

Chapter 2

Genetic Modification of the Mouse: General Technology – Pronuclear and Blastocyst Injection

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Abstract

Introduction of germ line mutations in mice via genetic engineering involves alterations of the structure and characteristics of genes. These alterations are mostly introduced via molecular genetic technology either in embryonal stem cells or in one-cell stage embryos. This chapter describes classic biotechnological methods used to generate mice from modified pre-implantation embryos.

Key words: Transgenic, Gene expression, Promoter, DNA construct

1. Introduction

Transgenesis, one of the first techniques specifically aimed at germ line alterations, makes use of introduction of exogenous DNA sequences, *transgenes*, into an organisms' genome. A transgenic animal is by definition an organism that has had extra, often times foreign DNA, artificially introduced in its genome. Transgenesis is applicable to not only a wide range of mammalian species including mice, rats, rabbits but also livestock such as sheep, pigs, cattle, and recently even primates. The focus of this chapter, however, will be on mice, because these are the most widely used laboratory animals for transgenic studies in experimental research. In addition to conventional transgenic technology described in detail below, mice are, as of yet, the only mammalian species suitable for gene targeting by homologous recombination in embryonic stem cells. Gene targeting allows for genetic germ line manipulation at predetermined genomic loci and is a very important and powerful extension of the current molecular genetic tools to generate experimental animal models to study gene function and disease.

This topic and some of its exciting applications are described in Chapters 8–10. In contrast to embryonic stem cell technology, there is no need for homology between the injected DNA and the host genome in this technique. Transgenic animals are generated by (retro)viral transduction of early embryos, introduction of transgenes in embryonic stem cells, or, more commonly, by microinjection of DNA directly into one of the pronuclei of a fertilized mouse egg (1–5). Typically, microinjected DNA will integrate at one site within the genome, often as a concatamer (a multi-copy insertion), arranged in a head-to-tail fashion.

Transgenesis may be used to study overexpression or ectopic expression of a gene of interest. Alternatively, the effect of mutation of a gene may be the subject of studies. In both instances, the basis of study is analysis of the resulting altered phenotype. Depending on the choice of regulatory sequences directing transgene expression (see Chapter 4), the expression of a transgene may follow the expression pattern of its endogenous counterpart or be limited to distinct cell types or particular developmental stages. Alternatively, transgene expression may occur in cell types where the endogenous gene is normally inactive (ectopic gene expression). Transgenes, which hold mutant forms of genes, either spontaneously occurring or genetically engineered, may exert dominant effects. Although genetic manipulation is possible in tissue culture, the interaction of transgenes with other genes, proteins, and other components of the intact organism provides a much more complete and physiologically relevant picture of the transgene's function than could be achieved in any other way. In addition to studies on gene function and pathology, transgenesis, therefore, represents an important and biologically relevant tool to complement *in vitro* gene expression studies aimed at, e.g., delineation of signal transduction pathways or identification of tissue-specific regulatory elements.

In Figure 1 below, an overview of the different experimental aspects involved in making genetically modified animals via conventional transgenic technology (i.e., pronuclear injection of one-cell stage embryos) or gene targeting (i.e., embryonic stem cell injection into blastocysts) is presented. In the following sections, some basic guidelines for the production of transgenic mice will be provided, including superovulation, microinjection of one-cell stage zygotes, and identification of transgenic founder mice. Since the animal and equipment use for embryonic stem cell injection into blastocysts are in essence very similar to microinjection of fertilized eggs, blastocyst injection, and uterine transfer are discussed in this chapter as well (see Figure 1 and Subheadings 3.2–3.4). For basic molecular cloning techniques and strategies, and molecular detection methods, such as polymerase chain reaction (PCR)-based or Southern blot analysis, we recommend additional reading in “Molecular Cloning” by Sambrook et al. (6). Transgenic construct design is discussed in Chapter 4.

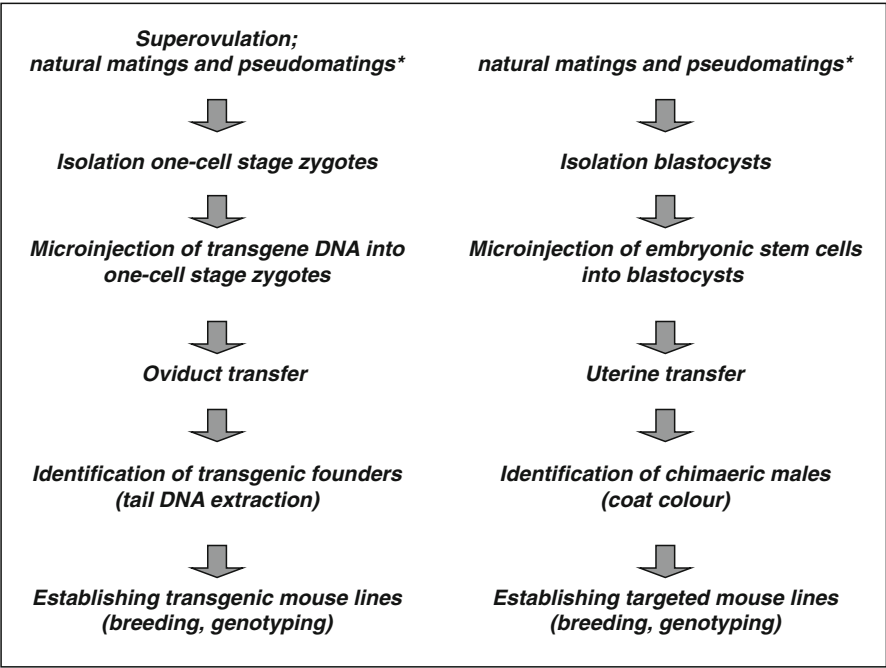


Fig. 1. Stepwise overview of the procedures for generating genetically modified mice by pronuclear injection of DNA (*left column*) and by gene targeting in embryonic stem (ES) cells, and subsequent injection of these ES cells into blastocysts (*right column*). *: Matings between foster females and vasectomized males.

**1.1. Mouse Husbandry,
Choice of Genetic
Background**

As transgenic technology and protocols are described in this chapter, it is assumed that the researcher has access to a professional and well-equipped laboratory animal facility, with ample experience in mouse handling, breeding, and surgical and/or dissection techniques required for the production of transgenic mice. Animals used to generate transgenic and targeted mouse lines comprise fertile donor females and fertile males for fertilized oocyte production, and fertile females and vasectomized males for oviduct or uterine transfer. Fertile females are either superovulated at a critical age and mated to studs, so as to obtain ample numbers of one-cell stage zygotes for pronuclear injection (see Subheadings 3.1 and 3.1.1), or mated naturally to studs for the production and isolation of blastocysts to reintroduce genetically modified embryonic stem cells into. Normal females are mated to vasectomized males to produce pseudopregnant females, which will foster transplanted, micromanipulated embryos after birth (see Subheadings 3.1 and 3.1.2). Procedures and requirements concerning animals used for fertilized oocyte production and for oviduct transfer and guidelines for equipment and microsurgical techniques are described in excellent detail in “Manipulating the Mouse Embryo” by Hogan et al. (1) and in the video guide “Transgenic Techniques in Mice” by Pedersen and Rossant (2). In addition, these media provide

comprehensive information on historical and genetic backgrounds of in- and out-bred strains, on mouse embryology, and dissection of specific developmental stages.

