7/98 **Applications** No. 12

PCR NO. 1

The use of the *Mastercycler® gradient* for single-cell PCR and preimplantation diagnosis

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Introduction
Preimplantation diagnosis (PGD) is a very early form of prenatal diagnosis: embryos obtained *in vitro* from couples at risk of contracting genetic disease are analyzed for the presence or absence of this disease and only the embryos which are shown to be free of the disease under consideration are transferred to the uterus. The PCR* (Polymerase Chain Reaction) has been the only tool available for diagnosing monogenetic diseases at the DNA level in single cells. At our centre, we have developed PGD protocols for numerous diseases, including cystic fibrosis (CF) where primarily the ΔF508 mutation was analyzed, Duchenne's muscular dystrophy (DMD) where deletions in the region of exon 45 were analyzed and myotonic dystrophy (DM) where the disease-causing repeat was examined. All these assays were developed on Perkin-Elmer-type PCR machines. I evaluated the possibilities of transposing our PCR protocols developed for the Perkin Elmer GeneAmp PCR System 9600 and 2400 to the Mastercycler® gradient from Eppendorf.

* The PCR (Polymerase Chain Reaction) is protected by patent. The patent is held by Hoffmann-La Roche.

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Materials and Methods

As a source of single cells, Lymphoblasts transformed with the Epstein-Barr-virus were used. The cells are a carrier of the ΔF508 mutation, a female heterozygous for the CA repeat in intron 45 of the DMD gene and an individual heterozygous for the DM-repeat (5 and 12 repeats) respectively. The cells were washed three times with PBS, after which individual cells were washed three times in 2-µl drops of Ca^{2+}- and Mg^{2+}- free medium and transferred to 200 µl PCR tubes (Eppendorf PCR tubes) containing 2.5 or 5 µl alkaline lysis buffer (ALB; 50 mM DTT and 200 mM KOH for CF and DMD or NaOH for DM).

Three µl from the first PCR were taken as a template in the second PCR. The reaction mix had the same final concentrations and volume as the first PCR. The PCR products were run on a horizontal 2 % agarose gel to check for amplification and contamination (figure 1), after which the samples showing amplification were separated on a vertical 4 % Metaphor Agarose gel (Sanver Tech, Boechout, Belgium), run at 350 V for 90 min (Figure 2). Primer sequences and PCR programmes are summarized in Table 1. The DMD sequence was amplified in a similar way to the CF sequence (Figure 3).

Table 1: Summary of primers and programmes used for the different PCRs

Locus/mutation analyzed	Primer sequences	PCR programmes for the *Mastercycler*®
CF(ΔF508), conventional PCR	5′-GCAGAGTACCTGAAACAGGA-3′ 5′-CATTCACAGTAGCTTACCCA-3′	1st PCR: 5 min 96 °C, (30 s 96 °C, 30 s 55 °C, 30 s72 °C) x 10, (30 s 94 °C, 30 s 55 °C,30 s 72 °C) x 15, 5 min 72 °C
	5′-GTTTTCCTGGATTATGCCTGGCAC-3′ 5′-GTTGGCATGCTTTGATGACGCTTC-3′	2nd PCR: 5 min 94 °C, (30 s 94 °C, 30 s 58 °C, 30 s 72 °C) x 30, 5 min 72 °C
CF (ΔF508), fluorescent PCR	5′-AATTGGAGGCAAGTGAATCC-3′* 5′-GTTGGCATGCTTTGATGACGCTTC-3′	5 min 96 °C, (30 s 96 °C, 30 s 55 °C, 30 s 72) x 10, (30 s 94 °C, 30 s 55 °C, 30 s 72 °C)x 27, 5 min 72 °C
DMD, CA repeat intron 45	5′-CAGGCTATAATTCTTTAACTTTGGC-3′* 5′-CTCTTTCCCTCTTTATTCATGTTAC-3′	5 min 96 °C, (30 s 96 °C, 30 s 62 °C, 30 s 72 °C) x 10, (30 s 94 °C, 30 s 62 °C, 30 s 72 °C) x 35, 5 min 72 °C
DM, triplet repeat 3′end	5′-CTTCCCAGGCCTGCAGTTTGCCCA-3′* 5′-GAACGGGGCTCGAAGGGTCCTTGTAG	5 min 95 °C, (30 s 95 °C, 30 s 65 °C, 1 min 70 °C) x 45, 5 min 72 °C

*** These primers are fluorescently labelled.**

The tubes were kept at -80 °C before being processed further. The cells were lysed by incubating them at 65 °C for 10 min, after which the ALB was neutralized with 2.5 or 5 µl neutralisation buffer (900 mM TrisHCl pH 8.3, 300 mM KCl, 200 mM HCl) if KOH was used and 2.5 µl 200 mM Tricine pH 4.9 if NaOH was used. For the detection of ΔF508 on conventional ethidiumbromide-stained gels, reaction mix was added to the cells to a final volume of 50 µl and final concentrations of 50 mM KCl, 100 mM TrisHCl pH 8.3, 2 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.2 mM dNTP,
1 mM primers and 1.25 U Taq polymerase.

