GV-SOLAS
Gesellschaft für Versuchstierkunde

Specialist information

from the Committee for Genetics and Laboratory Animal Breeding (GV-SOLAS)

Identification and genotyping of rodents

Status: November 2018

Authors: Boris Jerchow, Dirk Wedekind, Thorsten Buch, Peter Dobrowolski

Contents

Keywords:

Genotyping, Biopsies, Tissue sampling, Identification, Animal welfare

1. Background and objective

Laboratory rodents, especially the mouse (*Mus musculus*) and the rat (*Rattus norvegicus*), are traditionally important and frequently used laboratory animals. In the past 25 years, the development and establishment of methods for the genetic modification of these species has led to a huge increase in the number of lines with mutated alleles and transgenes. Today, many different lines are housed in parallel in laboratory animal facilities. It is therefore essential for these facilities to identify the animals of all these lines so that all individual animals can be clearly distinguished. In most cases, there is also a need to isolate genomic material of animals in order to determine their exact genotype. A variety of methods has been used for this purpose over the years. The aim of this guideline is to elucidate a selection of methods and to compare their practicability with the stress and harm inflicted on the animals. Here, we first describe the techniques that allow simultaneous marking and biopsy of the animals. Then methods are discussed that are used exclusively either for marking or for obtaining tissue samples. These are to be combined if the genotypes of rodents in a group need to be determined. Unless otherwise indicated, all methods are carried out without anaesthesia.

2. Simultaneous tissue sampling and marking

2.1. Ear notching

Compared with most other methods, ear notching has the advantage that the animals are marked, and a tissue sample obtained. The biopsy material can then be used for genotyping by means of molecular-biological methods. Based on a scheme with a combination of holes and notches over both ears, animals can be tagged with code numbers from 1 to 100[1]. To minimize the stress for the animals as far as possible, it is important to use a sharp tool to punch the holes and notches. In addition, care must be taken to thoroughly clean the tool between two animals to avoid cross-contamination.

2.2. Amputation of distal phalanx

The phalanx distalis is the outermost bone of the toe. If carried out precisely and at the right time using sharp microscissors, amputation of the distal phalanx allows early extraction of tissue for DNA isolation along with permanent marking, so any stress for the animal is kept to a minimum. No other method allows this at such an early stage. The toe must be large enough for this, but not yet ossified. At the same time, the young animals should as far as possible still be at a stage of development in which they show little motor activity. According to the data available, an age of 5 to 7 days has been found to be the optimum period[2-4]. It is assumed that both the amputation process itself and the absence of the toe pose only a mild degree of stress for the animal[2-3]. The same considerations apply to the assessment of pain as for the amputation of the tail tip. Accordingly, it is to be assumed that the perception of pain is markedly reduced in mice during the first 10 days after their birth. In addition to adhering to the optimal time window, care must be taken not to remove too much, but also not too little from the toe – i.e. that the distal phalanx is amputated with precision. Otherwise, the holding strength may be compromised, or identification of the animal made impossible, because the claw grows back. For this reason, experienced personnel should only carry out the technique after thorough training. When the toe-clipping procedure is first introduced into a facility, it should be borne in mind when teaching this method that it could meet with rejection among many animal technicians and scientists because it might be mistaken for the amputation of the whole toe

and be viewed from an anthropocentric perspective. Overall, the technique is recommended as a robust marking method in which tissue is obtained at the same time for genotyping and the stress on the animal is low.

3. Marking methods

3.1. Tattooing

Tattooing mice is relatively easy to learn. In the simplest case, tattoo ink is introduced into the dermis of the skin using a fine cannula without additional tools (micro-tattoo). Alternatively, tattoo pincers are also used to hold the cannula. In principle, all parts of the rodent body that are not hairy are suitable for tattooing. Usually, the tail or balls of the feet are used. There is sufficient space in particular on the tail of a rat, allowing also for multi-digit numbers. Many individual animals can be distinguished by a point code that covers all four paws or both ears. To tattoo the ears, however, the use of special tattooing pliers is required. It should be noted that marking the ears with tattoo pliers appears to cause greater stress on the animals than micro-tattooing the balls of the feet[5].

