A NON-INVASIVE SAMPLING METHOD OF GENOTYPING RODENT COLONIES INTEGRATING THE 4R PRINCIPLE

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charles river

INTRODUCTION

The 3R principle was developed by Russell and Burch in 1960 with the aim of improving animal wellbeing[1]. The three terms are defined as:

- Replacement: Avoiding or replacing the use of animals
- Reduction: Minimizing the number of animals used
- Refinement: Minimizing the pain, suffering, distress or lasting harm that research animals might experience

The Charles River 4 Rs Mission aims to advance science guided and driven by a foundationally important R - Responsibility. Genetically modified mice that are used in research are genotyped using an invasive ear or tail biopsy in 92% of cases[2]. In line with following the 4R principle and European regulations, there is growing interest in using non-invasive sampling methods for mice genotyping like oral swabs, hair, or feces. After having established the genotyping of oral swabs taken from rabbits for routine analysis several years ago in our EU Charles River genotyping laboratory, the sampling, extraction and PCR method was adapted and refined to swabs taken from mice and rats within the last years. There are several reasons why it is better to use a non-invasive method like oral swabs instead of biopsies which are summarized in Figure 1 – Benefits & Applications.

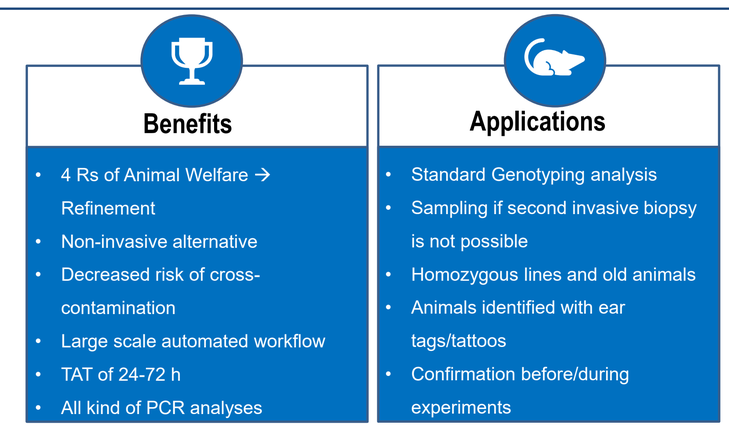


Figure 1: Benefits and applications of this established non-invasive sampling method for genotyping in mice.

METHOD

Swabs: Several different swabs were tested in terms of cotton head size and surface structure (data not shown). All data shown here were acquired using the optimal type of swab. A thorough sampling procedure is important to acquire sufficient animal tissue for further processing.

Mice: Both male and female mice from different transgenic lines were used. All animals were at least 16 days old due to animal wellbeing and size of swab head.

Sampling: Oral swab samples were taken as described in Figure 2. Briefly, swabs were autoclaved and brought into the animal barrier facility according to standard procedures. Swabs were placed into sterile tubes for easy access (Fig. 2A). Mice were securely scruffed and the swab was twirled around for 5-19 sec to collect the sample from the inside of the cheek (Fig. 2B). While swabbing was done and especially when the swab was brought into the mouth again, it must be ensured not to hurt the gums, incisors, and molars of the mice. After sampling, mice were placed back into their cages. The oral swabs were placed into the tubes, snapped off to completely fit into the tube (Fig. 2C), left to dry (Fig. 2D) and shipped to the genotyping facility (Fig. 2D). Ear biopsies taken for routine genotyping analyses from the same animals were used as controls.

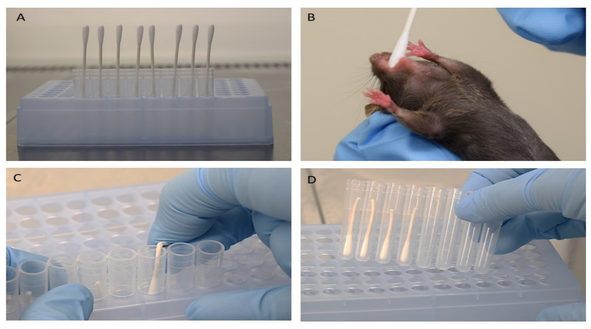


Figure 2: Autoclaved slim headed cotton swabs (A). The mouse was securely scruffed so that the head was not able to move. The oral swab was gently inserted into the oral cavity of the animal from an angle to collect the sample from the inside of the cheek (B). The oral swabs were snapped off and left to dry (C). The oral swabs were ready for transport in shipping tubes (D).

