CHAPTER 4

RESEARCH METHODS

4.1. RESEARCH FIELD

This research is in the field of molecular genetics related to clinical genetics.

4.2. SETTING/LOCATION, RESEARCH PERIOD

Research was conducted in Department of Clinical Genetics ErasmusMC Rotterdam The Netherlands for 1 year.

4.3. RESEARCH DESIGN

This is an explorative study using mouse as the model.

4.4. POPULATIONS AND SAMPLES

Populations in this research were mice with Janvier C57BL/6 background.

The samples were wild type, monogennic mice Tet-on-nCGG-eGFP, hnRNP-rtTA, GFA2-rtTA, PrP-rtTA; and bigenic mice Tet-on-nCGG-eGFP/ hnRNP-rtTA, Tet-on-nCGG-eGFP/ GFA2-rtTA, and Tet-on-nCGG-eGFP/ PrP-rtTA.

4.5. RESEARCH VARIABLES

1. Transgenic mice (independent variables)
    a. Tet-on-nCGG-eGFP transgenic mice
    b. PrP-rtTA transgenic mice
    c. GFA2-rtTA transgenic mice
    d. Tet-on-nCGG-eGFP / PrP-rtTA bigenic mice
    e. Tet-on-nCGG-eGFP / GFA2-rtTA bigenic mice
2. GFP expression (dependent variables)
    a. Fold change GFP RNA levels

        Scale: numeric

    b. Presence of GFP protein

        Scale: nominal

    c. Fold change GFP protein levels

        Scale: numeric

3. rtTA expression (dependent variables)
    a. Presence of rtTA protein

        Scale: nominal

    b. Fold change rtTA RNA levels

        Scale: numeric

4. Presence of ubiquitin inclusions (dependent variable)

    Scale: nominal

4.6. OPERATIONAL DEFINITIONS

1. Tet-on-nCGG-eGFP transgenic mice: mice containing transgene consisting of Tet-on operator, nCGG, and GFP. This transgene is injected to the nucleus of mice embryonic stem cell, and the transgene will be randomly combined with the mice genome resulting in transgenic mice Tet-on-nCGG-eGFP. Genotyping using specific primers is used to check whether or not the mice containing this transgene. The nCGG could be 99CGG which is a model for FXTAS transgenic mice, and 11CGG which is a model for normal transgenic mice. A certain PCR genotyping method is used to distinguish the number of CGG.
2. PrP-rtTA transgenic mice: mice containing transgene PrP driver promoter and rtTA. The transgene is made by DNA construction.This transgene is injected to the nucleus of mice embryonic stem cell, and will be randomly combined with the mice genome resulting in transgenic mice. This transgene will express the rtTA protein throughout the brain area, both neurons and astrocytes. Genotyping using specific primers is used to check the presence of the transgene in the mice genome
3. GFA2-rtTA transgenic mice: mice containing transgene with both GFA2 driver promoter and rtTA. The transgene is made by DNA construction. This transgene is injected to the nucleus of mice embryonic stem cell, and will be randomly combined with the mice genome resulting in these transgenic mice. This transgene will express the rtTA protein in specific area: in the Bergmann glia and astrocytes of the brain. Genotyping using

specific primers is used to check the presence of the transgene in the mice genome

4. Tet-on-nCGG-eGFP/PrP-rtTA bigenic mice: these mice contain both transgene Tet-on-nCGG-eGFP and PrP-rtTA. These mice result from breeding between Tet-on-nCGG-eGFP transgenic mice and PrP-rtTA transgenic mice. These mice will express the rtTA protein throughout the brain area, both neurons and astrocytes. With the presence of doxycycline, the rtTA will activate the Tet-on operator, and finally express the nCGG-eGFP throughout the brain area, both neurons and astrocytes. To ensure the bigenic mice, PCR genotyping is performed. One genotyping to check the Tet-on-nCGG-eGFP transgene and another genotyping to check the presence of PrP-rtTA transgene.
5. Tet-on-nCGG-eGFP/GFA2-rtTA bigenic mice: these mice contain both transgene Tet-on-nCGG-eGFP and GFA2-rtTA. These mice result from breeding between Tet-on-nCGG-eGFP transgenic mice and GFA2-rtTA transgenic mice. These mice will express the rtTA protein in Bergmann glia and astrocytes of the brain. With the presence of doxycycline, the rtTA will activate the Tet-on operator, and finally express the nCGG-eGFP in Bergmann glia and astrocytes of the brain. To ensure the bigenic mice, PCR genotyping is performed. One genotyping to check the Tet-on-nCGG-eGFP transgene and another genotyping to check the presence of GFA2-rtTA transgene.

6. GFP expression: GFP is widely used as a reporter as a proof-of-concept that a gene is expressed throughout a given organism. In this research, GFP is bound to nCGG sequence because the nCGG is hardly to be visualized. Thus the expression of nCGG is reported through the expression of GFP. The GFP protein can be detected through Western blot and immunohistochemistry, while the expression of its RNA can be measured by RT-Q-PCR.
7. rtTA expression: mice containing rtTA trangene will express the rtTA either in RNA or protein form. Determination of this rtTA can be performed RT-Q-PCR to measure the rtTA RNA levels, and immunohistochemistry to detect the presence of rtTA protein.
8. Presence of ubiquitin inclusions: the presence of ubiqutin inclusion is a primary hallmark of FXTAS. Immunohistochemistry with antibody against ubiquitin is used for the detection. Ubiquitin inclusion is indicated by the presence of ubiquitin protein within the nuclear of cell after ubiquitin immunohistochemistry.

