

The NEB Transcript

September 1992

A Scientific
Newsletter
from
New England
Biolabs

Deep Sea Hydrothermal Vents: Underwater Oases

Holger W. Jannasch

Woods Hole Oceanographic Institution, Woods Hole, MA 02543

The deep sea has long been thought of as a biological desert. Far removed from the photosynthetically productive surface waters, organisms living below a depth of 1,000 meters (arbitrarily defined as the deep sea) depend on the minute amounts of organic food particles that reach these waters by sedimentation. Most of the marine primary production, the photosynthetic organic carbon, is recycled in the upper 200 to 300 meters of water. Only a fraction, about 5%, reaches the deeper waters in the form of sinking particles and only about 1% of this food material reaches the ocean floor. Yet this space makes up about 75% of the biosphere of our planet by volume. The limited food supply is reflected by the low number of animals living in this permanently dark and cold (2-3°C) environment with a pressure of up to 1100 atm (16,000 psi) at its greatest depth.

It was a sensational discovery when, almost 15 years ago, dense animal populations were found at depths of 2,000 to 4,000 meters, tightly clustered around warm water (ca. 10-30°C) and hot water (ca. 350°C) vents. The vents are formed by tectonic plate movement at spreading centers which allows penetration of seawater several kilometers into the earth's crust. The underlying magma chambers heat the water to temperatures as high as 1,200°C before it ascends again to reach the seafloor as hydrothermal fluid. At hot water vents, the hydrothermal fluid (at temperatures of 350-360°C) shoots out of chimney-like structures known as "black smokers," so named because of the immediate precipitation of polymetal sulfides as the hot solution meets the cool ambient seawater (Figure 1). At warm vents, a premixing of ambient seawater and hydrothermal fluid takes place in the porous sub-seafloor lava beds prior to emission. It is the chemical composition of the hydrothermal fluid which provides the energy source for marine life around these vents.

The explanation for these deep sea oases was found in a

continued on page 2

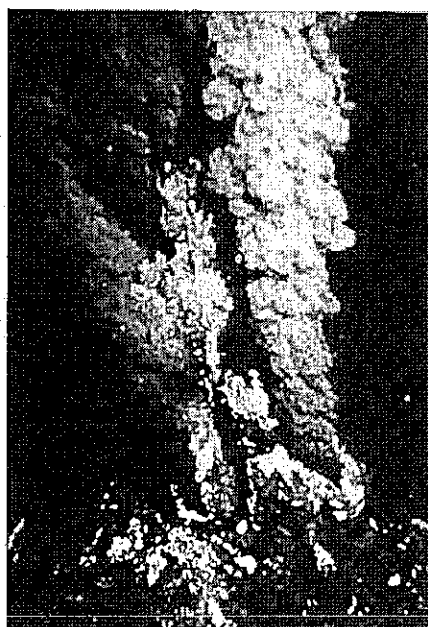


Figure 1. A group of black smokers. Hydrothermal fluid of 350°C is emitted at a rate of 2 m. 21°N hydrothermal vent site of the East (depth, 2,610 meters).

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Columbia Exhibit 2132

Illumina, Inc. v. The Trustees
of Columbia University in the
City of New York

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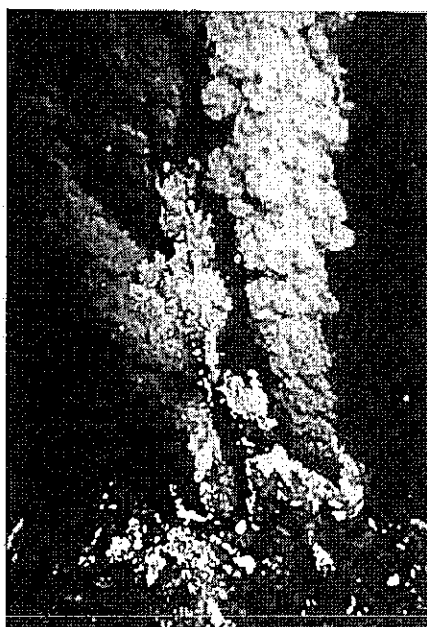
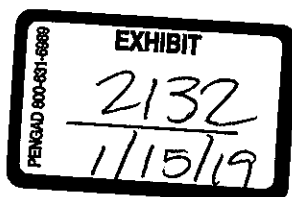


Figure 1. A group of black smokers. Hydrothermal fluid of 350°C is emitted at a rate of 2 m/s at the 21°N hydrothermal vent site of the East Pacific Rise (depth, 2,610 meters).

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food chain based on bacteria. A particular type of bacteria is capable of utilizing hydrogen sulfide, an inorganic dissolved gas contained in the hydrothermal emissions, as a source of energy when free oxygen is available. These microorganisms grow with high efficiency on an inorganic substrate using chemical oxidation energy, a process called "chemosynthesis" by analogy to plant "photosynthesis" that uses light energy (Figure 2). Although the chemosynthetic way of life in microorganisms was well known, it was a new discovery to learn that entire ecosystems can be based on this process. Our fundamental notion that life on this planet is supported solely by solar energy had to be amended. There are whole populations of very unusual organisms living in the permanently dark deep sea that are surviving on geothermal, i.e. terrestrial, energy. Although photosynthetically produced oxygen is required for aerobic chemosynthesis, anaerobic chemosynthesis (utilizing hydrogen) has also been observed.

Chemosynthetic bacteria are dispersed in vent plumes where they are eaten by small planktonic organisms and larger grazing animals, such as limpets and snails. They live on the surface of rocks that are exposed to vent plumes, which provide ideal conditions for bacteria to grow in dense mats. One of the most interesting riddles of the deep sea oases involves the two predominant species of large animals



Figure 3. Populations of white clams and brachyuran crabs located within cracks between boulders of "pillow lava" at the 21°N East Pacific Rise vent site (depth, 2,610 meters).

surrounding the vents: hitherto unknown gigantic clams of a foot in length and one pound of meat (Figure 3) and the never-before observed, 4-6 feet long tube worms (Figure 4). Clams commonly feed on particulate food materials, but nothing of this sort could be found in the surrounding waters. Yet the clams appeared to be healthy and growing fast (we notched them, put them in wire baskets and came back a year or more later to observe the growth of the shell). We do our work down there, by the way, in the research submersible ALVIN, (Figure 5), a newsletter topic all by itself. Ultimately, it was found that chemosynthetic bacteria were housed



Figure 4. Populations of tube worms up to 2 meters long with an additional 20 centimeters for the gill plume at the Galapagos Rift hydrothermal vent site (depth, 2,550 meters).

within the clams' gill cells, an association called "symbiosis". These bacteria grow within the animal's tissue on three inorganic materials, hydrogen sulfide, oxygen and carbon dioxide, and supply the animal "from within" with an organic food. How this latter process, the transfer of bacterial biomass into animal tissue, works, is not yet known.

While the clams still have a stomach and an intestinal tract, evolution has gone one step further with the tube worms. They have lost all the abilities and tools to eat and digest. Instead, each worm harbors a large mass of bacteria in its body cavity. It supplies the bacteria with hydrogen sulfide, oxygen and carbon dioxide through its well functioning blood system. Again, the worms are harvesting the product, the bacterial biomass, in an unknown manner. Here we have a brand new type of an animal that lives with the aid of symbiotic chemosynthetic bacteria on three dissolved gases, and this most efficient process produces more biomass per area (Figure 3 and 4) than we can find anywhere on the surface of the globe. The fascinating point here is that the source of energy for this process is essentially hydrogen sulfide, a strong poison and also a most problematic waste of our mining and power industries.

This leads us to the first biotechnological lesson from the deep sea vents. If

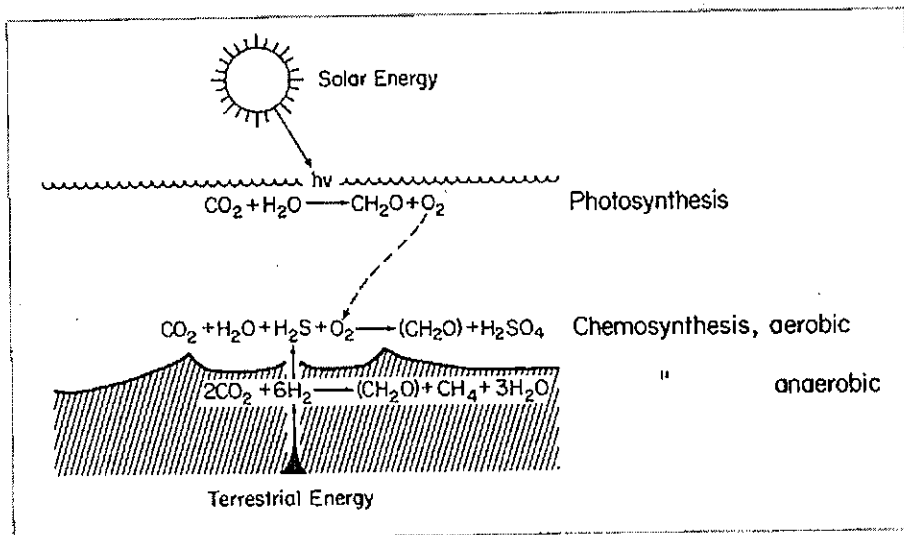


Figure 2. Scheme of energy supply for photo- and chemosynthesis and the role of free oxygen at deep sea vents.

