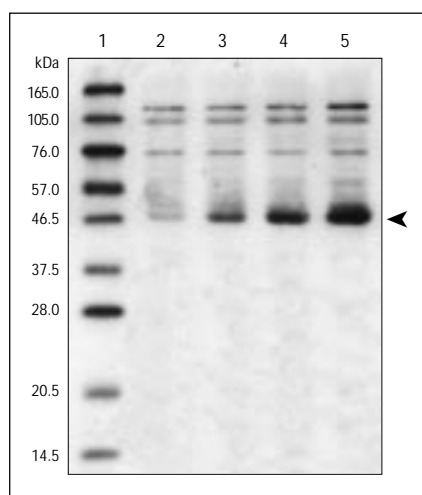
#7053-1	1.0 ml
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Anti-Biotin, AP-linked

#7051-2	1.0 ml
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500X CDP™-Star Substrate

#7001	2.5 ml
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Induction of SEK phosphorylation (arrow) detected using phospho-specific SEK antibody as analyzed by Western blotting. Biotinylated Protein Marker (lane 1), 293 cells either untreated (lane 2) stimulated with NaCl (lane 3), with NaVO₃/NaCl (lane 4), or with NaVO₃/anisomycin (lane 5). 12% SDS-PAGE developed using the Phototope-Star Western Blot Detection Kit.

Advantages of the Phototope™ Western Kits

- Complete System**
Biotinylated Protein Markers, Anti-biotin 2° Antibody, AP-linked 2° Antibody (anti-rabbit, mouse or human), Chemiluminescent Detection
- Sensitivity**
Detection of subpicogram quantities of protein
- Speed**
Less than 1 hour required for entire detection procedure. Exposure times of 2–10 minutes
- Multiple Exposures**
Light emitted at a constant rate for days, allowing multiple exposures to optimize signal intensity. Future re-exposure achieved by simply adding more reagent
- Stability**
A permanent record is generated which will not fade
- Quantitative**
X-ray films can be scanned to quantitate and record band intensities
- Versatility**
Kits available for rabbit, mouse and human primary antisera
- Simultaneous Detection of Biotinylated Molecular Weight Markers**

Linear Amplification Sequencing of Mycobacterial DNA

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Molecular studies of mycobacterial DNA suffer from several drawbacks, such as slow growth rates and massive cell envelopes. The high GC-content of mycobacterial DNA (56% to 69%) also causes problems when sequencing. The formation of strong secondary structures, such as hairpin loops in GC-rich regions, can block the DNA polymerase during the sequencing reaction, causing a strong stop in all four lanes of the sequencing gel. Furthermore, formation of hairpin loops in the single-stranded reaction products before or during electrophoresis can cause mobility compressions in the sequencing gel. Several solutions to these problems have been suggested, including sequencing with 7-deaza-dGTP or dITP (1), sequencing at 80°C (2), or electrophoresis in a high concentration of urea

(9.5 M instead of 7 M) which is only soluble at elevated temperatures. Despite the use of nonstandard techniques, the results are often unsatisfactory.

The use of thermophilic polymerases in a cyclic version of the dideoxy chain-termination method for sequencing DNA (3,4) has many advantages (5,6). The method is straightforward, fast, uses standard reagents and electrophoresis conditions, and allows the sequencing of GC-rich regions. The sequencing reactions, including primer labeling, can be completed within two hours.

Plasmids containing cloned sequences of mycobacterial DNA in an EMBL3 genomic library were prepared using standard methods for both large scale- and mini-preparations (6). Both EMBL3 sequencing primers and synthetic oligonucleotide primers derived from mycobacterial sequences were end-labeled with γ -[³²P] ATP and T4 Polynucleotide Kinase (New England Biolabs). Using the CircumVent™ Thermal Cycle DNA Sequencing Kit (New England Biolabs), 50 to 100 ng of plasmid DNA was sequenced following the recommended protocol (7). After 20 cycles of 20 seconds at 95°C, 20 seconds at 60°C, and 30

seconds at 72°C, 4 μ l of stop/loading dye was added to each tube. Samples were denatured for 5 minutes at 95°C before loading 3 μ l aliquots onto the gel. Reaction products were separated on 7M urea/8% polyacrylamide gels. After electrophoresis for 2.5 hours at 60 W, a second sample was loaded and electrophoresed for another 2.5 hours, followed by a third sample, which was electrophoresed for 1.5 hours. The gel was then transferred to Whatman paper and vacuum dried. Standard autoradiography procedures were followed (6).

