Picogram Cloning and Direct In Situ Sequencing of DNA from Gel Pieces

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ABSTRACT

We describe a simple and rapid procedure for cloning and sequencing of DNA fragments separated by gel electrophoresis, using novel hydrophilic gels, Clearose®, BG, Spreadex®, and Poly(NAT)®, that do not melt at 95°C. For cloning, a band of interest is excised precisely and incubated in an extraction buffer containing 5–10 mM MgCl₂ at 70°C for 15–45 min. The eluted DNA is added directly to the plasmid solution. Using a topoisomerase-based ligation system, we were able to transform bacteria with a few picograms of DNA and isolate recombinant clones. For in situ sequencing, the DNA in the gel serves as the template. No treatment before cycle sequencing is necessary for fragments up to 500 bp.

INTRODUCTION

The first steps in characterizing DNA fragments include size separation by gel electrophoresis, followed by extraction, subcloning, and sequencing. Optimal results require clear spatial separation of bands on the gel, precise excision, efficient extraction, and contamination-free subcloning. Submarine gel electrophoresis on agarose is widely used: it is inexpensive and easy, and DNA fragments are efficiently isolated by simple centrifugation (5) or by using chaotropic salts and silica beads (1). However, the resolving power of agarose gels is poor. It is insufficient for applications such as microsatellite analysis, single-strand conformational polymorphism (SSCP), or differential display (DD). Polyacrylamide gels offer increased resolution; however, DNA is difficult to extract from them, resulting in poor reproducibility (14) or requiring time-consuming procedures (3,6,15,16). Therefore, a PCR amplification step is usually carried out before cloning or sequencing of fragments excised from acrylamide gels. The extra PCR step increases the risk of subclone contamination with extraneous DNA. Recently, PCR-inhibiting contaminants have been reported to elute from polyacrylamide gels to a degree dependent on time and temperature of incubation (4).

Alternative gel matrices have been introduced to improve the resolution and extraction of electrophoresed macromolecules. Poly(NAT)® gels are prepared by polymerization of the monomer N-acryloyl Tris (11,12).
Clearose® BG gels are composed mainly of agarose cross-linked with 1,4-butanediol diglycidylether (7,9), whereas Spreadex® gels (Elchrom Scientific AG, Cham, Switzerland) are fully synthetic and are prepared by free-radical polymerization, resulting in a unique 3-D structure that prevents migration of DNA fragments larger than the exclusion limit (8,10). These alternative gel matrices increase the spatial separation of DNA fragments, and their thermostability permits in situ cycle sequencing. We now describe techniques using these matrices to support band excision, extraction, and direct cloning and sequencing of picogram amounts of electrophoresed DNA. Our methods require less DNA and less work compared to the existing protocols. Moreover, these techniques make re-amplification of gel-purified PCR fragments unnecessary in most cases for downstream applications such as subcloning or sequencing. Therefore, the risk of amplifying contaminating DNA sequences, which may lead to false-positive clones, is minimal.

MATERIALS AND METHODS

Gel Electrophoresis

Three PCR products of the porcine α(1,2)fucosyltransferase gene (FUT2) were investigated: a 1047-bp fragment with the primers FUT2p13G (5′-GC-CAATTACACGCTCCCGGAC-3′) and FUT2p30G (5′-GATAATCATCACGG-GCACCGG-3′), a 541-bp fragment with the primers FUT2p17 (5′-AACTGCACTGCCAGCTTCATGC-3′) and FUT2p12 (5′-GGTAGAAGGTCCAGGAGCAGG-3′), and a 114-bp fragment with the primers FUT2p11 (5′-GCAGGATCCCCTGGCAGAACT-3′) and FUT2p12. Depending on the DNA length and the separation needed, the fragments were loaded in various amounts onto Clearose BG (1047 bp), 6% Poly(NAT) (1047 and 541 bp) or Spreadex EL 400 (114 bp) gels. All gels were run in the SEA 2000® gel electrophoresis apparatus (Elchrom Scientific AG) for 45–60 min in 0.75× TAE buffer (containing 30 mM Tris-acetate and 0.75 mM EDTA) at an electric field strength of 10 V/cm. The Poly(NAT) and Spreadex EL 400 gels were run at 55°C, and Clearose BG gels were run at 20°C. Electrophoresis at 55°C reduces running time 2-fold and also eliminates sequence-dependent migration of DNA fragments. Control experiments were performed using either 6% and 8% polyacrylamide (Quantum Appligene, Europe Inter Services, Basle, Switzerland) or 1% and 2% agarose gels (Life Technologies AG, Basle, Switzerland).

