

# Preimplantation diagnosis of autosomal dominant retinitis pigmentosum using two simultaneous single cell assays for a point mutation in the rhodopsin gene

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**A couple requested preimplantation genetic analysis for a dominant form of retinitis pigmentosum caused by a C→A transversion in the rhodopsin gene. Since this point mutation does not alter a restriction endonuclease site we designed two separate analytical systems, one involving site-specific mutagenesis and the other involving allele-dependent length polymorphism. After establishing the accuracy and robustness of these assay systems we utilized both systems simultaneously in a heminested polymerase chain reaction (PCR) system. This allowed accurate preimplantation diagnosis to be performed. One embryo was transferred but a pregnancy did not occur.**

*Key words:* preimplantation genetics/retinitis pigmentosum

## Introduction

Preimplantation genetic diagnosis (PGD) was developed for couples at high genetic risk to avoid affected pregnancies and the potential for pregnancy termination following prenatal diagnosis. The first successful application of PGD involved removal of one cell from 5–8-cell embryos for gender determination for women who were carriers of X-linked diseases. This allowed transfer of female embryos who could not be affected with an X-linked disorder (Handyside *et al.*, 1990; Verlinsky *et al.*, 1996).

Specific mutation analysis by PGD was successfully performed to screen oocytes and pre-embryos for cystic fibrosis (CF) (Strom *et al.*, 1990; Handyside *et al.*, 1992). Our centre has been involved in developing PGD using analysis of polar bodies (PB) in addition to embryo biopsy (Strom *et al.*, 1990, 1992, 1994; Rechitsky *et al.*, 1996). The first polar body (PB1) is extruded following meiosis I. There are three genetic possibilities for the first polar body from a heterozygous woman. If no crossing over occurs, PB1 will be homozygous (either normal or affected), but in the event of a crossover PB1 will be heterozygous. If crossover does not occur and PB1 is homozygous for the mutant gene, the oocyte must contain two copies of the normal gene and any embryo developing from this oocyte can be transferred without risk of an affected child. If the PB1 is homozygous for the normal gene, the maternal contribution to any embryo must be the mutant gene. In the event that crossover occurs, the PB1 will be heterozygous and analysis of either the second polar body (PB2) or a blastomere is required to predict which maternal allele will be present in the mature oocyte.

Retinitis pigmentosum (RP) is a heterogeneous genetic disease causing progressive vision loss. A point mutation in the rhodopsin gene on chromosome 7 has been demonstrated

to cause many cases of autosomal dominant RP (Nathans and Hogness, 1984; Dryja *et al.*, 1990). A couple presented to our centre with a family history of the dominant form of RP due to this point mutation. The woman was affected and was suffering from deteriorating vision. They requested preimplantation genetic diagnosis (PGD).

PGD for Mendelian traits requires amplification of the gene using nested polymerase chain reaction (PCR). Since the mutation does not alter a restriction endonuclease site, we developed and tested two separate assays using published techniques for this point mutation. One method, site-specific mutagenesis (SSM), involves synthesis of PCR primers creating an artificial restriction site. The second method called allele-dependent length polymorphism (ADLP), employs allele-specific primers for the mutant and normal sequence in which tails of different lengths are added to each primer. Both assays could successfully genotype single fibroblasts, but sometimes one assay would give clearer differentiation between the two alleles than the other.

Both assays use nested PCR with identical conditions for the first round of PCR. Thus we were able to perform both assays simultaneously on the same single cell in order to increase the accuracy of diagnosis.

## Materials and methods

### Patients

A couple requested PGD for a dominant form of RP. The woman was affected as was her father. Previous analysis had established that she was heterozygous for the C→A transversion in codon 23 of the rhodopsin gene demonstrated to be associated with RP by Dryja *et al.* (1990). Family studies performed by another centre had confirmed that the disease segregated with this point mutation.

All protocols and consent forms were approved by the Institutional

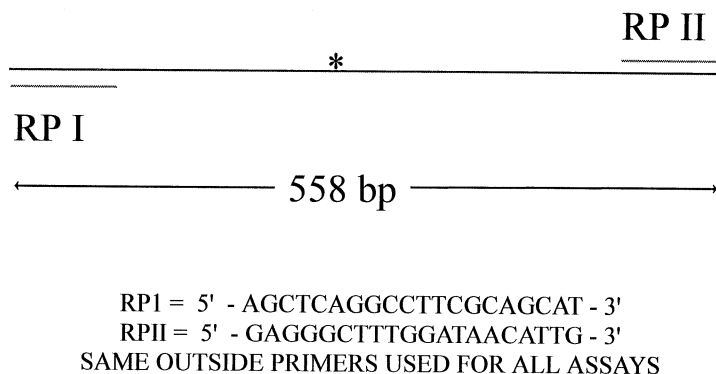


Figure 1. Diagram of outside primers for retinitis pigmentosum assays.

