A Protocol for Rapid and Inexpensive Genotyping of Mutant Mice from Dried Blood Spots: An Update

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Methodology

Keywords: Dried Blood Spots, genotyping, genomic DNA extraction, SNP-RFLP

Posted Date: September 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-895667/v1>

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Abstract

Background: Dried blood spot (DBS) testing is a well-known method of bio-sampling by which blood samples are blotted and dried on filter paper. The dried samples can then be analyzed by several techniques such as DNA amplification and HPLC. We have developed a homemade DBS method followed by an alternative protocol for genomic DNA extraction from a drop of blood adsorbed on paper support. This protocol consists of two separate steps: (1) organic DNA extraction from the DBS, followed by (2) DNA amplification by polymerase chain reaction (PCR). The PCR-restriction fragment length polymorphism (PCR-RFLP) is an advantageous and simple approach to detect single nucleotide polymorphisms (SNPs).

Results: We have evaluated the efficiency of our method for the extraction of genomic DNA from DBS by testing its performance in genotyping mouse models of obesity and herein discuss the sensitivity, specificity and feasibility of this novel procedure.

Conclusions: Our protocol is easy to perform, fast and inexpensive and allows the isolation of pure DNA from a miniscule amount of sample.

Background

DNA extraction is a routine procedure of molecular biology used in research laboratories and forensic analyses. The process is composed of a combination of physical and chemical steps [1]. Solution-based DNA extraction methods using organic solvents consists of the organic extraction and subsequent salting-out. The chemical steps include lysis, phenol-chloroform extraction, ethanol precipitation and washing. Organic extraction is mostly used in laboratories because it is affordable and allows the production of large quantities of pure DNA. The salting out method exploits the dependence of protein solubility on their chemical-physical characteristics, temperature, pH and saline concentration of the solution. Low salt concentrations increase protein solubility (salting-in), while high salt concentrations (e.g.: NaCl 3M) decrease it (salting-out) leading to protein precipitation. These two methods involve the use of toxic chemicals, like phenol and chloroform, and increase the risk of contamination because of the DNA transfer between multiple tubes. Other procedures of DNA isolation, such as commercially available kits (e.g. "QIAamp DNA Mini Kit" from Qiagen, Germany or "PureLink Genomic DNA Mini Kit" from Invitrogen, USA), Chelex 100 extraction (a chelating resin with a high affinity for polyvalent metal ions such as magnesium) and Solid Phase Extraction (SPE) are based on chromatographic principles. These methods differ in quality and quantity of extracted DNA but are all very expensive and poorly reproducible.

The choice of the most suited DNA extraction method depends on several factors, including cost, time, easiness of execution, quality and quantity of DNA isolated, safety and risk of contamination. In addition, the type of sample from which the DNA is extracted must also be considered [2, 3]. DNA can be extracted from diverse sources of samples through different methods that depend on the selected tissue. In

animals, peripheral blood leukocytes are the usual source of genomic DNA [4]. The most common blood collection procedures in mice (e.g. withdrawal from the orbital sinus) are usually painful and stressful for the animal; the performing personnel must be appropriately trained and compliant with guidelines for anesthesia and/or analgesic use and maximal sample volumes and phlebotomy frequency [5].

In detail, blood collection volume should not exceed 10% of total blood volume. Common DNA extraction methods require large amounts of blood (about 500 μ L), but mice have on average about 55 mL of blood *per* kg of bodyweight, so usually a mouse only has few milliliters of blood in total. For this reason, it is difficult to get satisfactory results with commercial kits.

Different mouse models are commonly used for the study of diabetes, including the non-obese diabetic (NOD) mice, a useful animal model for the study of the immunopathogenetic events leading to autoimmune type 1 diabetes development, and the obese mice (Lep^{ob} , referred to as *ob* or *ob/ob*) and their genotyping is of paramount importance. In the “single nucleotide polymorphism-restriction fragment length polymorphism” (SNP-RFLP) enzymatic method a region containing a specific SNP allele is targeted and amplified by PCR and subsequently digested using an endonuclease enzyme [6]; the length variation of the digested PCR fragments is then distinguished by gel electrophoresis [7]. It is a relatively simple, rapid, inexpensive, and convenient method frequently used for the detection of those small genetic differences between individuals which can lead to physiological or pathological changes [8–9].

In NOD/LtJ mice, a spontaneously G→T transversion mutation (designated as Lep^{db-5J}) occurs at position 640 of the leptin receptor gene (at the protein level, the mutation causes a valine-to-glycine substitution) and mutant mice become obese and hyper-insulinemic [10, 11].

