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Title: Mice genotyping using buccal swab samples – an improved method

Short title: Mice genotyping using buccal swab samples

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ABSTRACT

Routine methods used to genotype mice involve isolation of DNA from partially amputated neonate's tail, toe or ear. Inevitable drawback of such techniques is animal`s pain response and increased spending of time and funds because of obligatory DNA purification. In order to implement a non-invasive and simple protocol for mouse DNA isolation, we have improved the method based on samples collected by swabbing of the inner cheek. Combining alkaline and temperature lysis it was possible to isolate DNA solution ready for PCR in less than an hour. Testing the method on three different mouse lines showed that it is highly efficient, the volume of the PCR samples could be reduced to 25 μl and fragments up to 800 bp were successfully amplified. This protocol reduces animal discomfort, shortens the time for DNA isolation, and enables amplification of larger DNA fragments with optimal success rate, thus considerably facilitating large-scale genotyping of different mouse lines.

Key words: DNA isolation, buccal swabs, genotyping, PCR, mouse

Transgenic mice are an indispensable experimental model in biomedical research and their successful breeding requires a simple and reliable genotyping procedure, which is typically performed using PCR (polymerase chain reaction). Obtaining high quality genomic DNA is a critical for successful PCR amplification. Although large quantities of DNA can be isolated from a variety of samples, the routinely used methods for mouse genotyping involve partial amputation of a neonate's tail, ear or toe (Hanley et al. 1991, Ren et al. 2001, Malumbres et al. 1997). Although efficient, the procedures are invasive, mutilating and the animals regularly exhibit pain response. Moreover, additional step of DNA purification after tissue lysis represents a significant burden, especially in laboratories which need to genotype a large number of different samples. Therefore, every simplification of DNA isolation procedure is welcomed, as it follows the trend in refinement of animal handling procedures, and also saves both time and money (Council of Europe, 2006.).

Buccal swabs as a source of cells for DNA isolation was used to avoid the invasiveness of previously applied procedures. After swabbing of buccal mucosa, DNA could be isolated using commercially available kits (Mulot et al. 2005) or extensive procedures which involve collecting devices (Zhang et al. 2006), proteinase K and/or phenol/chloroform extraction (Cao et al. 2003). In addition, simple procedures based on alcaline lysis were reported, both in humans (Rudbeck et al. 1998) and mice (Meldgaard et al. 2004).

Here we report an improved simple method (Meldgaard et al. 2004) for mouse genotyping using buccal swab samples. By modifying the alkaline lysis and boiling method it was possible to reliably isolate the DNA from mouse buccal mucosa using simple cotton sticks and the basic, easily affordable chemicals. The PCR was performed in minimal volume (25 μL) and modified in order to achieve amplification of PCR products up to 800 bp.

MATERIALS AND METHODS

Animals and housing

3 mouse lines genotyped were *Nol1*^{gt1Gaj}, *Not*^{eGFP} and *Stam2*^{gt1Gaj}. *Nol1*^{gt1Gaj} and *Stam2*^{gt1Gaj} were generated by gene trap method, which was based on random insertion of nonhomologous DNA vector in the genome of embryonic stem cells (Ćurlin et al. 2002). The modified genes were Nucleolar protein 1 (Nol1) and Signal transducing adaptor molecule 2 (Stam2), respectively. *Not*^{eGFP} line is a knockout of Noto gene containing eGFP construct (Abdelkhalek et al., 2004). All lines were kept on C57BL/6NCrl (Charles River, Les Oncins, France) background and C57BL/6NCrl was used as control. The animals were kept in an animal room at temperature of 22±1°C, with 55±5% relative humidity, and 12/12 h light/dark cycle. Three to six animals were kept in polycarbonate cages (420 mm x 265 mm x 150 mm, Ehret, Emmendingen, Germany). Tap water in drinking bottles and pelleted food were given *ad libitum*. Wood shaving was used for bedding and changed twice a week.

