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# Potassium permanganate and tetraethylammonium chloride are a safe and effective substitute for osmium tetroxide in solid-phase fluorescent chemical cleavage of mismatch

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## ABSTRACT

**Whilst chemical cleavage of mismatch (CCM) detects all point mutations in DNA, its widespread use has been hampered by the complex multistage methodology and the need for toxic chemicals, in particular osmium tetroxide. Here we show that osmium tetroxide can be replaced by potassium permanganate, giving the same spectrum of mutation detection, but with greater sensitivity. The use of potassium permanganate is compatible with solid phase capture and fluorescent detection, giving a safer method of mutation detection. We present here a comparison of CCM with osmium tetroxide and with potassium permanganate, tested on a complete set of single base pair mismatches and a number of small insertion/deletions.**

The ability to detect point mutations and small insertion/deletions in large (≥1 kb) fragments of DNA is becoming increasingly important in the field of molecular genetic testing. Existing methods, such as single strand conformation polymorphism (SSCP), are often unable to cope with such sizes of DNA, do not detect all mismatches or are expensive and time-consuming. An alternative is chemical cleavage of mismatch (CCM) which is a reliable and sensitive method originally described by Cotton *et al.* (1) as a modification of the Maxam and Gilbert sequencing protocol (2). The technique involves the detection of mismatched cytosines and thymines by hydroxylamine and osmium tetroxide modification of the DNA respectively, followed by cleavage with piperidine. It has been refined in recent years to include solid phase chemistry and fluorescently-labelled products for easier manipulation and detection (3–5). These adaptations, using biotinylated products in conjunction with streptavidin-coated magnetic particles, circumvent a number of the most time-consuming manipulations. The use of fluorescent labelling has allowed the detection of products on an ABI Genescanner and thus increased the signal to noise ratio and allowed for the potential of multiplexing. Although these changes have meant that the method is much simpler to perform, there remains the problem of toxicity, an undesirable feature of a methodology in a routine testing environment. Osmium tetroxide is probably the most difficult to work with of the chemicals used in CCM.

As a potential replacement for osmium tetroxide, potassium permanganate was noted to detect single base mismatches of thymine and although the method was promising, it was still potentially hazardous as it involved the use of tetramethylammonium chloride, which is itself highly toxic (6). Therefore the advantages to using potassium permanganate over osmium tetroxide were not great.

Here we show that the potassium permanganate reaction is also efficient in tetraethylammonium chloride which is non-toxic, and this reaction compares favourably with osmium tetroxide in the detection of mismatches using solid phase capture and fluorescent detection.

Mismatches were generated from pUC19 clones containing a different nucleotide 145 bp from the biotinylated primer, 365 bp from the Tet-labelled primer. PCR products of 511 bp were amplified using either one biotinylated primer and the other fluorescently-labelled with TET, or both primers labelled with biotin. DNA was purified using Qiagen PCR Clean up kit. Heteroduplexes were formed in annealing buffer (50 mM Tris–HCl, pH 8.0, 25 mM NaCl, 20 mM $MgCl_2$) by heating to 95°C for 5 min and then cooling to 25°C at 1°C/min. DNA (~20–50 ng/15 µl) was mixed with 20 µl of washed streptavidin-coated magnetic beads (in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 2 M NaCl) and allowed to bind to beads at room temperature for 15 min. Both paramagnetic particles from Promega and Dynabeads from Dynal give similar results in this context (data not shown). The supernatant was removed from the beads, 20 µl of the reaction mix (a 1/100 dilution of a fresh 100 mM solution of potassium permanganate in 3 M tetraethylammonium chloride) was added and incubated at 25°C for 1 h. This supernatant was removed and the beads washed in 50 µl TE. The TE was removed and the tubes left open to dry for a few minutes. An aliquot of 5.5 µl of the following mix was added: 100 µl DIF, 25 µl piperidine, 12.5 µl TAMRA 2500 markers, and incubated for 30 min at 90°C. Tubes were placed on ice and 1.5 µl loaded on the ABI 373 Genescanner. Samples were run on a 6% denaturing polyacrylamide gel, in 12 cm well-to-read plates for 4 h at 2500 V. Data were analysed using the 672 software. Osmium tetroxide reaction was as described (4).

Results are shown in Figure 1 for a C/T*, A/G* mismatch in a 511 bp fragment, and for a 5 bp insertion in a similar fragment. Figure 1A shows the 511 bp homoduplex DNA reacted with osmium tetroxide, and Figure 1B shows the 511 bp DNA fragment that contains the mismatch at position 365 bp. Figure 1C shows a similar fragment containing a 5 bp insertion.