We have here developed an up-dated method for the extraction of genomic DNA from mouse whole blood, obtained by puncturing the tail tip, and letting blood drops to be adsorbed and dry on Whatman 3 MM paper. This method may substantially facilitate SNP-based genotyping by presenting many advantages such as: cost reduction, the employment of a less invasive practice for blood sample collection in small laboratory animals and the necessity of reduced blood volumes (about 50 μ L); furthermore, it also allows easy blood transportation among laboratories. We have tested this method for NOD Lep^{db5j} mice genotyping.

Methods

Reagents

- Whatman 3 MM paper support
- HCl 0,4 N
- NaOH 10N
- SDS 10%

- CHCl_3
- LiCl 4M
- Ethanol absolute
- 10X PCR buffer (usually supplied by the manufacturer with the Taq polymerase)
- Taq polymerase (Taq DNA Polymerase, dNTPack, Sigma-Aldrich, #4728904001)
- dNTP mixture (dATP, dCTP, dGTP, dTTP; usually supplied by the manufacturer with the Taq polymerase; Sigma-Aldrich, #4728904001)
- PCR primers (forward and reverse): 5 μM in Tris/EDTA (TE) buffer; primers are usually obtained from Integrated DNA Technologies, Inc.
- Nuclease-Free Water (not DEPC-Treated) (ThermoFisher Scientific, #AM9932)
- Template DNA (see Experimental design for further information)
- Restriction enzyme *Hae* III (10U/ μL) (BioLabs, #R0108S)
- Restriction digestion buffer CutSmart 10X (usually supplied by the manufacturer with the restriction enzyme)
- Agarose (UltraPure™ Agarose, ThermoFisher Scientific, #16500500) CRITICAL Usually discriminates DNA fragments of less than 1,000 bp. Within a given range of differently sized DNA fragments, the smaller DNA fragments of less than 1,000 bp are best separated in TBE buffer
- Tris base (Sigma, #T6066)
- Boric acid (Sigma, #B7901)
- 0.5 M EDTA pH 8.0 (Sigma, #E7889)
- EtBr (0.5 mg mL^{-1} ; Sigma, #E8751; stock solution is prepared as 10 mg mL^{-1} in water and stored in a light-resistant bottle at room temperature).
- Bromophenol blue (Sigma, #B0126)
- Xylene cyanol FF (Sigma, #X4126)
- Glycerol (Sigma, #G5516)
- An optimal DNA size marker used in the analysis of PCR products, 1Kb Plus DNA Ladder (Invitrogen, #10787018)

Equipment

- Scissors
- Tweezers
- PCR thermal cycler
- Pipettors and tips DNase and RNase free
- Horizontal minigel electrophoresis apparatus (Sub-Cell GT Cell, BioRad)
- Thermostatic bath

Solutions

- TE buffer (pH 8.0) 10 mM Tris-HCl, 1 mM EDTA
- NaOH 0,1 N / SDS 1% solution
- 10X stock solution electrophoresis buffer (TBE), 1 liter: 108 g Tris base, 55 g boric acid, 40 mL 0.5 M EDTA, pH 8.0
- Gel-loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol, 1 mM EDTA (pH 8.0)

Samples

Individual blood samples were taken from NOD/ShiLtJ-Lepr^{db-5J}/LtJ mice through a puncture of the tail tip. For each animal a blood drop was collected on pieces of Whatman 3 MM paper support, placed in sterile boxes and left to dry for at least 24 hours at room temperature. The area of each drop was about 80 mm². In addition, a clean piece of paper support (without blood) was used as negative control to evaluate the contamination degree of the procedure, while genomic DNA obtained through classical organic extraction from mice tails served as positive control (25 ng of DNA for each PCR reaction).

DNA extraction

A blood drop adsorbed on a Whatman 3MM paper support was used as starting sample (Fig. 1).

- Scissors and tweezer were sterilized through a treatment in HCl 0.4 N for 5 minutes, then washed in water;
- One fourth of the drop was cut with scissors. Then, excess of paper, without mouse blood, was carefully removed and the sample placed in a 1.5 mL conical tube containing 40 µL of TE buffer.
- Firstly, samples were incubated at 65°C for 15 minutes, then another incubation at 95°C for 2 minutes was required to allow the detachment of blood from the paper.
- Samples were centrifuged at 14,500 g for 30 seconds and the supernatant transferred to new tubes.
- Forty µL of NaOH 0,1 N and SDS 1% solution were added on the pieces of paper in the first tube, in order to lyse cells, degrade RNA and denature proteins.
- The previous steps were repeated (incubations at 65°C for 15 minutes and at 95°C for 2 minutes and centrifugation at 14,500 g for 30 seconds) and then the new supernatant was added to the former.
- Samples were incubated at 95°C for 2 minutes once again.
- TE buffer was added to reach the volume of 100 µL and then 1 volume of chloroform (100 µL) was added to the samples to remove lipids and hydrophobic proteins from DNA.
- Samples were mixed by inversion for 10 minutes (to promote the extraction of more DNA) and then centrifuged at 14,500 g for 10 minutes (to promote the separation of aqueous from organic phase).