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hydrogen sulfide is used so readily by bacteria which, in turn, produce biomass from carbon dioxide, why cannot we use this process for getting rid of the tremendous amounts of reduced sulfur, the hazardous by-product of our mining industries and power plants, rather than blowing it into the atmosphere where it turns into acid rain? We certainly can, and laboratory experiments have demonstrated the feasibility of such procedures. The resulting bacterial biomass can be used either as food for growing mussels in aquaculture, an idea directly derived from deep sea observations, or it can be fermented to synfuels, i.e. various types of alcohol. Why isn't it done? Because the price of fuel is still too low, and the incentive to protect the environment from acid rain is still too weak to overcome the threshold of the necessary investments.

This was not a problem for another biotechnological offshoot of the deep sea vent discovery. The above mentioned anaerobic chemosynthetic bacteria have one

surprising property: they are "hyperthermophilic". That means that they are growing at very high temperature ranges, namely between about 80°C and 110°C (ca. 180°F and 225°F). It is not the high temperature that kills these organisms but the boiling (gasification) of the water at about 100°C. If this boiling is prevented by applying pressure, as in a pressure cooker or in the deep sea, these organisms can grow beyond 100°C. The highest possible growth temperature is still not known. So far 110°C or slightly beyond seems to be the limit. Some of these hyperthermophiles are not chemosynthetic but use organic substrates and can be grown on conventional bacteriological media. They contain enzymes that, of course, are able to function at high temperatures and thereby provide highly desirable properties in a number of biochemical and molecular biological applications. Most notably, thermostable DNA modifying enzymes are obtained from hyperthermophilic bacteria that we have isolated from deep sea vents.

As often happens, basic research sooner or later leads to highly expedient and profitable applications, but rarely is there a case where the discoveries have been as serendipitous as in the case of deep sea vent explorations. ■

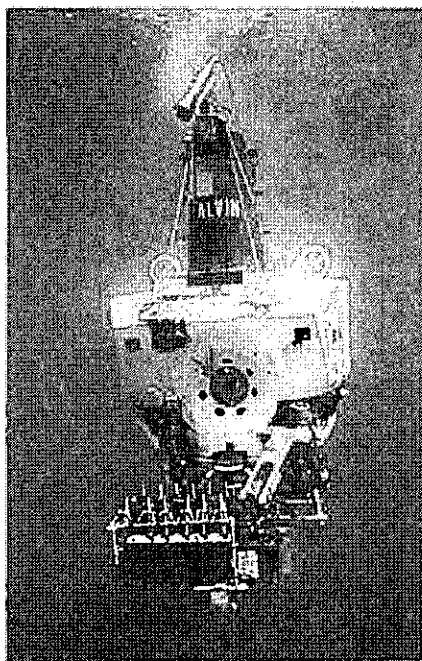


Figure 5. The submersible ALVIN has a maximum depth capacity of 4,000 meters and a payload capacity for samples or equipment of 300 pounds. ALVIN takes about 80 minutes to reach a depth of 2,500 meters and about the same time to come up. That leaves about 4 to 6 hours for the crew, a pilot and two scientists, to work on the bottom.

New Restriction Endonucleases

*Bsa*W I (*Bca*77 I)

(A/T)↓CCGG(A/T)
(T/A)GGCC↑(T/A)

#567S	100 units	\$55
#567L	500 units	\$220

*Bst*HKA I (*Hgi*A I)

G(A/T)GC(A/T)↓C
C↑(T/A)CG(T/A)G

#570S	1,000 units	\$50
#570L	5,000 units	\$200

*Bsp*120 I (*Apa* I⁺)

G↓GGCCC
CCCGG↑G

#ER0131S	3,000 units	\$55
#ER0131L	15,000 units	\$220

*Bsp*1407 I

T↓GTACA
ACATG↑T

#ER0931S	300 units	\$75
#ER0931L	1,500 units	\$300

*Bsr*B I

GAG↓CGG
CTC↑GCC

#102S	500 units	\$50
#102L	2,500 units	\$200

*Csp*6 I (*Rsa* I⁺)

G↓TAC
CAT↑G

#ER0211S	1,500 units	\$55
#ER0211L	7,500 units	\$220

*Ppu*10 I (*Ava* III, *Nsi* I⁺)

A↓TGCAT
TACGT↑A

#ER0861S	500 units	\$55
#ER0861L	2,500 units	\$220

*Psp*1406 I

AA↓CGTT
TTGC↑AA

#ER0941S	300 units	\$75
#ER0941L	1,500 units	\$300

Sap I

GCTCTTCN↓
CGAGAAGNNN↑

#569S	25 units	\$50
#569L	125 units	\$200

Isoschizomers with alternate cleavage sites are indicated with a "△"

Enzymes with catalog numbers that begin with "ER" are purified by Fermentas, a Lithuanian research company.

Biochemical Characterization of Vent_R[®] and Deep Vent_R[®] DNA Polymerases

William E. Jack, Rebecca B. Kucera and Huimin Kong. New England Biolabs, Inc.

One of the driving forces behind molecular biology has been the successful utilization of enzymes as reagents. Nucleic acid metabolizing enzymes have been essential tools in defining, characterizing, and refining DNA and protein structures. The ever-increasing repertoire of enzymes has extended the sensitivity of established protocols and led to the development of new technologies in molecular biology.

Several years ago New England Biolabs began isolating, characterizing, and cloning DNA polymerases from extremely thermophilic Archaea native to submarine thermal ocean vents (obtained from Holger Jannasch of the WHOI) (1). Currently, two such polymerases are available from New England Biolabs; the Vent_R[®] DNA polymerase, isolated from *Thermococcus litoralis* and the Deep Vent_R[®] DNA polymerase, isolated from *Pyrococcus* species. As expected from the extreme growth temperatures tolerated by the native organisms, both polymerases are extremely thermostable (see Table I) and well suited for applications requiring extended high

optimum between 75–80°C, which presumably reflects DNA primer/template stability rather than enzyme stability. Polymerization by Vent_R DNA polymerase is greatly decreased at low temperatures, with only 2% of the optimal activity noted at 30°C. Polymerization can, therefore, be done up to the maximum temperature consistent with primer/template stability, although most primer extensions are done at 72–75°C.

Vent_R DNA polymerase shows near maximal activity in a variety of buffer conditions. Less than two-fold differences in the rate of dNTP incorporation are observed between 0 and 0.1 M KCl and between 5 and 50 mM (NH₄)₂SO₄. This broad tolerance for different reaction conditions provides a cushion against sample to sample variation, allowing more consistent results. The reaction buffer provided with the enzyme encompasses the observed optima, and can be fine tuned for specific applications. In related studies, the fidelity of Vent_R DNA polymerase is less dependent on reaction conditions than is *Taq* DNA polymerase (2).

One of the most striking differences between *Taq*, Vent_R and Deep Vent_R DNA polymerases is the associated exonuclease activity. While *Taq* lacks a 3'→5' exonuclease, both Vent_R and Deep Vent_R DNA polymerases have a 3'→5' "proofreading" exonuclease activity. The contribution of this exonuclease to polymerase fidelity has been assessed by selectively inactivating the exonuclease domain. The native enzyme was found to have 5-fold higher fidelity than the exonuclease deficient form (3) and a 10-fold higher fidelity than *Taq* DNA polymerase (4). Thus, the exonuclease provides a significant contribution to fidelity, but other

elements of the polymerase are also critical. Fidelity studies on the Deep Vent_R DNA polymerase and the exonuclease deficient form of this enzyme are currently under way.

The Vent_R DNA polymerase 3'→5' double-strand dependent exonuclease activity is inhibited by dNTPs, with a 50% inhibition occurring at 1 μM dNTPs. Thus, under normal reaction conditions (200–400 μM dNTPs), polymerization vastly exceeds exonuclease degradation. Completed polymerization reactions can therefore be safely left at room temperature or 4°C.

The Vent_R DNA polymerase 3'→5' exonuclease also acts on single-stranded DNA. Unlike the double strand dependent activity, this exonuclease activity is not inhibited by dNTPs. Conditions can be chosen which minimize this activity: i.e., appropriate extension times, adequate dNTP pools, correct amounts of polymerase, etc. (see page 8). Of course, the exonuclease-deficient forms of the polymerases do not degrade single-stranded primers. This permits higher polymerase concentrations to be used, frequently increasing the yield of product.

Pseudo first-order kinetics have revealed an extremely low K_m for DNA and a relatively high K_m for dNTPs (Table II). Consequently, the enzyme is extremely efficient in polymerizing from minute amounts of template, but requires relatively high concentrations of dNTPs in the reaction. This efficiency is borne out in thermal cycle sequencing protocols, where linear amplification of extremely small amounts of DNA has been observed.

Extension rates of about 1,000 bases per minute have been observed in assays of the

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Table I: Thermal Stability

Polymerase	Half-life at 95°C	Half-life at 100°C
Vent _R	7 h	1.8 h
Deep Vent _R	23 h	8 h
<i>Taq</i>	1.6 h	<0.1 h

temperature incubations. High denaturation temperatures and extended reaction times can be used without fear of inactivating the polymerases. In addition, thermal cycler excursions which inadvertently go above the programmed temperature will not compromise enzyme activity.

