

The NEB Transcript

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The IMPACT™ of Protein Splicing Research

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The central dogma of “one gene one protein” was first challenged in the late 1970s following the discovery of introns, exons and RNA splicing (1,2). Recently, another exception has emerged from a novel method of gene expression—protein splicing—which has been observed in mesophilic bacteria, yeast and extremely thermophilic archaea (3-7). This time, processing occurs at the protein level instead of the RNA level (Figure 1).

Protein splicing involves the precise excision of an internal protein segment, the intein, from a precursor protein followed by the concomitant ligation of the flanking N- and C-terminal regions, the exteins, yielding two new proteins (8). Investigation into the chemical mechanism by which inteins break and make peptide bonds has not only helped us to understand the extraordinary phenomenon of protein splicing but has also evolved into a novel method for purifying native recombinant proteins in a single step.

Chemical Mechanism of Protein Splicing

Alignment of inteins from different organisms has revealed a set of highly conserved amino acid residues at both splice junctions (9). A hydroxyl or thiol containing residue (Ser, Thr, or Cys) is always present at the position that immediately follows both splice junctions. All inteins contain an invariant C-terminal asparagine, and, in most cases, a histidine residue in the penultimate position. Splicing occurs in heterologous expression systems when the intein plus the first downstream residue (or short flanking

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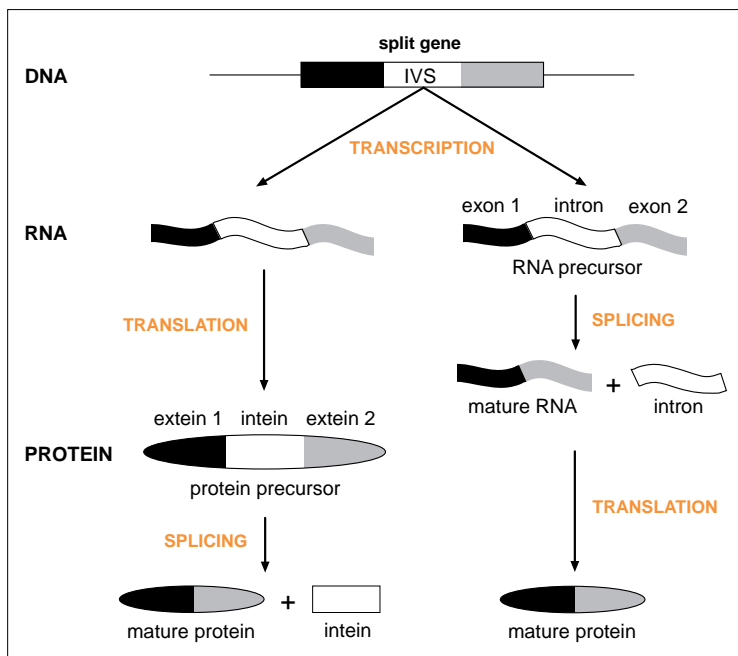


Figure 1: Protein splicing (left pathway) refines our view of genetic information transfer.

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sequences) are inserted in a foreign protein (10-13), implying that the intein plus the first C-extein residue contain sufficient structural and catalytic elements needed for the splicing reaction.

Early investigations of the chemical mechanism of protein splicing were hampered by the inability to purify precursor proteins or splicing intermediates for *in vitro* studies due to rapid splicing *in vivo*. In the past few years, scientists at New England Biolabs and their collaborators have worked out many details of the chemical mechanism (13-21). First, we took advantage of the fact that the *Psp* pol intein-1 from the DNA polymerase of the extreme thermophilic archaeon *Pyrococcus sp.* GB-D, when inserted in a foreign

protein, undergoes efficient splicing only at elevated temperatures (25–65°C). The fusion protein could be expressed in *Escherichia coli* at low temperatures (12–15°C) and purified as an unspliced precursor protein. Efficient *in vitro* splicing could then be induced at elevated temperatures (37–50°C). Mutagenesis studies and chemical analysis have identified several key splicing intermediates and defined the crucial steps in the complex protein splicing pathway (15-19).

Analysis of the mechanism of protein splicing in mesophiles presents a greater challenge because splicing reactions are extremely rapid and cannot be down-modulated by lower induction temperatures even in a foreign context. Recently, we have developed the first *in vitro* splicing system for an intein from a mesophile

using the *Sc*e VMA intein from the 69 kDa vacuolar ATPase subunit of *Saccharomyces cerevisiae* (21). Mutations were used to block various steps in the splicing pathway in order to trap precursors and intermediates for *in vitro* manipulation and chemical analysis. These studies indicate that the splicing pathway for mesophiles is the same as for extreme thermophiles. The mechanism involves a multistep reaction (Figure 2) and can be deduced from our *in vitro* studies of protein splicing using the *Psp* pol intein and the *Sc*e VMA intein:

Step 1: Formation of a thioester intermediate by an N-S acyl rearrangement at the conserved Cys residue at the N-terminal splice junction.

Step 2: Formation of a branched intermediate by transesterification involving attack by the

thiol side chain of the conserved Cys residue at the downstream splice junction on the thioester formed in Step 1.

Step 3: Branch resolution by cleavage of the peptide bond at the C-terminal splice junction by succinimide formation involving the intein C-terminal Asn residue.

Step 4: Spontaneous S-N acyl rearrangement of the transitory ligation product from a thioester to the more stable peptide bond.

Modulation of Protein Splicing

One important outcome of this work is the ability to modulate the rate of the splicing reaction through adjustments in temperature, pH or amino acid substitutions (13,18,21). For example, splicing of the fusion protein precursor containing the *Sc*e VMA intein proceeds rapidly *in vivo*. However, by introducing two substitutions (His453Leu and Cys455Ser) the splicing reaction can be slowed so as to allow isolation of unspliced precursor when expressed in *E. coli* at low temperatures (12–15°C). Efficient *in vitro* protein splicing could then be demonstrated to yield the final products (21).

Our understanding of the protein splicing pathway has enabled us to convert this process, which ordinarily consists of peptide bond cleavage tightly coupled to protein ligation, to peptide bond cleavage alone at either of the splice junctions. Dr. Shaorong Chong (in my laboratory) has found that mutation of the yeast intein by substituting the C-terminal asparagine with an alanine results in only the N-S acyl shift, the initial step in the splicing pathway, occurring at the N-terminal cysteine while the remainder of the splicing reactions are completely blocked (21). Protein precursors containing this modified yeast intein can undergo efficient cleavage at the N-terminal splice junction in the presence of a thiol reagent (i.e. β -mercaptoethanol [β -ME], 1,4 dithiothreitol [DTT] or cysteine) or hydroxylamine, even at 4°C. These nucleophiles induce a cleavage reaction by effectively shifting the N-S equilibrium in favor of the

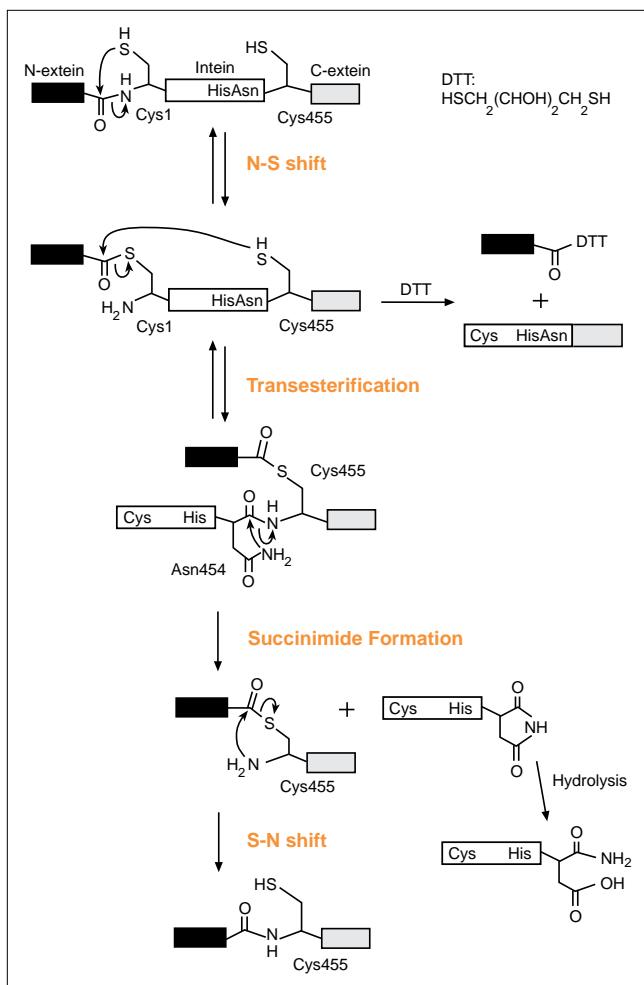


Figure 2: Proposed mechanism for protein splicing involving the *Sc*e VMA intein. For splicing of the *Psp* pol intein-1, the Cys residues shown in the diagram are replaced by Ser, so that steps 1 and 4 are N-O and O-N acyl shifts, respectively.

thioester with the net effect being cleavage of the linkage at the N-terminal splice junction.

