Chapter 9

Generation of Transgenic Mice Expressing FRET Biosensors

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Abstract

Transgenic mice play a significant role in modern biomedical research. They allow not only mechanistic insights into the functions of specific genes and proteins. Recent strategies have also established the use of transgenic mice as an exciting tool for the expression and in vivo or in situ analysis of fluorescent biosensors, which are capable of directly reporting second messenger levels and biochemical processes in real time and in living cells. In this chapter, we present a detailed protocol for the generation of plasmid vectors and transgenic mice expressing a Förster resonance energy transfer (FRET)-based biosensor for the second messenger 3′,5′-cyclic adenosine monophosphate. These tools and techniques should provide great potential for the analysis of second messenger dynamics in a more physiologically relevant context.

Key words Transgenic mice, FRET, Cardiomyocyte, cAMP, Biosensor

1 Introduction

Transgenic mice play an important role in contemporary biomedical research. Many biological and medical questions can be addressed in transgenic animals. For example, they allow detailed analysis of individual gene functions and can be used as genetic animal models of human disease [1–3].

Generally, a specific exogenous DNA fragment can be integrated into a host animal genome to overexpress, to knock in, or to knock out a specific gene in a constitutive or regulated fashion [4]. There are many approaches to establish transgenic animals, one of which is the microinjection of DNA [5–7]. Murine zygotes before the cell fusion can be harvested and manipulated with specific external DNA by injecting it in the male pronucleus. In this way, the external DNA can be permanently integrated into the host genome. However, the position where the external DNA is integrated is random. The injected zygotes are transferred into a surrogate mother, and some of the pups born carry the transgene integrated into their genome.

Manuela Zaccolo (ed.), *cAMP Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 1294,
DOI 10.1007/978-1-4939-2537-7_9,

Apart from using transgenic mice to study gene function, they can be an exciting tool for the expression of fluorescent biosensors capable of directly reporting second messenger levels and biochemical processes in living cells. For example, they have enabled direct visualization of the second messenger 3′,5′-cyclic adenosine monophosphate (cAMP) and its real-time monitoring in adult cardiomyocytes [8, 9]. Such biosensors are based on the principle of Förster resonance energy transfer (FRET) which occurs between the donor and acceptor fluorophores (e.g., cyan (CFP)- and yellow (YFP)-fluorescent proteins) when they come into close spatial proximity [10]. The simplest cAMP biosensor Epac1-camps is comprised of a cAMP-binding domain (derived, e.g., from the Epac1 protein) sandwiched between CFP and YFP [11, 12]. cAMP binding leads to a conformational change in the sensor molecule which can be visualized by a decrease of the FRET signal.

In this chapter, we describe a detailed protocol for the generation of plasmid vectors and transgenic mice expressing the cAMP biosensor Epac1-camps in a tissue-specific and ubiquitous manner.

2 Materials

2.1 Equipment

1. Thermocycler (e.g., SensoQuest, Göttingen).
2. Regular DNA electrophoresis chamber and power supply.
3. Gel documentation system.
4. Laboratory scale.
5. Microcentrifuge (e.g., Pico 17, Thermo Scientific).
6. Thermomixer (e.g., Eppendorf).
7. NanoDrop 2000 device (Thermo Scientific) or regular laboratory photometer.
8. FRET imaging system comprised of an inverted microscopy, light source, Dual-View beam splitter, CCD camera, and imaging software. An example can be found in a previously described comprehensive protocol [11].

2.2 Mice

FVB/NRj mice (Janvier Labs, Saint-Berthevin, France).

2.3 Vectors

1. α-MHC vector, empty [9].
2. CAG vector, empty [13, 14].
3. Epac1-camps plasmid [12].

