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Avoiding False Positives in Colony PCR

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Colony polymerase chain reaction (PCR) is a rapid PCR-based technique to determine the presence of insert DNA in plasmid vectors after ligation and transformation. Bacteria from single colonies growing on agar plates containing selective media are used directly for PCR amplification (3,6). With the advantage of being faster and more cost-effective, this technique is now widely used in many laboratories and offers a convenient alternative to the more traditional method of minipreps of plasmid DNA followed by restriction digestion. However, we recently found that if the two primers used in the PCR were derived from the insert alone and not the vector, a high frequency of false-positives resulted, presumably because of amplification of the unligated insert DNA present on the bacterial plate.

Full-length rat hepatocyte nuclear factor 4 (HNF-4) cDNA was amplified by PCR using the original clone pf7 as template and primers to the 5′ and 3′ ends of the coding region of HNF-4, Npf7 and Cpf7, respectively (2,5). The PCR product was digested with *Eco*RI and *Bam*HI (both from Life Technologies, Gaithersburg, MD, USA) gel-purified and ligated into an appropriately prepared vector pcDNA1.1/AMP (Invitrogen, Carlsbad, CA, USA). *Escherichia coli* strain DH5α™ (Life Technologies) cells were transformed by electroporation, and after a 1-h incubation in SOB (0.5 mL) (4), 100-µL aliquots were plated directly onto LB plates containing 50 µg/mL ampicillin (1). Fourteen ampicillin-resistant colonies from the ligation reaction were subjected to colony PCR as previously described (3) using primers Npf7 and

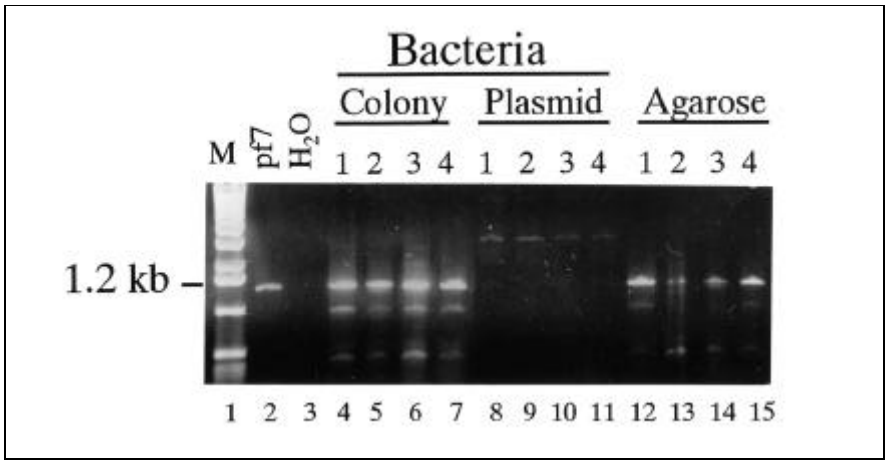

Figure 1. Colony PCR can amplify DNA from an agar plate. PCR was performed as described using the template pf7 (positive control) (lane 2), deionized water (negative control) (lane 3), single colonies from the original transformation plate (lanes 4–7), miniprep plasmid DNA from the corresponding single colonies (lanes 8–11), and agar samples obtained randomly from the original transformation plates (lanes 12–15). Lane 1: 1-kb DNA marker (Life Technologies). Shown is an ethidium bromide-stained 0.8% agarose gel.