Despite differing thermal stabilities, Vent_R, Deep Vent_R and *Taq* DNA polymerases have a common temperature

Table II: Kinetic Constants

Polymerase	K_m DNA	K_m dNTP	Processivity*
Vent _R	0.1 nM	50 μM	7
Deep Vent _R	0.01 nM	60 μM	—
<i>Taq</i>	1.5 nM	15 μM	40
<i>Pfu</i>	1 nM	60 μM	—
pol I (5)	5 nM	1 μM	50

*nucleotides per polymerase binding event

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enzymes. This rate should be used as a rule of thumb, allowing enough time to complete synthesis of the template. At the same time, care should be taken to avoid excessive extension times in order to minimize degradation of single-stranded primers.

The processivity, or number of nucleotides polymerized per polymerase binding event, for Vent_r DNA polymerase is low relative to several other polymerases (Table II). It has been argued that processivity is important for uniform, unimpeded replication through regions of DNA secondary structure (6). This is not the case with Vent_r DNA polymerase, as demonstrated by formation of extension products up to 13 kb in length and by the lack of non-dideoxy termination products in sequencing reactions (7). This behavior presumably relies on the reduction in DNA secondary structure at high reaction temperatures. Although a limited number of nucleotides are added per binding event, Vent_r DNA polymerase efficiently links multiple binding events to make a large product, and thus processivity alone is a poor predictor of the eventual size of an extension product. The low processivity can be an advantage in uniformly extending a population of templates with less than stoichiometric amounts of polymerase.

Primer extension products made with Vent_r DNA polymerase are predominantly blunt-ended. In contrast, products from a variety of other DNA polymerases have a non-template encoded single-base 3' addition (8). Accordingly, Vent_r DNA polymerase products made from 5'-phosphorylated primers are substrates for blunt end ligation without further modification (9). These termini are not, however, efficient substrates for ligation into 5' single-base overhang vectors. Approximately 30% of termini created by Vent_r (exo⁻) DNA polymerase have single base extensions, presumably due to the absence of 3'→5' exonuclease activity.

The reports above have focused largely on the Vent_r DNA polymerase since we have observed few differences between it and the exo⁻ form of the enzyme. We expect that properties of Deep Vent_r DNA polymerase

will parallel those of the Vent_r DNA polymerase, although its higher heat stability and lower K_m for DNA promise additional advantages in selected applications.

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Protein Fusion and Purification System: Update

The Protein Fusion and Purification System provides a method for the production of a protein expressed from a cloned gene or open reading frame. The gene of interest is fused to the *malE* gene (which encodes maltose-binding protein MBP) and expressed at levels constituting up to 40% of the total cell protein using the strong P_{mal} promoter and translation initiation signals of MBP. A one-step affinity purification for MBP is used to isolate the fusion protein. The following improvements have recently been made to the System.

pMAL-2 Vectors: The pMAL vectors have been upgraded to newer versions, pMAL-c2 (#800-64) and pMAL-p2 (#800-65). The polylinker now contains an *Xmn*I site in place of the *Sma*I site. This allows the same vector to be used for cloning blunt-ended inserts directly after the region coding for the factor Xa site and also for cloning into downstream sites in the polylinker without creating problems with factor Xa cleavage. The vectors also include the coding sequence for a poly-asparagine spacer between the *malE* gene and the cloning sites, which enhances binding of some fusions to the amylose resin. The *lacI* gene has also been substituted for the *lacZ* gene to lower the uninduced level of expression of the fusion protein. Finally, the vectors include the origin of replication of phage M13, which allows for the production of single stranded DNA when the vector is in an F' host.

These pMAL vector upgrades necessitate a few amendments to the original PF&P System instruction manual:

1. Choose pMAL-c2 for cytoplasmic expression of the fusion protein and pMAL-p2 for periplasmic expression. There is no longer a need for a third vector (e.g. pMAL-cRI) when cloning into sites downstream of the blunt-ended site.

2. When cloning blunt-ended fragments into pMAL-c2 and pMAL-p2, cut with *Xmn*I instead of *Sma*I.

Contact NEB to receive an updated version of the instruction manual.

New Buffer System: Some fusion proteins have a low affinity for the amylose resin in the presence of Tween 20. As a result, a revised affinity purification procedure was developed which, in most cases, outperforms the original protocol. In the new protocol, the original Lysis Buffer, Column Buffer, Low Salt Column Buffer and Factor Xa Buffer have been replaced by the following buffer:

20 mM Tris-HCl (pH 7.4),
200 mM NaCl, 1 mM EDTA
optional:
10 mM β-mercaptoethanol or
1 mM DTT
1 mM sodium azide

Ordering Information: The System includes; pMAL vectors, amylose affinity resin, factor Xa, *E. coli* host, markers and controls, and an instruction manual.

#800 PF&P System \$400

Catalog Corrections

Page 16: NEBuffer *Bam*HI contains 10 mM Tris-HCl.

Page 37: Only the large size (#189CL) of *Nor* I is available at high concentration.

Page 39: *Pml* I is not blocked by *dc*m methylation.

Page 89: The gel photos for the Lambda Ladder PFG Marker and the Low Range PFG Marker are reversed.

Page 126: The cleavage site for *Acl* I is G/CGG.

Page 136-171: All maps, *Sac* I/*Ecl*136 I should be *Sac* I/*Ecl*136 II.

Page 142: In the *E. coli* map legend, the "#?" indicates that a particular gene was not present in the 1990 Bachmann genetic map and that minute values were assigned to be consistent with the Map Search alignment.

Add this reference: K.E. Rudd in *A Short Course in Bacterial Genetics: A Lab Manual and Handbook for E. coli and Related Bacteria* Miller, J. (1992) Cold Spring Harbor Lab Press, NY pp. 2.3-2.43.

Page 146: *Agt*11 map, the membrane protein should be *lon* not *lon*.

Page 150: pACYC177 map, the orientation of the Kanamycin (Km) gene should be clockwise.

Page 152: pACYC184 map, the orientation of the Chloramphenicol (Cm) gene should be counter-clockwise.

Page 154: pBR322 map, at nucleotide 651 the restriction site should be *Hinc* II.

Page 156: pMAL™-p2 map, base number 2744 in the polylinker sequence should be an A: AAC CTC GGG.

The origin for single-strand DNA replication should be labeled "M13 ori," not "f1 ori," and the direction of the arrow should be reversed. DNA replication is initiated counterclockwise, producing single-strand DNA containing the antisense strand of the *malE* gene.

Page 216: The package sizes of *I-Tli* I (#698) are 100 and 500 units.

Which Vent DNA Polymerase Is Right for You?

Rebecca B. Kucera. New England Biolabs, Inc.

The *Vent*_R family of cloned and overexpressed extremely thermophilic DNA polymerases has redefined the standards by which thermal stable DNA polymerases are judged. Unparalleled thermal stability and high fidelity make *Vent*_R*, *Vent*_R*(exo⁻) and Deep *Vent*_R* DNA polymerases the enzymes of choice for primer extension. Biochemical differences between the three enzymes will dictate which one is best suited for your particular research needs.

*Vent*_R* DNA Polymerase

*Vent*_R DNA polymerase, the first member of the *Vent*_R family, was originally isolated from *Thermococcus litoralis*. Its thermostability ensures minimal loss of enzyme function over the course of an experiment, of particular importance when DNA with a high GC content necessitates high temperature incubations or when the DNA is methylated. *Vent*_R DNA polymerase has a 3'→5' proofreading exonuclease activity that results in a 5-15 fold greater fidelity than Taq DNA polymerase; it also has the capability of extending primers up to 13 kb in length. *Vent*_R produces blunt-ended DNA fragments, thus simplifying many cloning strategies.

It should be noted that the 3'→5' exonuclease activity, directly responsible for the enzyme's high level of fidelity, very slowly shortens primers from the 3' end over the course of an experiment (under normal reaction conditions, no primer shortening is seen after incubation for 20 minutes at 72°C). This primer shortening does not interfere with most uses of *Vent*_R DNA polymerase, although purposeful primer mismatches near the 3' end risk correction by *Vent*_R DNA polymerase upon annealing to the template. As a result of this proofreading exonuclease, it is important to follow NEB's suggested guidelines for primer extension with *Vent*_R DNA polymerase.

*Vent*_R*(exo⁻) DNA Polymerase

*Vent*_R*(exo⁻) DNA polymerase is the enzyme of choice for sequencing reactions and is also quite useful for primer extension reactions. It is the most "user-friendly" of the *Vent*_R DNA polymerases because it tolerates some deviation from recommended reaction conditions.

*Vent*_R*(exo⁻) DNA polymerase is particularly recommended when large quantities of primer extension product are desired. Since *Vent*_R*(exo⁻) DNA polymerase lacks any exonuclease activity, an increase in the polymerase concentration typically produces a proportionate increase in extension product yield even with target DNA sequences that are problematic in terms of secondary structure, length, etc.

It should be noted that the fidelity of *Vent*_R*(exo⁻) DNA polymerase is twice that of Taq DNA polymerase. When this level of fidelity is acceptable and high product yield is important, we recommend using *Vent*_R*(exo⁻) DNA polymerase for primer extension.