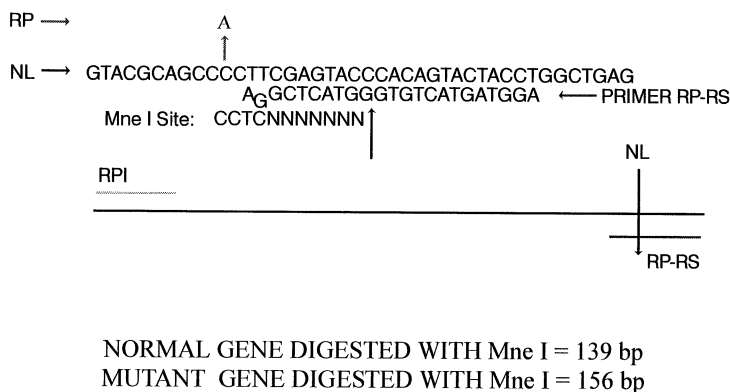


Figure 2. Diagram of site-specific mutagenesis retinitis pigmentosum assay. NL = normal (unaffected allele).

Review Board of Illinois Masonic Medical Center, IL, USA. Micro-manipulation and in-vitro fertilization (IVF) were performed as described in detail elsewhere (Verlinsky *et al.*, 1992, 1996; Strom *et al.*, 1994).

### Polymerase chain reaction

Three pairs of primer sets were designed to assay codon 23 of the rhodopsin gene using the published sequence of the rhodopsin gene (Nathans and Hogness, 1984). RP-1 and RP-2 are the outside primers and amplify a 558 bp region including codon 23 (see Figure 1). The sequence of RP-1 is (5'-3') AGCTCAGGCCTTCGCAGCAT and RP-2 is (5'-3') GAGGGCTTTGGATAACATTG.

Following the first round of PCR, two different assays were developed for the C→A transversion, one using SSM and a second using ADLP.

### Site-specific mutagenesis

The RP and normal rhodopsin gene sequences surrounding codon 23 are shown in Figure 2. Unfortunately, no restriction endonuclease site is created or destroyed by the mutation. We designed a primer (RP-RS) with a single alteration from the normal sequence (an A→G transversion in the penultimate 5' nucleotide) that creates a restriction site for the enzyme *Mne*I. The RP allele lacks the *Mne*I site. This primer design is shown in Figure 1. Following 20 rounds of amplification with the outside primers, 10 µl of a 100 µl reaction mixture were added to fresh buffer and the inside primer RP-RS was added along with RP1 to form a heminested PCR system. An additional 20 rounds of amplification were performed and the resulting PCR products were subjected to a 1-3 h restriction digestion with *Mne*I. The normal allele yielded a 139 bp band and the RP allele yielded a 156 bp band. These bands could be distinguished easily on ethidium bromide-stained polyacrylamide gels.

### Allele-dependent length polymorphism

Following the same first round of amplification, a second 10 µl aliquot was removed and subjected to ADLP analysis. The primers for this analysis are shown in Figure 3. In ADLP, two allele-specific primers were designed with the most 5' nucleotide corresponding to the transversion site. The RP specific primer has the identical sequence to the RP gene and the normal primer has the exact sequence of the normal allele. A run of non-specific nucleotides is added to the 3' ends of both primers. The lengths of the runs differ for each primer. In our design the RP primer (RP-RM) has a 20 bp tail and the normal primer has a 35 bp tail. This is illustrated in Figure 3.

Thus priming of the normal gene yields a 147 bp product whereas priming of the RP gene gives a 132 bp product. These could be distinguished easily on polyacrylamide gels.

### Results

A skin biopsy was taken from the affected woman and cultures of skin fibroblasts established. In a blinded fashion, single fibroblasts from this patient or a normal control were harvested by micro manipulation and analysed by the molecular laboratory. In all, 177 cells were analysed using the heminested simultaneous ADLP and SSM assays. Table I shows a summary of these analyses.