Sampling of buccal epithelial cells and isolation of DNA

The sampling of the buccal mucosa required some skilfulness and routine, as the sample should contain cells of buccal mucosa and not those of tongue. The mice were firmly held in the hand with their neck skin fixed between the thumb and the index finger. By pulling on the neck skin the mouth was opened and then a thin cotton stick (Aluminium Applicator 150 mm x 0.9 mm, Applimed SA, Châtel-St-Denis, Switzerland) was used to scrape one of the inner cheeks. The cotton stick was immediately soaked in 70 µL of sterile physiological solution (0,9% NaCl) in 1,5 ml microcentrifuge tube. After collection of all samples, the open microcentrifuge tubes containing sticks with the samples were briefly vortexed and sticks were discarded. After 8 minutes of centrifugation at maximum speed, liquid was removed, leaving scraped cells at the bottom. Cells were resuspended in 30 µL alkaline mixture of 25 mM NaOH and 0.2 mM EDTA and incubated for 20 minutes at 65°C.

Afterwards, samples were vortexed and incubated for 8 minutes at 98°C. Vortexing and boiling at 98°C were repeated once again, followed by cooling samples 2 minutes on ice. Finally, 30 μL of neutralizing solution containing 40 mM Tris-Cl pH 5.0 were added to every sample, making a total of 60 μL PCR-ready swab DNA solution. In some samples concentration of DNA was measured using spectrophotometer BioPhotometer (Eppendorf).

As an alternative, the DNA was isolated by two additional procedures. The first one required collection of cells in sterile water, lysis in 28 μL 0.1 M potassium hydroxide, incubation for 10 minutes at 75°C and dilution in 252 μL 20 mM Tris-HCl (pH 7.5) (according to Meldgaard). The second one required collection of cells in physiological solution, lysis in 25 mM NaOH and 0.2 mM EDTA, incubation at 98°C for 20 min, and dilution in 40 mM Tris–Cl (pH 5.0) (according to Zhang).

Genotyping of three mouse lines by PCR

PCR was performed using 1.5 - 3 μL of above described PCR-ready swab DNA solution in 25 μL of total reaction volume containing: GoTaq polymerase (1.5 unit, Promega), deoxynucleotide mixture (200 μM, Promega), primers (400 nM), $MgCl_2$ (2 mM), 5x Green Buffer already containing loading dyes (5 μL, Promega) and sterile water. Amplification temperature profile was: denatuaration at 94°C for 2 min, followed by 38 cycles of 94°C for 30 seconds, annealing at 54°C or 56°C for 30 seconds, extension at 72°C for 90 seconds, and final extension at 72°C for 7 minutes. Results were visualized using gel electrophoresis on 1.5% agarose gels, and the size of the product was determined by 100 bp DNA ladder (Promega).

Specific pairs of primers enabling the distinction between wild type, heterozygous and homozygous mice were used in the same reaction set:

For *Nol1*^{gt1Gaj} line: primer pair D6Gaj11 (5'tctgcctgccttgtttctt3', 5`ggacagcagcctccttaga3´) amplifying 401 bp indicative for the wild type allele, and primer pair D6Gaj11ß (5'tctgcctgccttgtttctt3', 5`cgccatacagtcctcttcac3`), amplifying 369 bp fragment of the mutant allele. Annealing temperature was 56°C.

For *Not*^{eGFP}: primers not-F (5`tgaccacctctctctcccattg3`) and not-wt-B (5`ccaccgcttccatactgatacc3`) amplifying 450 bp indicative for wild type allele and primer not-GFP-B (5`tgatgccgttcttctgcttgtc3`), which in combination with not-F amplifies 552 bp fragment indicative for the mutant allele (Abdelkhalek et al. 2004). Annealing temperature was 54°C.

For *Stam2*^{gt1Gaj} line: primer pair D2Gaj13 (5`gctttacagtggggatacat3`, 5`ttatggcttttaggcaatct3`) amplifying 600 bp indicative for the wild type allele and primer pair D2Gaj13ß (5`gctttacagtggggatacat3`, 5`ctgcaaggcgattaagttgg3`) amplifying 800 bp indicative for the mutant allele. Annealing temperature was 54°C.

Genotyping of *Stam2*^{gt1Gaj} because of its long amplified fragments was modified by varying $MgCl_2$ and deoxynucleotide concentrations and by addition of bovine serume albumine (BSA, 10 mg/mL, Promega). The optimal results were obtained with 1.5 mM $MgCl_2$, 280 µM deoxynucleotide mixture and 1.5 µL of BSA.