Linear amplification sequencing improved band resolution and determination of GC-rich sequences. Up to 300 bases could be read from a single gel. The gels also showed uniform distribution of band intensity and lower background of nonspecific banding compared to other sequencing methods. In contrast, when using the standard dideoxy chain-termination method, band compressions and "strong stops" hampered the unambiguous determination of the sequence.

Short reaction times, employment of standard equipment and techniques, and unambiguous results make linear amplification sequencing a valuable tool in mycobacterial genetic studies.

International Network of NEB

Scientists in Canada, the UK and Ireland, and Germany and Austria are serviced through NEB-owned subsidiaries—all committed to providing the same level of value, personalized customer service and technical support as our US headquarters. Our international subsidiaries are staffed by scientists who truly speak your language giving you access to all of the technical resources New England Biolabs has to offer.

Our subsidiaries can be contacted free of charge at the numbers listed below. For a complete listing of contact information, please refer to the back of the current NEB Catalog or visit our World Wide Web site <<http://www.neb.com>>.

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RNA Markers

New RNA Molecular Weight Mark-

NEB's RNA Molecular Weight Markers are produced by *in vitro* transcription of a mixture of 6 linear DNA templates. The sequence of each RNA molecule is identical for the first 461 bases, and all are transcribed from a template consisting primarily of lambda and pUC derived DNA. The relative amounts of each linear DNA template in the transcription mix have been optimized to give RNA bands of equal intensity.

The RNA Marker sizes are: 4061, 2193, 1701, 1280, 754, and 461 bases (Figure 1). These markers are suitable for use as an RNA size standard on denaturing or native agarose gels as well as polyacrylamide gels.

Traditionally, RNA markers are handled much like DNA markers and stored in water or TE buffer (Tris-HCl, EDTA). This storage practice is problematic because RNA is more susceptible to degradation than DNA due to its 2' hydroxyl groups that can act as an intramolecular nucleophile in both base- and enzyme-catalyzed hydrolysis of the phosphodiester linkages (1). Also, ribonucleases, such as ribonuclease A, exhibit optimum activity at neutral pH values (2). To minimize degradation of our RNA markers, they are supplied in an

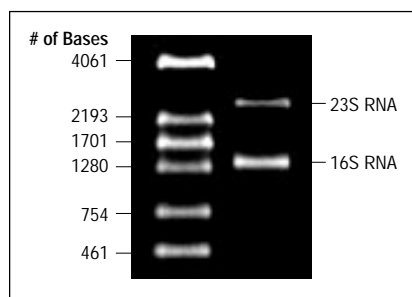


Figure 1. Lane 1, NEB's RNA Markers; Lane 2, 16S (1542 bases) and 23S (2904 bases) *E. coli* ribosomal RNA. Samples were heated at 65°C for 3 minutes in 1X Denaturing Sample Buffer and separated on a 1.6% native (TBE) agarose gel.

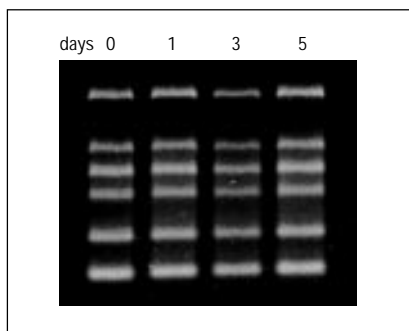


Figure 2. RNA Marker Stability: NEB's RNA Markers were incubated for 1, 3 and 5 days at room temperature and then heated at 65°C for 3 minutes in 1X Denaturing Sample Buffer and separated on a 1.6% native (TBE) agarose gel.

acidic storage buffer: 20 mM KOAc (pH 4.5). Figure 2 illustrates the stability of NEB's RNA Molecular Weight Markers after incubation at room temperature for 1, 3, and 5 days.

Denaturing Sample Buffer

RNA Markers are supplied with a 2X Denaturing Sample Buffer that can be used to prepare samples for native agarose gels or denaturing polyacrylamide gels. This buffer should not be used for gels containing formaldehyde or methyl mercuric acid.

Denaturing vs. Native Agarose Gels

It is common practice to electrophorese RNA on a denaturing agarose gel, such as one containing formaldehyde (3). However, in many cases it is possible to run RNA on a native agarose gel and obtain suitable results. In fact, it has been demonstrated that treatment of RNA in a denaturing sample buffer maintains the denatured state of RNA molecules during electrophoresis for at least 3 hours (4). Native agarose gels eliminate the use of toxic chemicals and difficulties associated with staining formaldehyde gels.

