Journal of Clinical Laboratory Analysis 18:176–181 (2004)

Single Cell PCR From Archival Stained Bone Marrow Slides: A Method for Molecular Diagnosis and Characterization

Stefanie Zanssen*

Department of Biochemistry, Medical Faculty, RWTH Aachen, Aachen, Germany

Molecular analysis of isolated single cells is a powerful tool for clarifying issues of cell origin and clonality. Previous reports have described PCR amplifications from total DNA and RNA extracted from archival bone marrow and peripheral blood smears and have also shown the feasibility of amplifications from single cells, microdissected from stained histological sections. In this study, a method is described for performing PCR from morphologically defined single cells isolated from archival May-Gruenwald-Giemsa-stained bone-marrow and blood smears. Using three DNA extraction procedures, the organic lysis showed reproducible high efficiencies of amplifications. With this method, we were able to amplify long range amplicons up to 14.5 kb from mitochondrial DNA as well as PCR products of conventional length. The usability of such products for molecular diagnosis is demonstrated by restriction fragment length polymorphism (RFLP) characterization of a mitochondrial disorder. In conclusion, this method has the power to perform molecular diagnosis and characterization of diseases on the single cell level, and should provide valuable information to aid disease treatment and prognosis of hematological disorders. J. Clin. Lab. Anal. 18:176–181, 2004. © 2004 Wiley-Liss, Inc.

Key words: single cell PCR; bone marrow; nuclear DNA; mtDNA; amplification

INTRODUCTION

The polymerase chain reaction (PCR) has evolved as a major research technique in molecular analysis of hematopoietic malignancies and solid tumors. However, tissue specimens are generally heterogeneous, containing not only the cells of interest (e.g., malignant cells) but also containing other cell types. Hence, analysis of isolated single cells is a powerful tool for studying molecular gene defects in neoplastic diseases.

Some previous reports have shown the feasibility of single cell PCR from stained histological slides (1–4). The use of microdissected cells from histological sections, however, had an efficiency below 40%, depending on the length of the PCR product (usually below 400 bp). Furthermore, there is the possibility of only picking cell fragments but not entire cells.

Other authors have described the extraction of amplifiable DNA and RNA from archival bone marrow and peripheral blood smears (5–8). These studies performed extraction from the entire smear but not at the single cell level. In a recent study, the detection of viral DNA in single peripheral lymphocytes was described, but this method was based on diluted peripheral blood smeared on thin polyester slides that were cut into tiny squares and eventually contained only single cells (9).

In this study, we present a new method for performing genome analysis at the single cell level on morphologically defined intact cells from routinely processed archival bone marrow slides. For the first time, the diagnosis of a mitochondrial disease is possible on the single cell level.

MATERIALS AND METHODS

Samples

After informed consent, peripheral blood and bone marrow samples were drawn from patients. May-Gruenwald-Giemsa-stained bone marrow slides were routinely processed and kept at room temperature in an

Grant sponsor: "START-Programm" of the Medical Faculty, RWTH Aachen; Grant number: S.Z. 07/96 SI.

*Correspondence to: Stefanie Zanssen MD, PhD, Columbia University, College of Physicians and Surgeons, Department of Neurology (Genetics and Development), 630 West 168th street, New York, NY 10032. E-mail: sz2106@columbia.edu; zaste3@hotmail.com

Received 25 July 2003; Accepted 16 December 2003
DOI 10.1002/jcla.20019
Published online in Wiley InterScience (www.interscience.wiley.com)

air-conditioned storage room in the archives of the hematology department. Prior to further analysis, the slides were microscopically observed to evaluate the quality of the specimens. All smears were well preserved without any cell damage. Cells were characterized and selected according to morphological criteria before micromanipulation.

Single Cell Micromanipulation

Cell areas were overlaid with 50 µl of TE-buffer (10 mM TrisCl, 1 mM EDTA, pH 8) and single cells were picked under an inverted microscope (Axiovert 135; Carl Zeiss, Jena, Germany) under 400-fold magnification by a glass capillary (Eppendorf, Cologne, Germany) using an electric micromanipulator (Patch-Man; Eppendorf). After capillary dissection, the single cell was transferred into microfuge tubes.