There were no diagnostic errors in this series. It is important to note that allele drop out (ADO), a phenomenon in single cell analysis where only one allele is amplified in a heterozygous cell was not seen. As others have noted, ADO is less common in fibroblast analysis than in polar body and blastomere analysis. In all cases where amplification failure occurred,

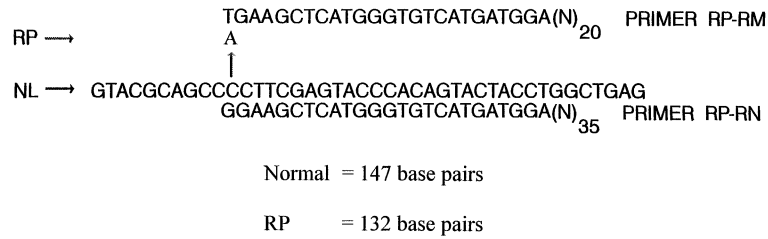


Figure 3. Diagram of allele-dependent length polymorphism retinitis pigmentosum assay.

Table I. Analysis of normal and retinitis pigmentosum (RP) single fibroblasts by polymerase chain reaction (PCR). Figures in parentheses are percentages

Cell type	No. analysed	No. amplified	Genotype R/N	Genotype N/N
RP/N	92	73 (79)	73 (100)	0
N/N	85	68 (80)	0	68 (100)

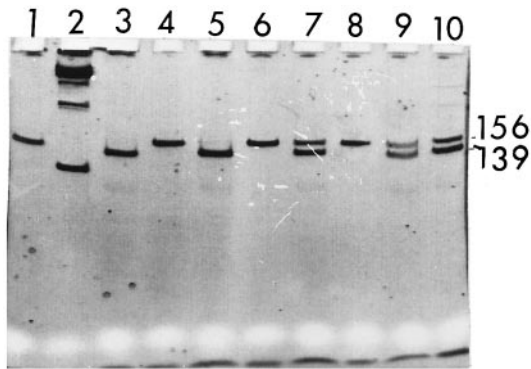


Figure 4. Preimplantation genetic diagnosis for retinitis pigmentosum (RP) using site-specific mutagenesis assay of first polar bodies. Ethidium bromide stained polyacrylamide gels. 156 bp band following *Mne*I digestion = RP allele; 139 bp band following *Mne*I digestion = unaffected allele. Lane 1: normal control, undigested; lane 2: size standard; lane 3: normal control, digested (N/N); lane 4: polar body # 3, undigested; lane 5: polar body # 3, digested (N/N); lane 6: polar body # 1, undigested; lane 7: polar body # 1, digested (RP/N); lane 8: heterozygous control, undigested; lane 9: heterozygous control, digested (RP/N); lane 10: overnight digestion of lane 7 (RP/N).

both the ADLP and SSM assays failed to yield amplified product. This observation is not unexpected as the same first round of PCR is used for both reactions prior to aliquotting the samples prior to the second round of amplification.

Another interesting observation was made during this series. For some experiments the ADLP analysis gave clearer results than the SSM analyses and for other experiments the converse was true, although there were no instances when the two methods gave conflicting results.

Confident that our system was sufficiently robust and accurate, we proceeded to perform a PGD analysis for this couple. Following stimulation, 11 oocytes were aspirated and nine first polar bodies were successfully removed for amplification. The remaining two oocytes were fragmented and not suitable for insemination or analysis.

Figures 4 and 5 show the results of the SSM assay for this case. Figure 4, lanes 1 and 3, represents a single fibroblast control known to be homozygous unaffected (N/N). Lane 1 is

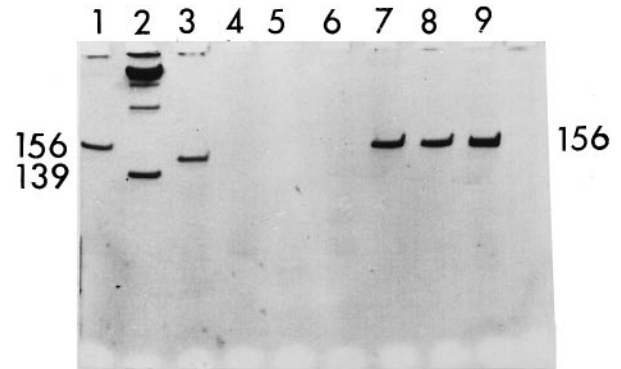
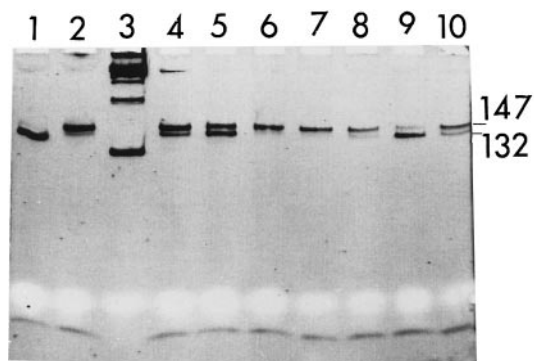


