Partitioned pulsed-field gel electrophoresis-PCR (PPF-PCR): a new method for pulsed-field mapping for STS and microsatellites

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Pulsed field gel electrophoresis (PFGE) is an important tool in the construction of physical maps of genomic DNA. The colocalization of probe DNA to a large restriction enzyme generated fragment demonstrates the contiguity of the selected markers. At present, the Southern blot and its subsequent hybridization are the only practical means to colocalize probes on a PFGE restriction. This method is time consuming and especially difficult when the hybridization probe contains repeat sequences. Two very important classes of probes are effectively excluded from direct use in probing PFGE: [1] Sequence Tagged Sites (STS) which yield PCR generated probes whose small size often causes a poor hybridization signal, and [2] Microsatellite repeats (MS), which are at the core of the largest class of genomic markers, yield PCR generated probes that are likewise small but also highly cross-reactive due to the core of tandem repeats. We present here a new non-radioactive method based on PCR that can incorporate these markers. We call this new technique Partitioned Pulse Field Gel Electrophoresis-PCR or PPF-PCR. To test this method we have applied it to the previously well mapped region of Charcot–Marie–Tooth disease type 1A, localized to chromosome 17p11.2 (1,2).

Human genomic DNA was restricted and prepared for PFGE by standard methods (1). PFGE was conducted on a BioRad CHEF-DR II on ordinary agarose. A switch time of 90 seconds for 15 hours and 60 seconds for 9 hours was used to achieve good separation between 0.2 and 1.2 megabases. A device was fabricated to cut the gel in small equal and indexed bands. Thirty-one single bladed stainless steel razor blades with aluminum spines were ganged on a threaded steel rod with brass spacers placed so that the cutting edges were parallel, planar, and spaced 0.10 inches apart. The gel was treated with Ethidium Bromide and the desired digest photographed (uv) along with a ruler, the adjacent molecular weight standard and a zero index notch. A single lane was excised from the gel, cooled to 4°C and placed on a flat hard surface. The multiplex cutter was soaked for 15 minutes in 0.5N NaOH and then washed thoroughly, first in sterile water and then ethanol.

The cutter was placed on the gel with the zero index notch aligned with the first individual blade. The cutter was forced through the gel with an even firm rocking motion. The gel slices (which remain sequestered in the cutter if cool) were transferred by sterile pipette tips (used as pushers) into labeled tubes. The closed tubes were put in a 95°C heat block for 10 minutes. Each

tube was removed one at a time and when it was estimated that the temperature had dropped to below 50°C, 0.5 µl of GELase (Epicentre Technologies) was added and the tube transferred to a 42°C heating block for 4 hours. A master mix was prepared which was 1× in supplied buffer, 1U/20 µl Taq polymerase (Gibco), 1 mM dNTP, 1.5 mM Mg₂Cl, and 0.3 µM in each primer. 20 µl of the master mix was dispensed into thirty-six labeled tubes. 5 µl of solubilized gel (from 150 µl of solubilized gel solution) or control was added to each tube with thorough mixing. PCRs were run on a Hybaid Omnigene: 94°C for 5 minutes at one cycle; 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, at 30 cycles. At the end of this regime 5 µl of loading dye was added to each sample which were then run out on a 2% agarose gel (Sea Kem, FMC).

Figure 1. Physical map of the chromosome 17p11-p12 site of the CMT1A-associated duplication used in this study. The bold bar represents the duplicated region. Location of the probes EW401 (D17S61), MFD41 (D17S261), and VAW412 (D17S125) are shown within the duplicated region.

Figure 2. A 2% agarose gel was used to examine the products of the D17S125 PCR using different slices of a genomic Ascl digest PPF-PCR library. Lane numbers correspond to the individual slices of the gel. Slice 20 is approximately 1020 Kbp. The positive lane is a genomic control for D17S125, the minus lane is a negative blank control. 'M' is a 100 bp standard.

Figure 3. The D17S61 PCR products using all 28 slices of an *AscI* digest is shown on a 2% agarose gel. The only significant bands observed were the expected 1020 kb band (slice 20) and those seen in the compression region (slices 25–27). The positive lane is a genomic control for D17S61, the minus lane is a negative blank control. 'M' is a 100 bp standard.

Figure 4. PCR results using D17S261 and selected slices of an *FspI* digest. Lower numbered slices have the smallest molecular weight DNA. Slice 25 corresponds to the compression band found on the pulsed-field gel of the digest. Slices 13 (700 Kbp), 14 (770 Kbp), and 11 (600 Kbp) correspond well with previously described fragment sizes. The positive lane is a genomic control for D17S261, the minus lane is a negative blank control. 'M' is a 100 bp standard.