Advantages of NEB's RNA Markers

- **Stability**
 Unique storage buffer ensures long term stability at temperatures above -70°C
- **Flexibility**
 Suitable for denaturing or non-denaturing (native) gels
- **Convenience**
 A broad range RNA size standard; supplied with 2X Denaturing Sample Buffer

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Ordering Information

RNA Molecular Weight Markers
 #361 25 µg

Companion Products

polyA Spin™ mRNA Isolation Kit
 #1560 8 isolations

T7 RNA Polymerase
 #251S 5,000 units
 #251L 25,000 units

SP6 RNA Polymerase
 #207S 200 units
 #207L 1,000 units

Protein Splicing: Mechanism and Possible Use in Molecular Biology

Dr. Donald G. Comb and

Dr. Francine B. Perler

New England Biolabs, Inc.,
Beverly, MA, 01915

I'll always remember the day we found DNA polymerase activity in the *Thermococcus litoralis* gene Fran Perler had cloned in *E. coli*. It had taken 18 months to coax Vent DNA polymerase activity out of this gene. The weekly in-house seminar had just ended and as we passed Fran's bench, we saw her jumping up and down with a Geiger counter in one hand and a "hot" paper disc in the other. The whole lab gathered around to congratulate her and later the champagne started to flow.

We have cloned hundreds of genes at NEB, but this one was the toughest because the DNA polymerase gene from this extreme thermophilic archaea (it grows at 90 to 96°C) contained two endonucleases embedded in-frame at different locations in the polymerase. In order to obtain active DNA polymerase, the two endonucleases had to be spliced out of the polymerase precursor—not at the RNA level, but from a large precursor protein (Figure 1). The three polymerase polypeptides had to be spliced together to form the mature enzyme. These two endonucleases made *E. coli* very sick by degrading its chromosome. As soon as Fran removed one endonuclease, the other

was able to splice itself out from the precursor protein in *E. coli*; this resulted in polymerase activity. One gene coding for three distinct proteins is an amazing addition to our genetic library!

Protein splicing, as the process is now called, was first described in yeast a year prior to our initial discovery (1,2) and later in eubacteria and archaea (3,4,5,6). To avoid confusion, a standard nomenclature has been adopted (7). The in-frame insertion is called an "intein" and the flanking protein sequences which are eventually spliced together are called "exteins". Inteins have similarity to homing endonucleases (8) and many have demonstrated endonuclease activity. Coding sequences for homing endonucleases have been found in the RNA introns of archaea, eubacteria and eukaryotes (8,9) and are designated with the prefix "I-" (i.e., I-*Ppo* I). Homing endonucleases that are spliced out at the protein level have been designated with the prefix "PI-" for protein intein (i.e. PI-*Tli* I) (7). Homing endonucleases have been shown to mediate intron and intein mobility and lateral transmission (9,10).

The seven protein splicing inteins described so far are from three different kingdoms (1,2,3,4,5,6,7,11). All contain highly conserved amino acids: cysteine or serine at the upstream splice junction and histidine followed by asparagine at the downstream splice junction (Figure 2). In addition, all downstream exteins start with either serine, threonine or cysteine; it's important to note that these three amino

acids contain hydroxyl or sulfhydryl side chains and, as nucleophiles, they presumably participate chemically in the splicing reaction.

Scientists at New England Biolabs have worked out many details of the mechanism of protein splicing *in vitro* by constructing a fusion protein consisting of the maltose binding protein of *E. coli* (M), a thermostable intein (I) and paramyosin (a truncated form of the parasite protein) (P). This allowed purification of a protein precursor which could then be induced to splice *in vitro* (12,13). The fusion protein was termed MIP (Figure 3).

The MIP construct could be over-expressed in *E. coli* and rapidly purified on an amylose column at 4°C. When the purified MIP is incubated at 37°C to 50°C it splices to yield MP and I. An example of protein splicing with purified MIP is shown in Figure 4. The numerous bands seen at time zero are *in vivo* cleavage and splicing products of MIP that co-purify on the amylose column because they either contain the maltose binding protein (MIP, MI and M) or because they aggregate with MIP (IP and I). Notice the appearance and disappearance of a band at about 180 kDa. Amino acid sequencing identified this band as a branched protein containing the N-termini of both M and I (Figure 5) (12,13).

