Allele identification using immobilized mismatch binding protein: detection and identification of antibiotic-resistant bacteria and determination of sheep susceptibility to scrapie

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ABSTRACT

A novel method for detection and identification of specific alleles has been developed utilizing immobilized mismatch binding protein (IMBP). The assay involves the use of biotin-labeled probes, which are prepared by PCR amplification of cloned fragments with known sequence. The use of probes avoids many of the problems associated with the extreme sensitivity of IMBP assays to errors in PCR amplification. The method can be used to monitor PCR fidelity and to genotype both diploid and haploid organisms and has been used to distinguish rifampicin-sensitive and -resistant strains of *Mycobacterium tuberculosis* and to detect and distinguish two alleles of the sheep prion protein gene involved in susceptibility to scrapie.

INTRODUCTION

Mutation detection can be divided into two broad categories: (i) detection of all mutations or polymorphisms within a particular segment of genomic DNA; (ii) detection of a specific mutation, generally one known to be of genetic significance. Similarly, mutation screening involves either: (i) detection of any sequence alterations in a specific DNA fragment, as would occur when screening affected populations for undiscovered disease-causing mutations; (ii) detection of a specific mutation in a large population, as would be required for diagnosis or genotyping.

Many mutation detection methods (1–9) have been designed to detect any (or at least most) mutations in a specific fragment of genomic DNA [e.g., single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and mismatch cleavage]. Other methods have been designed to detect specific mutations or mutations at a single base pair position in the DNA [e.g., oligonucleotide ligase assay (OLA) and allele-specific oligonucleotide hybridization]. Immobilized mismatch binding protein (IMBP) assays (7,8) can be used both to screen fragments for mutations and to detect and identify specific mutations. The assays are simple, inexpensive and easy to automate and do not require radioactivity, sequencing or gel electrophoresis.

The use of IMBP to detect mutations in specific DNA fragments has been described (8). In those experiments, labeled primers were used to amplify human genomic DNA. Following denaturation and annealing the PCR products were analyzed in IMBP assays. Heterozygotes were detected as strong positive signals. However, the homozygote background was significantly higher than the backgrounds obtained with synthetic oligonucleotides. This background appears to be the result of errors in PCR amplification, both misincorporation and mispriming. Mispriming, which presumably produces labeled single-strand molecules capable of adopting secondary structure with IMBP-recognizable mismatches or unpaired bases, appeared to be the largest contributor to background in those experiments. The results reported here demonstrate that the use of labeled probes, i.e., fragments prepared by amplification of cloned fragments or bacterial DNA, greatly reduces background signals. Labeled probes are mixed with test DNA samples prior to denaturation, annealing and exposure to IMBP. The observed background reduction presumably results from a reduction in mispriming due to the relatively high target to non-target ratio in the plasmid or bacterial DNA used as template for probe preparation. The use of probes also provides a means to use IMBP to detect and identify specific mutations. Alleles with sequence identical to the probe are identified by virtue of not being able to form IMBP-recognizable heteroduplexes with probes. Two model systems have been examined: (i) a region of the *Mycobacterium tuberculosis rpoB* gene containing sequence variants responsible for rifampicin resistance or sensitivity; (ii) a region of the sheep prion protein gene (*PrP*) containing sequence variants responsible for susceptibility or resistance to scrapie.

MATERIALS AND METHODS

Prion protein gene analysis

The fragment amplified for cloning, probe preparation and DNA testing was a 190 bp fragment of the sheep *PrP* gene spanning codons 149–212. Primers A (5′-GAGGACCGTTACTATCGT-GA) and B (5′-CCACTCGCTCCATTATCTTG) (Ransom Hill

Bioscience) were used for all amplifications, but contained a 5′ biotin label when used for probe preparation.

