ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 04, pp.14901-14908, April, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

APPLYING GENETIC TEST ON THE SINGLE CELL

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ARTICLE INFO

Article History:
Received 17th January, 2015
Received in revised form
22nd February, 2015
Accepted 27th March, 2015
Published online 30th April, 2015

Key words:
Single cell,
Cell lysis,
PCR,
DNA,
Allele dropout.

ABSTRACT

The molecular genetic analysis of microdissected cells by laser, a method for selecting a starting material of pure DNA or RNA uncontaminated. Our study focuses on technical pre-PCR (polymerase chain reaction) for the amplification of DNA from a single cell (leukocyte) isolated from human blood after laser microdissection and aims to optimize the yield of DNA extracted of this cell to be amplified without errors and provide reliable genetic analyzes. This study has allowed us to reduce the duration of cell lysis in order to perform the step of expanding genomic PEP (primer extension preamplification) directly after lysis the same day and the quality of genomic amplification and eliminate purification step of the product PEP, step with a risk of contamination and risk of loss of genetic material related to manipulation. This approach has shown that the combination of at least 3 STR (short tandem repeat) markers for genetic analysis of single cell improves the efficiency and accuracy of PCR and minimizes the loss of allele (allele drop out; ADO). This protocol can be applied to large scale and an effective means suitable for genetic testing for molecular diagnostic from isolated single cell (cancerous - fetal).

INTRODUCTION

Molecular genetic analysis of microdissected cells by laser, a process that allows the selection of a starting material of pure uncontaminated DNA and RNA (Bowen *et al.,*2009; Umar A, *et al.,*. 2009; Gagnon *et al.,*2010), this analysis was made possible by several molecular techniques: PCR (polymerase chain reaction), RT-PCR (reverse transcription polymerase chain reaction) and FISH (fluorescence in situ hybridization). The PCR amplifies trace amounts of DNA (eg DNA of a single cell), the quality of DNA after microdissection controlled by laser is sufficient for most tests based on PCR (Aaltonen *et al.,* 2011) and has been used for biomedical research, molecular biology and diagnosis of cancer (Rook *et al*., 2004) and monogenic genetic diseases: sickle cell anemia (Cheung *et al.,*1996) or other pathology of hemoglobin (Camaschella *et al.,*1990), SMA (Sekizawa *et al.,* 1996. Watanabe *et al.,*1998), ornithine transcarbamylase deficiency (Bianchi and al.1992). However, the development of techniques for extraction and amplification of cellular DNA is a challenge especially for molecular diagnostics carried out from a single cell, so the optimization of these techniques has become a necessity to simplify and improve the effectiveness and efficiency of reliable genetic information. The amplification characteristics of DNA derived from a microdissected single cell are different from those of extracted DNA from large amounts of many cells.

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The loss of allele "allele dropout" (ADO) is the extreme form of preferential amplification often encountered during the PCR of single cells. The ADO occurs randomly with equal probability for both alleles. Moreover, this phenomenon is sporadic and does not occur in all amplifications of single cells (Garvin *et al*., 1998). It has been shown that there is a correlation between cell lysis and the occurrence of ADO (Kim *et al*., 2009). The factors involved in this phenomenon may be due to the alteration of the genome during cell lysis. Our study aims pre-PCR techniques and aims to optimize the yield of extracted DNA from a single cell isolated from human blood after laser microdissection to be amplified without errors and provide reliable genetic analyzes.