4.7. RESEARCH PROTOCOLS

4.7.1. The Mice and its treatment carried out in the research

Transgenic mice were in Janvier C57BL/6 background. The mice were housed in standard condition. All experiments were carried out under permission of the local ethical committee. There were four transgenic mouse lines available: 1) tet-on-11CGG-eGFP, 2) tet-on-99CGG-eGFP, 3) PrP-rtTA transactivator, 4)

GFA2-rtTA transactivator. These mice were bred to generate bigenic mice which had both the tet-on promoter and the rtTA transactivator. This way would produce four main lines of bigenic mice: 1) tet-on-11CGG-eGFP/PrP-rtTA, 2) tet-on-11CGG-eGFP/GFA2-rtTA, 3) tet-on-99CGG-eGFP/PrP-rtTA, 4) tet-on-99CGG-eGFP/GFA2-rtTA. Mice were treated with dox in the drinking water at a concentration of 4 mg/ml, and IP injections with dox were 500 µg in PBS. Treatments were started when the mice were at 3-6 weeks old. Mice were sacrificed by cervical dislocation, and the tissues were collected and rapid frozen with liquid nitrogen and were kept in -80°C freezer before RNA and protein isolation. For immunohistochemistry experiments, the tissues were directly fixed overnight in 4% paraformaldehyde.

4.7.2. Isolating tail DNA & genotyping of the mice to ensure the genotype of transgenic mice

DNA isolation used salting out method. Mouse tail was incubated with 0.2 mg/ml Proteinase K (Roche Diagnostics) in 320 ml lysis buffer (1% SDS, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA) overnight at 55°C. After incubation, 100 µl NaCl 6 M was added and the suspension was centrifuged at 15000 g for 15 minutes. Supernatant was gently mixed with 2 volumes of 100% ethanol, followed by centrifugation at 15000 g for 1 minute. Supernatant was discarded, and 70% ethanol was added, followed by another centrifugation step. Supernatant was discarded and the pellet was dried and dissolved in 100 µl 0.1 X TE.

All genotyping carried out in this research used self-designed primers which never been used on other studies before.

Genotyping of mice containing the transgene Tet-on-(n)CGG-eGFP was performed using forward primer 5'-gcttagatctctcgagtttac-3' and reverse primer 5'-atggaggtcaaaacagcgtg-3'. One µl tail DNA was mixed with PCR mix containing 2.5 µl 10X PCR buffer, 1 µl 50 mM $MgCl_2$, 0.25 µl 1% W1, 0.25 µl 25 mM dNTPs, 0.25 µl 50 mM spermidine, 0.125 µl of 10 µm each primers, 0.06 µl 5 U/µl Taq polymerase, and 20 µl H_2O. Cycle parameters were 5 min at 95°C, followed with 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 58°C, and 90 sec extension at 72°C; final extension was set 5 min at 72°C. A band with the size of 422 bp (if the Tet-on-(n)CGG-eGFP transgene exists) was visualized using agarose gel electrophoresis.

For the transgene Tet-on-(n)CGG-eGFP, there are Tet-on-99CGG-eGFP and Tet-on-11CGG-eGFP. To ensure the number of CGGs in the transgene Tet-on-(n)CGG-eGFP, primer 5'-cgggtcgagtaggcgtgtac-3' was used as forward, and 5'-ccagtgcctcacgaccaac-3' as the reverse primer. One µl tail DNA was mixed with 2.5 µl 5x 5U/µl Expand High fidelity buffer-2, 12.5 µl 5 M betaine, 0.5 µl DMSO, 0.5 µl 100 µm forward primer, 0.5 µl 100 µm reverse primer, 0.25 µl 25 mM each dNTPs, 0.5 µl Expand High fidelity enzyme, and 4.25 µl H_2O. The PCR program was 10 min at 95°C, followed with 35 cycles of 1 min denaturation 95°C, 1 min annealing at 60°C, 5 min extension at 72°C; and 10 min final extension at 72°C. Visualization on a 2% agarose gel containing ethidium bromide should result in a

band of 657 bp in size for Tet-on-99CGG-eGFP, and 393 bp for Tet-on-11CGG-eGFP (if the transgene exists).

For genotyping the transgene containing rtTA (PrP-rtTA, GFA2-rtTA, and hnRNP-rtTA) the forward primer was 5'-cagcaggcagcatatcaaggt-3' with the reverse primer 5'-gccgtgggccactttacac-3'. One µl tail DNA was mixed with PCR mix containing 2.5 µl 10X PCR buffer, 0.75 µl 50 mM MgCl2, 0.25 µl 1% W1, 0.25 µl 25 mM dNTPs, 0.25 µl 50 mM spermidine, 0.125 µl of 10 µm each primers, 0.06 µl 5 U/µl Taq polymerase, and 20 µl H_2O. Cycle parameters were 5 min at 95°C, followed with 30 cycles of 10 sec denaturation at 95°C, 20 sec annealing at 60°C, and 30 sec extension at 72°C; final extension was 5 min at 72°C. The PCR products should be a band with 301 bp in size if the rtTA transgene exists, and were visualized in 2% agarose gel containing ethidium bromide.

Special for the transgene containing GFA2-rtTA, we used specific PCR to only detect this construct with disability to amplify PrP-rtTA. Forward primer was 5'-tgggcacagtgacctcagtg-3', and the reverse primer was 5'-gcctgtccagcatctcgatt-3'. One µl tail DNA was mixed with the same PCR mix for rtTA PCR (except for the primers). PCR program was 5 min at 95°C, followed with 30 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 58°C, 30 sec extension at 72°C; and 5 min of final extension at 72°C. Visualization using 2% agarose gel containing ethidium bromide should give a band with 412 bp in size if the GFA2-rtTA transgene exists.