2.4 Cells

1. Competent *E. coli* cells—Top 10 (C4040-10, Invitrogen).
2. 293A cells (R70507, Invitrogen).

2.5 Substances and Solutions

1. Agarose peqGOLD Universal (35-1020, Peqlab).
2. Ampicillin: 100 mg/ml stock solution in *aqua bidest.*
3. DirectPCR Tail (31-102T, PeqLab).
4. dNTPs: 100 mM (U1240, Promega) in nuclease-free water.
5. Ethidium bromide 1 % solution in water.
6. GoTaq DNA polymerase and GoTaq buffer (M3175, Promega).
7. KCM 5×: 500 mM potassium chloride, 150 mM calcium chloride, 250 mM magnesium chloride dissolved in nuclease-free water.
8. LB agar: powder by Miller (A0927, Applichem), dissolve in deionized water and autoclave. After cooling the solution down to ~50 °C, add the desired antibiotic (e.g., 0.1 mg/ml ampicillin) and mix well. Pour this solution into 10 cm petri dishes, cool down until the agar becomes solidified, and store at 4 °C.
9. LB medium: powder by Miller (A0954, Applichem), dissolve in deionized water, autoclave, and store at 4 °C.
10. Loading dye buffer DNA IV (A3481.0025, Applichem).
11. Midori Green advance (MG05, Nippon Genetics Europe GmbH).
12. TAE buffer for DNA electrophoresis (A1416, Applichem).
13. Proteinase K (A3830.0500, Applichem): 100 mg/ml solution in deionized water and store the aliquots at –20 °C.
14. Pfu polymerase and 10× Pfu buffer (M7745, Promega).
15. Quick-Load DNA Ladder (N0467S, New England Biolabs).
16. T4 DNA ligase and buffer (M0202S, New England Biolabs).
17. TE buffer: 5 mM Tris–HCl, pH 7.4, 0.1 mM EDTA.
18. Isopropanol.
19. Isoproterenol (I6504, Sigma). Prepare a 10 mM stock solution in water, aliquot, and store at –20 °C until use.
20. Nuclease-free water.
21. Transfection reagent Lipofectamine 2000 (11668-027, Life Technologies).

2.6 Kits

1. QIAquick Gel Extraction Kit (28706, Qiagen).
2. QIAfilter Plasmid Midi Kit (12243, Qiagen).

2.7 Primers

All primers were ordered from Eurofins MWG Operon, dissolved in deionized water upon arrival at final concentration of 100 pmol/µl, and subsequently stored at –20 °C.

For cloning of the CAG-Epac1-camps vector (*italic = linker sequence*):

Forward: 5′ *AAA GAT ATC TGC AGC GCC ACC* ATG GTG AGC AAG GGC G 3′

Reverse: 5′*AAA GAA TTC* CTT GTA CAG CTC GTC CAT GC 3′

For cloning of the α-MHC-Epac1-camps vector (*italic = linker sequence*):

Forward: 5′ *AAA GAT ATC* ATG GTG AGC AAG GGC G 3′

Reverse: 5′*AAA GAA TTC* CTT GTA CAG CTC GTC CAT GC 3′

For genotyping:

Forward: 5′ TGA CAG ACA GAT CCC TCC TAT 3′

Reverse: 5′ CAT GGC GGA CTT GAA GAA GT 3′

2.8 Restriction Enzymes

All restriction enzymes and buffers were from New England Biolabs and used in the following buffers, according to the manufacturer's protocol.

BamHI (R3136S): buffer 3 + bovine serum albumin (BSA).

EcoRI-HF (R3101S): buffer 4.

EcoRV-HF (R3195S): buffer 4.

KpnI-HF (R3142S): buffer 4.

SpeI (R0133S): buffer 4 + BSA.

XhoI (R0146S): buffer 4 + BSA.

2.9 Other Materials for DNA Purification

1. Slide-A-Lyzer Dialysis Cassette (66383, Thermo Scientific).
2. 0.2 μm filter FP 013/AS (462945, Schleicher & Schuell).

