Optimizing PCR for Mouse Genotyping: Recommendations for Reliable, Rapid, Cost Effective, Robust and Adaptable to High-Throughput Genotyping Protocol for Any Type of Mutation

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Genotyping consists of searching for a DNA sequence variation localized at a well-defined locus in the genome. It is an essential step in animal research because it allows the identification of animals that will be bred to generate and maintain a colony, euthanized to control the available space in the animal facility, or used in experiment protocols. Here we describe polymerase chain reaction (PCR) genotyping protocols for fast, sensitive, easy, and cost-effective characterization of mouse genotype. We discuss optimization of parameters to improve the reliability of each assay and propose recommendations for enhancing reproducibility and reducing the occurrence of inconclusive genotyping. All steps required for efficient genotyping are presented: tissue collection; sample verification and direct DNA lysis; establishment of a robust genotyping strategy with reliable, rapid, and cost-effective assays; and finally, transition to high-throughput automatized PCR, including mix miniaturization and automation. © 2019 The Authors.

Basic Protocol 1: Tissue sampling methods and procedure
Basic Protocol 2: Sample verification and DNA lysis
Basic Protocol 3: Design of a genotyping strategy
Basic Protocol 4: Moving to high-throughput genotyping

Keywords: 3Rs • replacement, reduction, refinement • automation • genotyping • molecular biology • mouse model • PCR • reproducibility

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INTRODUCTION

Many rodent genotyping protocols are based on polymerase chain reaction (PCR) amplification of genes or genetic markers, as PCR is easy, fast, sensitive, and cost effective. Wrongly, PCR genotyping is often considered as a straightforward and easy step; in reality, however, providing robust, accurate, and fast results is frequently more challenging that it seems. Because of its high sensitivity, PCR is subject to frequent false positive results (i.e., amplification of a contaminant). Conversely, because of its low tolerance of inhibitors, frequent false negative results are also encountered (i.e., no amplification; Bacich, Sobek, Cummings, Atwood, & O'Keefe, 2011; Schrader, Schielke, Ellerbroek, & Johne, 2012). PCR can also fail to amplify certain templates, such as GC-rich sequences or secondary structures. Although it is a routine technique in mouse research laboratories, the establishment of reliable, rapid, and cost-effective genotyping protocols for every mutation is generally low on the list of priorities for scientific researchers. Researchers are very attentive to the reliability of data from their experimental protocols, but the genotype of the animals used is not always verified.

Analysis of our genotyping over a 5-year period indicated that ∼6% of the animals that were sampled and genotyped twice had different genotype outcomes (Table 1). Additionally, when phenotyping cohorts (groups consisting of six to eight animals per genotype and sex) were sampled and genotyped twice, before and after phenotyping, 30% were found to include at least one animal with a discordant genotype (data not shown). These data are consistent with the finding that over 15% of lines deposited to public repositories, such as the Mutant Mouse Resource & Research Centers (MMRRCs) and the Jackson Laboratory (JAX), do not carry the mutation specified by the depositor (Lloyd, Franklin, Lutz, & Magnuson, 2015). Inconclusive genotyping is one factor that can impact preclinical studies and basic research reproducibility, contributing to the "reproducibility crisis" (Cinelli, Rettich, Seifert, Bürki, & Arras, 2007; Picazo & García-Olmo, 2015). Moreover, genotyping errors can lead to genetic contamination of stocks and even to the extinction of a genetically unique mouse line (Lloyd et al., 2015). Cryopreservation of mutant sperm or embryos and PCR quality control of the preserved stocks are therefore essential (Scavizzi et al., 2015). Finally, the development of CRISPR/Cas9 technology has enhanced the possibility of achieving mutations in mice (Birling, Herault, & Pavlovic, 2017; Birling, Schaeffer, et al., 2017) but also required further genotyping of the resulting mouse models (Birling, Schaeffer, et al., 2017; Mianné et al., 2017). The consequences of genotyping errors and animal misidentification should not be underestimated and must be controlled through efficient and robust PCR genotyping (Bonaparte et al., 2013).

Table 1 Genotyping Accuracy Over 5 Years[a]

Year	Total animals analyzed	Animals genotyped with two independent biopsies[b]	Comparison between the two results		
			Confirmed genotypes	Distinct genotypes	Inconclusive genotypes (%)
2013	56,331	4140	3867	273	6.6
2014	51,309	4588	4328	260	5.7
2015	58,250	7166	6673	493	6.9
2016	50,267	4411	4152	259	5.9
2017	51,272	6900	6576	324	4.7

[a]We analyzed the genotype outcomes of animals that were sampled and genotyped at least twice, for various different reasons: e.g., genotype verification before or after phenotyping experiments or before mutant line freezing or shipment.
[b]The analysis included only the animals for which the results of both genotypes were interpretable (no PCR inhibition or PCR contamination observed).

Based on our expertise in standardized and high-throughput genotyping (60,000 animals per year for hundreds of different genetic markers or gene mutations), we describe here some recommendations and protocols for reproducible PCR genotyping. The establishment of a robust genotyping strategy begins with the choice of tissue to be sampled, the verification of samples before direct DNA lysis, and finally reliable, rapid, and cost-effective testing. The last part of this article details how to translate a procedure to high-throughput PCR, including recommendations for reaction mix miniaturization and automation. The four protocols presented in this paper are optimized for the most common samples used for genotyping transgenic mice: DNA in crude extracted from tail, ear, or toe tissue, with specific authorizations according to your local ethical regulations.

TISSUE SAMPLING METHODS AND PROCEDURE

BASIC PROTOCOL 1

There are several ways to obtain DNA for mouse genotyping: tail biopsy, ear or toe clipping, hair, blood, or fecal or oral samples. The method selected depends upon several parameters, including the established practice in your laboratory or institute and the quantity of DNA required for the assay. Table 2 describes the different tissues typically used as samples for genotyping and the key considerations for choosing the most appropriate sampling method.

Material

70% (v/v) ethanol
0.75-ml screw-cap tubes (e.g., Matrix tubes, blank; cat. no. 446015, Dutscher) and caps (e.g., Sepra Seal Cap Mats for Matrix tubes; cat. no. 446045, Dutscher)
Sterile compresses
Animals for tissue collection

Class II microbiological safety cabinet or changing station
Personal protection equipment: lab coat, gloves
Ear puncher for mice (e.g., cat. no. AT7020, Agnthos) or sharp surgical scissors (e.g., cat. no. 14106-09, Fine Science Tools)
Clean cages

Preparation of materials

1. Five minutes before starting the procedure, turn on the safety cabinet or changing station.
2. Clean the work surface with 70% ethanol.
3. Place the following equipment on the work surface: rack of screw-cap tubes, clean cage, compress soaked in 70% diluted ethanol, and scissors or ear punch.
4. Place the cage containing the animals to be sampled under the safety cabinet or changing station and open it.
5. Transfer the parents (if present) to the clean cage. Animals to be genotyped will be placed individually in the cage after each biopsy.

   Check that the animals that need to be identified (number, sex) correspond to those listed on the genotyping request.

Preparation of sterile tools for biopsy procedure

Use of sterile tools for biopsy procedure is crucial (see Critical Parameters). The ear puncher or scissors must be sanitized using an appropriate method (e.g., with 70% ethanol).

6. Disinfect the scissors or ear puncher with the compress soaked in 70% ethanol.

Table 2 Comparison of Tissue Sampling Methods for DNA Genotyping

Sampling method	Choice of biopsy method depending on age of animal			Recommended sample size	Invasiveness of sampling	Possible repetition of sampling	Specific remarks
	<2 wk	3-4 wk	>4 wk				
Tail biopsy	Yes	Yes	Yes	0.3-0.5 cm	Amputation	Yes[1]	[1]At 14-17 days after birth, mouse tails are incompletely ossified; at >4 wk, anesthesia is mandatory to make the procedure less painful for the animal.
Ear punch	No	No	Yes	0.2-cm hole	Amputation	Yes[2]	Pinna ear contains mainly cartilage. [2]If ear sampling is also used as identification method, it will not be possible to do a second biopsy.
Toe clipping	Yes	No	No	Distal phalanx	Amputation	No	In young mice, the ossification process is not yet complete.
Fecal pellet	No[3]	Yes	Yes	10-50 mg[4]	Noninvasive	Yes	[3]Collecting feces from mice younger than weaning age (3 wk) is difficult, due to their milk diet, and results in poor DNA yield due to small sample size. [4]Feces weight from a 4-wk-old mouse (~10 mg) is much smaller than from an adult (~35 mg)
Blood sample	No	Yes	Yes	20-50 µl[5]	Minor	Yes[6]	Proper training is required to avoid inaccurate puncture and/or hemorrhages. [5] ≤10% of total blood volume should be taken at any one time. [6] ≤15% of total blood volume in a 28-day period.
Hair roots	No[7]	Yes	Yes	One tuft of hair[8]	Noninvasive	Yes	High risk of cross-contamination between samples from different animals because hairs stick electrostatically to instruments. [7]Hairs usually grow at ~10 days of age. [8]One tuft of hair represents ~3-30 fur hairs.
Oral swab	Yes	Yes	Yes	6-8 mm	Noninvasive[9]	Yes	[9]Although noninvasive, this appears to be as stressful as sampling from the tail or ear. Sample should contain cells of buccal mucosa. not tongue.

