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Vectorette PCR: A Novel Approach to Genomic Walking

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Vectorette PCR (or chemical genetics) is a method that enables the amplification of specific DNA fragments in situations where the sequence of only one primer is known.[1,2] Thus, it extends the application of PCR to stretches of DNA where the sequence information is only available at one end. In this report, we describe the chemical genetics method and demonstrate its use in one specific application. In addition, we demonstrate how fragments generated by this procedure can be sequenced rapidly and simply using methods that have the potential for automation.

The steps involved in the chemical genetics method, or vectorette PCR, are shown in Figure 1. There are three basic steps: (1) digestion of target DNA with a suitable restriction enzyme; (2) ligation of suitable synthetic oligonucleotides onto the digested DNA; and (3) PCR using a specific primer and a primer directed toward the synthetic oligonucleotides.

Nonspecific amplification of all digested fragments is avoided by the design of specific fragments of synthetic DNA, called vectorettes. Vectorettes are designed so that they can be amplified only if they are attached to the DNA sequence of interest. The vectorette is only partially double-stranded and contains a central mismatched region (see Fig. 2). The vectorette PCR primer has the same sequence as the bottom strand of this mismatched region and therefore has no complementary sequence to anneal to in the first cycle of PCR. In the first cycle of PCR, only the known primer, which is directed toward the sequence of interest, will prime DNA synthesis. This will produce a complementary strand for the vectorette PCR primer to anneal to in the second cycle of PCR. In the second and subsequent cycles of PCR, both primers can prime synthesis with the end result being that the only fragment amplified contains the sequence of interest.

To obtain single-stranded DNA from PCR products for sequencing, a recently described technique was utilized.[3] If the initiating primer in a PCR reaction is biotinylated, magnetic beads coated with streptavidin immobilize biotinylated DNA products, which can then be denatured with NaOH and separated with a magnet, leaving pure, single-stranded DNA for sequencing, attached to the magnetic beads.

MATERIALS AND METHODS

Preparation of DNA

Chlamydia trachomatis DNA was prepared as described previously.[4]

Digestion, Ligation, and PCR

Prepared DNAs were digested with appropriate restriction enzymes in suitable buffers at 37°C for 1 hr. ATP and dithiothreitol (DTT) were added to a concentration of 2 mM each, and appropriate vectorette units were added along with one unit of T4 DNA ligase (without a change of buffer). The samples were then incubated at 20°C for 1 hr followed by 37°C for 30 min. This incubation cycle was carried out a further two times, because the vectorettes have been designed so that the restriction enzyme site is not reformed on ligation of vectorette to target DNA; this incubation cycle leads to increased target–vectorette constructs. The incubation at 37°C leads to digestion of target–target DNA, but not target–vectorette constructs. PCRs were performed using the appropriate known biotinylated primer and vectorette PCR primer in 1x *Taq* PCR buffer with 2.5 units of *Taq* polymerase (Promega). PCRs were carried out on Techne PHC-1 unit, with 40 cycles of 96°C 1 min, 64°C 1 min, and 74°C 1.5 min. PCR products were visualized on a 1% agarose gel strained with ethidium bromide (see Fig. 3).

The biotinylated primer was synthesized using the biotin phosphoramidite, DMT-BIOTIN-C_6-PA, from CRB Ltd.

Sequencing

A total of 50 µl of magnetic beads were washed as described by the manufacturer (Dynal), followed by incubation with 25 µl of the biotinylated PCR product with gentle rotation for 10–15

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Tecan Ex. 2046, Page 1 of 5
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00029

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min at room temperature. The beads were then magnetized for 60 sec on the magnetic particle concentrator (MPC). The beads were then washed with 5 x 1 nl of 1 x *Taq* PCR buffer using the MPC. Then 0.15 M NaOH (1 ml) was added and the tube was incubated at room temperature for 5 min. The template was recovered and washed with 0.15 M NaOH and sterile distilled water (sdw). The beads were resuspended in 10 µl of sdw.

Next 0.5 pmole of vectorette sequencing primer was labeled with [γ-^{32}]ATP by standard methods and

