Detection of 100% of mutations in 124 individuals using a standard UV/Vis microplate reader: a novel concept for mutation scanning

Tania Tabone*, Georgina Sallmann, Elizabeth Webb and Richard G. H. Cotton

Genomic Disorders Research Centre, Fitzroy, Victoria 3065, Australia and the University of Melbourne, Parkville, Victoria 3052, Australia

Received January 2, 2006; Revised January 20, 2006; Accepted March 3, 2006

ABSTRACT

We report the development of a simple and inexpensive assay for the detection of DNA polymorphisms and mutations that is based on the modification of mismatched bases by potassium permanganate. Unlike the chemical cleavage of mismatch assay, which also exploits the reactivity of potassium permanganate to detect genomic variants, the assay we describe here does not require a cleavage manipulation and therefore does not require expensive or toxic chemicals or a separation step, as mismatches are detected using direct optical methods in a microplate format. Studies with individual deoxy-nucleotides demonstrated that the reactivity with potassium permanganate resulted in a specific colour change. Furthermore, studies with synthetic oligonucleotide heteroduplexes demonstrated that this colour change phenomenon could be applied to detect mismatched bases spectrophotometrically. A collection of plasmids carrying single point mutations in the mouse $\beta$-globin promoter region was used as a model system to develop a functional mutation detection assay. Finally, the assay was validated as 100% effective in detecting mismatches in a blinded manner using DNA from patients previously screened for mutations using established techniques, such as sequencing, SSCP and denaturing high-performance liquid chromatography (DHPLC) analysis in DNA fragments up to 300 bp in length.

INTRODUCTION

The detection and genotyping of polymorphisms and mutations has become one of the most challenging and often expensive and time-consuming obstacles to many molecular genetic applications including clinical diagnostics, pharmacogenomics and forensic analysis. The availability of a complete human genome reference sequence (1) has provided a valuable resource, allowing emphasis to now focus on the detection of novel mutations and the genotyping of established single nucleotide polymorphisms (SNPs) as the importance of knowledge of individual sequence variation to a broad range of disciplines is realized. However, the need for extremely reliable and efficient mutation detection has necessitated the continuous development of new methods and improvement of established methods to meet the requirements of individual applications (2).

The use of heteroduplex DNA is exploited in many methods for detecting sequence variants. Methods such as conformation sensitive gel electrophoresis (CSGE) (3), denaturing gradient gel electrophoresis (DGGE) (4) and constant denaturing capillary electrophoresis (CDCE) (5) involve observing a difference in the mobility of heteroduplex DNA compared to homoduplex DNA by electrophoretic migration. Similarly, methods such as chemical cleavage of mismatch (CCM) (6) and enzymatic cleavage-based methods (using CEL1, Mut enzymes, RNase, T4 endonuclease VII) (7–12) involve observing a difference in the mobility of heteroduplex DNA compared to homoduplex DNA by electrophoretic migration. Recently, a high resolution melting method (14) has been described that detects differences in DNA based on the melting profiles of matched and mismatched DNA using a double-stranded DNA binding dye. Each of these methods has advantages for particular applications. However, disadvantages include the need for a separation step, expensive enzymes, fluorescent primers, the use of toxic chemicals, specialized high precision instruments and the complexity of some methods, such as DHPLC, where quality control relies on determining the optimal assay
conditions for each amplicon to be analysed and the ability of a trained user to interpret results.

New methods for detecting sequence variants should provide high specificity, sensitivity and reproducibility, but should also avoid an electrophoretic separation step and the use of toxic reagents and be cost-effective and amenable to automation to allow high throughput analysis. We describe a method for the detection of heteroduplex DNA that meets all these requirements and in addition, does not require individual optimization for each new amplicon to be analysed, does not require a specialized detection instrument and is better suited to the detection of heterozygous mutations than current DNA sequencing methodologies.

The novel mutation detection method we have developed is based on the principles of CCM, first described by Cotton and colleagues in 1988. CCM requires the use of two chemicals, potassium permanganate (KMnO₄) and hydroxylamine (NH₂OH) to modify mismatched DNA and a third toxic chemical, piperidine, to cleave the modified products. Unlike CCM, the method we describe here is not based on a cleavage reaction to detect mismatched DNA, thereby eliminating the need for toxic chemicals, cumbersome incubation procedures and a time-consuming separation procedure, which is also required by many other techniques. Instead, the method relies on detecting the oxidative modification products of mismatched thymine (T) and cytosine (C) bases by potassium permanganate as it is reduced to manganese dioxide (MnO₂). Potassium permanganate has been shown to oxidise pyrimidine residues at the 5, 6 carbon-carbon double bonds to produce pyrimidine glycol residues (15,16). Potassium permanganate (pink solution) borrows electron density from these double bonds to form a bridged, unstable electron oxygen compound known as hypomanganate diester (yellow solution). Under normal pH and temperature conditions, this intermediate product is further oxidized by the permanganate ion to form the manganese dioxide (clear solution with brown precipitate) (17). The oxidation rate of the mismatched bases by potassium permanganate and hence the presence of a sequence variant is detected by measuring the formation of the hypomanganate diester intermediate, which absorbs at 420 nm visible wavelength (18) using a standard UV/Vis spectrophotometer. The hypomanganate diester intermediate product is further oxidized by the permanganate ion to form the manganese dioxide (pink solution) (19). Under normal pH and temperature conditions, this intermediate product is further oxidized by the permanganate ion to form the manganese dioxide (clear solution with brown precipitate) (17).