3 Methods

3.1 Cloning Strategy

To generate the ubiquitous transgenic Epac1-camps mice, we used the empty CAG vector (Fig. 1) as a backbone for the transgenic construct [13, 14]. CAG acts as a constitutively active promoter (any other well-established constitutively active promoter can be used instead), so that the genes downstream the promoter are transcribed in virtually every cell type. In this vector, a multiple cloning site is provided with several single-cutter restriction sites. For selection in bacteria, a gene encoding for ampicillin antibiotic resistance is also located in the vector backbone.

As template DNA, we used the previously described Epac1-camps sensor plasmid (Fig. 1), which includes the cAMP-binding site flanked by two fluorophores for FRET measurements [12]. Subcloning can be performed in one step but with two separate inserts which need to be ligated into one vector. First, we cut out the first insert DNA fragment (*Epac-Cfp*) with the restriction enzymes EcoRI and XhoI. These enzymes cut the DNA just before the *Epac* and after the *Cfp* fluorophore sequence. Second, the *Yfp*

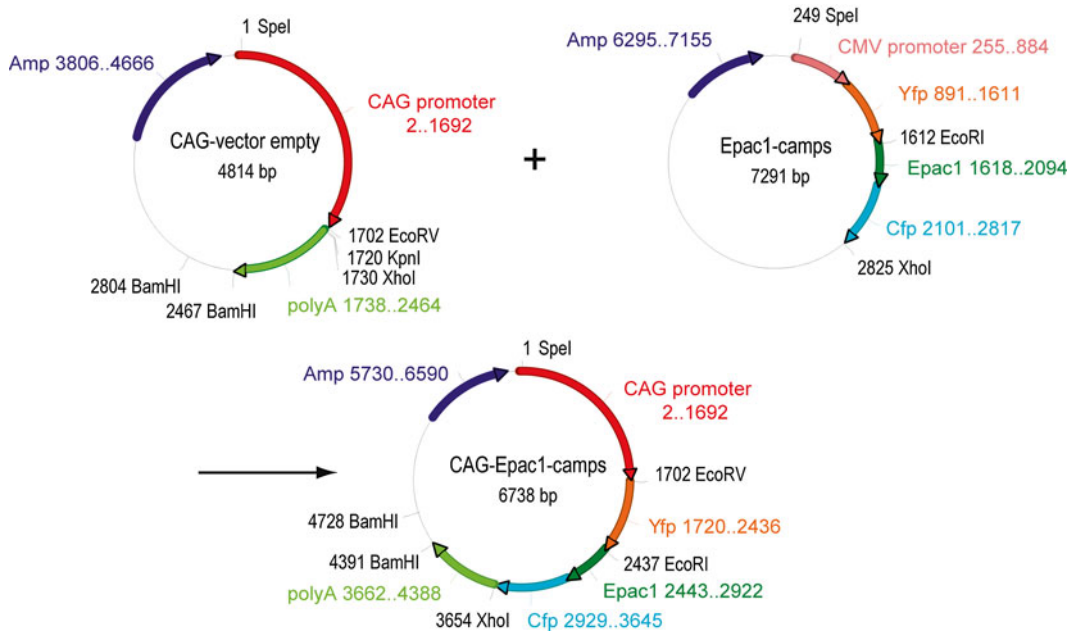

Fig. 1 Cloning strategy for the CAG-Epac1-camps construct. The empty CAG vector serves as a backbone. It is cut with the restriction enzymes EcoRV and XhoI. As an insert, two fragments are ligated into this backbone. They include the *Epac-Cfp* fragment cut out from the Epac1-camps plasmid with EcoRI and XhoI and the *Yfp* sequence amplified by PCR and digested using EcoRV and EcoRI