A Novel Protein Purification System

Our studies of protein splicing reveal many potential applications. One is the use of the inducible self-cleavage activity of a modified *See* VMA intein (Y) for a novel protein purification system. The idea was to develop a self-cleavable tag, capable of carrying out specific peptide bond cleavage upon induction. In this system, as illustrated in Figure 3, a target gene is inserted upstream of the yeast intein to form an in-frame fusion between the C-terminus of the target protein and the N-terminus (a cysteine residue) of the intein. A small affinity tag (chitin-binding domain [CBD or B]) from *Bacillus circulans* has been added to the C-terminus of the intein for affinity purification of the three part fusion. The fusion protein in the cell extract is absorbed onto a chitin column, while other *E. coli* proteins are washed through. The target protein can then be released by induction of the intein-mediated cleavage reaction at 4°C in the presence of a thiol compound while the fusion partner (intein-CBD) remains bound to the column.

To test this idea, a model system in which the *E. coli* maltose-binding protein (MBP) was used as a target protein was set up by Dr. Chong to study parameters of the inducible cleavage reaction. MBP-intein-CBD (MYB) was expressed as a stable fusion protein and absorbed onto a chitin column. MBP was eluted as a single band from the chitin column after inducing cleavage at 4°C overnight with DTT or β-ME. Non-ionic detergents had no effect on the binding or cleavage reactions.

Next, *E. coli* expression vectors were constructed with multiple cloning sites for insertion of the gene of interest. Using these vectors, a number of prokaryotic and eucaryotic proteins have been expressed in *E. coli* and purified free of their affinity tag after a single chitin column (Figure 4). Thus a novel protein purification system

called IMPACT™ (Intein Mediated Purification with an Affinity Chitin-binding Tag) has been developed at NEB. This is the first protein purification system that allows cloned proteins to be purified with their native sequence in a single step. We are currently developing eucaryotic expression systems to facilitate expression and purification of eucaryotic proteins.

Advantages

The IMPACT System allows purification of tag-free recombinant proteins through a unique on-column cleavage reaction. It has a number of advantages over other affinity tag systems (22) which usually require the use of expensive and sometimes nonspecific proteases. The IMPACT System relies on the highly specific intein-mediated cleavage reaction induced with inexpensive reagents (DTT or β-ME), and the entire procedure is carried out at 4°C which is an ideal temperature for protein purification. Since binding and cleavage are not affected by high salt concentrations or non-ionic detergent, these conditions can be used to improve the solubility and purity of a target protein. In addition, the use of chitin columns is cost effective as chitin, the second most abundant organic material, is extremely stable and can be reused

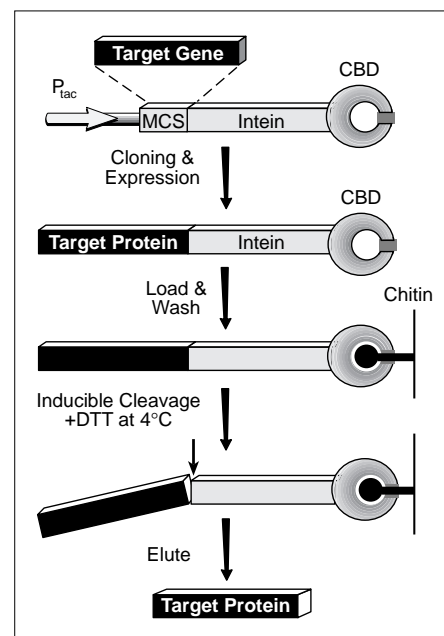


Figure 3: A schematic illustration of the IMPACT System. The target protein precursor is purified on a chitin column from a crude cell extract. Cleavage is induced by the addition of DTT and incubation at 4°C overnight. The target protein is eluted while the intein-chitin binding domain (CBD) fusion remains bound to the chitin column.

repeatedly. It is also possible to co-purify or trap a cofactor along with the protein of interest. A unique application for structural and functional analysis uses the IMPACT System to specifically label a target protein at its C-terminus. Follow-

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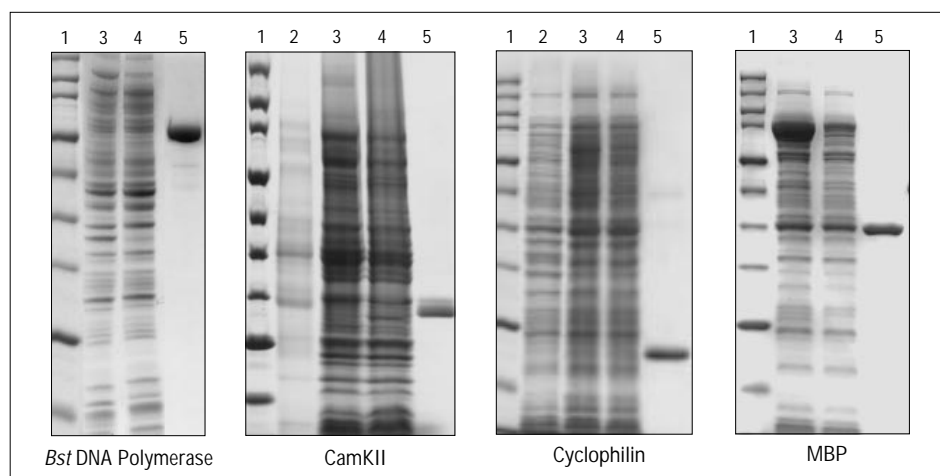


Figure 4: The IMPACT I System used to purify recombinant proteins in *E. coli*. In pCYB vectors with a T7 promoter, the following proteins were purified: Bst DNA polymerase-Large Fragment (Bst DNA Polymerase, 65 kDa) and Calmodulin-dependent protein kinase (CamKII, 33 kDa). In pCYB vectors with a P_{tac} promoter, the following proteins were purified: *Brugia malayi* cyclophilin PPIase N-terminal domain (Cyclophilin, 23 kDa) and *E. coli* maltose binding protein (MBP, 42 kDa). Lane 1: Protein Molecular Weight Marker, Broad Range (NEB #7701). Lane 2: uninduced cell extract. Lane 3: induced cell extract. Lane 4: flow through. Lane 5: eluted fraction after DTT-induced cleavage reaction (16 hr, 4°C).

impact™

One-step Native Protein Purification

Introducing IMPACT™, a new kit for obtaining recombinant proteins with native sequence after a single affinity step. Developed from basic research at New England Biolabs on protein splicing, IMPACT uses a new class of proteins, called inteins, which have been modified to undergo cleavage at 4°C when induced by thiol reagents such as DTT.

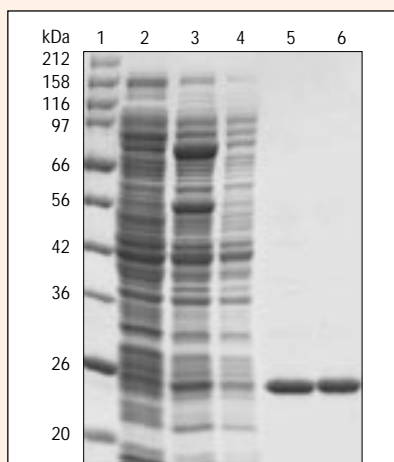
Simply clone your target protein into the supplied expression vector to create a *target protein-intein-chitin binding domain (CBD)* fusion. Bind the fusion protein to a chitin bead column, wash, induce on-column intein cleavage by adding DTT, and elute your target protein.