The PCR mixture (50 µl) contained 10 mM Tris–HCl, pH 8.3, 3.5 mM MgCl$_2$, 25 mM KCl, 5% glycerol, 100 µg/ml BSA, 0.2 mM dNTPs (Boehringer Mannheim), 1.0 µM each primer, 40 ng template DNA and 1.5 U Stoffel fragment enzyme (Perkin Elmer). Amplification was for 32 cycles: denaturation, 30 s 94°C; annealing, 1 min, four cycles at 66°C, four cycles at 64°C, four cycles at 62°C, four cycles at 60°C, 16 cycles at 58°C; extension, 1 min at 72°C; a final extension at 72°C for 10 min.

PrP fragment-containing plasmids were prepared using unlabeled PCR products prepared from genomic DNA of known genotype using the PCRscript Cloning Kit (Stratagene) according to the manufacturer's protocol. Clones with inserts were selected and DNA purified from them for sequencing and probe preparation. The sequence of the cloned fragments was confirmed by manual sequencing (dsDNA Cycle Sequencing System; Gibco-BRL).

Probes were prepared by PCR amplification of fragments from 0.2 ng plasmid DNA. Probe fragments were prepared with one biotin-labeled primer. Primer A was labeled for probes made from R DNA (R probe) and primer B labeled for probes made from Q DNA (Q probe). PCR conditions were essentially the same as for test DNA with the following exceptions; the annealing temperatures were 2° higher and the cycle number was limited to 30 cycles (14 cycles at 60°C). Unused primers were removed with Qiaquick spin columns (Qiagen) and probes were eluted in 50 µl Tris–HCl, pH 8.5, and adjusted to 100 mM NaCl by addition of 5 M NaCl. The probes were then quantitated by running them on 8% polyacrylamide gels and comparing them with known standards.

Mycobacterium tuberculosis antibiotic resistance analysis

The target for probe and test DNA preparation was a 190 bp fragment of the *rpoB* gene containing the region where most mutations associated with rifampicin resistance are located. Probe and target DNA were generated by PCR amplification using primers KY290 (5′-GGCGATCACACCGCAGACGT) and KY292 (5′-GGACCTCCAGCCCGGCA), which are specific for *M.tuberculosis*. Biotin-labeled primers were used for probe DNA synthesis and unlabeled primers were used for test DNA synthesis. Purified *M.tuberculosis* genomic DNA from one rifampicin-sensitive (wild-type) isolate and three rifampicin-resistant (mutant) isolates with known point mutations were used as templates for amplification. The mutant DNAs were provided by Dr B.Kreiswirth.

Test DNA fragments were generated by PCR amplification in reaction mixtures containing (final concentrations in 50 µl reactions) 10 mM Tris–HCl, pH 8.3, 5 mM MgCl$_2$, 25 mM KCl, 5% (v/v) glycerol, 100 µg/ml BSA, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.4 mM dUTP (Perkin Elmer), 1.0 µM each primer (unlabeled), 1.5 U Stoffel fragment of Taq DNA polymerase (Perkin Elmer) and ~1000 copies of *M.tuberculosis* DNA. Amplification was carried out for 32 cycles: two cycles of 30 s at 98°C; annealing, 1 min at 62°C; extension, 1 min at 72°C and 30 cycles of 30 s at 94°C, 1 min at 62°C; extension, 1 min at 72°C; a final extension at 72°C for 10 min, included to allow completion of DNA synthesis.

For probe preparation, PCR was performed using ~10 000 copies of either wild-type or mutant *M.tuberculosis* DNA as template. Probe fragments were prepared with both primers labeled with biotin. PCR conditions were essentially the same as for test DNA except that cycling was for 30 cycles: denaturation, 1 min at 94°C; annealing, 1 min, four cycles at 68°C, four cycles at 66°C, four cycles at 64°C, four cycles at 62°C, 14 cycles at 60°C; extension, 2 min at 72°C; a final extension at 72°C for 10 min. Probes were then purified and quantitated as described above.

Denaturing and reannealing conditions

All samples were denatured and reannealed as follows: 3 min at 100°C, 90 min at 75°C; cooling to 4°C (Perkin Elmer model TC1).