Deep *Vent*_R* DNA Polymerase

Deep *Vent*_R DNA polymerase is our most thermostable enzyme, with a 23 hour half-life at 95°C and an 8 hour half-life at 100°C. It possesses a 3'→5' proofreading exonuclease that is five times more active than that of *Vent*_R DNA polymerase. Its fidelity level is at least equal to that of *Vent*_R DNA polymerase and may be higher. Deep *Vent*_R DNA polymerase also leaves blunt-ended DNA fragments.

For a maximum level of thermostability, we recommend using Deep *Vent*_R DNA polymerase. ■

Comparison of NEB's Extreme Thermostable DNA Polymerases

	Vent _R [®] DNA Polymerase	Vent _R [®] (exo ⁻) DNA Polymerase	Deep Vent _R [®] DNA Polymerase
Enzymatic Activities			
Primer Extension	yes	yes	yes
Fidelity	high	moderate	high
Highly thermostable	yes	yes	yes
DNA Sequencing	no	yes	no
Reverse Transcription	experiments in progress	experiments in progress	experiments in progress
Exonuclease Activities			
3'→5' proofreading exonuclease	yes	no	yes
5'→3' exonuclease activity	no	no	no
Thermal Stability			
Half life at 95°C	6.7 hours	6.7 hours	23 hours
Half life at 100°C	1.8 hours	1.8 hours	8 hours
Primer Extension Characteristics			
Units/100 µl Primer Extension Rxn	1-2 units	4 units	1-2 units
Error Rate (x10 ⁻⁶ base insertions)	57	190	experiments in progress
Resulting DNA Ends	>95% blunt ends	70% blunt ends; 30% single-base 3' overhangs	>95% blunt ends
Strand Displacement	yes; temp. dependent	yes; temp. dependent	experiments in progress
Longest Primer Extension to Date	13.2 kb	15.0 kb	8.2 kb
Cloned Enzyme	yes	yes	yes
Ordering Information			
	#254S 200 units \$75	#257S 200 units \$75	#258S 200 units \$75
	#254L 1,000 units \$300	#257L 1,000 units \$300	#258L 1,000 units \$300

Taq DNA Ligase

Recombinant Taq thermostable DNA ligase is now available from New England Biolabs at an affordable price. Isolated from a strain of *E. coli* containing the cloned ligase gene from *Thermus aquaticus* HB8

(F. Barany), Taq DNA ligase catalyzes NAD-dependent ligation at temperatures up to the denaturation temperature of DNA.

Taq DNA Ligase specifically links two adjacent oligonucleotides when hybridized at 65°C to a complementary target only when the nucleotides are perfectly base-paired at the junction. By thermal cycling of the ligation reaction in the presence of a second set of adjacent oligonucleotides (complementary to the first set and the target), oligonucleotide products are increased exponentially. A single-base mismatch prevents ligation and is thus distinguished (1).

1. Barany, F. (1991) Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase, *Proc. Natl. Acad. Sci. USA* 88, 189-193.

Unit Definition: One unit is defined as the amount of enzyme that catalyzes the ligation of 50% of the cos sites (12 base-pair cohesive ends) of 1 µg of BstE II digested λ DNA in a total reaction volume of 50 µl in 15 minutes at 45°C. This cohesive End Unit is equivalent to the Nick-closing Unit of Barany.

Ordering Information:

#208S	2,000 units	\$60
#208L	10,000 units	\$240

Vent Price Reductions!

Due to successful cloning efforts we have reduced the cost of all our Vent_R DNA Polymerases. No matter which one best meets your needs, the price is just 30¢ per unit (based on large pack cost).

Take advantage of our price reduction and experience the unparalleled thermal stability and fidelity of Vent_R, Deep Vent_R, and Vent_R (exo⁻) DNA Polymerases.

Vent_R[®] DNA Polymerase

#254S	200 units	\$75
#254L	1,000 units	\$300

Vent_R[®] (exo⁻) DNA Polymerase

#257S	200 units	\$75
#257L	1,000 units	\$300

Deep Vent_R[®] DNA Polymerase

#258S	200 units	\$75
#258L	1,000 units	\$300

1992 Catalog Highlights

In case you haven't noticed, the New England Biolabs 1992 Catalog features over 150 restriction endonucleases, a wide range of new products and an easy-to-use reference appendix. Some of the new products include:

New 8-Base Cutters
Afr I (GG/CGCGCC)
Pme I (GTTT/AAAC)

25 New Restriction Endonucleases

Intron-encoded Endonucleases

Extremely Thermostable

DNA Polymerases

Vent_R[®], Vent_R[®] (exo⁻) and Deep Vent_R[®] DNA Polymerases

CircumVent[™] Thermal Cycle

DNA Sequencing Kit

(sequence with 10-fold less template, no "collapsing" of plasmid DNA, no centrifugation steps, no separate priming steps)

Phototope[™] Chemiluminescent DNA Probing and Sequencing

Biotinylated Primers, Linkers and Molecular Weight Standards

Random Priming System I

(produces labeled DNA probes of high specific activity; *Note: the System is now called the NEBlor[™] Kit*)

Appendix highlights include:
18 Restriction Maps

A Directory of Isoschizomers and Recognition Sequences

Properties of

Restriction Endonucleases

(NEBuffer activity chart, Performing double digests, Generating compatible ends, Heat inactivation, Survival times in a reaction, Cleavage close to the end of DNA fragments, Activity of thermophiles at 37°C, Star activity and more)

Genomic Analysis and Mapping Section
 (Protocols for embedding and digesting DNA in agarose, Comparative data examining units of enzyme required to cleave agarose-embedded DNA)

Cloning Strategies in *E. coli*

(Restriction of foreign DNA, Methylases, Genetic markers of interest, Strain genotypes and phenotypes)

If you would like to receive a copy of our 1992 Catalog, please fill out and return the enclosed reply card or call 1-800-NEB-LABS.

Vent_R[®] DNA Polymerases: Frequently Asked Questions

What Vent_R[®] support reagents are available from New England Biolabs?

Every Vent_R DNA polymerase vial is supplied with the following:

- 10X Vent Reaction Buffer
- 100 mM MgSO₄
- An applications sheet and data card

Non-acetylated BSA is available separately (free of charge, when placing an order) if you wish to perform primer extension reactions with BSA present. Do not use acetylated BSA with reactions above 85°C.

Also available separately:

- Vent Buffer Packs, including;
 4 vials of 10X Reaction Buffer,
 1 vial of MgSO₄ and
 1 vial of non-acetylated BSA
 (#007-254 or #007-258; \$8.00)
- 1X Vent Diluent/Storage Buffer
 (#008-254; \$8.00)
- 100 mM solutions of dNTPs
 (page 100 of the 1992 NEB Catalog)

What can I expect if I switch from another DNA polymerase to one of the Vent_R DNA polymerases?

Product yields should be similar to those seen with other thermophilic DNA polymerases and may be higher (especially if using Vent_R (exo⁻) DNA polymerase). You will need to use the 10X buffer accompanying the enzyme; while Taq DNA polymerase often works well in Vent_R buffer, Vent_R DNA polymerase does not work well in commercially available Taq buffers.

Positive results are usually achieved immediately by following the suggested guidelines for primer extension. Some template:primer systems may require optimization of annealing temperatures or MgSO₄ levels.

How can I get primer extension products in the 4-15 kb range with Vent_R DNA polymerases?

1. Use the right amount of enzyme. For the proofreading Vent_R DNA polymerases, less is sometimes better. For Vent_R (exo⁻) however, use 4-16 units per 100 µl reaction volume (for smaller reaction volumes, scale down the amount of enzyme appropriately).
2. Use the correct primer extension time calculated by the "one minute per kilobase extension product" guideline. It is especially important not to use an extension time that is more than 20% greater than the calculated extension time when using the proofreading Vent_R DNA polymerases.
3. Test at least 3 different MgSO₄ levels over the range of 2 mM to 6 mM. Primer extension products above 2 kb often require a higher level of magnesium than the basal level of 2 mM present in the 1X reaction buffer.
4. Use stringent (high) annealing temperatures so that nonspecific priming events do not occur.

What if my primer extension reaction with Vent_R DNA polymerase yields no product?

1. Review the primer extension guidelines for use of the reaction buffer, amount of enzyme and extension time.
2. Try different annealing temperatures. It is not uncommon for Vent_R DNA polymerase to require a higher annealing temperature than Taq DNA polymerase to achieve the same level of specificity—perhaps a reflection of the differences in their Km for DNA or in their buffer compositions. A higher annealing temperature should always be tried even if the gel lane appears blank by ethidium bromide staining, since nonspecific priming events are not always easily visualized.
3. Purify the DNA by phenol/chloroform extraction and alcohol precipitation. Avoid high salt carry-over into your Vent_R DNA polymerase primer extension reaction.
4. Check your dNTP solution to see if it has undergone hydrolysis.

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5. If using BSA, make sure it is non-acetylated BSA and not the acetylated BSA that accompanies our restriction endonucleases. Occasionally a template:primer system is sensitive to BSA. Try your primer extension in the absence of BSA.

What if my primer extension reaction with Vent_R DNA polymerase yields a smear instead of a specific product band?