Table 3 Average Yields and Percentages of PCR Failure Using Direct PCR Lysis Extraction

Sampling method[a]	Extraction method	Average quantity of DNA obtained[b]	Percentage of failed genotyping[c]	Easily adaptable to high throughput
Tail biopsies	Direct PCR lysis	26 ± 4 ng/μl	2.6	Yes
Ear punching	Direct PCR lysis	34 ± 5 ng/μl	2.8	Yes
Toe clipping	Direct PCR lysis	20 ± 2 ng/μl	3.3	Yes

[a]These three kind of tissue biopsies give enough DNA for PCR genotyping. Their performance in PCR is quite similar as the percentage of failed genotyping is very similar, indicating that similar levels of inhibitors are present in the crude extracts. Data were collected from 27,070 tail, 8470 ear, and 2900 toe biopsies to estimate genotyping failure.
[b]Amount of isolated DNA was determined by spectrophotometry with a NanoDrop ND-1000 system ($N = 5$).
[c]Percentage of biopsies that could not be genotyped (2018 data) because results were not interpretable. Causes are multifactorial and include uncalibrated biopsy, inefficient lysis, and presence of inhibitors in the crude extract.

7. Clean the scissors or ear puncher between mice to avoid sample contamination.

   Pay attention to be sure that you remove all tissue from the instruments after each animal (see Troubleshooting).

Sampling procedure

8. Check that the tube in which the sample will be placed is clean and correctly labeled.
9. Manually restrain the mouse between thumb and forefinger.

   Gentle handling is of major importance to reduce the stress of the intervention for animals (Cinelli et al., 2007).

10. Using the sanitized sharp scissors or ear puncher, precisely excise a piece of tissue, of homogeneous size relative to other samples (appropriate sizes: tail biopsy, 5 mm; ear punch, 2-mm hole; toe clipping, one distal phalanx; see Critical Parameters).
11. Place the sampled pup in the clean cage with its parents.
12. For each mouse, place the sample into the corresponding tube.
13. Close the tube and check that the biopsy is at the bottom of the tube.
14. Once all mice have been sampled, the tubes can be stored at −20°C for later genotyping.

SAMPLE VERIFICATION AND DNA LYSIS

BASIC PROTOCOL 2

There are many alternative protocols that can be used to prepare samples for PCR. They extend from the use of a raw lysate to a variety of purification protocols (e.g., organic extraction with phenol/chloroform or silica column) designed to remove contaminants and inhibitors. Purification protocols suffer the disadvantages that they are expensive, may have low performance (Miller, Bryant, Madsen, & Ghiorse, 1999), and are quite difficult to implement on a workstation. In contrast, direct lysis methods are quick, easy, and inexpensive, but do not remove inhibitors, and therefore cannot be used for all applications. Here, we will describe the use of DirectPCR Lysis Reagents with the addition of proteinase K for routine DNA isolation. In our hands, this approach provides a low level of PCR failure for standard PCR genotyping (Table 3, percentage of failed genotype) and good DNA yields (Table 3, average quantity of DNA obtained).

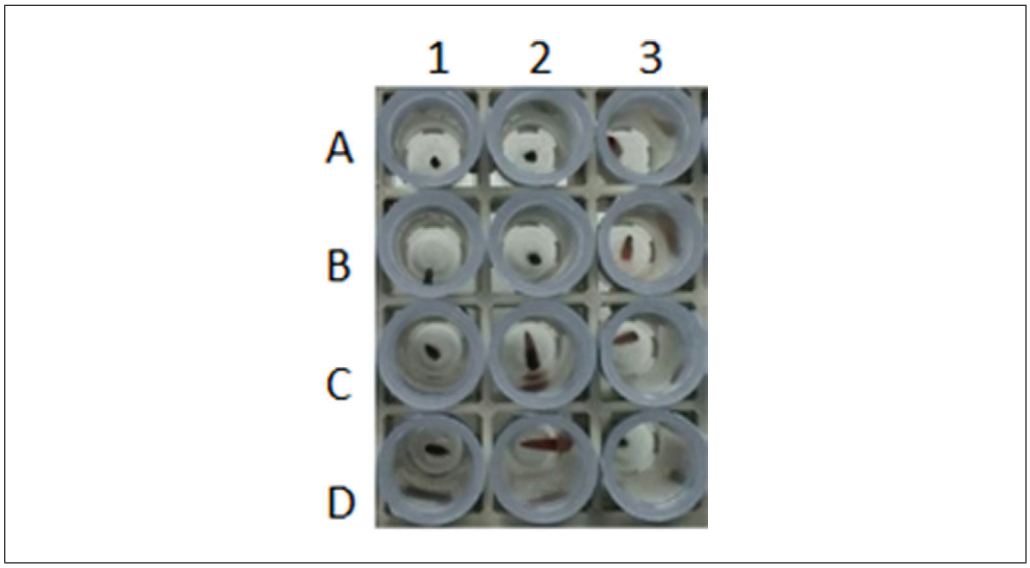


Figure 1 Picture showing ear and tail biopsies. Biopsies from a 96-well plate. Ear (A1, A2, B2, D3) and tail (B1, C1, C2, D1, D2, A3, B3, C3) biopsies can be recognized by visual inspection. Oversized tail biopsies can also be easily identified (C2 and D2).

Materials

Tubes containing mouse tissue samples (Basic Protocol 1)
DirectPCR Lysis Reagent (Mouse Tail; 102-T, Viagen)
10 mg/ml proteinase K (dissolve 1 g lyophilized proteinase K powder [cat. no. P6556, Merck] in 100 ml water to obtain a clear solution; if desired, store aliquots in 1.5-ml tubes (e.g., cat. no. 72.690001, Sarstedt) at −20°C)

Manual pipets
Centrifuge (e.g., Allegra 25R centrifuge Beckmann Coulter)
Heated water bath (e.g., GLF 1083), 85°C
Heating oven (e.g., Memmert), 55°C
Personal protection equipment: lab coat, gloves

Visually verify each tube to be processed

1. Check that only one sample is present in the tube.

   Check that the biopsy in the tube corresponds to the expected type of sample (i.e., tail, ear notch, etc.). See Figure 1 showing ear and tail biopsies.

2. Visually check that the size of the tissues corresponds to the recommended size (see Table 2 for recommended sizes). If not, mark the tube for later adjustment of the lysis buffer volume.

Prepare buffers

3. Prepare a premix lysis buffer: Add 200 µl DirectPCR Lysis Reagent (Mouse Tail) and 6 µl 10 mg/ml proteinase K solution (10 mg/ml) per reaction.

   Such a premix is stable for at least 24 hr at 4°C.

Incubate and lyse sample

4. To each sample, add 200 µl premix lysis buffer for a 0.5-cm tail biopsy or 100 µl for a 0.2-cm ear punch or toe biopsy.

   For uncalibrated biopsy, reduce or increase the premix lysis buffer volume proportionally.

5. Hermetically seal the tubes.

6. Centrifuge tubes 2 min at 4000 × *g*, room temperature.
7. Check that the biopsies are at the bottom of the tube and covered by the solution.
8. Incubate overnight at 55°C in a heating oven.
9. Remove tubes and check that they are still hermetically sealed.
10. Shake vigorously by turning over.

    The biopsy must dissociate in the tube, indicating that the lysis worked. If the biopsy does not dissociate (indicative of inefficient lysis), remove the lysis buffer and repeat steps 4-10 (i.e., perform sample incubation with a fresh premix buffer).

Deactivate proteinase K

11. Incubate tubes at 85°C in a heated water bath for 45 min to 1 hr to inactivate the proteinase K.
12. Centrifuge tubes 2 min at 4000 × *g*, room temperature.
13. Store tubes at 4°C.

    These crude extracts are stable for 2 weeks at 4°C.

DESIGN OF A GENOTYPING STRATEGY

BASIC PROTOCOL 3

A genotyping assay must provide a rapid and cost-effective method of identifying animals. It also needs to be reliable and robust, as it will be used to select mutant animals for experiments and for ensuring the integrity of a unique genetic resource (i.e., animals for future production, for cryopreservation, or to be received by a collaborator or resource).

Designing genotyping assays around markers that are found in many transgenic lines, such as the neomycin selection marker, GFP, or Cre, is discouraged as it does not verify that you are using the anticipated mutant line and is not suitable for genotyping double-transgenic strains that contain common transgenic markers. Instead, it is recommended that the PCR assay specifically identify each line and each possible allele derived from the stock mutant line. For example, the International Knockout Mouse Consortium is creating a catalog of mammalian gene function (Meehan et al., 2017) and reports the generation of over 5000 new mouse mutant strains all harboring a *lacZ* reporter cassette and providing conditional inactivation potential. An optimized protocol allows researchers to evaluate all possible genotypes and the integrity of the targeting event (Fig. 2A).

Defining PCR design rules that can be applied to all protocols (see Troubleshooting) is recommended. This simplifies genotyping because specific conditions (reaction mix, thermocycling program, agarose gel analysis) are not required for any project, and it thus allows multiple mutant lines to be assayed in the same experiment. This strategy is especially recommended when large volumes and/or high throughput are involved.