PCR for DM was carried out using the Extended Long Template Kit (Boehringer Mannheim) in a total volume of 25 µl with final concentrations of 5 % DMSO, 200 mM dNTPs, 1 x buffer 2 provided by the manufacturer, 20 mM tricine pH 4.95 and 1.4 U of DNA polymerase provided with the kit. Figure 4 shows a result of a PCR amplifying the triplet repeat at the 3′-end of the DM-gene.

Results

For the detection of the ΔF508 mutation with a conventional PCR, 23/26 (88 %) single lymphoblasts showed amplification (Figure 1 and 2). Of these, 4/23 (17 %) showed allele-drop out, i.e. one of both alleles in a heterozygous cell was not amplified (Table 2). Similar results were obtained for the detection of ΔF508 using fluorescent PCR: 25/27 (92 %) cells showed amplification using the Eppendorf Mastercycler® gradient (Figure 3), while 2/25 (8 %) cells showed ADO. For myotonic dystrophy, the results were also very good: 18/18 (100 %) amplification and 0 % ADO with the Mastercycler® (Figure 4). Finally, the DMD-sequence also gave quite good results (Figure 5): 18/18 (100 %) amplification and 2/18 (11 %) ADO for the Mastercycler® gradient.

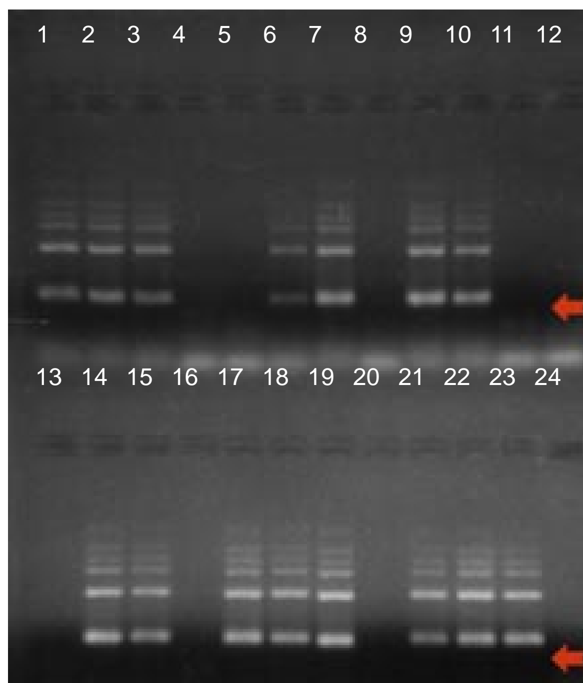

Figure 1: PCR fragments for CF(ΔF508) from single cells analysed on a 2 % agarose gel. Lanes 4, 8, 12, 16, 20 and 24 contain blanks; cells in lanes 5, 11 and 13 are not amplified. The smallest, brightest band represents the PCR product (see arrow) obtained after two PCR rounds; the larger products are from combination of primers from the first and second PCR rounds.

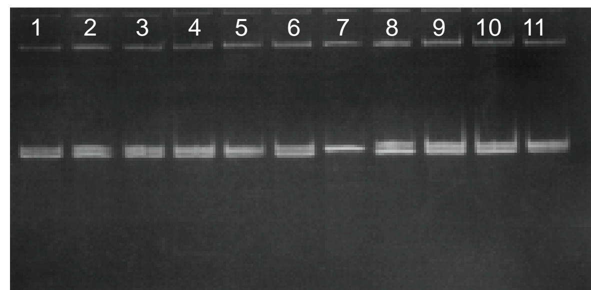

Figure 2: The same PCR products as in figure 1 analysed on a 4% Metaphor Agarose gel. The lowest band represents the mutated allele, the middle band represents the healthy allele (three bp larger), and the top band represents heteroduplexes. The larger bands are again the results from combinations of primers from the first and second PCR rounds. The cell in lane 7 shows ADO, i.e. only the healthy allele is amplified, such that this cell seems homozygous normal.

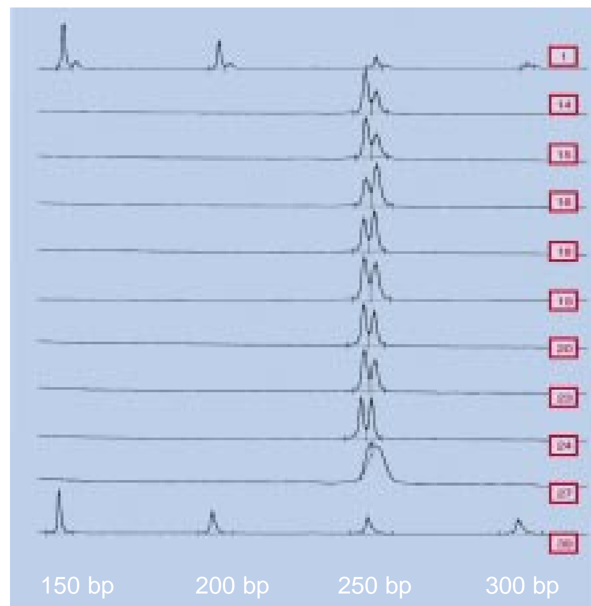

Figure 3: Cells heterozygous for the ΔF508 mutation after fluorescent PCR and analysis on an automated DNA sequencer. The cells in lanes 14 to 24 show two bands, while the cell in lane 27 only shows one band (i.e. ADO). Lanes 1 and 39 are molecular weight markers representing 150, 200, 250, and 300 bp.