Various companies also offer automatic tattooing devices especially for laboratory rodents, which make the tattooing itself even easier and also allow animals to be marked with easily readable alphanumeric codes. Some devices allow a fully automatic tattooing process, thus ensuring a standardized execution of the process. However, disinfection of the devices between individual animals sometimes poses a challenge and can become a hygiene problem in animal husbandry.

3.2. Microchips

In recent years, microchips or microtransponders have become ever smaller. As a result, the stress on the animals caused by implanting and wearing the chips is now very low. In a few cases, inflammatory reactions have been observed at the implantation site[6]. In rare cases, tumours may also develop at the implantation site[7-9]. However, the use of microchips is a costly method of marking rodents. The animal numbers are determined with the aid of a reading device. These devices must therefore be available in all areas in which work is carried out with chip-implanted animals. The programs for reading out the animal numbers allow a range of additional information to be linked to the animal. Particularly in complex studies, this can be very useful. If the animals are passed on to another unit and the recipient does not have a suitable reading device and is unable to unequivocally identify the animals by any other means, they may need to be marked again.

3.3. Colour markings

Newborn animals in particular can be easily identified using permanent markers or also livestock marker pens before a dense coat has grown, i.e. on the abdominal side up to the age of about ten days. Older animals can be marked on hairless sites, for example on the tail or the ears. The stress caused to the animals by the marking itself is negligible. Depending on the marking site, the animals do not even have to be touched in order to read the marking. The disadvantages of colour marking, however, are that it must frequently be repeated and the animals have to be restrained for the procedure. The colours may be absorbed on grooming

or directly through the skin. Harmful or irritant colours are labelled accordingly and must not be used. Possible effects must be considered in the context of experimental approaches.

3.4. Ear tags

Ear tags have the advantage that animals can be clearly marked with individual, relatively easy-to-read numbers or with machine-readable codes, even with large numbers of animals. Plastic – more precisely, nylon – ear tags have a number of advantages over metal tags. First, nylon tags are considerably lighter. Although the difference in absolute numbers is small, it does play a role in relation to the weight of the mouse. There are repeated reports of ear tags being torn out or the animals becoming entangled when grooming or fighting. Smaller, button-like ear tags remedy this. These not only reduce the risk of injury but also are also lighter because of their smaller size. In addition, there have been various reports of negative effects from inflammatory reactions to isolated cases of tumours possibly associated with metal ear tags[10-13].

4. Tissue sampling

4.1. Amputation of tail tip

Tail-tip amputation is easy to carry out and yields a lot of genomic DNA compared with other biopsy methods. For this reason, it is especially preferred when subsequent analytical methods depend on a large quantity of DNA, as is the case for example with Southern blot analysis. The claim sometimes made that other methods do not provide sufficient DNA for a PCR reaction, however, is not tenable.

The tail tip can also be easily removed in newborn animals as early as the first day following their birth, thus allowing very early genotyping.

Studies suggest that the perception of pain in tail-tip amputation increases with the age of the animals and the associated ossification of previously cartilaginous structures in the tail. The underlying maturation processes differ from one background strain to another. The frequently used background strain C57BL/6 in particular shows early ossification of the distal caudal vertebra. The optimum age for a tail biopsy is thus 1 to 16 days, when the amount of DNA to be isolated is at its maximum in relation to the amputated tail length[14, 15].

The stress on the animals as a result of tail-tip amputation has not been reliably measured to date. In seven-day-old animals, for example, effects that may possibly be caused by the pain stimulus are masked by the acute increase in serum corticosterone that is triggered by handling alone[2]. On day 15, however, an increase was observed as a result of amputating a 5 mm tail section[16]. No long-term effects on nociceptive stimuli were detectable in older C57BL/6J mice and only minimal effects in 129S6 mice[17]. Nor were any substantial effects detected with regard to anxiety (or fear) or the ability to climb or balance[18]. Based on a number of studies, we assume that the perception of pain is still underdeveloped in altricial animals such as mice and rats during the first 10 days after birth[19-21]. A review article from 2014 concludes that neurologically immature animals can be assumed to have a relatively undifferentiated experience of discomfort generated by neural stimulus processing at levels below the cortex[22]. However, the primary literature here is unclear, and to our knowledge, there are no targeted studies on the long-term consequences of biopsies performed in the first week of life.