In the branched intermediate, the bond between M and IP is stable to heating (65°C, 60 minutes) at pH 6.0, but rapidly hydrolyzes under the same conditions at pH 9.0, suggesting an ester type linkage between M and IP. Ming Xu, a scientist at New England Biolabs, has made over 85 mutations of amino acids essential for splicing in MIP and the behavior of these mutants has been critical in understanding this mechanism (12,13). These MIP mutagenesis studies indicate that if serine, the first amino acid of the downstream extein (P), is replaced by amino acids other than cysteine, there is no detectable branch formation. Therefore, we have proposed that upon cleavage at the upstream splice junction, a branched intermediate is formed containing an ester linkage between the C-terminal carboxy-

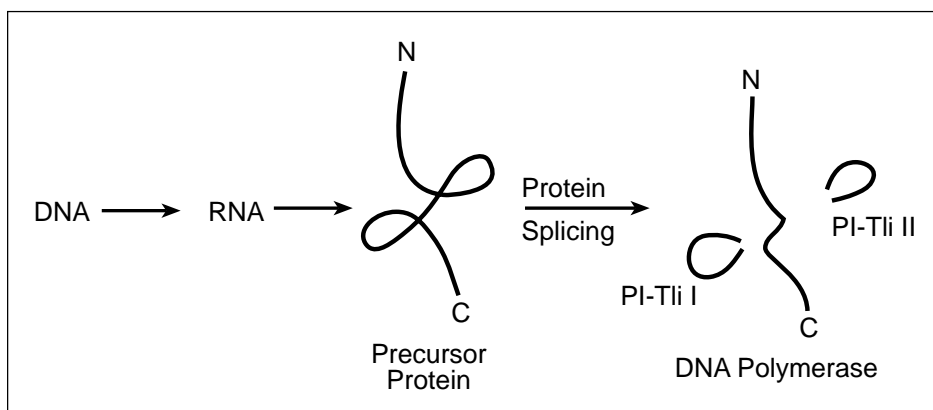


Figure 1. Protein splicing of the Vent DNA Polymerase precursor protein.

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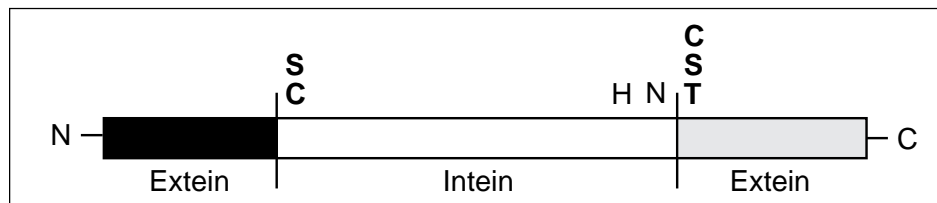


Figure 2. Conserved residues at protein splicing junctions (vertical lines).

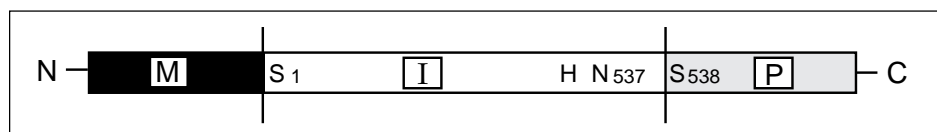


Figure 3. Structure of MIP. Numbering of amino acid positions begin with Ser1 of the Deep Vent intein.

late of M and the side chain hydroxyl of the serine at the downstream splice junction, with IP still intact.

The role of asparagine at the downstream splice junction is especially interesting, since mutations of this residue prevent splicing and inhibit C-terminal cleavage and branch resolution. We have isolated a small peptide from the C-terminus of the intein after *in vivo* or *in vitro* protein splicing and shown that the asparagine has been cyclized to form a succinimide ring (Figure 6) (13).

Our proposed mechanism of protein splicing is shown in Figure 7. We propose that the branch is resolved by cyclization of Asn resulting in cleavage of the peptide bond between I and M-ester-P. Finally, M-ester-P undergoes an O-N shift to form MP with a normal peptide bond between

M and P. Histidine is also conserved in all inteins. Mutation studies indicate that replacement with other amino acids often allows the formation of the branched intermediate but not splicing, suggesting that the amino acid His participates in resolution of the branched intermediate.

The role of the upstream serine is still unclear. Replacing it with alanine or a number of other amino acids results in no detectable splicing or N-terminal cleavage. Recent evidence from Dr. Henry Paulus' laboratory at Boston Biomedical Research Institute and Ming Xu's group at NEB indicates that when Ser1 is mutated to Cys, hydroxylamine can specifically cleave the upstream splice junction in the absence of branch formation (14). This suggests that the upstream Cys undergoes an acyl shift to form a thioester which can react with hydroxylamine. This data supports the model that the branch is formed by transesterification between this upstream ester generated by the acyl shift and the downstream serine hydroxyl. The mechanism in Figure 7 accounts for the broad features of protein splicing but many details remain to be worked out.