MATERIALS AND METHODS

The study was performed on leukocyte cells fixed on a filter (porous polycarbonate membrane), the peripheral blood (collected in EDTA) was diluted 1:10 with buffer lysis of red blood cells, which also sets the cells, then, blood is left at room temperature for 10 minutes before filtration (Mouawia *et al.,*2012;. Saker *et al.,* 2006; Beroud, *et al.,* 2003; Vona *et al.,* 2002; Vona *et al.,* 2000). Mild aspiration of different dilutions is carried out by a vacuum pump on the porous membrane. The cells in one ml of blood and whose diameter is greater than the pore size of the filter (8µm) are found spread over the filter surface. When the entire sample is passed through the filter, the membrane is rinsed with 1 ml of PBS (phosphate-buffered saline) and dried in air and finally colored by mixture of hematoxylin (dye nucleus) and 2% eosin

(cytoplasmic dye). This staining allows to visualize by microscopy, adherent cells on the membrane. At the end of these treatments, the filters are individually stored at - 20 ° C. By targeted laser microdissection using the NIKON system (Figure 1) interesting cells may be subject to molecular analysis by PCR to clarify conclusively the origin and nature of the cell for the diagnostic or scientific study.

Lysis of the cell single

Lysis can be achieved by different approaches. Alkaline lysis (Zhang *et al.,* 1992) is performed by applying on the cell 5 µl of a solution containing 200 mM KOH and 50 mM dithiothreitol, the assembly is subjected to a 10 min incubation at 65 ° C, the lysate is then neutralized with a buffer (900 mM Tris-HCl, 300 mM KCl). Enzymatic lysis is carried out in a final volume of 15µl (100 mM Tris-HCl pH 8 and 1mg/ml of proteinase K). Both approaches release DNA from their proteic environment, but no extraction is performed in order to minimize losses of the genome.

Accession to lysis of the cell

Access to the genetic information of the cell, protein lysis of each microdissected cell is performed. Product of microdissection is directly contacted with the lysis solution. Indeed, the two main methods of cell lysis performed on single cell are alkaline lysis (KOH) and enzymatic lysis (proteinase K). The first method is the method of choice, to perform lysis on fresh cells (Kim *et al.,* 2009), obtained to perform a genetic test because time is very short (10 minutes at 65 ° C). This lysis is normally done at the bottom of the PCR tube, since recovered cell is visualized under a microscope and transferred to the bottom of the tube, which ensures direct contact with the lysis buffer even if the final volume of lysis buffer is little (5 µl or 3 µl in some laboratories). However, in the case of fixed cells on membrane or filter, we are forced to perform lysis of the cell attached to the cap (adhesive membrane) of tube in an incubator and the tubes are turned upside down, hence the need of using a higher volume of lysis buffer (15 uL) to ensure contact between the lysis medium and the cell. Moreover, the isolated cells are not fresh cells, which makes time long to provide a "complete" cell lysis.

The enzymatic lysis protocol

The study of enzymatic lysis by proteinase K applied to single cells comprises the composition of the lysis buffer, the lysis time and temperature of incubation. Enzymatic lysis was performed in 15 µl final volume at 37 ° C for 16 h (Vona *et al.,* 2002 Beroud *et al.,* 2003). Despite the demonstrated effectiveness of the lysis protocol, we have realized that the incubation period was long (overnight). This can reduce and alter the cellular genome, and the risk of allele dropout during PCR. The challenge for us was the possibility to reduce the duration of lysis in order to perform genomic preamplification directly after cell lysis on the same day and improve the quality of amplification. To do this, we tested several protocols without changing the composition of the lysis buffer (15 µl). The tested cells derived from the same male individuals for whom blood was treated as described above, leukocytes "selected" on the filter were stained with hematoxylin - eosin (HE) and microdissected by NIKON system. For each protocol, we have microdissected 100 cells. The amplification efficiency was assessed by the number of unicellular genomes amplified by PCRY.