The use of anaesthesia and analgesia is the subject of some controversy. While general anaesthesia can be even more stressful than tail biopsy, the use of a local anaesthetic may be considered. The best analgesic effect has been detected with an ice-cold 70% ethanol solution, whereas lidocaine did not show a positive effect[16]. Anaesthesia should not be used up to the age of ten days but must be used from the age of 4 weeks at the latest.

To summarize, we consider a tail biopsy performed within the first week of life to be a method for obtaining tissue that is associated with very little stress. Yet, if possible, only a 1 mm section of the tail should be amputated. In older animals, up to 5 mm may be amputated if absolutely necessary.

4.2. Blood sampling

In the past few years, blood-sampling techniques in rodents have been optimized in such a way that their use involves relatively little stress for the animal. In the mouse, blood samples may be obtained, for example, from the saphenous vein, the facial vein or the tail vein without anaesthesia by appropriately trained personnel 23. Depending on the animal model, the blood may serve either as a source for genomic DNA24 or as a means of obtaining a phenotype classification of the animals with the aid of other parameters, for example by microscopic or cytometric analysis25. A note of caution: EDTA blood is not suitable for genotyping because it inhibits PCR.

As little blood as necessary should be taken when obtaining samples. In every case, however, maximum sampling volumes must be considered. These are 10% of the blood volume in the mouse and with repeated withdrawals 7.5% of the blood volume with corresponding regeneration times[23].

4.3. Non-invasive methods

There are various non-invasive methods of obtaining genomic material from an individual animal. It should be borne in mind here that every manipulation of a laboratory rodent involves a certain degree of stress for this animal, and the non-invasive methods presented here can therefore also prove a burden for the animal. For example, by plucking hair samples, follicles can be obtained that are suitable for DNA isolation. Problems with cross-contamination have been described in the literature[26,27] that can be prevented with attentive procedures. DNA can also be obtained by means of buccal swabs. Here it is necessary to proceed with great care so that the animals are not injured or do not injure themselves[26]. The personnel who take the samples must be appropriately trained so as to avoid injury to the animals and at the same time to obtain sufficient material for determining the genotype by PCR. The risk of cross-contamination can be practically excluded by using suitable swabs. Smears from the rectum are similar to buccal swabs[26,28]. In fact, DNA can be obtained from faecal pellets without any stress for the animals[29-32]. These pellets should not be older than 24 hours and at least two should always be collected in order to obtain a sufficient quantity of DNA. This method is suitable when only a few animals have to be genotyped. With larger colonies of laboratory rodents, all the animals would need to be separated, which clearly is not possible, rendering the method unsuitable for practical reasons.

What all of the methods subsumed under the heading of non-invasive methods have in common is that the quantities of DNA that can be obtained are small[33]. With modern analysis,

however, this should not present an obstacle. As a result of the very small quantities of DNA and the contamination risk, there is still a certain degree of uncertainty at present with regard to the robustness of genotyping large animal stocks using samples obtained with non-invasive methods. Here, further studies have to prove their suitability for daily use. Non-invasive methods can be recommended for cases in which a new DNA extraction is necessary – for example if a biopsy has been lost or there are doubts about the correct assignment.

5. Classification according to animal welfare legislation

According to Article 1 Paragraph 5 of EU Directive 2010/63/EU, procedures that serve primarily to identify animals are not explicitly included and therefore do not have to be regarded as animal experiments. Following a legal report by the National Committee for the Protection of Animals Used for Scientific Purposes at Germany's Federal Institute for Risk Assessment (BfR), "ear notching is not performed for the purpose of clarifying a scientific question (…), but has the objective of being able to distinguish between the animals". Therefore, "an animal experiment as defined in Section7.2 sentence 1 Animal Welfare Act (TierSchG) does not apply." By contrast, obtaining tissue for study purposes – i.e., for example, tail-tip amputation – is considered an animal experiment according to Section 7.2 sentence 2, no. 2, letter c TierSchG. However, such animal experiments do not require approval, but only notification. This exception is regulated by Section 8a.1 No. 3 letter b, since it is evidently a tried and tested procedure that serves purely diagnostic purposes[34].

