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Apr 01, 2022

# A real-time PCR method to genotype mutant mouse models with altered affinity for cardiotonic steroids on the Na,K-ATPase V.2

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dx.doi.org/10.17504/protocols.io.rm7vzym2rlx1/v2

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The highly conserved, cardiotonic steroid binding site (also termed ouabain binding site) on the primary α subunit of Na,K-ATPase 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 cardiotonic steroids on the α1 or α2 subunit isoform of Na,K-ATPase, 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. To further advance their use, we developed a rapid, real-time PCR method that amplifies digested tail clip samples without the need to isolate DNA, and detects mutant alleles using specific primers and fluorescent probes. PCR is performed in fast mode with up to 15 samples processed in 40 min. The method was validated by comparing results with a previous two-step method using PCR amplification followed by gel electrophoresis, and by Sanger sequencing. We also clarified inconsistencies in published sequences, updated numbering to current reference sequences, and confirmed 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 Na,K-ATPase and its cardiotonic steroid receptor.

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Peter W W Chomczynski, Kianna M Vires, Michal Rymascewski, Judith A. Heiny 2022. A real-time PCR method to genotype mutant mouse models with altered affinity for cardiotonic steroids on the Na,K-ATPase. **protocols.io** https://dx.doi.org/10.17504/protocols.io.rm7vzym2rlx1/v2
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SWAP mouse, ATP1A1, ATP1A2, Ouabain binding site, Mouse models, NaK ATPase

protocol ,

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

Mar 29, 2022

Apr 01, 2022

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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 (cat. no. 1725131) 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



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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**

1.2 20m

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 µM) | 20 µL |
| ATP1A1 REV primer (100 µM) | 20 µL |
| ATP1A1 WT probe (100 µM) | 10 µL |
| $ddH_2O$ | 150 µL |

**$\alpha 1^R$ assay mix**



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| Reagent | For 200 rxn |
|---|---|
| ATP1A1 FWD primer (100 µM) | 20 µL |
| ATP1A1 REV primer (100 µM) | 20 µL |
| ATP1A1 mutant probe (100 µM) | 10 µL |
| $ddH_2O$ | 150 µL |

**$\alpha1^S$ assay mix**

| Reagent | For 200 rxn |
|---|---|
| ATP1A2 FWD primer (100 µM) | 20 µL |
| ATP1A2 REV primer (100 µM) | 20 µL |
| ATP1A2 WT probe (100 µM) | 10 µL |
| $ddH_2O$ | 150 µL |

**$\alpha2^S$ assay mix**

| Reagent | For 200 rxn |
|---|---|
| ATP1A2 FWD primer (100 µM) | 20 µL |
| ATP1A2 REV primer (100 µM) | 20 µL |
| ATP1A2 mutant probe (100 µM) | 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



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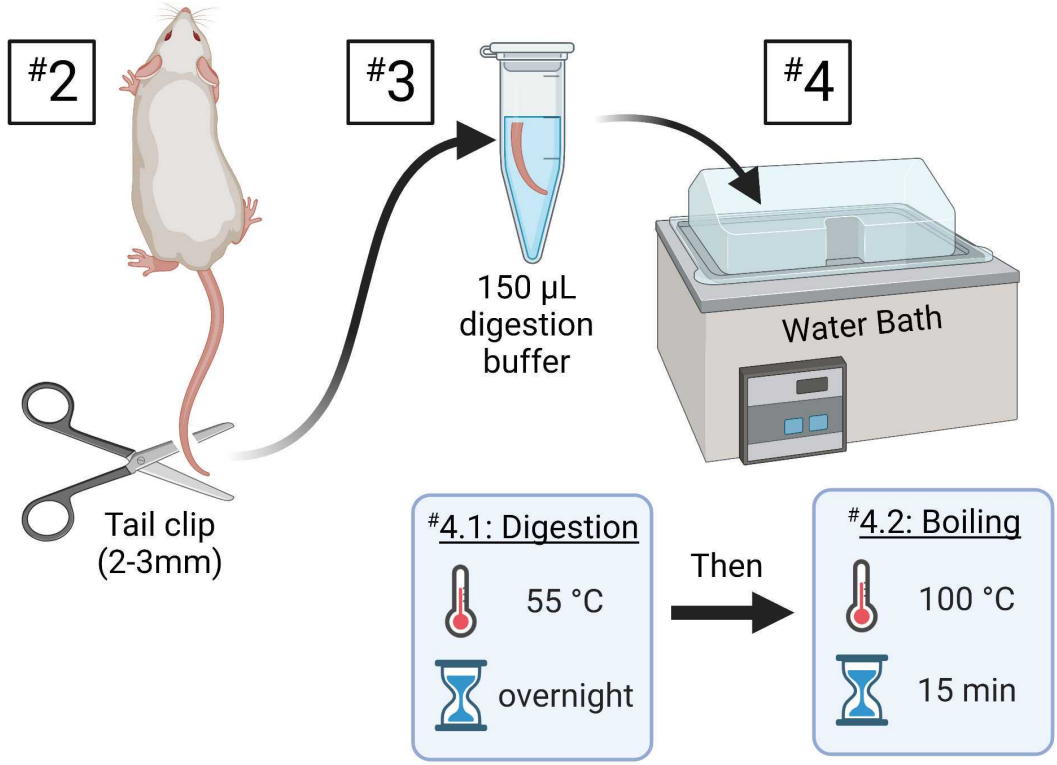
| Reagent | Vol. to make 10 mL |
|---|---|
| 10X Tail Lysis Buffer * | 1 mL |
| NP40 | 45 µL |
| Tween-20 | 45 µL |
| proteinase K (20 mg/mL) | 200 µL |
| ddH2O | to 10 mL |