For all animals involved in the experiment the genotype was in parallel determined using DNA isolated from the tails. Briefly, tissue was immersed in lysis buffer at 56 °C overnight. (100 mM TrisCl, pH 8.0; 5 mM EDTA; 0.2 % SDS, 200 mM NaCl, 100 µg/ml proteinase K). DNA was precipitated by ispopropanol and ethanol, dried and dissolved in sterile water.

RESULTS

In order to establish mouse genotyping on a large scale using DNA isolated from the mouse cheek swabs, several different protocols were tested. Isolation with KOH and incubation at 75°C was used as a starting point (Meldgaard et al. 2004). The method was based on a principle that alkaline solution and temperature cause lysis of cells and release DNA. Regrettably, in our hands the procedure was partially successful and in more than a third of samples the expected PCR products were not amplified. Therefore, further modifications were tested: in order to get better rate of amplification, the number of PCR cycles was increased, and in order to get higher concentration of DNA, it was precipitated with isopropanol or Na-acetate. However, results were not improved.

As an alternative, the procedure based on lysis with NaOH and subsequent boiling on 98°C was also tested (Zhang et al. 2006). Instead of using the cell collecting device as required by the original protocol, the samples were collected using cotton swabs. By applying this procedure the majority of samples were successfully amplified in 25 μL of PCR mixture, although a high amount of background amplification considerably aggravated the interpretation of results.

In order to improve the combination of alkaline and temperature lysis different temperature steps were tested. At first, samples were incubated at 65°C, which is the temperature expected to coagulate majority of cell proteins. This incubation was followed by an additional boiling at 98°C, which was assumed to disrupt the cell membranes. As the first round of experiments yielded good results (almost absolute rate of PCR success), additional boiling step at 98°C preceded by a vortex, was also tested. Finally, the best results were obtained when the samples were cooled on ice only after the last boiling step. An additional step of boiling at 98°C (fourth heating in the row) did not improve the procedure. The samples were tested in 25 and 50 μL of PCR mixture and observed results were the same. In this

way the optimal conditions for the DNA isolation procedure were established (Table 1), and such a procedure was further characterized and tested.

In order to compare the amount of DNA obtained by lysis of mouse tail and by inner cheek swabbing, concentration of DNA was measured using spectrophotometer. Tail isolation protocol regularly provided 30 μg of DNA, which dissolved in 100 μL of water yielded concentration of 300 ng/μL. Buccal swabbing provided 20 μg of DNA, which dissolved in 60 μL of water yielded concentration of 333 ng/μL. Therefore, buccal swab method yielded approximately the same concentration of DNA as isolation from the tails in our laboratory.

In order to test the reliability of the genotyping procedure 50 animals of *Nol1*^{gt1Gaj} line were analysed and the obtained genotype compared against those determined from the tail DNA. Of 25 heterozygotes and 25 of their wild type controls, all 50 samples were correctly genotyped using the buccal swab method, i.e. there were no wrong genotype recognitions and all the expected products were amplified (Figure 1). In the same way the method was tested on another transgenic mouse line *Not*^{eGFP}. All 12 mice, 6 heterozygotes and 6 wild types were as well correctly genotyped.

In order to test if the method could be used to amplify relatively longer PCR products, *Stam2*^{gt1Gaj} line was used (Figure 1). To achieve simultaneous amplification of 600 and 800 bp long products it was necessary to adjust the PCR conditions in order to avoid the background and in rare cases unsuccessful amplification of the expected bands. Nevertheless, testing of different $MgCl_2$ and deoxynucleotide concentrations, and adding of BSA yielded reliable results (for the choosen primers the concentrations were: 1.5 mM $MgCl_2$, 280 μM deoxynucleotide mixture and 1.5 μL of BSA). Seven homozygotes, 7 heterozygotes and 7 wild types were finally correctly genotyped using the adjusted protocol. Thus, we report the efficient amplification of up to 800 bp fragments, which represents considerable improvement (Figure 1). The presented method was proven suitable for large-

scale genotyping of transgenic mice, and it was subsequently applied for routine work in our laboratory with the same efficiency.