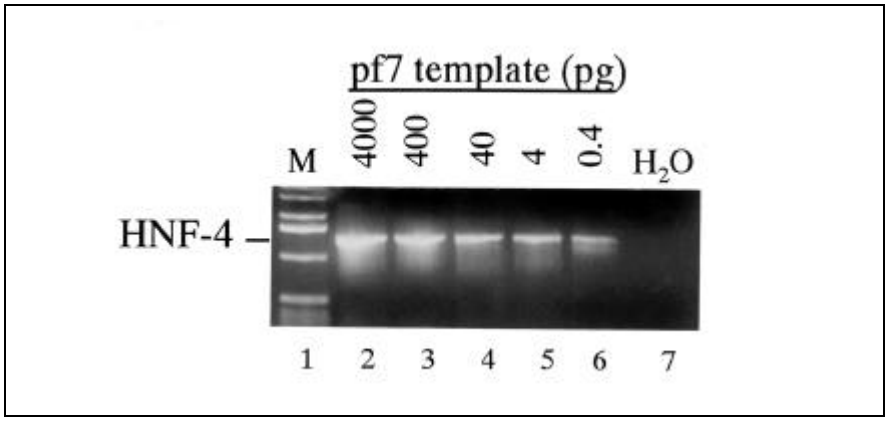

Figure 2. Template dilution experiment. PCR was performed and analyzed as described in Figure 1 using a series of diluted pf7 templates, so that the final amounts of template in the PCRs were 4000, 400, 40, 4 and 0.4 pg, as indicated. Lane 1: 1-kb DNA marker (Life Technologies). Lane 7: water used in place of template.

Cpf7 (92°C for 1 min, 54°C for 1 min and 72°C for 2 min; 32 cycles). The products were analyzed by agarose gel electrophoresis, and all were found to contain a 1.2-kb PCR product (gel not shown), suggesting, at least initially, that all the bacterial colonies contained the recombinant vector.

However, when minipreps were done on colonies that had been streaked to singles and the plasmid DNA analyzed by restriction digestion, no insert DNA was present in any of the minipreps (data not shown). These results suggested that the colonies did not contain recombinant vectors and that the colony PCR product was due to contamination, which, we hypothesized, resulted from the unligated HNF-4 insert DNA present on the agar plate. Control reactions lacking template verified that the PCR solutions were not contaminated with template.

To test the above hypothesis, colonies from the original transformation plate, the plasmid DNA from the minipreps of the corresponding single colonies and small amounts of agar samples obtained randomly from the transformation plates in places lacking any noticeable bacterial growth were subjected to PCR amplification using insert primers Npf7 and Cpf7. The results, depicted in Figure 1, show that full-length HNF-4 cDNA (1.2 kb) was amplified specifically from all of the original colonies (lanes 4–7) but not from any of the corresponding miniprep plasmid DNA (lanes 8–11). Surprisingly, the full-length HNF-4 cDNA was also amplified from the four agar samples devoid of bacterial growth (lanes 12–15). Neither a water control (lane 3) nor an agar sample from a plate of bacteria transformed with vector alone (not shown) yielded a PCR product. Therefore, we propose that the PCR product from the agar samples resulted from trace amounts of insert DNA that were present in the transformation culture that was spread on the selection plate.

To estimate the minimum amount of template required to give a signal in the PCR, a template dilution experiment was performed (Figure 2). pf7 DNA (4000, 400, 40, 4 and 0.4 pg) was used in PCR with primers Npf7 and Cpf7. As shown in lane 6, 0.4 pg of template was readily amplified in the PCR. Taking into account the amount of insert DNA in the ligation reaction (100 ng), the amount of culture spread on each plate (one-fifth of the total), the area of the plate (55.4 cm^2) and the size of the agar sample used in PCR (ca. 4 mm^2), we estimate that there could be as much as 10 pg of insert DNA present in the agar sample used in the PCR, which is more than sufficient to yield a detectable product.

In summary, this report shows that PCR can detect DNA from a ligation reaction spread as part of a transformation culture onto bacterial plates. To avoid this problem, a primer from the vector as well as the insert should be used in colony PCR to yield an overlapping product containing both vector and insert. Such a reaction serves the dual purpose of verifying that the insert is in the correct orientation. Centrifugation of the culture and resuspension prior to plating does not eliminate the problem of false positives because we have also detected PCR products under these conditions (not shown). It also suggests that the DNA might be bound to the cells. We have also found that the best positive control for colony PCR is a bacterial colony containing the appropriate target plasmid as opposed to purified plasmid DNA. A corresponding negative control of parental vector in a bacterial colony is also very helpful because different primers yield different nonspecific bands from the bacterial genome.

