Purification of DNA fragments from 2 kb to 18 kb using the Model 491 Prep Cell

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Introduction

The following procedure describes how the p53 (exons 3–11) gene was isolated from genomic DNA using continuous elution electrophoresis. After performing a restriction digest, the DNA fragments were loaded onto an agarose gel in the Model 491 Prep Cell. DNA fragments between 2–18 kb were resolved and isolated in a single run. Under the conditions presented (50 V constant, 9 cm gel height and genomic DNA restricted with *Bam*HI), the 7.5 kb fragment eluted in approximately 33 hours. Eluted fractions were then tested for the 7.5 kb p53 gene by PCR* and dot-blot analysis.

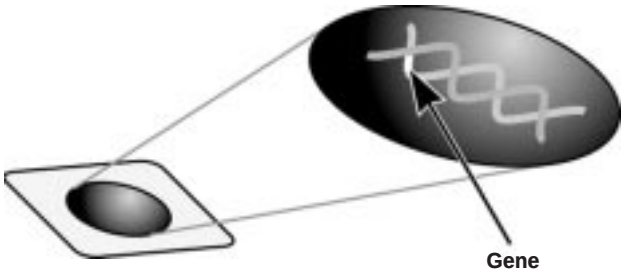


Fig. 1. Genomic gene isolation. Isolate individual genes as DNA fragment lengths.

Methods

MINI-PROCEDURE

1. Digest genomic DNA with appropriate restriction endonuclease. If DNA is digested with a restriction endonuclease having a high amount of recognition sequences, yielding a DNA with a low average Mr, gel puckering is reduced and more DNA starting material can be used.
2. Clean up the DNA by phenol, phenol-chloroform, chloroform extraction. Precipitate DNA and resuspend in 1x TE (pH 8) at a concentration of 1 µg/µl. Add 10x loading buffer dye to a final concentration of 1x.
3. Load onto preparative agarose column.
4. For high yields of purified genomic DNA fractions, an elution buffer rate of 50 µl/min, with a current of 50 constant volts, 2 mA is recommended. Collect 20 min fractions; each fraction yielding a 1 ml final volume.
5. Take 30 µl from each and run on standard agarose EtBr stained gel for viewing.
6. Precisely determine fractions containing the highest percentage of gene of interest by PCR or dot blot analysis.

CASTING THE PREPARATIVE AGAROSE GEL

1. Place the large gel tube assembly (37 mm ID) on the casting stand, aligning the four plastic screws with the holes in the casting stand. Hand tighten the screws. Level the stand with the aid of the leveling bubble using the leveling legs.
2. Insert the cooling core so that the two placement guides slide through the grooves of the cooling core collar in the gel tube assembly and the center pin on the casting stand is inserted in the elution tube of the cooling core. Turn the core 90° until it locks into place (this will prevent any vertical or lateral movement of the cooling core as well as gel traveling up the elution buffer outlet during polymerization).
3. Place the apparatus at 4 °C and pour the preparative gel formulation for agarose (see Table 1, Preparative Agarose Recipes, for agarose concentrations). Pouring the gel in the cold will assure uniform gels. Pour the agarose into the tube through the gap between the cooling core and the collar of the gel tube assembly. To assure good band separation, it is recommended that agarose be added up to the 8 cm marking on the gel tube assembly.
4. After hardening, pour a 0.25% agarose stacking gel on top of the preparative gel. This will prevent puckering of the main preparative gel. A stacking gel of 1 cm in length is recommended, as indicated by the graduated markings on the Gel Tube Assembly.
5. After the gel has solidified, remove the gel tube assembly with cooling core from the casting stand. Do this with the apparatus placed horizontally, to prevent the agarose from sliding out. Gentle force is required.
6. Place the gel tube assembly containing the gel on the elution chamber base. The frits and dialysis membrane must be completely wetted prior to use. To insure removal of entrapped air in the pores of the frits, place the frits and dialysis membrane in running buffer and place under vacuum for at least 10 min. Place the frits and dialysis membrane into the elution chamber base and screw the chamber base to

the gel tube assembly. Note: Do this with both apparatus placed horizontally, to prevent the agarose from sliding out.

7. Fill the upper electrophoresis buffer reservoir (300–600 ml) and the elution buffer reservoir (750 ml) with 50 mM TBE and check that all the lines are properly connected. Add 1,500 ml of 50 mM TBE electrophoresis buffer to the lower buffer reservoir (to cover at least the height of the gel). There is no need to degas the buffers.
8. Purge the Elution Chamber of air bubbles.
9. Cool the gel by using the buffer recirculation pump at a flow rate of ~100 ml/min (determine prior to setting up the apparatus).
10. Load the sample.

