

Multiple displacement amplification on single cell and possible PGD applications

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Multiple displacement amplification (MDA) is a technique used in the amplification of very low amounts of DNA and reported to yield large quantities of high-quality DNA. We used MDA to amplify the whole genome directly from a single cell. The most common techniques used in PGD are PCR and fluorescent in-situ hybridization (FISH). There are many limitations to these techniques including, the number of chromosomes diagnosed for FISH or the quality of DNA issued from a single cell PCR. This report shows, for the first time, use of MDA for single cell whole genome amplification. A total of 16 short tandem repeats (STRs) were amplified successfully with a similar pattern to the genomic DNA. Furthermore, allelic drop out (ADO) derived from MDA was assessed in 40 single cells by analysing (i) heterozygosity for a known β globin mutation (IVSI-5 C–G) and by studying (ii) the heterozygous loci present in the STRs. ADO turned out to be 10.25% for the β globin gene sequencing and 5% for the fluorescent PCR analysis of STRs. Moreover, the amplification accuracy of MDA permitted the detection of trisomy 21 on a single cell using comparative genome hybridization-array. Altogether, these data suggest that MDA can be used for single cell molecular karyotyping and the diagnosis of any single gene disorder in PGD.

Key words: PGD/whole genome amplification-comparative genome hybridization/CGH-array/multiple displacement amplification

Introduction

PGD is a technique developed more than a decade ago as an alternative to prenatal diagnosis for couples at risk of transmitting inherited diseases (Handyside *et al.*, 1990; Braude *et al.*, 2002; Hellani *et al.*, 2002). In this technique, embryos are generated by IVF and the diagnosis is usually made on cleavage stage embryos. This permits the transfer of only unaffected embryos thereby eliminating the need for abortion following the prenatal diagnosis in case of an affected fetus.

Despite the significant advantages provided by PGD, there are still technical limitations. PGD is being used for the detection of major chromosomal abnormalities or single gene disorders. Regarding chromosomal abnormalities, the main problem is the number of chromosomes that could be diagnosed in a single cell by fluorescent in-situ hybridization (FISH). Recently, the highest number reported was 15 chromosomes (Abdelhadi *et al.*, 2003). However, the accuracy of the technique is still debatable. For single gene disorders (Harper *et al.*, 2002), two techniques are widely used: whole genome amplification (WGA) and nested multiplex PCR. The main disadvantages of WGA are the generation of non-specific amplification artefacts (Cheung and Nelson, 1996), incomplete coverage of loci (Paunio *et al.*, 1996), inefficiency of microsatellite amplification (Wells *et al.*, 1999) and the generation of DNA less than 1 kb long (Telenius *et al.*, 1992), which makes its application limited in the microarray technology. On the other hand, the main disadvantage of nested PCR is the difficulty in choosing primers in multiplex PCR (Van de Velde *et al.*, 2004).

Due to these limitations, there is a need for a technique that would be able to amplify the single cell DNA with high fidelity that

suits microarray analysis or the diagnosis of any known single gene disorder by standard PCR technique.

Multiple displacement amplification (MDA) is a technique used in the amplification of very low DNA quantities in clinical samples (Dean *et al.*, 2002). In MDA, DNA is amplified isothermally by using Φ 29 enzyme (Blanco *et al.*, 1992). The MDA product was found to be favoured over other PCR-based WGA techniques regarding bias amplification, reproducibility and diagnosis (Hosono *et al.*, 2003). In this report, MDA was used to amplify single cell DNA, which was then utilized in several PGD applications.

Materials and methods

Genomic DNA from a family carrier of β globin mutation (IVSI-5 C–G) and a female Down syndrome patient (47XX; +21) was extracted using Dnazol kit (Invitrogen, USA).

