SWAP mouse genotyping V.1

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The highly-conserved, cardiotonic steroid (CTS) binding site (also termed ouabain binding site) on the primary α subunit of Na,K-ATPase (NKA) plays a receptor signaling role in a range of vital cell processes and is a therapeutic target for human disease. Mouse lines with altered affinity for CTS on the α1 or α2 subunit isoform of NKA, developed by the late Dr. Jerry Lingrel and collaborators, are a valuable tool for studying its physiological roles and drug actions. In one model, the normally ouabain resistant α1 isoform was rendered sensitive to ouabain binding. In a second model, the normally sensitive α2 isoform was rendered resistant to ouabain binding. Additional useful models are obtained by mating these mice. The Lingrel mice will soon become publicly available in a biorepository. To further advance their use, we developed a rapid real-time, direct PCR method that amplifies digested tail clip samples without the need to isolate DNA, and detects variant alleles using specific primers and fluorescent probes. PCR is performed in fast mode with up to 23 samples processed in 40 min. The method was validated using mice of known genotype and results were compared with a previous two-step method using PCR amplification followed by gel electrophoresis. We also performed Sanger sequencing to clarify inconsistencies in published sequences, update numbering to current reference sequences, and confirm the continued presence of the mutations in the colony. It is expected that the wider availability of these models and a more efficient genotyping protocol will advance studies of the NKA and its CTS receptor.

Peter W W Chomczynski, Kianna M Vires, Michal Rymascewski, Judith A. Heiny 2022. SWAP mouse genotyping. **protocols.io** https://protocols.io/view/swap-mouse-genotyping-b2kaqcse

SWAP mouse, ATP1A1, ATP1A2, Ouabain binding site, Mouse models, NaK ATPase

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Diagrams created by BioRender.com. Screenshots from ABI StepOne software.

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Common reagents:
1M Tris pH 8.0
0.5 M EDTA pH 8.0
5 M NaCL
SDS 10% w/v
NP40 detergent (10%)
Tween-20
proteinase K (20 mg/mL)
ddH2O
Bio-Rad iTaq Universal Probes Supermix (or compatible equivalent)

Protocol-specific primers and probes:
ATP1A1 FWD primer (100 mM): CAG CTC TTT GGA GGC TTT
ATP1A1 REV primer (100 mM): GCT ACC GTA ACT ACA CAA CTC
ATP1A1 WT probe (100 mM): /56-FAM/CA+T +CC+G +A+AG T+GC /3IABkFQ/
ATP1A1 mutant probe (100 mM): /56-FAM/TGG AAT +TC+A +G+AG T+GC /3IABkFQ/
ATP1A2 FWD primer (100 mM): TCC TCT GCT TCT TAG CCT ATG G
ATP1A2 REV primer (100 mM): CAG GGC TAT AAG CAG GTC CA
ATP1A2 WT probe (100 mM): /56-FAM/CAC ATT ATC /ZEN/GTT GGA TGG TTC GTC CTC C/3IABkFQ/
ATP1A2 mutant probe (100 mM): /56-FAM/CTC ACA TCA /ZEN/TCG TTC GAA GGC TCG TC/3IABkFQ/

Pre-experiment preparation

1 In the interest of time and consistency, it is recommended that certain stock solutions and buffers be prepared ahead.

1.1 Prepare a **10X stock solution of Tail Lysis Buffer** and store in freezer. 15m

Reagent	Vol. to make 10 mL
1M Tris pH 8.0	1 mL
0.5 M EDTA pH 8.0	2 mL
5 M NaCL	2 mL
SDS 10% w/v	5 mL

Tail Lysis Buffer, 10X stock solution

20m

1.2

Prepare **assay mixes**.
Primers and probes should be pre-mixed and stored frozen for convenience. Be mindful of the reagent stock concentrations to ensure a successful outcome. Mixes can be scaled as necessary.