The primer sequences used for the analysis were:

D17S261 (MFD41) A = CAGGTTCTGTCATAGGACTA B = TTCTGGAAACCTACTCCTGA (3)

D17S61 (VAW401) A = CGTATCTCTGATCTGTACAAG B = CTCAAGTATTCTACCTACAC

D17S125 (VAW412) A = ACCATGATGTGAGAGAACTT B = TCTAAGCCGTGCCAAGGCCAT (4)

The known PFGE map of the CMT1A region is shown in Figure 1 (2). To test the procedure we chose to use *FspI* and *AscI* digests and performed PCR on this digest library with the developed STS and MS primers. The STS from both D17S125 and D17S61 demonstrate a strong reaction in slice 20 of the *AscI* digest library, which corresponds to a band of approximately 1020 Kbp. These are shown in Figures 2 and 3 respectively. The molecular weights corresponding to the individual gel slices are based on the location of bands in a commercial yeast chromosome standard (BioRad). Figure 3 includes PCR reactions on the entire 28 slice *AscI* digest, demonstrating the absence of significant contamination in applying this approach. These two markers have been previously mapped (2) to a 1050 *AscI* fragment in the CMT1A region on 17p11 (Figure 1).

D17S261 is a microsatellite that contains a polymorphic C-A-repeat sequence (3). Previous mapping (2) has reported a major 720 Kbp fragment and a minor 600 Kbp fragment with an *FspI* digest of lymphocytic DNA. We observed strong reactions in *FspI* slice 13 (700 Kbp) and *FspI* slice 14 (770 Kbp) (Figure 4), corresponding well with the previously described major 720 Kbp band seen in radioactive hybridization. A faint D17S261 PCR product was also observed in slice 11 which corresponds with the 600 Kbp minor band.

Once the initial digest has been made, it can be used repeatedly as each PCR requires only a few microliters of solubilized gel per band. A second advantage of PPF-PCR is the speed with which multiple markers can be mapped. For example, a positive PCR by two probes in the same numbered band constitutes evidence of possible colocalization. If one has data to show that given primer pairs or triads yield their primary products without mutual interference, and if those products are sufficiently different in size, then multiplex reactions are possible (5). When this is possible, it represents a significant simplification of effort.

The combined use of PCR and PFGE has been previously described by Burright *et al.* (6,7), who fractionated PFGE gels of radiation hybrids to increase the specificity of IRS-PCR (Interspersed Repetitive Sequences). However, this technique was used for enrichment of the desired region rather than mapping of specific markers. Also, the direct use of template DNA from solubilized gel slices is far simpler and more effective than the use of electroelution.

One possible concern in employing this method is contamination. Care must be taken to avoid contamination of the individual bands by exogenous DNA and likewise, prior to electrophoresis, to avoid shearing of the DNA. This is a concern common to all PFGE, and a run with a mock digest will give an indication of the integrity of the DNA. However, in PPF-PCR it is a particular concern, because the PCR can amplify very small amounts of DNA. If restriction fragments degrade into smaller pieces, those pieces will run faster than their parent fragment and can support a misleading PCR reaction. A second major source of contamination could come from cross-contamination of the bands themselves. To reduce this possibility between cuts, the blades of the multiplex cutter are soaked in 0.6 N NaOH and rinsed thoroughly with water and alcohol. Care must be taken to remove the bands from the cutter in such a way that they do not contact each other or any non-sterile surfaces. However, as seen in Figure 3, using these precautions contamination has not been a significant problem with this approach.

Although we have developed this method for its utility in physical mapping of PFGE, an exactly analogous procedure could be applied to detection of probe-shared fragments in digests spanning the 1000 to 26,000 bp range. PPF-PCR is a tool which we believe will aid workers in the physical mapping of chromosomal areas of interest. It is intended to complement existing methods through the use of microsatellites as physical as well as genetic linkage markers.

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REFERENCES

1. Timmerman, V. *et al.* (1992) *Nature Genet.* **1**, 171–175.
2. Pentao, L. *et al.* (1992) *Nature Genet.* **2**, 292–300.
3. Weber, J.L. *et al.* (1990) *Nucleic Acids Res.* **18**, 4640.
4. Wright, E.C. *et al.* (1990) *Genomics* **7**, 103–109.
5. Ben Othmane, K. *et al.* (1993) *Hum. Mol. Genet.* **2**, 1625–1628.
6. Burright, E.N. *et al.* (1991) *Nucleic Acids Res.* **19**, 401–402.
7. Burright, E.N. and Gorski, J.L. (1993) *Nucleic Acids Res.* **21**, 175–176.