sequence was amplified by the polymerase chain reaction (PCR). We designed the forward primer which includes the EcoRV restriction site and a linker sequence in front of the Kozak sequence and the start codon (ACC ATG). The reverse primer contained an EcoRI restriction site which can be ligated together with the *Epac-Cfp* fragment. The CAG backbone vector was digested with the restriction enzymes EcoRV and XhoI. The vector backbone was then used for a triple ligation with the digested *Yfp* and *Epac-Cfp* fragments (Fig. 1). For transgenic mouse generation, the resultant construct was digested with the restriction enzymes SpeI and BamHI and ran in a 1 % agarose gel. The upper band (2,467 b.p.) was cut out and further used (*see* Subheading 3.7), while the lower two bands (2,010 and 337 b.p., which contain the antibiotic resistance and other unneeded sequences) were discarded.

To prove the functionality of the new construct, we transfected it into 293A cells using Lipofectamine 2000, according to the manufacturer's protocol. 24 h after transfection, these cells showed fluorescence and measurable FRET responses to the β-adrenergic receptor agonist isoproterenol, indicative of an increase in intracellular cAMP (Fig. 2).

For generating transgenic animals with cardiac-specific expression of Epac1-camps, a tissue-specific promoter is required. Alpha myosin heavy chain (α-MHC) is a contractile protein expressed in

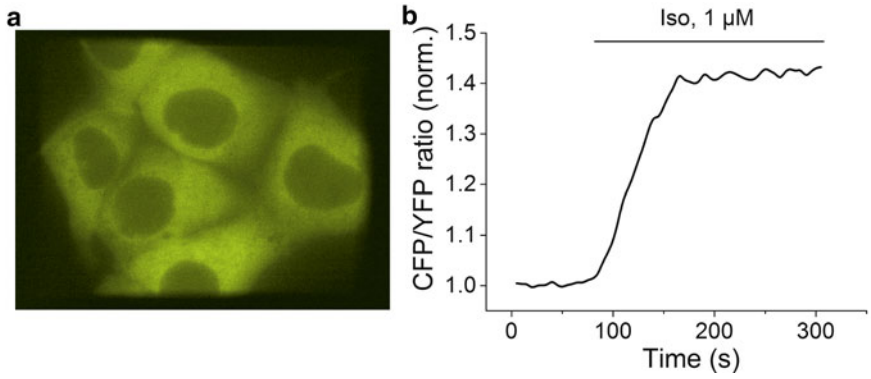

Fig. 2 Proof of functionality of the CAG-Epac1-camps construct. (**a**) shows the fluorescence of transfected 293A cells with the CAG-Epac1-camps vector. A FRET measurement is shown in (**b**). The CFP/YFP ratio is increasing after the addition of isoproterenol (Iso) which is indicative of a cAMP increase in cells