The need for genetic standardization of experimental animal models in experimental and applied research has been historically one of the reasons why inbred strains were established. A defined genetic context is important, for instance, to establish the genetics of cancer susceptibility, for studies on polygenic diseases, or for immunological studies. In these instances, inbred mice are preferred to generate transgenic mouse models, because of their strain homogeneity. A unique collection of inbred mouse strains is available worldwide. By definition, an inbred strain is derived by 20 generations of brother-to-sister matings and is essentially homozygous at all genetic loci (1). The choice of genetic background is determined by the aim of the experimental model. Sometimes a reason for widespread application is simply a historical one (i.e., best studied strain in a given context), while in other instances, there may be a clear advantage in using a particular strain because of a certain predisposition, although the exact underlying genetic cause (e.g., modifier loci, QTLs) is not always known. Although there is considerable choice in inbred strains, the most widely used strain is C57BL/6J, also known by the acronym B6. The C57BL/6J strain, for instance, appears more sensitive to diet-induced atherosclerosis, which makes this strain particularly valuable in cardiovascular research. The same inbred mouse strain is used frequently in immunological and behavioral studies as well. A common disadvantage of inbred strains, however, is their reduced reproductive capacity and relative poor yield of one-cell stage zygotes (fertilized eggs) upon superovulation compared to that in F1 hybrids. Furthermore, “inbred” zygotes often have an attenuated viability in vitro, microinjection, and transplantation. An exception may be the recently introduced FVB/N inbred strain, which does superovulate well yielding reasonable numbers of fertilized eggs (7). In most other instances, however, F1 hybrids are used to generate fertilized eggs for microinjection (sometimes up to 30 or more). A relatively large fraction of F1 hybrid-derived zygotes will develop to term. One of the most often used F1 hybrids is C57BL/6J × CBA (BCBA). Other F1-strain hybrids applied are C57BL/6J × SJL, C3H/HeJ × C57BL/6J, C3H/HeJ × DBA/2J, and C57BL/6J × DBA/2J (1).

2. Materials

2.1. Superovulation: Natural Matings and Pseudomatings

1. Light-cycle controlled mouse room.
2. Female mice (4–6 weeks of age).
3. Fertile male mice (8–12 weeks up to 8 months of age).

4. Foster mothers (preferably experienced mothers 3–6 months of age).
5. Vasectomized males (any strain; we use Swiss).
6. 1-ml syringes.
7. 26- or 27-G $\frac{1}{2}$ " needles.
8. Sterile 0.85% (w/v) sodium chloride solution or sterile water.
9. FSH analog: Pregnant mare serum gonadotrophin (PMSG); 1,000 IU.
10. LH analog: human chorionic gonadotrophin (hCG); 1,500 IU.

Hormones are available from Intervet, Boxmeer, the Netherlands: Folligon; Chorulon, or Sigma: PMSG: (1,000 IU); hGC: (2,500 IU).

2.2. Isolation of One-Cell Stage Zygotes; Isolation of Blastocysts

1. Mineral oil (Sigma).
2. M2 medium (Sigma).
3. M16 medium (Sigma).
4. Blastocyst isolation medium: 10% FBS, 10-mM HEPES in DMEM medium (+penicillin/streptomycin; see Note 13).
5. Blastocyst culture medium: 10% FBS in DMEM medium (+penicillin/streptomycin; see Note 13).
6. Penicillin and Streptomycin solution, 10,000 U/ml.
7. Pyruvate (Sigma).
8. Hyaluronidase type IV-S (Sigma).
9. Bovine serum albumin, fraction V (Sigma).
10. Phosphate-buffered saline (optional).
11. Depression slides (optional).
12. 10-cm petri dishes.
13. 35-mm petri dishes for microdroplets.
14. Fire-polished Pasteur pipet.
15. Transfer pipet (drawn Pasteur pipet with an internal diameter of ± 200 μ m).
16. Mouth pipet/tubing.
17. Syringes (1 ml)/needles (27 G; see Note 13).
18. Synthetic clay.
19. Incubator at 2.5% CO₂.
20. Dissection microscope.
21. Fiberoptic illuminator.
22. Diamond pencil.
23. Small iris scissors.
24. Set of tweezers.

25. 70% Ethanol.

26. Paper towels.

2.2.1. Preparation of Media

M2 and M16 media can either be prepared from separate stock solutions as described in Hogan et al. (1), or purchased prefabricated (Sigma). Individual researchers should consider and test personal preferences on location, since, e.g., composition and indications on storage conditions tend to vary. For the sake of simplicity, preparations below use prefabricated solutions. It is of importance to avoid dust collection in media and other liquids. For this reason, we adhere to not wearing powdered gloves, or at least washing off the powder and dust thoroughly before use.

- (a) M2 medium: to 50-ml of M2 medium, add 0.5 ml of penicillin/streptomycin solution. If necessary, adjust pH to 7.3–7.4 with 5 N NaOH. Add BSA to a final concentration of 4–5 mg/ml (see Note 3). Filter sterilize (0.22-mm filter). Pre-wash filters with PBS (sterile) or discard the first few milliliters. Make 2-ml aliquots in sterile tubes and store at 4°C until use. These aliquots can be used for a month according to the manufacturer's specifications.
- (b) M16 medium: to 50 ml of M16 medium, add 0.5 ml of penicillin/streptomycin solution. Filter sterilize (0.22-mm filter). Discard the first few milliliters. Make 2-ml aliquots in sterile tubes and store at 4°C until use. These aliquots can be used for 1 month according to the manufacturer's specifications. Incubate two small tissue culture dishes with 5–8 M16 drops (10–20 ml) under paraffin oil overnight at 100% humidity, 2.5% CO₂ (see Note 3).
- (c) Hyaluronidase solution: dissolve 50 mg of hyaluronidase in 50 ml of M2-Medium (Sigma). If necessary, adjust pH to 7.3–7.4 with 5 N NaOH. Filter sterilize (0.22-mm filter). Discard the first few milliliters. Make 0.5-ml aliquots and store at –20°C.
- (d) Pyruvate: dissolve 36 mg of pyruvate into 10 ml of water. Filter sterilize (0.22-mm filter). Discard the first few milliliters. Make 0.5-ml aliquots and store at –20°C.

The blastocyst isolation and culture media are prepared 1 day in advance. The culture medium is incubated overnight in 5% CO₂ at 37°C.

2.3. Microinjection of One-Cell Stage Zygotes: In Vitro Culture of Injected Zygotes – Injection of Blastocysts with Embryonic Stem Cells

1. Mineral Oil.
2. M2 medium.
3. M16 medium.
4. Blastocyst isolation medium: 10% FBS, 10-mM HEPES in DMEM (+penicillin/streptomycin; see Note 13).
5. Blastocyst culture medium: 10% FBS in DMEM (+penicillin/streptomycin; see Note 13).

6. 10-cm petri dishes.
7. 35-mm petri dishes.
8. Incubator at 2.5% CO₂.
9. Transfer pipet (drawn Pasteur or other glass pipet; internal diameter of $\pm 200\text{-}\mu\text{m}$).
10. Mouth pipet/tubing.
11. Synthetic clay.
12. Glass capillary tubes (A>520119; Leitz) for holding pipets.
13. Glass capillary tubes (G-1; Narishige) for blastocyst injection needles (see Note 13) or glass capillaries with inner filament (GD-1; Narishige).
14. Sigmacote (Sigma).
15. 96% Ethanol (analytical grade).
16. Pipet puller (e.g., PB-7; Narishige).
17. Microforge (e.g., MF-9; Narishige).
18. Microgrinder (e.g., E-40; Narishige; see Note 13).
19. Inverted microscope system (e.g., Nikon, Olympus, Leitz, and Zeiss).
20. Micromanipulators (e.g., Narishige).
21. Dissection microscope.
22. Diamond pencil.
23. Stage micrometer.
24. Fluorinert (Sigma).
25. Read-out type (see Note 13), motor-driven microinjector, or large 50-ml glass injection syringe.