The sensitivity and specificity of the mismatch oxidation assay was evaluated by genotyping, in a blinded manner, previously characterized anonymous DNA specimens. Mutations in these individuals were previously detected with SSCP and DHPLC analysis and sequencing. We report a new approach to mutation scanning that accurately and reliably detects insertions, deletions and nucleotide substitutions in heteroduplex DNA. The method will also be useful in fundamental studies of heteroduplex DNA.

**MATERIALS AND METHODS**

**DNA substrates**

High-performance liquid chromatography (HPLC) purified synthetic oligonucleotides (GeneWorks, Hindmarsh, SA, Australia) containing a single C:T mismatch (19) (Table 1) were used as a model system to develop a mutation detection assay. Plasmid pBR322 clones containing the wild-type as well as a collection of 14 different single point mutations in the 132 bp mouse β-globin promoter region (20), were used to evaluate the performance of the method in detecting each class of mismatch and in various positions on the DNA strand from the primer-binding site. Mitochondrial and genomic DNA samples (21,22) were used to evaluate the performance of the method in detecting a range of mutations in a variety of sequence contexts and compare the performance against established mutation detection methods.

**PCR amplification**

The sequences of the PCR primers used in the study are listed in Table 1. PCR amplification was performed in a 50 μl reaction volume containing 100 ng template DNA, 0.2 mM each dNTP (Bioline, Alexandria, NSW, Australia), 1.5 mM MgCl₂, 1 x reaction buffer [50 mM KCl, 20 mM Tris–HCl (pH 8.4)], 0.3 μM forward primer, 0.3 μM reverse primer (Sigma-Genosys, Castle-Hill, NSW, Australia) and 2 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Amplification was performed in 96-well PCR microplates (Axxygen Scientific, Union City, CA, USA) in a Mastercycler® gradient thermal cycler (Eppendorf, Hamburg, Germany). The cycling conditions were as follows: an initial DNA denaturation at

| Primer name | Forward sequence (5’→3’) | Reverse sequence (5’→3’) | Tₘ (°C) | Size (bp) |
|-------------|---------------------------|--------------------------|--------|----------|
| 38MER A     | GGAAAGGCAACGGATAGTGGTAACTAGGCGACGCGGACAAAT | —                      | —      | 38       |
| 38MER B     | ATTGTCGCTGCGTCTTACGTGATGTTACCCTGATGCTTCTTCC | —                      | —      | 38       |
| 38MER C     | ATTGTGCGCTGCGTCTTACGTGATGTTACCCTGATGCTTCTTCC | —                      | —      | 38       |
| β-globin    | GCAGGCGCTGCCCGGCGCAT | CACAATATAGTGACGCAAGC     | 58     | 150      |
| ACSL4       | GATTTCGAGCGAGGAGGAGT | GCAGCTTAAGAGTGACCCTCAG   | 63     | 100      |
| MtT1        | AGGCCCTTCCCCGTAATAATG | TTGTCTCGTGTAAAGCATTAGG  | 53     | 223      |
| MtDloop     | TCGCTCAGGCGGCCATACAC | GTGCGATAAAATAGGATTG      | 53     | 288      |
| CCR5-1      | CCCAGTGGGACGTGGGAGAAT | AACACACAGGCTAGGCGACAG    | 60     | 131      |
| CCR5-2      | TGTGGTCTGATCCTGAC    | TGTAGGGGACCCAGAAAGA      | 60     | 135      |
| CCR5-3      | GGATTACAAATGTCAGTCTACA | ATGTGCGCCAAAGGACCAG     | 60     | 144      |
| CCR5-4      | TCTTTTGTTTGGTGGGCAAC | AGCATAGTGACGGCACAGAAGG   | 60     | 146      |
| CCR5-5      | GCTGTGTTGCGTCTTCC   | GACACGCCCACAGTAGTCA      | 60     | 147      |
| CCR5-6      | TGCTATCACCACCATATCTCA | ATATTTCTGCCTCCCCAGT    | 60     | 175      |

The optimal primer annealing temperature (°C) and expected PCR product size (bp) for each primer pair are also given.
95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at optimal primer $T_m$ for 20 s (Table 1) and extension at 72°C for 20 s, with a final extension at 72°C for 10 min.