DNA Extraction

Thermic lysis

The micromanipulated single cell was resuspended in 100 µl of TE buffer and heated for 30 min at 95°C.

Alkaline lysis

The single cell, buffered in 2.5 µl TE buffer, was incubated for 30 min at 65°C with 2.5 µl of lysis buffer (200 mM KOH, 50 mM DTT) and then neutralized with 5 µl of N-buffer (900 µl 10 mM TrisCl + 20 µl fuming HCl + 80 µl distilled water).

Organic lysis

An amount of 100 µl of TE-buffer containing the single cell were incubated with 100µl of SDS-containing 2 × lysis-buffer (Applied Biosystems, Foster City, CA) and 10 µl of proteinase K (20 mg/mL) at 55°C for 2 hr. This solution was then extracted once with phenol-chloroform-water (Applied Biosystems), once with chloroform (Applied Biosystems), and finally ethanol-precipitated (10). The vacuum-dried DNA of one single cell was then totally resuspended in the specific PCR mix and, after transfer to a PCR reaction tube, amplification was performed.

Amplification of extracted DNA

The quality of the extracted DNA was evaluated by amplification of a 297 bp-fragment of the human β-actin gene from nuclear DNA and a 14.5 kb-fragment of the 16.5 kb mitochondrial genome. For the former, a 100 µl PCR reaction mixture containing 200 µM of each dNTP, 10 mM TrisCl, 50 mM KCl (pH 8.3), 1.5 mM $MgCl_2$, 1% BSA, 150 nM of each primer 5′TCACCCACACTGTGCCCATCTACGA and 5′CAGCGGAACCGCTCATTGCCAATGG, 2 U of Taq-Polymerase (Boehringer Mannheim, Mannheim, Germany), and 1µl of [$^{32}\alpha$]P dATP (10 µCi) was added to one single cell. A total of 40 amplification cycles were performed in a thermocycler consisting of a heating step at 94°C for 80 sec, followed by annealing at 58°C for 60 sec, and subsequent extension at 72°C for 120 sec. Prior to amplification, the reaction mixture was held for 5 min at 94°C, and the last incubation continued for 5 min.

For the long PCR amplification of the mitochondrial genome, the TaKaRa LA Taq system (TaKaRa, Gennevilliers, France) was used, which utilizes a mixture of Taq and Pwo-polymerases to allow amplification of DNA sequences larger than 20 kb. The whole DNA extracted from a single cell was submitted to PCR. The 50 µl PCR reaction consisted of 5 µl 10 × LA PCR buffer, 1 U TaKaRa LA Taq, 250 µM each dNTP, 0.25 µM forward primer 5′GATCACAGGTCTA TCACCCT, mitochondrial DNA (mtDNA) 1-20 and backward primer 5′GCGGTGTGGTCGGGTGTGTT, mtDNA 14571-14551, and 0.5 µl [$^{32}\alpha$]P dATP (5 µCi). PCR cycles consisted of 1 min at 94°C, 30 cycles of 20 sec at 98°C and 550 sec at 68°C, and a terminal step at 68°C for 2,000 sec.

Detection of PCR products and interpretation of results

PCR solution (5–50 µl) was separated in a 3.5% native polyacrylamide gel, exposed to an image screen, and imaged by a β-Scanner (Storm; Molecular Dynamics, Sunnyvale, CA).

Availability of radioactive labeled PCR products

A single cell 1.12 kb PCR product encompassing nt 11451 (forward primer 5′TTGCCGCAGTACT CTTAAAA) to nt 12570 (backward primer 5′TAG GGAGAGCTGGGTTGTTT) of the wild-type mitochondrial genome was restricted with SfaN1 (New England Biolabs, Frankfurt am Maim, Germany) in three fragments (679, 318, and 122 bp) according to the manufacturer's instructions and visualized as described above. The 11778 primary Leber's hereditary optic neuropathy (LHON) mutation destroys one SfaN1 restriction side and will only result in two fragments of 997 and 122 bp. The mutation load was estimated using Imagequant (Molecular Dynamics).