**2.4. Oviduct Transfer:
Uterine Transfer**

1. Mineral Oil.
2. Clean M2.
3. Blastocyst isolation medium: 10% FBS, 10-mM HEPES in DMEM (+penicillin/streptomycin; see Note 13).
4. Blastocyst culture medium: 10% FBS in DMEM (+penicillin/streptomycin; see Note 13).
5. Pair of small scissors (iris).
6. Two pairs of tweezers (size 5).
7. One pair of tweezers (size 4A).
8. Serafine clamp.
9. Suture and/or wound clip system.
10. Kimwipe tips.
11. Transfer pipets, 100–120- μm diameter.

12. Synthetic clay.
13. Inhalation anesthetic (see Note 5).
14. Small desiccator.
15. Injection sedative (see Note 5).
16. Syringes (1 ml)/needles (27 G; see Note 13)
17. 96% Ethanol.
18. Dissection microscope.
19. Fiberoptic illuminator.
20. Operation platform

2.5. Identification of Transgenic

Founders: Tail DNA Extraction

2.5.1. Rapid Procedure Tail-Tip DNA Extraction

1. Tail mix: 100-mM Tris-HCl, pH 8.5, 5-mM EDTA, 0.2% SDS, and 200-mM NaCl.
2. Proteinase K: fresh stock solution: 20 mg/ml in TE buffer.
3. TE buffer: 10-mM Tris-HCl, pH 7.2–7.6, 1-mM EDTA.
4. Pasteur pipets, flame-polished.
5. Reaction tubes, 1.5 ml.
6. 55°C oven.
7. Rotator.

2.5.2. Standard Procedure Tail-Tip DNA Extraction

1. Tail mix: 50-mM Tris-HCl, pH 8.6, 100-mM EDTA, 1% SDS, 100-mM NaCl.
2. Protease K: fresh stock solution: 10-mg/ml TE buffer
3. RNase: 10 mg/ml (heat inactivated; 10 min at 100°C).
4. Phenol–chloroform–isoamylalcohol (24:24:1; Phenol: saturated with demiwater (autoclaved) adjusted to pH 7.0 with 1-M Tris-HCl, pH 8.0.
5. TE buffer: 10-mM Tris-HCl, pH 7.2–7.6, 1-mM EDTA.
6. Pasteur pipets, flame-polished.
7. Reaction tubes, 1.5 ml.
8. 55°C oven.
9. Rotator.
10. Bench or wrist shaker.
11. 37°C waterbath.

3. Methods

3.1. Superovulation: Natural Matings and Pseudomatings

Animals have access to water and standard chow ad libitum and are housed under a 12-h day/night time regimen, most often comprising a 6 a.m. to 6 p.m. light period (see Note 1). We have

good experience using 4–6 weeks old B6CBA/F₁ females (Jackson labs) for superovulation. Tables 1 and 2 present a summary of the different actions in the procedures.

3.1.1. Superovulation:
Natural Matings

- 1. Lyophilized PMSG and hCG are dissolved in 0.85% NaCl solution or sterile water to a final concentration of 50 IU/ml. PMSG may be stored frozen at –20 or –80°C in 0.8–1.0-ml aliquots and thawed when needed. One aliquot will be enough for injection of six females. Alternatively, concentrated stocks of PMSG and hCG (200 IU/ml) may be stored at 4°C for a period of 2 months, but need to be diluted properly prior to use.

Table 1
Time table of superovulation and matings to obtain one-cell stage embryos for microinjection

Day	Time point	Action	Week day						
1	1.00–2.00 p.m.	PMSG injections ^a	Sa	Su	Mo	Tu	We	Th	Fr
3	12.00–1.00 p.m. 3.00–4.00 p.m.	hCG injections ^a Natural matings	Mo	Tu	We	Th	Fr	Sa	Su
4	10.00–11.00 a.m. Afternoon 3.00–4.00 p.m.	Isolation of fertilized eggs Microinjections Pseudomatings ^b	Tu	We	Th	Fr	Sa	Su	Mo
5	Morning	Oviduct transfer of two-cell stage embryos	We	Th	Fr	Sa	Su	Mo	Tu

^aDay/night rhythm: 6.00 a.m.: light, 6.00 p.m.: dark
^bMatings between foster females and vasectomized males

Table 2
Time table of natural matings to obtain blastocysts for injection

Day	Time point	Action	Week day						
1	3.00–4.00 p.m.	Natural matings ^a	Mo	Tu	We	Th	Fr	Sa	Su
2	3.00–4.00 p.m.	Pseudomatings ^b	Tu	We	Th	Fr	Sa	Su	Mo
3	Morning ^c	Pseudomatings ^c	We	Th	Fr	Sa	Su	Mo	Tu
5	Morning Afternoon	Collect blastocysts Blastocyst injection Uterine transfer	Fr	Sa	Su	Mo	Tu	We	Th
6	Morning	Uterine transfer	Sa	Su	Mo	Tu	We	Th	Fr

^aDay/night rhythm: 6.00 a.m.: light, 6.00 p.m.: dark
^bMatings between foster females and vasectomized males
^cOptional, in case not many copulation plugs are detected on day 3

2. Thaw a vial of PMSG and inject 4–6 weeks old females with 100- μ l of PMSG (5 IU) between 1.00 and 2.00 p.m. on the first day.
3. At day 3, 46–48 h after PMSG injection, thaw a vial of hCG and inject the same females with 100 μ l of hCG (5 IU; 12.00–1.00 p.m.).
4. The females are either transferred directly to fertile studs or in the afternoon (see Note 2). Use one male per female. To ensure maximum number of fertilized eggs, these male mice are used only once a week. At 8 months of age, or when the plugging ratio drops below 70%, the male mice are replaced.
5. Check for copulation plugs the next morning.

If natural matings are carried out for blastocyst production, use females of 2–4 months of age. In terms of numbers of mice used, the same guidelines apply as for “pseudomatings,” i.e., 2–3 females per male (see Subheading 3.1.2). Average yield per “plugged” female (C57blk/6J) is 5–7 blastocysts. To produce reasonable amounts of blastocysts for injection, superovulation is sometimes used in different laboratories. The quality of blastocysts obtained in this manner may, however, vary considerably.

3.1.2. Pseudopregnant Females

Mating females with vasectomized (or genetically sterile) males will generate pseudopregnant females required for re-implantation of microinjected zygotes or blastocysts (1). Basically, any genetic background may be used to generate pseudopregnant foster females, provided the females from this strain are known as “good mothers.” Most F1 hybrids or outbred strains can be used; for practical reasons we use females from the same background (B6CBA/F1) as those used for superovulation. If possible, experienced mothers are preferred. The females should be at least 2 months old (i.e., >20 g body weight) but should not weigh over 30 g: the older and heavier the females, the more problems can be expected in terms of fat accumulation, which can seriously hamper oviduct transfers. Since the females are mated in natural estrus, obtaining enough pseudopregnant females for oviduct transfer can be problematic. It is advisable to mate at least 5–6 females per intended oviduct transfer. To increase the chances of mating a female in estrus, several females (2–4) are placed in with one vasectomized male, in contrast to 1-on-1 matings between superovulated females and fertile studs. Experience in judging whether females are in estrus can be helpful in obtaining sufficient plugged pseudopregnant females. Females are placed in a cage with a resident male, *not vice versa*. Vasectomized males are housed separately for at least 1–2 weeks (see Note 2). Oviduct transfers take place on the day of copulation plug detection. The surplus of plugged, pseudopregnant fosters can be re-used after 2 weeks. The vasectomized male mice can be used twice a week for matings. The foster females are checked regularly for signs of pregnancy.

Table 3
Time table of preparations for pronuclear microinjection

Action	Time
Preparation of media	Day before, morning
Check plugs, kill superovulated females	9.00 a.m.
Isolation of one-cell stage zygotes	9.30 a.m.
Set up microinjection stage; holding pipet	10.30 a.m.
Injection needles	11.00 a.m.
Set up microinjection needle	1.00 p.m.
Pronuclear injection of one-cell stage zygotes	1.00 p.m. onward

Preparations for blastocyst isolation and injection are very similar. The microinjection system for ES cell injection is usually controlled by fluorinert or mineral oil

Depending on the strain, the litter will be delivered around 19–21 days after the oviduct transfer.