4.7.3. Isolation of RNA & protein from tissues to be used for gene expression determination

Mouse tissues (brain, kidney, intestine, lung, heart and liver) were homogenized on ice in 500 µl HEPES-buffer (10 mM HEPES, 300 mM KCl, 3 mM MgCl2, 100 µM $CaCl_2$, 0.45% Triton X-100 and 0.05% Tween-20, pH 7.6), with Complete protease inhibitor cocktail (Roche Diagnostics) and 40 U/µl RNase out (Invitrogen) and 1.5 µl 1 M DTT. After 30 minutes of incubation on ice they were re-homogenized. For RNA isolation, 100 µl of homogenates were mixed with 1 ml RNAbee, shaken and then 100 µl of chloroform was added. After incubation of 15 minutes the mixture was spun down at 4°C 15000 g for 10 minutes. The aqueous phase was taken and an equal volume of isoprophanol was added, followed by gently shaking to precipitate the RNA. Mixture then was spun down, supernatant was discarded and the pellet was washed 2 times with 80% ethanol. The RNA pellet was dissolved in 50 µl DEPC-treated water. For isolating the protein, the rest of homogenate was spun down 15000 g, 4°C for 15 minutes and the protein-containing supernatant was collected. Concentration of the protein was measured using the BCA kit (Tierce). The remaining pellet was used to extract the DNA by incubation with Proteinase-K and lysis buffer overnight and the same protocol was used as for isolating tail DNA. This DNA was used mostly for re-genotyping.

4.7.4. Measuring the RNA levels of GFP and rtTA transgene expression by Reverse transcriptase-Quantitative-PCR (RT-Q-PCR)

Expression of transgene can be detected and quantified from the RNA. In this research, besides using protein as the parameter of expression, RNA is also taken advantage. RT-Q-PCR is used to measure the RNA levels. Total RNA concentrations were measured using NanoDrop (NanoDrop Technologies). One µg of RNA was used to make cDNA by RT-PCR using iScript cDNA Synthesis Kit (BioRad) according to manufacturer instructions. Before cDNA synthesis, the RNA was treated with 1 µl 1 U/µl DNase (Invitrogen) for 30 min at 37°C, followed by inactivation for 10 min at 65°C. SYBR Green qPCR mix (Kapa Biosystems) was used for the Q-PCR reaction. For the GFP Q-PCR primer 5'-gagctgaagggcatcgactt-3' was used as the forward and 5'-ccatgatatagacgttgtggctg-3' as the reverse primer. rtTA Q-PCR was performed using primers 5'-ggaacaggagcatcaagtagc-3' and 5'-gcgtcagcaggcagcatatc-3' as the forward and reverse respectively.

For the internal control, Gapdh Q-PCR was conducted; the primers are 5′-cctggagaaacctgccaagtat-3′ as the forward, and 5′-ccctcagatgcctgcttca-3′ as the reverse. Total volume of each reaction was 15 µl containing 7.5 µl of 2X sybrgreen mix, 3 µl of 1 µm of primer mix, and 4.5 µl of 45 X diluted cDNA. The Q-PCR program was 3 min at 95°C, followed with 35 cycles of 5 sec at 95°C and 30 sec at 60°C. Each sample was assayed triplicate. To calculate the fold change we determined the ΔCt. ΔCt equals Ct(GFP/rtTA)-Ct(Gapdh). ΔΔCt was a result

from ΔCt(sample)- ΔCt(reference). From the ΔΔCt we calculate the fold change using formula $2^{-\Delta\Delta Ct}$.

4.7.5. Analyzing GFP, rtTA, and ubiquitin protein by Western blot and immunohistochemistry

4.7.5. 1. Western blot to quantify the levels of GFP protein

Expression of transgene can be measured from the levels of protein expressed. Western blot is widely used to compare and quantify the protein expression. Total protein 30 µg was loaded onto Criterion XT precast gel (4-12% bis-tris); Biorad, and was run in MOPS buffer (0.05 M MOPS, 0.05 M Tris-base, 3.5 mM SDS, 1 mM EDTA, pH 7.7) on 150 V. The gel was electroblotted to a nitrocellulose membrane in TG buffer (0.192 M glycine, 0.025 M Tris, 20% methanol) for 30 minutes. The membrane was incubated in PBS-Tween-milk (0.0005% Tween-20, 5% Elk (Campina)) for 30 minutes at 100 V. Membrane then was incubated at 4°C overnight with rabbit monoclonal GFP antibody (Abcam #ab290-50, 1:100000), and as loading control mouse anti Gapdh antibody was used (Chemicon #1924, 1:200000). After incubation with primary antibody, the membrane was incubated with secondary antibodies. The secondary antibody for GFP was goat anti-rabbit (Li-cor, 1:5000), and donkey anti-mouse (Li-cor, 1:5000) for Gapdh. The membrane was scanned with an Odyssey Infrared Imager. GFP was shown with red bands at 27 kDa and Gapdh with green bands at 37 kDa.

The density of the band was calculated using the Odyssey software. Western blot was not available for rtTA protein measurement.