1. Review the primer extension guidelines. A smear centered at an expected fragment length by gel analysis indicates insufficient magnesium; test higher levels of MgSO₄.

2. A smear directly below an expected fragment length indicates possible dNTP depletion which causes deregulation of the proofreading exonuclease moiety of Vent_R or Deep Vent_R DNA polymerase. Try stopping the experiment sooner or using less template DNA.

3. A smear extending down from the gel well usually indicates some reagent or condition in excess, or the annealing temperature is set too high. Use less enzyme (especially important if using the proofreading Vent_R DNA polymerases), primer or template.

4. Don't overdo the primer extension time (stick to the 1 minute/kb rule).

What causes an occasional smear in a "negative control" with no template present?

DNA polymerases with low K_m values for DNA, especially those with proofreading exonuclease functions, can cause primer artifacts to form if the DNA polymerase cannot bind to its preferred substrate (a 3'-end of an annealed primer). This primer artifact DNA possesses single-stranded and double-stranded regions, and can appear either as DNA barely migrating out of the gel well or as a smear originating at the gel well. Decreasing primer concentration or the length of the experiment, or using Vent_R (exo⁻) DNA polymerase can minimize this artifact. ■

Introducing Cloned and Overexpressed Glycosidases

New England Biolabs introduces cloned and overexpressed recombinant forms of PNGase F and Endo H (Endo H is offered in two recombinant forms, Endo H_f and Endo H_i; both forms exhibit equal activity [Figure 1]). They are produced and purified using NEB's pMAL Protein Fusion and Purification System (#800) which eliminates interfering contaminants such as proteases and other glycosidases and enables us to offer PNGase F and Endo H at prices unavailable elsewhere.

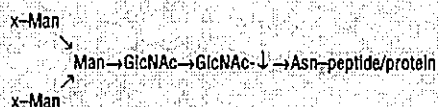
PNGase F, also known as peptide:N-glycosidase F, cleaves N-linked glycoproteins between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides. Endoglycosidase H cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins.

Both PNGase F and Endo H have been overexpressed and purified to homogeneity. They are supplied at high concentrations and affordable prices, making it practical to remove sugars from glycoproteins in only one hour (Figure 2).

PNGase F

Recombinant peptide:N-glycosidase F

#701S	50,000 units	\$50
#701L	250,000 units	\$200



Endo H

Recombinant Endoglycosidase H

#702S	10,000 units	\$50
#702L	50,000 units	\$200

Endo H_f

Recombinant fusion of Endoglycosidase H and maltose binding protein

#703S	100,000 units	\$50
#703L	500,000 units	\$200

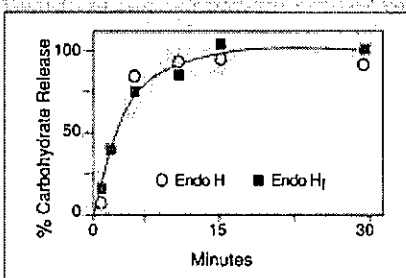
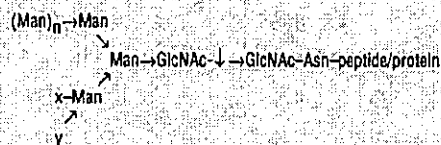


Figure 1. 60 mg of RNase B was incubated with 3,000 units of Endo H or Endo H_f under standard assay conditions. Aliquots were removed at various time points and measured for released carbohydrate. (Dubois et al. (1956) Anal. Chem. 28, 350-356).

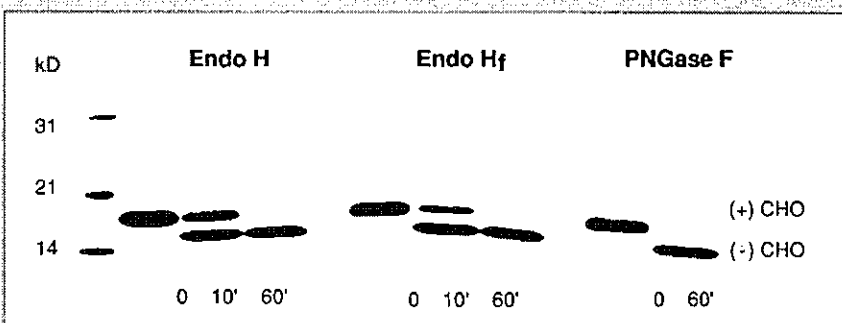


Figure 2. Mobility Shift Analysis. 1 unit of Endo H, Endo H_f or PNGase F was incubated per 10 µg of RNase B under standard assay conditions. At various time points, aliquots were removed and analyzed on a 10-20% SDS-gel for carbohydrate (CHO) release. 1 unit is defined as the amount of enzyme required to remove all of the carbohydrate from 10 µg of RNase B in one hour at 37°C.

Novel Gene Structures in Archaea DNA Polymerases

Francine B. Perler, Donald G. Comb, William E. Jack, Robert Hodges, Laurie S. Moran, Rebecca B. Kucera, Jack Benner, Barton E. Slatko, S. Kay Hempstead, and Clotilde K.S. Carlow. New England Biolabs, Inc.

In recent years, the desire for extremely thermostable, high fidelity polymerases has amplified as more applications develop. Archaea are found in high temperature niches and thus provide a fertile ground for systematic identification of thermostable enzymes. In 1985, a new species of extreme thermophile was isolated from a shallow submarine thermal vent near Naples, Italy (1). This organism, *Thermococcus litoralis*, can be cultured at up to 98°C and contains an extremely heat stable DNA polymerase, which we called Vent® DNA polymerase. In 1988, another marine thermophile, *Pyrococcus* species, (isolate GB-D) was isolated from a 2010 meter deep sea vent in the Gulf of California (2). *Pyrococcus* sp. can be cultured at up to 104°C and also contains a thermostable DNA polymerase, which we called Deep Vent® DNA polymerase. Both organisms are members of the Order Thermococcales, Kingdom Archaea (3).

The DNA polymerase genes from *T. litoralis* (4) and *Pyrococcus* sp. have been cloned and expressed in *E. coli*. Both DNA polymerase genes are interrupted by intervening sequences (IVS) comprising 55% of the Vent DNA polymerase gene and 41% of the Deep Vent DNA polymerase gene. The IVSs form a single, continuous open reading frame (ORF) with all the polymerase exons. All data obtained to date fails to support an mRNA splicing mechanism. We therefore propose that the entire Vent and Deep Vent DNA polymerase genes are expressed as 1702 and 1302 amino acid (aa) protein precursors, respectively, which are then spliced to form the mature 764 aa and 765 aa polymerases.

Our cloning strategy for Vent DNA polymerase was to screen λ gt11 genomic DNA libraries with sera raised against the purified native enzyme (4). Although many phage reacted with the sera, none synthesized active, thermostable polymerase. Initial

inserts were sequenced and used to clone larger genomic DNA fragments which contained the entire gene (see reference 4 for details). Sequencing a 5837 bp region (GenBank accession no. M74198) revealed a DNA polymerase ORF of 1702 aa, coding for a protein almost twice as large as the apparent molecular weight of the polymerase (~93 kDa).

Comparison to Other DNA Polymerases
DNA polymerases have a high degree of identity in small, interspersed regions, which enables classification of DNA polymerases into 2 families: (a) The Pol I-like Class A, whose prototype is *E. coli* DNA polymerase I and includes Taq polymerase, and (b) the Pol α -like Class B polymerases which are similar to human DNA polymerase α (5,6). These conserved regions are thought to compose substrate and metal ion binding domains, and the active site. Alpha-polymerase Regions I-VI (5) are always found in the same order within a polymerase (Figure 1). The region number denotes the degree of similarity among polymerases, with Region I being the most similar.

Region I in both Vent DNA polymerases (Vent: YADTDG, Deep Vent: YIDTDG) is less conserved than in other DNA polymerases, having only 4/6 consensus residues (YGD TDS). Region III of Deep Vent DNA polymerase is more

similar to Vent DNA polymerase than it is to *Pyrococcus furiosus* DNA polymerase (7). In fact, Deep Vent DNA polymerase from *Pyrococcus* sp. (isolate GB-D) is more similar to Vent DNA polymerase (*T. litoralis*) than to the limited published amino acid sequence of *Pyrococcus furiosus* (Figure 2).

Characterization of the IVSs

While we were able to identify Vent DNA polymerase regions IV, II, VI and V, identification of regions III and I proved more elusive. When these regions were eventually discerned, the motifs were found to be interrupted by IVSs (Figure 1). Initially, IVS1 was deleted and the polymerase was over-expressed in a derivative of pET11c in which IVS2 self-spliced (4,8). Next, IVS2 was deleted to improve expression.

Deep Vent DNA polymerase was cloned using the Vent DNA polymerase gene as a hybridization probe under moderate stringency. The polymerase gene DNA sequences are 67% identical, including IVS1; Deep Vent lacks IVS2. Deep Vent IVS1 self-splices in *E. coli*. Neither Vent DNA polymerase requires linkage to an *E. coli* promoter for expression.