After the run, the gels were stained
using either ethidium bromide (0.4 µg/mL) or SYBR® Gold (Molecular Probes, Eugene, OR, USA) diluted 1:10 000 in 0.25× TAE buffer, pH 8.2 (containing 10 mM Tris-acetate and 0.25 mM EDTA) and were subsequently destained for 30 min in double-distilled water. Polaroid® photographs were taken on a transilluminator (302 nm wavelength for preparative work, 254 nm for analytical work).

**Isolation of DNA Fragments**

The amount of DNA in PCR product bands was estimated either by a fluorescence method using Hoechst dye 33258 (13) and a fluorometer (Fluostar; Tecan, Hombrechtikon, Switzerland) or by comparing intensities of the bands to known standards of 200, 500, and 1000 bp (GenSura, San Diego, CA, USA), using a 2.1-megapixel digital camera (Samu- rai 2100DG; Kyocera Yashica AG, Thalwil, Switzerland) and quantification software (One-Dscan; Scanalytics, Fairfax, VA, USA). A newly developed gel excision device (BandPick™; Elchrom Scientific; patent pending) made possible precise isolation of individual bands. After staining, the gel was placed on a transilluminator (Vilber Lourmat, Torcy, France), and the BandPick was inserted into the band of interest. Pulling up the piston of the BandPick created a weak vacuum so that a gel piece remained inside the device. Pushing the piston back ejected the gel piece into a reaction tube for further analysis. A gel piece excised with BandPick has a volume of about 5 µL. If it is necessary to recover higher amounts of DNA, then the procedure can be repeated until the whole band is excised.

As an alternative to the BandPick device, one can use a 1-mL pipet tip. However, because it is difficult to create a vacuum in such a tip, the punched gel piece tends to slide out. Sometimes this can be prevented by making the inner surface of the tip rough by scratching it with forceps that have sharp teeth at the edges. In addition, gel pieces excised with pipet tips vary in size, making it difficult to reproducibly adjust concentrations in subsequent steps.

**Elution and Subcloning of DNA Fragments**

To the test tube containing the excised gel piece, an equal volume (5 µL) of solution containing 5 mM MgCl₂/10 mM Tris-HCl (pH 8.0) was added. Short fragments were then incubated at 70°C for 15 min, and long fragments were incubated at 70°C for 45 min in a thermal cycler (PCR Express; Hybaid, Teddington, UK) with heated lid. A drop of mineral oil needs to be added when using a cycler without a heated lid. The supernatant containing the eluted DNA fragment (2.5 µL) was added to 1 µL pCR2.1-TOPO vector and ligated according to the supplier’s recommendations (TOPO TA Cloning® kit; Invitrogen, Groningen, The Netherlands). One-third of the ligation reaction mixture was added to 50 µL chemically competent TOP10 OneShot™ E. coli cells (Invitrogen). After a 30-min incubation on ice, the bacteria were heat-shocked for 30 s at 42°C, incubated for 2 min on ice, mixed with 250 µL SOC medium (which contained 2% bacto tryptone, 0.5% bacto yeast extract, 10 mM MgCl₂, 8.55 mM NaCl, 2.5 mM KCl, and 26 mM glucose) and placed on a shaker at 37°C at 225 rpm for 30 min. A solution of X-Gal (40 µL, 20 mg/mL in dimethyl formamide; Flu- ka, Buchs, Switzerland) was evenly spread on top of 100-mm LB agar plates (containing 1.5% agarose, 1% tryptone, 0.5% yeast extract, 1% NaCl, and 50 µg/mL ampicillin) and incubated for at least 10 min before plating 10–30 µL of the suspension of transformed bacteria. Standard procedures for subcloning of PCR products or subcloning of DNA that was purified from agarose using silica particles (QIAEX® II, Qiagen AG, Basle, Switzerland) were followed as controls. Blue and white colonies were counted after 16 h at 37°C. The cloning efficiency was estimated by counting the number of white colonies (Table 1). For comparison, a commercially available TA cloning kit that uses a ligase instead of topoisomerase was also tested.

**In Situ DNA Sequencing**

Gels were stained and destained after the run as described above. Using the BandPick device, a gel piece was excised from hydrophilic gels and briefly rinsed with double-distilled water. (Because of the softness of polyacrylamide gels, their DNA fragments were cut out with a scalpel.) In all cases, gel pieces of approximately 5 mg were used. Then, 8 µL of the sequencing mixture were added (BigDye® Terminator Cycle Sequencing Kit; Applied Biosys-

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**Table 1. Subcloning of PCR Products**