Materials

Biological material: aliquot from crude sample lysate, to be used as template for PCR typing
Primers for mouse-specific target of interest
Master mix: e.g., FastStart PCR Master (50 ml; cat. no. 4710452001, Merck)
20× speed buffer (SB), prepared as described by Zhang, Wang, & Wang (2011) using boric acid (cat no. 5935, Euromedex), sodium hydroxide (cat. no. 06203, Merck), and ethidium bromide (cat. no. EU0170, Euromedex)
Water, PCR grade (for primer dilution and reaction fill-up)

Adjuvants (optional): 5% (v/v) dimethyl sulfoxide (DMSO; e.g., cat. no. D8418-100ML, Merck), 5% (v/v) glycerol (e.g., cat. no., 15524-1L-R, Merck), or 0.5 µg/µl bovine serum albumin (BSA; e.g., cat. no. B9001S, New England Biolabs) in final reaction volume

Agarose, DNA grade (e.g., cat. no. D5, Euromedex)

Homemade loading dye stock (prepared by dissolving 3 ml glycerol and 8 mg bromophenol blue in 7 ml H_2O)

DNA molecular weight marker: e.g., GeneRuler 50-bp DNA Ladder (cat. no. SM0372, Thermo Fisher Scientific)

Appropriate restriction enzymes with buffers recommended by suppliers

0.75-ml screw-cap tubes (e.g., Matrix tubes, blank; cat. no. 446015, Dutscher) and caps (e.g., Sepra Seal Cap Mats; cat. no. 446045, Dutscher)

1.5-ml Microtubes (e.g., cat. no. 72.690001, Sarstedt)

Computer with software for sequence analyses and tools for primer design, either commercial (e.g., Vector NTI, SnapGene, Geneious) or free (e.g., U-GENE, BioEdit, SeaView)

4titude FrameStar 96-well plates (cat. no. 44760, Dutscher) or 0.2-ml PCR tubes

Centrifuge for microcentrifuge tubes (cat. no. 016000, Dutscher), optional

PCR machine (e.g., Eppendorf thermal cycler)

Agarose gel electrophoresis equipment: Tank for electrophoresis, support where the gel is poured, combs for forming wells (where samples are deposited)

Acquisition imager using UV light as excitation source (e.g., U:Genius, Syngene)

Personal protection equipment: lab coat, gloves, safety glasses

Manual pipets

Search for targeted and wild-type sequence maps

1. Obtain from mouse provider the sequences of the wild-type and mutant alleles.

   To establish an efficient PCR genotyping strategy, it is essential to have access at least to the DNA sequence of the mutant allele. Without this information, no specific design can be done. Thus, if you are unable to get the recombinant sequence from the provider, we strongly advise generating the theoretical sequence yourself using information available in the corresponding published paper. If the recombinant sequence cannot be generated from a published paper, see the additional recommendations provided in Critical Parameters.

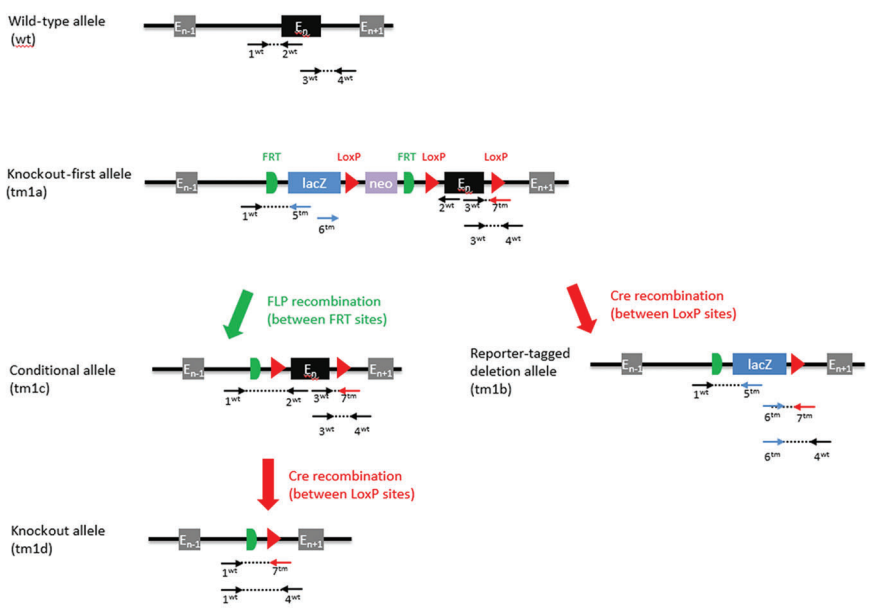
2. Verify that the wild-type sequence you have matches the latest release of the *Mus musculus* C57BL/6J reference genome assembly in NCBI (*https://www.ncbi.nlm.nih.gov/genome?term=mus%20musculus*) or Ensembl (*https://www.ensembl.org/Mus_musculus/*). If it does not, you will need to carefully check the wild-type sequence provided.

   Gene reannotation may change your gene structure. Other backgrounds than C57BL/6J are also available as wild-type references at https://www.sanger.ac.uk/science/data/mouse-genomes-project.

3. Use genomic sequence analysis software (such as commercial Vector NTI, SnapGene, Geneious or free U-GENE, BioEdit, or SeaView software) to construct all the sequence maps for each of the alleles you need to genotype.

   These software packages allow you to plan and simulate DNA manipulations, visualize open reading frames and primer binding sites, and share annotated sequence files with other researchers.

A Mouse genotyping strategy for knockout-first IMPC mutant



B Primer combinations to identify wild type and all IMPC mutant alleles

	forw	rev	Wild-type allele (wt)	Knockout-first allele (tm1a)	Reporter-tagged deletion allele (tm1b)	Conditional allele (tm1c)	Knockout allele (tm1d)
1	1^{wt}	2^{wt}	a	--	--	a + FRT/LoxP	--
2	1^{wt}	4^{wt}	*	*	--	*	b
3	1^{wt}	5^{tm}	--	c	c	--	--
4	3^{wt}	4^{wt}	d	d + LoxP	--	d + LoxP	--
5	6^{tm}	7^{tm}	--	--	e	--	--
6	1^{wt}	7^{tm}	--	--	--	*	d
7	3^{wt}	7^{tm}	--	f	--	f	--
8	6^{tm}	7^{tm}	--	--	h	--	--

Figure 2 Mouse genotyping strategy optimized to genotype mutant models generated by the IMPC consortium. With this strategy, the researcher need only design four different primers specific to their gene of interest in order to genotype any relevant allele combination. This design is based on the detection of a specific PCR product for each allele, and it thus avoids genotyping errors caused by false negative PCRs. PCRs 1 to 5 allow one to detect all possible combinations. PCRs 6 to 8 are additional combinations that can be used to confirm a genotype or if one PCR is not working. 5^{tm} (CTCCTACATAGTTGGCAGTGTTTGGG), 6^{tm} (GCACATGGCTGAATATCGACGGT), and 7^{tm} (ACTGATGGCGAGCTCAGACCATAAC) are universal primers that can be used for any knockout-first IMPC mutant model. –, no product expected; *, this PCR product will not be observed using the described PCR genotyping conditions ; tm1a, tm1b, tm1c, and tm1d correspond to allele nomenclature as defined by the IMPC consortium; En, exon; En+1, exon +1; En-1: exon −1.

Design of genotyping primers

Primers should be designed to fit the targeted sequences. It is possible to design primers using a variety of tools (genomic sequence analysis software), or even by eye following the rules below.

4a. Primer structure:

i. 20-24 nucleotides in length.

ii. G or C at 3′ end.

The bases G or C at the 3′ end serve as the starting binding site for the DNA polymerase.

iii. 40%-60% GC composition.

iv. Comparable melting temperatures (T_m) for both primers.

Comparable T_m (within 5°C of each other) will determine the stability of the hybrids once the match between primers and matrix is achieved.

v. Specific to the appropriate genomic DNA sequence.

Check the specificity of each primer against genomic DNA with NCBI Nucleotide blastn on the Mus musculus genome only (https://blast.ncbi.nlm.nih.gov/Blast.cgi). You do not need to modify any algorithm parameters or program selection (i.e., use megablast).

The specificity of a primer set is related to whether the primers will bind only to the sequence that we want to detect or also to additional sequences.

CAUTION: *It is not necessary that there be 100% homology between primers and a genomic sequence for nonspecific PCR amplification to occur. Primers with a few mismatches or with a nonspecific 5′ end can give rise to nonspecific amplifications.*

vi. No internal secondary or primer-primer annealing structures.

Internal secondary structure can be checked by using primer design software (e.g., OligoArchitect from Sigma-Aldrich) to analyze duplex formation.

Primer pairs should lack significant internal secondary structure to avoid internal folding. Primer-primer annealing caused by homology within the primer pair creates primer dimers and disrupts the amplification process and is thus to be avoided.

4b. Amplicon structure: 100-500 bp amplicon size.

An amplicon size of 100-500 bp is optimal for visualizing PCR fragments using 2% (w/v) agarose gel electrophoresis. Below 100 bp, PCR fragments are difficult to separate and visualize. Over 500 bp, PCR efficiency is lower as crude extract are usually more degraded than purified DNA. Moreover, classical Taq polymerase does not process amplicons >1 kb in size well.

If the sequence of the amplicon contains >60% GC, perform the PCR setup with and without adjuvants (as described in Troubleshooting) or even with a specific GC-rich polymerase (suppliers provide an array of specific DNA polymerase designed for specialized needs such as GC-rich amplification).

If possible, avoid dinucleotide repeats (e.g., GCGC or ATAT) and single-base runs (e.g., AAA or CCC) in the amplified sequence, as these can cause hairpin loop structures.