What are the practical applications of protein splicing? Can we use our understanding of the mechanism to produce a product? The behavior of Ming Xu's 85 mutations suggests several potential applications of protein splicing (12,13). Ming has obtained temperature sensitive mutants that splice poorly at 30°C but much faster at 37°C. Other mutants

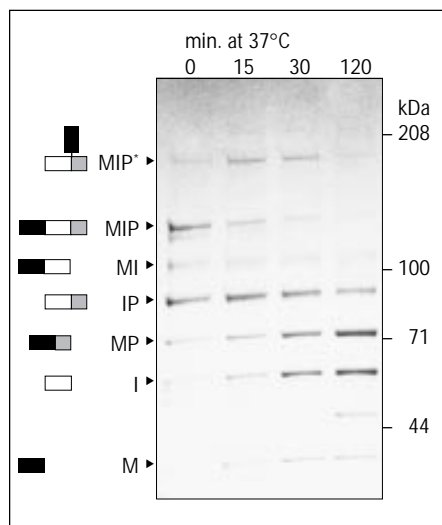


Figure 4. Time course of *in vitro* splicing of MIP (Coomassie blue stain).

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cleave at either the upstream or downstream splice junctions, but do not splice. Since the information for cleavage resides entirely in the intein, it should be possible to make vectors for purification of target proteins by substituting either of the exteins of MIP with the protein of interest. For example, MIX, where X is the protein to be cloned. This will allow a one step purification of the cloned protein on an amylose column. Warming the column to 37°C will cleave MIX to MI + X, and X may then be eluted in a highly purified form. At NEB, we have demonstrated all of these reactions and are now preparing a commercial version for the scientific community. No polyhistidine tags or proteolytic cleavage of the fusion protein—just warm it up!

Another important use may be for cloning toxic proteins. Certain proteins cannot be cloned in *E. coli*, yeast or other

expression systems, presumably because expression within a cell results in its death (e.g., restriction endonucleases in *E. coli*). By inserting the coding sequence for the intein in front of a serine residue in the toxic protein, it should be possible to inactivate the toxicity of the protein, synthesize large amounts of harmless fusion protein and then splice out the intein either in crude extracts or after purification. In the same manner, one may control protein function *in vivo* by inserting an intein into the gene of interest to inactivate its protein function or localization signal. An environmental or metabolic signal could be induced to initiate splicing; one could then observe the effect of the newly activated protein *in vivo*. We have not yet learned to precisely and quantitatively control the splicing reaction, especially *in vivo*, but we do have hints such as pH, temperature and mutation. Using a semisynthetic approach, Chris Noren's group at NEB has prepared a photoactivated protein splicing element. A protecting group on the upstream serine residue blocks splicing; visible light irradiation removes the group, unmasking the hydroxyl functionality and allowing intein excision to proceed normally (15). A major effort in these directions is underway at NEB.

In the future, inteins from these amazing extreme thermopiles may be

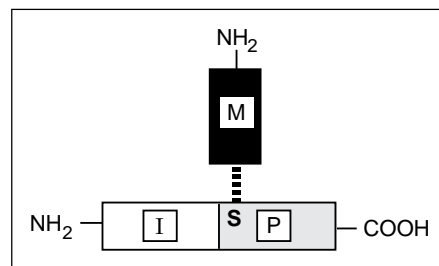


Figure 5. Structure of the MIP branched intermediate.

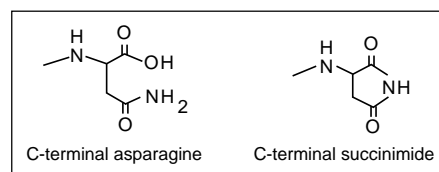


Figure 6. C-terminal asparagine cyclized to form a succinimide ring.

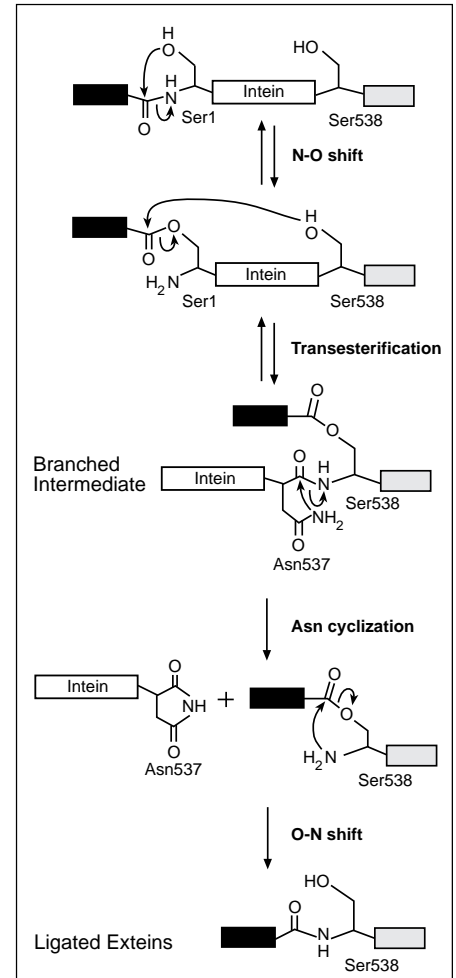


Figure 7. Proposed protein splicing mechanism.