Linear expansion of the genome of a single cell

After cell lysis is completed, the DNA of the cell is linearly amplified in a final volume of 60 µl.This expansion step of the DNA called PEP (Primer Extension Preamplification) uses a degenerate primer N 15, consisting of a degenerate base to each of the 15 positions (Dietmaier *et al.,* 1999; Zhang, *et al.,* 1992).With 15 µl of the product of cell lysis are added 5 µl of a solution at 400 µ M of degenerate primers (Kit gen PEPtm 75OD, Genetix, Boston, USA), 6 µl of 10 X PCR buffer without K + (25 mM MgCl2 / 1 mg gelatin / ml / Tris-HCl, pH 8 100 mM), 3 µl of a mixture of 4 dNTP (2 mM each) and 5 units of Taq polymerase (Applied Biosystems). The final ensemble is completed with sterile water to a reaction final volume at 60 µl. The expansion reaction requires 50 cycles of amplification, each cycle consists of a step of one minute at 94 ° C, a step of 2 minutes at 37 ° C, a gradual rise in temperature or ramping of 37 ° C at 55 ° C (0.1 °C / sec), a step for 4 minutes at 55°C and then a step of 30 seconds at 72 ° C. This preamp is performed with a thermal cycler Gene-amp type 9700® (Applied-Biosystems). PEP Product was the subject of a precipitation and concentration step (Vona *et al.,* 2002). This step aims to concentrate and purify the very low amount of DNA obtained by PEP. For Total volume of the expansion product 10 µl of NaCl (3M) and 3 volumes of refrigerated ethanol 100% (v / v) are added. The complex, incubated for 30 minutes at - 80 ° C, was centrifuged for 30 minutes at 15,000 rpm at 4 ° C and the supernatant was removed. After washing with 70% ethanol (v / v) of the pellet obtained, evaporation of residual liquid follows. At the end the dry residual was taken up in 10 µl of sterile water to be stored at -20 ° C. PCR amplification is then performed from 2 µl of purified product of PEP. Thus, the maximum number of PCRs that could be made from each purified PEP product is 5 PCRs. We considered eliminating the step of purification of the PEP, knowing the need to avoid such a step with a risk of contamination and a risk of loss of genetic material related to manipulation. In addition to that, removing this step allows us to gain more time. Unpurified product PEP (60 µl) must be divided into aliquots (10 aliquots each 6µl), which allows on one hand to minimize the risk of contamination and also to increase the number of PCRs that can be performed.

Optimization of the amplification step (nested PCR)

Amplification of microsatellite regions was confirmed on agarose gel, the PCR products were analyzed by sequencing. The integrity of the genome of the cell must be maintained in order to achieve a fine molecular analysis of the cell. A potential for increased sensitivity of PCR on single cells means is to apply the technique of nested PCR (Handyside *et al.,* 1990). Which are successive PCR for amplifying a specific sequence, a first PCR is carried out followed by a second PCR product of the first increase to the specificity ratio. This technique increases the sensitivity (102 -103) compared to the single PCR and the specificity and minimizes the effects of

inhibition of the first PCR. So in order to test this hypothesis, we configured external primers for the first PCR, capable of amplifying an area that can serve as support for internal amplification labeled primers (5'). We tested 100 leucocytes from the blood of five individuals. The blood of each individual was treated as described above. Leukocytes that remain on the porous membrane after filtration are colored by the hematoxylin eosin (HE). Then, using the NIKON laser microdissection system, we microdissected individual leukocytes well insulated. Cell lysis was performed by the enzymatic method (proteinase K) in a final volume of 15 mu.l. After incubation for two hours at 60 ° C, proteinase K was inactivated for 15 minutes at 94 ° C. After cell lysis is completed, the DNA of the cell is linearly amplified in a final (Zhang, *et al.,* 1992) volume of 60µl According to Zhang, the probability of having, by PEP, at least 30 copies of each genomic sequence is about 80%. Therefore, several PCRs can be made from a product of PEP from the same cell. Thus, to avoid any possible contamination, the product of each leukocyte microdissected PEP was divided into aliquots (6 mu.l each) were used as genetic material for possible PCR reactions. Microsatellite genotyping of single cells attached, requires solving the following problems: it requires both the identification of primers STR (short tandem repeat), ie able to amplify the genome of a single cell, and to obtain a minimum of allele dropout (ADO). We have tested and developed several microsatellite primers used in nested PCR. The development includes the choice of primers having high heterozygosity, the choice of marking for primers (internal), the reaction mixture and amplification conditions, especially regarding temperature and duration of annealing.

