5082–5084 Nucleic Acids Research, 1995, Vol. 23, No. 24 © 1995 Oxford University Press

Improved strategy for mutation detection—a modification to the enzyme mismatch cleavage method

Jeff J. Babon, Rima Youil and Richard G. H. Cotton*

Olive Miller Laboratory, Murdoch Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia

Received September 11, 1995; Revised and Accepted November 9, 1995

Despite the number of mutation detection systems currently available, the enzyme mismatch cleavage (EMC) method of mutation detection is one of a few techniques capable of quickly detecting and locating a single nucleotide mutation within kilobase stretches of DNA. EMC is analogous to the chemical cleavage of mismatch (CCM) technique in that it cleaves heteroduplex DNA at base pair mismatches however it does not require toxic chemicals or multiple precipitation steps. By melting and re-annealing wild-type (WT) and mutant DNA in the same tube, heteroduplexes are formed. EMC exploits the activity of a bacteriophage T4 enzyme, endonuclease VII that cleaves heteroduplex DNA at single base pair mismatches and deletions/insertions. The cleavage products can then be visualised by autoradiography after gel electrophoresis (1). The manner in which the heteroduplexes are formed can however, result in a large percentage of the denatured DNA re-annealing to form homoduplex, rather than heteroduplex, DNA. Whilst T4 endonuclease VII has a higher activity on mismatched DNA than on Watson–Crick paired DNA, it nevertheless cleaves homoduplex DNA at a low but significant rate. Therefore the high level of homoduplex DNA in solution acts a source of 'background cleavage'. This background cleavage decreases the signal-to-noise ratio which may lead to a mutation not being detected and we consider this to be the only major drawback to the technique. Here we describe a simple adaptation to the EMC method that significantly enhances the signal-to-noise ratio by removing the radiolabelled homoduplex DNA.

Under the traditional protocol (2), heteroduplexes between wild-type and mutant DNA are formed by mixing 5′ radioactively labelled WT DNA with excess unlabelled mutant DNA and then melting and re-annealing the DNA in the same vessel. Thus four species of duplex are produced, two different heteroduplexes and the two original homoduplexes. We have modified this procedure by using biotinylated primers to PCR amplify the mutant DNA and unbiotinylated primers of the same sequence to PCR amplify the WT DNA. The resultant biotinylated mutant DNA and the WT DNA (5′-radioactively labelled) are then melted and re-annealed as usual to form heteroduplex DNA (Fig. 1). The two different heteroduplexes formed are thereby distinct, being labelled with biotin on one strand and a radioactive moiety on the complementary strand. Streptavidin coated paramagnetic beads (Dynabeads M-280 streptavidin; Dynal, Norway) can be used to bind and extract all the biotinylated DNA. Thus the two heteroduplexes will be captured and detected, one homoduplex

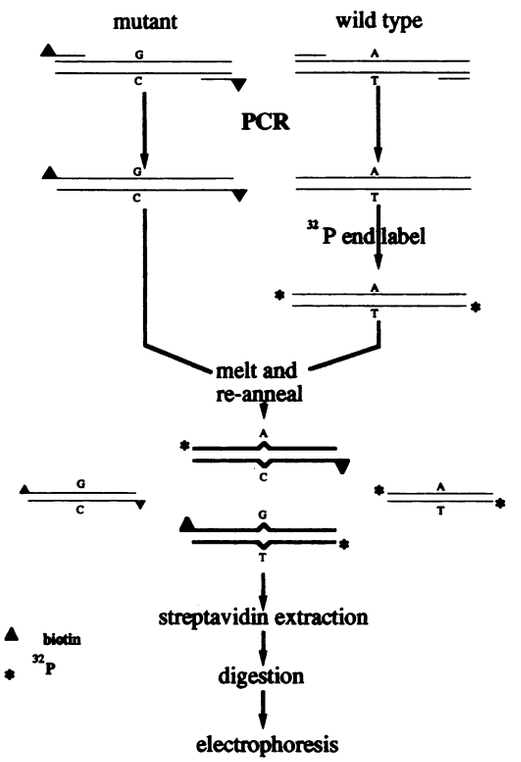

Figure 1. Schematic of the modification to the EMC technique. Mutant PCR amplifications are performed using biotinylated primers and the WT PCR is performed using non-biotinylated primers. After radiolabelling the WT DNA and annealing it to the mutant DNA to form heteroduplexes, the two heteroduplexes (shown in bold), contain a biotin, and a radioactive, moiety and are therefore distinct from the homoduplexes. By extracting the biotinylated DNA using Dynabeads M-280 Streptavidin, the radiolabelled homoduplex DNA can be discarded. Thus, after digestion and electrophoresis the two heteroduplexes will be sampled for base mismatches but no homoduplex DNA will contribute to the signal-to-noise ratio.

