

The NEB Transcript

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Newsletter
from
New England
Biolabs

Deep Sea Hydrothermal Vents: Underwater Oases

Holger W. Jannasch

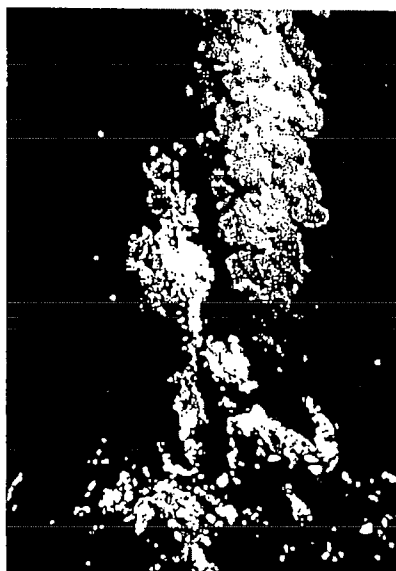
Woods Hole Oceanographic Institution, Woods Hole, MA 02543

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

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Vol. 4 No. 1

EXHIBIT 2084

FLOYD ROMESBERG, PH.D.

09-19-18

Patricia Y. Schuler CSR No. 11949
APTUS COURT REPORTING

Columbia Exhibit 2084

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

IPR2018-00291, 318, 322, 385 & 797

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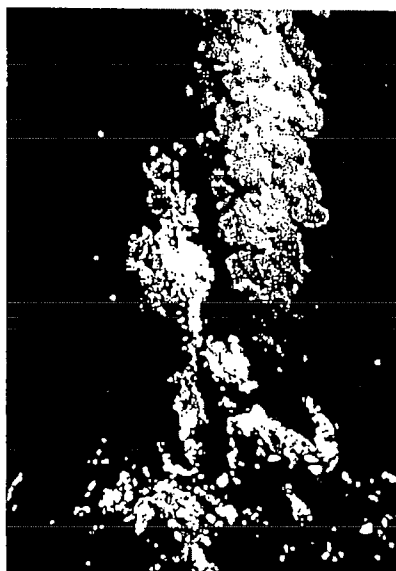


Figure 1. A group of black smokers. Hydrothermal fluid of 350°C is emitted at a rate of 2 ml/s at the 21°N hydrothermal vent site of the East Pacific Rise (depth, 2,610 meters).

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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_r[®] (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
#430-100 100 reaction kit

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.

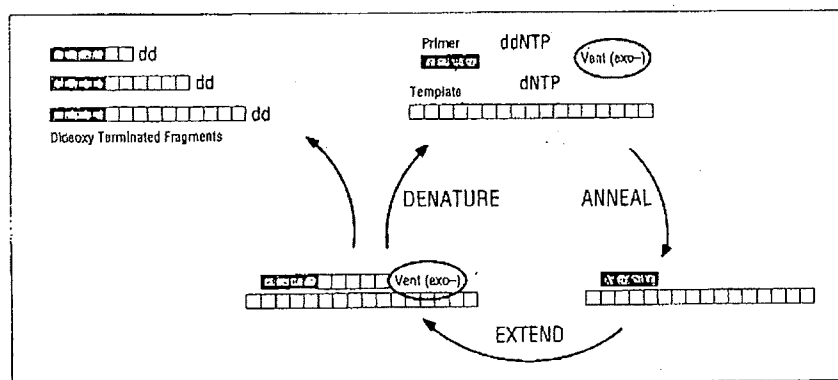
• Flexibility

For most applications, incorporation of ³⁵S or ³²P labeled dATP can be used to sequence single-stranded DNA templates (such as those derived from M13, φ, fd 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_r (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™ Thermal Cycle DNA Sequencing involves cycles of denaturation, annealing and chain extension.

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-

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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 micro-centrifugation 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_{ex} 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 ^{35}S 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_{ex} 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 NEN 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).