SSB treatment

After denaturation and reannealing samples were adjusted to assay conditions by the addition of 10× reaction buffer (200 mM Tris–HCl, 50 mM MgCl$_2$, 1 mM DTT and 1 mM EDTA). An aliquot of 1.2 µg SSB (Promega) was added to each sample. The tubes were then incubated at room temperature for 30–45 min. Samples were then added directly to the IMBP assay.

IMBP assays

All IMBP assays were performed with Genecheck Mutation Detection Kits or M$_2$B$_2$ (Magnetic Mismatch Binding Beads) according to the manufacturers' protocols.

RESULTS AND DISCUSSION

The results of experiments comparing the use of labeled primers with the use of labeled probes in IMBP assays for the detection of heterozygotes are presented in Figure 1. When labeled probes were used there was a clear reduction in background signal from homozygotes. In addition, the use of probes allowed an exact determination of the homozygote genotypes. The reduction in background signal observed with probes is presumably a consequence of the PCR conditions, including buffer and enzyme selection, used to prepare probes. Probes were amplified from fragments cloned into small plasmids where the ratio of target to non-target sequence is nearly six orders of magnitude higher than when the same fragment is amplified from genomic DNA. Therefore, the likelihood of mispriming is greatly reduced. Labeled probes prepared from cloned fragments should also contain fewer misincorporation errors than fragments prepared from genomic DNA, since it is possible to use more starting template and, therefore, fewer rounds of PCR to produce products. Mispriming, i.e., extension of primers at sites other than the target, may contribute to background by virtue of the fact that it produces labeled single-strand fragments which may adopt some secondary structure, frequently containing mispairs or regions with one to four unpaired bases, and thus be substrates for IMBP binding. The probe method also allows the use of a large excess of unlabeled test DNA with respect to probe, which may both increase the extent of annealing (which would leave less labeled single-strand DNA and, thus, reduce background) and allow the direct use of PCR products without quantitation. Support for the idea that single-strand DNA leads to increased background comes from the finding that pretreatment of samples with SSB (single-strand binding protein) after annealing and prior to exposure to IMBP decreases background signal in some samples (data not shown).

The success of probe preparation depends, to a large extent, on polymerase selection. Five different polymerases were tested for
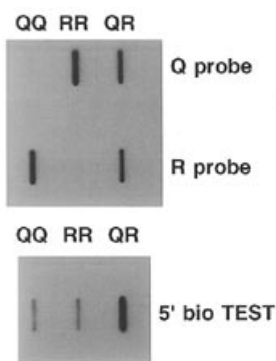
Figure 1. Comparison of the probe method with labeled primers. In the probe method sheep genomic DNA was amplified with *PrP*-specific primers to generate test DNA (see Materials and Methods). Aliquots of 10 μl each test DNA were mixed with 10 ng Q and R probes in 10 μl annealing buffer in separate reactions. In the labeled primer method Q/Q, R/R and Q/R genomic DNA (40 ng) were amplified with biotin-labeled primer A. The products were purified, quantitated on polyacrylamide gels, denatured, annealed and tested in an IMBP assay (10 ng/slot). In both methods identical concentrations of PCR fragments were used for denaturation and reannealing.