Table 1. Preparative Agarose Recipes[1]

AMOUNT OF AGAROSE IN GEL* (% [W/V])	RANGE OF SEPARATION OF LINEAR DNA MOLECULES
0.30%	5,000–60,000
0.50%	1,000–20,000
0.80%	800–10,000
1.00%	500–7,000
1.20%	400–6,000
1.5%	200–3,000
2.0%	100–2,000

* *For best results, use an agarose with high gel strength at low gel concentration.*

ENZYME DIGESTION

Fifty µg aliquots of DNA were digested in 250 units BamHI and 10 µg RNase A, in a volume of 500 µl at 37 °C for 4 h. Digestions were stopped by addition of 10 µl 0.5 M EDTA. Phenol/phenol-chloroform/chloroform extractions were carried out to remove the proteins. DNA was precipitated by a 10 min incubation on dry ice after addition of 50 µl of 3 M NaOAc, pH 7, and 1 ml of cold 100% ethanol. The air-dried pellets were resuspend in 50 µl 1x TE, pH 8.

CONTINUOUS ELUTION ELECTROPHORESIS

To 300 µg of BamHI digested genomic DNA in a volume of 300 µl TE, 30 µl of 10x agarose loading dye was added. The DNA sample was then loaded onto a Model 491 Prep Cell, containing a 0.5% preparative agarose gel and a 0.25% agarose stacking gel which was run in 50 mM TBE, at 50 constant volts (Bio-Rad PowerPac 300 Power Supply) at 4 °C.

FRACTION DETECTION BY STANDARD AGAROSE ELECTROPHORESIS

After Continuous Elution Electrophoresis, 30 µl from each fraction was mixed with 3 µl of 10x agarose loading dye and loaded onto 0.8% agarose gels which were run in 50 mM TBE, at 100 constant volts. Gels were stained by ethidium bromide (0.5 µg/ml) and photographed. DNA concentrations were determined by A260 measurements.

FRACTION SCREENING BY POLYMERASE CHAIN REACTION (PCR)

After continuous elution electrophoresis and fraction detection by standard agarose electrophoresis, desired fractions were precipitated by making two tubes each consisting of 500 µl eluted fraction, 50 µl 3 M NaOAc, pH 7, 1 µl glycogen (20 µg/µl), and 1 ml 100% cold ethanol. DNA was precipitated by a 10 min incubation on dry ice. Air-dried pellets were resuspend in 25 µl 1x TE, pH 8. Each respective pair was pooled together, and each fraction consisted of a 50 µl final volume.

1.7 µl (100 ng) from desired fractions were added to 98.3 µl of the Taq polymerase mix (2 mM $MgCl_2$, 0.01% gelatin, 10 mM tris-HCl, pH 8.9, 40 mM NaCl, 0.25 mM of each dNTP, 10 pmoles of upstream primer, 10 pmoles of downstream primer, and 3 units of Taq DNA polymerase (5 units/µl, Boehringer Mannheim). Primers for p53 (Exon 5) detection: (Upsteam Primer; 5' GTTGCAGGAGG TGCTTACACATGTTTG-3', and Downstream Primer; 5'-TGGTGTT GTTGGGCAGTGCTAGG-3'). Reactions were overlaid with mineral oil and underwent 25 PCR cycles of 95 °C for 1 min, 65 °C for 2 min and 74 °C for 3 min. After PCR, 20 µl from each sample was removed and added to 4 µl 5x agarose loading dye and loaded onto a 1% agarose gel which were run in 50 mM TBE, at 100 constant volts. Gels were stained by containing ethidium bromide (0.5 µg/ml) and photographed. Band intensities were determined by fluoroimager analysis.

QUICK FRACTION SCREENING BY DOT-BLOT ANALYSIS

After continuous elution electrophoresis, 10 µl from each fraction (representative of 30 ng of DNA) was added to 190 µl of 0.4 M NaOH, 10 mM EDTA solution. Samples were heated to 95 °C for 5 min by the aid of a thermocycler machine. Samples were immediately placed on ice and loaded onto a dot-blot apparatus (Bio-Dot®, Bio-Rad) containing a positively charged nylon membrane. Wells were rinsed with 200 µl of 0.4 M NaOH, 10 mM EDTA solution. Membrane was then soaked in 2x SSC for 5 min, UV-crosslinked (1,200 joules/m^2), and placed into a hybridization tube containing hybridization solution and radiolabeled probe.[2] Air dried membranes were exposed to Kodak XAR-5. The intensity of each dot was determined by PhosphorImager analysis.