Advantages of the IMPACT System

- One-step affinity purification carried out at 4°C
- Yield proteins with native sequence
- No polyhistidine tags
- No proteolytic cleavage of fusion protein
- Can label target protein C-terminus

The IMPACT I Kit Includes:

- **Vector DNA**
Four *E. coli* expression vectors with different multiple cloning sites
- **Control Plasmid (pMYB129)**
- **Chitin Beads**
An affinity matrix specific for chitin binding domain
- **Anti-Intein Serum**
For western blot analysis of crude extracts or purified samples
- **Sequencing Primers**
P_{tac} Forward and Intein Reverse
- **DTT and SDS-PAGE Sample Buffer**
- **Detailed Instruction Manual**



Purification of Bgl II with > 98% purity in one step.

Lane 1: Protein Molecular Weight Marker, Broad Range (NEB #7701).

Lane 2: uninduced cell extract.

Lane 3: induced cell extract.

Lane 4: flow through.

Lanes 5 and 6: eluted fraction after DTT-induced cleavage reaction (16 hr, 4°C).

Ordering Information

IMPACT™ I System
#6500

Kit Components Sold Separately

pCYB1 Vector DNA
#6501 10 µg

pCYB2 Vector DNA
#6502 10 µg

pCYB3 Vector DNA
#6601 10 µg

pCYB4 Vector DNA
#6602 10 µg

Chitin Beads
#6651S 20 ml
#6651L 100 ml

Anti-intein Serum
#6653 100 µl

P_{tac} Forward Sequencing Primer
#1260 0.1 A₂₆₀ units

Intein Reverse Sequencing Primer
#1261 0.1 A₂₆₀ units

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ing DTT-induced on-column cleavage, the C-terminal DTT moiety of purified proteins can be replaced by radiolabeled cysteine. The initial nucleophilic attack by the sulfhydryl group of cysteine followed by a spontaneous rearrangement (S-N shift) results in a peptide bond between the labeled cysteine and the C-terminus of the target protein (21,23).

Perspectives

Complete characterization of all determinants involved in protein splicing will require further extensive mutagenesis studies and awaits a precise crystal structure of an intein and its unspliced precursor. An effective genetic selection system or *in vitro* screening system will enable us to define the minimum intein sequence structurally and catalytically essential for splicing. Nevertheless, our research has suggested many useful technologies based on protein splicing. For example, controllable protein splicing could be used for synthesis of toxic gene products if an engineered intein is inserted in front of a serine, threonine or cysteine codon in the target gene. Specific gene knockout by insertion of an inducible intein may provide a novel approach to study the biological function of a target gene in a cell. Trans-splicing reactions could reconstitute a functional protein from two independently synthesized polypeptides. An understanding of the complex protein splicing process would not only open the way to novel routes of protein engineering, but may also have significant biological implications.

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Our Canadian Subsidiary Celebrates its 8th Birthday

New England Biolabs, Ltd. is the Canadian subsidiary of New England Biolabs, Inc. Located in the Toronto suburb of Mississauga, NEB Canada has been serving the national research community since 1990.

Celebrating its eighth year of operation, NEB Canada continues to focus on its main objectives: providing exceptional service, support and value to a dedicated and expanding base of research scientists.

By maintaining a well-stocked inventory, NEB Canada is able to offer next day delivery across Canada and same day delivery within the metropolitan Toronto area. In addition, NEB Canada has implemented its Molecular Biology Reagents Freezer Program in 30 universities and hospitals across the country—each program being tailored to the unique needs of the particular institution.

New England Biolabs continues to expand into new areas beyond its traditional restriction and modifying enzyme base. New and exciting products, such as phage display libraries, phospho-specific antibodies and IMPACT protein purification systems, enable NEB to be on the forefront of novel biomolecular technologies. Extensive in-house technical support capabilities ensure that Canadian researchers receive fast and accurate responses to their molecular biology queries in either French or English.

NEB Canada is dedicated to providing exceptional customer service. The team of Ian Clarke (Business Manager), Francesca Bahr (National Freezer Program Coordinator), Suzanne Cousineau (Customer Service) and Nicole Hearst (Office Manager) are always interested in discovering and implementing new ways to serve you better. Please contact us at <info@ca.neb.com>.



Summer Workshops in Molecular Biology and PCR

We are pleased to announce the 12th annual NEB Molecular Biology Summer Workshops held at Smith College, Northampton, MA, USA. In the past 11 years, over 1,000 scientists have attended this intensive "hands-on" workshop.

Experiments Include: Genomic and cDNA library construction and screening, PCR, RT-PCR, PCR subcloning, PCR labeling, DNA and RNA purification, restriction enzyme digestion, gel electrophoresis, bacterial transformation, Southern and Northern transfer and hybridization, methods for DNA labeling, and DNA sequencing.

Intended for Beginners: No previous experience in molecular biology is required or expected. Participants (48 per session) are selected from a variety of disciplines and academic backgrounds.

Fee: \$3,200/participant. Includes: lab manual, supplies, room (single) and board.

Applications: Submit a resume and brief statement explaining your reasons for applying to the course by March 10, 1997 to: Dr. Steven A. Williams, Clark Science Center, Smith College, Northampton, MA 01063. Please specify preferred session (1, 2 or 3) and indicate a second choice.

For More Information: Visit our web page: <http://math.smith.edu/~sawlab/neb.html> or contact us by phone at (413)-247-3004.

Session 1: June 1–June 14, 1997

Session 2: June 22–July 5, 1997

Session 3: July 13–July 26, 1997

The World of Restriction Enzymes

Rick Morgan, Carol Polisson,
Janos Posfai, Ph.D., Richard J. Roberts, Ph.D.
New England Biolabs, Inc.

Almost all of the restriction enzymes known today were found by random screening of bacterial isolates. This approach involves growing a small culture of a microorganism, preparing a cell extract and incubating the extract with a small phage or viral DNA. The DNA is then examined by agarose gel electrophoresis and the production of a specific fragmentation pattern is considered diagnostic of one or more Type II restriction enzymes. Once detected, the activity is partially purified to separate it from contaminating endo- and exonucleases, and its specific recognition sequence is then determined by biochemical or computational means. During the last 25 years, experience has shown that about 25% of all strains examined show evidence of one or more Type II restriction enzymes. Occasionally, several different Type II restriction enzymes are detected in a single strain; the record is currently held by *Dactylococcopsis salina* which has six different enzymes. As a result of this screening, more than 2,750 Type II restriction enzymes have been found, comprising 211 different specificities (1).

This screening method, which relies on the production of discrete banding patterns on small DNAs, is not well suited for the detection of Type III restriction enzymes and fails completely to find Type I restriction enzymes. As a result, these systems have usually been found because of the genetic phenomenon of phage restriction. Type I enzymes have been of less interest to molecular biologists because they cleave randomly and thus do not produce specific fragments. Only 29 different Type I enzymes and 5 Type III enzymes have been characterized (1). Until recently, it was thought that Type I enzymes were of rather limited distribution and might occur only among members of the *Enterobacteriaceae*.

The situation has recently changed dramatically with the announcement of complete sequences for *Haemophilus influenzae* serotype d, of 1.83 Mb (2), *Mycoplasma genitalia*, 0.58 Mb (3) and *Methanococcus jannaschii*, 1.66 Mb (4). Computer analysis of these sequences has revealed that they abound with genes that look remarkably like restriction-modification (RM) systems. In *H. influenzae*, previous biochemical evidence had indicated that four methylases and two restriction endonucleases were present (5-7). The well known examples, *Hind* II and *Hind* III, are typical Type II enzymes while one of the methylases was guessed to be the homolog of the *E. coli dam* methylase. The fourth methylase was postulated to be part of a different Type I or Type III RM system. Examination of the complete sequence of *H. influenzae* confirms these predictions, but surprisingly also reveals putative genes that minimally code another

Type II restriction system, two Type I restriction systems and a Type III restriction system. All of these predictions are based on a clear identification of new methylase genes, which can be made with confidence because similarity between methylases is particularly strong. In each case, open reading frames adjacent to the methylase genes seem likely to code for the corresponding restriction endonucleases.