Lysis and DNA extraction: DNA lysis buffer was added to the swabs and incubated for 2h at 56°C. Ear biopsies were incubated in lysis buffer overnight at 56°C. DNA was extracted using a Solid Phase Reversible Immobilization (SPRI) technology. Purified DNA was stored at +4°C or -20°C until PCR analyses.

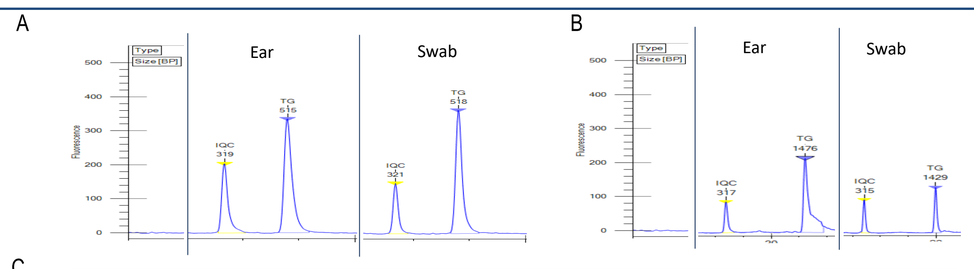
Polymerase Chain Reaction (PCR) and analysis of results: Amplification products from extracted DNA (Oral swabs or biopsies) were analyzed after conventional PCR by capillary gel electrophoresis (CE) (LabChip GX Touch, Perkin Elmer) or real-time PCR (quantitative PCR and endpoint analysis) by using StepOne Cycler (ThermoFisher Scientific). Only slightly adapted conditions were established, if needed e.g., increased number of PCR cycles, template or primer concentration.

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

RESULTS

1. Genotyping of oral swab samples led to clear results in conventional PCR analyzed by CE

We tested different oral swabs taken from transgenic lines (KO, KI, etc.) in conventional PCR with PCR amplicons from 100 to 1000 bp (Fig. 3A). All results were comparable in terms of fluorescence height with results obtained from ear punches and oral swabs (Fig. 3A & 3B). Furthermore, we could demonstrate a robust amplification of PCR fragments up to 1500 bp in size (Fig. 3B). 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 (Fig 3C).



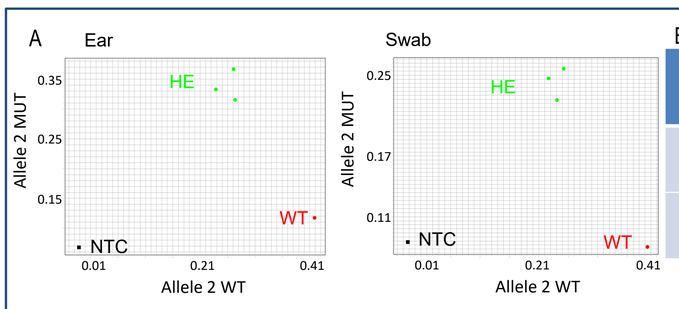
C

Gene	Oral swab result			Oral swab result compared to ear biopsy		
	Positive Results	Total no. of swabs	%	Positive Results	Total no. of swabs	%
X	533	541	99	529	533	99
Y	528	541	98	527	528	100

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 (A) and up to 1500 bp in size (B). IQC – internal quality control; TG – transgene (A+B). Table listing the number of oral swab samples tested for gene X or Y and number of oral swab tested compared to ear biopsies (C).