useful clinically. They could be useful in inactivating therapeutic proteins until they reach their target site. The endonuclease activity of the intein could be used for gene knockout at precise locations when inserted into proteins that enter the nucleus. It is our opinion that protein splicing and the ability to alter the splice junctions to achieve a variety of results will have undreamed of clinical and laboratory uses in the near future.

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New

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BsoB I (Ava I)

▼C Py C G Pu G
G Pu G C Py C ▲

#586S 500 units
#586L 2,500 units

BssK I (replaces ScrF I)

▼C C N G G
G G N C C ▲

#592S 250 units
#592L 1,250 units

Tse I

▼G C (A/T) G C
C G (T/A) C ▲

#591S 75 units
#591L 375 units

Now Cloned, More Units/\$

Ssp I

▲A T A T T
T T A T A ▲

#132S 1,000 units
#132L 5,000 units

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All of NEB's enzymes and kits are supplied with tubes of optimum reaction buffer. These buffer solutions and the enclosed gel-ice packs are frozen at –20°C before they leave our facility, however, they may have thawed upon arrival at their final destination. This need not be cause for alarm as long as the gel-ice pack is cool to the touch.

Storage

The recommended temperature for prolonged storage of most enzymes is –20°C. At this temperature enzymes will not freeze (due to the presence of 50% glycerol in the storage buffer) and will remain functionally active for at least one year, in many cases a great deal longer. Some enzymes, however, have recommended storage temperatures of –70°C in which case the 50% glycerol storage buffer will freeze. This lower storage temperature should be used for long term (greater than 60 days) storage only. For periods of frequent enzyme use, it is more desirable to store the enzyme at –20°C to avoid repeated freeze/thaw cycles which can be detrimental to enzyme stability.

We have experimented with a wide variety of enzyme storage options, including frost-free and non-frost-free freezers and temperature maintaining "devices" such as freezer blocks, special racks, etc. Our conclusion is that none of these measures will significantly increase the longevity of an enzyme. We recommend an upright freezer capable of –20°C.

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Improved Protocol for Kunkel Mutagenesis in Phagemid Vectors

Site-specific amino acid substitutions in proteins can be carried out in one of three ways: a) mutations can be introduced during PCR by incorporating appropriate mismatches in one or both primers; b) a synthetic duplex cassette containing the desired sequence can be ligated between a pair of restriction sites flanking the target codon (the Code20™ Kit [NEB #7520] is a combinatorial variation of this method); and c) a mutagenic primer can be annealed to single-stranded DNA and extended, yielding a heteroduplex that resolves upon *in vivo* replication into mutant and wild-type progeny. This latter method, oligonucleotide-directed mutagenesis, is the most general but the least efficient of the three methods, due to the presence of the parental wild-type DNA strand during replication. A number of improvements have appeared during the last decade, typically involving *in vitro* or *in vivo* selection against the parent strand. The Kunkel method (1) is probably the most commonly used, since it can be used with any vector with a single-stranded replication origin and yields mutagenesis efficiencies approaching 100%. The method works as follows: single-stranded DNA is isolated from an *E. coli* strain (typically CJ236) lacking dUTPase (*dut*) and uracil-DNA glycosylase (*ung*) activity. This results in multiple uridine incorporation in place of thymidines throughout the DNA. The mutagenic primer is annealed and extended in the presence of TTP. The resulting heteroduplex is transformed into a *dut*⁺ *ung*⁺ strain, and the U-containing parent strand is rapidly degraded and resynthesized using the mutant strand as a template. This results in both DNA strands harboring the mutation prior to DNA replication.