5. Select the best primer position on the wild-type or mutant(s) allele(s).

Ideally, primer position is optimized so that the primer can be used as one primer pair for different PCR assays. With this strategy, combining common primers with different, unique primers allows all possible alleles to be detected. Figure 2 provides an example of primer design optimization for genotyping mutant models generated by the International Mouse Phenotyping Consortium (IMPC https://www.mousephenotype.org; Meehan et al., 2017). Primers are positioned in the wild-type sequence on the DNA region that differs between the wild-type allele and the mutant allele(s). With this approach, only four different primers are required to genotype any relevant allele combination for a target gene. Three additional primers are designed on the mutant sequence. These three primers can be used for any knockout-first IMPC mutant model.

We recommend designing two independent set of primers per allele to be detected, which will enable mouse genotyping even if one primer set does not work.

6. *Optional*: Detect mutant alleles with single-nucleotide polymorphism (SNP) mutations.

If your mutant allele differs from your wild-type allele by only a single or few point mutations, wild-type and SNP mutant PCR amplicon will have exactly the same size. This will be particularly the case if CRISPR/Cas9 genome editing or ENU mutagenesis has been used to generate the mutant line (Birling, Herault, et al., 2017). In this case, PCR products will have to be sequenced using Sanger sequencing (Dorit, Ohara, Hwang, Kim, & Blackshaw, 2001) or a particular SNP detection method used (e.g., TaqMan, pyrosequencing, etc).

As PCR product sequencing remains expensive and time consuming, especially if numerous mutants need to be screened, it may be useful when creating the CRISPR/Cas9 mouse model to insert a restriction site that does not alter the gene protein sequence (a third-nucleotide triplet synonymous substitution that does not alter the amino acid encoded). This restriction site can then be used for diagnostic digest of the PCR product (step 25).

7. Verify that each PCR amplicon produces a size that can be readily separated on 2% agarose gel.

   Some primer pairs can be used to detect different alleles (see Fig. 2B), but the size of the PCR amplicon needs to differ by a minimum of 50 bp to correctly discriminate each amplicon.

8. Order primers from any provider.

   Standard PCR does not require high-quality primer synthesis. You can select any provider and use desalted purification.

9. Dilute primers to 100 µM in PCR-grade H_2O and store at −20°C.

Setup of polymerase chain reaction

The PCR strategy described below should be tested on three to five samples. Ideally, a biopsy from mutant animals is used for the test PCR. If no biopsy is available, embryonic stem cell clone DNA diluted in crude extract, targeting vector diluted in crude extract, or chimera biopsy can be used. A tissue biopsy from a wild-type animal should always be included as a control. Likewise, PCR reactions with no template act as a negative (or water) control to confirm the absence of PCR contamination.

10. Centrifuge tubes containing biological samples for 2 min at 4000 × *g*, room temperature, to sediment debris.

11. Prepare the PCR reactions as described below, either in a sterile 1.5-ml tube for a few samples or in a 96-well plate for larger sample numbers.

    As the enzyme used in this protocol is a hot-start polymerase, the PCR reaction mix can be prepared at room temperature (18°C-25°C). However, if any reagents have been frozen for later use (e.g., reaction mix or primers), the tubes should be thawed slowly on ice.

    The amount of each reagent added to the master mix is equivalent to the total number of volume reactions plus 10% rounded up to the nearest whole reaction (to accommodate pipet transfer loss).

    a. Add 14 µl master mix (FastStart PCR Master, Roche) per tube or well.

       Using a premade mixture of the enzyme, dNTPs, and reagents, such as FastStart PCR master, minimizes errors and contamination risk and reduce the time for PCR preparation.

    b. Add 0.2 µl each of 100 µM forward and reverse primers (from step 9) per tube or well.
    c. Add 7.6 µl sterile water per tube or well.

12. To each 0.2-ml PCR tube or each well of a 96-well plate, add 3 µl crude extract and then 22 µl PCR reaction mix.

Table 4 Recommended Cycling Conditions[a]

Temperature	Time	Number of cycles
95°C	4 min	1
94°C	30 s	
62°C	30 s	34
72°C	1 min	
72°C	7 min	1
20°C	5 min	1

[a]These cycling conditions work well with our protocols (Basic Protocols 3 and 4) but may require modification if other conditions (e.g., other Taq polymerase) are used.

13. Mix thoroughly by gently pumping the plunger of a micropipet up and down two or three times.
14. Prepare a negative control: Add all reagents with the exception of the DNA template (increase the water to compensate for the missing volume).
15. Seal the tubes or plate.
16. Centrifuge the reaction mixture briefly so that it falls to the bottom of the tube or plate.
17. Insert PCR tubes or plates into the thermal cycler and begin PCR program following the parameters described in Table 4.

    Developing standard conditions applicable to all mutant models allows all samples to be processed together regardless of the project.

Image acquisition and analyses

PCR amplicons are separated using a 2% agarose gel and the results are visualized using a digital camera.

18. Dilute 20× concentrated SB stock to 1× to be used to make and run the gel.

    This buffer allows you to run a DNA gel at high voltages without overheating and melting your gel.

19. Prepare a 2% (w/v) gel agarose containing 1× SB. Before polymerization, wearing gloves and safety glasses, add 15 µl 10 mg/ml ethidium bromide stock per 600 ml of agarose gel in the solution. Shake slowly without making bubbles.

    The greater the agarose concentration, the smaller the pores created in the gel matrix and the more difficult it is for large linear DNA molecules to move through the matrix. Changing the agarose concentration changes the size of the sieve matrix of the gel. 2% gel is well adapted for 100- to 500-bp PCR products.

20. To prepare PCR samples for migration, add 5 µl homemade loading dye to 15 µl of PCR reaction.
21. Carefully load samples into the wells of the gel.

    To approximate the size of the amplicons, commercially available DNA molecular weight marker is added at each end of a row (3.5 µl per well).

22. Run the gel at 280 V (400 mA, 100 W) until the dye line is ~80% of the way down the gel.

    A typical run time is ~45 min, depending on the gel size.

23. Use a digital camera (U:Genius) to visualize the DNA fragments.

If a PCR product is present, the ethidium bromide will intercalate between the bases of the DNA strands, allowing bands to be visualized with a UV illuminator.

Always save and archive the resulting image. For example, you may need to check for genotyping errors or provide this image for publication.

Assay validation

24. Check that each amplicon band corresponds to the expected size for each assay. Verify absence of additional bands.

    If yes, the PCR setting is validated, and the primer pairs should be selected for genotyping.

    The final protocol you have designed can be used now for genotyping your animals.

    If not, new primer sets should be tested.

    Refer to Critical Parameters for additional solutions for PCR setup.

Additional steps for SNP mutation detection via diagnostic digest

If the wild-type and mutant PCR amplicon are of the same size (see step 6), PCR product cleavage with restriction enzyme can be performed to differentiate the two amplicons.

25. Verify the presence of DNA amplicons after PCR by electrophoresis as detailed in steps 18-23 but using only 5 µl of the PCR reaction in step 20.

26. Prepare the digestion reaction mix, either in sterile 1.5-ml Microtubes for few samples or in a 96-well plate.

    a. Add components in the following order: 2.5 µl of the 10× buffer supplied with the enzyme, 1 µl restriction enzyme, and 11.5 µl water.

    The amount of restriction enzyme you use for a given digestion will depend on the amount of DNA you want to cut. By definition, one unit of enzyme will cut 1 µg DNA in a 50-µl reaction in 1 hr. Reactions are often performed with 0.5-1 µl enzyme.

    b. Add 10 µl of PCR reaction.

27. Incubate tubes at digestion temperature (usually 37°C) for 1 hr.

    Incubation time can range from 45 min to overnight. For diagnostic digests, 1-2 hr is often sufficient.

28. Visualize the digested PCR products by migration on an agarose gel as described in steps 18-23.

MOVING TO HIGH-THROUGHPUT GENOTYPING

BASIC PROTOCOL 4

All classical genotyping methods have relatively low throughput. Automating genotyping through the use of a workstation allows parallel genotyping of a large number of genetic modifications, at many genetic loci, in many individuals. In addition to improving throughput, automation reduces the potential for contamination and error by limiting pipetting steps and preventing tube switching. The conventional endpoint PCR method is easily adapted for automation and is indeed an effective, proven, and affordable method for high-throughput screening. Moreover, PCR assays can be miniaturized in 384-well plates on a workstation, reducing the cost of animal genotyping.

Developing a Laboratory Information Management System (LIMS) to effectively manage workstations, samples, and associated data is also essential (Fig. 3; Critical Parameters).