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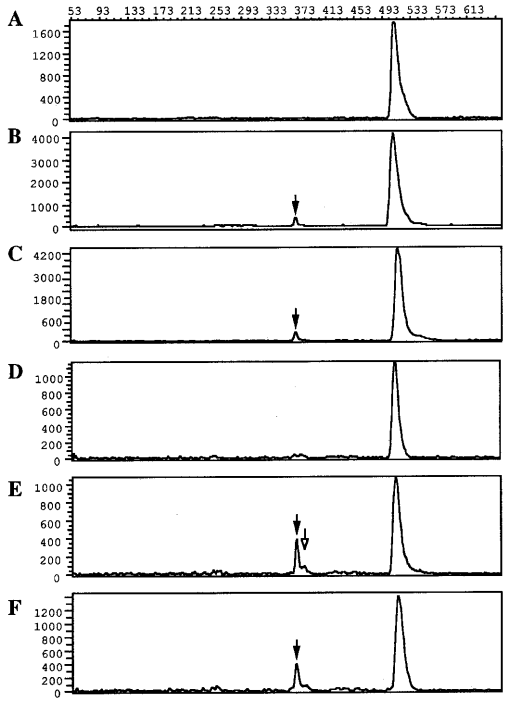


**Figure 1.** Chemical cleavage of a C/T*, A/G* heteroduplex and a 5 bp insertion. Homoduplex DNA reacted with osmium tetroxide as described. The scan shown is from an ABI 373 Genescanner, data analysed with 672 software. The intact PCR product is shown at 511 bp in both (**A**) and (**D**). DNA containing the mismatch reacted with (**B**) osmium tetroxide or (**E**) potassium permanganate. The intact PCR fragment is seen again, but in addition a cleavage product at 365 bp (indicated by a closed arrow) is seen. A secondary cleavage product is seen in (E) indicated by an open arrow. A 5 bp insertion/deletion reacted with (**C**) osmium tetroxide or (**F**) potassium permanganate. Cleavage products are indicated.

Figure 1D shows the same homoduplex DNA reacted with potassium permanganate as described above, Figure 1E the heteroduplex and Figure 1F the 5 bp insertion. The amount of cleavage product seen here with potassium permanganate is significantly greater than that seen with osmium tetroxide, for both the mismatch and the 5 bp insertion.

Table 1 gives a summary of cleavage reaction efficiencies for a range of mismatches and a number of insertion/deletions. These are all within the same sequence context of pUC19 and therefore allow an ideal situation for direct comparison of the two chemicals, osmium tetroxide and potassium permanganate, in detecting different mismatches. We find that whilst the potassium permanganate reaction gives similar amounts of cleavage each time the reaction is performed, the osmium tetroxide reaction is less robust. The chemical itself is unstable over long periods of time and therefore cannot be relied upon if infrequent use is to be made of this technique. In contrast, the potassium permanganate is extremely stable as a solid, and the required stock solution can be prepared as needed.

The potassium permanganate reaction is specific with a low background. There is occasionally a small amount of cleavage seen at adjacent T positions that are destabilised by the mismatch (Fig. 1E, open arrow) but this seems only to occur where there is a mismatch present and not in the homoduplex DNA.

Potassium permanganate is known to recognise mismatched bases other than thymine (6). To determine the contribution of the individual mismatches in the heteroduplexes shown in Table 1, we have performed PCR reactions using two biotinylated primers for one species and one biotinylated and one Tet-labelled primer for another. Heteroduplexes were formed from these, giving us only one labelled strand. We find that potassium permanganate will modify a range of mismatches, not only mismatched thymine. For the heteroduplex species in Table 1, each individual mismatch contributed equally to the figure obtained. This indicates that potassium permanganate would in fact detect an alteration in DNA, even though it may be that the mismatched base in the labelled strand is not thymine, giving greater confidence in this technique for mutation screening.

**Table 1.** Comparison of the efficiencies of osmium tetroxide and potassium permanganate on point mutations and small insertion/deletions in a 511 bp DNA fragment

| | C/T* A/G* | A/C* G/T* | A/A* T/T* | Insertion/ deletion 2 bp | Insertion/ deletion 3 bp | Insertion/ deletion 5 bp |
|---|---|---|---|---|---|---|
| Osmium tetroxide | 9% | 15% | 14% | 2% | 3% | 9% |
| Potassium permanganate | 32%[a] | 26%[a] | 15%[a] | 8% | 11% | 21% |

The amount of cleavage product for each of the mismatches is given as a percentage of the total input DNA. Data from duplicate experiments has been pooled for each mismatch.
*The strand labelled with a fluorophor. The insertion/deletions that are shown are those that have a mismatched T in the labelled strand.
[a]Equal contributions to this figure are made by each of the mismatches.

We have shown that potassium permanganate/tetraethylammonium chloride can be successfully used in place of osmium tetroxide; the sensitivity of mismatch and 1–5 bp insertion/deletion detection is at least as good and the potential hazard substantially reduced. Potassium permanganate is cheap and stable, easy to handle, store and dispose of, as is tetraethylammonium chloride, significantly reducing the toxicity of the CCM methodology.

CCM, using potassium permanganate/tetraethylammonium chloride and hydroxylamine, should be reconsidered as a viable alternative to current mutation detection methods as it is capable of detecting all point mutations and small insertion/deletions.

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