**Preparation of heteroduplex DNA**

Following PCR amplification, DNA samples were denatured at 95°C for 10 min and cooled slowly from 80°C to 25°C, decreasing at 1°C increments every 2 min in a thermal cycler, to promote heteroduplex formation. DNA samples from homozygotes were mixed approximately with equal ratios of wild-type and mutant DNA PCR products in a 50 μl final volume to generate heteroduplexes for mutation detection. The wild-type reference control consisted of wild-type DNA PCR products alone in a 50 μl final volume. Single-stranded synthetic oligonucleotides were duplexed by adding equimolar ratios of complimentary DNA strands and heating to 95°C for 10 min, followed by 45°C for 30 min and 25°C for 30 min.

**PCR purification**

PCR products were purified from residual primers and dNTPs that may react with KMnO$_4$ using the QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA), according to the manufacturer’s instructions. The DNA was eluted in 35 μl dH$_2$O.

**Mismatch oxidation assay**

The mismatch oxidation assay was performed in 96-well flat-bottom microplates (Greiner Bio-One, Frickenhausen, Germany) in a 200 μl final reaction volume. Individual deoxynucleotide triphosphates (dNTPs), synthetic oligonucleotides (1–25 μg) or purified PCR products (0.5–1.5 μg) were added to 3 M tetraethylammonium chloride, pH 8.0 (TEAC; Sigma, St. Louis, MO, USA). A 10 mM solution of KMnO$_4$ (Sigma) was added to a final concentration of 1 mM, the reaction mixed well by pipetting up and down several times and the absorbance measured immediately. A no-template control sample contained water instead of DNA.

**Analysis of oxidized reaction products**

The difference in oxidation rates between homoduplex and heteroduplex DNA was detected by measuring the change in absorbance at 420 nm at room temperature at 60 s intervals for 3 h, using the Cary 50 Microplate Reader (Varian, Walnut Creek, CA, USA) and comparing absorbance profiles against a wild-type reference standard.

**RESULTS**

**Potassium permanganate oxidation of deoxynucleotides: proof-of-principle for a mutation detection assay**

To demonstrate the degree of reactivity of the four deoxynucleotides with potassium permanganate, each base was individually tested in the standard reaction mixture and the resulting colour change observed. Figure 1A shows that the order of reactivity of the deoxynucleotides with potassium permanganate, as followed by the change in colour of solution, was determined to be in the order of $dT > dC > dG > dA$. Deoxythymidine was slightly more reactive than deoxycytidine, however both pyrimidine residues were more reactive than the purine bases, which is consistent with other published studies reporting the reactivity of potassium permanganate with DNA (15,23,24).

To determine that the unpaired deoxythymidine base was necessary for oxidation to occur with potassium permanganate, deoxythymidine and deoxyadenosine nucleotides were hybridized together by incubating equimolar ratio of bases at room temperature for 30 min. The reactivity of the $dT:dA$ duplex with potassium permanganate was compared to the unpaired deoxythymidine and deoxyadenosine nucleotides. Figure 1B shows the order of reactivity was found to be $dT > dT:dA > dA$. The pairing of deoxyadenosine to deoxythymidine significantly reduced the oxidation rate by potassium permanganate compared to the unpaired deoxythymidine base as observed by the decreased rate in the colour change of the $dT:dA$ duplex solution. The deoxyadenosine again showed no reactivity and appeared equivalent in colour to that of the potassium permanganate control without deoxynucleotide bases.

![Figure 1](image-url)

**Figure 1.** Visual colour change observed by the potassium permanganate oxidation of nucleotide bases. (A). Potassium permanganate reactivity with $dT$ (1), $dA$ (2), $dC$ (3), $dG$ (4) and no-template control (5). (B). Potassium permanganate reactivity with $dA$:$dT$ (1), $dA$ (2), $dT$ (3) and no-template control (4).
Oxidation as a means of detecting mismatches formed using oligonucleotides: validation of the mismatch oxidation assay

The reactivity of potassium permanganate with mismatched DNA was examined using 38mer synthetic oligonucleotides containing a single C.T mismatch (Figure 2A). Using 25 μg of synthetic DNA, a difference in the colour of the solution was observed between the matched and mismatched duplexes. The solution of the mismatched DNA turned from a pink to a yellow colour suggesting a significant level of oxidation of the heteroduplex, whereas the matched DNA remained pink and showed only a slight colour change. Adjusting the concentration of the synthetic DNA used in the assay to a level commonly used in mutation detection applications (1 μg) did not produce a visible colour change. However, the use of a UV/Vis spectrophotometer showed there was a difference in absorbance at 420 nm between matched and mismatched DNA that corresponded to the change in colour used to visually discriminate between the two duplexes (Figure 2B). The mismatched DNA (red trace) showed a greater rate of oxidation compared to the matched DNA (blue trace), as determined by the increased rate of absorbance at 420 nm.