For the sake of simplicity, here we present the results concerning the microsatellite marker D7S486.These primers flanking the repetitive nucleotide sequence (CACA) on chromosome 7 (Figure 2). The outer primers (Figure 2, in red) to amplify a fragment of 228 base pairs (bp), whereas the internal fluorochrome labeled primers (6-FAM), which is associated with the sense primer (or forward) to amplify a fragment of 145 bp (Figure 2, in blue).

The first reaction: From 6 µl of PEP Product from isolated leukocytes, we completed three reactions, each in a different final volume of the other. The first is performed in 100 µl, 60µl in the second and 40 µl in the third. The final concentrations for all reagents in the reaction mixture were identical (10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl2, 200 mM of each dNTP, 0.5 mM of each external marker and 2U of Taq Gold). Then, the reactions were performed with a thermal cycler of Gene-amp 9700® type (Applied-Biosystems) according to the standard profile (initial denaturation at 94 ° C for 5 min, 40 cycles consisting of denaturation at 94 ° C for 30 seconds, hybridization at 55 ° C for 30 seconds and elongation at 72 °C for 30 seconds. Finally, a final elongation step at 72 ° C for 5 minutes). After migration on an agarose gel (2%) of PCR products (a volume of 10µl of each product is deposited), we observed that in the case of PCR performed in a final volume of 60 µl, results (number of unicellular genomes and amplified signal strength of the gel) were better than those obtained with the other PCRs.

Reaction Mixture: The final concentration of each reagent in the reaction mixture is the factor key that may be the aim of obtimization of PCR. Among these reagents, we focused on the development of the final concentration of MgCl2 and primers. Thus, from 6 µl of PEP Product from 50 isolated leukocytes, and in a final volume of 60 µl, we conducted a series of PCRs with three different final concentrations of MgCl2, 1.5 mM, 2 mM and 2.5 mM and with two different primers for final concentrations, 0.25 and 0.5 microns.

The temperature and duration of hybridization: We tested three different annealing temperatures, 55 ° C, 58 ° C and 61 ° C for two times, 30 seconds and 45 seconds.

The second reaction: The product of the first PCR-STR nestled we developed the subject of a preamp stage by a second PCR using internal primers labeled 5 'to amplify the fragment interest (see Figure 2 as an example for the marker D7S486). Regarding the temperature and duration of hybridization, each internal primer was tested with several conditions. The second PCR reaction is performed in a final volume of 20 µl. 2µl of the first PCR product diluted 1:10 are used as genetic material (dilution is to avoid excess DNA from the first PCR).

For example, the marker D7S486, we used the internal labeled primers to perform the second PCR reaction with temperatures and times different hybridization. This second reaction was carried out from the first PCR product previously developed.

RESULTS

Step pre-PCR: cell lysis and genomic expansion: We compared the amplification efficiency for three different lysis protocols performed at 37 ° C for 16 h (control), 4 hours, and 2 hours, respectively. In the latter 2 protocols, the PEP was performed directly after lysis (the same day) as opposed to the control case. We have not seen any improvement, but a decrease in amplification efficiency in the last two protocols compared to the control protocol. We concluded with incubation at 37 ° C, with proteinase K is more effective than the increasing of lysis time. This finding allowed us to combine the factor "temperature and duration ". Thus, we conducted 4 lysis protocols at 50 ° C and 60 ° C for 4 hours and 2 hours, respectively. These protocols were also compared with that of the control (at 37 ° C for 16 h). We saw that there was no significant difference between these protocols (Table 1). These results allowed us to conclude that the effectiveness of proteinase K is also obtained with a short incubation period when the temperature is relatively increased. Thus, we selected the cell lysis protocol for 2 h at 60 ° C as it saves a lot of time without altering the amplification efficiency. Finally, we can deduce that we are able to achieve all the above PCR steps in one day. Figure 3 shows a comparison test between the purification and cleansing of the non-product preamp (PEP). The loss of genetic material related to the purification is shown.