Cell lysing and MDA protocol

Single leukocytes from the Thalassemia family (20 from father and 20 from mother) and the Down syndrome female (5) were collected in 0.5-ml PCR tubes containing 5 μ l of lysing buffer (1% Tween 20, 1% Triton 100X, 1X PCR buffer and 20 mg/ml proteinase K). Aliquots from the last washing droplets were taken to serve as blanks (10 tubes). Samples and blanks were incubated at 45°C for 15 min, followed by proteinase K inactivation at 96°C for 20 min. Cell lysates were used directly for MDA (Molecular Staging, USA) by adding 45 μ l of the master mix in a total volume of 50 μ l. The mix was then incubated at 30°C for 6 h followed by heat inactivation at 65°C for 3 min. MDA products were then digested with *Eco*R1 (Invitrogen, USA) in order to produce a homogeneous smear extending from 20 kb to approximately 600 bp. The digestion product was then purified using a Zymo

clean-up kit (Zymo Research, USA), re-suspended in 100 μ l of sterile water and quantified using a fluorometer.

Short tandem repeat (STR) analysis

MDA product was used for STR analysis according to the Applied Biosystems Identifier kit protocol (ABI, USA). This experiment was performed on the genomic DNA of the Thalassemia parents in addition to 40 MDA products derived from their single lymphocytes (20 from father, 20 from mother). Briefly, 10 μ l of the genomic DNA and the MDA product containing 0.5 ng DNA were added to 15 μ l of the ABI identifier kit master mix. The PCR protocol was as follows: 11 min incubation at 95°C, 28 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C, followed by 1 h incubation at 60°C and then held at 4°C.

Sequencing analysis

MDA product weighing 100 ng was added to the PCR master mix with final concentration of 10% dimethylsulphoxide (DMSO), 50 mM KCl, 100 mM Tris-HCl pH 8.3, 2 mM MgCl₂, 0.1 mg/ml gelatin, 0.2 mM dNTP, 1 μ M primers (forward 5'-CAG GGC AGA GCC ATC TA-3' and reverse 5'-GTC TAT GGG ACC CTT GAT G) and 1.25 IU *Taq* polymerase (Life technology, USA). PCR was carried out on a Biometra thermocycler using the following programme: 5 min denaturation at 96°C followed by 28 cycles of 20 s at 96°C, 60 s at 60°C, and 20 s at 72°C, followed by 6 min at 72°C. The PCR products were purified, analysed and sequenced using the forward primer in order to detect the β globin mutation (IVSI-5 C-G).

Comparative genome hybridization (CGH)-array technique

For this study, a human genomic microarray containing 2600 bacterial artificial chromosome (BAC) clones with an average of 1 MB resolution (Human BAC Array-1MB system, Spectral Genomics Inc, USA) was used. This microarray includes subtelomeric sequences as well as the most common microdeletion regions. The DNA clones used for 1MB microarray production were obtained from public libraries and were controlled by FISH, PCR and sequencing.

The DNA of clones was modified to be fixed covalently onto the glass without coating slides. Each DNA clone was printed in duplicate. To reduce non-specific hybridization and the background, the array was blocked by Human cot DNA and Salmon sperm DNA before hybridization.

Normal reference (control samples from the mother single cell of our Thalassemia patient) and 500 ng (MDA product, test sample) were processed blindly and the identity of the tubes was revealed upon interpretation of the results. Samples were digested for 1 h with 80 units of *Eco*R1 at 37°C and then purified by Zymo Research's column (Orange, CA, USA). The test and reference DNAs were both labelled with Cy3 and Cy5 using a random primer labelling kit (Invitrogen, USA).

For the hybridization solution, Cy5-labelled test DNA and Cy3-labelled reference DNA samples were mixed with 65 μ g of Cot-1 DNA and 35 μ g of Salmon sperm DNA and then the mix was precipitated and washed with ethanol. The same experiment was repeated by counter labelling, test and reference DNA with Cy3-labelled test and Cy5, respectively. The pellets were dissolved homogeneously in 10 μ l of distilled water and mixed with 50 μ l of hybridization solution [50% formamide, 10% dextran sulphate in 2 \times saline sodium citrate (SSC)]. The hybridization solution was denatured at 73°C for 12 min and followed by 40 min at 37°C for annealing.