Detection of mismatches in PCR amplified DNA

The studies with deoxynucleotides demonstrating oxidation of free deoxythymidine and deoxycytidine residues suggested that an assay to detect all four classes of mismatches (C/T/A/G, C.A/T.G, C.C/G.G, T.T/A.A) was possible. As two alleles will form two alternate mismatched pairs, the observation that potassium permanganate oxidizes mismatched pyrimidines ensures that at least one mismatch in each pair will be recognized and modified. Furthermore, experiments with synthetic oligonucleotides demonstrating a difference in absorbance at 420 nm between matched and mismatched DNA showed a spectroscopic method could be developed and applied for the detection of heteroduplex DNA. To determine whether the assay could further be applied to detect mutations in research or diagnostic applications, 14 different plasmid clones, each containing a single point mutation in a 132 bp fragment of the mouse β-globin promoter region, were initially examined. This model system was used to determine the optimal conditions for a reliable and reproducible mutation detection assay. The broad distribution of mutations across the DNA fragment (Figure 3) allowed the determination of the assay to detect mutations close to the primer-binding site and at various positions on the DNA strand, which can often be a limiting factor in methods, such as sequencing. As an example, Figure 4 shows the spectrophotometric analysis of 150 bp PCR amplicons with a G.T/A.C mismatch at position −57, a C.T/A.G mismatch at position −43, a C.C/G.G mismatch at position −76 and an A.A/T.T mismatch at position −2 of the mouse β-globin promoter region. Duplexes were also formed between two mutant DNA fragments to produce amplicons with two mismatches (−2/−76; −57/−43 and −13/−27). Figure 5 shows the presence of two mismatches (A.A/T.T and C.C/G.G) in the same amplicon resulted in an

![Figure 2.](image-url)

![Figure 3.](image-url)

**Figure 2.** Potassium permanganate oxidation of 38mer synthetic oligonucleotides (A). Visual colour change observed by the potassium permanganate oxidation of synthetic oligonucleotides with heteroduplex DNA (left), homoduplex DNA (right) and no-template control (middle). (B). Spectroscopic scan at 420 nm of 38mer synthetic oligonucleotides (1 μg) after treatment with potassium permanganate (1 mM final concentration) at room temperature. The top trace (red) shows the oxidation rate of the mismatched duplex, the middle trace (blue) shows the oxidation rate of the matched duplex and the bottom trace (green) shows the oxidation rate of the no-template control sample.

**Figure 3.** Location of mutations in the mouse β-globin promoter sequence. Primer-binding sites underlined.

5' GGACCCCGCTG GAGCCGCTAC GATTCCTAG AAGCACAANCC TGGTAAGGCG NAATCTGTC C

ACACAGGGATN GAGAGGCGCTG GAGNNAGGGC AGANTCATAN AGGINAGGTA GGGNCAGTTG T

CTCONCACAT TTGCTTCTGA CATACTGGTC-3'
increase in the oxidation rate of the DNA compared to amplicons containing a single mismatch, as detected by the greater absorbance at 420 nm, suggesting the assay could be used to distinguish between samples containing a mutation and a common polymorphism.

When the optimal assay conditions were determined, each of the mutations in the mouse β-globin promoter DNA were analysed with a minimum of four repeats for each sample to ensure accurate reproducibility of the data could be achieved with the optimized assay conditions. The results showed the assay to be completely reproducible with all mutations detected on each occasion. In order to show that the developed method was applicable to the detection of mutations in real samples, the assay was used to successfully detect a G.T;A.C mismatch in the ACSL4 gene (MIM 300157) by analysing 10 genomic DNA samples, an A.C;G.T mismatch present at 40% heteroplasmy in the mitochondrial TL1 gene using 40 mitochondrial DNA samples and a G deletion and T.T;A.A mismatch also present at a 40% heteroplasmic frequency in the mitochondrial D loop gene with 32 mitochondrial DNA samples (MIM 590050) (Table 2). Finally, analysing 28 genomic DNA samples in a blinded fashion, the assay was applied to successfully detect mismatches in

Figure 4. Spectroscopic scan at 420 nm of 150 bp PCR products (1 µg), representing all four classes of mismatches, after treatment with potassium permanganate (1 mM final concentration) at room temperature (A). G.T/A.C mismatch at position −57 (B). C/T/A.G mismatch at position −43 (C). C.C/G.G mismatch at position −76 (D). A.A/T.T mismatch at position −2. In each scan, the top trace (red) shows the oxidation rate of the mismatched duplex, the middle trace (blue) shows the oxidation rate of the matched duplex and the bottom trace (brown) shows the oxidation rate of the no-template control sample.