In *M. genitalia* there are two clear RM systems, while in *M. jannaschii* there are at least eleven systems. In the latter case, only two had previously been discovered biochemically (8). Examination of other new bacterial sequences in GenBank shows that methylase genes are quite common. Frequently, they lie adjacent to open reading frames with no homologs in the database, the present best indicator for a restriction-modification system. It is not yet known whether

Type I restriction enzymes recognize a specific sequence, but cleave randomly. They contain three subunits: a specificity subunit (S) responsible for sequence recognition, a methylation subunit (M) which in all known cases forms N⁶-methyladenine, and a restriction subunit (R). When a Type I enzyme encounters hemimethylated DNA, it acts as a methylase. With unmodified DNA it acts predominantly in the restriction mode, but occasionally will methylate. These enzymes require ATP, Mg⁺⁺ and S-adenosylmethionine to cleave DNA.

Type II restriction enzymes are the best known of the restriction enzyme systems. They produce specific fragments of DNA and are key to the production of recombinant DNAs. They consist of one protein which acts as a restriction enzyme and a second protein that acts as a methylase. Typically, the restriction enzyme requires only Mg⁺⁺ as a cofactor. Most of the known enzymes recognize symmetric sequences and the dimeric restriction enzyme cleaves symmetrically. The methylase is a monomer. Several subtypes of these enzymes are recognized; best known are the Type IIS enzymes, which recognize asymmetric sequences and generally cleave a few nucleotides away from the recognition sequence. A typical type IIS enzyme has two methylases, one specific for each strand of the recognition sequence. Other subtypes require more than one copy of the recognition sequence for efficient cleavage (e.g. *Nae* I or *Eco*R II) or have more complex subunit arrangements (e.g. *Bcg* I or *Eco*57 I).

Type III restriction enzymes have properties intermediate between Type I and Type II. They are multisubunit enzymes, recognize specific sequences, but typically cleave 20 to 25 nucleotides away from that sequence and rarely give complete cleavage. The restriction activity requires ATP, and is enhanced by S-adenosylmethionine. Only five such enzymes have been characterized.

these systems are cryptic and able to be activated in response to environmental conditions, or whether they are merely evolutionary relics.

It was not too surprising that additional RM systems might be found through genomic sequencing, since extensive work with *Neisseria gonorrhoea* had indicated that within that organism there were both multiple methylases, as well as cryptic restriction systems that could be found by cloning (9). The major surprises have been the extensive occurrence of Type I systems and the density of restriction systems per megabase of bacterial DNA, which is currently running at one system per 200 kb. The present findings point out the limitations of using only a biochemical approach to screening. It will be of interest to discover if some of the new systems, identified by computer, are merely isoschizomers of known enzymes or harbor new specificities, perhaps with new kinds of properties that will be useful for the preparation and analysis of recombinant DNAs. In the past, screeners began with an organism and an agarose gel, using the computer only to help characterize the recognition sequence of any enzyme found. In the future, screeners may begin their search with a good computer program to scan GenBank and only use their agarose gels to confirm that a restriction enzyme is present.

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Biotinylated RNA Molecular Weight Markers

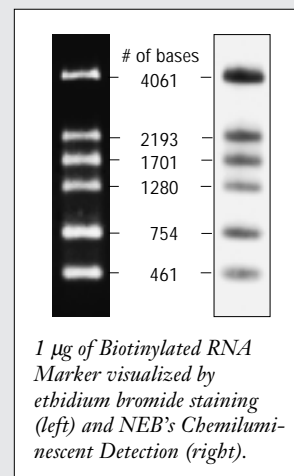
NEB introduces Biotinylated RNA Molecular Weight Markers (#361BT) to complement our RNA Molecular Weight Markers (#361).

The biotinylated markers are made by *in vitro* transcription of six linear DNA templates in the presence of biotin-14-CTP. The level of biotinylation has been optimized such that 1 µg of RNA marker results in a clearly visible pattern by ethidium bromide staining and an appropriate chemiluminescent signal after Northern blotting and detection.

Both RNA Markers are supplied in 20 mM KOAc (pH 4.5) for enhanced stability. They are suitable for use on native or denaturing agarose or polyacrylamide gels.

RNA Molecular Weight Markers
#361 25 µg

Biotinylated RNA Molecular Weight Markers
#361BT 25 µg



cloned
more units/\$

Tsp509 I (10X more/\$)

▼ A A T T
T T A A ▲

#576S 1,000 units
#576L 5,000 units

Improved production efficiency has allowed us to introduce substantial price reductions for the following recombinant enzymes.

DNA Polymerase I, Large (Klenow) Fragment (60% more/\$)

#210S 200 units
#210L 1,000 units

Klenow Fragment (3' → 5' exo-) (60% more/\$)

#212S 200 units
#212L 1,000 units

T7 DNA Polymerase I (unmodified) (50% more/\$)

#256S 300 units
#256L 1,500 units

new!
endonucleases

BclV I

G T A T C C N₆▼
C A T A G G N₅▲

#596S 200 units
#596L 1,000 units

BstZ17 I (replaces Bst1107 I)

G T A T A C
C A T A T G ▲

#594S 1,000 units
#594L 5,000 units

Pfl I (Tth111 I)

G A C N₄▼ N N G T C
C T G N N N ▲ C A G

#595S 1,000 units
#595L 5,000 units

Sml I

▼ C T P y P u A G
G A P u P y T C ▲

#597S 200 units
#597L 1,000 units

For over 20 years, the NEB Catalog has been a resource for scientists around the world.

Now, we have dedicated some of its pages to raise awareness about issues that threaten the beauty, diversity and productivity of the earth's unique environments—this year, we invite you to join us as we celebrate coastal habitats.

As a follow up to our 96/97 Catalog's theme of endangered coastal zones, Elliott Norse of the Marine Conservation Biology Institute provides insight into one of the major threats to coastal and shelf fisheries around the world.

Bottom Trawling: The Unseen Worldwide Plowing of the Seabed

*Elliott A. Norse, Ph.D., President,
Marine Conservation Biology Institute
Redmond, WA*

In Gilbert and Sullivan's *Mikado* (1885), Pitti-Sing happily expresses the prevailing belief that “there's lots of good fish in the sea.” Sadly, the intervening century has proven her wrong. In the USA and worldwide, most fisheries are much less productive than they once were. The teeming schools of Atlantic cod and haddock that fueled the economies of New England and the Maritime Provinces are now gone. Their population has declined so steeply that the USA and Canada finally closed cod fishing on Georges Bank and Grand Bank. In warm-temperate and tropical waters, snappers and groupers as large as turkeys and sheep that once swarmed above reefs have all but disappeared; those few that remain now fit in a frying pan.

The obvious cause is overfishing: catching fish faster than they can replace themselves. The remedy (at least in theory) is simple: catch fewer, allowing fish time to grow, reproduce and rebuild their populations, then catch only as many as can be sustained in perpetuity. Unfor-

tunately, fishery management processes in nations worldwide have one thing in common: most have failed to prevent overfishing. But while there is no doubt about the impact of overfishing, there is another, more insidious cause: people are altering the habitats where fish feed, breed and grow to maturity. We do it in many ways. Among the better-known are killing coral reefs with dynamite or bleach, polluting estuaries to the point where even tough species cannot survive, paving over mangrove forests and salt marshes to build housing, shopping centers and roads and dredging harbors to accommodate bigger ships. But the most widespread way that humans alter fish habitat has received astoundingly little scrutiny. It is bottom trawling.