Oligonucleotide-directed mutagenesis has historically been carried out by cloning a portion of the gene to be

mutagenized into the single-stranded phage M13, going through the procedure, and then cloning the piece back out. A number of cloning and expression vectors (e.g. the LITMUS™ and pMAL phagemid vectors) contain M13 single-stranded replication origins, allowing mutagenesis to be carried out directly in the vector without additional cloning steps. A phagemid culture is superinfected with helper phage, resulting in single-stranded phagemid being packaged and secreted into the culture medium, where it is easily isolated by PEG precipitation and phenol extraction. The following procedure for Kunkel mutagenesis in phagemid vectors has been developed at NEB (2) and reproducibly yields mutagenesis efficiencies of 70–100% for phagemids bearing the pUC replication origin (e.g. LITMUS). Lower copy number plasmids (e.g. pMAL) give lower yields (10–30%), as a result of greater relative amounts of helper phage being packaged.

Preparation of single-stranded uracil-containing template

1. Transform phagemid vector into CJ236 (*dut ung*) (expect low transformation efficiency with RbCl or CaCl₂ methods).
2. Inoculate 50 ml LB (no amp), containing 0.25 µg/ml uridine, with a fresh colony, grow at 37°C with vigorous aeration until slightly turbid (<10 Klett).
3. Add M13KO7 helper phage (NEB #315) to a final concentration of 1 X 10⁸ pfu/ml, continue vigorous aeration for 60–90 minutes.
4. Add kanamycin to a final concentration of 70 µg/ml, grow overnight (14–18 hours) with vigorous aeration.
5. Spin culture at 8,000 rpm for 10 minutes. Transfer supernatant to a new tube and spin again.

6. Pipet upper 90% of supernatant into a new tube, add 0.2 volume 2.5 M NaCl/20% PEG, let sit at 4°C for 60 minutes or overnight.
7. Recover phage by centrifugation at 8,000 rpm for 10 minutes. Discard supernatant.
8. Resuspend pellet in 1.6 ml TE, divide into 2 microfuge tubes.
9. Spin in microcentrifuge for 5 minutes to pellet any remaining cells, transfer supernatants to new tubes.
10. Add 200 µl PEG/NaCl to each, let sit at room temperature for 5 minutes, spin in microcentrifuge for 5 minutes.
11. Decant supernatant, spin briefly, remove last traces of supernatant with pipetman.
12. Resuspend each pellet in 300 µl TE, extract with phenol (let sit 15 minutes before spinning), then phenol/chloroform (twice), then chloroform. Add 30 µl 2.5 M NaOAc, pH 4.8 and alcohol precipitate.
13. Suspend dried pellets in 25–50 µl TE. Yield should be >50 µg single-stranded phagemid. For lower copy number vectors, the bulk of the single-stranded DNA will be helper phage at this point.

Phosphorylation of mutagenic primer

1. In a microfuge tube combine:
 - 1 µg Synthetic Primer*
 - (~100 pmol for 25-mer)
 - 1 µl 10X Kinase Buffer (NEB)
 - 1 µl 10 mM ATP
 - H₂O to 9 µl
 - 1 µl T4 Polynucleotide Kinase (10 units)

*Note: primer need not be gel-purified, but it should be purified by reverse-phase HPLC or C18 Sep-Pak™ (Waters) prior to use.
2. Incubate at 37°C for 30 minutes, 65°C for 10 minutes. Store at –20°C.

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Annealing of primer

- In a microfuge tube combine:
 - 1 pmol Single-stranded Template (~1 µg for 3 kb plasmid)
 - 10 pmol Phosphorylated Primer (1 µl of kinase reaction)
 - 1 µl 10X Annealing Buffer
 - H₂O to 10 µl
- 10X Annealing Buffer:
 200 mM Tris-HCl, pH 7.5
 100 mM MgCl₂
 500 mM NaCl
 10 mM DTT
 Store -20°C

- Float tube in beaker of 90°C water, allow to cool slowly to <40°C.

Extension/ligation

- In a microfuge tube combine, *on ice*, *in this order*:
 - 26 µl H₂O
 - 4 µl 10X T7 DNA Pol Buffer (NEB)
 - 2.5 µl 1 mg/ml BSA
 - 5 µl 10 mM ATP
 - 0.5 µl 10 mM dNTPs
 - 10 µl Annealing Reaction
 - 1 µl T7 DNA Polymerase* (10 units)
 - 1 µl T4 DNA Ligase (400 units)
- * T4 DNA Polymerase can be used.