In Switzerland, the least stressful method must be used when marking laboratory animals (TSchV Section120.2). Section 5 of the Swiss animal welfare ordinance regulates the marking of animals and taking of biopsy for genotyping in detail. In breeding, the use of invasive marking methods, such as tattoos, microchips, ear notching or toe-tip amputation is possible, but the combination of marking and biopsy is compulsory. Ear notching and amputation of the first phalanx are thus the only methods usefully permitted in the breeding of transgenic animals (yet only in case a biopsy is required). Experiment-specific applications must be submitted for exceptions to this rule. Marking outside of breeding, for experimental reasons, also requires justification for the individual case. Marking with ear tags is generally not permitted. While it is permitted to take blood samples for genotyping according to Section10.1 of the Animal Welfare Ordinance, Section 10.2 stipulates that tail biopsies up to a maximum length of 5 mm are only permitted in individual cases for experiment-specific reasons.

In Austria, the Animal Welfare Act does not apply to "practices that are mainly used for the identification of animals" according to Section 1.2 No. 4. This applies to ear notching and amputation of the distal phalanx if they are used simultaneously for marking. This also applies to the combination of non-invasive techniques for obtaining material combined with ear tags, tattoos, and the like.

6. Recommendations

As a rule, genetically modified rodents in the laboratory animal facility must be both identified and genotyped. In order to keep the animal´s stress as low as possible, we recommend choosing a method in which a tissue sample is obtained in addition to the marking. This is the case both for amputation of the distal phalanx and for ear notching. Alternatively, we also consider the combination of tattooing or nylon ear tags with a non-invasive method of

genotyping to be a low-stress approach. This is obviously only possible if the analysing laboratory is able to reliably detect the genotype with such small amounts of DNA.

The aim should be to determine the genotype within the first three weeks of life. This is the only way to form stable groups of animals on weaning. Otherwise, it regularly occurs that animals have to be regrouped after removing those with undesirable genotypes, which entails additional stress for the animals. This can be avoided if the genotype is determined earlier (for the legal assessment of killing animals with the wrong genotype, see[35]). A biopsy should be taken as early as possible, since it can be assumed that younger animals feel less pain simply because they are less mature. In this respect, the tail biopsy in combination with the micro-tattoo is a recommended method. This combination can be performed safely in the first week of life. During this time, the animals are not fully developed neurologically, and it is therefore to be assumed that the perception of pain will be much lower.

7. References

1 Donovan J, Brown P. 2006. Animal identification. In: Coligan JE et al. (eds), Current protocols in immunology, Chapter 1, Unit 1 5, doi:10.1002/0471142735.im0105s73.

2 Schaefer DC, Asner IN, Seifert B, Burki K, Cinelli P. 2010. Analysis of physiological and behavioural parameters in mice after toe clipping as newborns. Lab Anim 44:7-13, doi:10.1258/la.2009.009020.

3 Castelhano-Carlos MJ, Sousa N, Ohl F, Baumans V. 2010. Identification methods in newborn C57BL/6 mice: a developmental and behavioural evaluation. Lab Anim 44:88-103, doi:10.1258/la.2009.009044.

4 Vachon P. 1998. Anatomical and histological observations of fore- and hind limb toes in adult mice after amputations performed at the age of two weeks. Can J Vet Res 62:311-313.

5 Kasanen IH, Voipio HM, Leskinen H, Luodonpaa M, Nevalainen TO. 2011. Comparison of ear tattoo, ear notching and microtattoo in rats undergoing cardiovascular telemetry. Lab Anim 45:154-159, doi:10.1258/la.2011.010113 (2011).

6 Gruda MC, Pinto A, Craelius A, Davidowitz H, Kopacka WM, Li J, Qian J, Rodriguez E, Kuspiel E, Mandecki W. 2010. A system for implanting laboratory mice with light-activated microtransponders. J Am Assoc Lab Anim Sci 49:826-831.

7 Le Calvez S, Perron-Lepage MF, Burnett R. 2006. Subcutaneous microchip-associated tumours in B6C3F1 mice: a retrospective study to attempt to determine their histogenesis. Exp Toxicol Pathol 57:255-265, doi:10.1016/j.etp.2005.10.007.

