Short report

Chemiluminescent detection of blotted PCR products (CB-PCR) of two CAG dynamic mutations (Huntington's disease and spinocerebellar ataxia type 1)

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Abstract

We have used a non-isotopic PCR assay based on the chemiluminescent detection of blotted PCR products (CB-PCR) for two dynamic mutation diseases (Huntington's disease and spinocerebellar ataxia type 1). This gives an accurate sizing of alleles and permits a rapid analysis of at risk persons. The system involves PCR of the samples, separation of alleles on polyacrylamide gels, Southern blotting, and hybridisation with specific primers 3' labelled with fluorescein (Fl)-dUTP as probes. CB-PCR retains the isotopic sensitivity for accurate allele determination, avoids isotopic manipulation, and provides the advantages of safety, long term storage of probes, and recycling of hybridisation solutions.

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Trinucleotide repeat sequences have become one of the more striking discoveries in human molecular genetics of the last two years. This kind of sequence has so far been implicated in six neurological diseases: myotonic dystrophy, fragile X syndrome, spinobulbar muscular atrophy, Huntington's disease, spinocerebellar ataxia type 1, and FRAXE mental retardation.¹⁻³

Such peculiar stretches of repeated nucleotides have been considered to be dynamic mutations⁴ since they show instability of alleles through generations and the mutation rate is related to the copy number of repeats. Therefore, the mutability of one of these sequences in offspring is different from that in their parents. We have applied a non-isotopic PCR assay based on the chemiluminescent detection of blotted PCR products⁵ (CB-PCR) for two of these diseases (Huntington's disease (HD) and spinocerebellar ataxia type 1 (SCA1), which gives an accurate sizing of alleles and permits a rapid analysis of at risk persons. The system involves PCR of the samples, separation of alleles on polyacrylamide gels, Southern blotting, and hybridisation with specific primers 3' labelled with fluorescein (Fl)-dUTP as probes.

Isolation of genomic DNA and PCR reac-

tions were performed according to methods previously described.^{6,7} PCR was carried out using the following primers: HD: HD1 5'-ATGAAGGCCTTCGAGTCCCTCAAGTCCTCC-3', HD2 5'-AAACTCACGGTCGTGCAGCGGCTCCTCAG-3',⁸ and SCA1: Rep1 5'-AACTGGAAATGTGGACGTAC-3', Rep2 5'-CAACATGGGCAGTCTGAG-3'.^{2,9}

Reaction mixtures contained the following components: 250 ng of DNA, 10 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 2 mmol/l MgCl₂, 200 µmol/l dATP, 200 µmol/l dCTP, 50 µmol/l dGTP, 200 µmol/l dTTP, 150 µmol/l 7-deaza-dGTP (Pharmacia), 2% formamide, 5 pmol of each primer, and 1 U *Taq* DNA polymerase (Promega, storage buffer A) in a total volume of 25 µl. The samples were subjected to amplification on a DNA thermal cycler 480 (Perkin Elmer Cetus, Norwalk, CT) with previous denaturation at 95°C for five minutes, and 25 cycles with denaturation at 95°C for one minute 30 seconds, annealing/extension at 65°C for two minutes (HD) or annealing at 55°C for one minute and extension at 72°C for two minutes (SCA1). A final extension at 72°C for seven minutes was performed. PCR products were concentrated in a Univapo centrifuge or cold ethanol precipitated, combined with loading dye (95% formamide), heated at 95°C for two minutes, and electrophoresed on a 5% denaturing polyacrylamide gel at 1500 V for three to four hours. Samples containing alleles previously sized by sequencing were used as size markers. The area of the polyacrylamide gel that contained the samples was blotted for 90 minutes according to the following procedure: the gel was lifted onto a Whatmann 3MM filter paper and laid, gel upwards, over another Whatmann soaked in 1 × TBE. A smaller piece of Hybond-N membrane, also soaked in 1 × TBE, was set on top. Finally, three dry sheets of Whatmann 3MM were laid over the membrane and a glass plate was placed on top. The blot was prehybridised with ECL hybridisation buffer (Amersham) at 42°C for one hour and then hybridised at the same temperature for two hours, adding Fl-dUTP 3' labelled primers HD1 or Rep1 as probes. Washing, membrane blocking, antibody incu-

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Figure 1 Chemiluminescent blotting PCR (CB-PCR) analysis of the CAG repeat at the HD locus. The analysis was on a 5% denaturing polyacrylamide gel in eight controls (lanes 1, 3, 7, 10, 11, 12, 14, and 15) and eight HD patients (lanes 2, 4, 5, 6, 8, 13, 16, and 17). There is clear cut difference between normal and mutated alleles. The allele sizes are given in number of CAG repeats. Lane 9 is the negative control.

Figure 2 CB-PCR analysis of the CAG repeat at the SCA1 locus. The study was on a 5% denaturing polyacrylamide gel in four controls (lanes 3, 5, 6, and 7) and eight SCA1 patients (lanes 1, 2, 4, 8, 9, 11, 12, and 13). The allele sizes are given in number of CAG repeats. Lane 10 is the negative control.

bations and washes, and signal generation and detection were performed according to the ECL 3'-oligolabelling and detection system protocol (Amersham). The autoradiographic film was exposed between one and three hours to obtain the optimum presentation of the result.

The analysis of several persons is shown in fig 1 (HD) and fig 2 (SCA1). PCR amplification of these repeats is both rapid and accurate for

obtaining the exact number of repeats in both the normal and affected range. However, in cases of extreme trinucleotide expansion, PCR may amplify the region with a lower efficiency. We have tried to solve this problem by using 7-deaza-dGTP instead of dGTP (1:4, dGTP:7-deaza-dGTP), as successful amplification of large alleles was found to be dependent upon this.¹⁰ When 7-deaza-dGTP was used, we achieved a better amplification of large alleles than when it was not. Poor specificity is another problem when amplification takes place in such segments. DMSO is frequently used to enhance the amplification of large repeats (for example, for the CGG repeat of FMR-1¹¹), although it seems to decrease the *Taq* polymerase activity and it was found to be only partially effective.¹² Formamide has been found to be a better alternative,¹³ and this has also been confirmed in this report.

Radioactive analysis of trinucleotide repeats requires special facilities and handling, limiting its use to laboratories with isotopic facilities. Although non-radioactive PCR amplification of the CAG repeats in HD has previously been described,¹⁴ the method does not give the size accuracy obtained by radioactive methods.²⁸ CB-PCR retains the isotopic sensitivity for accurate allele determination, avoids isotopic manipulation, and provides the advantages of safety, long term storage of probes, and recycling of hybridisation solutions.

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