Figure 5. Spectroscopic scan at 420 nm of a 150 bp PCR product (1 µg) containing two mismatches in the same amplicon after treatment with potassium permanganate (1 mM final concentration) at room temperature. The top trace (red) shows the oxidation rate of the mismatched duplex, the middle traces (blue and green) show the oxidation rate of the matched duplex and the bottom trace (brown) shows the oxidation rate of the no-template control sample.
five separate amplicons of the CCR5 gene (MIM 601373). All of these mutations are summarized in Table 2. Evaluation of the method with plasmid DNA and human DNA detected all the mismatches present in a total of 124 DNA samples with a 100% sensitivity and 100% specificity rate (Table 3). The method did not detect any false positives and did not report any false negative heteroduplexes, suggesting the method is comparable in sensitivity and specificity to currently established mutation detection methods.

**DISCUSSION**

There is an ever-increasing range of mutation detection technologies available. These methods have several advantages and disadvantages according to a particular requirement or laboratory. However, the vast majority of methods are both suitable for high throughput analysis and therefore not economical for small sample sized analysis or are time-consuming to perform and require extensive optimization to enable sensitive and accurate detection. There is a clear, unmet need for a mutation detection method that is simple, sensitive and cost-efficient to perform for both low and high throughput applications. We have developed a new approach to detect DNA variants based on the potassium permanganate oxidation of mismatched bases, eliminating the need for a cleavage and separation step (25). We report the first description of a simple and sensitive spectroscopic-based mutation detection method that utilizes the reactivity of potassium permanganate with mismatched pyrimidine bases by measuring the difference in absorbance at the 420 nm wavelength between heteroduplex and homoduplex DNA to detect the presence of a mutation.