DNA precipitation and recovery

- Aqueous phase was transferred to new tubes and DNA was precipitated with LiCl 400 mM and 2.5 volumes of absolute Ethanol (final concentration 70%) (Fig. 2).

Lithium Chloride interacts with the phosphates of the DNA, while ethanol removes a solvation shell around the DNA, promoting its precipitation.

- DNA precipitation was carried out at -20°C for 1 hour or at -80°C for 20 minutes.

Samples can be stored overnight at -20°C.

- Samples were centrifuged at 14,500 g for 15 min, the supernatant was removed by a vacuum pump and DNA pellet was washed twice with ethanol 70 % (centrifugation at 14,500 g for 15 minutes is repeated)
- The DNA pellet was dissolved in 20 µL of TE buffer.
- DNA samples were stored at 4°C until further use or submitted to quantitative and qualitative analysis. The yield of DNA was approximately 3 micrograms.

DNA amplification by PCR

PCR-RFLP analysis always needs specific amplification primers pairs and appropriate restriction enzymes to identify the SNP in the PCR product. In this mouse model, *Lep^{db-5j}*, we have designed a primer pair that amplifies a part of the extracellular domain of mouse leptin receptor, including *HaeIII* restriction enzyme site, to identify the presence of a specific SNP (G→T transversion mutation). In particular, the amplified region comprised exon 13 (primers' sequences are shown in Fig. 1). Primer sequences were: Forward 5' -CGAGAACATGTGTCTGACTCAG - 3', Reverse 5' -GGTAATGACAGGGCTATGAACA - 3'.

The PCR reaction was set up in a final volume of 30 µL for each DNA sample and, to amplify more than one sample, a master reaction mixture was prepared in a 1.5 mL tube, then the mixture was aliquoted in 0.2 mL micro-amplification tubes, and each template DNA was added individually to PCR tubes. The total volume of the master mixture was calculated considering an extra 10% to account for loss of liquid during pipetting steps. The reaction mixture for 1 sample was prepared as follows:

Component	Initial Conc.	Final Conc.	Volume
PCR reaction buffer	10X	1X	3.0 μ L
dNTP mix	2 mM	200 nM	3.0 μ L
Primer forward	5 μ M	250 nM	1.5 μ L
Primer reverse	5 μ M	250 nM	1.5 μ L
Taq polymerase	5 U/ μ L	0.5 U/30 μ L	0.1 μ L
Sample DNA	-	10–50 ng/reaction	4.0 μ L
H ₂ O	-	-	19.9 μ L
Total (Vf)			30.0 μ L

PCR-amplification of template DNA is performed using an automated thermal cycler (SimpliAmp, Applied Biosystems) and the amplification program is shown in the table below. The program was designed according to the manufacturer's instructions of Taq polymerase and primers used in PCR.

	Time and Temperature	Number of cycles
Initial denaturation step	5 min 95 °C	1
Denaturation	45 sec 95 °C	} 35
Annealing	1 min 60 °C	
Extension	1 min 72 °C	
Final extension	10 min 72 °C	1
Chilling	∞ 4 °C	

Restriction enzyme digestion of PCR products

To detect the point mutation, the PCR-amplified product was digested adding a restriction enzyme to a reaction tube containing an aliquot of the PCR product. The restriction enzyme we used is *Hae* III; this enzyme recognizes a specific palindromic sequence of 4 nucleotides and performs a symmetrical cut on DNA, producing flat ends and allowing SNP-genotyping. The palindromic sequence recognized is shown in Fig. 3 (panel a). The digestion mixture solution is prepared as follows:

Component	Initial Conc.	Final Conc.	Volume (for 1 sample)
Enzyme buffer	10X	1X	2.5 μL
Restriction enzyme	10 U μL^{-1}	0.2 U μL^{-1}	0.5 μL
PCR product	/	/	20 μL
DNase-free water	/	/	2.0 μL
Total (Vf)			25.0 μL

Reaction mixture was incubated on thermostatic bath at optimal temperature (depending on restriction enzyme, in this case 37°C) for appropriate time (1 hour and 30 min).