Evaluation of nested PCR: In the first PCR reaction, the final concentrations of $MgCl_2$ and 2.5 mM of each primer at 0.5 µM had the best results (Table 2). As for the marker D7S486 (external), the temperature and duration of optimal hybridization are 55 ° C and 45 seconds, respectively

(Table 3). In the second PCR reaction, the final concentrations of reagents in the reaction mixture were demonstrated identical to those developed for the first PCR reaction (especially $MgCl_2$ 2.5 mM of each primer and 0.5 microM). We observed an amplification of 84% of leukocytes (42/50) with a temperature of 55 ° C and a duration of 30 seconds. Evaluation of development of the nested PCR (STR) comprises firstly amplification efficiency compared to a single PCR reaction (number of unicellular genomes amplified signal strength on an agarose gel) next to the accuracy of the PCR (amplification of the two alleles, no ADO). As for the STR-PCR, we observed with the marker D7S486, an amplification efficiency increased for the second PCR (42/50, 84%) compared to the first (37/50, 74%). Figure 4 compares the two reactions of the nested PCR-STR (marker D7S486), the first reaction was performed from 6µl Product PEP 8 from leukocytes. The signals of the second PCR reaction were intense relating to those of the first PCR which were low (Figure 4). In conclusion, nested PCR-STR is the method of choice to increase the efficiency and PCR signal on single cells. From 42 Leukocytes whose genome were amplified, there were 34 (81%) with two allelic profile (19% ADO). The efficiency and accuracy of the amplification were subjected to an optimization step in order to maximize the number of genomes amplified and minimize the loss of allele.

cells. We performed nested PCR and evaluated the effectiveness of amplification migration of the second PCR product on a 2% agarose stained with ethidium bromide gel. From the product of PEP (6 µl for each PCR), the first reaction is performed in a final volume of 60 µl We then took 2 µl of diluted product of the first PCR to achieve 1/10th second PCR reaction 20 µl final volume using labeled internal markers. Regarding the PCR-STR, we studied the efficiency of PCR performed with three STR markers (M1, M2 and M3), the heterozygosity was confirmed on 50 unique leukocyte microdissected cells. We obtained amplification efficiencies of 80% (40/50), 76% (38/50) and 72% (36/50) for STR markers M1, M2 and M3 respectively. When we combined the results obtained with at least 2 STR markers (M1 + M2 + M3 or M1 or M2 + M3), efficiency has increased on average to 88%. Finally, the combination of the results of three markers (M1 + M2 + M3) has brought efficiency to 93%. We concluded that the use of 3 STR markers provides a very high efficiency of the PCR genotyping (STR) rates. The results showed an efficiency of amplification in 91 cells (91%).

Accuracy of PCR genotyping (STR): We then evaluated the accuracy of PCR (the percentage of single cells whose DNA was amplified without ADO) by STR primers, by fragment analysis using the automated sequencer ABI Prism 3100.

Table 1 . Development of enzymatic cell lysis protocol

Temperature(C°)	Duration (hours)	No of cells	Nb of positive cells by Y PCR (%)	Difference of control tube
37	16h	100	80 (80%)	
37	4h	100	70 (70%)	NS
37	2h	100	72 (72%)	NS
50	4h	100	82 (82%)	NS
50	2h	100	81 (81%)	NS
60	4h	100	79 (79%)	NS
60	2h	100	84 (84%)	NS

NS: non significance

Table 2. Development of final concentrations of $MgCl_2$ and STR primers

	Final Concentration		Nb of positive cells in 1st PCR D7S486
PCR	$MgCl_2$ (mM)	primer (µM)	(%)
1	1.5	0.25	29 (58%)
2	1.5	0.5	28 (56%)
3	2	0.25	33 (66%)
4	2	0.5	32 (64%)
5	2.5	0.25	33 (66%)
6	**2.5**	0.5	35 (70%)

Table 3. Development of temperature and the duration of hybridation of external STR primers (D7S486)

