Non-invasive sampling methods for genotyping: improvements towards the 4r principle

SIVATHARSINI THASIAN-SIVARAJAH, REBECCA MOHR, MARIA WALTER, JOHN GBADEGOYE and MIRIAM HOPFE

Charles River

Correspondence: John.Gbadegoye@crl.com

Introduction

The Charles River (CRL) 4Rs Mission strives to advance science by improving upon the 3Rs principle which was introduced by Russell and Burch[1] in 1960 and focussed on enhancing animal wellbeing through emphasis on an imperative R – Responsibility.

The four terms are:

- Replacement: to avoid or replace the use of animals.
- Reduction: to minimise the number of animals.
- Refinement: minimise pain, stress and suffering.
- Responsibility: leading progressive change regarding Animal Welfare.

In research involving genetically modified mice which is usually to perform genotyping using invasive ear or tail biopsies in 92% of cases,[2] there is a growing shift towards non-invasive sampling techniques such as oral swabs, hair, faeces and tears. Our European Union (EU) Charles River genetic testing laboratory has successfully tested and proven the efficacy of genotyping using oral swabs (from mice and rabbits) and hair (from mice), aligning with the 4R principle and European regulations. The advantages of using non-invasive methods such as oral swabs and hair instead of biopsies is shown in Figure 1.

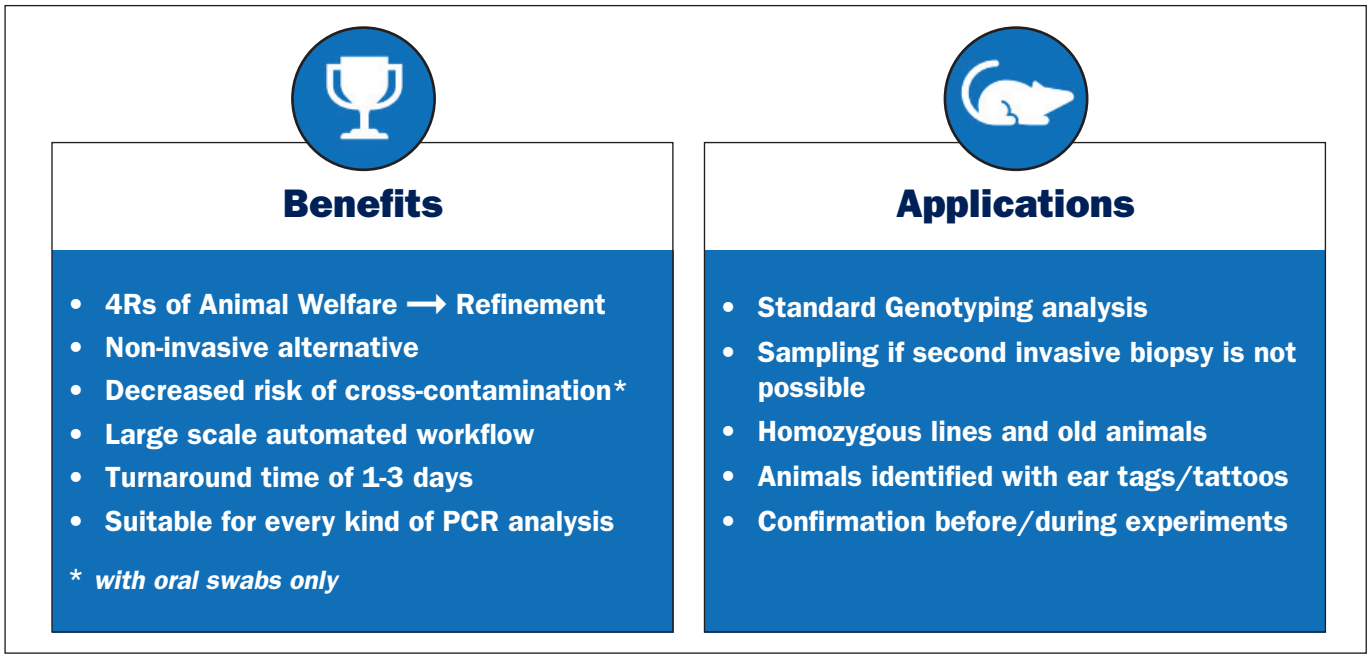

Figure 1.

Method

Research Conditions

For the data represented here, both male and female mice from different transgenic lines were used. Due to animal wellbeing and the size of the swab head all animals were at least 16 days old while oral swab and hair follicle samples were taken. Several swab-types were tested in terms of the cotton head size, the surface structure and the quality of the genotyping results. Based on this pre-test, the optimal type of swab regarding our whole genotyping process was determined. Besides that, a thorough sampling procedure is important to acquire sufficient animal tissue for further processing.