Reagent	For 200 rxn
ATP1A1 FWD primer (100 mM)	20 µL
ATP1A1 REV primer (100 mM)	20 µL
ATP1A1 WT probe (100 mM)	10 µL
ddH_2O	150 µL

$\alpha1^R$ assay mix

Reagent	For 200 rxn
ATP1A1 FWD primer (100 mM)	20 µL
ATP1A1 REV primer (100 mM)	20 µL
ATP1A1 mutant probe (100 mM)	10 µL
ddH_2O	150 µL

$\alpha1^S$ assay mix

Reagent	For 200 rxn
ATP1A2 FWD primer (100 mM)	20 µL
ATP1A2 REV primer (100 mM)	20 µL
ATP1A2 WT probe (100 mM)	10 µL
ddH_2O	150 µL

$\alpha2^S$ assay mix

Reagent	For 200 rxn
ATP1A2 FWD primer (100 mM)	20 µL
ATP1A2 REV primer (100 mM)	20 µL
ATP1A2 mutant probe (100 mM)	10 µL
ddH_2O	150 µL

$\alpha2^R$ assay mix

1.3 Prepare **Tail Digestion Buffer** fresh for each experiment, per the following table. Add proteinase K last from a frozen and thawed aliquot. 15m

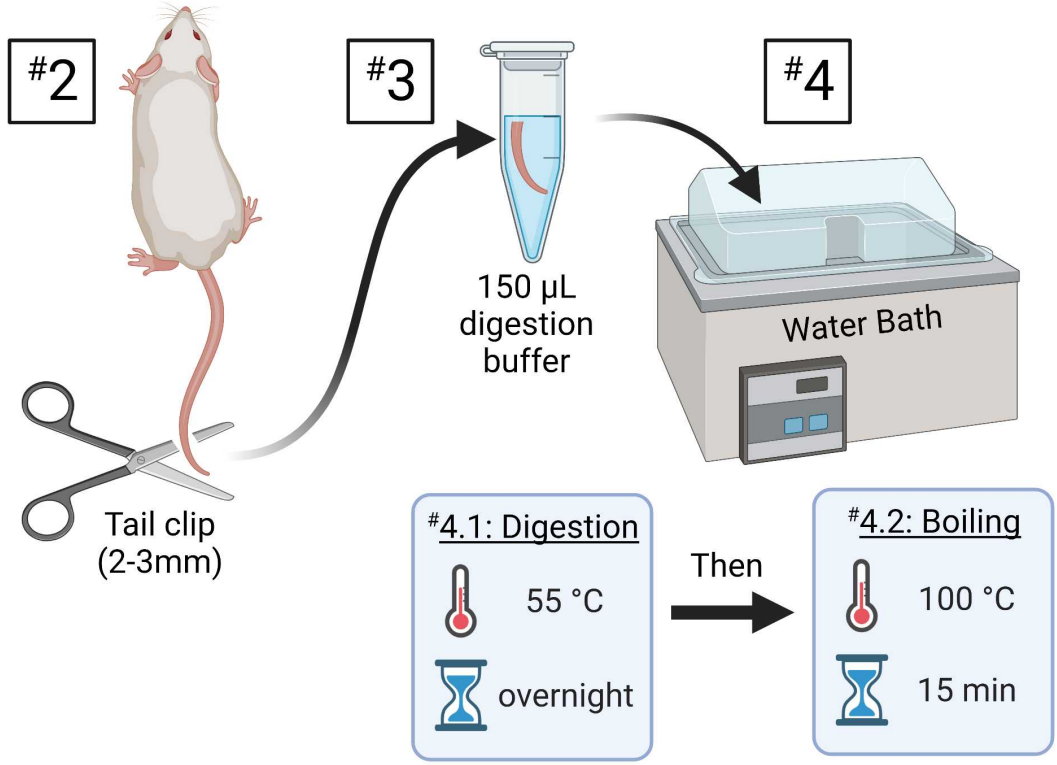
Reagent	Vol. to make 10 mL
10X Tail Lysis Buffer *	1 mL
NP40	45 µL
Tween-20	45 µL
proteinase K (20 mg/mL)	200 µL
ddH2O	to 10 mL

Tail Digestion Buffer

* final working concentrations are: 10 mM Tris pH 8, 10 mM EDTA, 100 mM NaCl, 0.5% SD