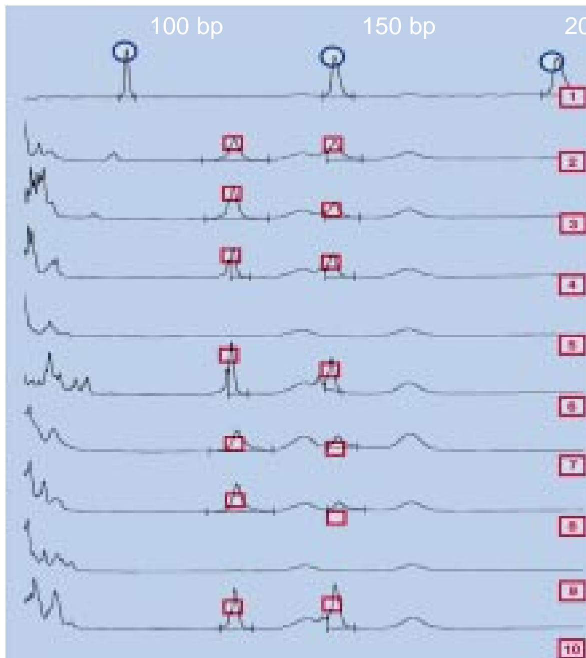

Figure 4: Single-cell fluorescent PCR for the 3′ triplet repeat in the DM gene in an individual heterozygous for 5 and 12 repeats. Lanes 2, 3, 4, 6, 7, 8 and 10 show single cells, while lanes 5 and 9 represent blank samples.

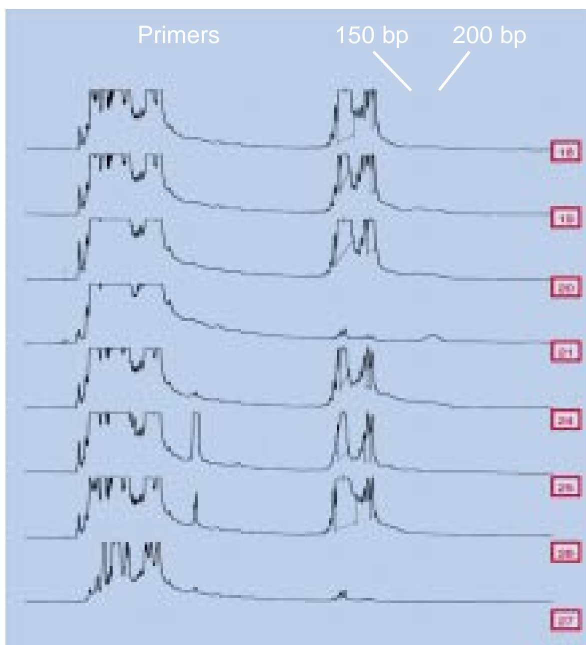

Figure 5: The result of an amplification for intron 45 of the DMD gene. Lanes 18, 19, 20, 24, 25, and 26 show heterozygous cells, while lanes 21 and 27 show blanks. The high peaks on the left of the diagram represent primer peaks.

Table 2: Results obtained with Eppendorf *Mastercycler® gradient*

Locus/mutation	**Eppendorf *Mastercycler®***	
	Amplification (%)	**ADO (%)**
CF (ΔF508), conventional PCR	23/26 (88 %)	4/23 (17 %)
CF (ΔF508), fluorescent PCR	25/27 (92 %)	2/25 (8 %)
DMD, CA-repeat intron 45	18/18 (100 %)	2/18 (11 %)
DM, triplet repeat 3′	18/18 (100 %)	0/18 (0 %)

Discussion

The aim of this small-scale study was to show the efficiency and accuracy of the new Eppendorf Mastercycler® gradient in single-cell PCR.
To achieve this, we compared the results obtained with the Eppendorf Mastercycler® with our results previously obtained using Perkin Elmer GeneAmp PCR systems. None of the programmes used for the Perkin Elmer PCR machines had to be altered, with regard to both the incubation temperatures and the incubation times.

References

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Prenat. Diagn. **10**, 925-932

Ordering information

Article	**Order no**
Mastercycler® gradient	5331 000.010
Mastercycler® gradient US 115 V	5331 000.045

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