possible use in these experiments. Testing involves selecting optimum conditions for each polymerase. Conditions were optimized in a two-step fashion. Each of the tested polymerases was used independently to amplify fragments from plasmid templates. These PCR products were run on polyacrylamide gels (data not shown). (Plasmid DNA was used as template to limit the possibility of mispriming.) Those products revealing only a single band were then tested in IMBP assays. Cycling temperature and buffer conditions producing the lowest background signal were selected independently for each polymerase. (Twelve different buffers varying in pH and Mg^{2+} and KCl concentrations were tested.) Five different polymerases were compared following individual optimization (Fig. 2). A Q plasmid, an R plasmid and a mixture of Q and R plasmids were amplified, using identical primers, with each of the five polymerases. The amplified fragments from the Q and R plasmids differ by only a single base pair substitution, which in heteroduplexes gives G:T and A:C mismatches. The PCR products were run on a polyacrylamide gel (Fig. 2A). The product of each enzyme gave a single band and the bands from all of the enzymes were of nearly equal intensity. However, IMBP assays revealed a wide range of signals (Fig. 2B). In those cases where background is especially high it is impossible to distinguish heteroduplex-containing preparations from those containing only homoduplexes. The best results were obtained with Pfu polymerase, a known high fidelity polymerase. The Stoffel fragment of Taq polymerase also gives very low background signals. The Stoffel fragment may have a higher fidelity compared with Taq because of its inability to extend mispaired termini. Based on these results, probes for the experiments reported below were prepared with the Stoffel fragment enzyme. Note that lane 6 of the gel and row 6 of the slots (Vent buffer B) provides an example of PCR conditions (in this case it is a different buffer) that, although producing products which appear as a single band, generate a very strong background signal in the IMBP assay. Thus IMBP assays provide an ideal method for monitoring PCR fidelity.

The use of probes in IMBP assays allows accurate, rapid and inexpensive allele genotyping. Allele identification in haploid organisms, e.g., pathogenic bacteria, could, for example, involve
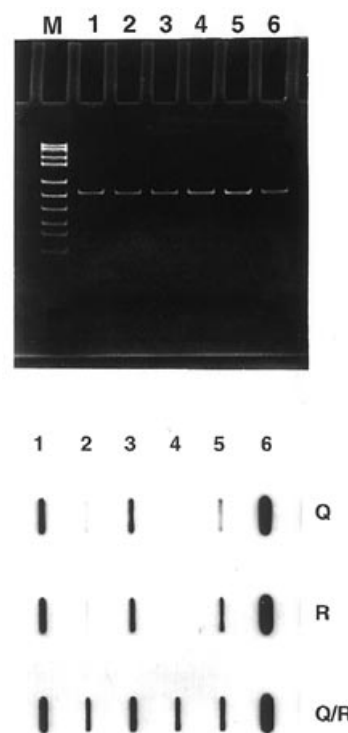

Figure 2. Comparison of five different polymerases in an IMBP assay. PCR was optimized for each enzyme: (i) 12 different buffers of varying pH and Mg^{2+} and KCl concentration were tested with each polymerase for conditions producing single band products on polyacrylamide gels; (ii) following denaturation and reannealing, IMBP assays were run to determine conditions giving the lowest background signal with products of amplification of Q plasmids with primers A (biotinylated) and B (unlabeled) (data not shown). The optimum conditions for each polymerase were used to amplify both Q plasmids, R plasmids and a mixture of Q and R plasmids. (**A**) Polyacrylamide gel electrophoresis. Each lane contains ~100 ng PCR products from the Q plasmid template. The results with products from the R plasmid and the Q and R plasmid mixture are identical (data not shown). Lane M, 1 μg *Msp*I digest of puc18 DNA; lane 1, Ampli-Taq™; lane 2, Stoffel fragment™; lane 3, rTth; lane 4, Pfu™; lane 5, Vent™ (buffer A); lane 6, Vent™ (buffer B). (**B**) Aliquots of 25 ng each PCR product were denatured, reannealed and tested in an IMBP assay. Numbers correspond to gel lane numbers.

an array of probes to determine the strain or species of a given organism or to detect the presence of specific bacterial chromosomal antibiotic resistance mutations (e.g., Rif[R], Nal[R] and Strep[R]) which tend to occur in one of a few neighboring codons (10). In these cases the probes would be perfectly complementary to a particular strain or variant such that the presence of a particular strain or variant would be revealed by a negative signal in the probe array. By using an array of probes such that only one in the array would be negative also provides an internal control to determine the success of PCR without gel electrophoresis. Should all probes give positive signals the test DNA fragment must contain a previously unknown mutation.