Tail clip digestion 12h 45m

2 Clip 2-3 mm from the tail of each mouse to be genotyped. Place in a clean, labelled 1.5 mL microcentrifuge tube. 30m



Steps 2-4 visual overview

3 Add **150 µL** of **Tail Digestion Buffer** containing proteinase K to each tube. 5m

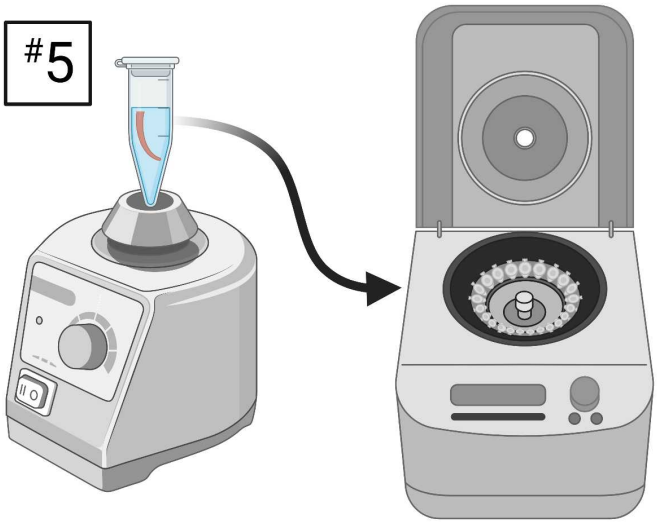
4 Place the tail samples in a heat block or water bath. 3m

4.1 Incubate **Overnight** at **55 °C** . 15m

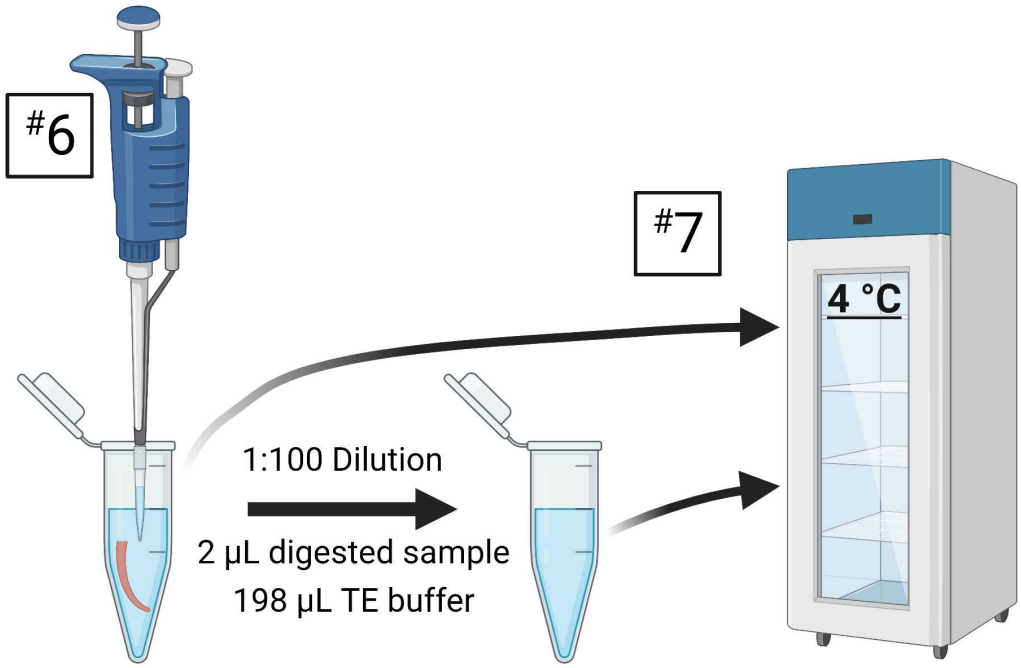
4.2 Raise the temperature to **100 °C** for **00:15:00** . 15m

4.3 Remove the tubes and allow to cool to **Room temperature** . 10m

5 Briefly vortex and centrifuge the tubes to pellet insoluble material. DNA will remain in the supernatant. 5m



6 Create a 1:100 dilution of each sample in a new, labelled 1.5 mL microcentrifuge tube. Add **198 µL** of TE buffer (or ddH2O) to each tube, then add **2.0 µL** of supernatant from the digested sample. 10m



7 Store samples and dilutions at **4 °C** until ready to genotype. If storing samples longer than 14 days, keep in **-20 °C** freezer.