DISCUSSION

As DNA is present in every sample containing nucleated cells, the presence of inhibitors was considered as a main obstacle for its successful amplification by PCR. The presence of different cell products (heme, immunoglobulins, proteases) and extracellular components (fibers) degrades DNA or inhibits Taq polymerase (Lantz et al. 2000). Therefore, additional steps of DNA extraction and purification using different alcohol solutions and/or phenol/chloroform became a part of the routine work.

The main aim of this work was to establish simple and reliable method for DNA isolation, which would avoid obstacles of already established procedures. In order to collect the sufficient amount of cells for DNA isolation and reduce distress of animal, inner mouse cheek was chosen. Stratified sqamous nonkeratinized epithelium is rich in cells that can be easily scraped off and the amount of extracellular components, as common inhibitors of PCR, is scarce (Lantz et al. 2000). Moreover, the aim was to design a protocol which could be performed in less than an hour using basic and easy affordable chemicals. The presented protocol was based on collection of cells in physiological solution followed by a simple alkaline lysis of cells at 3 temperatures. Since our procedure sampled a sufficient amount of cells, no special scoop device or additional tissue damage was necessary (Zhang et al. 2006).

Multistep incubation was considered crucial for the success of the presented procedure. Three steps at different temperatures yielded much better results than boiling at one temperature. In the first boiling step, the tempereature of 65°C was assumed to coagulate proteins which could degrade DNA and to partially disrupt cell membranes. During the second boiling step the temperature at 98°C combined with sudden cooling on ice and vortexing, completed the disruption of cell membranes and degraded possible inhibitory molecules without reducing the final amount of isolated DNA. Possible reasons for a better yield of DNA when using two temperatures could be that the direct boiling at 98°C

suddenly lysis all of the membranes thus allowing the mixing of enzymes with DNA and resulting in degradation. Incubation at 65°C prior to lysis at 98°C causes the enzymes to coagulate before the complete lysis occurs thus preventing them from damaging the DNA.

Some of the aspects of the procedure were considered critical for its success. The first one was proper scraping of the mouse cheek and sampling the adequate amount of buccal cells. Interestingly, scraping two cheeks versus one cheek showed that better PCR amplification was obtained when single cheek was scraped. Possible reason for that is that although more cells (i.e. DNA) could be collected, the amount of reaction inhibitors was higher, especially from the keratinized epithelium of the surface of the tongue. Thus, scraping of one cheek and avoiding the tongue is advisable.

After sampling, the cotton buds were immediately soaked in physiological solution rather than in sterile water (Meldgaard et al., 2004), since it is possible for the epithelial cells to rupture in the hypotonic solution and cause the DNA to become entangled in the cotton fibres. This would make the DNA more difficult to recover back in to the solution. Similarly, drying cotton buds before DNA isolation was unnecessary. Drying should be used only if samples need to be transported, and moreover the recovery of cells from dried cotton could represent a problem. In addition, the use of 5x Green Buffer (Promega) which contains loading dyes together with GoTaq polymerase probably helped the method to work as well. This combination of the buffer and polymerase was designed already to overcome the inhibitory effects of the loading buffer (Glebs et al. 2003).

The presented protocol regularly yields on average 20 μg of DNA, which is more efficient than protocols that use direct boiling at one temperature ((maximum total DNA yield: 5 μg, (Mulot et al. 2005)) or even some reported phenol/chloroform extractions (Garcia-Closas et al. 2001). Obtained DNA concentrations were comparable to average DNA concentration of 300 ng/μL obtained by isolation of DNA from the mouse tail in our laboratory. Garcia-Closas et al. reported simple swabbing protocol that yielded even higher amounts of DNA (total DNA yield: 126 μg), but because

of very low success rate of PCR, they suggested that isolation of DNA by cheek swabbing is not suitable for genotyping.