Sampling

Oral swab samples of the mice were taken, as shown in Figure 2. Briefly, swabs were autoclaved and brought into the animal barrier facility according to standard procedures. Mice were securely scruffed and the swab was twirled around for 5 to 19 seconds to collect the sample from the inside the cheek (Figure 2A). While swabbing it was ensured to not hurt the mice. Hair follicle samples were obtained by carefully plucking a small amount of hair (10-20 units) (Figure 2B). After sampling, mice were placed back into their cages. The oral swabs were left to dry before placing each swab and hair sample into individual tubes. Ear biopsies taken for routine genotyping analyses from the same animals were used as controls. Finally, the samples were shipped to the genotyping facility. All samples were taken at AAALAC accredited CRL sites according to Animal Welfare rules and guidelines.

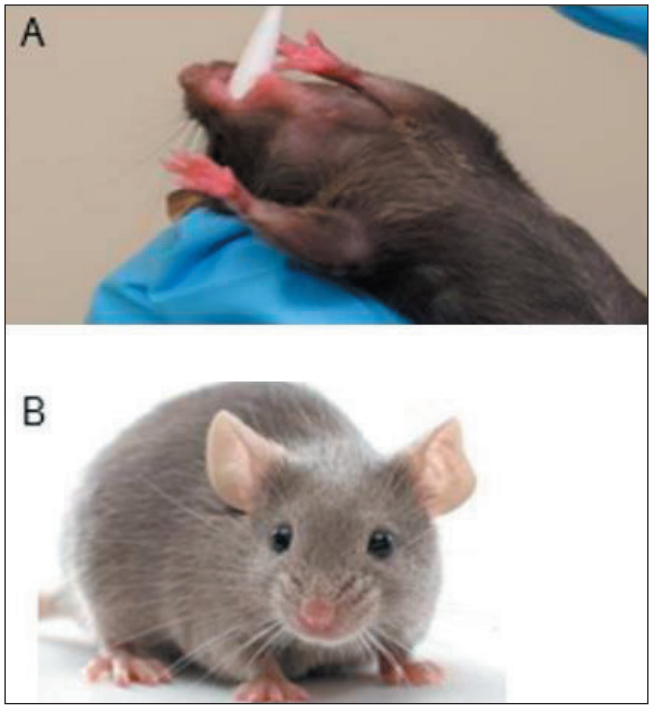

Figure 2A and 2B. Sampling methods.

The mouse was securely scruffed to prevent it from moving its head. (Figure 2A) The autoclaved oral swab was gently inserted into the oral cavity of the animal from an angle to collect the sample from inside the cheek. The oral swabs were snapped off, dried and placed into shipping tubes for transport. Hair follicle samples were obtained by carefully plucking a small amount of mouse hair (10 to 20 units), (Figure 2B) followed by placing them into shipping tubes for transport.

Processing:

Throughout the whole processing workflow, samples were kept inside a 96-well format to avoid potential mix up and to allow the processing of a large number of samples.

Lysis and DNA extraction:

The oral swabs were incubated in lysis buffer for 2 hours at 56°C. Hair and ear biopsies were incubated under the same conditions but overnight. Deoxyribonucleic acid (DNA) was extracted using Solid Phase Reversible Immobilisation (SPRI) technology. Purified DNA was stored at +4°C (short term) until polymerase chain reaction (PCR) analyses.

Polymerase Chain Reaction (PCR) and analysis of results:

The DNA extracted from the samples (oral swabs, hair and biopsies) was subjected to either conventional PCR and analysis using capillary gel electrophoresis (CE) (LabChip GX Touch, Perkin Elmer) or real-time PCR (quantitative PCR and endpoint analysis) and analysis using StepOne Cycler (ThermoFisher Scientific). Slightly adapted conditions were established, if needed e.g. increased number of PCR cycles, template or primer concentration. Amplification products were analysed and compared between the different sample types.

Results

1. Oral swab genotyping – suitable for every kind of PCR

Oral swabs taken from transgenic lines (KO, KI, etc.) were tested in conventional PCR with PCR amplicons ranging from 100 to 1500 bp in length (Figure 3A), real-time endpoint analysis (allele discrimination plot (qPCR: n=11 samples Figure 3B) and in zygosity testing for transgene lines (Figure 3C), leading to the correct genotyping determination and comparable signal intensity. In our study we could show that >98% of oral swab samples led to clear results. Furthermore >99% of the results from oral swabs matched those from corresponding ear biopsies.