Extreme care was taken throughout the procedure to avoid contamination by DNA: gloves were changed frequently, separate equipment and working space were used for pre- and post-PCR manipulations, and aerosol resistant pipette tips were used. Negative controls (sterile water aspirated from the nonmanipulated slide) were systematically included to monitor possible DNA

contamination. Positive controls were performed using 5 pg DNA.

RESULTS

Single cells of defined morphology were microdissected using our micromanipulation system, and individual cells chosen for picking were photodocumented (Fig. 1a, b) and correlated to molecular results (Fig. 1c). Amplifiable DNA was obtained with all of the three extraction methods presented in this study. Fig. 2a exemplifies the results of several trials of the three extraction procedures with different bone marrow cells and slides. Only the time consuming organic extraction yielded high quality DNA with high amplification efficiencies. Long amplicons could be obtained not only from cells with high mtDNA content like megakaryocytes, but also from cells with normal mtDNA content like lymphocytes. The 14.5 kb mitochondrial DNA signal from organic extractions could be obtained after short exposure times of about 2 days. The rougher extraction procedures like thermic or alkaline lysis showed low efficiencies and required inappropriately long exposure times to yield a signal on the phosphoimager. In Fig. 2a, only one thermally lysed single cell showed the 14.5 kb amplicon whose intensity was much lower than that of the organic extracted cells. Using the alkaline lysis, long amplicons could not be detected. We were able to amplify genes from organically extracted cells with a high efficiency of about 80% (26 out of 33 PCRs, not all data shown).

The β-actin gene single cell amplification (Figs. 1c and 2b) exemplified that single cell PCR can also be transferred successfully to nuclear genes. Depending on the cell type, the mitochondrial genome can be concentrated 1,000-fold higher than the nuclear genome, but amplification efficiencies were comparable (Fig. 2b, c). Fig. 2a also shows that it was possible to average PCR findings over a larger number of cells (e.g., erythrons) microdissected from discrete slide segments.

Fig. 2c shows that, in a patient with LHON, restriction endonuclease SfaNI does not cut a single cell amplicon of 997 bp into the wild-type 679 and 318 bp-fragments. Furthermore, a quantification of the mutation load on the single cell level revealed that a lymphocyte contains 100% of the LHON mutation, but a megakaryocyte only 5%. This further analysis of DNA fragments amplified from single cells was an additional proof that targeted genes were specifically amplified.

The usefulness of the stored bone marrow slides was excellent. We show that single cell amplification with high efficiency was possible over a time range from 0.5 to 3 years. Amplification of long genes was possible even after a storage duration of 25 months (Fig. 2a).

DISCUSSION

Until recently, the analysis of human DNA mutations from single cells was limited to short gene fragments and only yielded a poor efficiency. In this study, we discussed the applicability of conventional and long PCR to the smallest tissue unit, the single cell. May-Gruenwald-Giemsa-stained bone-marrow smears provided excellent features for retrospective molecular examination.

For the first time, this study showed that molecular genetic diagnosis can be performed on the single cell level and can give important additional implications for treatment and prognosis of hematological disorders. This was exemplified by a patient with the mitochondrial disorder LHON, a subacute bilateral vision loss, who required chemotherapy for a malignant lymphoma (11). Because this patient, homoplasmic for the 11778 LHON mutation in peripheral blood and lymphoma tissue, did not show an altered sensitivity to cytotoxic drugs, determination of the heteroplasmy level at the single cell level was performed. Fig. 2c demonstrates that the mutation load can vary between lymphatic and myelopoietic cell lineages. Cells of the bone marrow, such as megakaryocytes, still contained rescuing wild-type molecules in comparison to lymphocytes, for example, which were 100% mutant. The heteroplasmy of bone marrrow cells may explain why the 11778 LHON mutation did not interfere with the hematological response to cytotoxic drugs in a detrimental manner. Single cell PCR may give new insights into mitochondrial disorders (12) with hematological expression like Pearson's marrow pancreas syndrome or myelodysplastic syndromes (13). Furthermore, long PCR from single cells will improve retrospective hematological studies of long genes. Tumorogenic viruses, e.g., HTLV-I, can now be traced in their suspected target cells. In addition, the effects of cytostatic or differentiating therapy on the neoplastic clone can be monitored on the single cell level (e.g., methylation status of tumor suppressor genes).