Results

PCR-RFLP analysis of mouse DNA from *Lepr^{db-5j}* mice

We analysed 31 samples of *Lepr^{db-5j}* mice by PCR and subsequent enzymatic digestion. The PCR-amplified region spanning exon 13 of mouse leptin receptor gene (Fig. 3b), in the extracellular domain of leptin receptor and it includes the point mutation (G→T transversion). After the PCR reaction, samples were subjected to electrophoresis analysis (Fig. 3c). The results were visualized using UV-transilluminator (GelDoc system, Biorad) comparing the PCR products with DNA size marker. In addition to the samples, positive (lanes 15 and 16) and negative controls (lanes 14 and 17) were loaded. Positive controls are represented by the amplification of DNA extracted from mice tails through classical organic extraction protocol (25 ng of DNA used for each PCR), while as negative controls we amplified the sample eluted from paper without blood in lane 14 and a sample without template DNA in lane 17. The presence of an intense band of 200 bp for all the samples and the two positive controls indicates the successful amplification.

In alleles carrying the G→T mutation at position 640 of the leptin receptor gene, a *HaeIII* restriction enzyme site is lost, and this leads to a replacement of glycine by valine in the protein sequence (Fig. 4a). To determine the genotype for each mouse specimen, the remaining volume of PCR samples (20 μL) was digested with *HaeIII* enzyme (5 enzymatic units for each sample) for 90 minutes. After the digestion was completed, samples were loaded onto 2% (wt/vol) agarose gels (stained with EtBr 0,4 $\mu\text{g/mL}$). The results were visualized comparing the digestion products with DNA size marker (Fig. 4b and c). In wild type mice, the *HaeIII* site is preserved in both alleles, so the amplified band of 200 bp is cut in two bands of 150 bp and 50 bp. This is what happened for 11 mice and a positive control: lanes 2, 11, 12 and 13 in the first gel (Fig. 4b), lanes 2, 3, 4, 8, 10, 11, 12 and 15 in the second gel (Fig. 4c). In heterozygous mice only one allele is cut by the restriction enzyme, generating the two bands of 150 and 50 bp. The other allele presents the

point mutation hence the restriction site is lost and the band of 200 bp cannot be cut. In these cases, we have three bands of 200 bp, 150 bp and 50 bp. Fourteen mice had this genotype: lanes 3, 4, 5, 6, 7, 8, 9, 10, 16, 18 and 19 in the first gel and lanes 5, 9 and 13 in the second gel. Finally, if mice present the point mutation on both alleles (homozygous mutants) the restriction enzyme recognition site is lost and the digestion cannot occur. As a result, in these samples only the band of 200 bp (undigested PCR product) is detectable. This was the case of six mice and the other positive control: lane 14, 15, 17 and 20 in the first gel, lanes 6, 7 and 16 in the second gel.

Discussion

This study aimed at developing a sensitive, rapid and cost-effective method for the extraction of DNA from mouse whole blood on filter paper. Compared with conventional plasma sampling, DBS (Dried Blood Spots) requires much smaller blood volumes (50 vs 500 µL). The method described here aims to improve the TE buffer DNA extraction method, since in addition to drop blood incubation with TE buffer, there is a second incubation with a NaOH/SDS solution which allows to extract greater quantities of DNA from DBS (Dried Blood Spots). NaOH is a DNA chemical denaturant and allows to remove DNA molecules stuck on the paper. In addition, extraction of samples with an organic solvent such as chloroform and their precipitation with ethanol allow to obtain DNA that can be used for SNP-RFLP genotyping studies. Previous studies developed similar extraction methods for detection and genotyping of parasites [12, 13], but the advantage of our method is the ability to obtain significant quantities of DNA, in fact mean DNA yields are 2–3 µg, a result until now obtained with commercial kits [14, 15]. In 2018, Panda *et al* compared three methods for DNA extraction from mosquitoes dried blood spots: Chelex-100, QIAamp DNA mini kit, and TE (Tris EDTA)-Buffer, obtaining the lowest yield with TE buffer method [13]. The TE buffer-based DNA extraction method described here has instead shown better results, compared with traditional protocols. The only limitation of the procedure described here is that extracted DNA is not so pure to be stored for long periods. In addition to NOD *Lep^{db5j}* mice we performed similar DNA extraction and PCR reactions for *ob/ob* mice (*Lep^{ob}*) genotyping (data not shown). In these mice a nonsense mutation in codon 105 of the leptin precursor gene resulting from a C to T point mutation make them unable to produce functional leptin and creates a *DdeI* restriction enzyme site [16, 17].