**3.2. Isolation
of One-Cell Stage
Zygotes: Isolation
of Blastocysts**

*3.2.1. Isolation of Fertilized
Oocytes*

When microinjections are planned, the order of procedures should have some (chrono)logic to it. Although the planning of a microinjection session is highly personal, some suggestions are presented below in Table 3. Day/night rhythm is as in Table 1(see Note 4).

1. On the day of isolation, add 20 µl of the pyruvate stock to 2 ml of M2 and M16 aliquots and mix by swirling gently.
2. Prepare the depression slides: one with 0.2 ml of M2, two depression slides with 0.2 ml of hyaluronidase solution, and three depression slides with 0.2 ml of M16 medium. Place each depression slide in a 9-cm petri dish and incubate them briefly at 37°C in the incubator. Alternatively, droplets of media may be prepared in 35-mm dishes.
3. Collect females with a clear copulation plug. Kill the mice by CO₂ asphyxiation or cervical dislocation and transfer the animals to the location where zygotes are isolated.
4. Place the females’ abdomen upward on a paper towel, wet the abdomen with 70% ethanol, and make an incision over the xiphoid (see Figure 2).
5. Grasp the skin with both hands and firmly pull back in opposite directions (rostrally and caudally), essentially skinning the mouse completely (see Figure 2).
6. Make incisions in body wall by lifting it with tweezers and cutting it with scissors, and expose complete body cavity.
7. Move intestines aside gently and grab ovaries by fat pad, remove ovaries plus oviducts by cutting through the transition to the uterus horn, and transfer to a dish with PBS to wash off blood or debris.

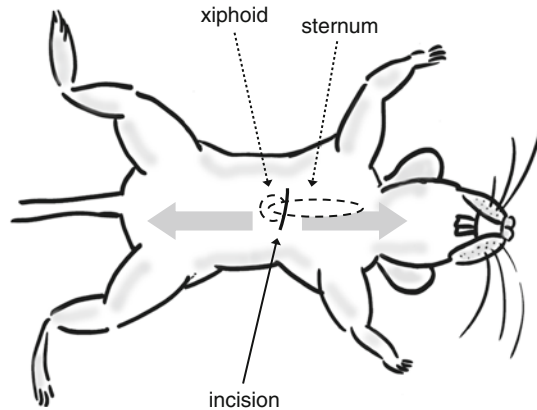


Fig. 2. Dissection of donor females and isolation of fertilized eggs. An incision is made over the xiphoid, after which the skin is firmly pulled back in the indicated direction (*gray arrows*).

8. Swollen ampullae (1) are ruptured with sharp tweezers or a needle in M2 medium containing hyaluronidase (e.g., in a dish or on a depression slide). Cumulus masses are released into the medium and slowly fall apart by enzymatic action. Once freed of cumulus cells, the one-cell stage zygotes are washed free of hyaluronidase right away. Use pipets (I.D. 200 μm) and fresh M2 medium, and subsequently wash and transfer to M16 droplets under mineral oil in a 35-mm dish (37°C; 2.5% CO_2) until further use. Handling 35-mm dishes is the easiest on top of the lid of a 10-cm dish, depression slides are best placed inside a 10-cm dish.

3.2.2. Isolation of Blastocysts

1. Dissect the uterus by a cross section through the cervix and subsequent separation of the uterus from attached mesenteries, blood vessels, and fat.
2. Expose the lumen of the uterus horns by a transverse section below the transition to the oviduct. Make cross incisions at the opening to prevent constriction of the created opening.
3. One uterus is transferred to a dish with clean isolation medium. Disconnect the uterus horns from the cervix by making a cut just above the bifurcation point.
4. Carefully insert a short 27-G needle at the opening (closest to the cervix) and flush the blastocysts out using 0.5–1.0 ml of medium.
5. Collect the blastocysts with a wide-bore pipet (200–250 μm) and transfer to a drop culture with clean medium, wash, and transfer to culture medium at 37°C and 5% CO_2 .
6. Fully expanded blastocysts are the easiest to use for injection; culturing blastocysts for some hours at 37°C and 5% CO_2 may increase the percentage of useable embryos.

3.3. Microinjection of One-Cell Stage Zygotes: In Vitro Culture of Injected Zygotes – Injection of Blastocysts with Embryonic Stem Cells

3.3.1. Preparations

The availability of an operational microinjection set-up is considered a prerequisite to apply transgenic technology successfully. If no microinjection unit is available, several types of microscopes, micromanipulators, injectors, and peripheral equipment, to make injection needles and holding pipets for either pronuclear or blastocyst injection (see Chapter 8), such as needle pullers, microforges, and grinders, are commercially available (Leitz, Narishige, Nikon, Olympus, Sutter, and Zeiss). We refer to Hogan et al. (1) for a detailed description of a microinjection set-up. Microinjection of one-cell stage zygotes and subsequent transplantation are essentially carried out as described in ref. (1). It is highly recommended to consult the video guide “*transgenic techniques in mice*” by Pedersen and Rossant (2) for a visual reference to the protocols and procedures outlined in this chapter. In practice, one will see that slight deviations from an existing protocol are possible and sometimes necessary in order to make things work for the individual user. Therefore, the protocol below only presents some of the most essential steps in the microinjection procedure.

Use dust-free gloves when preparing holding pipets and injection needles. Holding pipets are heat-polished until an opening of about 10–20 μm remains. Holding pipets and depression slides can be siliconized and rinsed extensively with clean, dust-free 96% ethanol; siliconized holding pipets can be re-used. Also, injection needles can be treated similarly: dip the needle tip into silicon solution and rinse it with alcohol before the needle is opened.

Several types of injection chambers may be used for microinjection. These chambers may consist of a slide and a Perspex ring (outer diameter slightly smaller than the width of the slide; 1.5 mm in height), which is fixed in position on a siliconized microscopic slide with 2% agarose. Alternatively, a depression slide can be used as an injection chamber. The injection chamber contains a droplet of 10 μl of M2 under light mineral oil. Depending on the manner in which DNA is loaded into the injection system, a 3–5- μl DNA droplet may be positioned next to the M2 droplet (also under oil), or a needle with an inner filament is back-filled. The chambers are prepared just before use and kept in a 90-mm petri dish in the CO_2 incubator until use. Several injection chambers may be set up in parallel in this fashion and be used alternately. Figure 3 depicts an example of a microinjection set-up.

3.3.2. Pronuclear Injection

1. Place the microinjection chamber on the microscope. Position the holding pipet. Positioning both the holding pipet and the needle is usually carried out at a magnification of 100 \times .
2. Just before injection, a number of one-cell stage zygotes (20–40) are transferred into the M2 droplet of the injection chamber. Transferring one-cell stage zygotes between injection chambers and culture droplets is best done under a dissection

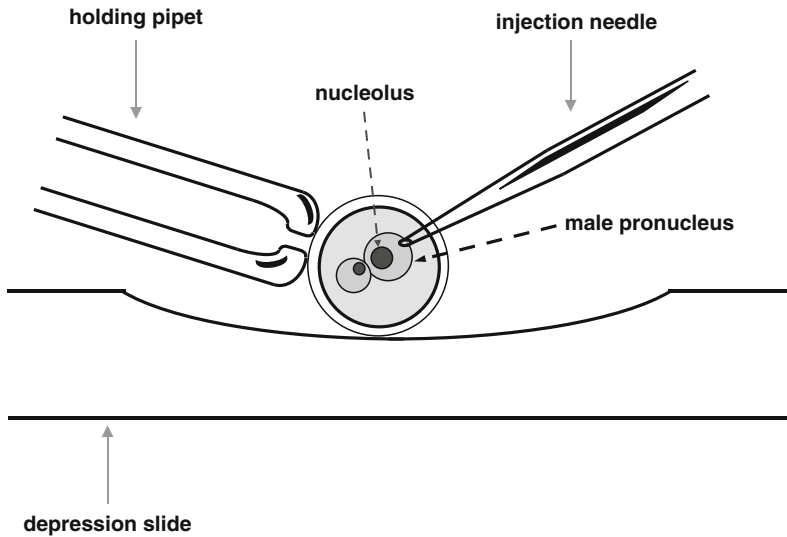


Fig. 3. Schematic representation of a microinjection chamber (*side view*). The basis of the injection chamber in this figure is a depression slide. Microinjection is carried out in a droplet of M2 medium.

microscope. Since culture conditions inside the injection droplet are suboptimal, take no more zygotes than can be injected within 20–30 min.