Figure 5. Preimplantation genetic diagnosis for retinitis pigmentosum (RP) using site-specific mutagenesis assay of first polar bodies (continued) Ethidium bromide-stained polyacrylamide gels. 156 bp band following *Mne*I digestion = RP allele; 139 bp band following *Mne*I digestion = unaffected allele. Lane 1: normal control, undigested; lane 2: size standard; lane 3: normal control, digested (N/N); lane 4: negative control; lane 5: negative control; lane 6: negative control; lane 7: polar body # 8, undigested; lane 8: polar body # 8, digested (RP/RP); lane 9: overnight digestion of lane 8.

the predigestion PCR products showing the expected 156 bp band. Following *Mne*I digestion (lane 3) all the material was digested to the smaller 139 bp products confirming that the cell is homozygous N/N. There is a very faint band at a lower molecular weight representing the 17 bp cleavage fragment. This can be seen because all the primers have been used up in the single cell PCR reaction leaving no primer or primer-dimer artefact visible on the gel. The absence of this band in the undigested lanes (1, 4, 6, 8) confirms this observation.

Lanes 8 and 9 are the undigested and digested PCR products respectively of a known heterozygous cell. The presence of two clear bands, an undigested one at 156 bp and the digested one at 139 bp, confirms the N/RP genotype. Lanes 4 and 5 show the analysis of polar body number 3. The complete digestion of the PCR products to the 139 bp bands establishes the genotype as N/N. Lanes 6 and 7 represent the analysis of polar body number 1 and reveals a heterozygous RP/N genotype for this polar body.

Figure 5 contains negative 'false biopsy' controls in lanes 4, 5, and 6 showing the lack of contamination. These tubes contain 1–2 µl of fluid used to bathe the oocyte during the biopsy process. Lanes 7 and 8 are the analyses of polar body number 8 demonstrating no digestion and therefore a genotype of RP/RP. Lane 9 is an overnight digestion confirming the lack of digestion shown in the shorter incubation in lane 8. Lanes 1 and 3 are undigested and digested PCR products of normal control DNA showing complete restriction digestion.



**Figure 6.** Preimplantation genetic diagnosis for retinitis pigmentosum (RP) using allele-dependent length polymorphism assay of first polar bodies. Ethidium bromide-stained polyacrylamide gels. 147 bp band = unaffected allele; 132 bp band = RP allele. Lane 1: polar body # 8 (*RP/RP*); lane 2: polar body # 3 (*N/N*); lane 3: size standard; lane 4: polar body # 1 (*RP/N*); lane 5: control heterozygote (*RP/N*); lane 6: polar body # 6 (*N/N*); lane 7: polar body # 9 (*N/N*); lane 8: polar body # 11 (*N/N?*); lane 9: polar body # 5 (*RP/RP*); lane 10: heterozygous control (*RP/N*).

Figure 6 represents the ADLP analysis in this case. On this gel the polar body number 8 is in lane 1 and shows a single band at 132 bp indicating a *RP/RP* genotype. Lane 2 is polar body number 3 showing a single 147 bp band indicating a genotype of *N/N*. Lane 4 is polar body number 1 and lane 5 is a heterozygous control. Both lanes show two bands indicating an *RP/N* genotype. Lanes 6 and 7 display unambiguous results with a single band at 147 bp establishing a genotype of *N/N* for polar bodies 6 and 9. However, lanes 8–10 show ambiguous results using ADLP. In lane 8 (polar body # 11) there is an intense band at 147 bp and a faint band at 132 bp. We interpreted this as probably indicative of *N/N* genotype with a potential to be *RP/N*. The SSM analysis demonstrated that this cell was clearly homozygous *N/N* and the faint extra band was most likely due to non-specific priming (data not shown). Since all false biopsies and reagent controls were negative these faint bands are unlikely to be due to contamination. Due to the unambiguous results from the same polar bodies from the SSP analysis we were confident in our determinations of the genotypes of these polar bodies.