2. Reliable results for real-time qPCR and endpoint analyses

Reliable amplifications in addition to the conventional PCR were also detected from oral swab samples tested in real-time endpoint and qPCR analyses. The real-time Endpoint analysis led to distinct genotyping results shown in the allele discrimination plot (qPCR: n=11 samples Fig.4A) where heterozygous (HE), Wild type (WT) and negative samples/control (NTC) could be separated precisely. In the zygosity testing for transgene lines, the cycle threshold (Ct) values were a little bit higher in samples from oral swab testing compared to ear biopsies however clear results were obtained (n=6 samples (Fig. 4B)) leading to the correct genotyping determination via the ΔΔ Ct-calculation method.



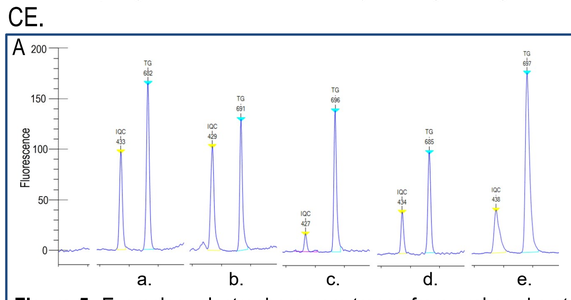
B

Gene		Ear Ct (mean)	Swab Ct (mean)
V	Endo	28.4	29.9
	TG	28.6	29.6
W	Endo	27.5	31.1
	TG	27.4	31.4

Figure 4: Allele discrimination plot from ear biopsies (left) and swab samples (right) in endpoint analysis; HE – heterozygous; WT – wild type. No amplification was detected in the negative control samples (NTC) (A). Table displaying the mean Ct values and standard deviations (SD) of ear and swab samples for two genes from a quantitative PCR; Gene V: n=5; Gene W: n=6; Endo – endogenous control; TG – transgene (B).

3. Swabs can be stored for up to 1 week before shipment at +4°C or -20°C

We tested the robustness of oral swabs in terms of storage before shipment, shipment times and conditions. We saw strong amplification peaks in the capillary gel electrophoresis (CE) for all different conditions tested (Fig. 5A). The best results were obtained when the samples were stored and shipped at room temperature (RT) or +4°C if the analysis after sampling does not exceed 6 days. Long storage and shipment lead to a decrease in the fluorescence signal in the CE.



B

	Storage time before shipment [d]*	Shipping time [d]	Shipping condition	Analysis after sampling [d]
a.	1	4	+4°C	6
b.	3	1	-20°C	5
c.	7	1	-20°C	9
d.	8	4	+4°C	13
e.	3	1	RT	5

Figure 5: Exemplary electropherogram traces from oral swabs stored for each of the above conditions; IQC – internal quality control; TG – transgene (A). Table listing five different storage times, shipping times and shipping conditions tested; n=5-8 per condition (B). *Oral swabs were stored at the same temperature as the shipping temperature. Room temperature (RT)

CONCLUSION

We have optimized and expanded the possibilities of genotyping mice using oral swabs in our automated workflow. Considering the 4Rs and a growing interest in refining genotyping sampling methods, the oral swab sampling and genotyping method provide an alternative that can be used for large-scale routine genotyping especially if no invasive biopsy is allowed (e.g., for animals with ear tags or toe tattoos) or no further biopsy is possible.

Non-invasive oral swabs…

- ✓ are a good alternative to ear biopsies
- ✓ can be taken from mice aged 16 days or older
- ✓ can be used before/during experiments to confirm the correct genotype
- ✓ can be stored for up to 1 week at +4°C; for longer storage -20°C is advisable
- ✓ can be shipped on cool packs, dry ice or at room temperature
- ✓ can be used for conventional PCR, real-time qPCR and endpoint analysis
- ✓ can be used in conventional PCR with PCR fragments up to 1500 bp
- ✓ can be processed on a large scale leading to faster turnaround times

REFERENCES

[1] Russell and Burch Methuen & Co. Limited, 1960
[2] Mazlan et al., Animal Technology and Welfare, 2014

ACKNOWLEDGEMENTS

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