**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



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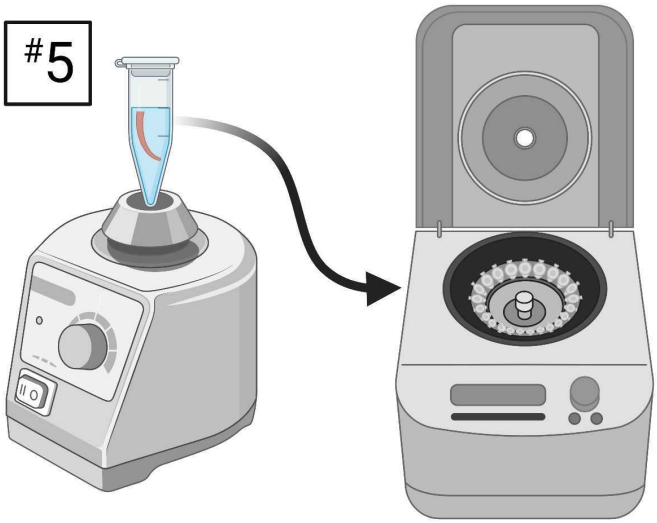
4 Place the tail samples in a heat block or water bath. 3m

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



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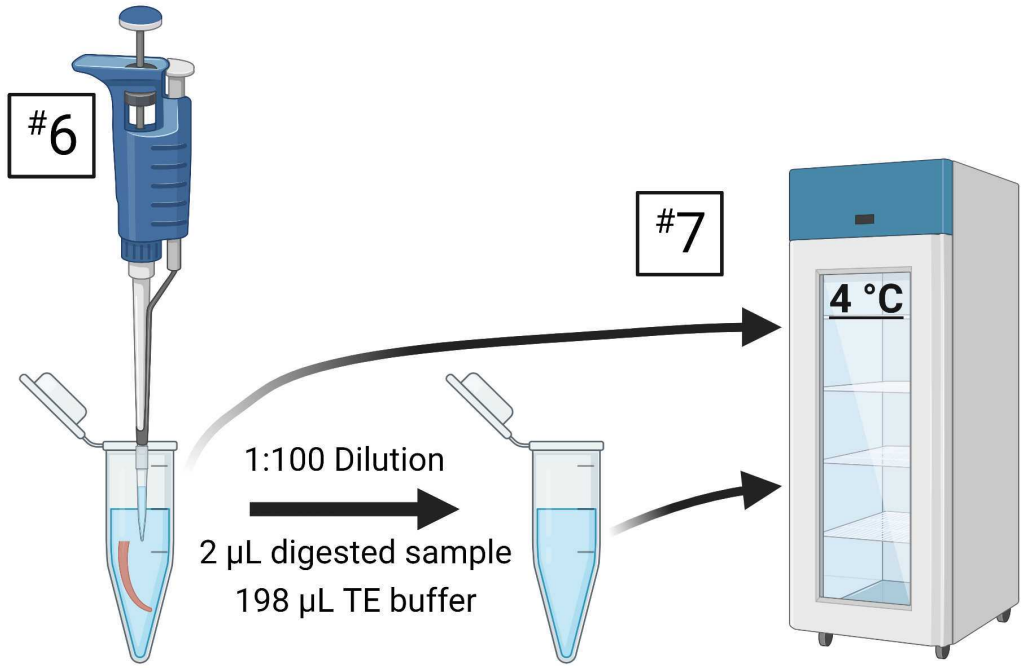


7 Store samples and dilutions at **4 °C** until ready to genotype. If storing samples longer than 14 days, keep in **-20 °C** freezer.