3. Fill the injection needle with DNA solution from the DNA droplet and position the injection needle. In case of a back-fill system (i.e., glass capillary tubes with inner filament), an opening is created in the injection needles by gently tapping it against the holding pipet and breaking off the very tip of the needle. Microinjection is done at 300–400× magnification.
4. Fix an egg in position with the holding pipet.
5. The injection needle is gently pushed through the plasma membrane and brought close to the nuclear membrane of one of the pronuclei. In most cases, the male pronucleus is the best accessible, because it is larger. If at this stage a small burst of DNA invaginates the plasma membrane further, this indicates that the membrane was not properly pierced. Reposition the zygote on the holding pipet and try again.
6. Carefully penetrate the nuclear membrane, avoid touching the clearly visible nucleoli and inject the DNA solution into the pronucleus. Stop injecting at the slightest swelling of the pronucleus.
7. Gently withdraw the injection needle. If any material is accidentally pulled out of the nucleus, or when injected zygotes lyse frequently, the needle should be replaced.
8. The zygotes are placed within the optic field strategically, so that yet to be injected, abnormal and lysed zygotes, and

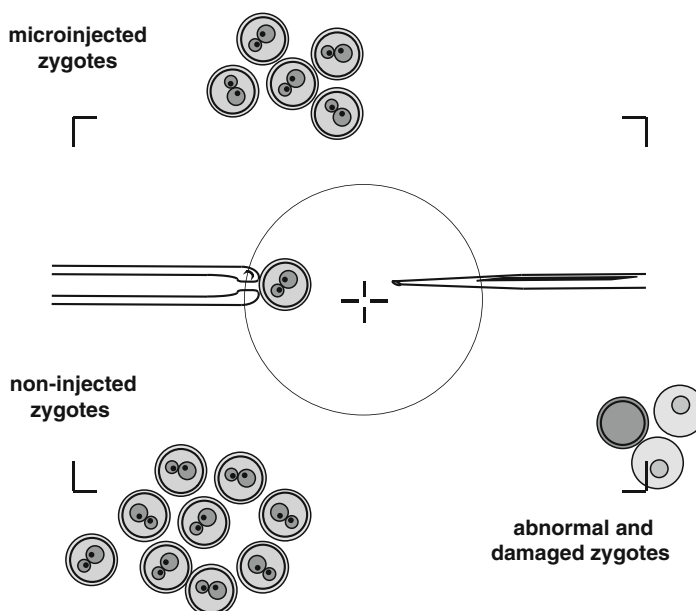


Fig. 4. Overview of the microinjection field (*top view*): non-injected, successfully injected, and abnormal zygotes, or zygotes that were damaged during microinjection are all kept apart throughout the injection procedure.

successfully injected zygotes are conveniently separated (see Figure 4).

9. Successfully injected zygotes are washed free of M2 medium, transferred to a 35-mm dish containing a few CO₂-buffered M16 droplets, and kept in an incubator at 37°C and 2.5% CO₂.
10. Repeat the microinjection procedure until all one-cell stage zygotes are injected.
11. Keep successfully injected zygotes overnight at 37°C and 2.5% CO₂.

Although after a successful injection session, as much as 90% of zygotes may survive the microinjection procedure, on average 60–75% of the zygotes can eventually be used for oviduct transfers. Transfer of zygotes is possible on the same day as the microinjection (one-cell stage). However, we prefer to culture the one-cell stage embryos overnight. During this time, the embryos will undergo the first cleavage and develop into two-cell stage embryos. In this manner, it becomes possible to make a selection of properly developed embryos before transfer into the recipient females' reproductive tract.

3.3.3. Blastocyst Injection

1. Place the microinjection chamber on the microscope. Sometimes a cooling stage (4–10°C) is used to make the blastocysts more rigid. Not all laboratories use this application.

Positioning the holding pipet and injection needle is carried out at a magnification of 100 \times . Optimal needles are bevelled to an opening of 12–14 μ m at 30–35°C. Injection needles may be siliconized and re-used.

2. Just before injection, a number of blastocysts (20–25) are transferred into the injection droplet. Injection is carried out in isolation medium or HEPES-buffered ES cell culturing medium (see Chapter 8).
3. ES cell suspensions are made by trypsinizing and preplating the suspension to get rid of feeder cells, which support ES cell growth in culture (see Chapter 8). We typically keep a few small ES cell cultures at hand to be able to repeat this procedure throughout the injection day. Wash ES culture twice with Ca²⁺/Mg²⁺-free PBS and trypsinize the feeders plus ES cells. Sediment cells (5 min, at 1,000–1,200 rmp at ambient temperature) and suspend in ES cell medium; preplate on gelatinized culturing surface (see Chapter 8) and harvest after 15–20 min. Most feeder cells will have attached, whereas ES cells will not. If so desired, the preplating procedure may be repeated. Collect the ES cells, sediment, suspend well in a small volume of Ca²⁺/Mg²⁺-free PBS, and transfer to a petri dish: make several drop cultures in either PBS or isolation medium.
4. Transfer single ES cells into the injection chamber and load the injection needle with a fair number of ES cells (e.g., 75–120 ES cells). Typically, 12–15 ES cells are injected into one blastocyst.
5. Fix a blastocyst in position with the holding pipet, with the inner cell mass (ICM) located away from the injection site (see Figure 5). Find a junction (window) between two trophoblast cells and insert the needle through the zona pellucida through the junction into the blastocoel; gently expel the ES cells.
6. The injection needle is gently removed from the blastocoel, the blastocyst will visibly collapse shortly afterwards; move the injected blastocyst aside (as in Figure 4) and inject the next one.
7. The successfully injected blastocysts are transferred to a 35-mm dish containing a few CO₂-buffered culture medium and kept in an incubator at 37°C and 5% CO₂.
8. Repeat the injection procedure until all blastocysts are injected. ES cell suspension will tend to aggregate; repeat the preplating procedure with fresh suspension.

The successfully injected blastocysts are either transferred to pseudopregnant females the same day or cultured overnight at 37°C and 5% CO₂.