The forward and reverse hybridization reactions were added onto duplicate microarray slides and placed at 37°C for 16 h. After hybridization, the slides were washed at room temperature in 2 \times SSC for 5 min, then 20 min in 50% formamide/2 \times SSC at 50°C with shaking. The washing steps were repeated at 50°C with shaking in 0.1% NP40/2 \times SSC for 20 min and 0.2 \times SSC for 20 min. Finally, the slides were rinsed briefly with distilled water at room temperature and centrifuged for 3 min at 500 rpm for complete drying.

Hybridized microarrays were analysed with a GenPix scanner (Axon Ins. CA, USA).

Cy3 and Cy5 images were scanned separately through two different channels. Two 16-bit TIFF images were created per array. Then, the obtained data were analysed by spectralware software (Spectral Genomics). The software recognizes the regions of fluorescent signal, determines signal intensity and compiles the data into a spreadsheet that links the fluorescent signal of

every clone on the array to the clone name, its duplicate position on the array and its position in the genome. The software was also used to normalize the Cy5: Cy3 intensity ratios for each slide and each data point. The slide was normalized such that the total Cy5 signal equalled the total Cy3 signal. The normalized Cy3: Cy5 intensity ratios were computed for each of the two slides and plotted together for each chromosome. The linear order of the clones is reconstituted in the ratio plots consistent with an ideogram, such that the p terminus is to the left and the q terminus is to the right of the plot.

For data analysis, we assigned a ratio plot such that gains in DNA copy number at a particular locus were observed as the simultaneous deviation of the ratio plots from a modal value of 1.0, with the blue ratio plot showing a positive deviation (upward) and the red ratio plot showing a negative deviation at the same locus (downward). DNA copy number losses show the opposite pattern.

Results

Single cell DNA amplification

To demonstrate the feasibility of MDA for single cells, 45 individual lymphocytes were utilized for WGA. The amount of the MDA product was $35 \pm 5 \mu$ g DNA (1 000 000 times more than single cell DNA). The amplification profile on 1% agarose gel after restriction cut and column purification showed a smear starting from more than 10 kb and extended to 2 kb, very similar to the genomic DNA (Figure 1).

STR and β globin gene amplification from MDA product

In order to detect the homogeneity of the amplification, MDA products of 40 single cells (20 from a father and 20 from a mother who were carriers of β globin mutation) were used for the amplification of 16 loci and the β globin gene. The result showed that STRs and the β globin gene were both amplified on genomic DNA in 39 of the 40 single cells tested (Figure 2A). It is noteworthy that the double head bands observed in the STR loci are commonly seen in single cell PCR.

Allelic drop out (ADO) evaluation of the MDA technique

ADO assessment was performed by examining the heterozygosity status of single cells regarding the β globin mutation and the STRs.

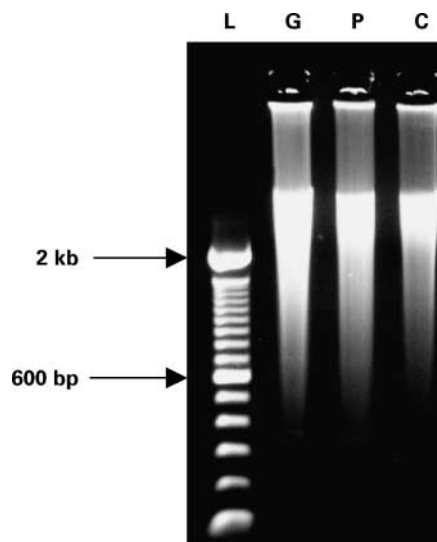


Figure 1. Profile of MDA amplification on 1% agarose gel. L: 100 bp ladder; G: genomic DNA. P and C are patient and control DNA derived from a single cell. The smear extension is starting from 600 bp.