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Digestion of Terminal Restriction Endonuclease Recognition Sites on PCR Products

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One of the common methods for cloning polymerase chain reaction (PCR) products is overhanging-end cloning (also known as sticky-end or directional cloning). Frequently, it is not possible to use restriction enzyme sites already present in the amplified product, and primers that encode recognition sites of restriction endonucleases in addition to the specific sequence have to be designed. After amplification of the target sequence with these primers, the PCR products are purified, digested with restriction enzymes and cloned into vectors treated with the same enzymes. However, it has been found that many restriction enzymes fail to cleave at the end of PCR fragments (1–5). To circumvent this problem, the addition of at least three more nucleotides at the end of a restriction site (1) was suggested. In contrast to this, it was claimed in one study that even 3–4 extra bases might be insufficient for reliable cutting (3). Some publications (4,5) and commercial catalogues contain lists of base pair extensions required for efficient restriction endonuclease cleavage near DNA termini. One major disadvantage of these lists is that they are all based on experiments in which a plasmid vector has been subsequently digested with two enzymes specific for neighboring cleavage sites. Because the distance of the two restriction sites in a plasmid vector is predetermined, these lists give no information about the minimal number of base pairs really necessary for the restriction enzyme to successfully attach to its cleavage site; furthermore, the experiments have not been performed with PCR products. We per-

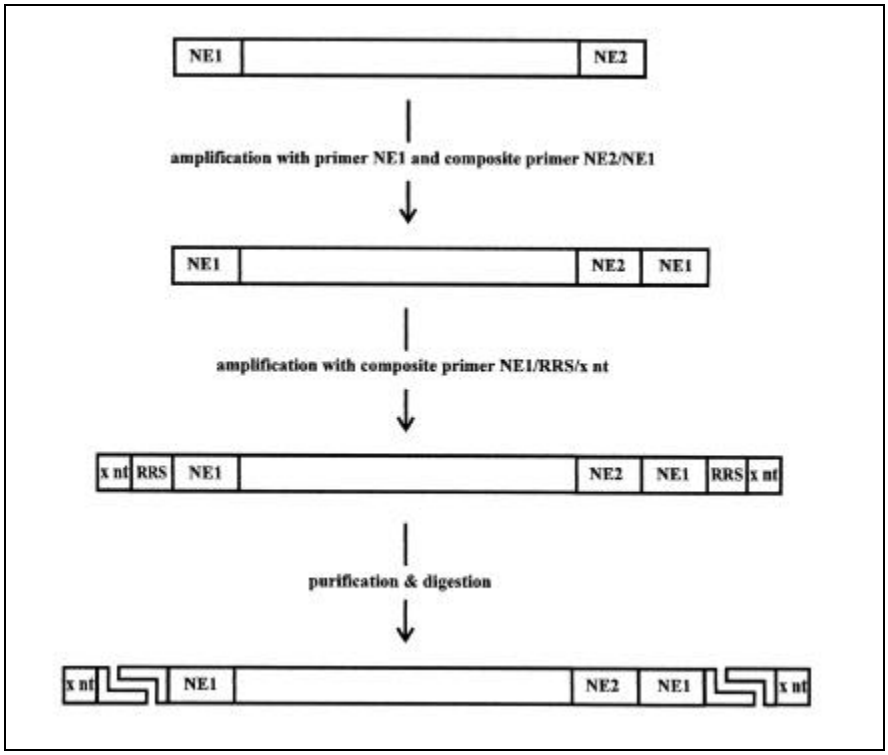

Figure 1. Scheme of the assay for reproducible cleavage of PCR products. The target sequence was amplified with primer NE1 and composite primer NE2/NE1. The resulting chimeric target sequence containing two terminal NE1 primer recognition sequences was then amplified with one primer composed of the sequence NE1, the restriction enzyme recognition site and 0–3 additional nucleotides at the 5′ terminus (NE1/RRS/xnt). After purification by microconcentrators or ethanol precipitation, the PCR products were digested with the respective restriction endonucleases and analyzed on an ethidium bromide-stained agarose gel.