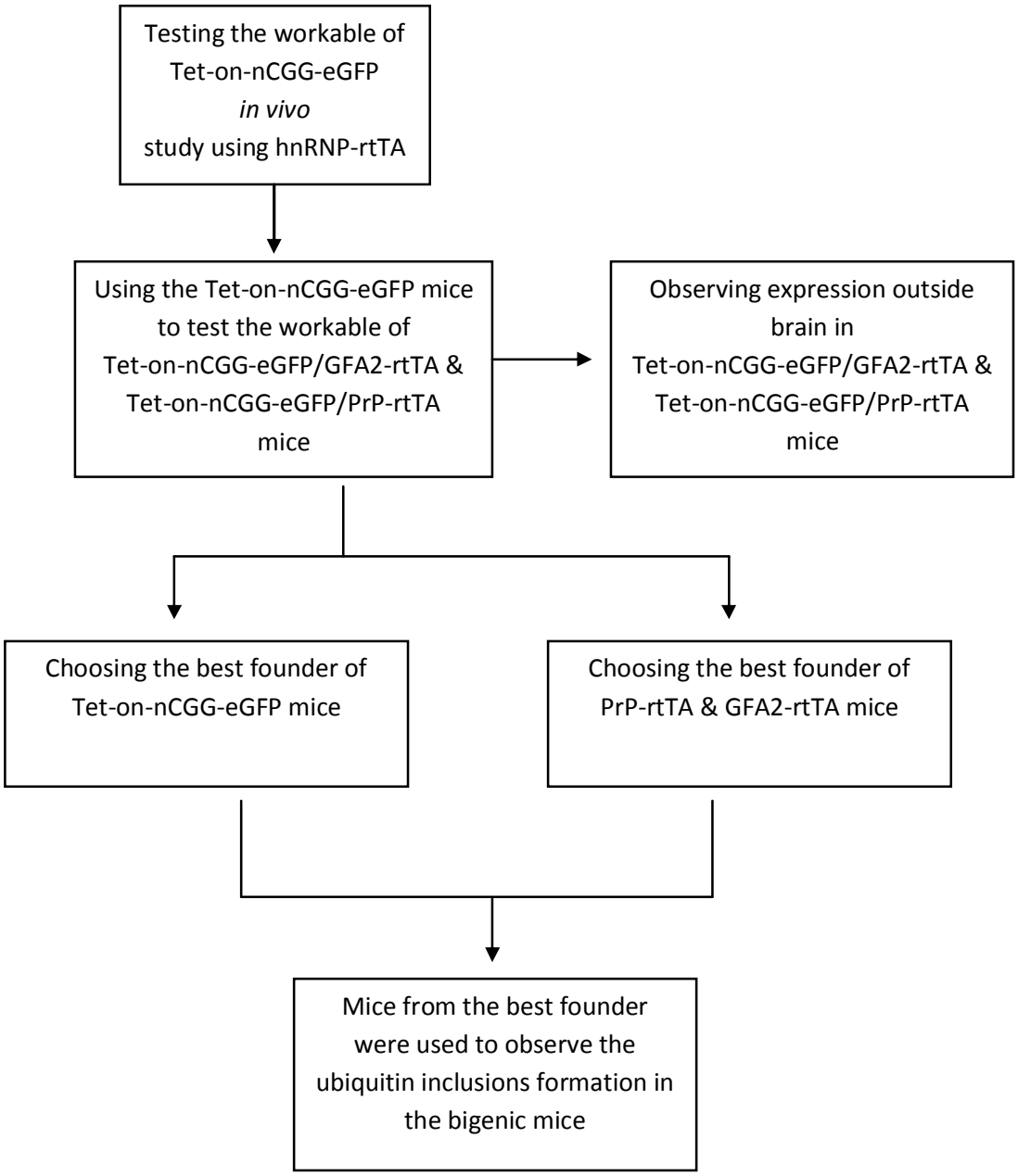
4.7.5.2. Immunohistochemistry to detect the GFP, rtTA protein as well as ubiquitin inclusions

Immunohistochemistry is used to detect the expression of protein directly in the tissue. Immunohistochemistry cannot be used for quantification; it only detects the presence of the protein. The tissues that had been fixed overnight in 4% paraformaldehyde were embedded in paraffin according to standard protocols. Sections were cut at 6 μm, followed by drying at 37°C overnight. Sections then were deparaffinized in xylene for 2X2 min, ethanol 100% for 2X1 min, ethanol 90% for 2X1 min, ethanol 80% for 1 min, ethanol 70% for 1 min, ethanol 50% for 1 min, and aquadest for 1 min. Antigen retrieval was achieved by microwave treatment in 0.01 M sodium citrate (pH 6.0) solution. Endogenous peroxidase was blocked using PBS-hydrogen peroxide-sodium azide solution (0.067% H_2O_2, 0.08% sodium azide). Primary antibody was added and incubated overnight at 4°C. The primary antibody for GFP staining was mouse anti GFP (Roche #1814460, 1:2000), followed with Histostain plus Broad Spectrum (Invitrogen #85-8943), in which 30 min of incubation in room temperature with Alpha-biotin as the secondary antibody, and another 30 min of streptavidin as the third antibody.