Similarly, polar body number 5 shown in lane 9 has a dark band at 132 bp and a faint band at 147 bp while the SSM assay revealed the cell to be homozygous affected. Lane 10 is a known heterozygous control but the 147 bp band is clearly darker than the 132 bp band. Thus, in this particular analysis the SSM was more easily interpreted than the ADLP. However, this is not always the case.

Table II is a summary of the PGD analysis in this case. Two oocytes had a first polar body homozygous for the RP mutation meaning that the oocyte contained the normal allele. Only one of these oocytes fertilized and was transferred. A pregnancy did not develop. The unfertilized oocyte was analysed but did not reveal any amplification product.

Four oocytes had homozygous normal first polar bodies predicting that the oocyte would contain the affected gene. Since RP is a dominant disease in this family, any embryos resulting from these eggs would be affected. The four resulting

**Table II.** Summary of genotype analysis in preimplantation genetic diagnosis for retinitis pigmentosum (RP)

Oocyte no.	PB genotype	Embryo genotype (#)	Oocyte genotype
1	<i>RP/N</i>	<i>N/N</i> (1) No Amp (1)	
2	No Amp		
3	<i>N/N</i>	<i>RP/N</i> (7) No Amp (1)	
5	<i>R/R</i>		No Amp
6	<i>N/N</i>	No Amp.	
7	No Amp		
8	<i>R/R</i>	Transferred	
9	<i>N/N</i>	<i>RP/N</i> (4) No Amp (2)	
11	<i>N/N</i>	<i>RP/N</i> (3) No Amp (4)	
Neg. Controls = 8		No Amp = 8	

embryos were dissociated into individual blastomeres and analysed to confirm the diagnosis. In all, 14 blastomeres from three embryos were successfully analysed and the affected status was confirmed in all cases.

## Discussion

Point mutations that do not alter a restriction site provide challenges to accurate genotyping, especially in the context of single cell analysis. Several techniques such as allele-specific oligonucleotide hybridization, ADLP (initially called ARMS), SSM, and ligation PCR can be used in such situations. These assays, when used alone, can yield ambiguous results because of the sensitivity of each system to minor changes in reaction conditions. We specifically chose ADLP and SSP because they involved different mechanisms of specificity with ADLP relying on allele specific priming and SSP using the same priming sequences but with a primer mismatch. We felt that for any given reaction, if one assay failed, the other would succeed. Additional independent assays, such as SnuPE or SSCP could also have been used. We chose SSP and ADLP because we had the most experience with such assays, but there is no reason why all four assays could not be used simultaneously. Another potential method for increasing accuracy would be to perform replicate amplifications in the second round. This could potentially further reduce any ambiguity of results. However, we felt that the two assays, as currently performed, yielded accurate genotyping of single cells without the additional labour and time that these amendments would entail.

This report demonstrates the successful application of a new technique for PGD of Mendelian disorders caused by point mutations that do not alter restriction sites. The simultaneous use of two assays using the same outside primer pair allows genotyping to be made with confidence, especially when using assays that rely on allele-specific priming or SSM. This technique, however, does not prevent or detect allele drop out. Another multiplexed outside primer pair looking at a linked polymorphism would have the ability to detect this phenomenon.

The ADLP assay sometimes gave ambiguous results apparently due to non-specific priming. The SSM assay sometimes

yielded insufficient PCR product for unambiguous genotyping presumably due to inefficient priming by the mismatched primer. Since the two assays fail for different reasons, in any single analysis, at least one assay should succeed.

In the case of an autosomal dominant disease when the female has the affected gene, polar body diagnosis is far preferable to blastomere biopsy. We have observed a 3-fold higher rate of ADO in blastomeres than in polar bodies (unpublished data). Ray *et al.* (1996) and Ray and Handyside (1996) have also observed high rates of ADO in blastomere analysis for cystic fibrosis even after conditions have been optimized. If ADO occurs following a blastomere biopsy in a dominant disease, an affected embryo could be misdiagnosed as unaffected. This situation is analogous to performing blastomere biopsy for a recessive disease when the parents have different mutations. In such cases, ADO has led to misdiagnoses in three cases of cystic fibrosis (Handyside and Delhanty, 1996; Lissens and Sermon, 1997). An excellent review of this issue can be found in Lissens and Sermon (1997). Therefore we do not offer PGD by blastomere analysis for dominant diseases or for recessive diseases (both autosomal and X-linked) when the parents have different mutations.

We feel that the use of two assays increases the accuracy of single cell genetic diagnosis, but could also be used in standard genotyping applications such as prenatal diagnosis, diagnostic testing and carrier detection.

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