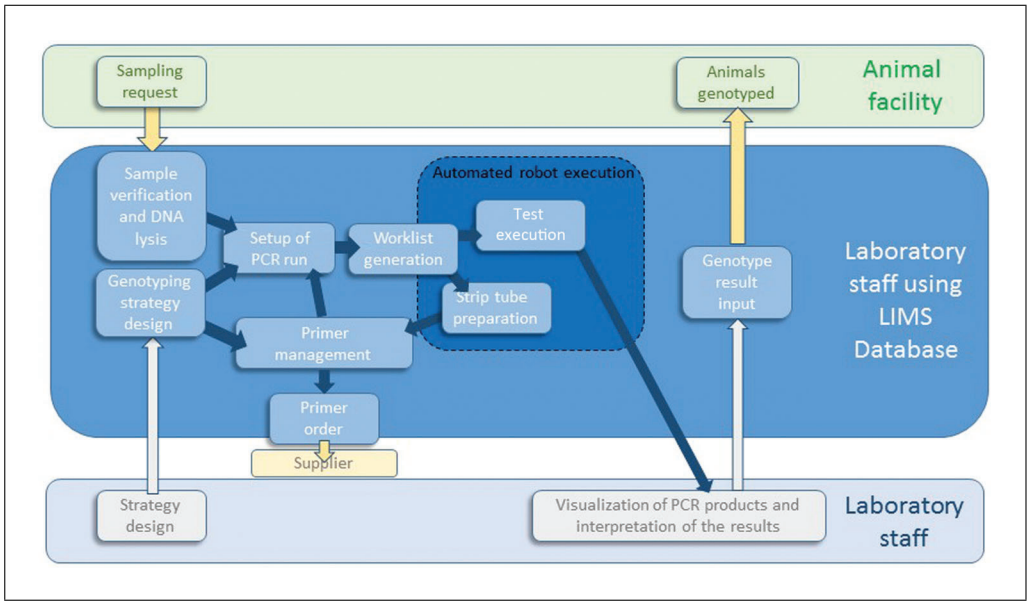


Figure 3 Laboratory Information Management System (LIMS). Example of a database controlling the flow of samples, the primer bank, and the operation of the workstation with worklists. The database is connected to the animal management database so that it can receive genotyping requests and transfer animal genotypes at the end of the analyses.

Materials

Biological material: Crude sample lysates in 96-tubes racks
Primers for mouse-specific target of interest
Water, PCR grade (for primer dilution and reaction fill-up)
FastStart PCR Master (50 ml; cat. no. 4710452001, Merck)
Adjuvant: 5% (v/v) DMSO (cat. no. D8418-100ML, Merck), 5% (v/v) glycerol (cat. no. 15524-1L-R, Merck), or 0.5 µg/µl BSA (cat. no. B9001S, New England Biolabs)

Workstation or liquid handler (e.g., Freedom EVO200, Tecan, or STARplus, Hamilton Microlab)
4titude FrameStar 384-well PCR plates (cat. no. 384 44751, 4titude)
8-Strip PCR tubes with caps (e.g., cat. no. 016000, Dutscher)
15-ml conical tube (e.g., cat. no. 352097, Corning)
Integrated centrifuge (e.g., Sias)
Integrated heat sealer (e.g., PlateLoc, Agilent
Sealable film clear seal (cat. no. 4Ti-0542, 4titude)
PCR thermocycler with motorized heated lid (e.g., T-robot, Biometra)
Manual pipets

Design of genotyping strategy and assay validation

1. Follow steps 1-9 of Basic Protocol 3 to design a high-throughput genotyping strategy.

   We also strongly advise following the recommendations given in Critical Parameters (section on important recommendations for designing genotyping strategy for high-throughput workflow) to guarantee successful high-throughput automation.

2. For assay validation, follow steps 10-24 of Basic Protocol 3. Use conditions described in Table 5 for 384-well plates.

   High-throughput genotyping strategy validation must be done in 384-well plate format as the PCR conditions of 96-well plates are not always proportionally applicable in 384 wells.

Table 5 Comparison of Reaction Volumes Between 384- and 96-Well Plates

Reagent	Volume for 384-well plate	Volume for 96-well plate
FastStart PCR Master (Roche)	7.5 µl	14 µl
Crude extract	1.5 µl	3 µl
5′ primer (100 mM)	0.06 µl	0.2 µl
3′ primer (100 mM)	0.06 µl	0.2 µl
Sterile H_2O	Up to 15 µl	Up to 25 µl

Prepare the automatized PCR run

We present here the protocol used on our two workstations (the Tecan Freedom EVO 200/8 and the Hamilton Microlab STARplus). This protocol must be adapted to each installation according to your particular workstation specifications.

3. Turn on the workstation, the associated computer, and all integrated instruments.
4. Open the software controlling the workstation (e.g., Freedom EVOware for Freedom EVO 200/8 Tecan or VENUS software for STARplus Hamilton).
5. Initialize the instrument.

   The initialization sequence is used to calibrate the robotic arm movements, i.e., to determine the reference (zero) positions along the x, y, and z axes.

6. Flush the instrument.

   This function uses a wash station to flush the diluters and the tubing (fill them with system liquid) and to wash the fixed-steel washable needles (or tips) on the pipetting arm.

7. Load or generate the worklist.

   The worklist is a file that contains commands telling the instrument what and where to pipet. The file contains information on source and destination positions and the volumes to be pipetted.

   a. If you have developed a LIMS to manage your sample (see Critical Parameters), load your worklist using the software controlling the workstation.
   b. If not, generate the worklist as indicated by the workstation provider.

Load reagents, tubes, and plates onto the robot worktable

A visualization of the worktable (in worktable windows) is generated by the software controlling the workstation following step 7. This windows represents the working surface (deck) of the instrument. Follow the indications on the screen to place all the reagents, tubes, and plates.

8. Place all 96-tube racks containing crude extract on the workstation as indicated by the worktable windows.
9. Position the primer sets as described in the worktable windows.

   We recommend preparing the primer sets in an 8-tube strip (see Critical Parameters).

   a. Prepare 12-24 tube strips per mutant line and store them at −20°C.
      - Add 6.5 µl each of 100 mM forward and reverse primers per microtube.
      - Supplement with 187 µl sterile water per microtube.
      - Repeat this step for all PCR sets used for genotyping the mutant lines (up to eight PCRs per mouse line).

b. Place all 8-tube strips needed for the PCR run on the workstation as indicated by the worktable windows.

10. Prepare the reaction mix.

    a. Add 3.3 ml FastStart PCR Master and 1.7 ml sterile water in a 15-ml conical tube for each 384-well PCR plate to be generated during the run. An extra 15% of reaction mix is included to accommodate pipetting loss.
    b. Place the 15-ml tube as indicated on the worktable windows.

    The reaction mix volume is based on the total number of samples, number of PCR amplifications to be conducted per sample, and required reagent dead volumes.

11. Place 384-well PCR plates in the indicated locations.

Set up a PCR run on an automated workstation

12. Start the appropriate pipetting script (e.g., method for PCR with 384-well plates and sealing).

13. Visually check that the pipetting process has started correctly before leaving the workstation.

    In our configuration, the liquid handler will distribute to each well of the plate, in order:

    a. 11.5 µl of reaction mix;
    b. 2 µl of the relevant primer set;
    c. 1.5 µl of the relevant sample crude extract.

    This pipetting order (reaction mix then primers and sample crude extracts) is optimized to reduce pipetting steps and duration, decontamination steps (bleaching), flushing, and risk of contaminations.

Post-run process

14. Each 384-well plate is automatically sealed, then centrifuged (2 min at 4000 × *g*) and placed in a thermocycler by the gripper arm. The liquid handler software controls the thermal cycler and starts the thermal cycling program (program parameters are described in Table 4).

15. At the end of a run, it is possible to perform a visual inspection to check that all the wells of the plate have the same and expected volume.

Image acquisition and analyses

16. Follow steps 18-23 of Basic Protocol 3.

Decontamination

We recommend thoroughly decontaminating the instrument at the end of each week either by pipetting a 10% diluted bleach solution or by using a UV lamp.

COMMENTARY

Critical Parameters

Age of animals for biopsy

Table 2 shows the age(s) at which each tissue sampling method can be used. Sampling should, where possible, be done on young animals for the reasons listed below:

- DNA from tissues of young mice is more optimal for genotyping than that from older animals (Picazo & García-Olmo, 2015).
- In a newborn mouse (particularly before 12 days of age), discomfort due to the sampling is reduced because the sampled tissue is

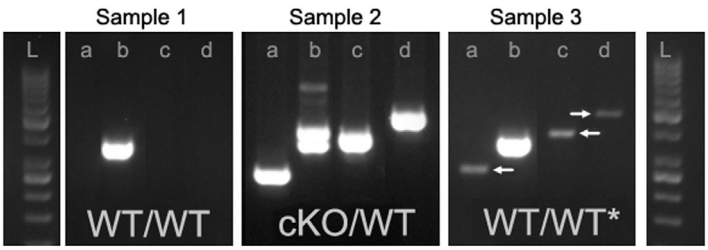


Figure 4 Sample contamination caused by improper scissors cleaning during tissue collection. Three samples were harvested consecutively and processed in the same experiment. In this example, PCRs a, c, and d identify the conditional knockout (cKO) allele; PCR b shows a lower band for a WT allele and an upper band for the cKO allele. The first collected sample is positive for PCR b only (lower band), indicating a wild-type (WT/WT) genotype. The second collected sample is positive for PCRs a, c, and d and show two bands for PCR b. This sample is genotyped as a heterozygote animal (cKO/WT). The third collected sample is strongly positive for the lower band in PCR b and more weakly positive for PCRs a, c, and d (white arrow). Contamination during tissue collection explains this result and is clearly affecting the genotype determination. Negative and positive controls were also performed but are not shown in this figure. All four possible amplicons are absent from the negative (water) control, whereas the positive control (wild-type DNA) contains the PCR b product only (lower band; data not shown).

not fully ossified and because the nociceptive stimulus may not result in the conscious perception of pain due to the lack of a competent pain pathway at this age (Hankenson, Garzel, Fischer, Nolan, & Hankenson, 2008; Silverman & Hendricks, 2014; Wever, Geessink, Brouwer, Tillema, & Ritskes-Hoitinga, 2017).

- If genotyping is completed before weaning, extra animals or those of nondesired genotype can be sacrificed before separation of young animals into different cages.

