A one-hour universal protocol for mouse genotyping

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Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines."

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None of the authors has any conflict of interest to disclose

Abstract

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Introduction: Transgenic animals are widely used for research and for most of them, genotyping is unavoidable. Published protocols may be powerful but may also present disadvantages such as their cost or the requirement of additional steps/equipment. Moreover, if more than one strain must be genotyped, several protocols may need to be developed.

Methods: we adapted the existing amplification-resistant mutation protocol to develop the 1-hour universal genotyping protocol (1-HUG), which allows the robust genotyping of genetically modified mice in 1 h from sample isolation to PCR gel running.

Results: This protocol allows the genotyping of different mouse models including mdx mouse, and FLExDUX4 and HSA-MerCreMer alone or in combination. It can be applied to different types of genomic modifications and to sexing.

Discussion: The 1-HUG protocol can be used routinely in any laboratory using mouse models for neuromuscular diseases.

Key words: Genotyping, mouse model, FSHD, DMD, neuromuscular diseases

Introduction

Genetically modified mice are routinely used in many laboratories across the world and the most popular modifications are deletion, insertion in a known or unknown location and point mutation. Many methods have been proposed to genotype these mice, and those developed for the mdx mouse perfectly illustrate the difficulty in finding the best one. This mouse is the animal model for Duchenne muscular dystrophy (DMD) and carries a nonsense point mutation (C-to-A mutation) in exon 23, leading to a truncated and non- functional protein [1].

These methods include an allele-specific oligonucleotide (ASO)-based hybridization method[2], a SNaPshot assay[3], a high resolution melting PCR[4], a restriction fragment Length polymorphism (RFLP)[5], a dideoxy fingerprinting (ddF)[6] and an amplification resistant mutation system (ARMS) method[7,8]. These methods may be powerful but many of them also present disadvantages such as their cost, the requirement of additional steps, or the purchase of specific expensive equipment. For example, in the ARMS protocol, the PCR reaction is performed with 3 primers in the same tube: 1 forward primer and 2 reverse primers that differ only in the last nucleotides. In order to be able to distinguish the WT and mdx allele, the WT primer is 17 bp longer than the mdx one [7]. A 3% agarose gel and a long running time are required to allow the correct discrimination between mdx and WT samples. Moreover, numerous laboratories that use the mdx mouse also need to genotype other genetically modified mouse models and having one general method for different kinds of modification, including sexing, is valuable.

Here we have optimized the ARMS protocol for the mdx genotyping and developed a one hour universal genotyping protocol (1-HUG protocol). This protocol can be easily implemented in laboratories and allows the robust genotyping of different modified strains carrying either a point mutation or insertion in a known or unknown location, using a simplex or multiplex PCR.

Materials and methods

Animals.

Mice were bred in the Biological Services Unit of the Great Ormond Street Institute of Child Health and University College London in accordance with the Animals (Scientific Procedures) Act 1986, under Home Office Licence 70/8389. All experiments were performed following the United Kingdom and European guidelines (Directive 2010/63/UE of the European Parliament and of the Council) and approved by relevant committees. Mdx, the B6(Cg)-Gt(ROSA)26Sortm1.1(DUX4*)Plj/J also called the FLExDUX4 and HSA-MCM mice were purchased from the Jackson Laboratory (#001801, #028710, #025750). This strain, which may be useful for studying facioscapulohumeral muscular dystrophy (FSHD), was created after the insertion of a 2.3 kb insertion in the Gt(ROSA)26Sor locus [9]. All collected tissues came from mice bred for independent studies. Phalanx removals and tail biopsies were performed on dead animals.

Fast genotyping protocol

DNA was extracted from fresh tissue (ear punch, 3 mm diameter) using 100 µL of lysis buffer (10mM NaOH, 0.1 mM EDTA) and incubated at 95°C for 5 min. PCRs were performed on a Veriti 96-well Thermal Cycler (Applied Biosystems) in a final volume 15 µL with 1 µL of lysate, 1 µL of primer pre-mix, and GoTaq G2 Hot Start Green Master Mix (Promega). Primers were ordered from Eurogentec and are listed in Supplemental Table 1 and their concentration in pre-mix is 5 µM except DL1509 at 2.5 µM. The PCR conditions were set as (1) an initial denaturation at 95°C for 2 min; (2) 10 cycles of the touchdown stage with 5 sec of denaturation at 95°C, 5 sec of annealing at 67°C (decrease by 0.5°C per cycle) and 5 sec of extension at 72°C; (3) 30 cycles with 5 sec of denaturation at 95°C, 5 sec of annealing at 62°C and 5 sec of extension at 72°C. PCRs were loaded in a TAE 2% agarose gel and the electrophoresis was run at 140 V for 12 min. Sexing of animals was realized using primers targeting the *Rbm31* gene as previously described [10]