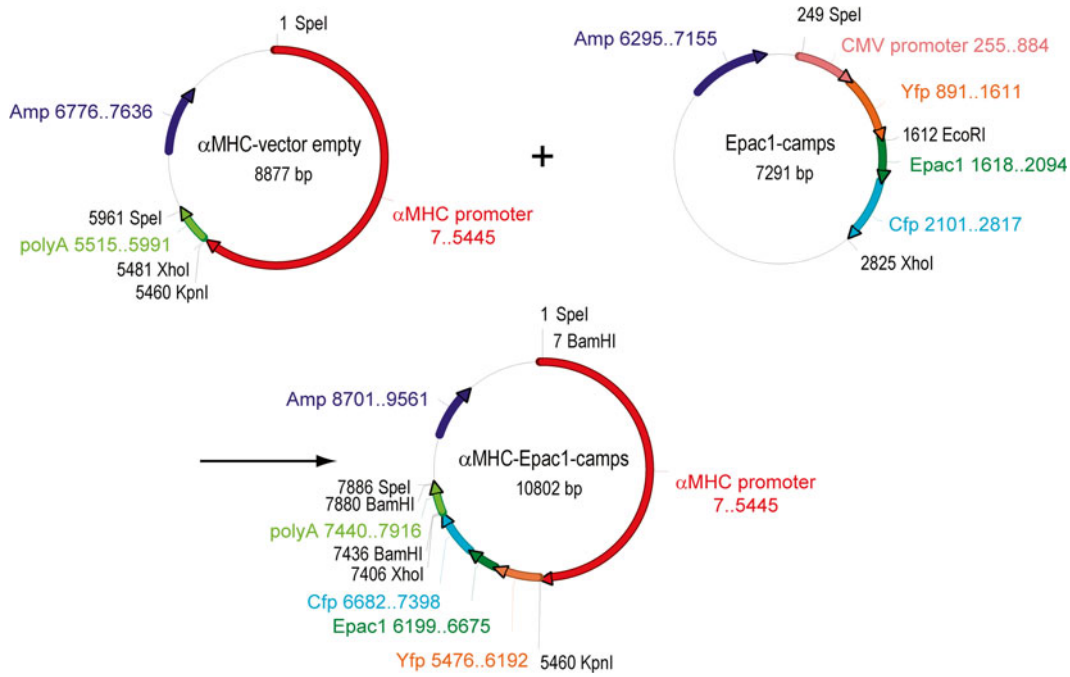

Fig. 3 Cloning scheme of the α-MHC-Epac1-camps construct. The empty α-MHC vector serves as a backbone. It is cut with the restriction enzymes KpnI and XhoI. As an insert, two fragments are ligated into this backbone. They include the *Epac-Cfp* fragment cut out from the Epac1-camps plasmid with EcoRI and XhoI and the *Yfp* sequence amplified by PCR and digested using KpnI and EcoRI

adult cardiomyocytes, and its promoter is routinely used for tissue-specific expression in adult myocardium. To clone such a construct, we choose the α-MHC vector as a backbone (Fig. 3) [9]. We digested this vector with KpnI and XhoI. The restriction sites are located behind the α-MHC promoter and before the

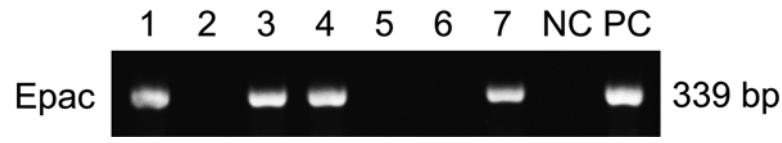

Fig. 4 Example of a genotyping PCR analysis. Shown are representative results from a genotyping PCR analysis of transgenic founder offspring. Mice with the numbers 1, 3, 4, and 7 contain the Epac1-camps construct in their genome. NC = negative control, PC = positive control, bp = base pairs

poly-A tail. The Epac1-camps vector was digested as described above with EcoRI and XhoI to excise the *Epac-Cfp* fragment. The *Yfp* sequence was again amplified by PCR. In this case, the KpnI restriction site was introduced in front of the start codon and an EcoRI restriction site after the *Yfp* sequence to triple ligate it with *Epac-Cfp* fragment and the α-MHC vector backbone. The resulting vector α-MHC-Epac1-camps (Fig. 3) was control digested with BamHI. This enzyme has three restriction sites in this vector, so three bands are detectable on a gel. For microinjections, we digested the α-MHC-Epac1-camps construct with the restriction enzyme SpeI and ran in a 1 % agarose gel. The upper band (7,885 b.p.) was cut out and further used (*see* Subheading 3.7), while the lower band (2,916 b.p., which contains the antibiotic resistance and other unneeded sequences) was discarded.