Tail biopsy is ideally performed between ~10 and 21 days of age. At this age, the distal tail is not fully ossified in most mouse strains, making the procedure less painful and reducing the likelihood of complications (Hankenson et al., 2008). After 4 weeks of age, anesthesia is mandatory for this procedure. Removal of more than 5 mm of tail must be avoided, as the bone is thicker in more proximal parts of the tail and this increases the likelihood of causing tissue trauma and suffering.

Ear clipping (also ear notching or ear punching) should not be carried out on mice <2 weeks of age because the ear is not yet fully developed and the removal of even a small piece of tissue can represent a significant proportion of the pinna. Ear clipping is the preferred sampling method for animals >4 weeks of age because it produces little discomfort in older mice (Picazo & García-Olmo, 2015) and therefore does not require anesthesia. During sampling, care should be taken not to accidentally drop or lose the very small ear tissue sample.

Toe clipping consists of the removal of the distal phalanx of a neonatal animal as a means of identification. This can be used as a source of sample for genotyping. Toe clipping of very young mice (ideally up to postnatal day 7) is an acceptable method as the ossification process is not yet completed and the peripheral nervous system is not yet fully myelinated (and thus the nerve conduction of pain is dramatically reduced; Dorit et al., 2001; Lee et al., 2012).

Sample calibration

Sample calibration is verified by visual inspection of each tube (see Basic Protocol 2, step 2).

Ear punching (0.9% total oversized samples; 2018 data corresponding to more than 40,000 samples visually checked) and toe clipping (1.0% oversized samples) provide more calibrated samples than tail biopsies (8.7% oversized samples), as shown in Figure 1. Respecting recommended and calibrated sample size reduces the risk of false negative results due to either increased inhibitors or insufficient template concentration.

Impact of improperly cleaned instruments

When collecting tissue samples, the instruments must be cleaned between individual animals to avoid cross-contamination of genetic material. As shown in Figure 4, false positive PCR amplification can result from sample cross-contamination.

Table 6 Guide to Evaluating Sample Quality

Observation	Possible cause	Solution
More than one sample in tube	Toe biopsy cut for identification of the animal remained stuck to the scissors and fell into the tube with the sample to be genotyped	Remove the incorrect sample (if can be distinguished: e.g., toe biopsy in a tail sample) or ask for a new biopsy.
	An earlier sample was mistakenly placed in the tube	Discard the tube or even the request, if there is any doubt about a possible shift in tubes, and ask for a new biopsy. Misidentification (see Critical Parameters for discussion of error rate).
No sample in the tube	Sample tissue was accidentally dropped or lost	Discard the tube or even the whole genotyping sample set, if there is any doubt about tube switching, and ask for a new biopsy.
Type of biopsies in the tube different from what is mentioned by the animal facility	Writing error occurred	Modify the type of biopsy indicated on the genotyping request and add the corresponding premix lysis buffer.
Uncalibrated biopsy: Sample too large	Part of the ear was torn off	Cut the sample to obtain a calibrated size or adjust the premix lysis buffer.
	Tail biopsy is too big (Fig. 1)	
Uncalibrated biopsy: Sample too small	Tail biopsy is too small	Adjust the premix lysis buffer.
	Tail biopsy is from embryonic day 8.5 mouse	

Sample storage

Tail, ear, or toe biopsies contain DNase that will slowly reduce the quantity and integrity of genomic DNA after tissue collection (Al-Griw et al., 2017). To slow this process, samples are usually stored at −20°C. However, native high-molecular-weight DNA is not required to amplify a target sequence by PCR: as only the target sequences are required to be intact, partially degraded or denatured DNA could be successfully used for PCR applications (Wever et al., 2017). From our experience, tissues stored at room temperature can be used if storage is for <24 hr and temperature does not exceed 20°C (for example, avoid shipment of samples at room temperature in summer).

Quality check of each tube before lysis

Before starting lysis, we recommend each tube be checked for absence of (or presence of additional) biopsy or oversized samples (see guide in Table 6). This quick quality check will reduce inconclusive genotyping (see section on error rate, below) and allow you to adapt the lysis buffer volume if needed.

Error rate

Inconclusive or incorrect genotyping can occur for a variety of reasons, including errors during the genotyping procedure itself and the misidentification of samples or animals. Genotyping errors can result in irreproducible results and genetic contamination or loss of a mouse line. They are sometimes detected

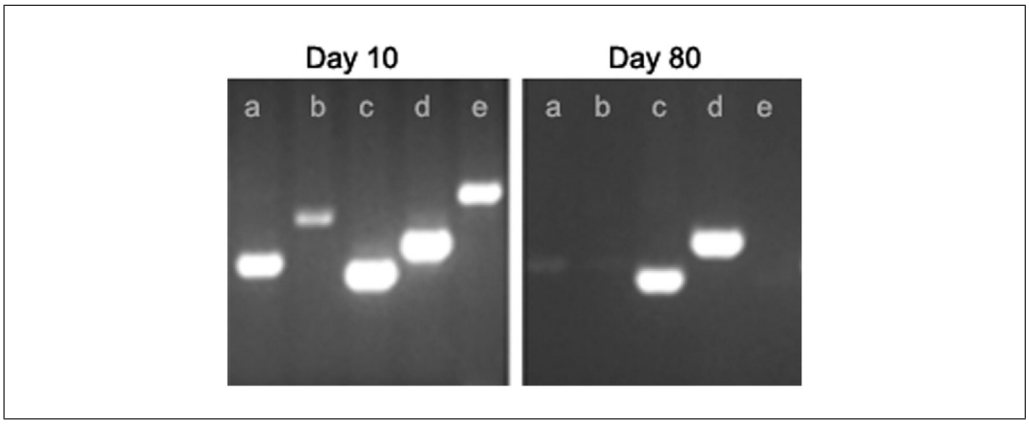


Figure 5 Genotyping divergence observed in animal sampled at day 10 and day 80. The same animal was sampled at the newborn stage (day 10) and at its shipping date (day 80). The first biopsy at day 10 amplified all of the PCRs (a-e), indicating a transgenic animal genotype; the second biopsy at day 80 amplified only the WT PCRs (c and d), corresponding to a WT animal genotype. This wrong genotype could be due to any of several different types of errors: for example, during tissue biopsy, the wrong mouse was sampled because of misreading the identification code; during tissue biopsy, tubes or samples were inverted; during PCR, a sample was inverted; during analysis, the wrong line was labeled.

because of inconsistent Mendelian patterns in pedigrees (i.e., the observed genotypes are not consistent with the transmission pattern). In these cases the parents are biopsied again to confirm their genotypes.

Genotype verification is advised for all key animals: that is, before or after phenotyping experiments or freezing or shipment of mutant lines (Fig. 5). Without verification of the genotype during a phenotyping study, in our experience 30% of the cohorts will have at least one animal whose genotype does not correspond to the one expected (data not shown). Likewise, >15% of lines deposited to public repositories do not carry the mutation specified by the depositor (Lloyd et al., 2015).

How to design a genotyping strategy with minimal sequence information

To ensure accurate assay design, it is crucial to know the precise sequences of the wild-type and mutant alleles. The sequence of the wild-type allele can easily be retrieved from the Ensembl or NCBI databases. However, the sequence of the mutant allele is not systematically provided when you receive a new mutant model. Where possible, insist on obtaining this key information from the researcher who generated the mouse.

In cases where you do not know the mutant allele sequence, you may have received a genotyping protocol. You can create a hypothetical mutant map by aligning the primers from this protocol onto the wild-type sequence, which will allow you to determine the position of modified sequences (such as *loxP* sites or selection cassettes). Additionally, the PCR products can be sequenced to obtain a partial mutant sequence. If necessary, a new assay can be designed once this information is available (see Basic Protocol 3 for recommended design strategy). Sequencing the mutant allele PCR product can also provide quality control of the mutant model (presence of an SNP mutation or *loxP* site).

If the primers provided do not give you accurate information on the genomic structure of the targeted allele, you will only be able to design your strategy to target marker sequences, such as the neomycin selection marker, GFP, or Cre. These sequences are very frequent in many genetically modified mouse strains, meaning that you will not be able check reliably for animal misidentification (critical parameters) or genotype double-transgenic models.

Genotyping assay optimization

The genotyping protocol presented here is very robust in most cases. Of >4000 different PCR primer sets tested in our lab, only 9% were not validated (data not shown). Instead of changing PCR parameters when a PCR primer pair does not work, we advise first testing a different primer pair.

It is often possible to retrieve the genotyping protocol from the researcher who generated the mouse. This protocol can of course be used for genotyping. As it will be necessary to optimize it to adapt this protocol to your laboratory conditions, however, we recommend instead designing a new genotyping protocol

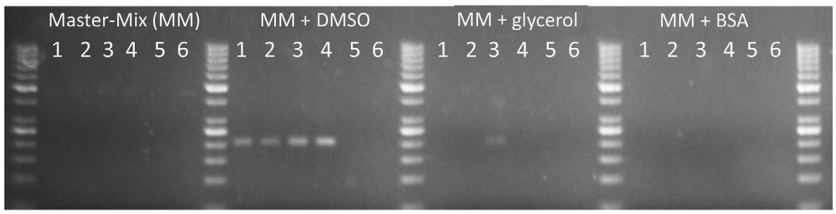


Figure 6 PCR efficiency can be improved by additives, as exemplified by the case of a GC rich template. PCR using the conditions described in this protocol was performed on a GC-rich target for four mutant samples (lane 1 to 4), one wild-type control (lane 5), and a negative control (blank without DNA; lane 6) under four different conditions: (**A**) without any adjuvant, (**B**) with 5% DMSO added, (**C**) with 5% glycerol added, and (**D**) with 0.5 μg/μl BSA added.