A model system used to demonstrate allele identification in haploid organisms is the detection and identification of rifampicin-resistant mutants of *M.tuberculosis*. Since 1984 there has been an increased incidence of TB accompanied by the emergence of multidrug-resistant strains, including strains resistant to rifampicin (Rif), a major component of multidrug regimens used for treating TB. Rif resistance in *M.tuberculosis* is largely
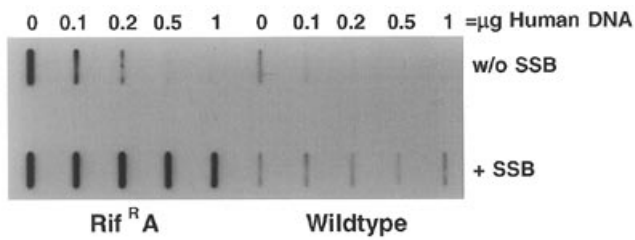
Figure 3. Testing of *M.tuberculosis* isolates with wild-type, Rif[R] A, Rif[R] B and Rif[R] C probes. *Mycobacterium tuberculosis* DNA was amplified with *rpoB*-specific primers to generate probe and test DNA (see Materials and Methods), after which 40 ng probe (3 μl) were mixed with 20 μl test PCR product (100–120 ng). The DNA was denatured and allowed to reanneal, then treated with SSB (see Materials and Methods). Aliquots of 20 μl each sample were then tested with a Genecheck Mutation Detection Kit according to the manufacturer's protocol except that 1 μg MutS was used in each slot. Exposure time of the film was 30 s. The labels across the top of the film indicate the test genotype and the labels down the side indicate the probe genotype.

Figure 4. Detection and identification of *M.tuberculosis* variants with M_2B_2 (Magnetic Mismatch Binding Beads). Samples were prepared as for the nitrocellulose assay. After SSB treatment the samples were tested with M_2B_2 according to the manufacturer's protocol. After color development a 1:10 dilution of sample was prepared in 1 ml H_2O for OD readings. The data are the average of three separate experiments.

Figure 5. Detection of bacterial mutants in the presence of excess human genomic DNA and the effects of pretreatment with SSB. Aliquots of 40 ng biotin-labeled wild-type probe (3 μl) were mixed with 20 μl wild-type or Rif[R] A PCR product amplified in the presence of the indicated amounts of human DNA. The mixtures were denatured and allowed to reanneal, treated with SSB and tested in an IMBP assay. Film exposure time was 30 s.

associated with point mutations in the *rpoB* gene, which codes for the RNA polymerase β subunit. Rif specifically interacts with prokaryotic RNA polymerase to inhibit transcription, which ultimately leads to cell death. Over 30 separate point mutations mapping to 14 codons of the *rpoB* gene have been identified in rifampicin-resistant isolates of *M.tuberculosis* (10–14). The mutations are clustered within a 27 amino acid (81 bp) region of the *rpoB* gene. Reports indicate that >95% of *M.tuberculosis* isolates exhibiting rifampicin resistance harbor mutations within this region.

Four strains of *M.tuberculosis*, three Rif-resistant and one wild-type, were used to test the use of probes in allele identification in haploid organisms. Because of the relatively small genome size of bacteria, probes were prepared directly from genomic DNA rather than from cloned fragments. 5′-Biotin-labeled probes were prepared from all four bacterial strains and used in IMBP assays with test DNA prepared by amplification of bacterial DNA with unlabeled primers. Following amplification the test products were mixed with labeled probes, denatured and allowed to reanneal. The reannealed DNA was then analyzed with an IMBP assay to reveal the presence or absence of mismatches. The experiments were performed with both nitro-cellulose- and magnetic bead (M_2B_2)-based assays. The results are presented in Figures 3 and 4. As expected, negative signals were obtained only when the probe was identical to the test DNA. Similar results were obtained when the *M.tuberculosis* DNA was amplified in the presence of a large excess of human genomic DNA (Fig. 5), which mimics the situation expected when working with clinical samples such as sputum. Note that in the experiment with excess human DNA SSB was used to enhance the signal, which was otherwise reduced in the presence of excess human genomic DNA, presumably from competition for IMBP binding from incompletely annealed human DNA.