PCR machine setup

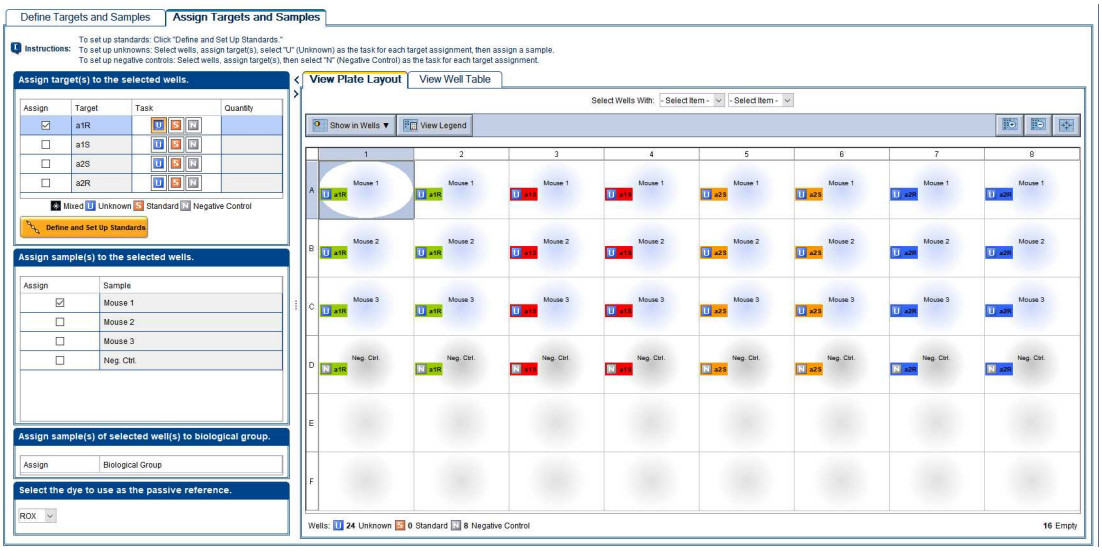
8 Create a new experiment on your PCR machine's software. Templates for ABI StepOne and StepOnePlus machines are attached.

SWAP mouse genotype StepOne template.edt

SWAP mouse genotype StepOnePlus template.edt

9 Enter your list of probes and samples into the machine software. Assign 8 wells per sample; 2 wells for $a1^R$, 2 wells for $a1^S$, 2 wells for $a2^S$, and 2 wells for $a2^R$.

A sample plate layout for genotyping 3 mice is shown (ABI StepOne software).



10 Set the thermocycling program as per the following table, then start the cycling.

Stage	Duration (m:ss)	Temperature	Cycles
Initial denaturation	3:00	95 C	1
Denaturation	0:03	95 C	35 Cycles
Annealing/Extension	0:30	95 C	

PCR reaction setup 2m

15m

11 Prepare 4 reaction mixes as follows, 1 for each of the assay mixes.
Create enough mix for the total number of reactions, plus 2 extra to account for potential pipetting loss.

Reagent	1 rxn	50 rxn	100 rxn
iTaq Probe mix (2X)	10.0 µL	500 µL	1000 µL
Assay mix	1.0 µL	50 µL	100 µL
ddH2O	4.0 µL	200 µL	400 µL

PCR reaction mix

12 Pipet **15 µL** reaction mix into each well of a 96-well plate. 5m

13 Pipet **5 µL** of each diluted sample into its assigned wells. Pipet **5 µL** ddH2O into each negative control well. 7m

