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A novel method for the parallel analysis of multiple mutations in multiple samples

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In a novel method for analysing mutations, allele specific oligonucleotides (*ASOs*) are synthesised in stripes on the surface of a glass plate and single-stranded ^{35}S labelled RNA probes applied in orthogonal stripes. We have tested the approach using the well studied example of the sickle cell mutation in the human β-globin gene.

Detection of sequence variation in DNA has applications in linkage analysis, in the analysis of inherited diseases, in genetic fingerprinting, and in studies of evolution. As full sequence analysis is time consuming and expensive, several methods have been developed to analyse variation directly. Some are based on the hybridisation of short oligonucleotides to the test sequence (1). One advantage of this approach is that it can detect both the mutant and the wild-type sequence in a single analysis. In others the oligonucleotides may be bound to a solid support and probed with the labelled test sequence (2, 3).

In some applications, such as the analysis of a common mutation as may be the case in the haemoglobinopathies (4), there is a need to analyse many samples with a few oligonucleotides; in others, for example in linkage analysis using *ASOs* instead of RFLPs (5), there is a need to analyse a few samples with many oligonucleotides; and in yet others, for example population screening for mutations in genes such as the *CFTR* gene with many alleles (6), there is need to analyse many samples with many oligonucleotides. This communication describes a versatile approach which can be adapted to any of these applications.

Our method for synthesising oligonucleotides on glass plates (7, 8) was used to produce stripes of *ASOs* 15 nt long for the *A*, *C* and *S* alleles of the β-globin sickle cell locus. The stripes were 2 mm wide and 150 mm long made using the device shown in Figure 1 on 3 mm thick window glass.

Four different single-stranded RNA probes covering the site of the β-globin mutation were prepared as follows: Carrier and patients' DNAs (a gift from Dr J.Old, John Radcliffe Hospital, Oxford) and wild-type control were amplified using a standard PCR procedure (25 cycles; 55°C, 2 min; 72°C, 2 min; 94°C, 2 min; Cetus PCR machine), Figure 2, to give a 162 bp product. The 46 nt upstream primer consisted of 20 nt of β-globin sequence and a 26 nt T7 polymerase promoter clamp at the 5′ end, and the downstream primer of 20 nt of β-globin sequence and a 26 nt SP6 RNA polymerase clamp to allow separate transcription of either the 'sense' or 'anti-sense' strand.

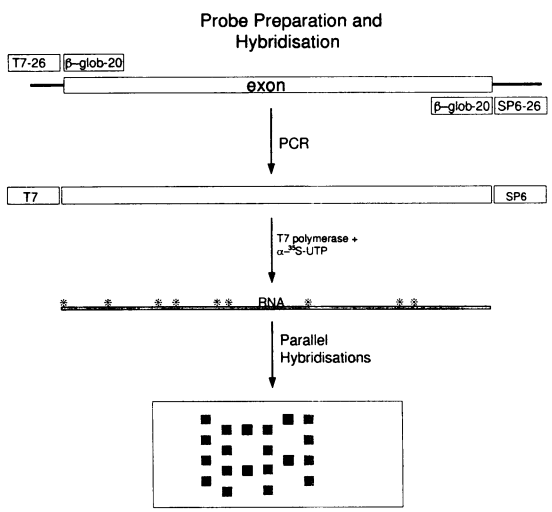

Figure 2. Strategy used to generate a labelled single-stranded RNA probe from genomic DNA. The primers were: upstream primer, TTC TAA TAC GAC TCA CTA TAG GGA GA ACA CAA CTG TGT TCA CTA GC; downstream primer, CTT AAT TAG GTG ACA CTA TAG AAT AG CAA CTT CAT CCA CGT TCA CC. Standard PCR buffer containing 2 mM Mg^{2+} and AmpliTaq polymerase were used.

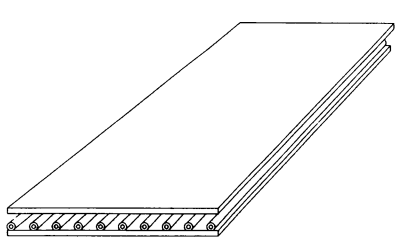

Figure 1. Set-up used to synthesise oligonucleotides in lines on a glass plate.

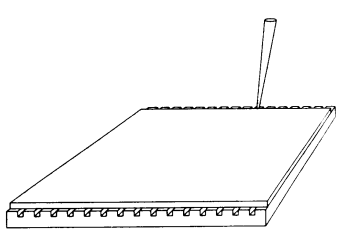

Figure 3. Device used to apply solutions in channels orthogonal to the oligonucleotide lines. Individual channels were 3.5 mm wide.

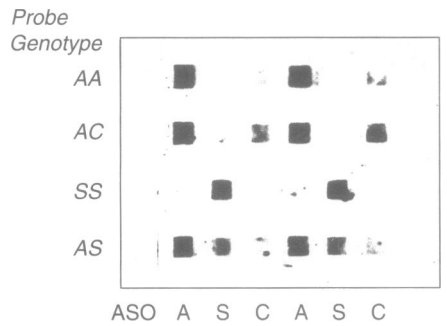

Figure 4. 'Multiplex' analysis of sickle cell mutations. The order of *ASO* lines is ASC ASC, from left to right. Four single-stranded probes were applied in columns perpendicular to the stripes. The determined genotypes are, from top to bottom, *AA*, wild-type control; *AC*, a carrier of the *C* allele; *SS*, sickle-cell patient; *AS*, carrier of the sickle trait.

Transcription was carried out using the Promega Riboprobe kit according to manufacturer's instructions, with 120 μCi α-^{35}S-UTP (Amersham) and no unlabelled UTP, for 30 min at 37°C. The mixture was spun through a Sephadex G-25 STE column. Equal amounts of radioactive transcript were used in each hybridisation.

Several probes can be analysed simultaneously by applying solutions in stripes across the stripes of oligonucleotides on the surface of the microscope slide. The stripes were formed by putting the plate on the device shown in Figure 3, made from plexiglass. The hybridisation solution (10 μl, 0.1 M NaCl in TE pH 7.5, containing 0.2% SDS) was run into the line of contact between the plexiglass and the glass by capillary action. Hybridisation was for 2 hrs at room temperature. The plate was rinsed in 0.1 M NaCl, eluted at 43°C for 10 min, and exposed to a PhosphorImager storage phosphor screen overnight, scanned on a Molecular Dynamics PhosphorImager and printed (Figure 4). The results for all individuals are clear and as expected.

The method has several advantages over alternatives. It allows for multiple comparisons to be carried out in a single simple experiment. The number of oligonucleotides that could be synthesised and the number of probes analysed are determined by the size of the glass plate and the width of the stripes. On a 200 mm×200 mm plate it should be possible to synthesise 100 different sequences and test probes from 50 different individuals. This level of highly parallel analysis is potentially a lot higher than the method introduced by Erlich *et al.* (9) which involves the hybridisation of one PCR product at a time to oligonucleotides UV crosslinked to strips of membrane.

The manipulations are simple to carry out manually, although the method would lend itself to automation. The analysis is rapid; we have carried out a complete procedure, starting with genomic DNA to having the final result in less than a working day. It is versatile and can be applied to any locus for which there is sufficient information to produce oligonucleotides for test and amplification, e.g. cystic fibrosis testing or scanning for mutations in the *p53* gene. The glass plates, unlike filters, are stable; we have reused them more than thirty times with no loss of performance. The interpretation of the result is straightforward and can readily be automated and quantified.

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