IMBP assays may also be used for genotyping diploid organisms and are particularly useful in situations where only a small number of alleles is involved, e.g., hemochromatosis in humans (15) or scrapie susceptibility in sheep. Scrapie is a neurodegenerative disease of sheep that appears to be caused by infectious protein particles (prion protein). Homozygosity for glutamine at codon 171 of the *PrP* gene appears to predispose sheep to natural scrapie and there is a growing body of evidence
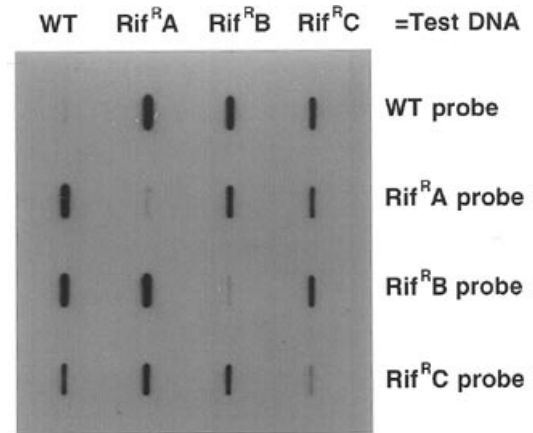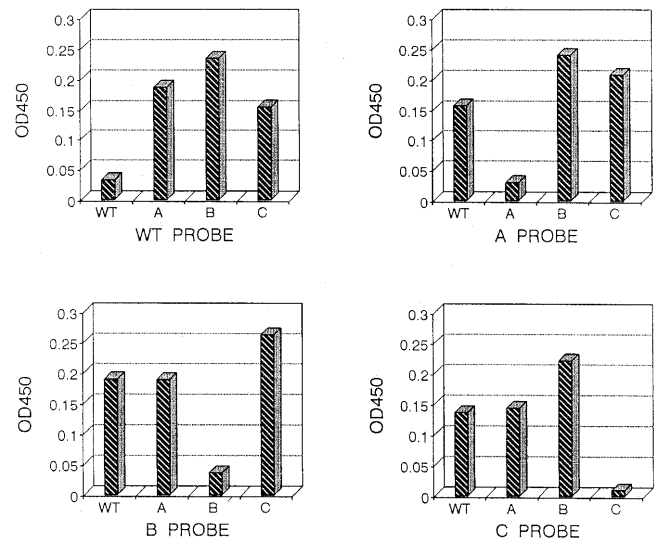
suggesting that arginine at codon 171 (either homozygous or heterozygous) confers resistance to at least some forms of scrapie (16–18).

An IMBP assay has been developed as a method for accurate and rapid genotyping at codon 171. Genomic DNA of known sequence was used to prepare PCR products for cloning fragments containing codon 171 variants. Plasmid DNA from these clones were used as templates for PCR to produce biotin-labeled probes corresponding to either glutamine (CAG-Q) or arginine (CGG-R) at codon 171. Test DNA was produced
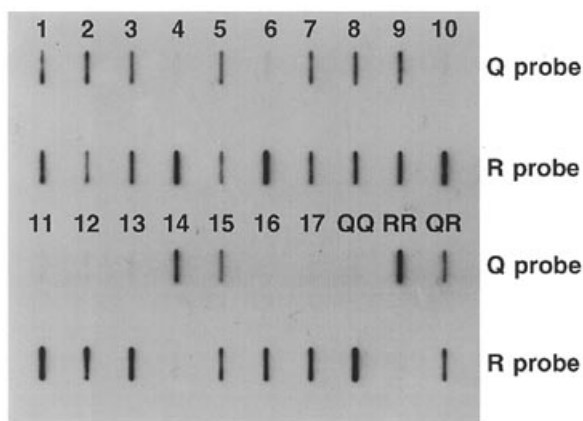
Figure 6. Testing of 17 different sheep (and controls) for genotype at codon 171. Sheep genomic DNA was amplified with *PrP*-specific primers (see Materials and Methods). Samples of 8 ng Q probe (first and third rows) or 8 ng R probe (second and fourth rows) in annealing buffer were mixed with 10 µl test PCR product. The mixtures were denatured and reannealed and tested in an IMBP assay according to the manufacturer's protocol except using 1 µg MutS in each slot. Exposure time for the film was 30 s.