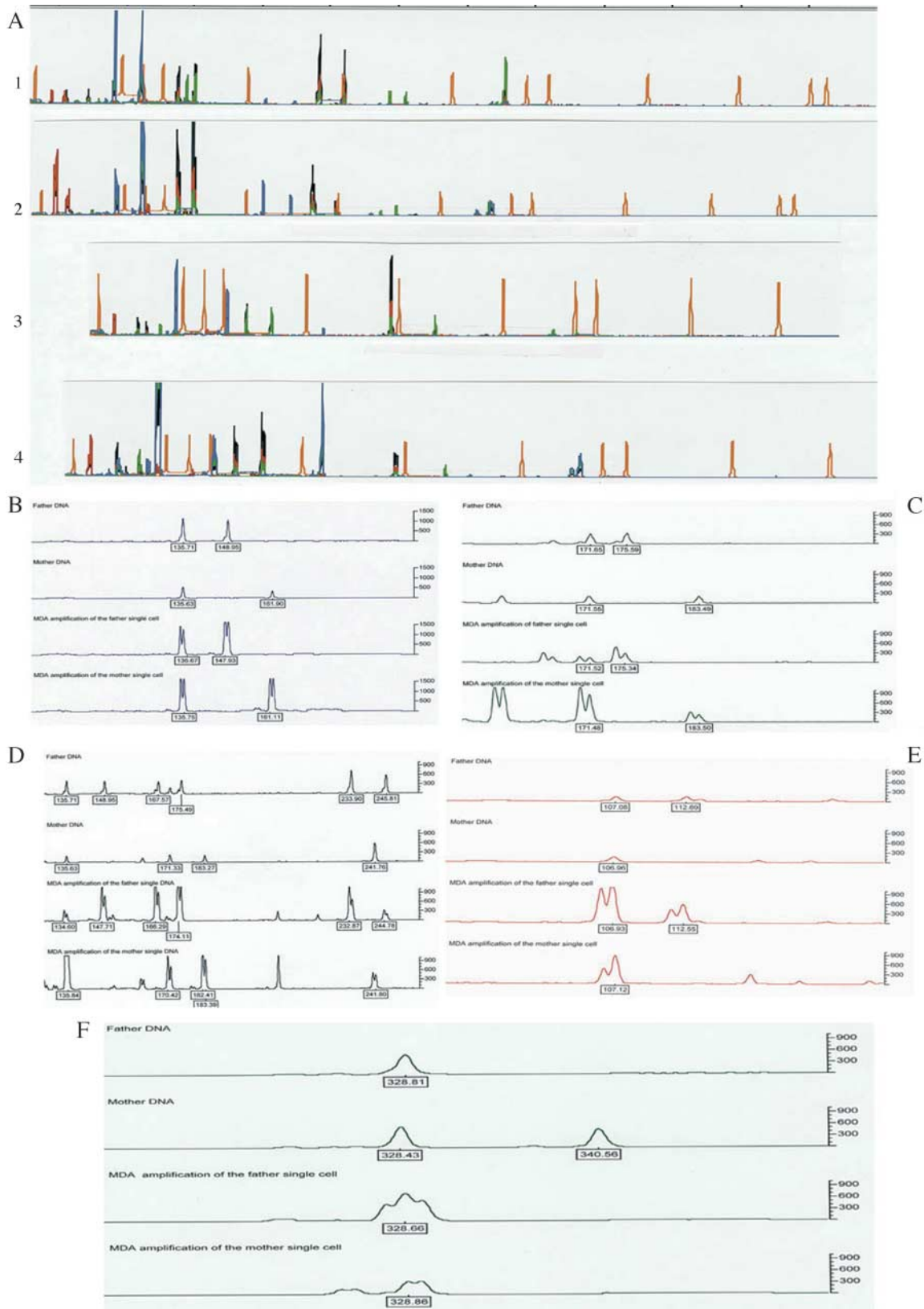


Figure 2. Result of the STR amplification analysis (ABI Identifier kit) on the father, the mother genomic DNA and their single cell DNA amplified by MDA. (A) Raw data of the father genomic DNA (1), father single cell (2), mother genomic DNA (3) and mother single cell (4); (B) amplification of the STR D8S1179; (C) amplification of the STR TH01; (D) amplification of the STRs D19S433, vWA and TPOX; (E) amplification of the amelogenin locus; (F) ADO observed in the STR D2S1338 of the mother sample. B, C, D, E, F rows from top to bottom; father DNA, mother DNA, MDA amplification of the father single cell, MDA amplification of the mother single cell.