14 Cover the plate with optical sealing film. Ensure that the film is fully adhered to the plate. 1m

15 Briefly vortex the plate. 1m

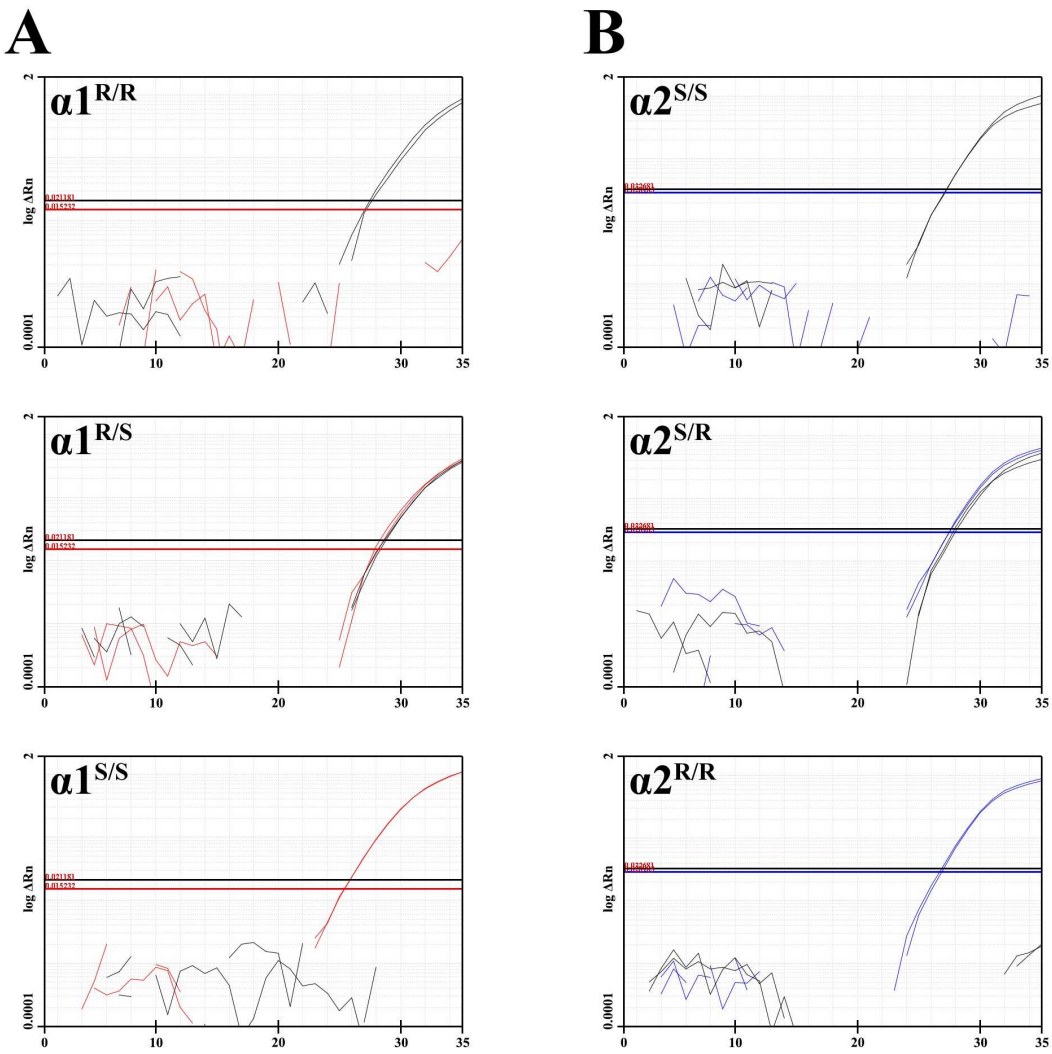
16 Centrifuge the plate at **1700 rpm, 00:02:00** . 2m

17 Insert the plate into the instrument and initiate the run. 1h 45m

Interpretation

18 Analysis of the PCR results begins by checking the outcome of the negative control wells. If all negative controls show no amplification, you may proceed with the analysis. If one or more negative control wells show amplification, the results are invalid and the experiment must be repeated. An invalid outcome is typically due to contamination of the reaction mix or of a reagent.

19 Genotyping is based on the presence or absence of signal from the 2 probes of each gene. The example below shows representative amplification plots for the possible genotypes of α1 (**A**) and α2 (**B**). The graphs show results from a sample's $\alpha1^{R}$ (black) and $\alpha1^{S}$ (red) wells plotted together (**A**), and a sample's $\alpha2^{S}$ (black) and $\alpha2^{R}$ (blue) wells plotted together (**B**).



Representative amplification curves

Samples considered positive for a specific allele show C_T values of 26-31 for the corresponding probe, while negative samples do not reach threshold in 35 cycles. Heterozygous samples show amplification of both probes within 2 C_T of each-other.