One of the primary problems associated with standard applications of extra long PCR (XL-PCR) is the requirement for a high quality template (14,15). Also, template quantity becomes a critical limitation in XL-PCR from single cells. Organic lysis maximized recovery and purity, and minimized template damage. It is advisable to keep cycle numbers relatively low using about 40 cycles and to radiolabel the PCR products, since increasing the number of PCR cycles improved sensitivity at the expense of specificity (Fig. 1c). Specific PCR inhibitors, e.g., hemoglobin, may lead to an

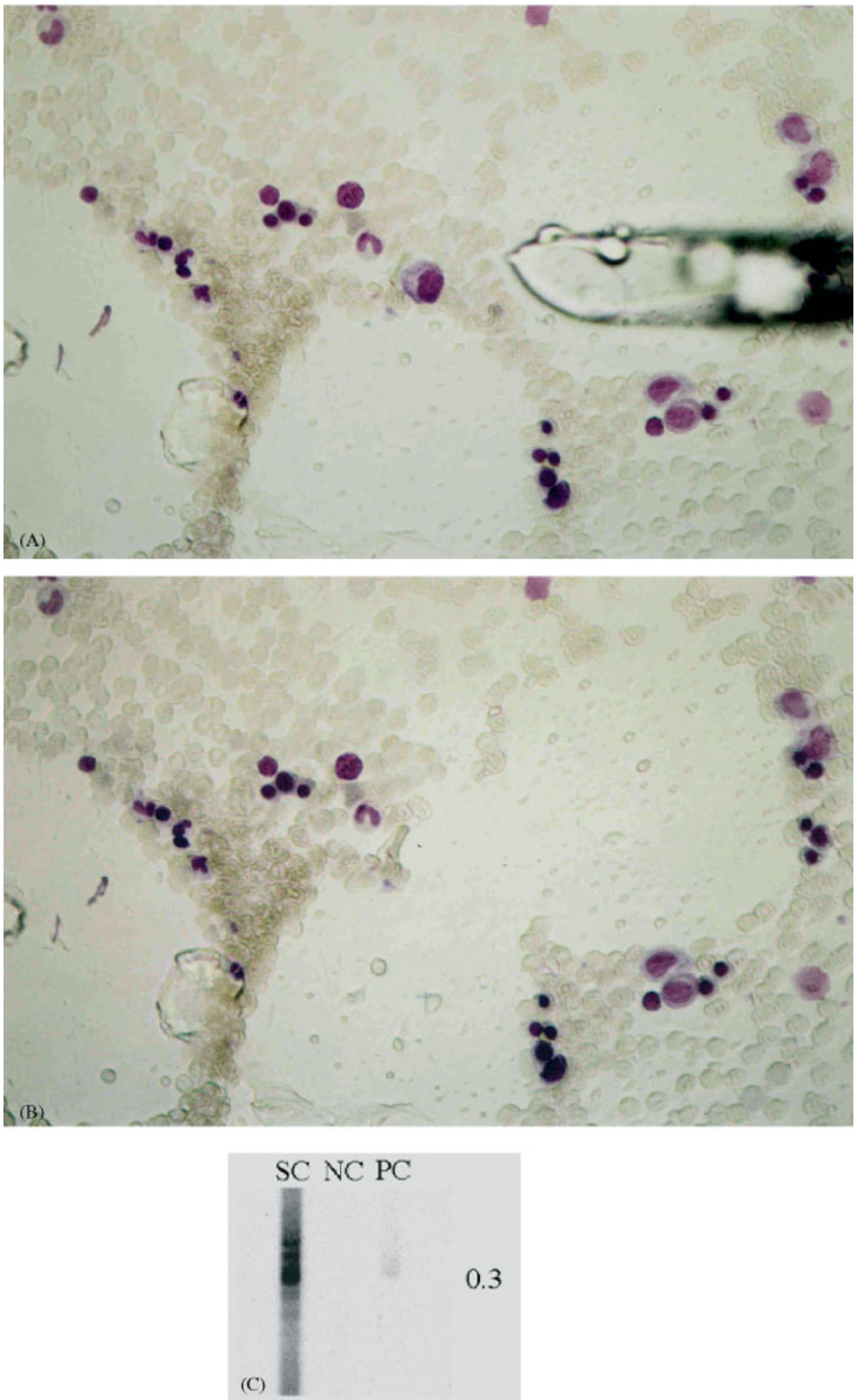

Fig. 1. Isolation of a single cell from an 8-month-old stained bone marrow smear of a 73-year-old woman. **a:** The dissection capillary is pointing at a promyelocyte. **b:** The promyelocyte is harvested, while the surrounding cells remain intact. **c:** β-actin amplicon of this promyelocyte extracted by organic lysis. Separable additional unspecific bands are also observed. Lanes: SC, single cell; NC, negative control; PC, positive control; size in kb.

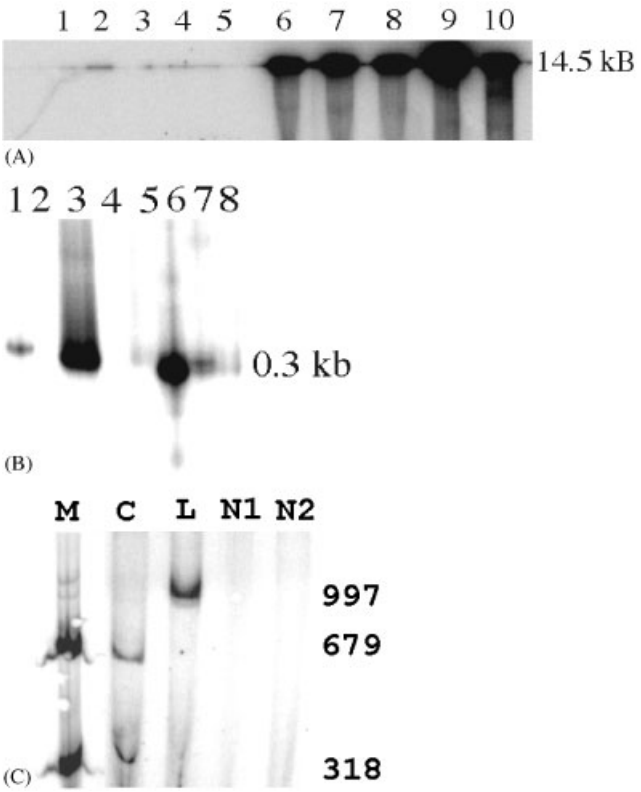

Fig. 2. **a:** XL-PCR analysis of the mitochondrial genome from single cells of a 25-month-old bone marrow smear from a 72-year-old individual with anemia. The 14.5 kb PCR products were labelled and detected as outlined in the text. Thermic lysis lanes: 1–promyelocyte, 2–erythron. Alkaline lysis lanes: 3–eosinophilic granulocyte; 4–polychromatic normoblast; 5–lymphocyte. Organic extraction lanes: 6–lymphocyte; 