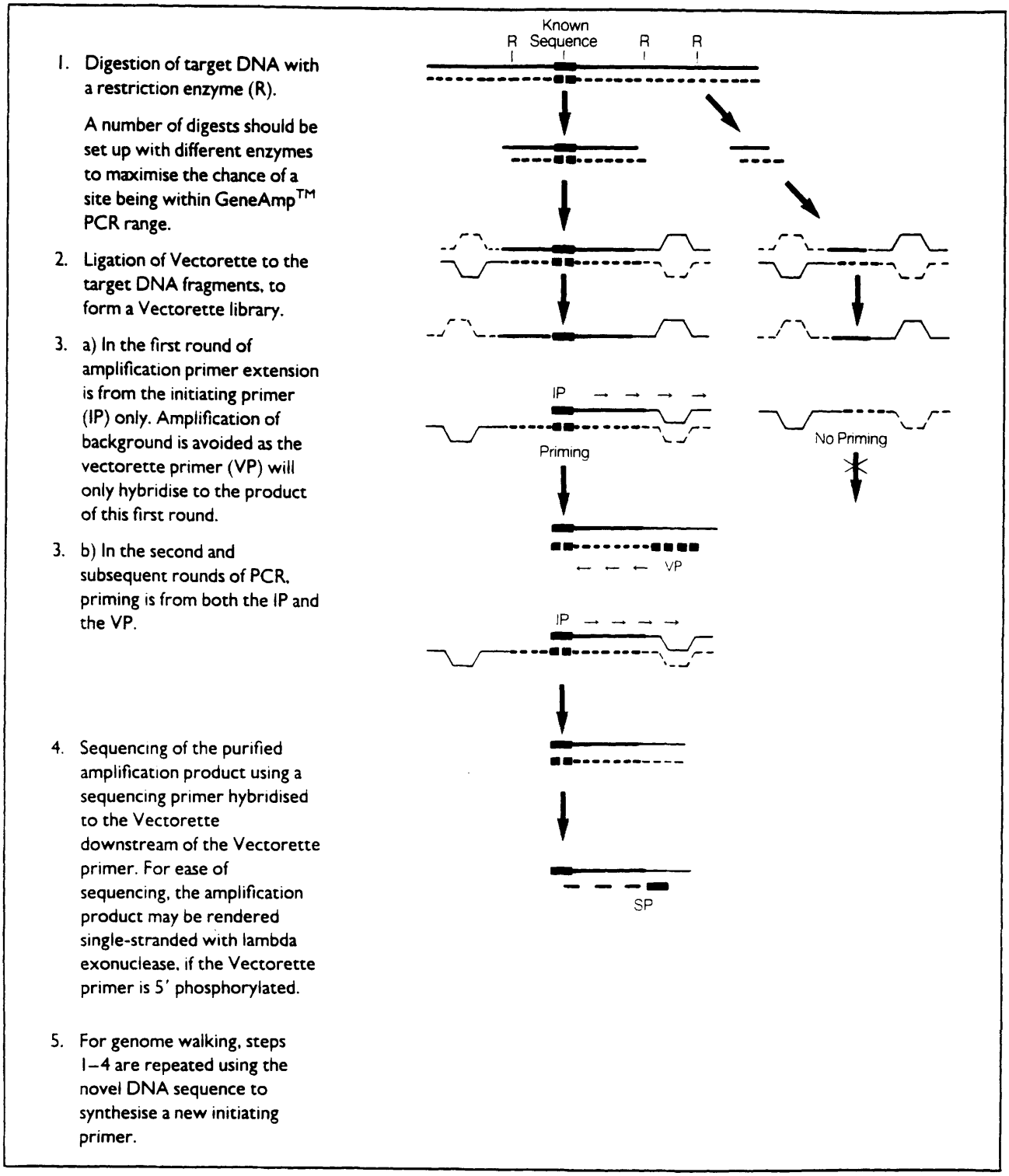

FIGURE 1 The chemical genetics system.

Tecan Ex. 2046, Page 2 of 5
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00029

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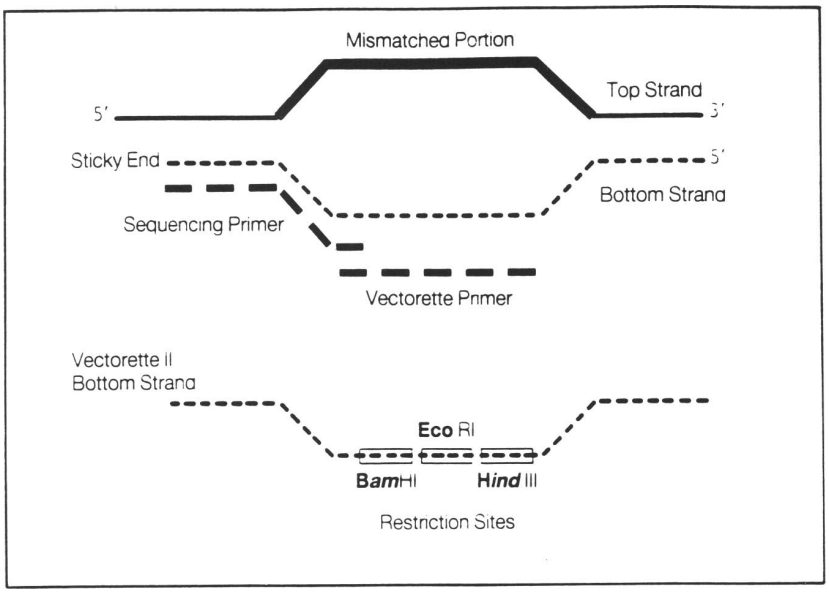

FIGURE 2 Schematic of a vectorette unit.

used to sequence the PCR product using the Sequenase sequencing kit (USB). After the reactions were complete the beads were isolated, washed with sdw, and then resuspended in 3 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). After the addition of 3 μl of sdw, the samples were heated to 96°C for 1 min and 3 μl of each reaction loaded onto a 6% denaturing acrylamide gel which was electrophoresed for 2 hr at 1400 V. Autoradiography gave the sequence shown in Figure 4.