A thermostable endonuclease was detected during early steps of Vent DNA polymerase purification. The N-terminus of the endonuclease was sequenced yielding 30 aa which are identical to the polymerase

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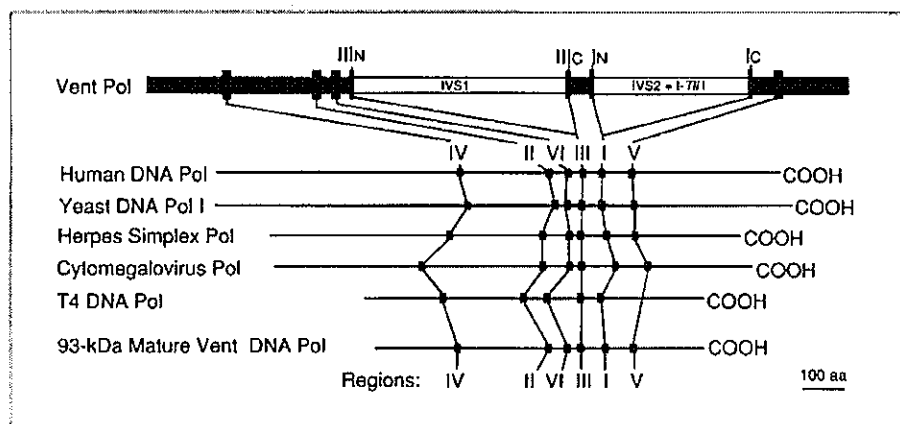


Figure 1. The structure of the Vent DNA polymerase gene and the mature protein are depicted and compared to other DNA polymerases (4). Hatched boxes indicate polymerase exons and white boxes represent IVSs. Vent Region III is split into Regions IIIIN and IIIC by IVS1 and Vent Region I is split into Regions IN and IC by IVS2. IVS2 encodes I-Tli. The schematic relationship of conserved alpha polymerase regions I through VI is also indicated (5).

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RIII	D..Q.A.K... NS.YG..G... ..A...T..GR
Vent	EKKMLD YRQRAIKLLA NSYYGYMGYP KARWYSKECA ESVTAWGR
Deep Vent	EKKMLD YRQRAIKLLA NSYYGYGYA KARWYCKECA ESVTAWGR
Pfu	kKiILD YRQRAIKLLA NSFYGYGYA K
Human Pol α	lilqyD iRQAIKLLtA NSmYGcIGfs ysrfYaKp1A alVTykGR

Figure 2. Comparison of Region III (also referred to as Region 2a, see ref. 6) from Vent, Deep Vent and Pfu polymerases and Human Pol α to the consensus sequence (RIII). Residues which differ from Vent DNA polymerase are in lower case.

ORF beginning with the first residue of IVS2. The endonuclease was called I-Tli I to designate its intervening sequence origin. I-Tli I is similar (29% identity) to the HO endonuclease from *Saccharomyces cerevisiae* (9), and to group I intron endonucleases, especially at the conserved dodecapeptide motifs (10,11). Furthermore, like intron endonucleases, deletion of IVS2 creates an I-Tli I site at the exon2/exon3 junction (referred to as the "homing site").

However, although IVS2 encodes a group I intron-like endonuclease, it lacks the secondary structures and sequences which define group I, group II and Archaea pre-tRNA introns (10-12).

Intron endonucleases are thought to mediate intron mobility by a mechanism similar to nonreciprocal replicative homologous recombination initiated when the intron endonuclease makes a site-specific double-stranded break at the homing site in genes which lack the intron (10,11). Eddy and Gold have shown that any endonuclease, including *EcoR* I, can potentially act as a mobile DNA element when flanked by sequences homologous to the artificial homing site (13). Generally, acquisition of a mobile self-splicing intron in an essential gene is not lethal because the intron and endonuclease sequences are spliced out of the target mRNA, allowing production of a functional product.

RNA or Protein Splicing?

We have been unable to gather any data which would support an RNA splicing mechanism in production of Vent DNA polymerase (4). Only non-spliced mRNA has been detected in cDNA/PCR experiments, although this negative result could be due to technical limitations or to immediate degradation following or

coincident with translation. We have, however, been able to demonstrate a requirement for in frame expression within IVS2, which is not complemented by I-Tli I *in trans*. We have also demonstrated the lack of an effect of multiple silent mutations (which maintain the native amino acid sequence) at the exon2/IVS2 junction. The exon/intron junctions in previously described group I, group II and Archaea pre-tRNA introns form secondary and tertiary structural elements required for splicing (10-12). The silent mutations are likely to disrupt these structural elements, but have no effect on splicing. This result, coupled with the lack of similarity to known intron structures, indicates that if RNA splicing occurs, it must differ significantly from previously described mechanisms. It is also possible, but seems unlikely, that the silent mutations occur in non-critical regions, or that I-Tli I, acting as a maturase, is insensitive to the silent mutations.

One of the most unusual properties of these IVSs is that they form a single reading frame with the polymerase exons. Although introns often contain ORFs which are in frame with either the 5' or 3' exon, they are rarely in frame with both exons. We propose that the entire gene, including IVSs, is expressed as a precursor protein and that the IVS sequences are then spliced out of the protein. Protein splicing is becoming a more widely observed phenomenon. An intron in the *S. cerevisiae* *TFPI* gene also forms a continuous ORF with the surrounding exons is spliced at the protein level (14,15). The *recA* gene of *Mycobacterium tuberculosis* is split by a 440 aa insertion which is in frame with the surrounding exons and which the authors propose is spliced from the protein (16).

Consensus Protein Splicing Elements?

There are similarities at the 5' and 3' ends of the Vent DNA polymerase IVSs, the Deep Vent polymerase IVS, the *M. tuberculosis recA* IVS, and the *TFPI* "spacer". All 5 IVSs end in His-Asn, followed by either Ser, Thr or Cys in the downstream exon. All 5 IVSs begin with either Ser or Cys. We believe that these amino acids are critical in the protein splicing mechanism because of their special chemical reactivities. We hope in the future to be able to dissect this mechanism by studying the effect of amino acid substitutions *in vivo* and *in vitro*.

Finally, since we believe that the Vent IVSs are spliced out of a protein precursor and not out of mRNA, I-Tli I has no need to be contained within a self-splicing RNA intron in order to yield active polymerase.

References

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CircumVent™ Thermal Cycle DNA Sequencing Kit

CircumVent™ thermal cycle DNA sequencing is a simple and rapid method which generates sequence information from less template than any other protocol. The kit uses the highly thermostable Vent_{ex}® (exo-) DNA polymerase. The method involves cycles of denaturation, annealing and chain extension with small amounts of template in the presence of primer excess, dNTPs and ddNTPs. In each cycle, the reaction is raised to 95°C to denature double-stranded templates or secondary structure regions of single-stranded DNA templates, lowered to 55°C for the annealing of the primer to the template, and subsequently raised to 72°C for the elongation step of enzymatic synthesis. Subsequent cycles of denaturation, annealing and extension occur in which the excess primer anneals to the identical denatured template molecules as in the first cycle. Cycling of the three steps achieves a linear amplification and high sequencing signal.

Using ³²P or ³³P end-labeled primers, only femtomoles of template are required (as little as 10 ng of M13 single-stranded DNA, 20 ng of double-stranded pUC plasmid DNA, or 300 ng of λgt11 DNA). CircumVent™ thermal cycle sequencing with ³⁵S dATP incorporation requires more template to achieve the same signal, but still much less than in any previously described protocol.

Ordering Information:

#430-50	50 reaction kit	\$125
#430-100	100 reaction kit	\$180

Advantages of CircumVent™:

• Sensitivity

The method requires much less template than a standard reaction due to the linear amplification of labeled product.

• Convenience

The reactions are rapid and easy to perform. The method is efficient for both manual and automated DNA sequencing.

• Speed

There is no need to denature ("collapse") double-stranded DNA template before sequencing. There is no separate annealing step preceding the sequencing reaction.

• Flexibility

For most applications, incorporation of ³²S or ³³P labeled dATP can be used to sequence single-stranded DNA templates (such as those derived from M13, φ1, φd phage or from phagemid vectors) and double-stranded templates (such as plasmid DNA, PCR products, or large linear double-stranded DNA such as bacteriophage λ). Since the kit components are also designed for use with end-labeled primers, much smaller amounts of template can also be sequenced.

• No Secondary Structure Effects

The highly thermostable Vent_{ex} (exo-) DNA polymerase allows sequencing at high temperatures, an advantage for obtaining sequence from DNA templates which have high degrees of secondary structure and which may be recalcitrant to lower temperature sequencing methods.

• Direct Sequencing of Phage Plaques and Bacterial Colonies ■

CircumVent™: Questions and Answers

The control template and primer sequence looks better than my experimental sequence. Why?

In the vast majority of cases, the trouble lies in the template preparation. Often, an additional phenol/chloroform step or alcohol precipitation followed by a 70% alcohol rinse will eliminate the problem.

Another common problem is the substandard performance of thermal cyclers. Temperature ramp times vary from one machine to another often necessitating longer reaction times (increasing each step from 20 sec to 1 min in every cycle). In addition, if one or two lanes of one reaction are lighter than the others or if one lane fails, suspect the cycler. In several cases, the primer has been found to be improperly purified, giving rise to less than optimal results.

Can using too much primer or template be detrimental?