The linearized and purified DNA sequences were injected into the male pronuclei of the FVB/N mouse zygotes. Microinjections are normally performed by transgenic mouse facilities which we supply with the specially prepared transgenic construct DNA (*see* Subheading 3.7). Please, refer to a previously published comprehensive protocol if you need to establish this procedure in your own laboratory [15]. The genotypes of the newborn mice were analyzed by PCR (Fig. 4). The primers for the genotyping selected to bind both parts of the ligated constructs. We choose a forward primer which binds in the α-MHC promoter sequence and the reverse primer which binds in the middle of the *Yfp* gene. Mice where the construct was integrated in the host genome were classified as founder and used for further breedings. The integration of the external DNA is random. Therefore, the F1 generation has to be analyzed by PCR and FRET measurements to test the functionality of these construct in the descendants. We usually chose one or two founders within the F1 generation showing robust sensor fluorescence and FRET responses. This founder is then selected to establish a new transgenic mouse line.

Below, the cloning steps are described in more detail.

3.2 PCR and DNA Extraction

The specific DNA fragment to be integrated into the host DNA must first be amplified by a polymerase chain reaction (PCR). Therefore, the required DNA fragment is amplified using specific primer pairs.

1. For the PCR mix, pipette 10 µl Pfu buffer, 2 µl dNTPs (10 mM), 2.5 µl each primer forward and reverse (pre-diluted to 10 pmol/µl), 0.5 µl template DNA (0.1–0.3 µg), 83.5 µl nuclease-free water, and 1 µl Pfu polymerase in a PCR reaction tube. Prepare this mix on ice.
2. Mix carefully and spin down the solution.
3. Start the following program in a thermocycler:

   94 °C—3 min.
   94 °C—15 s.
   55 °C—15 s] ×30 cycles.
   72 °C—1.2 min (*see* **Note 1**).
   72 °C—7 min.
   4 °C—∞.
4. Purify the PCR product by running the PCR product in a 1 % agarose gel supplemented with Midori Green (4 µl/50 ml of the gel) or ethidium bromide stock solution (3 µl/50 ml of the gel).
5. Mix 100 µl PCR product with 10 µl loading dye and transfer all on the agarose gel. Start the electrophoresis for 30–60 min at 100 V.
6. Cut out the specific DNA fragment under UV light (*see* **Note 2**), determine the weight of the gel piece, and transfer it into a 1.5 ml reaction tube.
7. Extract the DNA out of the gel piece with the QIAquick Gel Extraction Kit. Add a threefold amount of the QG buffer to the gel piece and melt it in a thermomixer at 50 °C. Add one-fold amount of isopropanol to the reaction sample and transfer it to a spin column. Follow the instruction provided by the manufacturer. Elute in 40 µl nuclease-free water.

For insertion of a specific DNA fragment in a target vector, both components have to be digested with the same restriction enzymes. Since restriction enzymes generate “sticky ends” (in the majority of cases), the specific DNA fragment (insert) can be easily ligated into the target vector.

3.3 Restriction Digest and Ligation

1. Prepare two reaction samples: (a) 6 μg of the target vector (fill up to 40 μl with nuclease-free water) and (b) 40 μl of the insert (PCR product; see above) in a reaction tube.
2. Add 5 μl of a 10× restriction buffer specific to the selected restriction enzyme to each preparation. Add 0.5 μl 100× BSA, if required by the restriction enzyme.
3. Add 2.5 μl of each of the two reaction enzymes required.
4. Mix the samples and incubate it for at least 2 h at 37 °C in a thermomixer.
5. Visualize the DNA digestion on a 1 % agarose gel. Cut out the desired fragments under UV light.
6. Purify the DNA with the QIAquick Gel Extraction Kit. Elute the vector in 50 μl and the insert in 25 μl EB buffer.
7. For ligation, mix 11.5 μl of the digested insert and 1 μl of the linearized vector (corresponds to a ~5:1 insert/vector ratio) in a separate reaction tube.
8. Add 1.5 μl of 10× ligase buffer and 1 μl of the T4 DNA ligase.
9. Mix the sample and incubate for at least 2 h (optimally overnight) at 14 °C.

For amplification of the ligated reaction sample, transform them into competent *E. coli* cells. Only bacteria, which absorbed a correctly ligated vector, are able to grow under antibiotic specific pressure.