	Hybridation conditions		Nb of positive cells in 1st PCR D7S486 (%)
PCR	T° (°C)	Duration (")	
1	55	30	34 (68%)
2	55	45	37 (74%)
3	58	30	34 (68%)
4	58	45	35 (70%)
5	61	30	30 (60%)
6	61	45	30 (62%)

Optimizing the efficiency and accuracy of PCR

Efficiency of PCR genotyping (STR): We first evaluated the effectiveness of PCR (the percentage of single cells whose DNA was amplified) by STR primers, from microdissected

From the product of the second PCR-STR of each marker (M1, M2 and M3), we conducted an analysis of fragments by genotyping.

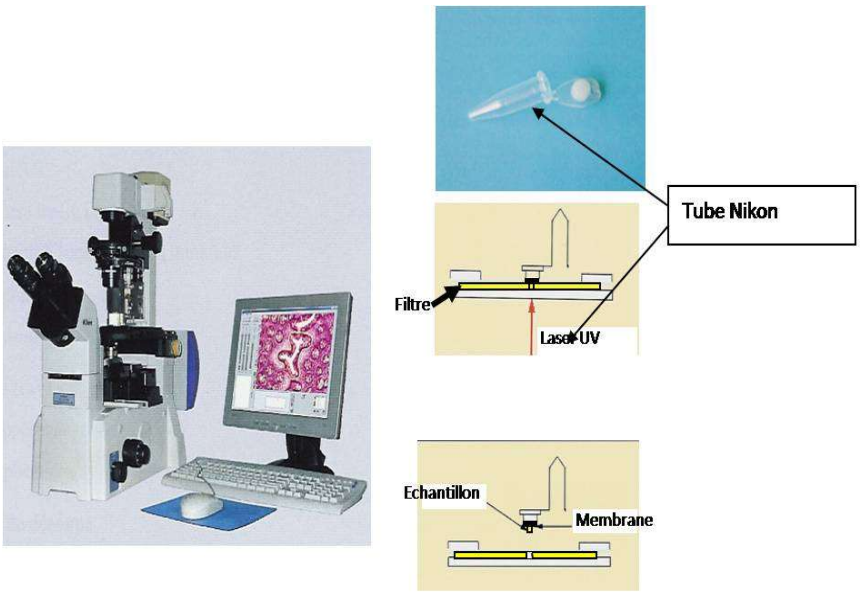

Figure 1. Isolation of single cells by laser microdissection (Nikon® System)

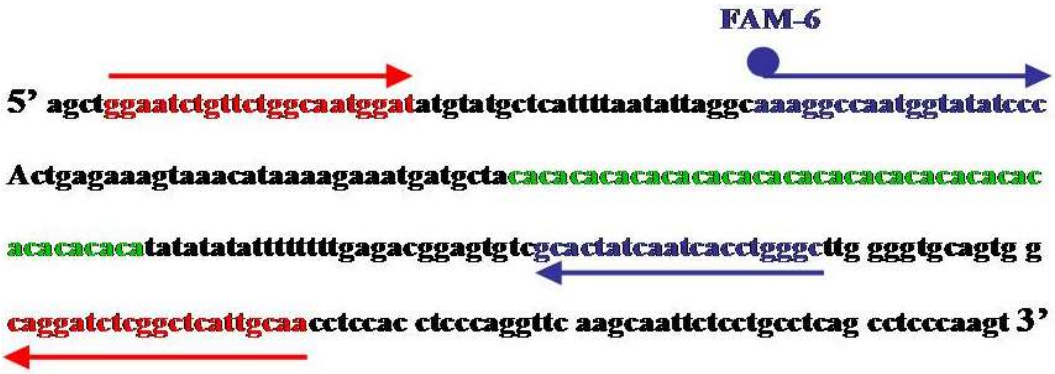

Figure 2. Repetitive nucleotide sequence CACA (green) flanked by D7S486 primers, external (red) and internal labeled at 5' (blue)