Trawling is a method of catching fish and crustaceans that accounts for more of the world's catch than any other. In bottom trawling, individual boats or ships from 10-100 meters long (or, in some cases, pairs of ships, called pair trawlers) pull large weighted nets across the seabed. Most often the nets are held open by a pair of otter boards (also called doors) that can weigh anywhere from hundreds of kilograms to a ton or more. A line stretched across the bottom of the net's mouth can be weighted with many metal bobbins each weighing tens to hundreds of kilograms, or can be armed with metal tickler chains that disturb the seabed and cause fish or shrimp to rise from the bottom and get caught in the net. Rock-hopper or roller trawls are equipped with heavy wheels that roll over obstructions such as boulders or coral heads, reducing the risk that nets will hang and be lost. Another type of trawl, the beam trawl, is held open by a massive steel beam instead of otter boards. Scallops are caught by towing dredges (heavy chain bags attached to massive steel frames) through the seabed. A typical boat pulls two 4-5 meter wide dredges that plow through the sediment and funnel sand, gravel and

animals through the chain bag.

When trawlers (sometimes called draggers) and other mobile gear fishers fish for “groundfish,” shrimp or scallops, they plow the seabed churning sediments to a depth of 10-15 centimeters, leaving deep gouges and overturning rocks. This would not be a problem if the seabed were lifeless, but like a forest, prairie or desert, the seabed is actually a diverse, spatially complex realm, with hundreds or thousands of species living in and on it. Some tunnel in it; others extend their tubes, tentacles or gills into the water column to capture drifting food particles and oxygen. They are important in their own right and because they provide food and hiding places for other organisms, including the fish and shrimp we catch.

Trawling crushes, buries and exposes marine organisms (such as lobsters, amphipod crustaceans, clams, tubeworms, moss animals, corals and sponges) that live on or in the seabed, damaging or killing them. Some animals whose homes are destroyed cannot reconstruct them, which makes them vulnerable to scavenging fish and crabs. Others that are less vulnerable nonetheless show the effects of trawling; starfish in more heavily trawled areas have a higher incidence of damaged and regenerating arms.

No type of seabed—whether clays and silts (muds), sands, gravels, cobbles, boulders or rocky reefs, seagrass beds, worm reefs, mussel beds, sponge reefs or coral reefs—is immune from trawling.





Shrimp trawlers can catch up to 20 kilograms of animals they do not want for every kilogram of shrimp. But this is only part of the story—bottom trawling for shrimp and fish crushes, buries and exposes to predation countless numbers of seabed animals that are not brought on deck. Photo by Elliott Norse.

Indeed, on bottoms with lots of “hangs” (e.g., coral reefs), trawlers sometimes drag heavy gear expressly to pulverize them; they call it “conditioning the bottom.” Bottom trawling traditionally occurred on the continental shelf at depths of tens to hundreds of meters, but as more and more continental shelf fish stocks have been overfished, fishermen seeking “underexploited” species have trawled the continental slope in depths of 1,000 meters and more. In recent decades, trawling has also extended geographically, from traditional fishing grounds such as Georges Bank along the continental margins of industrialized nations, to waters of sparsely-inhabited developing nations and even remote oceanic seamounts of the Southern Ocean surrounding Antarctica.

This would be a nonissue if trawling were limited in scope, but trawlers sweep a vast area of seabed. A small fleet of 100 shrimpers towing nets 25 meters wide, at

5 kilometers/hr for 10 hours a day, for 200 days/year would cover 25 billion m², or 25,000 km² each year, an area larger than the state of Massachusetts. The US alone has thousands of bottom trawlers. And some places get trawled many times a year. Trawling's effects are similar to terrestrial activities such as plowing, clear-cutting or strip-mining, yet its impacts are so little-appreciated that it is even allowed in marine protected areas, such as US National Marine Sanctuaries.

To gain a comprehensive understanding of the effects of bottom trawling on the world's marine ecosystems, the Marine Conservation Biology Institute held a scientific workshop at the University of Maine's Darling Marine Center in Walpole, ME (USA) on June 28-30, 1996. Participating were 17 marine scientists (systematists, ecologists, fishery biologists, biogeochemists and geologists), from Australia, New Zealand, Canada, USA and United Kingdom. More than 20 others from government agencies, the fishing industry, environmental organizations and the electronic and print media observed the proceedings and interacted with the participants.

The scientists agreed that trawling is the most important source of human-caused physical disturbance on the world's continental shelves. We concluded that, by crushing marine animals and their habitats, trawling greatly reduces structural complexity of the seabed. This harms organisms (including commercial fish and lobsters) whose early life history stages benefit from complex seabed structures. Trawling also alters seabed biogeochemistry and causes clouds of sediment to rise into the water column, where they can affect processes that depend on water clarity, from feeding by fish to photosynthesis. We acknowledged that different places are trawled at very uneven rates, with some spots getting dragged as many as 100 times a year. We recognized that trawling in some places

might actually maintain conditions that favor certain commercially fished species, but that in others it likely contributes to declining fisheries. Nonetheless, all bottom trawling alters the seabed.

Humans are uniquely able to affect the world around us and to recognize the consequences and change what we do. With 5.8 billion people to feed, fish is an important source of animal protein and fishing provides a way of life for several million people worldwide. But fishers, the rest of us and the rich diversity of marine life all pay the cost of destructive fishing practices. Surely there are ways to lessen the impact of trawling. And surely there are some places in the sea so important that we should protect and restore them. With our fisheries collapsing, it wouldn't hurt to try.

Marine Conservation Biology Institute

Established in 1996, the Marine Conservation Biology Institute (MCBI) is a nonprofit environmental organization dedicated to safeguarding life in the sea by advancing the multidisciplinary science of marine conservation biology. MCBI holds scientific workshops on emerging marine conservation issues and is organizing the first Symposium on Marine Conservation Biology at the annual meetings of the Society for Conservation Biology at the University of Victoria in Victoria, British Columbia, Canada on June 6-9, 1997.

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Sfi I: An Unconventional Restriction Enzyme

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Restriction enzymes remain essential tools for the analysis and reconstruction of DNA molecules, without which the progress of Molecular Biology during the last twenty years would not have occurred. Consequently, considerable efforts have been extended to find new restriction enzymes, particularly those in the Type II category that cleave DNA specifically at or next to their recognition sequences, as opposed to the Type I and III enzymes that cleave far away from their recognition sites (1). Historically, restriction enzymes were first isolated by purifying the activities responsible for the restriction of phage growth in bacteria but nowadays they are almost always discovered by looking for activities in cell-free extracts that cleave DNA into discrete fragments. The latter strategy has to date yielded over 2,700 restriction enzymes from many different species of bacteria although only a few of these have ever been shown to act in phage restriction (2). Between them, the 2,700 known restriction enzymes recognize over 210 distinct DNA sequences, often palindromic sites 4 or 6 base pairs (bp) long (2).

Some progress is currently being made towards understanding how restriction enzymes recognize and act at their target sites on DNA, although this has focused on just a handful of examples (1). Crystal structures have been solved for *Bam*H I, *Eco*R I, *Eco*R V, *Pvu* II (3) and *Cfr*10 I. These are all dimeric proteins that interact with 6 bp sequences in symmetrical fashion, so that the two active sites in the dimer are positioned against the two scissile bonds in the DNA, one on each strand. They have very different amino acid sequences

(4) but the structural studies and other studies on their mechanism of action indicate two different classes, one typified by *Eco*R V and *Pvu* II and another by *Eco*R I and *Bam*H I (3). Nevertheless, many restriction enzymes have recognition sites that deviate from the conventional pattern of 4-6 bp palindromes (1,2) and these might interact with DNA by different mechanisms from either the *Eco*R V or the *Eco*R I modes. One such enzyme is the *Sfi* I endonuclease, which was discovered at New England Biolabs (5) where its gene was subsequently cloned, sequenced and overexpressed (4).

The recognition sequence for *Sfi* I, GGCCnnnn/nGGCC (where “n” is any base and “/” the point of cleavage), consists of two 4 bp symmetry-related elements interrupted by a 5 bp spacer of unspecified bases (5). This site looks more like a target sequence for a site-specific recombination enzyme, such as FLP or *cre* (6), than a typical restriction site. Indeed, recent biochemical studies on *Sfi* I have shown that its mechanism of action has more in common with recombination enzymes than most restriction enzymes and that it certainly falls outside either the *Eco*R V or *Eco*R I families (7-9).