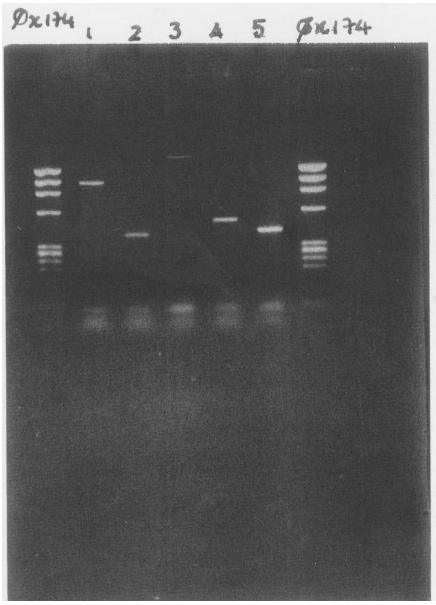

FIGURE 3 Vectorette PCR of *Chlamydia trachomatis* DNA fragments. *Chlamydia* DNA was digested with the restriction enzymes *Bam*HI (lane *1*), *Cla*I (lane *2*), *Eco*RI (lane *3*), *Hin*dIII (lane *4*), and *Hae*III (lane *5*). It was then ligated to the appropriate vectorette I ends, followed by PCR. The PCR primers were the vectorette I universal PCR primer and the initiating primer from the 5′-flanking region of the *Chlamydia* 16S ribosomal RNA gene.

RESULTS

Vectorette libraries using a number of different restriction enzymes were constructed from chlamydial genomic DNA. PCRs were carried out on these libraries using a primer from the 5′ end of the 16S rRNA gene[5] and a vectorette PCR primer. A number of different size bands were produced (see Fig. 3). The authenticity of these bands (produced by the various libraries) was confirmed by Southern blotting, and subsequently by DNA sequencing.

We used a number of different methods to sequence the PCR products produced. We have found that the use of magnetic beads to purify one strand of the PCR product to be particularly convenient. In each case the *Chlamydia*-specific primer was biotinylated, and a vectorette-specific primer was used for sequencing. This enables the vectorette terminal portion of the PCR product to be sequenced. A typical sequencing gel is shown in Figure 4.

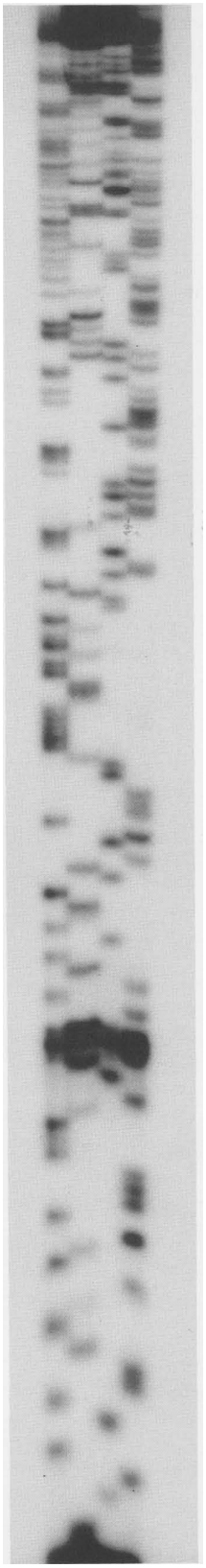

FIGURE 4 Example of sequencing using magenetic beads.

Tecan Ex. 2046, Page 3 of 5
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00029

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By combining the sequence data, it is possible to sequence up to 1.5 kb of previously uncharacterized DNA, using only one biotinylated PCR primer and one sequencing primer in a period of 3–4 days.

DISCUSSION

In this report, we describe a novel approach to the amplification of previously uncharacterized sequence. We have described how the method can be used to generate novel sequence rapidly, starting from a known DNA sequence.

This technique has also been used (1) to sequence the termini of yeast artificial chromosomes (YAC) clone inserts,[6] providing a rapid method of characterizing the large inserts produced in YAC cloning, and (2) to amplify human genomic DNA (Arnold and Hodgson, unpublished). Due to the complexity of the human genome, a second cycle of nested PCR is often required to achieve the desired specificity.

The chemical genetics method can also be used for: (1) genomic walking; (2) sequencing of YAC insert termini; (3) mapping of introns in genomic DNA from cDNA clones; (4) sequencing of large clones without subcloning; and (5) mapping and sequencing of regions containing deletions, insertions, translocations, and so forth.

Advantages of the magnetic bead method include 100% single-strand conversion, high purity, and no interference from unreacted label on the gel (excess label is removed by magnetic separation prior to gel loading). This combination of protocols leads to the generation of novel sequence data from vectorette PCR products within 2 days.

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Tecan Ex. 2046, Page 4 of 5
QIAGEN Sciences, LLC v. Tecan Genomics - IPR2025-00029

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