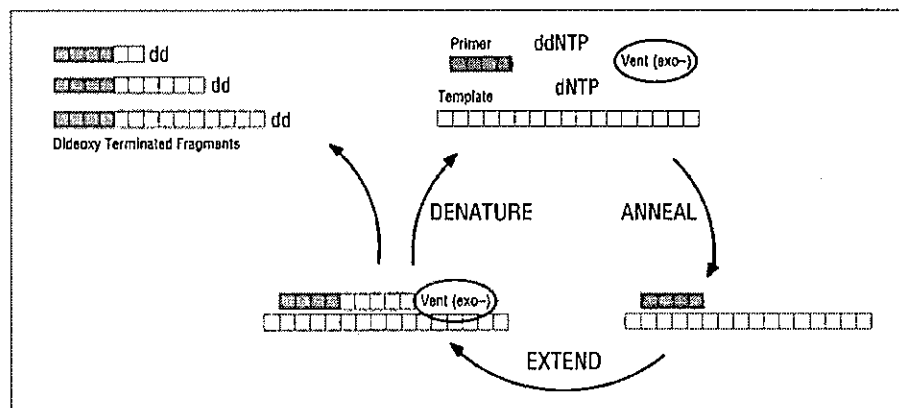
The pmol amounts provided in the protocols have been empirically worked out to provide the best results. Increasing the template or primer amounts may lead to increased shadow banding on resulting autoradiograms.

Is it possible to alter the number of cycles for the reaction?

Yes, the number of cycles can be lowered, especially if the amount of template DNA being used is more than recommended. However, increasing the number of cycles to over 30 has seldom been successful in sequencing smaller amounts of DNA; it tends to increase shadow banding on resulting autoradiograms.

Is it possible to alter the cycle temperatures and/or times for the reaction?

Templates have been successfully sequenced using only a 2-step cycle (a 95°C denaturation step and a 75°C annealing and extension step) even with primers that have apparent T_m values of as low as 55°C. The signal intensities with this approach may be weaker than with the 3-step cycle recom-



CircumVent™ Thermal Cycle DNA Sequencing involves cycles of denaturation, annealing and chain extension.

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mended in the manual. It is possible to lower the annealing/extension temperature, corresponding to the primer T_m , and then perform sequencing cycles consisting of two steps, a 95°C denaturation step and a 55°C annealing and extension step.

If weak sequence signal is observed with the standard 3-step method and it is suspected that the temperature of the annealing step is too high for the primer being used, the annealing step can be lowered to correspond to the individual primer T_m . As mentioned previously, successful use of some cyclers has required longer reaction times (increasing each step from 20 sec to 1 min in every cycle).

When directly sequencing colonies and plaques, is it necessary to make up the resuspension mix fresh each time?

Ideally, the solution should be made fresh each day as needed. However, we have observed that a frozen proteinase K stock solution can be defrosted and refrozen numerous times without losing activity.

When loading the final reactions on the gel, what can be done about the cap of oil on the top of the reactions?

We recommend a 15 second microcentrifugation of the reaction after the stop/loading dye has been added (or just before heating the reactions to load on the sequencing gel) to ensure that the aqueous layer fully separates from the oil layer covering the reaction.

Other techniques that actually involve removing the oil from the top of the reaction include the use of silicone oil followed by precipitation of the reaction products from the oil (Ross, J. and Leavitt, S. (1991) *BioTechniques* 11, 618-619) or the use of parafilm to remove the oil (Whitehouse, E. and Spears, T. (1991) *BioTechniques* 11, 616-618).

Another approach is to use a "hot-top" apparatus, set to at least 15°C hotter than the highest step in the cycle reaction (~115°C). This eliminates evaporation during the reaction and precludes the requirement for putting oil on the reactions. However, we have never observed any

sequence aberration due to oil being loaded into the sequencing gel lane.

Is it possible to use the CircumVent Kit with chemiluminescent detection methods?

Yes. The CircumVent Phototope™ Kit, a non-radioactive version of the successful CircumVent Kit, incorporates chemiluminescent detection into thermal cycle DNA sequencing.

The radioactive incorporation protocol recommends using 2 µl of radioactive nucleotide. Can I use less? Do I have to use the exact specific activities as recommended in the manual? What about for the kinase reactions in the end-labeled protocol?

Less radioactive material can be used. In our experience, 0.5 µl of 500 Ci/mmol ^{35}S label gives a somewhat lighter sequence, whereas little difference is observed with 0.5 µl of 500 Ci/mmol ^{32}P [dATP]. With respect to specific activity, the lower and higher specific activity ^{35}S [dATP] and ^{32}P [dATP] and (the only currently available specific activity) ^{32}P [dATP] all function equally well in the reactions. It is important to ensure that enough dATP is present in the reaction so that DNA synthesis is not encumbered.

With respect to the kinase reactions for preparing end-labeled primers, we recommend the use of the higher specific activity (3000 Ci/mmol) ^{32}P or ^{32}P rATP, as it provides stronger end-labeled primer signal for sequencing. In all protocols we recommend using radioactive material within two half-lives.

Can the CircumVent Kit be used for sequencing templates to be used on automated fluorescent sequencers?

Yes, given the following: In any automated sequencing system which uses end-labeled primers, the CircumVent Kit gives excellent results. However, when fluorescent tagged dideoxy dNTPs are used in the sequencing method, the results can be mixed. Inefficient incorporation leads to different intensities in peak heights on the resultant fluorograms; thus, the software has more difficulty calling correct bases.

However, researchers do use the

CircumVent™ Kit with tagged dideoxynucleotides because the Vent_x (exo⁻) DNA polymerase has been shown to relieve compressions in sequence information, in direct comparisons with Klenow fragment, Taq polymerase (thermal cycling) and T7 Sequenase™ reactions.

Can ^{32}P [rATP] or ^{35}S [rATP] be used for the end labeling protocols?

^{32}P or ^{33}P end-labeled primers are recommended for sequencing small amounts of DNA template (0.004 - 0.01 pmol) or for sequencing directly from bacterial colonies or phage plaques. ^{35}S [rATP] is not recommended since it is inefficiently incorporated by T4 Polynucleotide Kinase.

After cycle sequencing, can reactions be left in the thermal cycler at room temperature overnight?

Yes, since no difference in sequence has been observed between reactions removed immediately after the cycling procedure has been completed and those left at room temperature overnight. This is due to the reduced activity of Vent_x (exo⁻) DNA Polymerase at room temperature. ■

45% Discount on Radionucleotides

With the purchase of a CircumVent™ Thermal Cycle DNA Sequencing Kit, you automatically qualify for a 45% discount on α -[^{32}P] dATP (#NEG-312H) and γ -[^{32}P] ATP (#NEG-302H) from DuPont NEN Research Products. Used in conjunction with the CircumVent Kit, these radionucleotides provide high resolution electrophoretic banding patterns and strong sequence signals.

To receive your 45% discount off the list-price, call DuPont NEN at 1-800-551-2121 and mention code NB-10 and the NEB purchase order number of your CircumVent Kit.

This offer expires December 31, 1992 and is subject to radioactive license verification. Valid only in the US, Canada and Western Europe (excluding Ireland).

Direct DNA Sequencing of Genomic Repeats without Cloning or Amplification

John F. Elder, Jr. and Bruce J. Turner
Dept. of Biology, Virginia Polytechnic Institute

In our studies of the evolution of a highly repetitive satellite DNA sequence (9.5×10^3 copies per haploid genome) in the Atlantic Sheepshead Minnow (*Cyprinodon variegatus*), we wished to estimate the rate at which molecular drive was able to homogenize these repeat sequences within individuals, within populations and among populations (1). We needed to determine the sequence of a very large number of individual repeats and traditional cloning proved to be too labor intensive. Data from collected material suggested that for this sequence there was a high degree of homogeneity between repeats within individuals and highly conserved regions among populations. Primers were designed with the hope that there would be sufficient copies of the sequence per individual to provide a strong sequencing signal using the CircumVent™ Thermal Cycle sequencing method. Sequence homogeneity would thus provide clear unambiguous sequence patterns and heterogeneity would manifest as ambiguous sequence patterns; in either event, we would be able to survey the degree of homogeneity resulting from the mechanisms of molecular drive.

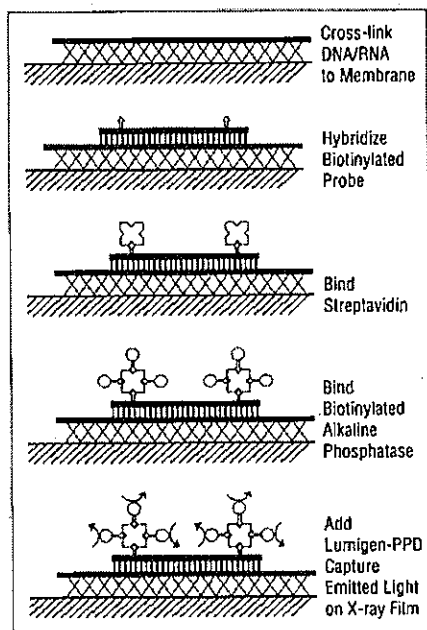
The satellite repeat was cleaved with *Hind* III and isolated from the remaining genomic DNA in a 1% (TAE) Sea-Plaque™ low-melt agarose gel (2). The agarose band containing the repeat DNA was excised and briefly microcentrifuged. Three volumes of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA were added and the mixture was heated at 70°C for 5 minutes to melt the agarose. The slurry was briefly vortexed, quick frozen at -70°C for 5 minutes and then microcentrifuged for 1 min. 8.5 µl of the supernatant was directly used in the CircumVent™ sequencing reaction, using the ³²S dATP label incorporation protocol. The results have provided both individual, interpopulational and intrapopulational estimates of sequence diversity.