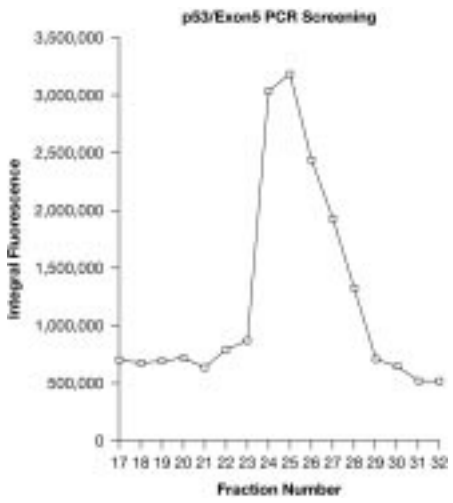


Fig. 2. After PCR screening specific fractions, 10 µl from each PCR reaction was loaded onto an agarose gel and EtBr stained. Band intensities were determined by fluoroimager analysis and plotted. These fractions correspond to gels not shown.

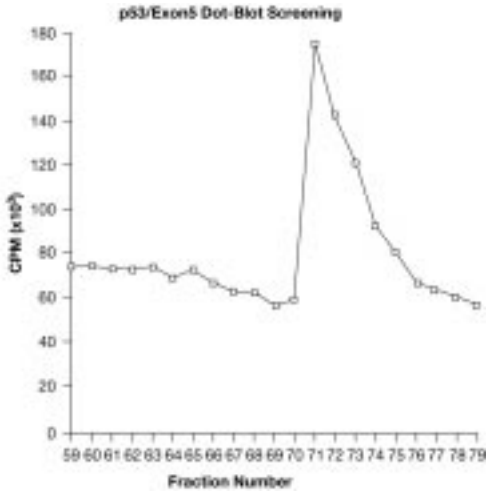


Fig. 3. After dot-blot screening specific fractions, signal intensities were determined by Phospholmager analysis and plotted. The fractions correspond to the gels in Figure 5.

PROBE PREPARATION

[^{32}P]dCTP-labeled single-stranded probes were prepared by repeated linear primer extension, between 30 and 35 cycles, by Taq polymerase on the p53 gene (Exon 5) made by using HeLa cell DNA as a template.[3] The 150 µl mix consists of: 0.01% gelatin, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.9, 40 mM KCl, 250 µM of dATP, dGTP, and dTTP, 40 ng of template, 75 pmol of primer (p59/Exon 5: 5'-CAACTCTGTCT CCTTCCTCTTCCTACAG-3', T_m of 58 °C), 2.5 units of Taq polymerase and 10 µl of [^{32}P]dCTP (3,000 Ci/mmol). The probe was precipitated with 37.5 µl of 10 mM ammonium acetate, 20 µg of glycogen, and 420 µl of ice-cold ethanol, resuspended in 100 µl TE buffer (TE, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), then added to 6 ml of the hybridization buffer. After

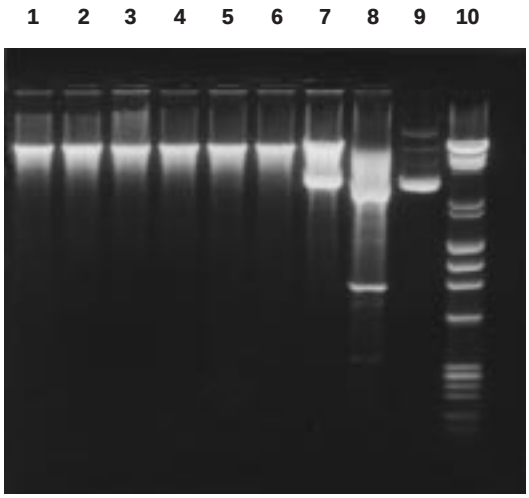


Fig. 4. Lane 1–6. Genomic DNA (500 ng) digested with Bam*HI. Lane 7. From one tube (lane 1) containing Total 50 µg DNA and* Bam*HI, 1 µg of DNA containing* Bam*HI was taken and mixed with 1 µg PGK1 plasmid. Lane 8. From 1 tube (lane 2) containing total 50 µg DNA and* Bam*HI, 1 µg of DNA containing* Bam*HI was mixed with 1 µg PGK1 plasmid and 5 units of Eco RI. Lane 9. PGK1 Plasmid (1 µg) uncut. Lane 10. Marker (500 ng Lambda Hind III/500 ng* Phi*X174* Hae *III). Note: Lanes 7–8 incorporated a plasmid as a means to verify terminal digestion conditions.*

overnight hybridization at 66 °C, membrane was washed 5 min in Buffer A (20 mM $NaPO_4$, 1 mM EDTA, 2.5% SDS, 0.25% BSA) at 66 °C, and 5 min in Buffer B (20 mM $NaPO_4$, 1 mM EDTA, 1% SDS) at 66 °C. Buffer B wash was repeated two times.

