Same day diagnosis of Down’s syndrome and sex in single cells using multiplex fluorescent PCR

I Findlay, P Matthews, T Tóth, P Quirke, Z Papp

Abstract
The major reason for prenatal diagnosis lies in the detection of trisomies, particularly trisomy 21 (Down’s syndrome). Current techniques require lengthy laboratory procedures and high costs. Furthermore, diagnosis is often not possible if the sample is of small size or is contaminated. An alternative method, quantitative fluorescent polymerase chain reaction (PCR) of short tandem repeats (STRs), can also be used to diagnose trisomies and it has the advantage that a result is obtained within five to eight hours. However, this method is currently limited to relatively large amounts of sample, which restricts diagnostic confidence and value. Recently, genetic diagnosis using fluorescent PCR has been applied at the single cell level but is limited to sex or single gene defect diagnosis. This study, using quantitative multiplex fluorescent PCR, provides for the first time simultaneous diagnosis and confirmation of sex and trisomy in single cells. Two markers for chromosome 21 increase diagnostic confidence, informativeness, and confirmation. This system is rapid (five hours), reliable, and accurate and we believe that it will be more cost effective than alternative methods. The technique has direct application to preimplantation genetic diagnosis, early prenatal diagnosis, and other diagnostic systems where sample size is limited.

Keywords: Down’s syndrome; trisomy; prenatal diagnosis; multiplex fluorescent polymerase chain reaction; short tandem repeats

Chromosomal abnormalities are the most frequent genetic disorders seen in both newborn babies and miscarriages. Trisomies, the most frequent chromosomal disorder, account for ~53% of all chromosomal abnormalities in early fetal deaths. The most frequent trisomy is Down’s syndrome (trisomy 21), which occurs in about one in 600 newborns, and is a major reason for prenatal diagnosis. Such diagnosis is usually performed by means of karyotyping and depends on analysis at 11–18 weeks of gestation. As well as high costs (approximately £150 for karyotyping), the lengthy culture procedure results in a significant delay (generally until 13–20 weeks of gestation) until the diagnosis can be made. This results in either a long delay for the mother before reassurance about a healthy pregnancy or the prospect of a late termination in the second trimester, which is both emotionally and physically traumatic. Second trimester terminations require surgery, rather than medical treatment, which exposes the patient to an increased risk of mortality.

In addition, karyotyping is not always possible, especially when the numbers of cells obtained are limited, where cell culture fails (in 1–2% of patients), or when the culture is contaminated. Maternal contamination rates of up to 10–14% have been reported even in the most experienced laboratories. In these cases, diagnosis is extremely difficult if not impossible, even with molecular genetic techniques such as the polymerase chain reaction (PCR) or fluorescent in situ hybridisation (FISH).

Therefore, an alternative method providing rapid (same day) diagnosis of small numbers of cells would be extremely valuable. One such technique, which is both rapid and inexpensive, is the quantitative fluorescent PCR (F-PCR) amplification of short tandem repeats (STRs). This method, using STRs specific for chromosome 21, has previously been used to diagnose trisomy 21. The quantitative nature of this technique allows the amount of PCR product to be determined and thus the amount of each PCR product from each allele to be compared. This allows the amount of one allele in relation to the other to be calculated.

Although this method was first described by Mansfield in 1993, there have been only a few reports applying the technique clinically to trisomy detection in prenatal diagnosis. This has been mainly the result of the relatively high numbers of cells required and the relatively low amount of information produced by such a diagnosis when using a single STR marker. Although an F-PCR system was used recently by Perl et al on amniotic fluid, this method was subsequently shown to be suboptimal because of the primers used.

An alternative multiplex F-PCR method has been used previously for genetic diagnosis of sex and single gene defects in single cells, and this method was adapted for trisomy detection. This system can also be used to determine the origin of the extra chromosome and, if maternally derived, whether the extra chromosome is derived from meiosis I or meiosis II.

Materials and methods
 Thirty five single dissociated cells from four known trisomy 21 fetal livers and 18 single dissociated cells from two known disomic fetal livers were analysed.
Cell lysis was not necessary for single cell samples. Three fluorescent PCR primers were used in each PCR, one primer for sexing and two for trisomy 21 detection. The amelogenin gene, used previously for single cell sexing, and two primer sets, D21S11 and D21S167, were used for trisomy 21 detection. Heterozygosity rates of the D21S11 and D21S167 STRs are 89% and 82%, respectively. Details of the primers are given in Table 1.