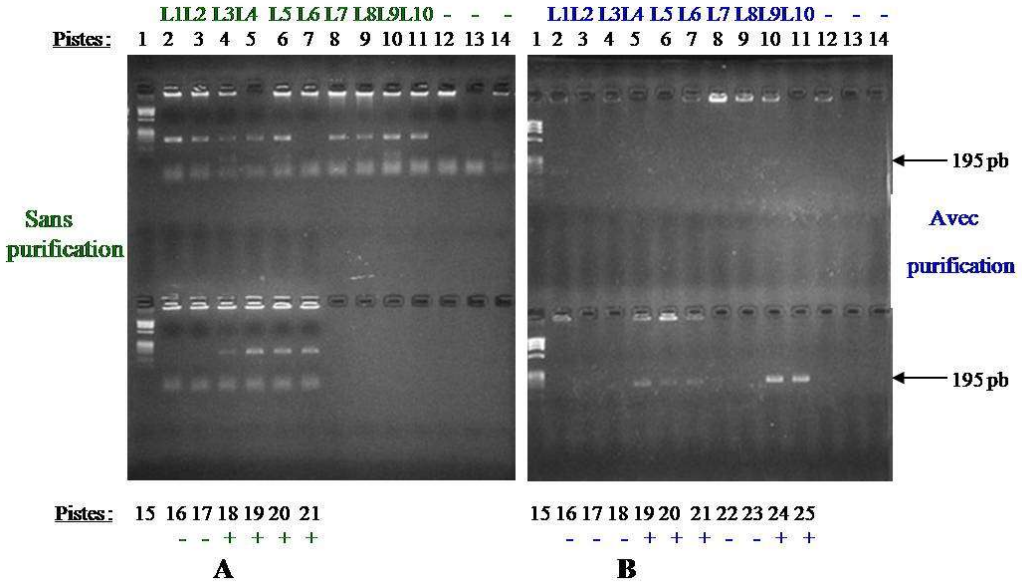

Figure 3. Comparative test between non purified and purified product of PEP

A: Migration on agarose gel 2% of the SMN1 PCR (a single reaction) made from 6µl of unpurified PEP product from 10 lymphocytes. The size marker is placed in track 1, tracks 2-11 correspond to the amplification of 10 cells L1 to L10, track 12 matches negative control tube microdissection, tracks 13 and 14 present the negative control tube of lysis, track 16 corresponds to the negative control of PEP, the track 17 to the negative control of SMN1 PCR, tracks 18, 19 and 20 correspond to the positive controls of PEP (100 pg) and finally the track 21 has a positive control of PCR (10 ng). 10 µl from 20 µl of PCR product are deposited on gel

B: Migration on agarose gel 2% of SMN1 PCR (a single reaction) formed from 2 µl l purified solution of PEP of the same 10 cells used in the previous approach. Purification was carried out from 54 µl remaining product PEP of each cell, the recovery is made in 8 µl of sterile water. Tracks1 to 17 are identical to those of Figure 3 A. The track 18 corresponds to the control negative of purification; tracks 19, 20 and 21 show positive controls PEP (100 pg); the tracks 22 and 23 negative controls PCR; and tracks 24 and 25 to positive controls for PCR (10 ng). 10 µl from 20 µl of PCR product are deposited on gel

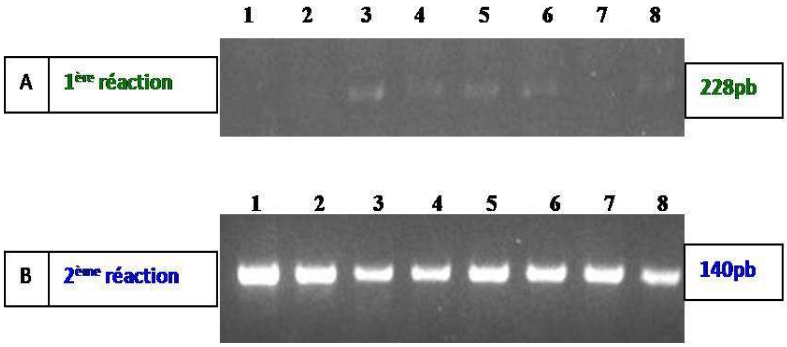

Figure 4. Comparative test between the 2 reactions of nested PCR-STR

A: Migration on agarose gel (2%) of the 1st PCR reaction D7S486 from PEP product of 8 leukocytes.
B: Migration on agarose gel (2%) of the 2nd PCR reaction D7S486 from the product of the first reaction (A).