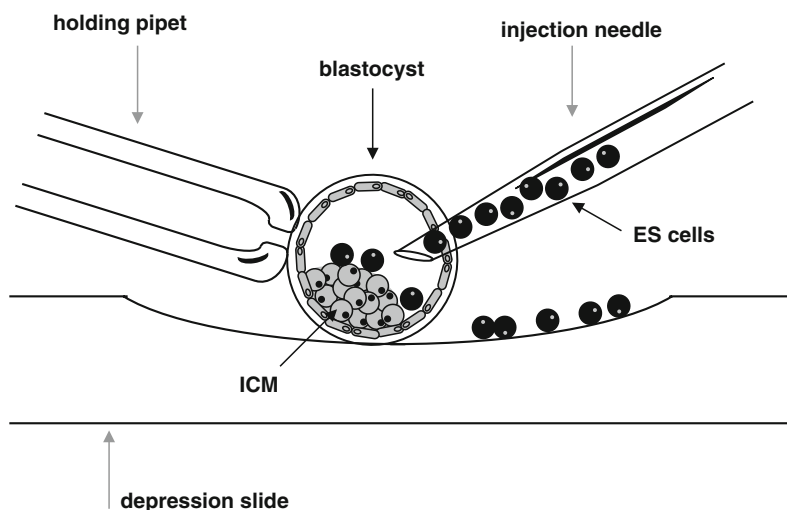


Fig. 5. Schematic representation of a blastocyst-injection chamber (*side view*). Genetically manipulated ES cells (*black*) are injected into a recipient blastocyst (*gray*). ES cells will adhere and become part of the inner cell mass (ICM) and partake in development. The resulting newborn will be a coat color chimera.

3.4. Oviduct Transfer: Uterine Transfer

3.4.1. Preparations for Oviduct Transfer

Injected zygotes are transferred either on the same day of micro-injection, or the next day. If the oviduct transfer is carried out the same day, about 2×20 one-cell stage zygotes are transferred bilaterally to one recipient pseudopregnant female (i.e., 20 on each side). If two-cell stage embryos are transferred 1 day after microinjection, usually a smaller number of embryos suffice (2×12 – 14 ; see Notes 5 and 6).

1. Load transfer pipets as depicted in Figure 6: draw in mineral oil until it reaches the part where the pipet widens (to avoid capillary suction) – M2 medium – air bubble – M2 – air bubble – $20 \times$ closely stacked embryos – air bubble – M2.
2. Fix the loaded transfer pipets with synthetic clay to the bench, placing their shafts containing the embryos over a wetted kimwipe, so as to prevent evaporation of medium, and clogging of the opening. Take care not to knock over the loaded pipets.
3. Pre-anesthetize mouse using an inhalation anesthetic in a small desiccator (see Notes 7 and 8) and subsequently inject the female with a sufficient dose of sedative to anesthetize the animal for at least 30 min (see Notes 7 and 8).
4. The mouse is placed on an operation platform (10-cm dish with a plastic elevation positioned at the center).
5. Wet one flank with 96% ethanol and, with tweezers, clear a short horizontal “parting” in the fur of about 1-cm in length parallel to the spinal column just below the rib cage (see Figure 7a); grasp and lift skin with forceps and make a small

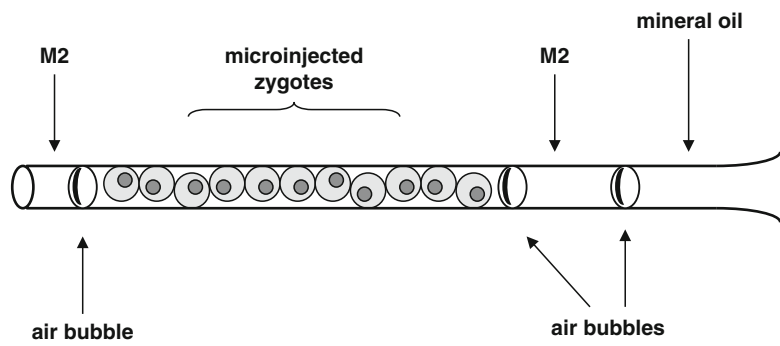


Fig. 6. Transfer pipet: microinjected zygotes are surrounded by small air bubbles, which function as markers during the oviduct transfer.

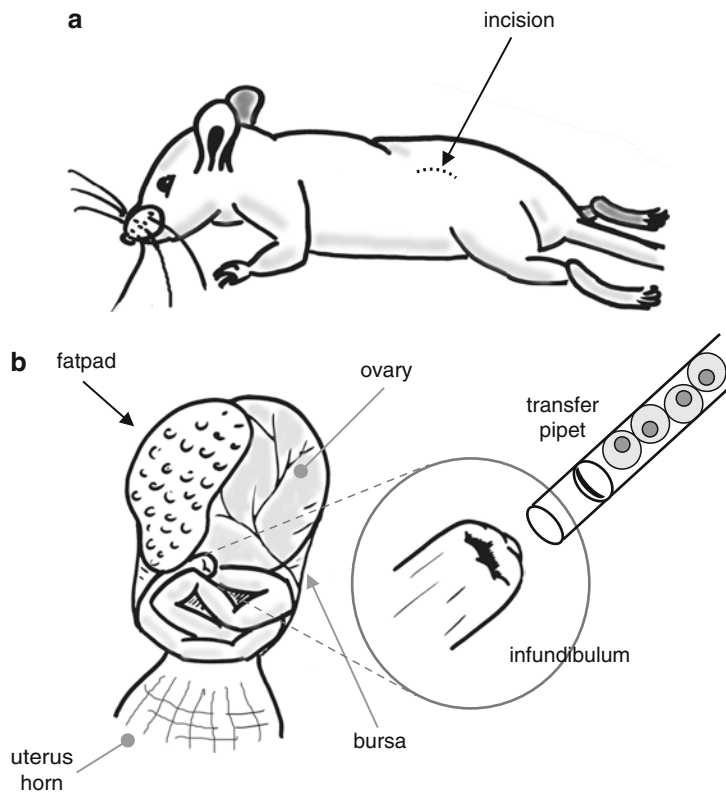


Fig. 7. Oviduct transfer. (a) An incision is made in the flank of the pseudopregnant female, just below the rib cage. (b) The ovary and oviduct are surrounded by the bursa, which needs to be ruptured for oviduct transfers. Inset: infundibulum and transfer pipet (see Fig. 5) magnified.

incision (scissors); insert the scissors into the incision and tear open the skin along the “parting” in the fur until an opening of approximately 6–8 mm is generated.

- 6. Grasp and lift body wall with forceps and make an incision with the scissors avoiding blood vessels; tear open in same

direction as the previous tear and attach suture to the upper “lip” of the wound (body wall).

7. Pull out ovary and oviduct gently by the fat pad attached to the ovary; attach a serafine clamp to the fat pad, pull out the uterus a bit more, and position the clamp over the flank of the mouse in such a manner that the infundibulum (i.e., the entrance to the fallopian tube), still covered by bursa, is visible (see Figure 7b).
8. Transfer the mouse under a dissecting microscope; magnification 15–20×. Use fiberoptic illuminators for lighting during surgery.
9. At this stage, take the mouth-piece of the transfer pipet in your mouth, while the other end remains in synthetic clay on the microscope or table surface.
10. Gently tear an opening into the bursa covering the ovary and infundibulum with 2× 5-tweezers, avoid tearing blood vessels; if bleeding is caused inadvertently, use kimwipe tips to soak up excessive fluids.
11. Locate the infundibulum, pick up transfer pipet, and bring into position (i.e., line-up with the position of the infundibulum and fallopian tube). Probe the infundibulum carefully with the transfer pipet and when certain of deep enough insertion, gently expel the injected embryos by blowing.
12. While expelling, the air bubbles surrounding the embryos become visible in the fallopian tube: if two or more are observed, the embryo transfer was successful. Pull out the pipet completely and transfer the mouse back under the fiberoptic illuminator.
13. Gently maneuver uterus horn back into body cavity using the blunt tip of the serafine clamp, close the body wall with one stitch and a tight knot, and use suture or a wound clip to close the wound in the skin; proceed with the other side (see Notes 5 and 6).
14. When completed, ear-mark the female, if so desired (see Note 9), and return the animal to a cage.
15. Clean all equipment used with water and soap, and 70% ethanol, and sterilize.