3.4 Transformation

1. To transform the ligation reaction product into competent *E. coli* cells, add 65 μl nuclease-free water and 20 μl of the 5× KCM buffer to the ligation sample.
2. Store this preparation for 5 min on ice.
3. Then add 100 μl of competent *E. coli* cells, mix gently, and incubate 20 min on ice and afterwards 10 min at room temperature.
4. Add 1 ml LB medium (without antibiotic) and incubate for 50 min in a thermomixer at 37 °C by 700 rpm.
5. Centrifuge the suspension for 2 min at 2,400 × *g*.
6. Collect 100 μl of the supernatant and remove the rest of it. Resuspend the pellet with 100 μl of the supernatant and then transfer and spread the suspension on LB agar plates (with ampicillin). Incubate the plates by 37 °C overnight.
7. Pick individual bacteria colonies with a sterile pipette tip and transfer it into a 15 ml reaction tube with 3 ml LB medium.
8. Add the desired antibiotic in a 1:1,000 dilution.
9. Incubate this suspension at 37 °C in a shaker overnight.

To harvest the vector DNA, purify the plasmid DNA from bacteria by using QIAfilter Plasmid Midi Kit (Qiagen) according to the manufacturer's protocol. Elute the DNA with 100 μl of nuclease-free water.

To make sure that you have picked a positive colony with the correctly integrated construct, perform a control digest.

3.5 Control Digest

1. Take 8 μl out of a plasmid DNA sample and add 1 μl of 10× specific restriction enzyme buffer.
2. Add two restriction enzymes (0.5 μl each) to the reaction tube, which leads to a specific banding pattern after digestion.
3. Incubate the tubes for at least 30–60 min at 37 °C.
4. Add 1.5 μl loading dye to each sample and visualize the specific banding pattern on a 1 % agarose gel via electrophoresis.

Positive samples can be amplified to obtain higher amount of plasmid.

3.6 Proliferation of the Constructed Vector

1. Take 100 μl out of the bacterial suspension after transformation (Subheading 3.4, **step 4**) and inoculate 50–60 ml LB media containing the specific antibiotic. Incubate this bacteria suspension at 37 °C in a shaker overnight (*see* **Note 3**).
2. Centrifuge the whole suspension at 11,385 × *g* and 4 °C for 5 min.
3. Remove the supernatant and purify the DNA with the QIAfilter Plasmid Midi Kit following the manufacturer's protocol.

This generated purified plasmid DNA could be now used for different applications. For generating transgenic animals via microinjection, the DNA has to be linearized and purified in a special way.

3.7 Preparation of the DNA for the Microinjection

1. Linearize 40 μg of the DNA with a specific restriction enzyme (*see* **Note 4**).
2. Separate the linearized DNA on a 1 % agarose gel, 30–60 min at 80–100 V.
3. Cut out the band of the right size under UV light (*see* **Note 5**).
4. Purify the gel pieces by the QIAfilter Plasmid Midi Kit. Use 4 spin columns and elute each in 100 μl sterile TE buffer.
5. Sterile filter the DNA with a 1 ml syringe and a 0.2 μM filter carefully (*see* **Note 6**).
6. For effective removal of salts, ethidium bromide, and further contaminations, the DNA has to be dialyzed. Use the Slide-A-Lyzer cassettes (Thermo Scientific) according to the manufacturer's protocol (*see* **Note 7**).
7. Measure the concentration of the dialyzed DNA with a NanoDrop device or a photometer.

8. Adjust the concentration of the sample with sterile TE buffer. For pronucleus injection, a DNA concentration of ~30 ng/μl in TE buffer and a minimum volume of 200 μl have to be submitted to the transgenic mouse unit. This is usually diluted down to 3 ng/μl with the same buffer for injections.

After injections and embryo transfer, the mouse pups are genotyped at the age of 3–4 weeks to identify the founder animals for the further breeding.