FRACTIONATING THE GENOME FOR THE GENE OF INTEREST

We were interested in isolating the p53 gene; therefore, we used *Bam*HI which cleaves outside Exons 3–11. Thus, the resulting fragment length we were interested in was 7.5 kb. This was the reason for using a preparative agarose concentration of 0.5% in the gel tube assembly.

Final eluted volume per fraction was 1 ml. An average of 3 µg total DNA per fraction was obtained; a concentration of 3 ng/µl (starting DNA material prior to continuous elution electrophoresis was 300 µg). Note: The distribution of DNA amount per fraction depends on the average fragment length of the enzyme-restricted DNA, which in turn depends upon the average frequency of the recognition sequence in genomic DNA. The DNA concentration in fractions corresponding to the 7.5 kb range would be greater if a restriction endonuclease with more frequently occurring recognition sequences than *Bam*HI was used.

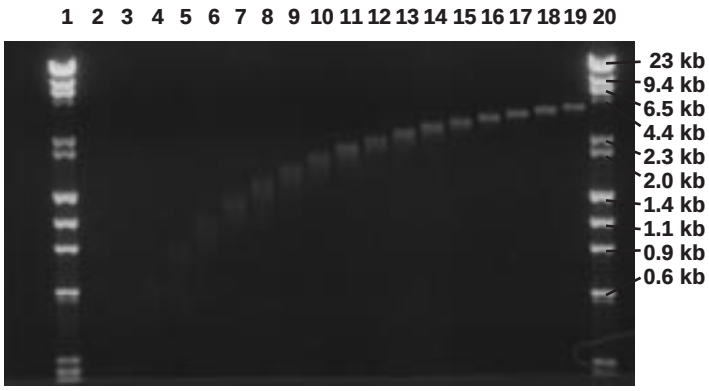
Conclusions

Continuous elution electrophoresis provided an easy and cost-effective means of isolating preparative amounts of genomic DNA fragments ranging in size from 2 kb to greater than 18 kb. The prep cell's run time for the 7.5 kb fragment was approximately 33 hours with minimum set-up time and little to no hands-on time during the run. Run times may vary depending on sample viscosity, power conditions, height of the gel, and restriction enzyme parameters. Recoveries averaged between 60–70%; typical for electro-elution techniques. With up to 300 micrograms of genomic DNA starting material, multiple genes can be isolated from one run on the Model 491 prep cell. This method can be used to purify recombinant plasmid inserts on a large scale and is applicable to other techniques requiring gene purification.

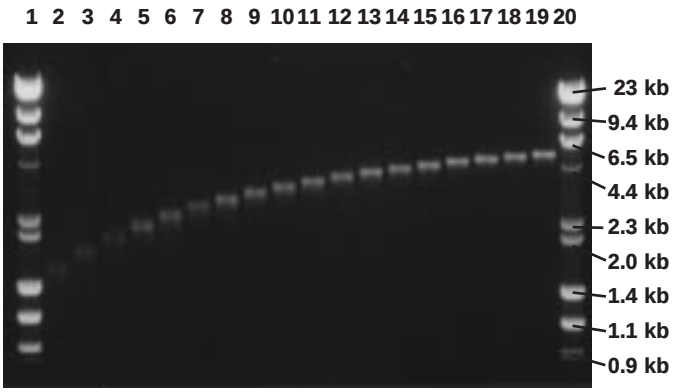
References

1 *Maniatis, T.,* et al., Molecular Cloning, *1, 6.5 (1989).*
2 *Rodriguez, H.,* et al, J. Biol. Chem., *270, 17633–17640 (1995).*
3 *Tournaletti, S.,* et al, Oncogene, *8, 2051–2057 (1993).*
4 *Singer-Sam, J.,* et al., Gene, *32, 409–417 (1984).*