The PCRs were performed in 25 µl reaction volumes using 1× PCR buffer (reaction buffer IV; Advanced Biotechnologies Ltd, Leatherhead, Surrey, UK), 200 µM of each dNTP (MBI, Lithuania), 1.5 mM of MgCl₂, 0.6 U of Taq polymerase (Thermoprime Plus; Advanced Biotechnologies Ltd). For the D21 multiplex, primers were added such that the final amount of each primer in each PCR tube was 3 pmol of amelogenin, 15 pmoles of D21S11, and 3 pmoles of D21S167. A 25 µl aliquot was added to each sample undergoing the PCR and mixed well. The PCR comprised an initial denaturation step of 95°C for five minutes, followed by 41 cycles of 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, then a final extension step of 72°C for 10 minutes. The PCR products were stored at 4°C until analysis.

DNA was analysed by the method reported by Findlay and Quirke, except that samples were not co-loaded and only ROX 350 was used as the internal standard. Samples that gave an overamplified response were run again at a 10% dilution.

Trisomy was defined as either a triallelic signal or cell ratio (amount of PCR product from the first allele divided by the product from the second allele, where ratios >4 or <0.25 in three cases were disregarded as a result of extreme preferential amplification) of <0.7 or >1.3. Disomy was defined as a ratio between 0.7–1.3. Where triallelic signals were not seen, the aggregated ratios for three or more cells were used. Preferential amplification is an important consideration in trisomy diagnosis using double dose responses because overamplification of one allele might result in misdiagnosis, either because of enhanced amplification of a smaller allele falsely indicating disomy status or decreased amplification in a disomic allele falsely indicating trisomy status.

All cells were run “blind”—the operator did not know the trisomy status until all results were completed and tabulated. Trisomy and sex results were then obtained from karyotype data.

Results

One or more D21 markers were seen in all of the trisomic and disomic cells (35 of 35 and 19 of 19, respectively). Both D21 markers were used.

### Table 1 Primer details

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Fluorescent label</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUMAMGXA</td>
<td>Xp22.1–p22.3</td>
<td>AMEL A</td>
<td>CCCTGGGCTCTGTAAAGAATAGTG</td>
<td>FAM</td>
</tr>
<tr>
<td>HUMAMGY</td>
<td>Yp11.2</td>
<td>AMEL B</td>
<td>ATCAGAGCTTAAACTGGGAAGCTG</td>
<td></td>
</tr>
<tr>
<td>D21S167</td>
<td>21q22.2</td>
<td>D21S167 1</td>
<td>TGCCCTGAGCACTGGTG</td>
<td>HEX</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D21S167 2</td>
<td>TCC TTC CAT GTA CTC TGCA</td>
<td></td>
</tr>
<tr>
<td>D21S11</td>
<td>21q21</td>
<td>D21S11 1</td>
<td>ATATGTGAGTCAATTCCCAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D21S11 2</td>
<td>TGT ATT AGT CAA TGT TCT CCA G</td>
<td>FAM</td>
</tr>
</tbody>
</table>

Figure 1  Double dose is determined by comparing peak areas (rather than peak size) of each allele. This figure shows trisomy diagnosis at the single cell level using multiple trisomic markers. The sex of the cell is also shown. The D21S167 marker indicates trisomy status by having double dose results (1:2 ratio). Trisomy status is confirmed by the D21S11 marker, which has a triallelic signal.

Figure 2  All calculations were based on peak areas, which were estimated automatically as a measure of product yield by the Genescan software. This figure shows that a range of efficiency is seen for each allele. The trend bar indicates that the graph is similar to a normal distribution curve and that most of the ratios are between 40% and 60%.
seen in 26 of 35 of the trisomic samples and 15 of 19 of the disomic samples. In all cases, the STR results confirmed the known karyotype and there were no cases where one STR indicated trisomy and the other disomy. Figure 1 shows trisomy diagnosis at the single cell level using two trisomic markers. The need for two or more chromosome 21 markers was demonstrated because preferential amplification was seen in both STRs (fig 2).