3.4.2. Uterine Transfer

A uterine transfer is very similar to an oviduct transfer, with the exception that injected blastocysts are transferred directly into the uterus lumen. To this end, gently pull out the uterus horn a little further compared to that in an oviduct transfer (see Figure 7b); carefully insert a sterile 27-G needle into the uterus lumen through the uterus wall. The insertion direction should be almost parallel to the uterus horn, to avoid puncturing the uterus. It is imperative not to stretch the uterus walls too much during needle

insertion, since the inner and outer muscle layers of the uterus will move in respect to each other when the needle is withdrawn, and the uterus relaxes again; the consequence will be that entrance through the hole will be blocked. Remove the needle and carefully insert a glass transfer pipet containing the injected blastocyst through into the uterus lumen. Gently aspirate the blastocysts into the uterus lumen; push back the uterus into the abdominal cavity and close the animal (see Subheading 3.4.1).

3.5. Identification of Transgenic Founders: Tail DNA Extraction

In essence, genotyping of mice, whether derived by transgenic technology or via targeted mutagenesis in ES cells, is carried out by similar procedures. “Genotyping” founders is a lot more straightforward in case of germ line chimeras (ES cell manipulation and blastocyst injection) since this can be done visually (i.e., by coat color mosaicism). The analysis of chimeras and their offspring is discussed in Chapter 8. Transgenic founders will have to be genotyped by DNA analysis.

If expression of transgenic constructs *in vivo* is not embryonically lethal, litters resulting from microinjected zygotes will typically comprise 10% or more transgenic pups. Transgenic founder (F0) mice are identified through Southern blot or by PCR analysis of purified mouse-tail DNA. Southern blot analysis, as opposed to PCR, offers the possibility to study actually the inserted transgene in terms of copy number (i.e., number of transgenes inserted; see Notes 10 and 11), rearrangements, multiple integration sites, and even genomic position. On occasion, genomic integration of a transgene happens after the first cell division. In essence, this results in mice mosaic for transgene integration and expression. In such instance, the F0 animal will have a lower copy number than its F1 offspring. In case mosaic animals do transmit the transgene in a sub-Mendelian ratio to their F1 offspring, their F2 should of course show a normal Mendelian segregation. Alternatively, in case of multiple chromosomal integration sites, F1 offspring within a given line may reveal a lower copy number than the F0, because multiple integration sites tend to segregate upon breeding. Sporadically, unstable integrations may present similar copy number discrepancies between F0 and F1 mice. Transgene rearrangements and sub-haploid copy numbers may be indicative for poor or absent expression and germ line transmission. Such mice would show up positive in a PCR-based identification assay, but are worthless for study since no breeding lines can be established from these animals. Nevertheless, once transgenic founders have been identified, subsequent identification of transgenic offspring does not require Southern analysis, but can be done by PCR. Southern blot analysis and PCR analysis are described in detail elsewhere (6). Here, a number of procedures for tail-tip DNA extraction are provided, described elsewhere. In addition to DNA analysis, it is wise to include a transgene

expression analysis on several tissues of F1 animals from different founder lines. This may include both RNA and protein analyses, such as for instance the detection of apolipoproteins in circulation, provided that immunological detection reagents and/or biochemical assays are available.

3.5.1. Rapid Procedure for Tail-Tip DNA Extraction

This method will allow for a rapid DNA extraction from mouse tails, which can be used for PCR-based screens without problems and was even reported to yield DNA of sufficient quality for Southern analysis (8). However, to get rid of contaminating proteins and RNAs for an exact quantitative Southern analysis, a more thorough DNA purification might be required (see Notes 10 and 11). A relatively quick method for DNA isolation is described below; in the next section (see Subheading 3.5.2), a tail DNA purification method is outlined employing phenol extraction. Mice are genotyped by tail DNA analysis (see Notes 10 and 11). Genotyping can be done from 10 days postpartum (pp) onwards. To identify the individual mice following genotyping, animals need to be marked at the time of the tail cut. Relatively young animals (i.e., 10–12 days pp) are toe-marked, on older animals (3 weeks of age) ear-marking is an option. Consult the animal facility to adhere to the marking system used on location.

1. Collect mouse tail-tips, about 0.5 cm (10 days pp) to 1.0 cm (3 weeks pp), in 1.5-ml reaction tubes on ice and either store dry at -80°C or in a tail mix at -20°C if not processed immediately. It is convenient to sedate mice lightly using an inhalation sedative, especially when older mice are marked. It is possible at this time to take a small blood sample for biochemical analysis.
2. Add 660- μl of tail mix and 10- μl of proteinase K (20 mg/ml). Incubate overnight at 55°C , preferably while rotating. This will greatly help dissolving and subsequently isolating the tail DNA.
3. Pellet the remaining debris (hairs and bone) in a microfuge at 14,000 rpm for 10–15 min at ambient temperature. Transfer the supernatant to a fresh microfuge tube.
4. Add 0.6 \times volume (i.e., $\pm 400\text{ }\mu\text{l}$) of isopropanol and mix by inverting the tubes *gently* (25 \times), allowing the DNA to precipitate in visible threads (vigorous shaking will hamper visible precipitation).
5. Either pellet the DNA briefly (15–20 s, 14,000 rpm) or fish the DNA clump out using a flame-polished glass Pasteur pipet.
6. Rinse the DNA sticking to the glass tip in 70% ethanol, let the DNA briefly air dry, and transfer to 500 μl of TE.

7. Dissolve the DNA thoroughly by incubating it for 10–15 min at 55–65°C, followed by firm vortexing or shaking. Alternatively, we use the rotator at an ambient temperature to dissolve the DNA overnight.
8. Store the DNA samples at 4°C until needed.
9. A volume of 1.5 µl of DNA suffices for a PCR. If a Southern analysis is attempted at this stage, 30–50 µl can be used.

3.5.2. Standard Procedure for Tail-Tip DNA Extraction

1. Collect mouse tails and add 700 µl of tail mix, or store (see Subheading 3.5.1); add 25-µl Proteinase K to each tube and incubate at 55°C overnight (in a rotator).
2. Add 10 µl of RNase and incubate for 1–2 h at 37°C.
3. Transfer the content of the tubes to new tubes¹ containing 400 µl of phenol–chloroform–isoamylalcohol using a cut blue tip and shake firmly for 15 min (bench or wrist shaker) at an ambient temperature.
4. Centrifuge at 14,000 rpm for 30 min, at an ambient temperature.
5. Transfer the supernatant to tubes containing 400 µl of phenol–chloroform–isoamylalcohol and shake (bench or wrist shaker) for 15 min at an ambient temperature.
6. Centrifuge at 14,000 rpm for 30 min at an ambient temperature.
7. Transfer the supernatant to tubes containing 400 µl of chloroform to remove phenol traces and shake for 5 min at an ambient temperature.
8. Centrifuge at 14,000 rpm for 5–10 min at an ambient temperature.
9. Transfer the supernatant to tubes containing 0.6× vol (±440 µl) of isopropanol.
10. Mix by inverting the tubes *gently* (25×), allowing the DNA to visibly precipitate (see Subheading 3.5.1).
11. Fish out DNA with (flame-polished, slightly bent at the tip) Pasteur pipet and wash in 1 ml of 70% ethanol. If the expected amount of DNA is too low, to fish out, pellet at 14,000 rpm for 10 min at an ambient temperature and wash the pellet with 70% ethanol (2×).
12. Let the DNA air dry for a while, then place the pipet in tube (50–100 µl of TE) until DNA comes off; DNA is dissolved by incubating for 10–15 min at 55–65°C, and subsequent incubation at 37°C, mixing firmly and repeatedly by hand until DNA is fully dissolved.

¹Tubes that have been incubated o/n at 55°C with SDS inside tend to leak when phenol–chloroform–isoamylalcohol is added.

13. Determine the DNA concentration: dilute 5 μl of DNA sample in 600 μl of water. Concentration (in $\mu\text{g}/\mu\text{l}$) is: $\text{OD}_{260\text{nm}} \text{ DNA} = 50 \mu\text{g}/\text{ml}) \times 6$; typical yield: 50–200 μg of DNA
14. Perform a quantitative Southern analysis (see Notes 10 and 11).
15. DNA samples may be stored at 4°C or –20°C.