Results

We designed a secondary primer (RJ-WT) that hybridizes with the non-specific tail of the DL1509 primer. The 4 primers were mixed together in the reaction at equimolar concentration. For the WT allele, a 165 bp amplicon was expected in the presence of the RJ-WT primer compared to the 134 bp when only primers DL1577 and DL1509 were used. The amplification of the mdx allele is always 117 bp (Fig. 1). Using the standard PCR program described in Fig. 2, the influence of the concentration of agarose on the band separation was investigated on genomic DNA isolated from either WT or Heterozygote (WT/mdx, heterozygous) animals using the 4 primers. As expected, a single 165 bp band was observed in the WT lane, whereas 2 bands (165 bp + 117 bp) were visible in the heterozygous lane (Fig. 2A). A 2% agarose gel was selected for further investigation. The influence of different ratio between primers DL1509 and RJ-WT was evaluated on genomic DNA isolated from either WT, heterozygous or mdx animals. Using ratio 1/1 and 0.1/1 the WT and mdx bands were not well separated in the heterozygous animals but ratio 0.5/1 gave rise to 2 discrete bands with similar intensity and consequently was selected (Fig. 2B). The genomic DNA was also extracted from different types of samples including ear punch, tail biopsy, finger/phalanx removal and non-invasive ventral skin swab from either WT (+/+) , heterozygous (+/-) or mdx (-/-) 3-weeks old animals and digested for either 5 or 10 min (Fig. 2C, upper and lower panel respectively). The best results were obtained with ear punching and finger removal (Fig. 2C). Indeed, PCR fragments were not well amplified with the tail biopsy but when the incubation time is longer (10 min), bands were visible (Fig. 2C lower panel). The ear punch was selected because it is a low invasive protocol. Next, incubation time of the ear punch into the lysis buffer at 95°C was also investigated and 5 minutes was selected (Fig. 2D). Finally, the consequence of the addition of the 4th primer (RJ-WT) was assessed on band separation (Fig. 2E). After an electrophoresis of 10 minutes, the use of the RJ-WT primer allowed a good discrimination of the WT and mdx alleles, even in the heterozygous animals. Without the RJ-WT primer, at least 30 minutes were required to distinguish the 2 bands in the heterozygous animals.

Thermocycler conditions were optimized to reduce the protocol time. Two alternative PCR programs were evaluated and compared to our standard touchdown program (Fig. 3).

The PCR program was optimized to reduce the running time without reducing the specificity. In the standard PCR, the running time is 73 min with an annealing temperature of 65°C (Fig. 3A). In the optimized PCR, the annealing temperature was increased to 67°C to be more specific and the global running time was decreased to either 40 or 38 minutes by changing the denaturation, annealing and elongation time (Fig. 3B-D). These PCR were performed on WT, heterozygous or mdx animals. As expected, a 165 bp band is visible when genomic DNA is isolated from WT animals, whereas a 117 bp band is visible on mdx mouse. A thin band of 134 bp might be also sometimes visible, which corresponds to an amplification product of the DL1577 and DL1509 primers. The Opt 3 program was selected because all the bands are clearly visible with a running time of only 38 min.

This 1-HUG protocol was applied to genotype different kinds of genetic modifications and for sexing.. Interestingly, whereas the *Rbm31* gene is present on both chromosome X and Y, sequence alignments have revealed an 84 bp deletion in Rbm31x compared with Rbm31y. By using 2 primers flanking this region and performing the 1-HUG protocol, it is easy to distinguish female (269 bp long PCR product) and male (269 + 353 bp long PCR products) (Fig. 4A).

The 1-HUG protocol was also applied to reveal the presence of the long insertion present within the mouse strain B6(Cg)-Gt(ROSA)26Sortm1.1(DUX4*)Plj/J also called the FLExDUX4 .. Because 5 sec extension is not enough to amplify the whole insertion using flanking primers, an insertion specific primer was added (Fig. 4B). The PCR reaction was thus performed using 3 primers producing a 198 bp product in WT animals, 198 bp and 124 bp products in heterozygote animals and 124 bp product in homozygote transgenic animals (Fig. 4B).