| GenBank ID | PCR amplicon | Amplicon size (bp) | Mismatch class | Mutation | WT A420 180 min (OD) | Mut A420 180 min (OD) | % Increase in heteroduplex oxidation |
|------------|--------------|--------------------|----------------|----------|---------------------|---------------------|-----------------------------------|
| J00413     | β-globin     | 150                | G.T/A.C        | −22G>A   | 0.81                | 1.12                | 38                                |
|            |              |                    |                | −27A>G   | 0.82                | 1.10                | 34                                |
|            |              |                    |                | −42C>T   | 0.75                | 1.09                | 45                                |
|            |              |                    |                | −57A>G   | 0.84                | 1.14                | 36                                |
|            |              |                    | G.A/T.C        | −14A>C   | 0.81                | 1.11                | 37                                |
|            |              |                    |                | −33G>T   | 0.74                | 1.08                | 46                                |
|            |              |                    |                | −42C>A   | 0.81                | 1.14                | 41                                |
|            |              |                    |                | −43A>C   | 0.75                | 1.13                | 51                                |
|            |              |                    |                | −89C>A   | 0.71                | 1.04                | 46                                |
|            |              |                    | C.C/G.G        | −42C>G   | 0.72                | 1.02                | 42                                |
|            |              |                    |                | −76C>G   | 0.73                | 1.06                | 45                                |
|            |              |                    |                | −13T>A   | 0.76                | 0.99                | 30                                |
|            |              |                    |                | −48A>T   | 0.82                | 1.02                | 24                                |
|            |              |                    |                |          |                     |                     |                                   |
| NM_004458  | ACSL4        | 100                | G.T/A.C        | g.13958G>A | 0.66                | 0.99                | 50                                |
|            |              |                    |                | g.13958G>A | 0.66                | 1.02                | 55                                |
| NM_001807  | MrTL1        | 223                | A.C/G.T        | m.3243A>G | 0.42                | 0.57                | 36                                |
|            |              |                    |                | m.3243A>G | 0.42                | 0.56                | 33                                |
|            |              |                    | A.C/G.T        | m.3243A>G | 0.42                | 0.57                | 36                                |
|            |              |                    |                | m.3243A>G | 0.42                | 0.60                | 43                                |
|            |              |                    | A.C/G.T        | m.3243A>G | 0.42                | 0.55                | 31                                |
|            |              |                    |                | m.3243A>G | 0.42                | 0.61                | 45                                |
| NM_001807  | MtDloop      | 288                | G.T/A.A        | m.66delG | 0.56                | 0.76                | 36                                |
|            |              |                    |                | m.72T>A   | 0.56                | 0.72                | 29                                |
| NM_000579  | CCR5-1       | 131                | T.T/A.A        | g.303T>A  | 1.01                | 1.29                | 28                                |
| NM_000579  | CCR5-2       | 135                | G.T/A.C        | g.668G>A  | 1.06                | 1.30                | 23                                |
| NM_000579  | CCR5-3       | 144                | AAG/-          | g.228delK | 1.06                | 1.32                | 25                                |
| NM_000579  | CCR5-4       | 146                | T.T/A.A        | g.48T>A  | 0.99                | 1.20                | 21                                |
| NM_000579  | CCR5-5       | 147                | T.T/A.A        | g.164T>A  | 0.80                | 1.00                | 25                                |
| NM_000579  | CCR5-6       | 175                | C.T/A.G        | g.492C>A  | 0.90                | 1.10                | 22                                |
|            |              |                    |                | g.2lp del | g.5s4del5s8 | 0.90                | 1.13                | 26                                |
|            |              |                    |                | C.T/A.G   | g.893delC | 0.72                | 1.11                | 54                                |
|            |              |                    |                | g.900C>A  | 0.72                | 1.12                | 56                                |
|            |              |                    | G.A/T.C        | g.902G>T  | 0.72                | 1.12                | 56                                |
|            |              |                    |                | g.1004C>T | 0.72                | 1.00                | 39                                |
|            |              |                    | A.A/T.T        | g.1016A>T | 0.72                | 1.09                | 51                                |
The novel mutation detection method is cost-effective to perform and can be automated to allow analysis of large numbers of sequences. Unlike other automated approaches, our method does not require the initial purchase of an expensive precision instrument, as only a standard UV/Vis microplate reader in required and the on-going running costs of the assay are minimal. The DNA preparation procedure uses standard PCR reagents and primers, no special reagents such as biotinylated or fluorescent primers or radioisotopes are required. The mismatch detection assay is then performed with two cheap and commonly available reagents, potassium permanganate and TEAC and there are no enzymes or expensive chemicals required. The method is not labour intensive or time-consuming to perform and does not require any complicated pre-assay set-up such as, the design of primers and probes for mutation detection. Following PCR amplification and purification, regular, unlabelled PCR amplicons are added directly to the potassium permanganate and TEAC in the microplates and analysis begins. Following completion of the analysis, the traces are visually inspected, by comparison of the test samples to the wild-type reference sample. No complicated computer analysis, normalization or other manipulation of the data are required. In addition, the universal reaction condition for all amplicons allows a mixture of samples to be analysed in the same microplate, further reducing the time and cost involved with performing the assay. These characteristics are all important to the successful implementation of a new method into laboratories performing mutation scanning for the first time or laboratories looking to introduce such an assay. It is highly sensitive and specific, does not require specialized reagents, or other manipulation of the data are required. In addition, the universal reaction condition for all amplicons allows a mixture of samples to be analysed in the same microplate, further reducing the time and cost involved with performing the assay. These characteristics are all important to the successful implementation of a new method into laboratories performing mutation detection assay for the first time or laboratories looking to introduce such an assay. It is highly sensitive and specific, does not require specialized reagents, and the presence of 1 mM Tris or 0.1 mM EDTA will strongly reduce the amount of noise.

Table 3. Summary of the detection specificity and sensitivity for the mismatch detection assay

| Mismatch class | Mismatches detected/total number of mismatches present |
|----------------|-------------------------------------------------------|
| G.T/A.C        | 11/12                                                 |
| G.A/T.C        | 10/10                                                 |
| C.C/G.G        | 2/2                                                   |
| A.A/T.T        | 12/12                                                 |
| C del          | 1/1                  |
| G del          | 1/1                                                   |
| 3 bp del       | 1/1                                                   |
| 32 bp del      | 1/1                                                   |