Regarding the β globin mutation, ADO was present on four occasions of 39 cells successfully amplified by PCR after MDA (Table I). On the other hand, STR analysis showed that six and five loci for the father and the mother samples, respectively, were heterozygous. Consequently, they were used in ADO analysis (Table II). Four loci were common in both parents where no ADO was found (Figure 2B–E). However, one of the mother's heterozygous loci (D2S1338) showed one ADO out of 20 (5%) (Figure 2F). It is important to note that preferential amplification was observed in many single cell DNA, amplified by MDA during STR analysis (Figure 2C–E) or sequencing (data not shown). Moreover, ADO observed in the STR analysis was not complete. It might be preferential amplification since traces at the same size were present (Figure 2F). Overall, the image of STR amplification by MDA is similar to the genomic DNA with over-representation of most of the loci and 5% of ADO.

CGH-array

To test the reliability and the accuracy of the amplification, we performed single cell molecular karyotyping on the MDA product. Human BAC Arrays-1MbV1.2 for high resolution genome profiling were used in order to detect trisomy 21. The result confirmed the presence of trisomy 21 in the MDA product obtained from single cells (Figure 3).

Discussion

The current report showed that MDA yielded 35 μ g of DNA (1 000 000 fold) confirming the previous observation (Dean *et al.*, 2002), which would be of major interest for genetic analysis in PGD. The STR analysis from a single cell showed a similar pattern

to the genomic DNA. However, an over-representation of locus was observed in some samples. Such an observation is in accordance with a previous report (Dean *et al.*, 2002), which showed an average of 150% locus representation.

Assessment of ADO is critical to evaluate any new technique for PGD. The presence of ADO was assessed by two different tests: (i) β globin mutation and (ii) STR analysis. It is important to mention that ADO was observed only with an STR locus where the size was bigger than 300 bp. This might explain the absence of ADO in the father STR where no heterozygous loci were detected in this size range. The case of ADO observed in STR analysis would most probably be a severe form of preferential amplification because some traces of amplification could be noticed at the expected size (Figure 2F). Moreover, the preferential amplification phenomenon was observed in many STR loci as shown in Figure 2C (father and mother locus), Figure 2D (father's first and last loci) and Figure 2E (father and mother locus). preferential amplification was present in β globin mutation analysis as shown by the different sized peaks for each nucleotide (C and G) at the mutation site (data not shown). A previous report (Hosono *et al.*, 2003) showed a reliable and accurate amplification of nine STR loci plus amelogenin (ABI profiler kit) with complete absence of ADO from MDA product. However, the amount of DNA used was from approximately 300 cells. Although these data would not be applicable to single cells, they could be helpful when considering blastocyst biopsy in PGD where a high number of cells are available. The phenomenon of the microsatellite slippage (change microsatellite repeat numbers) is a major disadvantage of the current WGA technique (Wells *et al.*, 1999). As a consequence of this slippage, an error in the amplification of microsatellite would lead to a misdiagnosis and a discrepancy with the genomic DNA. Such a misdiagnosis could be of major negative impact on the success of PGD mainly when STR analysis is used

Table I. Assessment of ADO and amplification efficiency of the MDA on a single cell

	Total		Diagnosed		FA		ADO	
	Father	Mother	Father	Mother	Father	Mother	Father	Mother
β Globin mutation (IVSI-5 C–G)	20	20	19	20	1/20	0/20	2/19	2/20
Analysis of STR	20	20	19	20	1/20	0/20		1/20 (5%)

Amplification efficiency and heterozygosity of the MDA product on single cells regarding β globin mutation (IVSI-5 C–G) and STR analysis were assessed in 40 single cells. FA, failure of amplification.