3.8 Genotyping

1. Take tail biopsies (by cutting a 3–5 mm piece of tail) from mice aged 3–4 weeks and store them at −20 °C.
2. Extract the DNA out of the tissue with 190 μl direct PCR tail lysis buffer and 10 μl Proteinase K in the thermomixer at 55 °C overnight under vigorous mixing.
3. Heat the samples for 45 min to 85 °C without shaking.
4. Cool down the samples for 30 min at 4 °C.
5. Pipette 0.5 μl of the sample in a PCR reaction tube and add 14.7 μl nuclease-free water, 4 μl 5× GoTaq buffer, 0.5 μl dNTPs, 0.05 μl of the forward primer, 0.05 μl of the reverse primer (both at 100 pmol/μl concentration), and 0.2 μl of the GoTaq polymerase (*see* **Notes 8** and **9**). Prepare these steps on ice.
6. Mix carefully and spin down the reaction mix.
7. Start the PCR in the thermocycler:

   94 °C—4 min.

   94 °C—30 s.
   56 °C—30 s ×35 cycles.

   72 °C—1.2 min.
   72 °C—7 min.
   4 °C—∞.
8. Analyze the results on a 1 % agarose gel.

Usually, about 20–30 % of the newly generated mice are PCR positive. Positively identified founders can then be used for further breedings with wild-type mice to establish several heterozygous transgenic mouse lines. The functionality of the construct in the F1 generation can be analyzed by FRET measurements in single isolated cells. Founders which give rise to newborns with a sufficient amount of fluorescence and good FRET responses can be chosen to establish new transgenic mouse lines. There might be a certain variability in the percentage of fluorescent cells and in the intensity of fluorescence. We normally prefer working with the lines in which virtually all desired cells are brightly fluorescent to obtain good signal-to-noise ratios in FRET experiments.

4 Notes

1. The PCR elongation time is calculated as 2 s per kilobase of DNA.
2. Make sure that you switch on the UV light only for a very short time. Otherwise, strand disruptions may occur.
3. Check the cloudiness of the suspension. For optimal results, the suspension should become cloudy and have an optical density above 1.
4. Due to high amount of DNA, mix this reaction in the total volume of 200 μl. Add water at 160 μl to the DNA, 20 μl of the restriction enzyme buffer, and 20 μl of the restriction enzyme or enzyme mixture.
5. Make sure that you only use the UV light for a very short time. Otherwise, strand disruptions could occur.
6. Rinse the reaction tube, the syringe, and the filter with 50 μl TE buffer to make sure that you do not lose DNA. Pipette and filtrate very carefully; otherwise, the DNA may shear.
7. The membrane of the dialysis cassette has to be hydrated in the TE buffer for 30 s before the sample is loaded into the cassette. Make sure that you use an 18–19 gauge needle for adding the sample; otherwise, the DNA could shear. Dialyze for 2 h at room temperature in TE buffer, change the dialysis buffer, and dialyze for another 2 h. Change again the dialysis buffer and dialyze overnight at 4 °C.
8. It is advisable to prepare a master mix containing all PCR components except for the tissue DNA. Upscale the quantities of the components according to the number of samples and pipette 19.5 μl of master mix to each DNA sample right before starting the reaction.
9. The primer pair should include parts of the original vector and of the insert. Each primer should have a base pair length between 15 and 30, resulting in a melting temperature of around 55 °C. Avoid presence of repeats containing more than four identical nucleotides, especially guanosine phosphates.

Use also a positive (plasmid or transgenic mouse DNA) and a negative (water or a wild-type mouse DNA) control for the PCR reaction.

Acknowledgment

The work in authors' laboratories is supported by the Deutsche Forschungsgemeinschaft (grants NI 1301/1, FOR 2060, SFB 1002 TP A01, IRTG 1816), the German Centre for Cardiovascular Research (DZHK), and the University of Göttingen Medical Center ("pro futura" grant).

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