<table>
<thead>
<tr>
<th></th>
<th>32</th>
<th>10.7</th>
<th>3.6</th>
<th>1.2</th>
<th>0.39</th>
<th>0.13</th>
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<tr>
<td>Recovered (ng)</td>
<td>1.6</td>
<td>0.53</td>
<td>0.18</td>
<td>0.059</td>
<td>0.020</td>
<td>0.007</td>
</tr>
<tr>
<td>For DNA ligation (pg)</td>
<td>800</td>
<td>267</td>
<td>89</td>
<td>30</td>
<td>10</td>
<td>3.3</td>
</tr>
<tr>
<td>For DNA transformation (pg)</td>
<td>267</td>
<td>89</td>
<td>30</td>
<td>10</td>
<td>3.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Bacteria plated with 1/10 volume (pg DNA)</td>
<td>26.7</td>
<td>8.9</td>
<td>3.0</td>
<td>1.0</td>
<td>0.33</td>
<td>0.11</td>
</tr>
<tr>
<td>Recombinant colonies</td>
<td>121</td>
<td>114</td>
<td>34</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Different amounts of the 541-bp FUT2p17/FUT2p12 PCR fragment were separated on a 6% Poly(NAT) gel stained with SYBR Gold (Figure 2). After excising the bands with the BandPick device, the DNA was extracted from gel pieces by simple incubation at 70°C in 5 µL buffer containing 5 mM MgCl₂. Supernatant (2.5 µL) was ligated into a pCR2-TOPO cloning vector, and recombinant (white) colonies were selected using agar plates containing ampicillin and X-Gal.
RESULTS AND DISCUSSION

Recovery of DNA

Depending on the gel type and the amount of DNA per band, the fraction of the band excised by the BandPick device was approximately 1/5 for Spreadex EL 400 and Clearose BG gels, and 1/10 to 1/5 for Poly(NAT) gels. Figure 1 shows the recovery of 200-, 500-, and 1000-bp fragments from a Clearose BG gel. Dark spots within the bands show the part excised using the BandPick device. Starting with 50 ng DNA per band and one excised gel piece, the recovery of the 200-bp fragment was 4.9 ± 1.0 ng, that of the 500-bp fragment was 4.2 ± 0.8 ng, and that of the 1000-bp fragment was 2.6 ± 0.3 ng, based on three independent experiments. The recovery of the same DNA fragments from a 6% Poly(NAT) gel was 4.8, 2.6, and 0.8 ng, respectively (not shown). Clearose BG gels gave the highest recovery, but their resolving power is lower than that of Poly(NAT) and Spreadex gels.

Control experiments using polyacrylamide gels were only successful with small fragments of around 100 bp and using 2 vol 5 mM MgCl₂ solution/mg gel. The excision efficiency was strongly dependent on the fragment size. It was 30% for the 100-bp fragment (8% gel), less than 10% for a 500-bp fragment, and less than 3% for a 1000-bp fragment (6% gel). However, in every case, re-amplification of the DNA fragments by PCR was possible.

Subcloning

Figure 2 shows a 1:3 serial dilution of the 541-bp PCR fragment. The DNA fragments were separated on a 6% Poly(NAT) gel and were stained with SYBR Gold. The visible cut areas are smaller than the excised pieces because the gel is elastic and shrinks after a piece is removed. Table 1 summarizes the results of the subcloning experiments when the excised 541-bp fragment was ligated into the pCR.2-TOPO vector. Starting with 32 ng DNA in the band in lane 2, we estimate the excised gel piece to be approximately 1/10 of the whole band. When the gel piece is incubated in an equal volume of extraction buffer, then the maximum possible amount of eluted DNA in the supernatant equals the DNA amount that remained in the gel. As indicated above, starting with 50 ng of a 500-bp fragment, the recovery was 2.6 ng, so we estimate that from 32 ng the recovery was 1.6 ng. One-half of this amount, 800 pg, was present in the ligation reaction. One-third of this DNA (267 pg) was transformed into E. coli cells. Since we plated only 1/10 of the bacterial suspension, corresponding to 26.7 pg DNA, the 121 white colonies (>97% of total colonies) corresponded to around 3625 recombinant colonies/ng DNA taken for ligation. We also performed cloning experiments with bands containing less DNA (Figure 1). Recombinant colonies were detected even from scarcely visible bands containing about 0.1 ng DNA, which corresponded to plating of less than 1 pg DNA (Table 1). When working with these extremely low amounts of DNA, we recommend that all transformed bacteria be plated and/or that the whole band of interest be excised. For ligation, we always used the same amount of plasmid DNA and one-half volume of the eluted DNA, whose concentration varied over 200-fold. Recombinant colonies were obtained in all cases, but it is possible that adjusting the ratio of plasmid to insert DNA would be beneficial.

The subcloning of the 114-bp fragment from Spreadex EL 400 gels was also very efficient (4996 colonies/ng DNA). We observed a large number of light blue colonies. However, 10 out of 10 light blue colonies turned out to be real recombinants, whereas PCR showed that the dark blue colonies contained no insert.