Instead of being a dimeric protein that interacts symmetrically with one copy of its recognition site, *Sfi* I exists in solution as a tetramer of identical subunits (7). The tetrameric structure might have reflected its recognition site, with possibly two subunits being needed for each GGCC palindrome. But *Sfi* I reactions on a plasmid with two *Sfi* I sites result in the majority of the DNA being converted directly to the product cut at both sites: only a small fraction of the DNA is liberated after cutting at just one site and, since this is formed more slowly than doubly-cut DNA, it cannot be an intermediate on the main route to the final product (7,8). In addition, plasmids with one *Sfi* I site are cleaved more slowly than plasmids with two sites, although the rates on DNA with one site

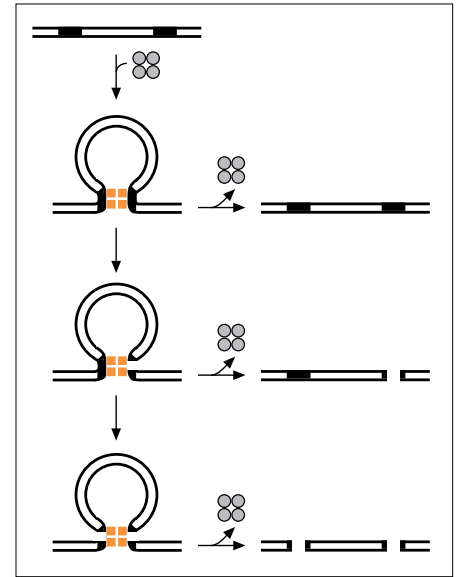


Figure 1: The binding of the tetrameric *Sfi* I endonuclease (gray circles) to two *Sfi* I sites on the DNA (filled segments in black lines) loops out the intervening DNA and produces a conformational change in all 4 subunits (orange squares). The protein will then dissociate from the DNA, before cutting the DNA or after cutting either one or both sites, depending on the relative rates for cleavage and dissociation.

can be enhanced by adding a 20 bp duplex containing the recognition site for *Sfi* I (7).

The *Sfi* I nuclease thus needs to interact with two *Sfi* I sites before cleaving DNA (7). The binding of the tetrameric protein to two sites *in cis*, on the same DNA molecule (Figure 1), loops out the intervening DNA (9). In most instances, both strands of the DNA at both sites are then cleaved before the loop dissociates, although sometimes the loop falls apart before both sites are cut: the latter accounts for the small fraction of singly-cut DNA from substrates with two sites (8). Alternatively, the *Sfi* I tetramer appears to be able to act *in trans*, bridging two DNA molecules by binding to a site on each molecule. However, *trans* interactions between sites on separate DNA molecules are disfavored relative to *cis* interactions (10). This explains why substrates with one *Sfi* I site are cut less efficiently than substrates with two sites (7).

These views were validated by experiments on catenanes made from two rings of DNA, with one *Sfi* I site on each ring (Figure 2): the catenane was cleaved by *Sfi* I more rapidly than the two separate

rings and gave as its principal initial product two linear fragments, rather than one linear DNA and one circle (9). The need for two *Sfi* I sites may be due to a concerted conformational change in all 4 subunits (Figure 1) since the enzyme has a very low (perhaps zero) activity when bound to one copy of its recognition sequence (8).

With 8 specified bp in its recognition sequence, *Sfi* I sites occur on most DNA molecules at lower frequencies than either 4 or 6 bp sites. Consequently, *Sfi* I is a widely-used enzyme in the analysis of large genomes, even though it has the reputation of being a “difficult” protein that often gives partial rather than complete digests. The enzymology of *Sfi* I explains why it might have acquired this reputation even though the protein is extraordinarily stable (5). Looping interactions with sites *in cis* on linear DNA become progressively less stable as the length of DNA between the sites is raised above 1 kb, though such interactions can still take place on supercoiled DNA (10). Most applications of *Sfi* I will therefore utilize its *trans* reactions, spanning sites on separate molecules. In contrast to *cis* interactions, *trans* reactions by *Sfi* I occur more readily with linear

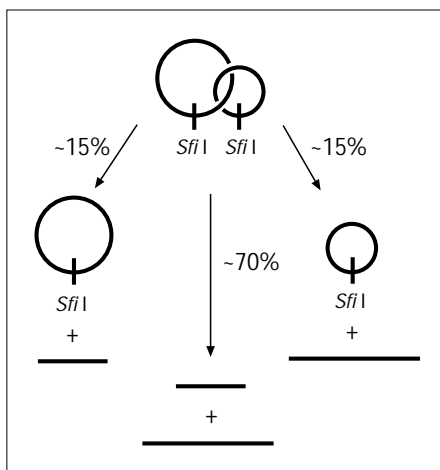


Figure 2: A catenane made from two DNA circles of different sizes, with one *Sfi* I site on each ring, can be cleaved by *Sfi* I at one site to give either a large linear and a small circular product (right-hand route) or vice-versa (left-hand route), or it can be cleaved concertedly at both sites to give two linear products (central pathway). The fraction of the DNA cleaved by each route is shown (%) by each arrow.

substrates than supercoiled substrates, presumably because a linear DNA can penetrate the volume occupied by another DNA molecule more easily than a supercoiled DNA (7). *Trans* reactions are also aided by increasing the total DNA concentration but, perversely, *Sfi* I reactions can be hindered by increasing the enzyme concentration. In reactions with enzyme in excess of DNA sites, each site becomes loaded with a *Sfi* I tetramer, thus preventing the protein from binding the two sites necessary for full activity (9).

The characteristics of the *Sfi* I endonuclease suggest that it would be inefficient at restricting the growth of most phage that might infect its host, *Streptomyces fimbriatus*. While many restriction enzymes cleave DNA at sequences like GGCC, *Sfi* I has to first find two GGCC sequences separated by 5 bp followed by another pair of GGCC elements separated by 5 bp. The minimal sequence needed for a *Sfi* I reaction must therefore occur on phage chromosomes at much lower frequencies than individual GGCC sites. If a phage chromosome has a *Sfi* I site, then it still might be possible for this endonuclease to act against infections at high multiplicities by using *trans* reactions to destroy two chromosomes at a time (7), but *Sfi* I is unlikely to work efficiently with sites *in trans* under intracellular conditions (8) and the multiplicity would have to exceed the number of molecules of *Sfi* I endonuclease in the cell (9).

The temperature optimum for *Sfi* I is 50°C (5) while *S. fimbriatus* can only be cultured *in vitro* below 30°C. The rates of *Sfi* I reactions at 50°C are similar to those for *EcoRV* or *EcoRI* at 37°C but the turnover rate for *Sfi* I at 30°C is remarkably slow, solely because the cleaved DNA at the end of the reaction takes about 30 minutes to dissociate from the enzyme. Thus, under *in vivo* conditions, *Sfi* I appears to hang on to the DNA termini that it generates, which might allow these ends to be acted upon by other cellular factors to bring about some kind of genome rearrangement. Certainly,

the way in which the *Sfi* I endonuclease brings together distant DNA sites before carrying out its reaction is shared with many enzymes mediating site-specific recombination (6), but whether this is the biological function of the *Sfi* I “restriction” endonuclease has yet to be determined.

Acknowledgments

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phage display

Introducing the Ph.D.-12™ Phage Display 12-mer Peptide Library

Phage display creates a physical linkage between a vast library of variants of a binding function (short peptides or larger proteins) to the gene encoding each variant, permitting identification of individual binding clones by DNA sequencing. Over the last 7 years random peptide libraries displayed on phage have been extensively used to select for peptide ligands specific for a variety of targets (antibodies, receptors, enzymes, etc.) by multiple cycles of *in vitro* selection (biopanning) and *in vivo* amplification (1,2).

New England Biolabs is pleased to offer a second combinatorial phage display peptide library, the Ph.D.-12™ Kit. Like our popular Ph.D.-7™ Kit, the Ph.D.-12 Kit is based on a highly diverse library of random linear peptides displayed on the surface of M13 phage, but with the diversity spread out over a "window" of 12 residues rather than 7. This permits affinity selection of peptide ligands for target binding sites that require more than 7 ligand residues for binding, or require interaction with fewer residues spaced too far apart to be contained within a 7-residue peptide. Additionally, the increased length of the displayed peptide allows folding into small structural elements (short helices, β -turns, etc.) that may be required for target binding.