The PCR efficiency with samples obtained by the presented protocol was optimal. Actually, after necessary adjustments all tested samples worked even with primers producing amplicons up to 800 bp, which is higher than in other published protocols (Mulot et al. 2005, Zhang et al. 2006). The advantage of the presented protocol is that only 1 – 3 μL of DNA solution is required for PCR, compared with e.g. 30 μL recommended in the protocol of Meldgaard et al., therefore diminishing the amount of inhibitors in the reaction mixture.

In conclusion, a highly reliable method for mouse genotyping using DNA isolated from buccal swab samples was presented. It is based on cell lysis in alkaline solution and 3 subsequent steps of boiling. The main advantages are: non-invasive approach to the animal, obtaining of good quality DNA in less than an hour using basic chemicals, simplicity of the approach, and high success rate of PCR.

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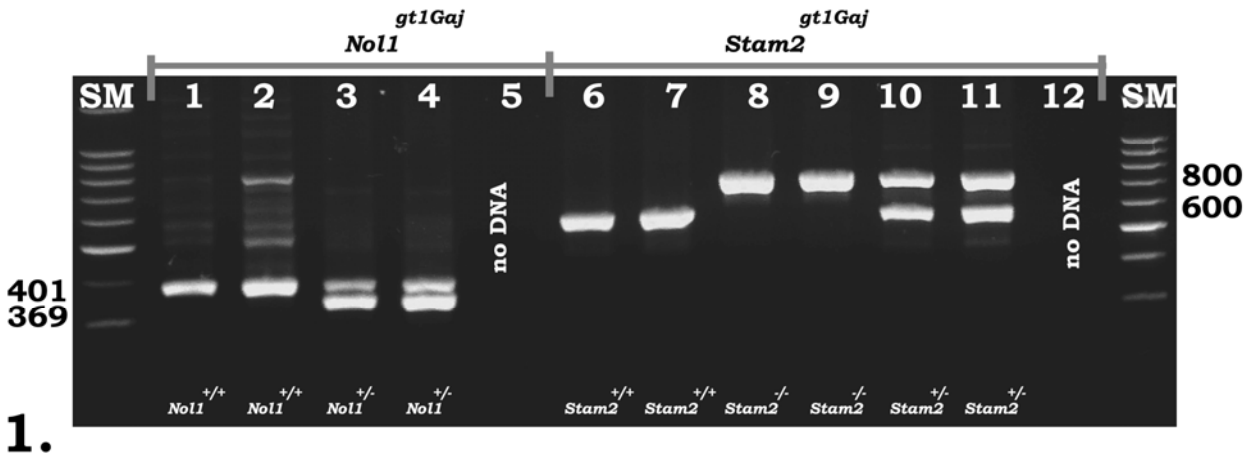
Table 1: Isolation of DNA from mouse buccal swabs

1. scrape one mouse cheek
2. soak the cotton bud in 70 µL of sterile physiological solution (0.9% NaCl)
3. brief vortex together with cotton bud
4. discard cotton bud
5. centrifugation for 8 minutes at maximum speed
6. remove liquid leaving scraped cells at the bottom
7. resuspend in 30 µL of alkaline solution (25 mM NaOH and 0.2 mM EDTA)
8. incubate for 20 minutes at 65°C
9. vortex and incubate for 8 minutes at 98°C (2 x)
10. cool 2 minutes on ice
11. add of 30 µL of neutralizing solution (40 mM Tris-Cl pH 5.0)
12. use 1-2 µL of the sample for PCR

Figure 1:

Gel electrophoresis of PCR products obtained in genotyping of *Nol1*^{gt1Gaj} and *Stam2*^{gt1Gaj} transgenic mice. Lanes 1 and 2 contain 401 bp amplified fragment, specific for *Nol1*^{gt1Gaj} wild type genotype, while lanes 3 and 4 contain 369 and 401 bp long fragments, which reveal *Nol1*^{gt1Gaj} heterozygotes. Lanes 6 and 7 contain 600 bp long fragments, specific for *Stam2*^{gt1Gaj} wild type genotype, lanes 8 and 9 contain 800 bp fragments, specific for *Stam2*^{gt1Gaj} homozygotes and lanes 10 and 11 contain 600 and 800 bp which reveal *Stam2*^{gt1Gaj} heterozygotes. SM – 100 bp DNA ladder as a size marker.



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