by PCR amplification of sheep genomic DNA using unlabeled primers. As in the *M.tuberculosis* test described above, a negative result indicates that all sequences in the test DNA are identical to the probe sequence; a positive result indicates that there is a sequence difference between at least some of the test DNA molecules and the probe. The results for testing 17 sheep of unknown genotype and three controls of known genotype are presented in Figure 6. When the test DNA was from a known Q/Q homozygote no signal was obtained with the Q probe and a strong signal was obtained with the R probe. When the test DNA was from a known R/R homozygote the results were reversed. When the test DNA was from a known Q/R heterozygote positive signals were obtained with both the Q and R probes. As expected, the signal obtained with Q/R DNA was less than that obtained when the same probe was mixed with an equal amount of test DNA of the opposite homozygous genotype (e.g., R probe with Q/Q DNA) because, in a heterozygous sample, 50% of the probe DNA will anneal to test DNA of the same genotype and will, therefore, not produce any signal in the IMBP assay. In other words, there are 50% as many mismatches in each Q/R + probe mix as in a Q/Q + R probe or R/R + Q probe combination. The results of the unknown samples indicate that samples 4, 6, 10, 11, 16 and 17 are from Q/Q sheep, sample 14 is from an R/R sheep and samples 1–3, 5, 7 and 8 are from Q/R sheep.

The accuracy of the scrapie genotyping test was confirmed with a blind study involving 20 samples (provided by the Animal Research Service, USDA, Pullman, WA). The assay correctly determined the genotype of all 20 samples (data not shown).

The IMBP assay is ideally suited to genotype determination in two allele systems of any diploid organism, including humans. It is possible to design allele identification systems where there are more than two alleles of a given gene by reducing the size of the fragment examined to the point that the fragment becomes a two allele system. Thus a diagnostic array might be produced for genes known to have multiple disease-causing variants, such as *p53*, *BRCA*1 and *CFTR*. The array would consist of primer pairs

designed to amplify multiple regions of the gene in which only one mutation was known to occur. The primers could cover regions in which other small mutations were known to occur and PCR could be multiplexed. Probes could be only of wild-type sequence, to limit the number of test slots required, such that a positive signal would indicate the presence of the mutant allele of that fragment.

One potential limitation of the IMBP assay for allele identification in diploids is its inability to determine genotypes in a heterozygote. In other words, it is possible that those samples judged to be heterozygous might contain a previously unknown allele, in either homozygous or heterozygous form. However, as discussed above, true heterozygotes will produce signals of lesser intensity than homozygote positive signals and of equal intensity for both probes. If the test mutation is homozygous, the presence of a heterozygous unknown mutation or polymorphism will cause both probes to produce signals, but the probe which does not form a mismatched heteroduplex with the test mutation will produce a signal of lesser intensity. A homozygous unknown mutation or polymorphism will produce signals from both probes, but stronger signals than those obtained with test heterozygotes. In addition, the likelihood of interference from unknown mutations or polymorphisms clearly diminishes as the fragment size is reduced.

Thus the IMBP assay can provide a simple, accurate, inexpensive and easily automatable method of allele identification in both diploid and haploid organisms and does not require use of gel electrophoresis, radioactivity or expensive instrumentation. In addition, the ability to do sensitive, low cost assays with extremely high throughput makes IMBP a logical choice for use in human diagnostics, where multiple disease-causing or predisposing mutations are found in multiple regions of a gene (e.g., *BRCA*1 and *BRCA*2). In these applications IMBP can be used to pre-screen fragments in a sequencing-based diagnostic system. Only positives in an IMBP assay, which should be relatively uncommon, will require actual sequencing. (Note: IMBP assays have recently been adapted to a 96-well microtiter plate format; A.Dean and R.Wagner, personal communication.)

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