The Ph.D.-12 library consists of 1.9×10^9 independent clones amplified once to yield ~70 copies of each sequence in 10 μ l of the supplied library. Like the Ph.D.-7™ library, each 12-mer sequence is fused to the N-terminus of the minor coat protein pIII, with the first position of the mature displayed fusion protein being the first randomized position. Each peptide is followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild-type pIII sequence. Extensive sequencing of the naive library has revealed a wide diversity of side chains at each position

with no obvious biases. The library has been successfully used at NEB to screen for peptide ligands for streptavidin, monoclonal antibodies and cell-surface receptors.

The Ph.D.-12 Kit Includes:

- **Phage Library**
12-mer Phage Display Library, complexity $\sim 1.9 \times 10^9$ sequences. 1.4×10^{12} phage supplied, 1.4×10^{11} phage used per experiment
- **Sequencing Primers (100 pmol)**
–28 gIII Sequencing Primer, for manual sequencing

–96 gIII Sequencing Primer, for automated sequencing
- **Host Strain**
- **Control Target (Streptavidin) and Elutant (Biotin)**
- **Detailed Protocols**

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Ordering Information

Ph.D.-12 Phage Display Peptide Library Kit (Introductory Price until March 1, 1997)
#8110 10 Biopanning Exp.

Ph.D.-7 Phage Display Peptide Library Kit
#8100 10 Biopanning Exp.

–28 gIII Sequencing Primer
#1258 0.1 A₂₆₀ units

–96 gIII Sequencing Primer
#1259 0.1 A₂₆₀ units

ER2537 Host Strain
#801-N glycerol culture

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Identification of a B-cell Epitope of β -endorphin with the Ph.D.-12™ Library

Mapping the immunogenic surfaces of a protein antigen (epitope mapping) has historically been carried out by immunoblotting a systematically constructed series of deletion and point mutants of the antigen. This will identify which residues are required for antigen recognition. The advent of phage display peptide libraries has greatly simplified the epitope mapping process: a single random peptide library can in principle be used for any antigen/antibody combination. By biopanning the library against each antibody, the epitope sequence recognized by each antibody can be identified in as little as one week.

The Ph.D.-12 library was biopanned in solution (10 nM antibody) against a monoclonal antibody raised against the opioid neuropeptide β -endorphin, followed by affinity capture of the antibody-phage complexes onto Protein A-agarose (rounds 1 and 3) or protein G-agarose (round 2) beads. Selected third round sequences are shown aligned with the first 14 residues of β -endorphin. Two of the sequences appeared twice each as indicated in parentheses. The results clearly show that the epitope for this antibody spans the first 7 residues of β -endorphin (YGGMTS), and the conserved position of the selected sequences within the 12-mer indicates that the free α -amino group of the N-terminal tyrosine of β -endorphin is part of the epitope. The results also suggest that most of the binding energy for the antigen-antibody interaction is contributed by the first 4 residues of the epitope (YGGF), with some flexibility allowed in the third position.

β -Endorphin

Y	G	G	F	M	T	S	E	K	Q	T	P	K	V	...
Y	G	G	F	M	T	T	P	S	H	V	P	(2)		
Y	G	G	F	I	S	Q	T	Q	H	Y	S	(2)		
Y	G	G	F	G	N	S	L	V	M	P	V			
Y	G	G	F	S	M	P	F	L	P	A	L			
Y	G	A	F	D	V	T	T	G	V	T	S			
Y	G	V	F	N	P	H	Y	L	P	S	L			

A P S T D K Q A T M P L
A S V A V S S R Q D A A

New Vectors for Gene Targeting

Roberto D. Polakiewicz, Ph.D.

Rachel Friedman

New England Biolabs, Inc.

Gene targeting in mice via homologous recombination is one of the most powerful tools available today for studying gene function in higher organisms (1,2). The procedure involves the production of mice carrying a precise mutation in a gene of interest. Gene targeting has also been used to generate mouse models to study human genetic diseases (3), as well as to shed light on other important biological questions such as genomic imprinting (4), X-chromosome inactivation (5), and learning and memory (6).

In addition to generating loss-of-function mutations, a wide range of sophisticated genetic manipulations can be performed—from subtle modifications such as small deletions or point mutations to complex schemes that result in chromosomal deletions and translocations. A new generation of inducible “knockout” plasmids enable the silencing of particular genes at a precise development time and specific tissue or cell lineage. This will provide insight into the function of these genes in the adult while bypassing the developmental effect. This “knockout” technology is extending our ability to modify the genome of an experimental animal to limits hardly imaginable only a few years ago (7).

Gene targeting initially requires the use of standard recombinant DNA technology to create a mutation in cloned DNA. This involves extensive restriction mapping and subcloning of genomic DNA segments into a construct where a positive selection cassette is often used to disrupt the integrity of the transcription unit. Once the targeting vector containing the mutated gene is made, it is introduced into embryonic stem (ES) cells by transfection, followed by selection and screening of clones that have undergone homologous recombination, replacing the wild-type copy of the gene with the mutated one.

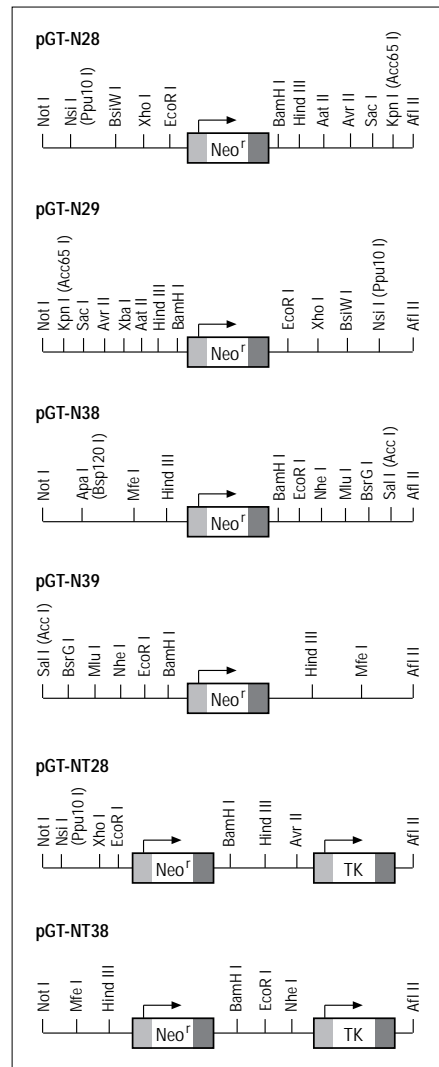


Figure 1: Polylinkers of the pGT vectors. Only unique sites are indicated.

Correctly targeted ES clones are micro-injected into mouse blastocysts to generate the founders of the new mouse strain, which must be then bred to homozygosity at the mutant locus.

This long and labor intensive process usually requires several months until homozygous mutant mice are ready for phenotypic analysis. To facilitate this process, we have developed the pGT series of plasmids that make the design and construction of gene targeting vectors easier and faster. Features of the plasmids (see inset) include positive and negative markers for selection in ES cells and the necessary elements for propagation in bacteria as found in our LITMUS cloning vectors (8).

Features of the pGT Plasmids:

- Multiple cloning sites surrounding the positive selection cassette facilitate direct cloning of genomic DNA (Figure 1)
- Selection markers: neomycin resistance gene (Neo^r) for the positive and HSV thymidine kinase gene (TK) for the negative—both driven by the phosphoglycerate kinase gene promoter (PGK)
- *Not I* and/or *Sal I* sites facilitate plasmid linearization prior to transfection into ES cells
- M13 origin of replication allows generation of single stranded DNA for rapid site-directed mutagenesis and sequencing
- All plasmids are tested for their ability to generate G418 resistant ES cell clones and gancyclovir sensitive clones when applicable

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Ordering Information

pGT-N28 #307-28	20 µg
pGT-N29 #307-29	20 µg
pGT-N38 #307-38	20 µg
pGT-N39 #307-39	20 µg
pGT-NT28 #308-28	20 µg
pGT-NT38 #308-38	20 µg
pGT Vector Pack (all six) #309	20 µg each

In vivo, in vitro, in coli

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With the advent of recombinant DNA technology in the mid-70's, there was suddenly the promise of producing large amounts of previously inaccessible proteins by expressing their genes in *E. coli*. In large measure this promise has been fulfilled as witnessed by dramatic research advances and the boom of the biotechnology industry. NEB has long been committed to cloning and overexpressing the enzymes that we sell and *E. coli* is usually the host of choice. For most of our restriction enzymes, the native source is a poorly-characterized microorganism and so we have employed rigorous quality control measures to ensure that the recombinant product is functionally equivalent to the native product. No significant differences have been seen between native and recombinant restriction endonucleases.