1. Turner, B.J. et al (1991) *J. of Fish Biology* supplement 39.

2. Quian, L. and Wilkinson, M. (1991) *BioTechniques* 10(6): 736-737.

Phototope™ Chemiluminescent Kits

All of the Phototope™ Kits are based on chemiluminescence, an enzyme-catalyzed reaction which emits a yellow-green light. This new detection method is replacing radioactive detection due to sensitivity, convenience, safety and cost.



The Chemiluminescent Process

This process is carried out in a sealed hybridization bag through serial incubations with streptavidin, biotinylated alkaline phosphatase, and the dioxetane substrate. After the substrate is added, the membrane is briefly exposed to X-ray film to capture the image. Lumigen-PPD is a trademark of Lumigen Inc.

In the Phototope chemiluminescent kits, biotin associated with the target DNA provides the handle for the chemiluminescent detection. Biotinylated DNA is detected on a membrane support by first exposing the membrane to streptavidin, which binds to the biotinylated DNA. Next, a biotin/alkaline phosphatase complex is added. This complex binds to the streptavidin. The result is the creation of a conjugate between the alkaline phosphatase and the DNA on the membrane. In the final step, the Lumigen-PPD® reagent is added. Alkaline phosphatase catalyzes the removal of the phosphate from the Lumigen (phenylphosphate substituted 1,2 dioxetane)

to yield a moderately stable intermediate which then spontaneously decays to emit light at 477 nm. The emitted light is detected by exposing the membrane to X-ray film for 5 to 10 minutes.

The biotin handle can be associated with your target DNA either directly (covalently) or by hybridization. Biotin can be incorporated directly into DNA by priming enzymatic polymerization of DNA with a biotinylated primer or by polymerization in the presence of biotinylated nucleotide triphosphates.

NEB introduces the Phototope family of products designed for the non-radioactive detection of nucleic acids:

CircumVent Phototope™ Kit

A non-radioactive version of the successful CircumVent™ Thermal Cycle DNA Sequencing Kit which uses Vent_R (exo-) DNA Polymerase

Klenow Phototope™ DNA Sequencing Kit

Incorporates chemiluminescence into standard dideoxy DNA sequencing protocols using the Klenow fragment of DNA Polymerase I

NEBlot Phototope™ Kit

Random primer biotin labelling for chemiluminescent Northern and Southern blotting and plaque hybridizations

Phototope™ Detection Kits

Reagents for the chemiluminescent detection process (streptavidin, biotinylated alkaline phosphatase, and Lumigen-PPD®). The Detection Kits are designed to be used in conjunction with the other three Phototope™ Kits ■

Phototope™ DNA Sequencing Kits

The **CircumVent Phototope™ Kit** is the non-radioactive version of the successful CircumVent Kit which uses Vent_r (exo⁻) DNA polymerase for thermal cycle dideoxy DNA sequencing. In short, thermal cycle DNA sequencing involves cycles of denaturation, annealing and chain extension with small amounts of template in the presence of an excess of biotinylated primer, dNTPs and ddNTPs (see page 12 for a more complete description of thermal cycle DNA sequencing). This method requires much less template than conventional sequencing and saves time by eliminating the need to denature double-stranded templates or perform separate annealing steps prior to the reactions. A standard sequencing gel is run and the resulting gel is blotted and permanently bound to a membrane. The DNA bands are then detected using one of the Phototope™ Detection Kits.

The **Klenow Phototope™ DNA Sequencing Kit** enables conventional Sanger DNA sequencing with chemiluminescent detection and requires minimal adjustment to previously existing techniques. Standard dideoxy DNA sequencing reactions are performed using M13 or denatured plasmid vectors without radioactive isotopes. Instead, a biotinylated primer is annealed to the template prior to the sequencing reactions. A standard sequencing gel is run and the resulting gel is blotted and permanently bound to a membrane. The DNA bands are then detected using one of the Phototope™ Detection Kits.

Phototope Chemiluminescent Sequencing offers the following advantages:

- **Sensitivity**

Equal or greater sensitivity than radioactive detection.

- **Speed**

Produces films that are identical to radioactive detection in only 70 minutes after running the gel.

- **Convenience**

No fixing or drying of gels.

- **Stability**

Biotinylated primers and reagents are stable for extended periods.

- **No Radioactivity**

NEBlot Phototope™ Kit

The **NEBlot Phototope™ Kit** is a complete system for the chemiluminescent detection of nucleic acids in standard Southern and Northern blotting applications. With the NEBlot Phototope Kit, biotin is incorporated into hybridization probes in two ways—first, by utilizing a unique biotinylated random octamer primer and second, by incorporating biotinylated dUTP during polymerization. The biotinylated probes are hybridized to target DNA immobilized on a membrane, and the target DNA is detected by chemiluminescence using one of the Phototope Detection Kits.

The NEBlot Phototope Kit offers the following advantages:

- **Sensitivity**

Detection of single gene copies in mammalian DNA (<1 pg).

- **Speed**

Only 40 minutes is required for the entire detection procedure.

Exposure times of 5-10 minutes.

- **Multiple Exposures**

Light is emitted at a constant rate for a day, so you can perform multiple exposures to optimize signal intensity. Re-exposure at a future date is achieved by simply adding more Lumigen-PPD reagent.

- **Simple Reprobing**

If you want to search the same membrane for a different set of fragments, simply strip the membrane and rehybridize with a different probe.

- **Stability**

Biotinylated probes and reagents are stable for extended periods unlike ³²P-labelled probes which are only stable for a few days.

- **Simultaneous Detection of Biotinylated Molecular Weight Standards**

- **Versatility**

The Kit can be used for Northern blotting, Southern blotting and plaque hybridizations.

- **No Radioactivity**

Ordering Information for Phototope™ Kits

CircumVent™ Phototope™ Kit

Reagents sufficient for 100 sets of thermal cycle DNA sequencing reactions
#7430 \$200

Klenow Phototope™ DNA Sequencing Kit

Reagents sufficient for 50 sets of DNA sequencing reactions
#7409 \$150

NEBlot™ Phototope™ Kit

Reagents sufficient for 25 random primer biotin labelling reactions
#7550 \$175

Phototope™ Detection Kits

Provide reagents for the chemiluminescent detection process, including: streptavidin, biotinylated alkaline phosphatase, and Lumigen-PPD®. The Detection Kits are designed to be used in conjunction with the other three Phototope™ Kits

Phototope™ Detection Kit 6K

Reagents sufficient for chemiluminescent detection on 6,000 cm² of membrane
#7006 \$250

Phototope™ Detection Kit 30K

Reagents sufficient for chemiluminescent detection on 30,000 cm² of membrane
#7030 \$1,000

Phototope™ Detection Kit 60K

Reagents sufficient for chemiluminescent detection on 60,000 cm² of membrane
#7060 \$1,750

A Note about Membranes

Phototope™ Kits require transfer of DNA from a gel to a solid support membrane. The kits were developed using Millipore Immobilon-S membranes; however, other nylon based membranes with a neutral or slightly positive charge can be substituted. Nitrocellulose does not give acceptable results.

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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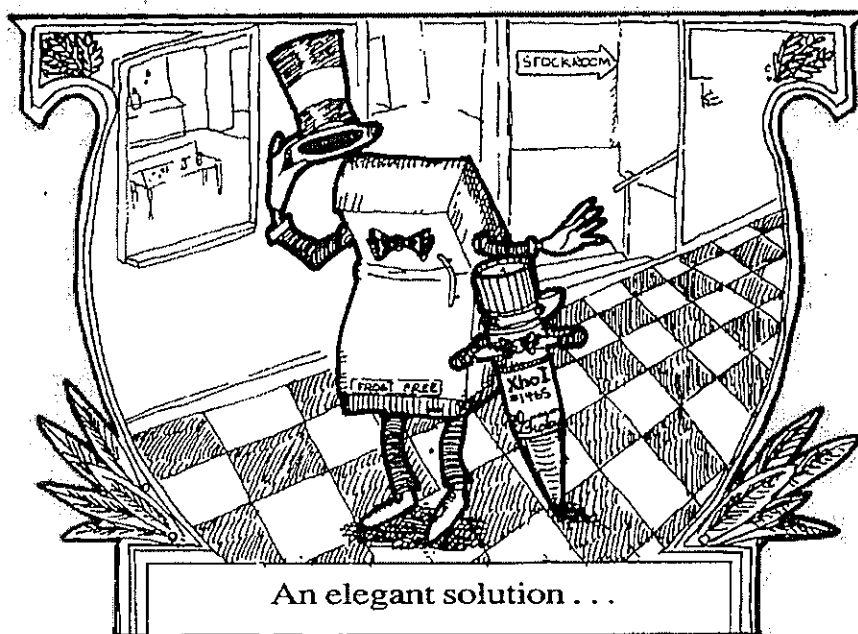
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