2. Shipment and storage condition for oral swab – (RT (+ 20°C), + 4°C and – 20°C)

The robustness of oral swabs genotyping in terms of shipment and storage time/condition were tested for up to 25 days using capillary gel electrophoresis (CE) as

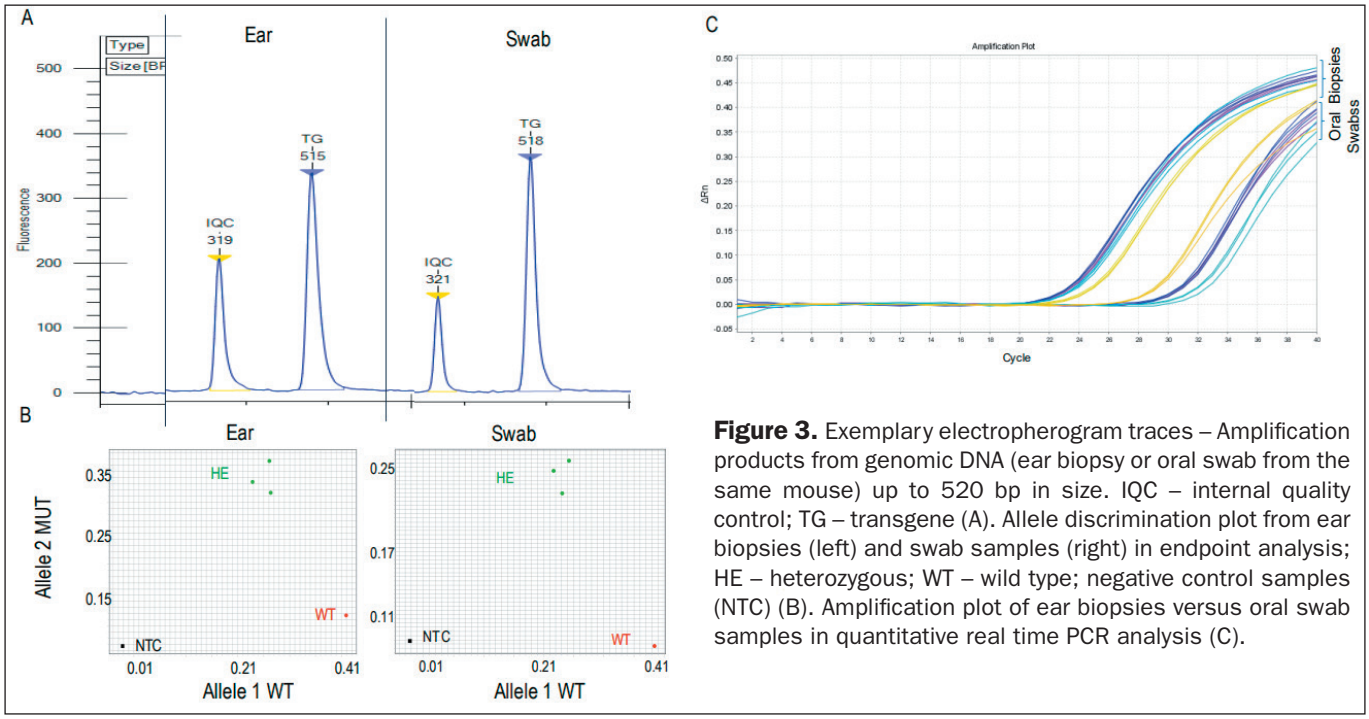

Figure 3. Exemplary electropherogram traces – Amplification products from genomic DNA (ear biopsy or oral swab from the same mouse) up to 520 bp in size. IQC – internal quality control; TG – transgene (A). Allele discrimination plot from ear biopsies (left) and swab samples (right) in endpoint analysis; HE – heterozygous; WT – wild type; negative control samples (NTC) (B). Amplification plot of ear biopsies versus oral swab samples in quantitative real time PCR analysis (C).

analysis method. The percentage ratio of the PCR amplicon in ng/µl to biopsy is shown in Figure 4. Signals could be detected up to 18 days after sampling if the oral swabs were shipped and stored at room temperature (RT) (+20°C). The best results were obtained when the samples were stored and shipped at -20°C. Visible signals and evaluable results were also detected at +4°C up to 25 days after sampling.

3. Swabs versus hair

In this pilot study we developed a simple, economic and efficient strategy to extract DNA from hair follicles of mice which are suitable for genotyping. When comparing oral swab and hair follicle samples, we were also able to demonstrate consistent genotyping results from hair follicles.