* To whom correspondence should be addressed

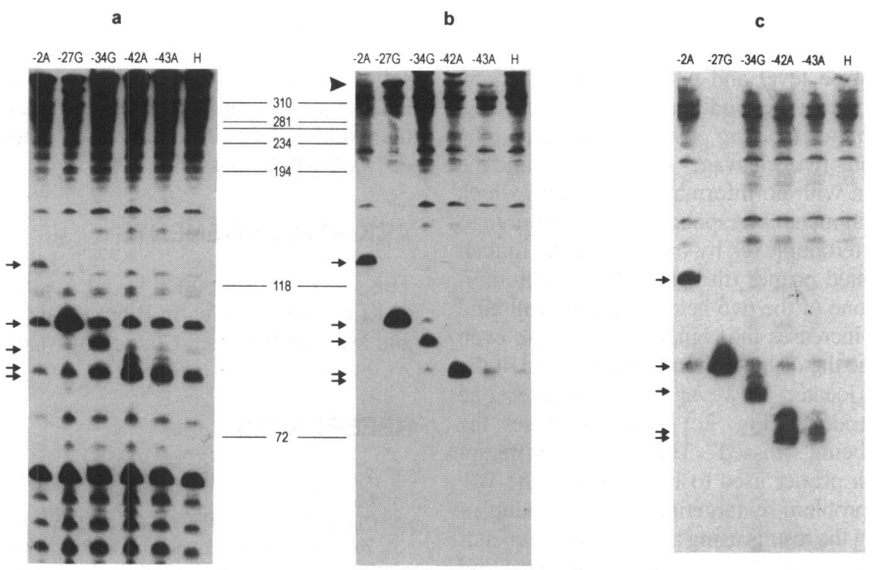

Figure 2. A comparison of the signal-to-noise ratio of the conventional and modified EMC techniques. EMC was performed on five different heteroduplexes (–2A, –27G, –34G, –42A, –43A) and a homoduplex control (H) under (**a**), conventional and (**b**) modified EMC conditions (see text), sizes are indicated in base pairs. (**c**) Modified EMC was performed on the same five mutations but only one biotinylated primer was used to PCR amplify the mutant clones (see text). The expected cleavage fragment sizes are as follows: –2A; 421 and 125 bp, –27G; 446 and 100 bp, –34G; 453 and 93 bp, –42A; 461 and 85 bp, –43A; 462 and 84 bp. Each mutation results in a small (→) and large (▶, not well resolved) cleavage fragment, however the method used in (c) will only detect one of the two cleavage products. The large cleavage products are not easily visible in (a) due to the high background cleavage level and are thus not marked.

molecule will be non-radioactive and hence not detected whilst the other homoduplex molecule is not biotinylated and will not be captured.

To qualitatively assess the increased signal-to-noise ratio we chose to test this technique on five different plasmid clones, each containing a point mutations in a 132 bp segment of the mouse β-globin promoter region (3). The mutations are designated (–2A, –27G, –34G, –42A, –43A) and this DNA was previously shown to give a significant level of background cleavage (3). These mutations were chosen because they gave a strong (–27G), medium (–34G, –42A) or weak (–2A, –43A) signal after conventional EMC.