The possibility of multiplexing several STRs on chromosome 21 in the same PCR provides:

- Increased accuracy. If allelic dropout occurs in one STR, then the other STR should provide a result. Allelic dropout, where one allele fails to amplify, is an important problem in single cell analysis and can result in misdiagnosis. Allelic dropout occurs at a rate of 5–10%, depending on the primer used. However, when using two STRs, the possibility of allelic dropout should be reduced from 10% to 1% (10% of 10%) for both STRs.
- Confirming the diagnosis. If both STRs give concordant results, the confidence that the result is correct will be increased.

However, for multiplexing to succeed multiple cells from the same sample must be used when triallelic results are not obtained. This increases time and cost and reduces the number of individual tests possible from one sample. However, these negative aspects are compensated for by the fact that concordant results confirm the diagnosis.

If the cell is known to be heterozygous, perhaps by previous testing of the parents in preimplantation genetic diagnosis or prenatal diagnosis, then the usefulness of trisomy diagnosis will increase. For example, if the mother is known to be heterozygous for both D21S11 and D21S167, the trisomy detection rates for triallelic results will increase from 68% to 75%, and by double dose from 23% to 25%. Uninformative results will decrease from 10% to 0%. This demonstrates the importance of selecting STRs with as high a heterozygosity rate as possible and the need for multiple markers.

This technique has important applications in conventional prenatal diagnosis and preimplantation diagnosis of genetic disease. In addition, it has potential for use in the diagnosis of trisomies from fetal cells in the maternal circulation.

Table 2: D21S167 and D21S11 short tandem repeat amplification ratios in single cells

<table>
<thead>
<tr>
<th>Sample number (number of cells)</th>
<th>D21S167 ratio range</th>
<th>D21S11 ratio range</th>
<th>Trisomy status by PCR</th>
<th>Sex by PCR</th>
<th>Trisomy status by karyotyping</th>
<th>Sex by karyotyping</th>
</tr>
</thead>
<tbody>
<tr>
<td>203 (8)</td>
<td>1.06-2.43</td>
<td>0.86-2.71</td>
<td>Disomic Female</td>
<td>Male</td>
<td>Trisomic Female</td>
<td>Male</td>
</tr>
<tr>
<td>211 (40)</td>
<td>1.11-3.5</td>
<td>1.40</td>
<td>Disomic Male</td>
<td>Male</td>
<td>Trisomic Male</td>
<td>Male</td>
</tr>
<tr>
<td>204 (7)</td>
<td>1.21-3.14</td>
<td>1.61</td>
<td>Disomic Female</td>
<td>Female</td>
<td>Trisomic Female</td>
<td>Female</td>
</tr>
<tr>
<td>133 (10)</td>
<td>0.64-2.1</td>
<td>3.5</td>
<td>Trisomic Male</td>
<td>Male</td>
<td>Trisomic Male</td>
<td>Male</td>
</tr>
<tr>
<td>201 (9)</td>
<td>0.86-3.5</td>
<td>1.40</td>
<td>Trisomic Male</td>
<td>Male</td>
<td>Trisomic Male</td>
<td>Male</td>
</tr>
<tr>
<td>124 (9)</td>
<td>1.34-3.78</td>
<td>1.82</td>
<td>Trisomic Female</td>
<td>Female</td>
<td>Trisomic Female</td>
<td>Female</td>
</tr>
</tbody>
</table>

Discussion

This technique is rapid and has high reliability, accuracy, and increased confidence for single cell trisomy 21 detection because it can provide simultaneous confirmation. This system has several significant advantages over conventional karyotyping for trisomies:

- Time taken for diagnosis. Diagnosis can be obtained within five to six hours rather than two weeks. This same day diagnosis would allow rapid patient reassurance or pregnancy termination if required.
- Reduced costs. The costs of karyotyping (£150–200/sample) are significantly higher than for multiplex fluorescent PCR (approximately £30/sample). Although fluorescent PCR does require an intial outlay of approximately £50 000 to purchase the DNA sequencer, it allows a wide range of diagnoses to be undertaken.
- Number of cells required. Karyotyping needs very high numbers of cells (thousands) for reliable results, whereas fluorescent PCR requires as few as three cells (or even single cells). Results can be obtained even when conventional methods have failed.
- The effects of contamination are reduced. Because these analyses are performed on single cells, results can be obtained even if the sample is heavily contaminated with maternal or other cells.