7–band-form neutrophil; 8–megacaryocyte. Positive controls lanes: 9–muscle mtDNA from control; 10–muscle mtDNA from control. **b:** Demonstration of the high amplification efficiency using β-actin as an example. The amount of amplicon varies in different cells. Single cells of a 54-year-old patient with iron deficiency anemia from a 14-month-old bone marrow smear are organically extracted. Lanes: 1–erythroblast; 2–erythron; 3–promyelocyte; 4–promyelocyte; 5–metamyelocyte; 6–metamyelocyte; 7–lymphocyte; 8–human male DNA as control. **c:** Autoradiograph of the amplified 1.12 kb region encompassing parts of the NADH Dehydrogenase subunit 4 gene. Restriction fragments of 679 and 318 bp (wild-type) as well as 997 bp (mutant) are shown (122 bp short fragment not shown). Single cells were picked from the bone marrow smear of a 36-year-old patient with centroblastic non-Hodgkin's lymphoma and LHON as well as a control smear. Single cells were organically lysed. M, megacaryocyte; C, muscle mtDNA from control; L, lymphocyte; N1, negative control; N2, buffer put an the patients smear after cell picking; sizes in bp.

inconsistent amount of amplicon (Fig. 2b), which does not correlate with the cell type. Contaminating proteins as well as the inability to extract good quality template DNA may also decrease the efficiency to amplify DNA extracted by simpler rough methods. Boiling makes long amplifications ineffective by introducing numerous breaks into mtDNA and may favor the amplification of nucleus-embedded mtDNA pseudogenes.