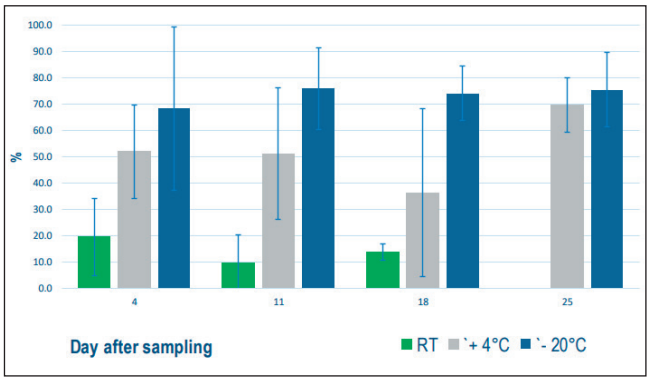

Figure 4. Bar chart. Value was determined from the capillary gel electrophoreses using TG-PCR fragment amplicon amount in ng/µl for biopsies versus oral swabs 4, 11, 18 and 25 days after sampling. Samples were shipped and stored at room temperature (RT: +20°C), +4°C and -20°C. Standard deviation is shown in the Figure. TG – transgene.

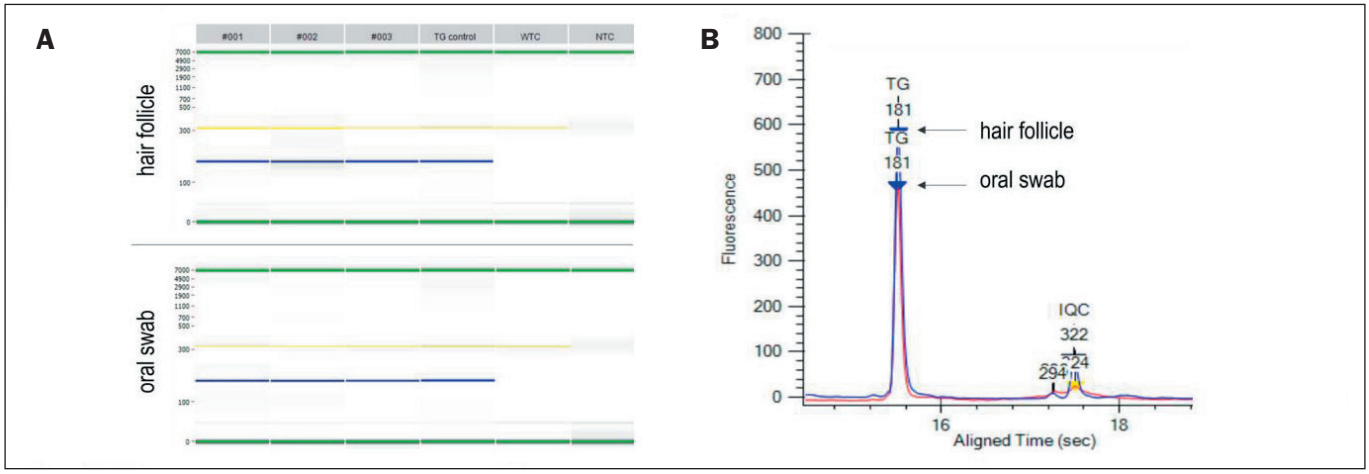

Figure 5. Genotyping using murine hair follicle and oral swab samples. Results of capillary gel electrophoresis showing the presence or absence of transgene in both biological materials – hair follicle and oral swab from same mouse (A). Exemplary electropherogram traces – amplification products from genomic DNA (oral swab and hair follicle from same mouse) up to 500 bp in size (B). IQC – internal quality control; TG – transgene (A+B).

Conclusion

In accordance with the 4R principle we have optimised and expanded the possibilities of genotyping mice using non-invasive methods in our automated workflow. This involves the non-invasive collection of oral swabs and hair follicles to replace stressful and painful biopsies. Since hair sticks electrostatically to instruments, there is a risk of cross contamination between hair samples from different animals. Considering the 4Rs and a growing interest in refining genotyping sampling methods, oral swab and hair follicle sampling provide alternatives that can be used for large scale routine genotyping especially if no invasive biopsy is allowed (e.g. animals with ear tags or toe tattoos) or no second biopsy is possible.

Non-invasive sampling methods:

- alternative to ear biopsies
- sampling of mice aged 16 days or older
- confirmation of genotype before/during experiments
- storage up to 25 days at +4°C; for longer storage -20°C advisable*
- shipping at RT, on cool packs or dry ice
- suitable for conventional PCR, real-time qPCR and endpoint analysis*
- large scale routine genotyping leading to faster turnaround times*

**only tested for oral swabs so far*

Acknowledgements

We would also like to thank our German and French colleagues for establishing the oral swab and hair sampling method at our Charles River breeding facilities (AAALAC accredited). Further thanks to our European Genetic Testing Services technicians for their support.

References

1 Russell and Burch Methuen & Co. Limited, 1960.

2 **Mazlan, N., López-Salesansky, N., Burn, C., Wells, D.** (2013). Mouse identification methods and potential welfare issues: a survey of current practice in the UK. *Animal Technology and Welfare* Vol 13 No 1, 1-10.