The optimal potassium permanganate and TEAC concentrations was critical to the development of a successful mutation detection assay. The optimal concentration of potassium permanganate was found to be 1 mM. Insufficient concentrations of potassium permanganate resulted in low efficiency of oxidation and a weaker signal, whereas higher concentrations were too erratic and specificity of oxidation for the mismatch was lost. Similarly, insufficient TEAC concentrations resulted in inadequate buffering of the DNA, whereas high concentrations of TEAC resulted in loss of specificity of oxidation for the mismatched DNA. These findings are consistent with published studies on the specificity of potassium permanganate for mismatched DNA. Potassium permanganate alone without a buffer, probably because it is a strong oxidant, does not discriminate between homoduplex and heteroduplex DNA. However, when a solution of 2 M TEAC or 3 M tetramethylammonium acetate (TMAC) is used, the modification of mismatched bases is enhanced (24). Discrimination between heteroduplex and homoduplex DNA was not improved with the addition of 0.5 M or 1 M NaCl, presumably because NaCl stabilizes the reactivity of potassium permanganate with pyrimidines (24). To ensure consistent and reproducible results, the assay was performed at 25°C, although temperature ranges from 20–30°C produced optimal results. Reaction temperatures lower than 16°C resulted in a very slow oxidation rate of the heteroduplex DNA and reaction temperatures greater than 30°C resulted in incorrect oxidation rates, presumably due to the oxidation of both matched and mismatched DNA by potassium permanganate. The pH of the reaction was maintained at pH 8.0 by the TEAC solution. Due to the strong oxidation properties of potassium permanganate, care must be taken to completely remove any residual ethanol from any post-PCR purification procedures used and the DNA cannot be eluted or rehydrated in TE or other buffers containing Tris or EDTA. Ethanol present in a concentration of 1% or higher in the reaction and the presence of 1 mM Tris or 0.1 mM EDTA will strongly
react with potassium permanganate and interfere with the DNA oxidation signal.

As the mutation detection assay does not provide details about the nature or location of the mismatch, sequencing would be required to characterize an unknown mutation. However, one of the many benefits of the assay is that it significantly reduces the amount of sequencing that would otherwise be performed on samples that do not contain a mutation. The assay is also especially suited to the detection of heterozygous mutations, which are easily missed with current sequencing techniques. The method we propose should be valuable for rapid mutation screening, followed when appropriate by sequencing to characterize a novel variant that is detected. The method is applicable to the detection of insertions, deletions and point mutations. To further expand the scope of the method, it will be useful to determine the sensitivity rate of the assay to detect mismatches in PCR fragments greater than 288 bp, the upper limit tested so far.

The detection sensitivity of the assay was determined by testing 39 different mutations representing all four classes of mismatches and in different sequence contexts from 124 cloned mouse DNA and human genomic and mitochondrial DNA samples. The assay was found to be 100% efficient in detecting all the mismatches present and validated the performance of the assay to detect mismatches in multiple DNA targets. The specifications of the assay, including fragment length and accuracy, was determined by testing different mutations in various sized PCR products, ranging from 100–288 bp in length. The assay was able to detect mismatches in all sized amplicons without loss of performance sensitivity, with an increase in the susceptibility of oxidation of the heteroduplex by KMnO₄ of 21–56% compared to the homoduplex. DHPLC analysis.

CONCLUSION
This new approach to mutation detection has many advantages over current methods as it does not require fluorescent primers or probes labels, electrophoresis, toxic chemicals and individual assay optimization or post-PCR modification. The assay is based on a simple optical assay providing easy to interpret data. As the assay only requires a PCR amplicon to detect unknown mutations or genotype known mutations, minimal information about the mutation and sequence is required. The assay has one universal protocol that can be applied to detect all types of mutations, which eliminates a time-consuming optimization process for each new individual amplicon to be analysed, thereby saving time and increasing throughput productivity and increasing the detection rate of multiple mismatches.

ACKNOWLEDGEMENTS
This research was supported by Seed funding from the CASS Foundation (Genvartec Pty Ltd) and a fellowship from the National Health and Medical Research Council to D. C. The authors would like to thank Varian Australia for the donation of the Cary 50 Microplate Reader and Michael Hammer (Varian Australia), Daren Fyfe (Varian Australia) and Eric Gerber (formerly Varian USA) for experimental advice and helpful discussions during the development of this assay. The authors would like to thank Michael Dean (NCI), Gregor Berger (ORYGEN) and Rosetta Marotta and Robert Kapsa (NMDRC) for the generous donation of human genomic and mitochondrial DNA samples. Funding to pay Open Access publication charges for this article was provided by National Health and Medical Research Council.

Conflict of interest statement. None declared.

REFERENCES
1. Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. et al. (2001) Initial sequencing and analysis of the human genome. Nature, 409, 860–921.
2. Cotton, R.G.H. (2002) Mutation Detection. In Creighton, T. (ed.), Encyclopedia of Molecular Medicine. Wiley, NY, pp. 2159–2162.
3. Ganguly, A., Rock, M.J. and Prokop, D.J. (1993) Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. Proc. Natl Acad. Sci. USA, 90, 10325–10329.
4. Myers, R.M., Maniatis, T. and Lerman, L.S. (1987) Detection and localization of single base changes by denaturing gradient gel electrophoresis. Meth. Enzymol., 155, 501–527.
5. Khrapko, K., Hanekamp, J.S., Thilly, W.G., Belenkii, A., Foret, F. and Karger, B.L. (1994) Constant denaturing capillary electrophoresis (CDCE): a high-resolution approach to mutational analysis. Nucleic Acids Res., 22, 364–369.
6. Cotton, R.G., Rodrigues, N.R. and Campbell, R.D. (1988) Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proc. Natl Acad. Sci. USA, 85, 4397–4401.
7. Youil, R., Kemper, B.W. and Cotton, R.G.H. (1995) Screening for mutations by enzyme mismatch cleavage using T4 endonuclease VI. Proc. Natl Acad. Sci. USA, 92, 87–91.
8. Youil, R., Kemper, B. and Cotton, R.G.H. (1996) Detection of 81 of 81 known mouse β-globin promoter mutations with T4 endonuclease VII–the EMC method. Genomics, 32, 431–435.
9. Till, B.J., Burtner, C., Comai, L. and Henikoff, S. (2004) Mismatch cleavage by single-strand specific nucleases. *Nucleic Acids Res.*, **32**, 2632–2641.