3.6. Establishing Transgenic Lines: Breeding and Analysis

Following identification of valuable transgenic founders and F1 mice, transgenic mouse lines will have to be established. It may be useful to genotype the animals relatively early (10 days pp; see Subheading 3.5.1), for instance when rapid onset of disease in a given mouse necessitates early diagnosis or compromises life span. PCR-based genotyping on 1 day pp mice and even on mouse blood, hair, or toes has recently been described (8–12). In addition, early genotyping will allow the researcher to sort out valuable transgenic mice from litters before weaning and cull the mouse colony accordingly, which reduces housing expenses significantly.

If the mouse model needs to be studied in a particular genetic background, the transgenic lines need to be backcrossed to the strain of choice. Eight to ten backcrosses of a transgenic line to an existing inbred strain, although not inbred by definition (see Subheading 1.1 (1)), produce a genetic background sufficiently homogeneous for experimental purposes. A mouse line may be bred to “homozygosity” to increase transgene expression (i.e. double copy number); such double transgenic mice are produced by transgenic male-to-transgenic female matings. If this is done within one mouse line, disease or altered behavior, and even (embryonic) lethality, all unrelated to the expression of the transgene, may be the consequences: such conditions may be the result of recessive mutations caused by transgene integration into specific loci. X chromosomal integrations can be particularly cumbersome in this respect, although such integrations have been known to result in unexpected and exciting discoveries (13). Y chromosomal transgene integrations, conversely, if not silenced, make genotyping a fairly straightforward task. Sex-chromosome targeting, on the contrary, can speed up the creation of null-mutant models significantly.

Once transgenic mouse lines have been established, a typical initial screen would include a complete analysis by a qualified mouse pathologist, a full spectrum expressional and biochemical analysis to evaluate the effect of transgene activity. Ideally, several independently derived transgenic mouse lines should be studied to validate the animal model.

The above mentioned clearly points out that establishing mouse lines can be complex at times. It is, therefore, advisable to keep clear records of the history of all animals used in breeding programs (see Note 9).

4. Notes

1. Although the light period is most often 6.00–7.00 a.m. to 6.00–7.00 p.m., the day–night rhythm at which animals are kept may be adjusted to local needs/convenience. A light period from 2.00–3.00 a.m. to 2.00–3.00 p.m. is an option. The authors' experience is that fertilized oocytes, which are a bit more advanced in development at the time of microinjection, are often more successfully microinjected: slight ruffling of the plasma membrane makes it easier to penetrate it undamaged. However, since membrane ruffling signals the onset of the first cleavage, this methodological variation may be an option mainly for the experienced. Since mouse ovulation, fertilization, and subsequent development of zygotes follow a preset time course, hormone injection regimens should be adjusted accordingly, as should the time of fertilized oocyte isolation.
2. For matings, female mice are always placed in with a male, not vice versa. Introducing a male into an unfamiliar environment will significantly affect their performance. Surplus of plugged pseudopregnant females may be used again after 2 weeks. Unsuccessfully superovulated females (i.e., without a visible copulation plug) may be kept aside for later use as foster as well.
3. Several measures are taken to reduce stickiness of the embryos and glass injection needles. Adding BSA to incubation media is one. Reducing the CO₂ concentration in the incubator is another factor that increases injection efficiency in our hands. We typically use 2.5% instead of 5% CO₂.
4. In order to keep animals SPF, all equipment and working surfaces should be sterilized before isolation of fertilized oocytes, microinjection, and oviduct transfer. If the aforementioned experimental procedures are not carried out within the confines of the animal facility, foster mice should be transported in filter-top cages.
5. For oviduct transfers, use transfer pipets with an inner diameter only slightly wider than that of zygotes. Too wide a transfer pipet will be relatively hard to insert into the infundibulum. We typically use transfer pipets with a shaft of 100–120 μm (O.D.) maximum. Ruler slides (slides with an engraved millimeter/micrometer scale) are very useful to estimate the bore size of transfer pipets.
6. It is advisable not to pull the fallopian tube out by grabbing the infundibulum: its fragility will cause it to be damaged very easily. Prying and maneuvering of the infundibulum are possible

- if carried out extremely gently. Some laboratories prefer unilateral oviduct transfers, other adhere to bilateral transfers, since sometimes unilateral transfers will not result in pregnancy.
7. To reduce stress and excessive bleeding, a brief sedation with an inhalation anesthetic may be preferred. Several inhalation and injection sedatives are approved of by animal welfare committees and are commercially available; we use ether or metofane (methoxyflurane) as inhalation anesthetic and 2.5% avertin, described in Ref. (1), as the sedative during microsurgery. A volume of 20 μ l of avertin/g body weight suffices, although less is preferred and possible if proficient in fallopian tube transfers; on the whole, 250–300 μ l per average-sized female will do. To stop bleeding, epinephrin can be used (1).
 8. An indicator for full sedation is the absence of a blinking reflex when blown (puffed) on the eyes softly. Care should be taken to exert only minimal peri-operative stress on the animal; eyes can be covered with a paper towel wetted in 0.85% sodium chloride, or should be closed manually at regular intervals. Animals should be kept warm after surgery until fully awake.
 9. It is advisable to keep meticulous records of the history of all animals (i.e., transgenic lines, individual mice within a line, experimental and autopsy records, pedigree, etc.) at all times. We also record success rate of superovulations, natural matings, pseudomatings and oviduct transfers, litter sizes, and % transgenic animals obtained in a given session. A range of software is available for this purpose.
 10. To carry out a quantitative Southern analysis, 15 μ g (or less) of DNA is fragmented by restriction endonucleases (REN) in a 50- μ l reaction volume. If REN digestion of DNA is still hampered by contaminants in solution, 1.5 μ l of a 0.1-M spermidine solution may be added (cat. # S0266; Sigma) to the reaction mix. Alternatively, the reaction volume may be increased to 300 μ l and spermidine may be added (9 μ l, 0.1 M); however, the DNA should be precipitated before loading it onto an agarose gel. When preparing the REN digestion in the presence of spermidine, care should be taken to mix all components at room temperature; spermidine is not useful in restriction buffers without salt, for it will precipitate the DNA.
 11. To estimate the copy number of a particular transgenic founder or line, 1 ng of purified, linearized transgenic construct of 15 kb will roughly equal a haploid copy number of 1 in 15- μ g control tail DNA. We typically run 0.65–0.7% agarose gels. The quality of the Southern blot after hybridization (i.e., straight bands and good separation) is greatly improved when electrophoresis is carried out at low power over a few days.

12. Several companies provide a wealth of mouse-related information via the Internet: (<http://www.criver.com>, <http://www.harlan.com>, <http://www.jax.org>, <http://www.taconic.com>). A source providing a particularly extensive database on knock-out and transgenic mice and related topics is "TheTransgenic/Targeted Mutation DatabaseTBASE" at <http://tbase.jax.org>. In addition, useful web sites and Internet links can be found at <http://www.bioresearchonline.com> and at Websites of transgenic facilities at a number of Universities in Europe and USA. Important information regarding the status of the mouse genome map, including available YAC clones, is provided by the Whitehead Institute at <http://www-genome.wi.mit.edu/cgi-bin/mouse/index>; the National Center for Biotechnology Information at <http://www.ncbi.nlm.nih.gov/>; and by The European Collaborative Interspecific Mouse Backcross (EUCIB) consortium at <http://www.hgmp.mrc.ac.uk/MBx/MBxHomepage.html>.
13. Methods and procedures described are specifically used for blastocyst and ES cell manipulation, not for pronuclear injection and oviduct transfer.

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