The 1-HUG protocol was next used to genotype the Tg(ACTA1-cre/Esr1*)2Kesr/J strain (JAX #25750), also known as HSA-MerCreMer. Two sets of primers were designed: one recognizing the transgene and producing a 102 bp product and one internal control producing a 198 bp product. Because the transgene insertion site is unknown, this protocol did not allow the distinction between homozygote

and hemizygote animals. WT animals produced a single band (198 bp) whereas in transgenic mice, 2 bands (102 + 198) were observed (Fig. 4C).

Finally, the 1-HUG protocol was applied to a multiplex PCR. When FLExDUX4 and HSA-MerCreMer animals were mated together, different genetic combinations can be observed depending on the parent genotypes. The primers used for the Gt(ROSA)26Sor locus were used as the internal control for the Cre PCR. Consequently, 3 bands can be amplified: the FLExDUX4 product (300 bp), the WT Rosa26 (198 bp) and the Cre transgene (102 bp). The multiplex PCR allowed the amplification of the different bands depending on the matings (Fig. 5). A summary of the protocol workflow is presented in Fig. 6.

Discussion

Here we developed a robust genotyping protocol for mouse from sample isolation to electrophoresis. Several protocols have been published over the years but they show limitations because they only address few types of modifications such as transgene insertion [11], or propose alternative methods such as southern blot [12]. Our protocol, named the 1-HUG protocol, allows sexing and genotyping in one hour of most of the genetic modifications encountered in transgenic mice.

For sexing, when newborn pups must be injected a few hours or days after birth, sexing by visual examination of the ano-genital distance is not possible [13]. An external pigmentation method can be used but is not applicable to light colored mice [14]. Many labs now use a PCR of the Sry gene to indicate a male [15]. But as this gene is not present in females and to be sure that the lack of Sry band is not a PCR failure, a 2nd set of primers must be added to amplify an internal PCR control.

For genotyping, we used the previously described ARMS model as a starting point for primer design, but each step of the protocols was improved including sample type, incubation in lysis buffer, PCR program etc. Concerning the sample isolation, we focused on 4 different types (ear punch, tail biopsy, finger removal and ventral skin swab) but the ventral skin swab did not give satisfactory

results. Another non-invasive method is the use of fecal pellets [16] but this requires special animal husbandry with one animal per cage and this is not in accordance with the principles of the replacement, reduction and refinement (3Rs). Blood sample collection [17] for genotyping is highly invasive and is not recommended. Finally, ear punching allows genotyping of the animals but also allows their individual marking with one manipulation only (in accordance with the 3Rs).

Isolation of gDNA was also improved in the 1-HUG protocol. The use of a lysis buffer for the isolation of genomic DNA of different origins including ear, tail, toe and spleen was previously published[18] but there are several major difference between that protocol and this one: the concentration of the 2 lysis buffers are not identical and in our case, the pH doesn't need to be adjusted to 12. Moreover, a neutralizing reagent is not used, and samples are heated for 5 minutes only and not chilled to 4°C. However, in some occasions, genomic DNA may be isolated from tail biopsies leading to a longer and more expensive protocol since proteinase K may be added [19].

Concerning the primers for the FLeXDUX4 mouse, the previously published primers gave a 409bp amplicon that is difficult to amplify using the Opt3 PCR program because of the very short extension time used (5 sec). The new primers generate a 300bp and 200bp amplicons that are easily distinguishable on a 2% after 10min of running. These new primers can also be combined with the Cre-specific primers to generate 100, 200, and 300bp amplicons.

For the mdx mouse, our working hypothesis was that a longer WT amplicon would be better for sample discrimination. Two possibilities were envisaged to generate this longer fragment. In the 1st one, the size of the WT primer could have been increased, but we excluded this possibility because the size of the DL1509 is already 50 bp and having a longer primer would increase the probability of generating non-specific amplicon, primer dimers and hairpins. This is the reason we decided to use a secondary primer that recognizes the unbound tail of the 1st one. The results clearly demonstrates the advantage of the addition of the RJ-WT primer over the previous ARMS protocol.

In conclusion, the 1-HUG protocol (5 min lysis, 38 min PCR, 12min electrophoresis) can be used routinely in any laboratory to genotype most standard mouse strains.