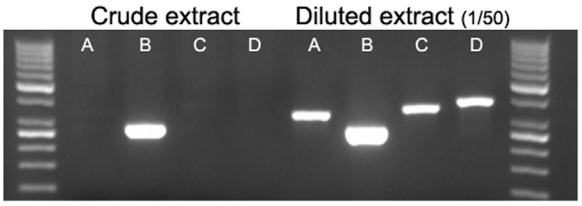


Figure 7 Comparison of PCR done with either crude or diluted extract as template. The same sample was analyzed from undiluted crude extract or after 1/50 dilution. In this example, PCRs A, C, and D only worked when the sample was diluted.

according to the standardized parameters we described here. This will allow you to verify the line you received with different PCRs. The PCR design rules we describe here can also be applied to any mutant line. If you systematically follow them, all steps from DNA preparation to gel agarose analysis will be standardized and can thus be performed in parallel for all your mutant models, saving time and effort.

The ramping conditions of your PCR thermocycler is a critical parameter. The ramping conditions used here are a heating rate of 4°C/s and a cooling rate of 3°C/s. Differences in ramping conditions between two thermal cyclers can explain why the same PCR protocol may work or fail in different laboratories.

Optimizing PCR for complex template (GC or AT rich template, repeated sequence, secondary structure)

There are many parameters to optimize in a PCR, such as magnesium, dNTP, and Taq concentrations, as well as cycling conditions. Optimization of such parameters is described, for example, by Lorenz (2012). Our approach is to keep the PCR protocol as standard as possible. We therefore propose, as a first step, merely trying different additives, such as DMSO, glycerol, or BSA. Such adjuvants improve PCR amplification efficiency and specificity. Figure 6 illustrates an instance in which adding 5% (v/v) DMSO substantially improved the amplification of a GC-rich region. Multiple additives usually need to be tested for a complex template, as the most efficient additive will depend of the sequence to be amplified and cannot be anticipated.

Presence of inhibitors in reaction or template too concentrated (oversized biopsy)

For samples that produce inconclusive results, a second trial can be performed with a sample diluted 1/50 (Fig. 7). Dilution of the crude extract is an easy solution to reduce inhibitor or template concentration.

Purchasing a liquid handling workstation

Ideally, the workstation should accommodate all steps from sample lysis to gel electrophoresis. Among these, automation of sample lysis is far from essential, as this is a very quick step. Automation of gel electrophoresis (or other PCR reading methods) is very complex and thus not advised. We therefore recommend purchasing a workstation that can prepare PCR mix and perform thermal cycling. This will be composed of a liquid handler with a robotic gripper arm (for instance, a Tecan or Hamilton workstation), an automated thermal cycler, and an automated heat sealer. In addition, including a centrifuge is highly recommended (see section on centrifugation

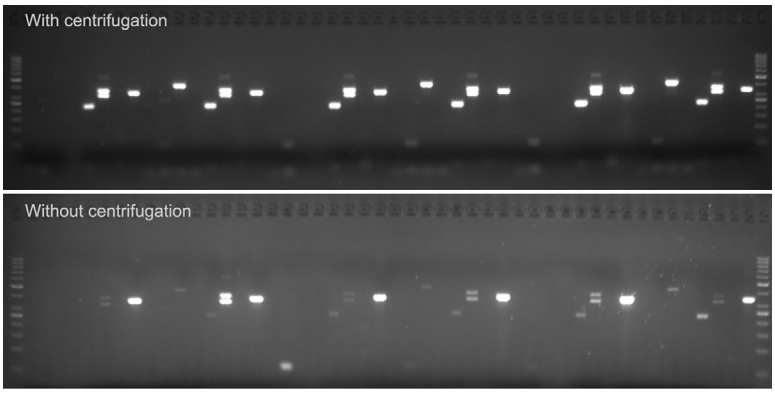


Figure 8 Centrifugation of the plate after dispensing by pipetting platform greatly improves PCR reactions. Two identical 384-well plates were prepared by a workstation and placed in a thermocycler after prior centrifugation (top row) or without centrifugation (bottom row). Even through the automated liquid handling mixes the solution by pipetting few times, we have found that centrifugation greatly improves the homogenization of the PCR mix and reduces the incidence of failed or weak PCR.

before PCR amplification, below). The volume of samples that the multipurpose liquid-handling automated workstation can manage is one feature to consider when making a purchase.

The programming of sampling, mixing, and combining of liquid samples automatically on the workstation, called the worklist, is usually done by the liquid handler supplier, but as you may have to perform protocol optimization, the ease of use of the provided software interface is another important feature to consider.

Use of disposable tips versus fixed-steel washable needles

Workstation suppliers advise the use of disposable tips to reduce risk of cross-contamination between biological samples. From our experience with more than 600,000 samples, fixed-steel washable needles produce very reproducible results for PCR genotyping and do not induce contamination of PCR assays. When using these needles, the workstation needs to be programmed to include a decontamination step (parameters are specific for each workstation): aspiration of bleach followed by flushing of the system with water (the water flow will efficiently clean the needles) after each sample or primer dispensing.

Specific plasticware used with workstations

Not all plasticware can be adapted for use on a workstation. For example, some 384-well plates are not suitable for handling by robotic gripper arms: the plates must be rigid enough for the arm to grasp them. Plates must also be thermosealable: i.e., resistant to the temperature used for the sealing step (165°C).

For tissue sampling, using individual tubes is easier for animal caretakers. Choosing 0.75-ml microtubes with an independent cap that can be adapted to use in a 96-tubes rack (for instance Matrix tubes; cat. no. 446015, Dutscher) simplifies the processing of a very large number of biopsies.

Any changes to consumables or reagents must be tested before being used for high-throughput genotyping.

Laboratory Information Management System

Workstation suppliers will develop scripts for their automate piloting software according to your specifications. This will manage the sampling, mixing, and combining of liquid samples. We also recommend developing an in-house LIMS that manages sample traceability (Fig. 3). This reduces genotyping errors as it avoids transposition of data between samples. When a high number of different mutant lines are to be genotyped, a LIMS will also allow easy communication to the workstation, via worklists.

Important recommendations for designing genotyping strategy for high-throughput workflow

Reduce the number of primers used per mutant line: Primers are used in multiple PCR combinations in order to detect all possible alleles and minimize the number of primers ordered and stored. Figure 2 illustrates this genotyping strategy.

Table 7 Troubleshooting Guide for PCR Assay Design

Observation	Most probable cause(s)	Solution(s)
No product obtained with most of the PCR sets	Missing reaction component	Check the experimental plan and repeat reaction setup.
	Degraded reaction component (due to multiple freeze-thaw cycles or bad storage)	Repeat the PCR with reagents from another provider, or change batch number.
	Low quality of reaction component batch sent by the provider, or issue during shipment	Repeat the PCR with reagents from another provider or batch number.
	Thermal cycler malfunction	Validate performance of your thermal cycler (Kim, Yang, Bae, & Park, 2008).
	Problem with agarose gel	Check that the agarose gel was loaded correctly and stained properly. Check the connections of the electrophoresis tank (check for inversion of anode and cathode connection).
	Presence of inhibitors in plasticware	Check the compatibility of plasticware for PCR reaction.
No product obtained with more than one PCR set	High levels of inhibitor in the template (oversized biopsy)	Dilute the sample (see Fig. 7).
	Poor biopsy lysis	Check if the biopsy is dislocated, add proteinase K, or start from a new biopsy.
	Poor template quality (DNA too degraded or too old)	Start from a new biopsy.
	Poor genotyping design	Check the experimental plan and repeat reaction setup.
No product obtained with one PCR set	Poor primer design	Design and order new primer.
	Inefficient PCR amplicon or primer pair	Design and order new primer pair.
	Poor quality of primer synthesis	Order new primers.
	Complex template (GC- or AT-rich template, repeated sequence, or secondary structure)	Try an additive like DMSO or betaine (see Fig. 6), or even a specific GC-rich DNA polymerase.
	Missing primer or missing template	Check the experimental plan and repeat reaction setup.

continued

Table 7 Troubleshooting Guide for PCR Assay Design, *continued*

Observation	Most probable cause(s)	Solution(s)
Multiple or nonspecific band amplification	Problem with primer specificity	Run a blast search on the NCBI website to check the target specificity of the primers.
	Amplification of related pseudogenes or homologs	Run a blast search on the NCBI website to check the target specificity of the primers.
	Complex template (GC- or AT-rich template, repeated sequence, or secondary structure)	Try an additive like DMSO (see Fig. 6) or even a specific GC-rich DNA polymerase.
	Contamination of reagent, pipets, or working area with other PCR products	Use new reagents; clean the pipets and working area. Always use filter tips.
	Mutant allele sequence that is inaccurate	Sequence the mutant allele by an appropriate method.
	Sample contamination by another biopsy during tissue sampling (see Fig. 4)	Start from a new biopsy.
	Poor template quality (DNA too degraded or too old)	Start from a new biopsy.
Nonspecific band amplification in blank	Contamination of reagent, pipet, or working area with other PCR products	Use new reagents; clean the pipets and working area. Always use filter tips.
Weak target amplification	Poor genotyping design	Check the experimental plan and repeat reaction setup.
	Complex template (GC- or AT-rich template, repeated sequence, or secondary structure)	Try an additive like DMSO or betaine (see Fig. 6), or even a specific GC-rich DNA polymerase.
	Template insufficiently concentrated (for example biopsy too small)	Increase volume of crude extract per PCR.
	Template too concentrated (biopsy oversized)	Dilute the sample 1/50 in water (see Fig. 7).
	Presence of inhibitors in reaction	Dilute the sample 1/50 in water (see Fig. 7).
	Poor biopsy lysis	Check if the biopsy is dislocated, add proteinase K, or start from a new biopsy.
	Poor template quality (DNA too degraded or too old)	Start from a new biopsy.

continued

Table 7 Troubleshooting Guide for PCR Assay Design, *continued*

Observation	Most probable cause(s)	Solution(s)
Nonreproducibility of genotyping protocol or smearing of amplification	Use of a different thermal cycler for reaction	Use the original cycler or optimize PCR program.
	Reaction component degradation (due to multiple freeze-thaw cycles or incorrect storage)	Repeat the PCR with reagents from another batch number.
	Low quality of reaction component batch sent by the provider, or issue during shipment	Repeat the PCR with reagents from another batch number.