Conclusions

The protocol described here is rapid, simple and confers a reduced risk for cross-contamination due to minimum manipulation of samples during extraction; moreover, it requires relatively limited equipment or funds resulting very inexpensive. All these aspects contribute to make it very advantageous in genotyping studies in which the model organisms (such as mice) have limited amount of blood, moreover it involves the use of a less invasive practice (puncture on the tail) resulting less stressful and painful for the animals.

Abbreviations

DBS, dried blood spots; PCR, polymerase chain reaction; SNP-RFLP, Single Nucleotide Polymorphisms-Restriction Fragment Length Polymorphism

Declarations

Ethics approval

All of the experiments were performed according to the international guidelines for animal research and approved by the Animal Care Committee of “Federico II” University of Naples, Italy and Ministry of Health, Italy.

Consent for publication

Not applicable

Availability of data and materials

All relevant data generated during this study are included in this published article.

Competing interests

The authors declare no competing interests.

Funding

This study was partly funded by Consiglio Nazionale delle Ricerche (CNR-CEOS) and by Università di Napoli “Federico II.” A.R. was supported by a postdoctoral fellowship from the Department of Medicine and Surgery, University of Salerno. G.M. is funded by Fondazione Italiana Sclerosi Multipla (FISM; grants 2016/R/18 and 2018/S/5), Progetti di Rilevante Interesse Nazionale (PRIN; grant 2017 K55HLC 001), and Ministero della Salute (grant RF-2019-12371111). P.d.C. is supported by FISM (grant 2018/R/4).

Authors' contributions

AP designed the experiments. AR, GZ, RG and CZ performed the experiments. GZ and CZ prepared figures. AR, GZ, SM, PdC and GM were involved in the writing of the manuscript. All authors were involved in the review and editing of the manuscript.

Acknowledgements

Not applicable

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Figures

Figure 1

DNA extraction from dried blood spots Schematic representation of experimental workflow for extraction of DNA from dried blood spots (DBS). Steps from 1 to 8 describe the procedure from the cut of the filter paper to the recovering of the aqueous phase containing DNA after chloroform extraction

PRECIPITATION OF THE EXTRACTED DNA (A)

Figure 2

DNA precipitation and recovery Schematic representation of experimental workflow to recover DNA extracted in previous steps. Steps from 9 to 13 describe the protocol to precipitate DNA with LiCl and ethanol and to recover it after centrifugation and TE resuspension

Figure 3

PCR analysis for *Lepr^{db-5J}* mice. (a) The restriction enzyme sequence recognized by *HaeIII* (b) A schematic representation of the PCR-amplified region spanning exon 13 of mouse leptin receptor gene is shown, the cleavage site for the restriction enzyme *HaeIII* is indicated. Primer positions are represented as blue arrows. (c) The gel picture displays the PCR-amplified DNA of the region of the extracellular domain of leptin receptor that includes the point mutation (G→T transversion); the presence of a band of 200 bp indicates the successful amplification. In upper gel, lane 1 shows the molecular weight marker 1 Kb plus, lanes 2 to 20 contain PCR-products obtained by the amplification of DNA extracted from blood's drops; in lower gel, lanes 1 and 18 show molecular weight marker 1 Kb plus, lanes 2 to 13 contain PCR-amplified DNA relative to mice samples, lane 14 and 17 correspond to negative controls (amplified from paper without blood in lane 14, PCR sample without template DNA in lane 17), lanes 15 and 16 represent positive controls (amplified-DNA extracted from mouse tails).

Figure 4

NOD-*Leprdb-5J* mice genotyping by PCR-RFLP. (a) Single nucleotide change (a G to T transversion in position 640) causes a glycine to valine substitution (G640V). This substitution is discriminated using a mismatch PCR-RFLP method because this SNP deletes the restriction site of HaeIII enzyme. (b,c) Representative results from gel electrophoresis analysis of 31 DNA samples after digestion of the PCR products by HaeIII enzyme. Lanes 1 (first gel, b), 1 and 17 (second gel, c) display molecular weight marker 1 Kb plus; lanes indicated as WT are wild-type mice in which the enzymatic cut produces two bands of 145 bp and 55 bp respectively. In NOD-*Leprdb-5J* heterozygous mice (lanes indicated as E) HaeIII digestion produces three bands of 200 bp (allele with SNP), 145 bp and 55 bp (wild type allele) respectively; in NOD-*Leprdb-5J* mutant mice the lack of the restriction enzyme site means that the only visible band is the 200 bp one (lanes indicated as M). Lane 14 in (c) represents a negative control and lanes 15-16 are positive controls: genomic DNA (from mouse tails) extracted from a wild type mouse (lane 15) or a mutant mouse (lane 16), amplified and subjected to HaeIII digestion.