Abbreviations used in the manuscript

1-hour universal genotyping protocol (1-HUG)

Duchenne Muscular Dystrophy (DMD)

restriction fragment Length polymorphism (RFLP)

dideoxy fingerprinting (ddF)

amplification resistant mutation system (ARMS)

B6(Cg)-Gt(ROSA)26Sortm1.1(DUX4*)Plj/J (FLExDUX4)

facioscapulohumeral muscular dystrophy (FSHD)

WT (+/+) , heterozygous (+/-)

Tg(ACTA1-cre/Esr1*)2Kesr/J (HSA-MerCreMer)

replacement, reduction and refinement (3Rs)

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Figure legends

Figure 1: PCR strategy

Schematic outline of PCR used to genotype mdx mice. In mutant mice, the forward DL1577 primer and the reverse mutant-specific DL1573 amplify a 117 bp fragment. In wild-type (WT) mice, the reverse WT-specific primer DL1509 produces a 134 bp fragment. The annealing of the RJ-WT primer on the non-matching DL1509 tail increases the size of the WT amplicon to 165 bp.

Figure 2: PCR setting-up

(A) Agarose concentration effect on band separation. PCR with the RJ-WT primer were run in 0.8, 1, 1.5 and 2% agarose gels at 150 V for 10 minutes. WT and heterozygote samples were used.
(B) Optimization of primer ratio. Since the double annealing of the DL1509 and RJ-WT primers on the WT amplificon could be complex, several ratios of these two primers were tested (DL1509:RJ-WT).
(C) Different types of samples were used to genotype mice: ear punch (E), tail biopsy (T), finger/phalanx removal (F) and non-invasive ventral skin swab (NI) from the same mouse. Samples were digested at 95°C for 5 min or 10 min (upper and lower panel respectively).
(D) Incubation time: ear punch samples of the same heterozygous mouse were digested in lysis buffer at 95°C for 0, 2, 5, 10 min.
(E) Electrophoresis time effect on separation with or without the RJ-WT primer. Two different samples of WT, heterozygote or homozygote were used. PCR were loaded in a 2% agarose gel and run at 150 V. Pictures of the same gel were taken after 10, 20 and 30 minutes without changing camera settings.
WT: wild-type, Hz: heterozygous, Mdx: homozygote, NC: negative control

Figure 3: Optimization of the PCR

The standard protocol (A) used in our laboratory was modified to reduce the running time without losing the specificity (B to D). Ear punches of 3 week old WT, heterozygous, or mdx animals were

used a template. Protocols A and B differ by the temperature used during the touchdown PCR. In protocol C, denaturation and annealing time were decreased while elongation time was supressed. In protocol D, denaturation, annealing and elongation times are 5 s each. A longer electrophoresis was used to distinctly see all potential amplified bands. The expected PCR products are 165 bp for the WT, 117+165 for the heterozygous and 117 for mdx. A weak 134 bp corresponding to the WT amplification without RJ-WT primer is also detectable.

Figure 4: Applying the HUG-1 protocol to different strains.

(A) Genotyping a small insertion or deletion. Sexing of pups or young offspring can be achieved using Rbm31 gene, located in both X and Y chromosomes. Rbm31x has a small deletion of 84pb compared to Rbm31y. Using flanking primers (F+R), males have two bands for X and Y isoform and females only one band for X.

(B) Genotyping a long insertion. FLExDUX4 mice (JAX #028710) have a 2.3kb insertion in ROSA26 locus. In mutant mice, two flanking primers (F1+R1) don't allow this sequence to be amplified. A third primer specific to the insertion (R2) is used for the mutant allele.

(C) Genotyping an unlocated insertion. HSA-MCM mice (JAX #025750) express MerCreMer protein. The insertion site of the transgene is not well characterized. To genotype mice, two sets of primers are used: one specific for the transgene (F3+R3) and one targeting another part of the genome as PCR positive control (F4+R4). Carrier have two bands and WT only one. This type of genotyping is not able to distinguish homozygous from heterozygous.

Specific primers of each example are indicted in Supplemental Table I. WT: wild-type, Hz: heterozygous, Tg: transgenic, NC: negative control.

Figure 5: Applying the HUG-1 protocol to a multi-transgenic mouse using a multiplex PCR.

FLExDUX4 and HSA-MCM animals are mated to generate tamoxifen-inducible DUX4 mice. The primers specific for the Cre transgene (F3, R3) are added to those of FLExDUX4 genotyping (F1, R1,

R5). Three bands can be amplified: 300 bp for the FLExDUX4 allele, 198 bp for the WT Rosa26 locus, and 102 bp for the Cre transgene.

F1, F3, R1, R3, R5 indicate specific primers of each example (Supplemental Table I). WT: wild-type, Tg: transgenic.

Figure 6: Protocol summary

After ear punch, tissues are incubated in lysis buffer for 5 min at 95°C. The touchdown PCR is performed with 1 μL of lysate and during 10 cycles, the temperature is decreased by 0.5°C/cycle. Finally, PCR products migrated on a 2% agarose gel for 12 min.