A 546 bp fragment (including the 132 bp β-globin promoter region) was amplified from plasmid clones by PCR. The WT clone was amplified using the 5′ primer [5′-GCACGCGCTGG-ACGCGCAT] and the 3′ primer [5′-AGGTGCCCTTGAGGC-TGTCC]. The five different mutant clones (and the WT clone for use as a homoduplex control) were amplified using primers of the same sequence but with biotinylated 5′ ends. The PCR conditions in all cases were 95°C for 2 min, 65°C for 2 min and 72°C for 2 min for 30 cycles. The PCR products were then purified (1) and the unbiotinylated WT PCR product radiolabelled as described previously (1). Heteroduplexes were then formed by annealing radiolabelled WT DNA to excess mutant DNA in an approximately 1:2 molar ratio under conditions described previously (1). A sample of the substrate was taken at this stage to represent the substrate used in traditional EMC reactions. Those reactions to be performed under the modified conditions were then subjected to a streptavidin based extraction as follows: 7.5 μg of Dynabeads M-280 Streptavidin (pre-washed and resuspended in PBS/0.1% albumin at a concentration of 0.5 mg/ml) was added to 100–250 ng of heteroduplex DNA. This was incubated at room temperature for 30 min on a rotator. The Dynabeads (now bound to biotinylated DNA) were then pelleted using a Dynal magnet, washed two to three times with TE buffer, pH = 8.0 and resuspended in TE, pH = 8.0. The substrate, whether prepared using the traditional or modified procedure, was then incubated in the presence of 100 U T4 endonuclease VII as previously described (1) but in a 37°C shaker for 45 min. The reaction was stopped by adding a half volume of formamide containing loading dye (1) and heating to 72°C (allowing dissociation of the biotinylated DNA from the Dynabeads). The beads were pelleted using a magnet and the supernatant loaded onto an 8% denaturing (19:1, acrylamide:bis-acrylamide, 8.3 M urea) polyacrylamide gel. The samples were then electrophoresed in 1× TBE buffer at 45 W for 90 min, the gel dried and the results visualised by autoradiography (Fig. 2).

The five β-globin promoter mutations when investigated using conventional EMC (3) gave results as typified by those in Figure 2a. The noise level using conventional EMC can obscure the mutation signal which could result in the mutation not being detected, especially when the mutation signal lies at the same position on the gel as a background signal (compare lanes –43A and H in Figure 2a). Using our modification the signal-to-noise ratio of these mutation signals were increased in all cases (Fig. 2b). However the background is reduced, not eliminated and mutation signals that lie at the same position as background signals (compare lanes –43A and H in Figure 2b) will only be detected due to the increase in the strength of the mutation signal compared with the homoduplex control. T4 endonuclease VII cleaves many secondary structures in DNA (4); therefore the background cleavage observed is most likely due to DNA secondary structure in the heteroduplexes.

This modification ensures that a poor heteroduplex yield after the melting/re-annealing step will not result in the signal being obscured by the high noise level and could be useful to any technique that relies on optimal heteroduplex yield. As shown in Figure 2b, depending on the resolving power of the gel and the position of the mutation, two cleavage fragments should be visible for each mutation with no information regarding which end of the DNA each fragment corresponds to. It is possible to target just one of the heteroduplexes by amplifying the mutant using only one biotinylated primer (the other being unbiotinylated). In this way only one of the two heteroduplexes will bind to the Dynabeads. This increases the signal to noise ratio even further and will determine the distance from a specific end of the DNA that the mutation is located. However it will only detect one of the two cleavage products (Fig. 2c) which increases the chances of a mutation being 'missed'. The use of a different fluorescent label on each primer used to PCR amplify the WT DNA should solve this problem by targeting each heteroduplex separately and visualising the results using an automatic sequencing apparatus. This should detect both cleavage fragments and accurately determine the position of the mutation relative to one end of the DNA. In fact because the reaction is performed on a solid phase the whole process should lend itself well to automation. In conclusion, our new adaptation to EMC increases the likelihood that 100% mutation detection in kilobase lengths of DNA will be possible.

ACKNOWLEDGEMENTS

This work was supported by an Australian Postgraduate Award to J.J.B. and by a Centre Block Grant from the National Health and Medical Research Council of Australia.

REFERENCES

1 Youil, R., Kemper, B.W. and Cotton, R.G.H. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 87–91.
2 Cotton, R.G.H. (1991) *Methods Mol. Biol.* **9**, 39–49.
3 Youil, R., Kemper, B. and Cotton, R.G.H. *Genomics*, in press.
4 Pottmeyer, S. and Kemper, B. (1992) *J. Mol. Biol.* **223**, 607–615.