Table II. STR name, size and heterozygosity in both father and mother genomic DNA

STR name	STR size	Heterozygosity father	Heterozygosity mother	ADO after MDA
D8S1179	120–180	Yes (135; 147)	Yes (135–161)	No
D21S11	190–240	Yes (210–225)	No (210)	No
D7S820	250–300	No (260)	No (270)	
CSF1PO	310–340	No (330)	No (330)	
D3S1358	100–150	Yes (135–148)	No (135)	No
TH01	160–200	Yes (167–175)	Yes (171–183)	No
D13S317	210–250	Yes (233–275)	Yes (232–244)	No
D16S539	260–290	No (270)	No (270)	
D2S1338	300–360	No (328)	Yes* (328–340)	Yes*
D19S433	100–140	No (120)	No (120)	
vWA	150–210	Yes (171–175)	Yes (171–183)	No
TPOX	220–250	No (230)	No (240)	
D18S51	260–340	No (320)	No (270)	
D5S818	130–180	No (130)	No (130)	
FGA	210–360	No (250)	No (250)	
Amelogenin X	107	107	107	No
Amelogenin Y	112	112		No

Note that ADO (*) was observed in mother sample only.

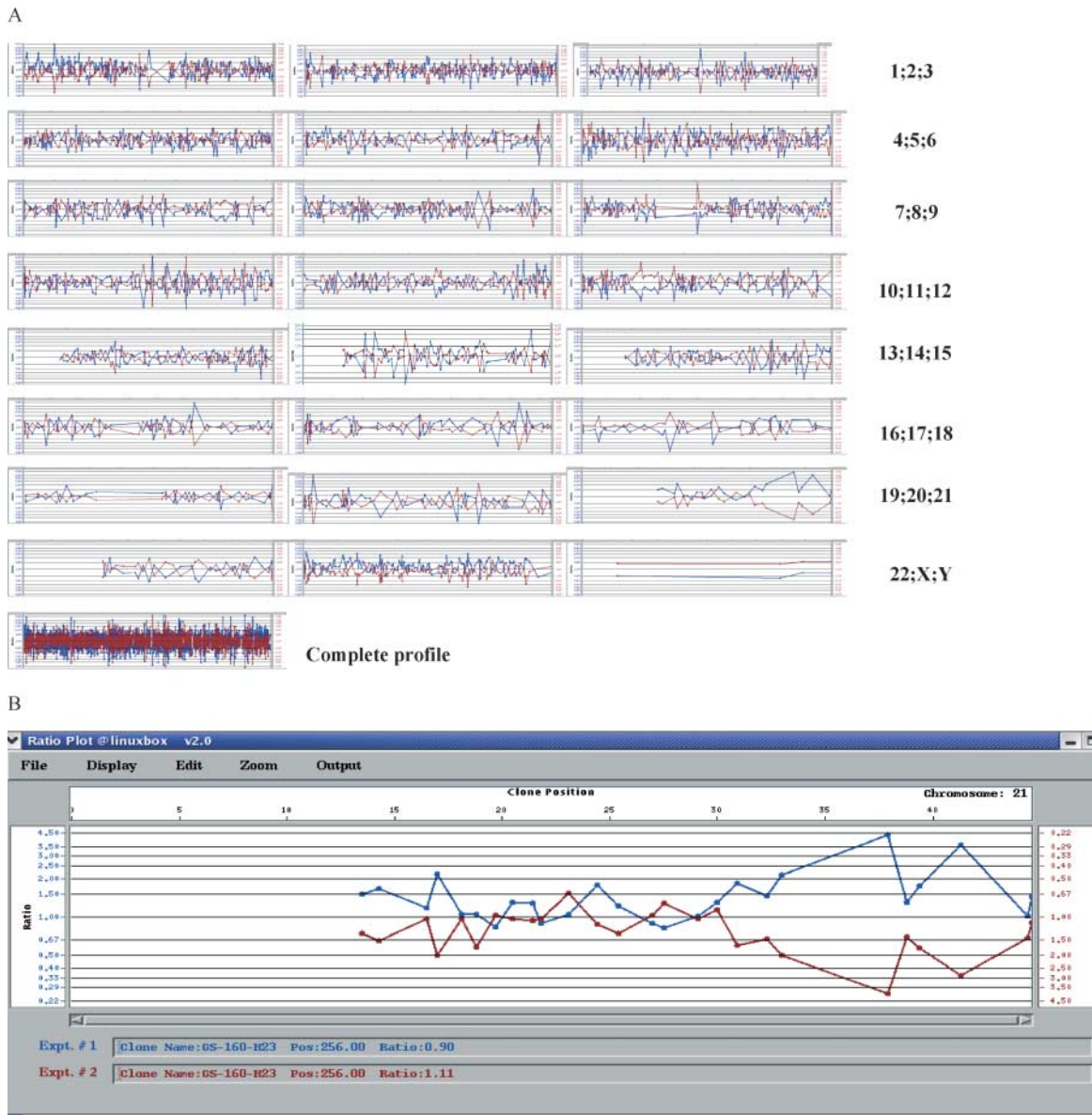


Figure 3. Representative schema of the CGH-array result showing the genome profile of the whole single cell chromosomes (A) with the number of the chromosome on the right side of the figure. The abnormality found in chromosome 21 is shown in (B). The DNA is derived from a single lymphocyte of a patient having the karyotype 47XX (+21) and a control normal female karyotype. The experiment from single cell to CGH was run three separate times.

(Grewal *et al.*, 2004). Results shown in this report indicate that the slippage of the MDA enzyme on microsatellite does not exist. However, there were double-headed peaks with a 1 bp difference from each other in the majority of STR loci.

The alternatives to FISH in assessing chromosome abnormalities are CGH (Wells *et al.*, 2002) and microarray technologies (Lage *et al.*, 2003; Lovmar *et al.*, 2003; Chung *et al.