In conclusion, our study showed reproducible PCR results from archival stained bone marrow slides at the single cell level applying organic extraction procedures.

ACKNOWLEDGMENT

I thank Dr. Buse (Professor of Biochemistry, RWTH Aachen, Germany) for making the mitochondrial genetics laboratory available to our work. I thank Dr. Osieka (Professor of Hematology and Oncology, RWTH Aachen, Germany) for the gift of samples and also for critically reading the manuscript. I would like to thank Dr. Galm and Dr. Lorenzen for helpful comments regarding the manuscript. The excellent technical assistance of Roswitha Anglmayer is acknowledged.

REFERENCES

1. Becker I, Becker KF, Rohrl MH, Hofler H. Laser-assisted preparation of single cells from stained histological slides for gene analysis. Histochem Cell Biol 1997;108:447–451.
2. Gravel S, Delsol G, Al Saati T. Single-cell analysis of the t(14;18)(q32;q21) chromosomal translocation in Hodgkin's disease demonstrates the absence of this translocation in neoplastic Hodgkin and Reed-Sternberg cells. Blood 1998;91:2866–2874.
3. Kuppers R, Zhao M, Hansmann ML, Rajewsky K. Tracing B cell development in human germinal centers by molecular analysis of single cells picked from histological sections. EMBO J 1993;12:4955–4967.
4. Roehrl MH, Becker KF, Becker I, Hofler H. Efficiency of single-cell polymerase chain reaction from stained histologic slides and integrity of DNA in archival tissue. Diagn Mol Pathol 1997;6:292–297.
5. Fey MF, Pilkington SP, Summers C, Wainscot JS. Molecular diagnosis of haematological disorders using DNA from stored bone marrow slides. Br J Haematol 1987;67:489–492.
6. Grunewald K, Lyons J, Hansen-Hagge TE, Janssen JW, Feichtinger H, Bartram CR. Molecular genetic analysis of DNA obtained from fixed, air dried or paraffin embedded sources. Ann Hematol 1991;62:108–114.
7. Schoch R, Jenisch S, Haferlach T, Muller Ruchholtz W, Gassmann W, Loffler H. Glass slide smears are a suitable source for RT-PCR-based analysis of chromosomal aberrations in leukemias. Br J Haematol 1996;92:140–142.
8. Vince A, Poljak M, Seme K. DNA extraction from archival Giemsa-stained bone-marrow slides: comparison of six rapid methods. Br J Haematol 1998;101:349–351.
9. Miyagi T, Murakami K, Sawada T, Taguchi H, Miyochi I. A novel single cell PCR assay: detection of human T lymphotropic virus type I DNA in lymphocytes of patients with adult T cell leukemia. Leukemia 1998;12:1645–1650.
10. Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW, Wallace DC. Myoclonic epilepsy and ragged-red fibre disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. Cell 1990;61:931–937.

11. Zanssen S, Buse G. Successful chemotherapy in a male patient with malignant lymphoma and Leber's hereditary optic neuropathy (LHON). Am J Hematol 2003;72:263–266.
12. DiMauro S. Lessons from mitochondrial DNA mutations. Semin Cell Dev Biol 2001;12:397–405.
13. Gattermann N. From sideroblastic anemia to the role of mitochondrial DNA mutations in myelodysplastic syndromes. Leuk Res 1999;24:141–151.
14. Cheng S, Higuchi R, Stoneking M. Complete mitochondrial genome amplification. Nat Genet 1994;7:350–351.
15. Reynier P, Malthiery Y. Accumulation of deletions in mtDNA during tissue aging: analysis by long PCR. Biochem Biophys Res Commun 1995;217:59–67.