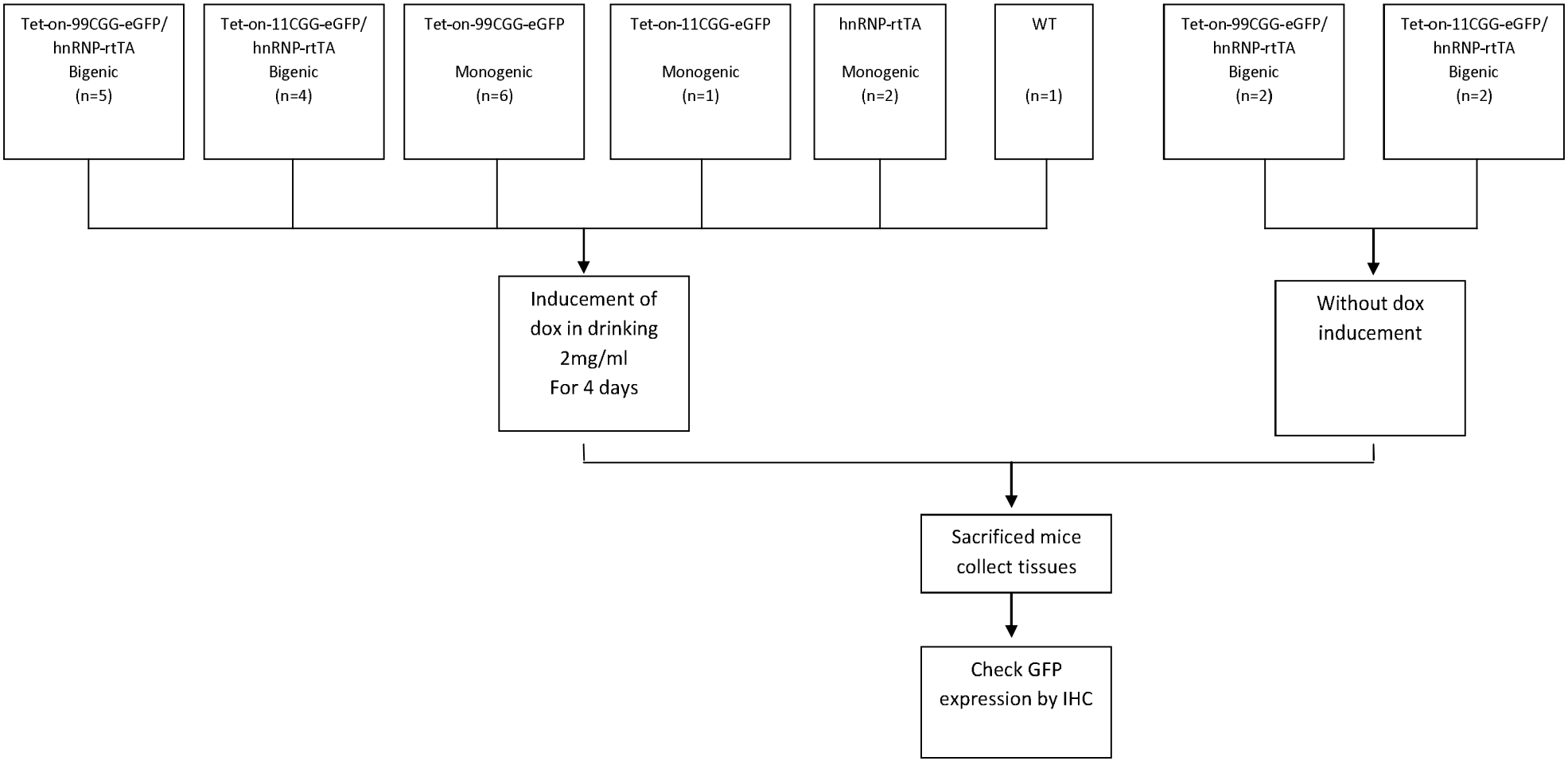
Ubiquitin staining was performed by incubation overnight at 4°C with a polyclonal rabbit antibody against ubiquitin (Dako #Z0458, 1:500) for the first antibody, followed by 1 hour incubation of swine anti-rabbit Hrp (Dako #P0217, 1:100) for the second antibody. Antigen-antibody complexes were visualized by incubation with DAB-substrate (DAKO Liquid DAB substrate-chromagen system #K3466, 2% DAB solution in DAKO buffer) for 8 minutes. The slides were then counterstained with haemotoxylin, followed by dehydration in ethanol 96% for 1 min, ethanol 100% for 2X1 min, and xylene for 2X2 min. The slides were mounted with Entellan. All dilutions were made in PBS+ (PBS 0.1 M, 0.5% milk Protifar plus (Nutricia), and 0.15% glycine). Immunohistochemistry for rtTA was performed using primary antibody against rtTA (1:5000), and followed with Histostain plus Broad Spectrum (Invitrogen) which has the similar steps as GFP staining. Brown staining indicates a positive result which is observed using optical microscope.