*, 2004). CGH has been successfully applied to PGD (Wells and Delhanty, 2000). However, the time required for CGH exceeds the culture period of embryos, ending up with freezing and thawing them before transfer. Such a procedure results in the survival of only 75% of the embryos (Veeck, 2003) and might negate the benefits of the screening procedure. In this report, microarray technology was utilized successfully with much less time (6 h amplification; 1 h cut; 2 h labelling, overnight hybridization, washing and scanning the following day). For PGD cycles, embryo transfer can be done on the next day after biopsy. This is a revolutionary protocol in the field of PGD since molecular karyotyping of a single cell is now possible.

Our data showed, for the first time, the use of CGH-array successfully by detecting trisomy 21 on single cells. However, the profile of chromosome 21 on our microarray panel is not full trisomy. As reported before (Dean *et al.*, 2002), MDA is unable to amplify heterochromatic sequences (centromere and telomere sequences). This observation explains why only the long arm of chromosome 21 showed the abnormality. To override such artefacts, new microarray slides can be designed by spotting MDA product. A recent report (Hu *et al.*, 2004) showed that spotting of the product deriving DOP-PCR (the same technique was used in the amplification of a single cell) enabled detection of many trisomies (13,15,18) by CGH-array. Their report has two disadvantages: the absence of any result on trisomy 21 and the failure to detect the amplification of the Y chromosome in 71% of cases. Although we used only female cells in microarrays, the Y chromosome locus was consistently amplified in father cells and trisomy 21 was successfully detected. We believe that the success of our data is due to MDA.

In conclusion, MDA could produce enough DNA from a single cell for multiple PCR analyses including STR, HLA typing and microarrays. Compared to nested multiplex PCR, MDA offers a broader choice of application with a high DNA yield including CGH-array, while at the same time providing very high amplification efficiency and a constant ADO.

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