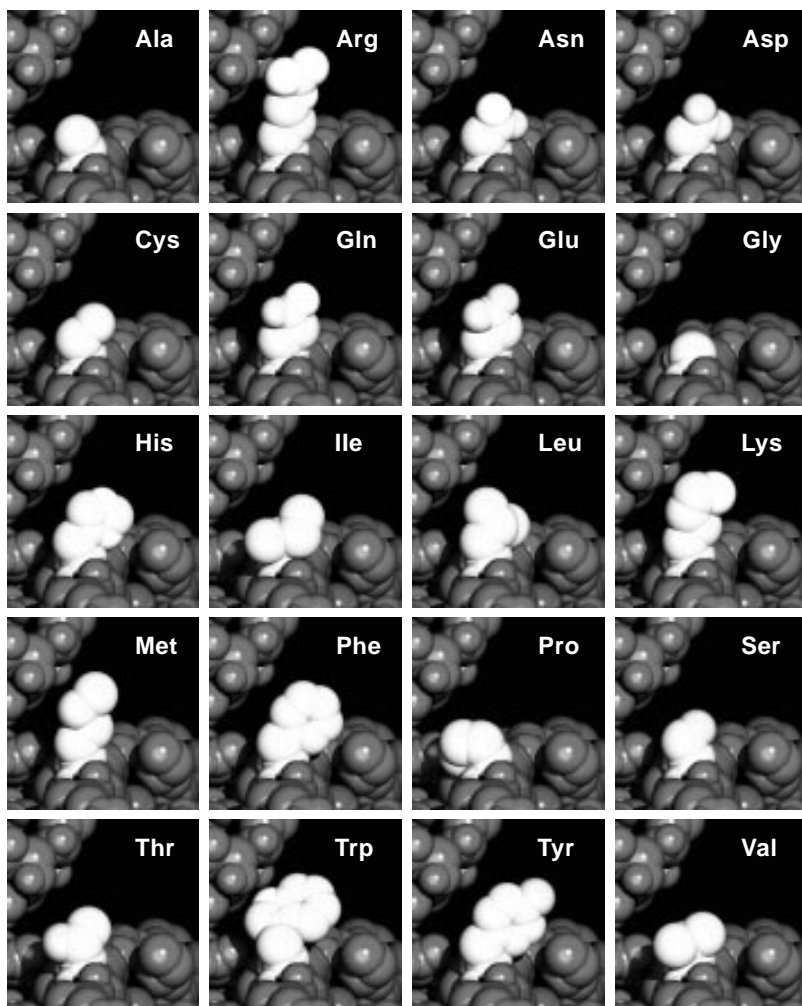
- Keep on ice for 5 minutes, then at room temperature for 5 minutes, then 37°C for 2 hours.
- We recommend transforming 5 µl and 0.5 µl of the extension reaction into a dut⁻, ung⁻ strain (we use NM522), plate on selective medium.

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- pLITMUS 28, a multisite polylinker vector with no *Sap* I recognition sequences
- Instruction Manual

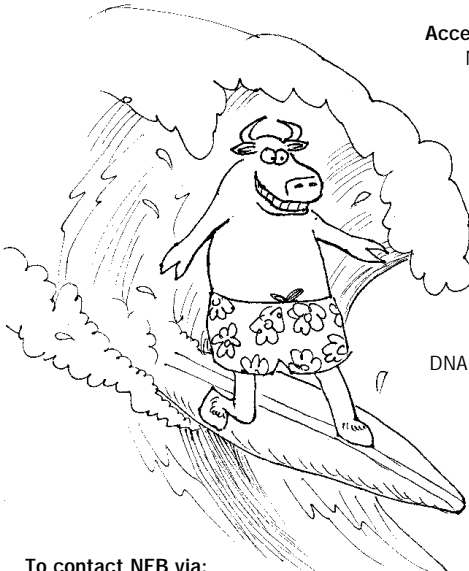
Code20™ Ordering Information:

#7520 20 substitutions

The NEB Transcript

The NEB Transcript is designed, written, and edited by the employees of New England Biolabs, Inc. It is intended to offer technical information and ideas that are useful and thought-provoking. We encourage you to share your comments with us.

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DNA Sequence Error Detection Program (SUN)

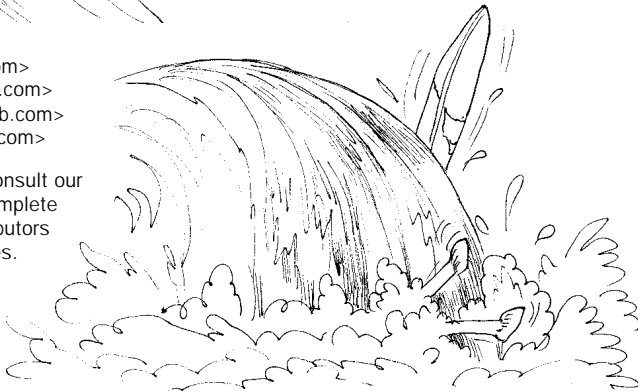
Plasmid/Vector Maps and Nucleotide Sequence Files

Crystal Structure Coordinate Files

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