The heterozygous profile of these three markers allowed us to identify the proportion of amplification giving origin to the ADO. Our results showed that ADO was observed in 20% (8/40), 15% (6/38) and 17% (6/36) of PCR with markers M1, M2 and M3, respectively, which means accuracy amplification 80%, 85% and 83% respectively. When we combined the results obtained with at least two STR markers (M1 + M2 + M3 or M1 or M2 + M3), the percentage of ADO decreased on average to 10% (accuracy amplification of 90%). Finally, the combination of the results of three markers provides the accuracy of the PCR on average in 95% of cases. We concluded that for a very high probability (95%) STR biallelic profile (ie d. Absence of ADO) in at least one STR PCR, use at least three STR markers on the product PEP in the same cell.

DISCUSSION AND CONCLUSION

Technical exploiting physical and biological properties of the cells were used to enrich circulating fetal cells from maternal blood: the density gradient centrifugation (Al-Mufti *et al.,* 1999; Bianchi *et al.,* 2002; de Graaf *et al.,* 1999; Sitar *et al.,* 2005. van Wijk *et al.,* 1996), the selective lysis of nucleated erythrocytes (Voullaire *et al.,*2001), flow cytometry (FACS: fluorescent-activated cell sorting) (Bianchi *et al.,*1990; Bianchi *et al.,*1993; Herzenberg *et al.,*1979; Iverson, *et al.,*1981; Price *et al.,*1991; Simpson.....and Elias, 1993; Wachtel *et al.,*1991) and separation by immunomagnetic beads (MACS magnetic-activated cell sorting) (Ganshirt-Ahlert *et al.,*1993; Ganshirt-Ahlert *et al.,* 1992). The last two techniques exploit antigenic differences between the cells to select the cells of interest. However, there is no specific surface antigen of fetal erythroblasts. It is still not possible to identify cells of fetal origin on the basis of immunostaining of cell surface antigens (Ho *et al.,* 2003; Tjoa *et al.,* 2007). Advances in molecular biology techniques makes genotype analysis possible for one or more cells. This can be used in preimplantation genetic diagnosis, not only of chromosomal aneuploidy, but also monogenic syndromes and hereditary cancer diseases (Raszyková, *et al.,* 2012. Zanssen, 2004 Rook *et al.,* 2004).

This study contributes to the development and optimization of a technique that is applicable at a large scale and can be an appropriate effective way to genetic testing and molecular diagnostics from the single cell attached to porous membranes. It shows the functional role of the development of pre-PCR steps. The optimization of this procedure allowed us to reduce the time of lysis in order to perform the PEP preamp directly after lysis the same day and improve the quality of genomic amplification and eliminate the step of purification of the preamplifier PEP, given the need to avoid such a step with a risk of contamination and a risk of loss of genetic material related to manipulation. Furthermore, the elimination of this step allows us to gain more time. This approach has improved the efficiency of PCR in order to maximize the number of genomes amplified minimize allele dropout (ADO) and eliminate the risk of contamination, the genetic test result (from the single cell) based on PCR techniques has more accuracy and reliability of genomic information. We believe that this molecular technique of single cell can be applied to try the clinical and molecular diagnosis of cancer or fetal cells.

Acknowledgement

We thank INSERM (National Institute of Health and Medical Research) and the Medical Genetics Laboratory at the Necker-Enfants Malades Hospital, Paris - France) for their help in the realization of this work. This work was supported by grants from INSERM, Université Paris Descartes.

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