4.8. FRAMEWORK OF RESEARCH

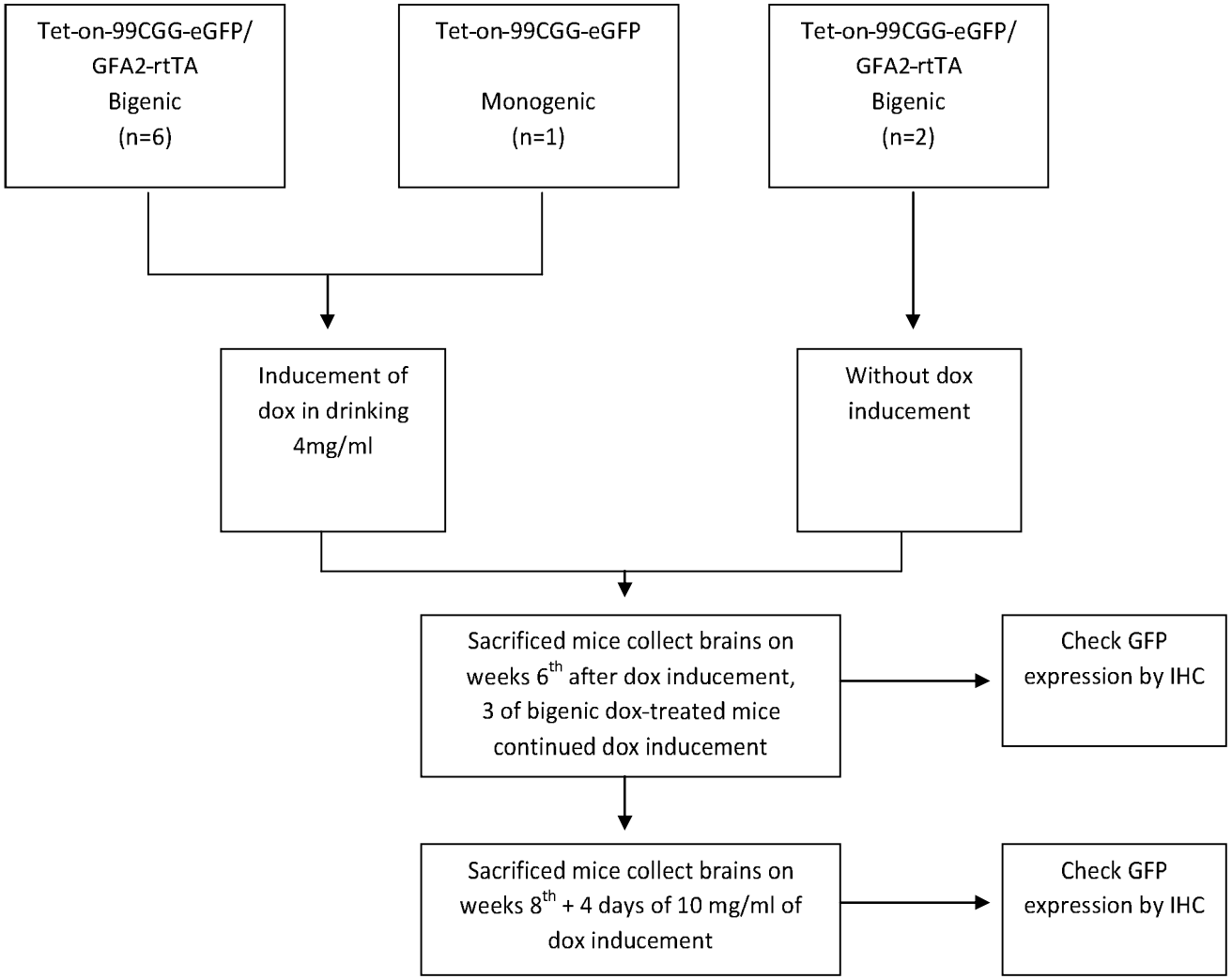
4.8.1. General research flow framework



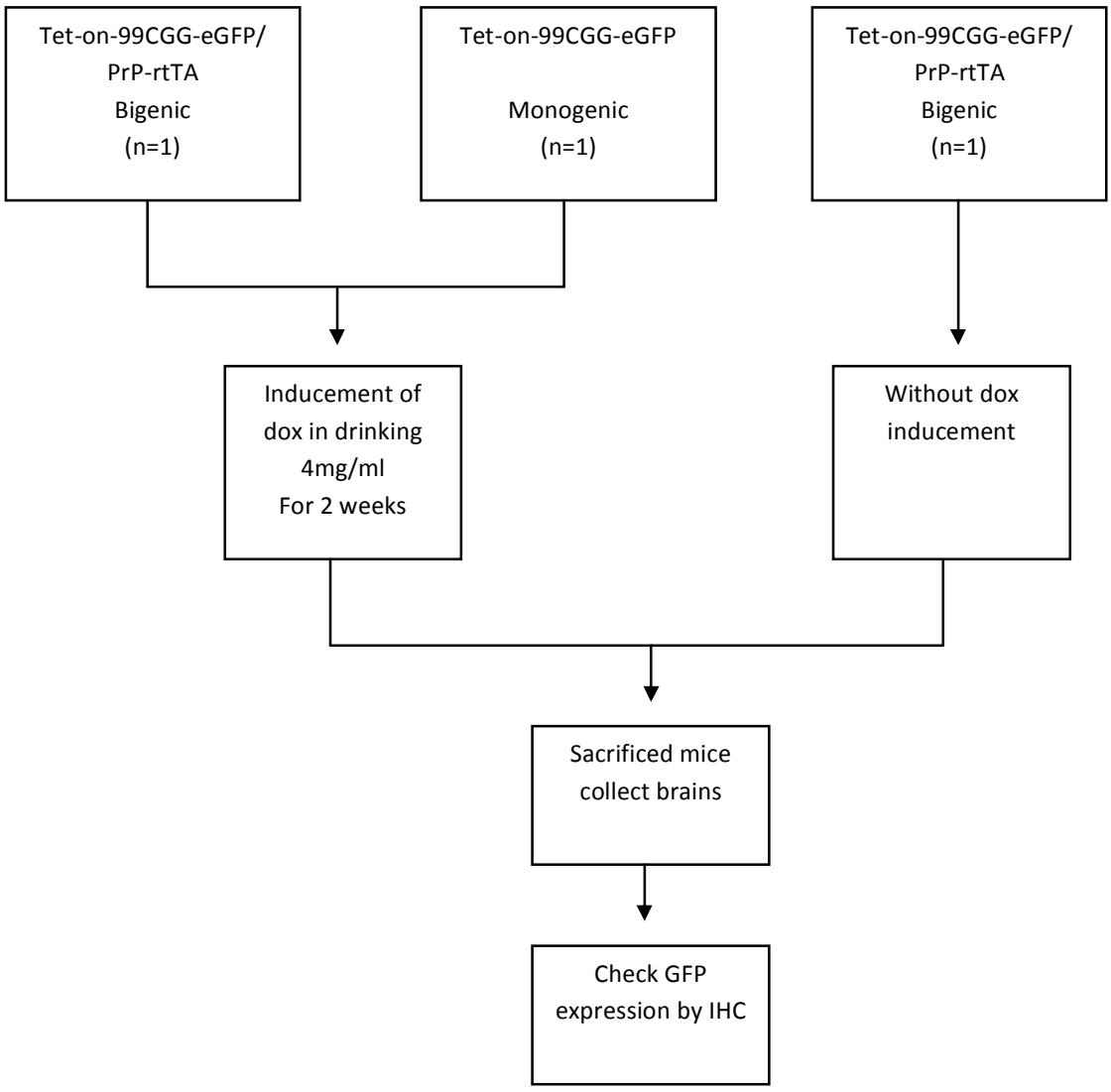
4.8.2. Testing the workable of Tet-on-nCGG-eGFP system