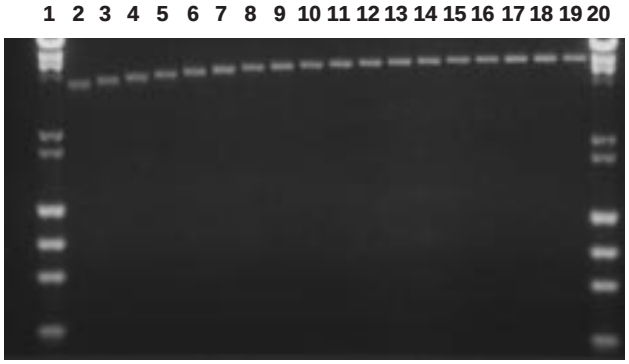
* *The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffmann-LaRoche. Use of the PCR process requires a license.*



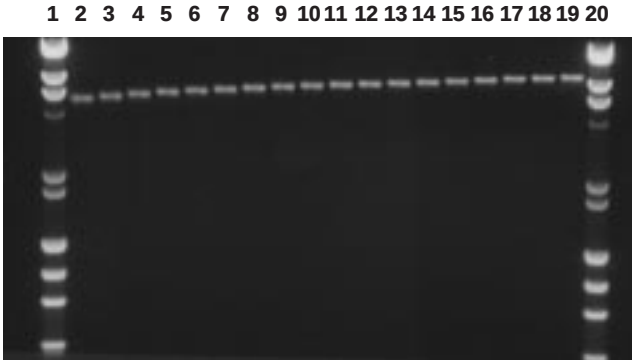
5A. Lane 1 and 20 marker; Lanes 2–19 correspond to every other fraction # 20–54.



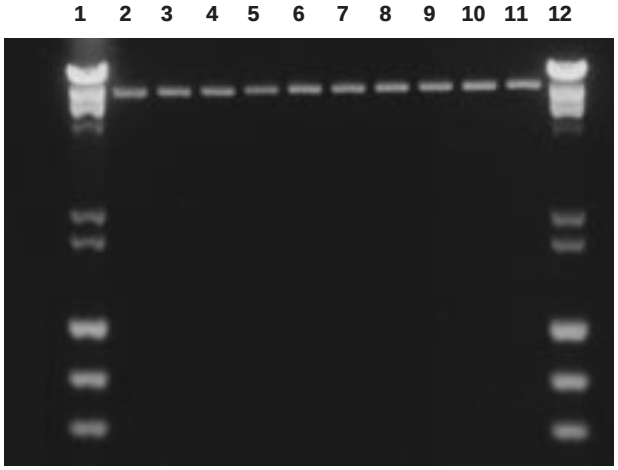
6A. Lane 1 and 20 marker; Lanes 2–19 correspond to every other fraction # 18–52.



5B. Lane 1 and 20 marker; Lanes 2–19 correspond to every other fraction # 56–90.

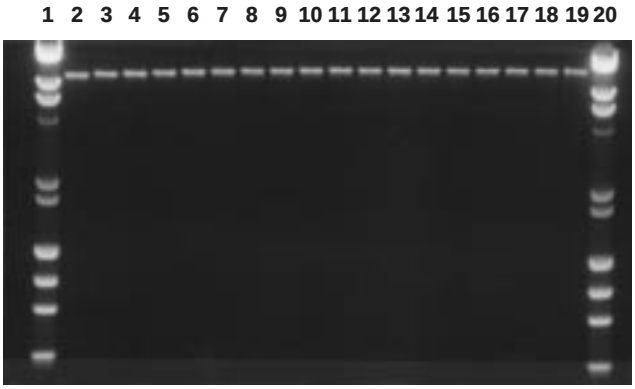


6B. Lane 1 and 20 marker; Lanes 2–19 correspond to every other fraction # 54–88.



5C. Lane 1 and 12 marker; Lanes 2–11 correspond to every other fraction # 92–110.

Fig. 5. Marker Lanes: 500 ng Lambda Hind *III, 500 ng PhiX174* Hae *III*
Fraction Lanes: 30 µl from fraction
Preparative Gel: 0.5%, Stacking Gel: 0.25%
Loaded fraction onto a 1% agarose EtBr stained gel.



6C. Lane 1 and 20 marker; Lanes 2–19 correspond to every other fraction # 90–124.

Fig. 6. Marker Lanes: 500 ng Lambda Hind *III, 500 ng* Phi*X174* Hae *III*
Fraction Lanes: 30 µl from fraction
Preparative Gel used: 0.5%, Stacking Gel used: 0.25%
Loaded fraction onto a 0.8% agarose EtBr stained gel.

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