10. Oleykowski, C.A., Bronson Mullins, C.R., Godwin, A.K. and Yeung, A.T. (1998) Mutation detection using a novel plant endonuclease. *Nucleic Acids Res.*, **26**, 4597–4602.

11. Smith, J. and Modrich, P. (1996) Mutation detection with MutH, MutL and MutS mismatch repair proteins. *Proc Natl Acad Sci USA*, **93**, 4374–4379.

12. Goldrick, M.M. (2001) RNase cleavage-based methods for mutation/SNP detection, past and present. *Hum. Mutat.*, **18**, 106–112.

13. Oefner, P.J. and Underhill, P.A. (1995) Comparative DNA sequencing by denaturing high-performance liquid chromatography (DHPLC). *Am. J. Hum. Genet.*, **57**, A266.

14. Wittwer, C.T., Reed, G.H., Gundry, C.N., Vandersteen, J.G. and Pryor, R.J. (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin. Chem.*, **49**, 853–860.

15. Hayatsu, H., Atsumi, G., Nawamura, T., Kanamitsu, S., Negishi, K. and Maeda, M. (1991) Permanganate oxidation of nucleic acid components: a reinvestigation. *Nucleic Acids Symp. Ser.*, **25**, 77–78.

16. Hayatsu, H. (1996) The 5,6-double bond of pyrimidine nucleosides, a fragile site in nucleic acids. *J. Biochem.*, **119**, 391–395.

17. Freeman, F., Fuselier, C.O. and Karchefski, E.M. (1975) Permanganate ion oxidation of thymine: spectrophotometric detection of a stable organomanganese intermediate. *Tet. Letters*, **25**, 2133–2136.

18. Bui, C.T., Sam, L.A. and Cotton, R.C. (2003) UV-visible spectral identification of the solution-phase and solid-phase permanganate oxidation reactions of thymine acetic acid. *Bioorg. Med. Chem. Lett.*, **14**, 1313–1315.

19. Miret, J.J., Parker, B.O. and Lahue, R.S. (1996) Recognition of DNA insertion/deletion mismatches by an activity in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **24**, 721–729.

20. Myers, R.M., Larin, Z. and Maniatis, T. (1985) Detection of single base substitution by ribonuclease cleavage at mismatches in RNA:DNA duplexes. *Science*, **230**, 1242–1246.

21. Dean, M., Carrington, M., Winkler, C., Huttley, G.A., Smith, M.W., Allikmets, R., Goedert, J.J., Susan, P., Buchbinder, S.P., Vittinghoff, E. *et al.* (1996) Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CCR5 structural gene. *Science*, **273**, 1856–1862.

22. Carrington, M., Kissner, T., Gerrard, B., Ivanov, S., O’Brien, S.J. and Dean, M. (1997) Novel alleles of the chemokine-receptor gene CCR5. *Am. J. Hum. Genet.*, **61**, 1261–1267.

23. Howgate, P., Jones, A.S. and Tittensor, J.R. (1968) The permanganate oxidation of thymidine. *J. Chem. Soc C*, **1**, 275–279.

24. Gogos, J.A., Karayiorgou, M., Aburatani, H. and Kafatos, F.C. (1990) Detection of single base mismatches of thymine and cytosine residues by potassium permanganate and hydroxylamine in the presence of tetralkylammonium salts. *Nucleic Acids Res.*, **18**, 6807–6814.

25. Cotton, R.C., Bui, C. and Lambrinakos, A. (2002) Method of identifying differences between nucleic acid molecules. Patent 20040146875; PCT/AU02/00171.

26. Iida, S. and Hayatsu, H. (1970) The permanganate oxidation of deoxyribonucleic acid. *Biochim. Biophys. Acta*, **123**, 1–13.

27. Rubin, C.M. and Schmid, C.W. (1980) Pyrimidine-specific chemical reactions useful for DNA sequencing. *Nucleic Acids Res.*, **8**, 4613–4619.