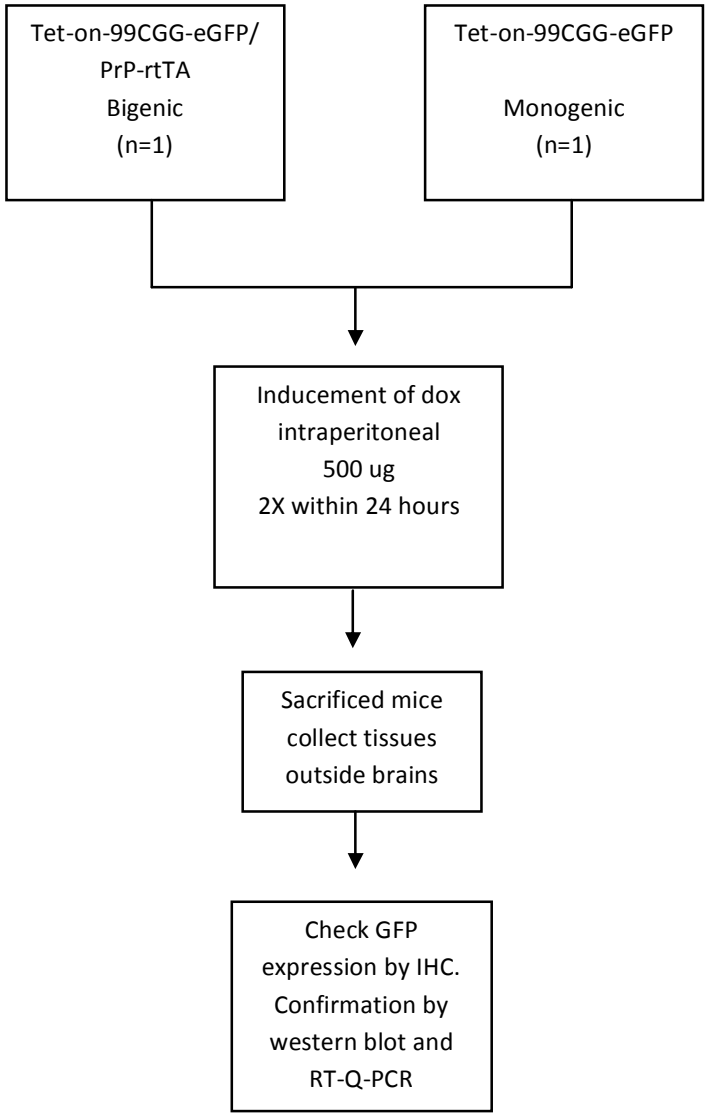
4.8.3. Testing the workable of bigenic mice Tet-on-99CGG-eGFP / GFA2-rtTA