8 Elcock LE, Stuart BP, Wahle BS, Hoss HE, Crabb K, Millard DM, Mueller RE, Hastings TF, Lake SG. 2001. Tumors in long-term rat studies associated with microchip animal identification devices. Exp Toxicol Pathol 52:483-491, doi:10.1016/S0940-2993(01)80002-6.

9 Tillmann T, Kamino K, Dasenbrock C, Ernst H, Kohler M, Morawietz G, Campo E, Cardesa A, Tomatis L, Mohr U. 1997. Subcutaneous soft tissue tumours at the site of implanted microchips in mice. Exp Toxicol Pathol 49:197-200, doi:10.1016/S0940-2993(97)80007-3.

10 Baron BW, Langan G, Huo D, Baron JM, Montag, A. 2005. Squamous cell carcinomas of the skin at ear tag sites in aged FVB/N mice. Comp Med 55:231-235.

11 Cover CE, Keenan CM, Bettinger GE. 1989. Ear tag induced Staphylococcus infection in mice. Lab Anim 23:229-233.

12 Waalkes MP, Rehm S, Kasprzak KS, Issaq HJ. 1987. Inflammatory, proliferative, and neoplastic lesions at the site of metallic identification ear tags in Wistar [Crl:(WI)BR] rats. Cancer Res 47:2445-2450.

13 Kitagaki M, Hirota M. 2007. Auricular chondritis caused by metal ear tagging in C57BL/6 mice. Vet Pathol 44:458-466, doi:10.1354/vp.44-4-458.

14 Hankenson FC, Garzel LM, Fischer DD, Nolan B, Hankenson KD. 2008. Evaluation of tail biopsy collection in laboratory mice (Mus musculus): vertebral ossification, DNA quantity, and acute behavioral responses. J Am Assoc Lab Anim Sci 47:10-18.

15 Hankenson FC, Braden-Weiss GC, Blendy JA. 2011. Behavioral and activity assessment of laboratory mice (Mus musculus) after tail biopsy under isoflurane anesthesia. J Am Assoc Lab Anim Sci 50:686-694.

16 Dudley ES, Johnson RA, French DC, Boivin GP. 2016. Effects of Topical Anesthetics on Behavior, Plasma Corticosterone, and Blood Glucose Levels after Tail Biopsy of C57BL/6NHSD Mice (Mus musculus). J Am Assoc Lab Anim Sci 55:443-450.

17 Morales ME, Gereau RW. 2009. The effects of tail biopsy for genotyping on behavioral responses to nociceptive stimuli. PLoS One 4, e6457, doi:10.1371/journal.pone.0006457.

18 Sørensen DB, Stub C, Jensen HE, Ritskes-Hoitinga M, Hjorth P, Ottesen JL, Hansen AK. 2007. The impact of tail tip amputation and ink tattoo on C57BL/6JBomTac mice. Lab Anim 41:19-29, doi:10.1258/002367707779399383.

19 Baccei ML, Bardoni R, Fitzgerald M. 2003. Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol. J Physiol 549:231-242, doi:10.1113/jphysiol.2003.040451.

20 Gupta A, Tsai LH, Wynshaw-Boris A. 2002. Life is a journey: a genetic look at neocortical development. Nature Rev Genet 3:342-355, doi:10.1038/nrg799.

21 Ayala R, Shu T, Tsai LH. 2007. Trekking across the brain: the journey of neuronal migration. Cell 128:29-43, doi:10.1016/j.cell.2006.12.021.

22 Campbell ML, Mellor DJ, Sandoe P. 2014. How should the welfare of fetal and neurologically immature postnatal animals be protected? Anim Welf 23:369-379, doi:10.7120/09627286.23.4.369.

23 GV-SOLAS, Committee of Animal Welfare Officers and Working Group 4 of the TVT. 2009. Recommendation for blood sampling in laboratory animals, especially small laboratory animals. http://www.gv-solas.de/fileadmin/user_upload/pdf_publikation/Tierschutzbeauftragte/tie_blutentnahme17_e.pdf

24 Horváth A, Sántha P, Horváth V, Török N, Nagy I, Jancsó G, Vágvölgyi C, Somogyvári F. 2013. Rapid genotyping of genetically modified laboratory animals from whole blood samples without DNA preparation. Acta Biol Hung 64:262-265, doi:10.1556/ABiol.64.2013.2.11.