However, our characterization efforts have led to an interesting discovery about Protein Phosphatase 1 (PP1), the catalytic

Recombinant PP1

The recombinant Protein Phosphatase 1 (PP1) we offer for sale has activity against phosphoserine/threonine residues that is comparable to that of the native enzyme. Use of the recombinant enzyme is recommended in all instances where concomitant dephosphorylation of phosphotyrosine residues is unimportant. However, it should not be used to distinguish phosphoserine/threonine residues from phosphotyrosine residues in proteins, unless it is used in combination with our tyrosine-specific phosphatases (LAR, T-Cell and YOP).

Protein Phosphatase 1 (PP1)
#754S 40 units
#754L 200 units

subunit of the α -isoform of type 1 protein phosphatase 1 from rabbit skeletal muscle, when expressed in *E. coli*. PP1 is probably the best understood and most extensively characterized of all of the protein phosphatases. It has been studied in detail following purification from native sources (1) and the gene has been cloned and overexpressed in *E. coli*. (2). It was noted early on that the recombinant enzyme expressed in *E. coli* differed from the native enzyme (3). Particularly, recombinant PP1 required Mn^{++} ions to obtain maximal activity. This recombinant material was also used to obtain crystals of the enzyme, complexed with the inhibitor microcystin, and structures were recently reported (4). These structures demonstrated an unexpected property of the enzyme in that one cysteine residue (Cys-127) was present in an oxidized form (either a sulphanyl or sulphonyl). It was suggested that because Cys-127 was located at some distance from the active site it might explain the Mn^{++} ion requirement, but a possible functional effect was not investigated. It remains unknown if the enzyme produced in rabbit skeletal muscle also carries this oxidized cysteine residue.

In more recent experiments (T. Barshevsky, New England Biolabs, unpublished) yet another difference has been found between the native enzyme and the recombinant enzyme. As part of our continuing quality control of PP1, we have tested as substrates various proteins containing either phosphoserine/threonine or phosphotyrosine residues. This was to ensure that the specificity of native PP1 was maintained in our recombinant preparation. The native enzyme prefers phosphoserine/threonine more than 2,000-fold over phosphotyrosine residues in proteins (Figure 1). Surprisingly, recombinant preparations of both α and γ isoforms from *E. coli* differ dramatically from the native enzymes in their substrate preference (Figure 1). Depending upon the substrate, the ratio of phosphoserine/

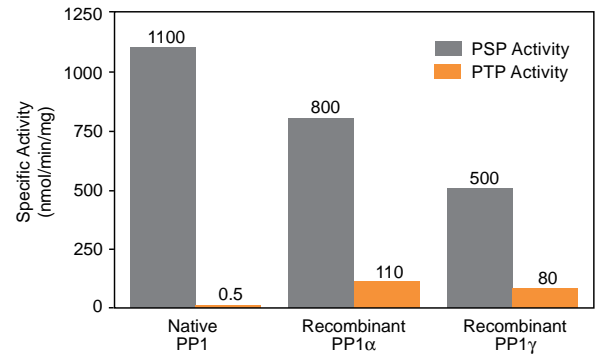


Figure 1. Myelin Basic Protein (MyBP) was either phosphorylated on Ser/Thr residues with [γ - ^{33}P] ATP using cAMP-dependent Protein Kinase (NEB #6000) or on Tyr residues using Abl Protein Tyrosine Kinase (NEB #6050). After incubation of PP1 with phospho-MyBP, TCA-soluble counts (representing released phosphate) were measured.

threonine over phosphotyrosine activity is reduced to a mere 5 to 20-fold in many cases.

We do not know if the oxidized cysteine residue is responsible for this alteration in specificity, or if it is rendered accessible to oxidation as a result of unfolding. Given that the oxidized cysteine lies some distance from the active site, the latter possibility seems most likely and suggests that a crystallographic comparison with the native protein, isolated either from rabbit skeletal muscle or in reduced form from *E. coli* might be highly informative about the mechanism by which PP1 discriminates phosphoserine/threonine from phosphotyrosine.

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CircumVent® Thermal Cycle Sequencing of Eubacterial 16S Ribosomal DNAs

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Accurate characterization of bacteria is of interest for determination of phylogenetic and taxonomic relationships, for assessment of the composition of microbial populations within different ecosystems, and for application to clinical diagnosis and food analysis. Analysis of 16S ribosomal sequences (rRNA or rDNA) provides a tool for bacterial classification (1). For the determination of these sequences from different species of eubacteria, one of the easiest and most rapid methods involves amplification and direct sequencing of the amplification products (2-4).

We have developed a simplified method to obtain nearly the entire 16S rDNA sequence from a wide variety of eubacterial species using only four universal primers for both amplification and direct sequencing. The primers 27f (5' GAGAGTTTGATCCTGGCTCAG) and 1495r (5' CTACGGCTACCTTGTACGA) allow the amplification of nearly the entire 16S rDNA gene starting from crude DNA preparations.

For most of the bacterial samples, freezing and boiling treatment is sufficient to release DNA suitable for amplification. A more reliable procedure requires heating at 90°C for 10 minutes, treatment at 55°C for a few hours with proteinase K (100 µg/ml) followed by incubation at 90°C for 10 minutes to inactivate the proteinase K. Products from the subsequent amplification reaction are electrophoresed and then the amplification product is purified from a low-melt agarose gel with TAE buffer containing 0.5 µg/ml (w/v) ethidium

bromide. A small agarose slice is excised under long-wave UV (312 nm), frozen and microcentrifuged for a few minutes. An aliquot of the supernatant containing at least 30 ng of DNA is directly used in the sequencing reactions. The external primers 27f and 1495f with two internal primers 704f (5' GTAGCGGTGAAATGCGTAGA) and 765f (5' CTGTTTGCTCCCCACGCTTTC) are used to obtain the sequence of the amplified region. The reactions are performed according to the instructions of the CircumVent™ Thermal Cycle DNA Sequencing Kit (NEB #430). We have found that this kit generates clear and unambiguous sequence information from even small amounts of template DNA (5). The protocol works well even when the eubacterial template DNA is mixed with other non-eubacterial DNAs. On a standard sequencing gel it is possible to

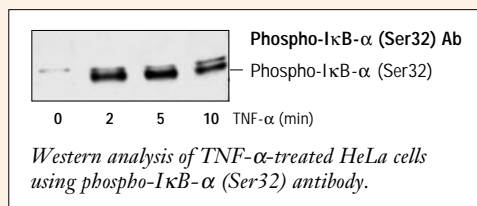
read up to 500 nucleotides from each primer. We have amplified and obtained unambiguous nucleotide sequences of the 16S rDNA from a variety of eubacterial species. Thus, this method improves the use of 16S rDNA as a standard and rapid tool for bacterial characterization.

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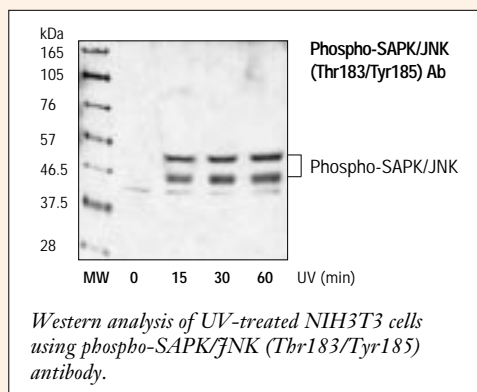
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