4.8.4. Testing the workable of bigenic mice Tet-on-99CGG-eGFP / PrP-rtTA

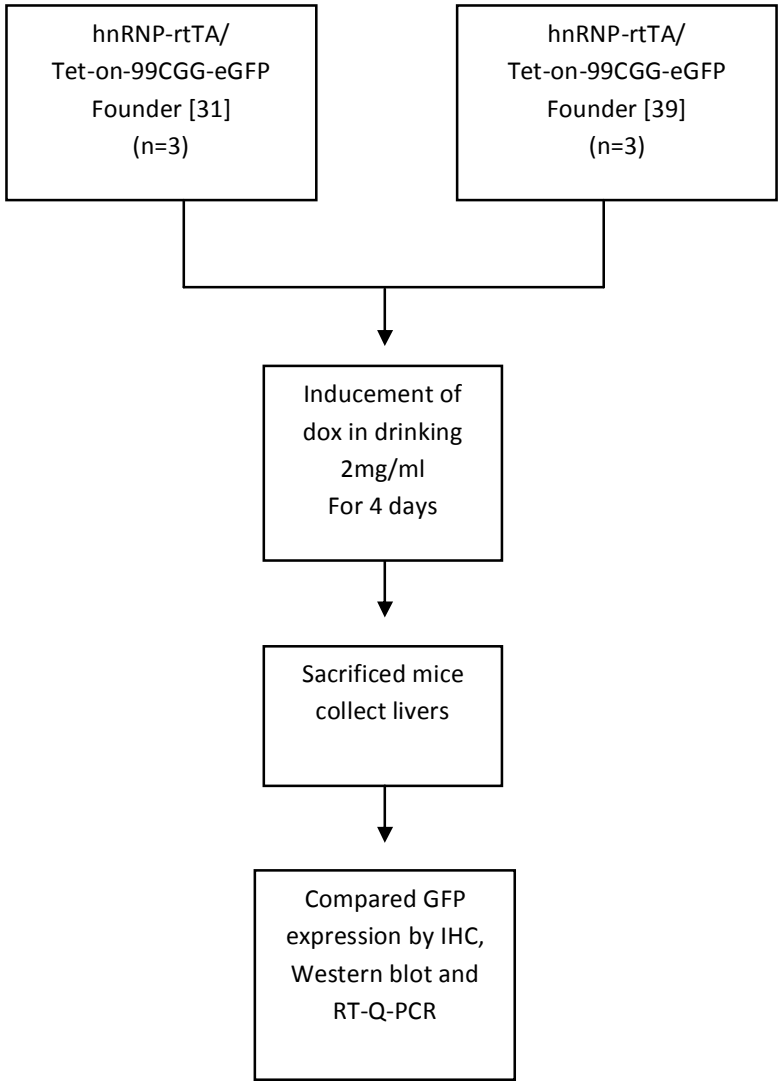


4.8.5. Observation leakage of expression outside brain of bigenic mice Tet-on-99CGG-eGFP / PrP-rtTA

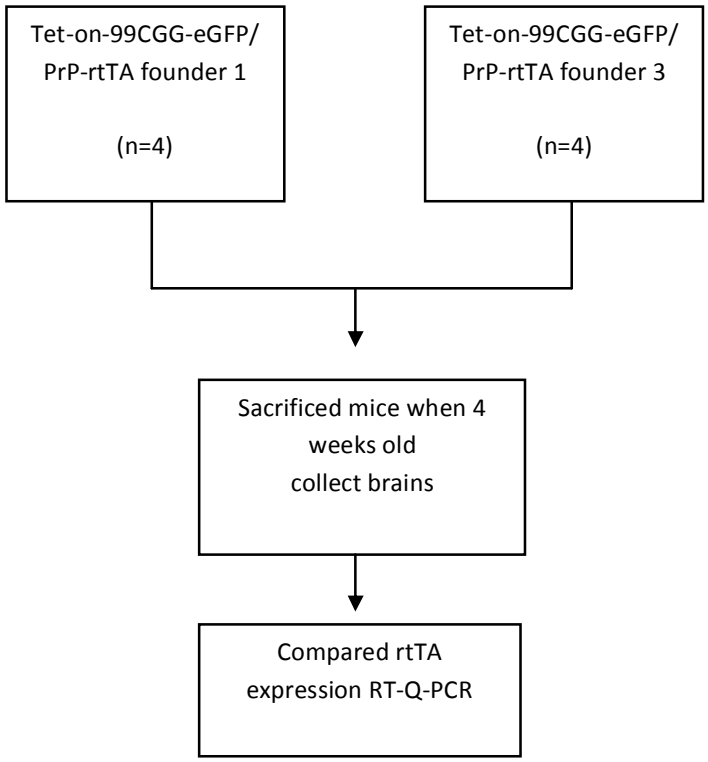


4.8.6. Choosing the best founder of each transgenic line

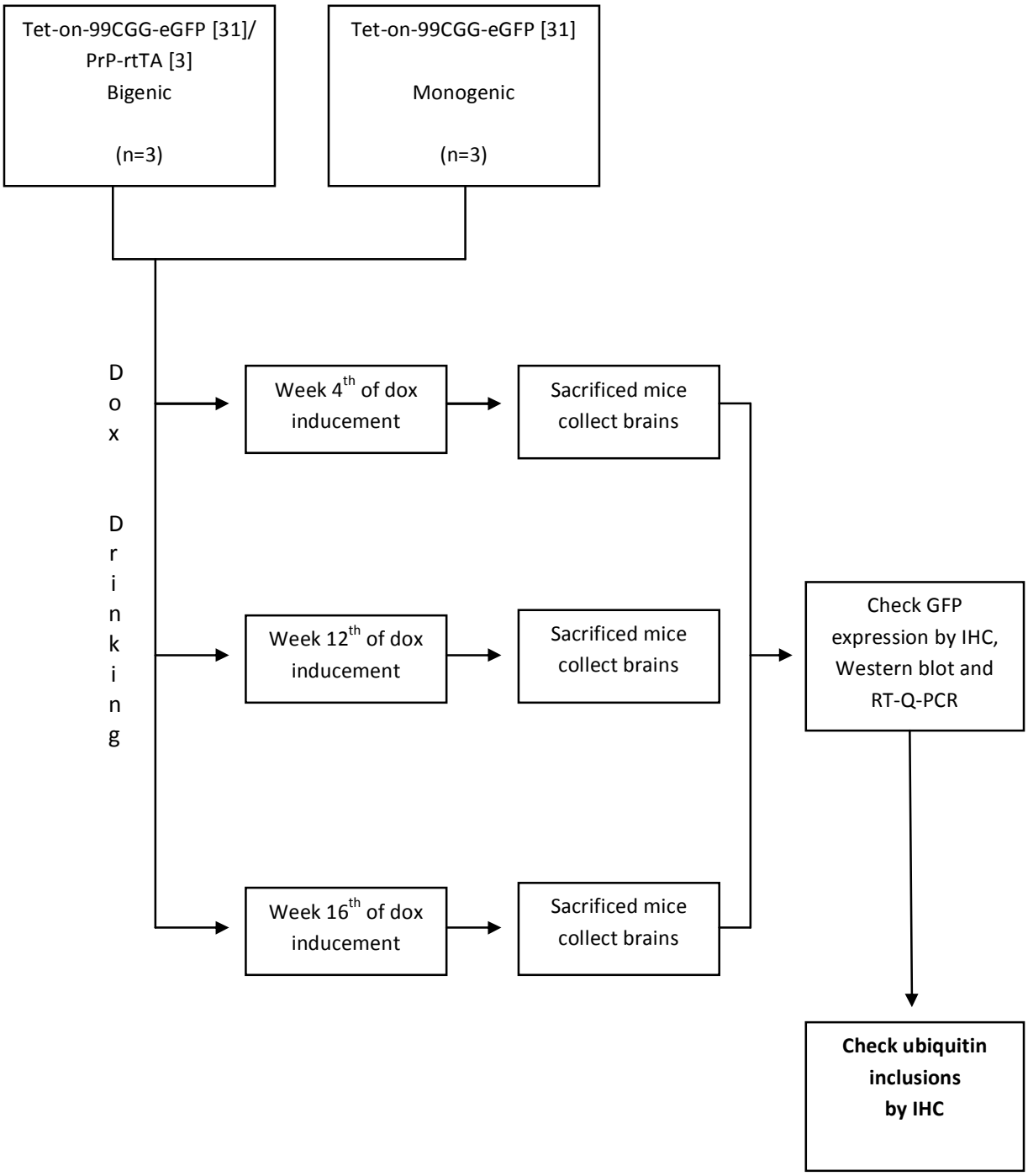
4.8.6.1. Choosing the best founder of Tet-on-nCGG-eGFP transgenic mice



4.8.6.2. Choosing the best founder of PrP-rtTA transgenic mice



4.8.7. Observing the formation of ubiquitin-positive intranuclear inclusions in bigenic mice Tet-on-99CGG-eGFP / PrP-rtTA



4.9. DATA ANALYSIS

Data was analyzed using Microsoft Office Excel. RNA and protein levels were presented in graph.

4.10. ETHICAL IMPLICATION

All experiments were carried out under permission of the local ethical committee Erasmus Medical Center Erasmus University Rotterdam, The Netherlands.