As expected, the subcloning of the longest DNA fragment (1047 bp) was the most difficult. This fragment was eluted from a Clearose BG gel using the procedure described above. With a prolonged extraction time (1 h) and prolonged ligation time (20 min instead of 5), we were able to get 630 recombinant colonies/ng DNA. The poor cloning efficiency seemed to be due to poor elution of the DNA, as direct subcloning of the PCR product into the pCR2 Topo vector was very efficient (about 20,000 colonies/ng DNA). The subcloning efficiency using gel-purified DNA fragments from agarose gels was slightly lower because the elution...
efficiency was only 70%-90%.

We also tested a ligase-based TA cloning system for cloning of DNA fragments extracted from Spreadex and 6% Poly(NAT) gels. The results with the 114-bp fragment were comparable to those obtained using the topoisomerase-based system, except for a higher background (70% white colonies). We successfully subcloned the 541-bp DNA fragment using the ligase system; however, the number of recombinant colonies was only 6% of that in the topoisomerase-based system, and the background was higher (14% white colonies).

**In Situ Sequencing**

The in situ sequencing of the 114- and 541-bp fragments succeeded with all three ethidium bromide-stained gel types (Elchrom Scientific AG) using the BandPick device. A maximum of two gel pieces per reaction, not exceeding 10 µL or 50% of the total sequencing volume, was optimal (Figure 3A). The DNA amount in the sequencing reaction was not critical. Using the 541-bp DNA fragment in Clearose BG gels as a template, we obtained comparable signals starting with 38–150 ng DNA/band, equivalent to 7.5–30 ng DNA in the excised gel pieces. The sequence quality and the number of nucleotides identified were comparable to those obtained with DNA templates purified from agarose gels using standard procedures [amount of DNA used per sequencing reaction (ng): fragment size (bp)/20]. For comparison, we tested direct sequencing with agarose and polyacrylamide gels. No readable sequence was obtained after in situ sequencing with agarose gel. With 8% polyacrylamide gel, readable sequence was obtained with the 114-bp fragment (15–70 ng DNA/gel piece), but the sequence of the 541-bp fragment (in 6% polyacrylamide gel) was readable only up to 200 bp. We observed significant gel swelling so that little free liquid was left at the end of the cycle sequencing reaction. This could be the reason for the poor sequencing results. Alternatively, the release of polymerase inhibitors from the polyacrylamide gel may have occurred (4).

Importantly, there was a consid-

erable difference in the sequence quality depending on the dye used for gel staining. With SYBR Gold-stained gels, direct in situ sequencing produced many sequence ambiguities and errors after 100 bp of sequence (Figure 3B). GA sequences often appeared mutated to GX; however, we could not detect a clear pattern. At present, we cannot explain this difference between the two dyes. The same errors occurred when pure DNA was used for sequencing and SYBR Gold was added separately, indicating that the errors were related to the presence of SYBR Gold, and not the gel, in the sequencing reaction. Sequence quality was not improved by prolonged destaining of SYBR Gold-stained gels. Therefore, we recommend that ethidium bromide be used for staining of gels before direct sequencing.

Taken together, these studies clearly demonstrate that DNA fragments from electrophoresis gels can be used directly for cloning and sequencing when the gels are made of hydrophilic, thermally stable materials. The new gel excision device BandPick allows precise cutting and picking of gel pieces containing pure DNA fragments. Picogram amounts of DNA are sufficient for cloning. The proposed techniques may save considerable time in identifying and characterizing DNA fragments, particularly in DD analysis. Use of polyacrylamide gels for direct sequencing and subcloning is restricted to small fragments of around 100 bp and high-quality gels because of inefficient extraction of DNA and inhibiting contaminants. Larger fragments have to be amplified by PCR, and the purified fragments may be contaminated by unrelated DNA sequences, leading to false-positive clones when the band of interest is re-amplified (2). Our methodology

![Figure 3. In situ sequencing. A 541-bp PCR fragment (90 ng) was separated on a 6% Poly(NAT) gel at 55°C and 10 V/cm for 45 min and stained with either ethidium bromide (A) or SYBR Gold (B). After destaining, the bands were punched out using the BandPick device and sequenced by adding the sequencing mixture directly to the gel pieces. Sequences were analyzed on an ABI Prism 377 Sequencer. Occasionally, sequencing errors [GA to GX mutations, indicated by an asterisk (*)] occurred when SYBR Gold was present in the sequencing reaction (B). Such errors were not observed using gels stained with ethidium bromide (**)(A). Direct sequencing from Clearose BG and Spreadex gels was also successful (not shown).](image-url)
makes re-amplification unnecessary because DNA is extracted efficiently from the hydrophilic matrix. Its combination of spatial separation, precise excision, efficient extraction, and contamination-free subcloning provides high-quality characterization of DNA fragments.

REFERENCES


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