Use as many common primers as possible: In a high-throughput workflow, it is important to use as many common primers as possible. Most mutant models contain selection markers, tags, and reporter genes. These sequences can be used to design primers that will be suitable on multiple mutants. However, as stated in the Basic Protocol 3 introduction, designing genotyping primers only for markers that can be found in many transgenic lines is discouraged. Using common primers means using one common primer in combination with a mutation-specific primer for each PCR assay (see Fig. 2 for an example).

Define a standard annealing temperature (T_m) and thermal cycling conditions for all mutant lines: See Table 4 for recommended conditions. This is especially important when multiple mutant lines are genotyped. Without standardization of this step, PCR cannot be automated.

Reducing PCR failure in high-throughput genotyping

In a high-throughput workflow, making a duplicate or even a triplicate for each PCR point is common and ensures reliable and robust results. We do not advise making a duplicate of the same PCR design, but rather using two independent PCR designs for each allele being genotyped, to further increase genotyping reliability and robustness.

Reducing cost by multiplex PCRs

Another way to optimize a high-throughput workflow is to set up multiplex PCRs: The different PCR sets are combined into one tube to detect all the relevant alleles for a mutant line. Optimization is usually required for PCR multiplexing.

For multiplex PCRs, we recommend checking the parameters below.

- *Sequence structures*: All primers must not contain complementary regions of more than 3 nucleotides in 3′.
- *Amplicon lengths*: The choice of primer must be made so that the sizes of the sequence to be amplified are sufficiently distinct from each other to be identified by gel electrophoresis.

Setting up a multiplex PCR is done in two steps: First, validate each PCR set by simplex PCR, and then multiplex the PCR sets.

If the intensity of each amplicon is not similar on an agarose gel, weak PCR signals can be improved by increasing the concentrations of the primers that produce weak signals and/or decreasing the concentrations of the primers that give strong signals.

Managing a large number of primer sets

By analyzing our genotyping dataset (~1500 mutant lines genotyped using ~10,000 primers), we observed that thawing/freezing cycles lead to variable and unpredictable degradation of primers. We thus recommend the use of a master primer bank (that is used as backup) and a working primer bank (smaller single-use aliquots). This will reduce primer degradation issues by decreasing the number of thawing cycles to only two.

Using 8-tube strips (discarded after each thawing) to optimize the working primer bank improves large-volume primer management. Each tube of a strip contains the primer pair that is used to detect a specific allele, so that up to eight different PCRs can be done for a mutant line genotyping. Using 8-tube strips reduces the time needed to prepare the workstation for PCR genotyping and the number of pipetting steps on the automated workstation (as PCR pairs are already mixed together), and avoids repeated primer thawing-freezing cycles and the risk of primer contamination.

Table 8 Automation Troubleshooting

Observation	Possible cause(s)	Solution(s)
Automated workstation stops during the run	Gripper stopped and plate fell	Check if plasticware (plates) is adapted for automation. Verify errors in worklist program.
	Arm collision occurred	Check if plasticware (plates) is adapted for automation. Verify errors in worklist program. Verify that each plate and tube is in a good position on the workstation.
	Loss of connectivity with peripheral devices occurred	Possible power or network failure: add a power inverter.
	Plate was incorrectly positioned on the workstation	Verify that each plate and tube is in a good position on the workstation.
	Pipetting or dispensing error occurred	Check for problems such as clogged or loose tips, faulty O-rings, or poorly optimized pipetting settings.
Wrong volumes in PCR plate	Evaporation of PCR mix occurred	Check if cooler is broken or was not turn on. Verify that sealer is well configured.
	Level of PCR mix in the tube was too low	Verify that enough reaction mix and primers are added to perform all reactions.
	Pipetting or dispensing errors occurred	Check problems such as clogged or loose tips, faulty O-rings or pipetting setting poorly optimized. Check for errors in worklist program.
No PCR band	Fixed-washable needle is clogged	Run a washing program and reboot the robot.
	Fixed-washable needle is broken	Replace needle.
	Inversion of plates on the workstation: reagents such as primers and crude extract 96-well plates are incorrectly positioned on the workstation	Check position of each plate, and if wrong, change and restart the run.
	Incorrect worklist was loaded	Check which worklist was used; restart with the appropriate worklist.
	No centrifugation step occurred before thermocycling	Check that the centrifuge is working properly. Check for errors in worklist program.
	Level of PCR mix in the tube was too low	Verify that enough reaction mix and primers were added to perform all reactions.
	Reagents were incorrectly dispensed into PCR plates	Check for errors in worklist program.

continued

Table 8 Automation Troubleshooting, *continued*

Observation	Possible cause(s)	Solution(s)
Contamination (PCR amplicon in the negative control)	No decontamination step was included in worklist	Program into the worklist a decontamination step that include bleach aspiration followed by flushing the system with water.
	During run, decontamination between dispensing did not occur	Verify that enough bleach and water are available to perform all decontamination steps between each sample and primer.
	Drop is visible on pipet tip when dispensing samples	Check liquid class, dispenser seals, and dispensing position in the well.

Table 9 Time Considerations for Sample Validation and DNA Lysis

Steps	Time (min)
Biopsy quality check	30
Premix preparation and dispensing into tubes	15
Incubation of the lysis buffer	Overnight
Proteinase K inactivation and storage	50

Automation using 384- plate versus 1536-well plates

384-well plates allow a volume of 10 to 130 µl per well, whereas 1536-well plate are adapted to volume of 3 to 10 µl. Thus, using 1536-well plate further reduces the volume of the reaction mix used and increases the throughput. However, 1536-well plates are not adapted for PCR genotyping if multiple PCR sets are to be used. If you need not only to genotype many individuals for one mutation but to analyze numerous genetic modifications and/or genetic loci, the pipetting step will last more than 1 hr and some samples will evaporate in 1536-well plates before the start of the PCR thermal cycling.

Centrifugation before PCR amplification

After dispensing of all PCR components into the 384-well plates and sealing, centrifugation of each 384-well plate will strongly reduce failed or weak PCR by improving homogenization of the PCR mix (Fig. 8).

Avoiding sample cross-contamination in high-throughput design

The large number of samples analyzed, and particularly the resulting PCR amplicons, logically increases the risk of contamination for high-throughput platforms. It is therefore crucial to use negative controls and follow good laboratory practices (wearing gloves, using filter tips) in all experiments. Daily sterilization of the workstation using UV light (to break contaminating DNA molecules) is also recommended.

No PCR products should be opened or otherwise handled near workstations. It is very important that a room be dedicated to the migration of PCR products on agarose gel. A PCR tube that has been opened and then closed again is very contaminating and should not be thrown into a refuse bin near workstations.

Troubleshooting

A troubleshooting guide for PCR assay design (Basic Protocol 3) is provided in Table 7. A troubleshooting guide for the use of the automated workstation (Basic Protocol 4) is provided in Table 8.

Time Considerations

Tissue sampling methods and procedure

Sampling (including preparation of the materials, instrument sterilization, restraint of the animal, and tissue biopsy) lasts around 2 to 3 min per animal.

Sample verification and DNA lysis

The duration of the protocol does not increase linearly with the number of treated samples. In Table 9, we detail experiment

Table 10 Time Considerations for One Standard Assay

Steps	Time (min)
Primer design (sequences validation, design, primer order)	60
Samples preparation	30
PCR cycling (cycler and target size dependent)	90
Gel electrophoresis (loading of samples and migration)	60
Image acquisition with a digital camera	5
Interpretation of results	30

Table 11 Time Considerations for High-Throughput Genotyping

Steps	Time (min)
Run preparation in the database	15
Workstation preparation	15
Reagent preparation	30
Run timing (automates pipetting, sealing, centrifugation, PCR)	Overnight[a]
Gel electrophoresis (loading of samples and migration)	85
Image acquisition with a digital camera	30
Interpretation of results	180

[a]A run of six 384-well plates requires about 17 hr with our configuration.

duration per 96-sample plate. Note that the incubation in lysis buffer is done overnight.

Design of a genotyping strategy

Table 10 shows the experiment duration for 96 samples.

High-throughput genotyping

The deployment of a high-throughput platform requires months from workstation design to efficient automation. Table 11 describes the duration of a run that generates six 384-well PCR plates.

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