25 Hellrung DJ, Rossi G, Link CJ. 2006. High-throughput fluorescent screening of transgenic animals: phenotyping and haplotyping. Cytometry A 69:1092-1095, doi:10.1002/cyto.a.20328.

26 Cinelli P, Rettich A, Seifert B, Burki K, Arras M. 2007. Comparative analysis and physiological impact of different tissue biopsy methodologies used for the genotyping of laboratory mice. Lab Anim 41:174-184, doi:10.1258/002367707780378113.

27 Schmitteckert EM, Prokop CM, Hedrich HJ. 1999. DNA detection in hair of transgenic mice-- a simple technique minimizing the distress on the animals. Lab Anim 33:385-389.

28 Lahm H, Hoeflich A, Rieger N, Wanke R, Wolf E. 1998. Identification of transgenic mice by direct PR analysis of lysates of epithelial cells obtained from the inner surface of the rectum. Transgenic Res 7:131-134.

29 Hamann M, Lange N, Kuschka J, Richter A. 2010. Non-invasive genotyping of transgenic mice: comparison of different commercial kits and required amounts. Altex 27:185-190.

30 Broome RL, Feng L, Zhou Q, Smith A, Hahn N, Matsui SM, Omary MB. 1999. Non-invasive transgenic mouse genotyping using stool analysis. FEBS Lett 462:159-160.

31 Kalippke K, Werwitzke S, von Hornung M, Mischke R, Ganser A, Tiede A. 2009. DNA analysis from stool samples: a fast and reliable method avoiding invasive sampling methods in mouse models of bleeding disorders. Lab Anim 43:390-393, doi:10.1258/la.2008.008057.

32 Murgatroyd C, Bilko D, Spengler D. 2006. Isolation of high-quality DNA for genotyping from feces of rodents. Anal Biochem 348:60-162, doi:10.1016/j.ab.2005.10.004.

33 Garzel LM, Hankenson FC, Combs J, Hankenson KD. 2010. Use of quantitative polymerase chain reaction analysis to compare quantity and stability of isolated murine DNA. Lab Anim 39:283-289, doi:10.1038/laban0910-283.

34 Chmielewska J, Bert B, Grune B, Hensel A, Schönfelder G. 2017. Probleme aus der tierversuchsrechtlichen Praxis: Rechtliche Einordnung der Genotypisierungsmethoden sowie der Zucht immunmodifizierter Tiere. Natur und Recht 39:385-392, doi:10.1007/s10357-017-3190-4 (GERMAN).

35 Chmielewska J, Bert B, Grune B, Hensel A, Schönfelder G. 2015. Der “vernünftige Grund” zur Tötung von überzähligen Tieren. Eine klassische Frage des Tierschutzrechts im Kontext der biomedizinischen Forschung. Natur und Recht 37:677-682, doi:10.1007/s10357-015-2903-9 (GERMAN).

Disclaimer

Any use of GV-SOLAS publications (specialist information, statements, booklets, recommendations, etc.) and application of the information contained therein are at the express risk of the user. Neither GV-SOLAS nor also the authors can accept liability for any accidents or damages of any kind arising from the use of a publication (e.g. resulting from the absence of safety instructions), irrespective of legal grounds. Liability claims against GV-SOLAS and the author for damages of a material or non-material nature caused by the use or non-use of the information or by the use of erroneous and/or incomplete information are in principle excluded. Legal claims and claims for damages are therefore excluded. The work, including all content, was compiled with utmost care. However, GV-SOLAS and the authors assume no responsibility and no liability for the currentness, correctness, completeness or quality of the information provided or for printing errors. GV-SOLAS and the authors accept no legal responsibility or liability in any form for incorrect statements and consequences arising therefrom. Responsibility for the content of the internet pages printed in these publications lies solely with the owner of the websites concerned. GV-SOLAS and the authors have no influence on the design and content of third-party websites and therefore distance themselves from all third-party content. Responsibility within the meaning of press legislation lies with the board of GV-SOLAS.