## PCR machine setup

8 Create a new experiment on your PCR machine's software. The instructions here are based on ABI StepOne machines but other manufacturers' procedures are generally similar. 2m

Templates for ABI StepOne and StepOnePlus machines are attached, which have the probe list and thermocycling program pre-set. To use the templates, download the version corresponding to your machine type. Open StepOne Software and choose [File] -> [New Experiment] -> [From Template...] and select the template file.

**SWAP mouse genotype StepOnePlus template.edt**

**SWAP mouse genotype StepOne template.edt**

9 Set the thermocycling program (run method) as per the following table. 2m



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| 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 | |

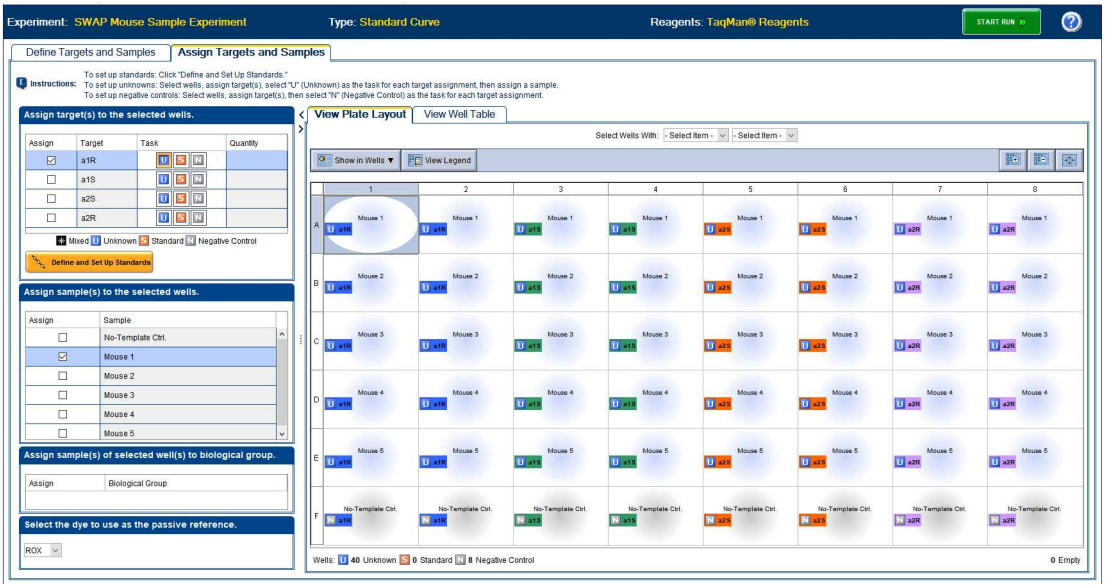
10 Enter the 4 probes into the targets list as per the following table. Populate the sample list with your mouse sample numbers, plus a no-template control (NTC) sample. 2m

| Probe | Reporter | Quencher |
|---|---|---|
| a1R | VIC | None |
| a1S | FAM | None |
| a2S | FAM | None |
| a2R | FAM | None |

Genotyping probe configurations

11 Assign 8 wells for each sample (including the NTC); 2 wells for $a1^R$, 2 wells for $a1^S$, 2 wells for $a2^S$, and 2 wells for $a2^R$. For the NTC, mark the wells as negative controls for the respective targets. 5m

A sample plate layout for genotyping 5 mice is shown here (ABI StepOne).



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## PCR reaction setup 2m

12 Prepare 4 reaction mixes as follows, 1 for each of the assay mixes. Create enough mix for the total number of reactions of each assay on your plate map, plus 2 extra to account for potential pipetting loss. 15m
For example, the plate shown in Step 11 (having 5 samples + 1 NTC) would need a 14 rxn mix for each assay. A full 96-well plate with 11 mouse samples and 1 NTC would require a 26 rxn mix for each assay.

| Reagent | 1 rxn | 14 rxn | 26 rxn |
|---|---|---|---|
| iTaq Probe mix (2X) | 10.0 µL | 140 µL | 260 µL |
| Assay mix | 1.0 µL | 14 µL | 26 µL |
| ddH2O | 4.0 µL | 56 µL | 104 µL |

PCR reaction mix

13 Pipet **15 µL** reaction mix into each well of a fresh PCR plate. 5m

14 Pipet **5 µL** of each diluted sample into its assigned wells. Pipet **5 µL** ddH2O into each no-template control well. 7m

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

16 Briefly vortex the plate. 1m

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

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



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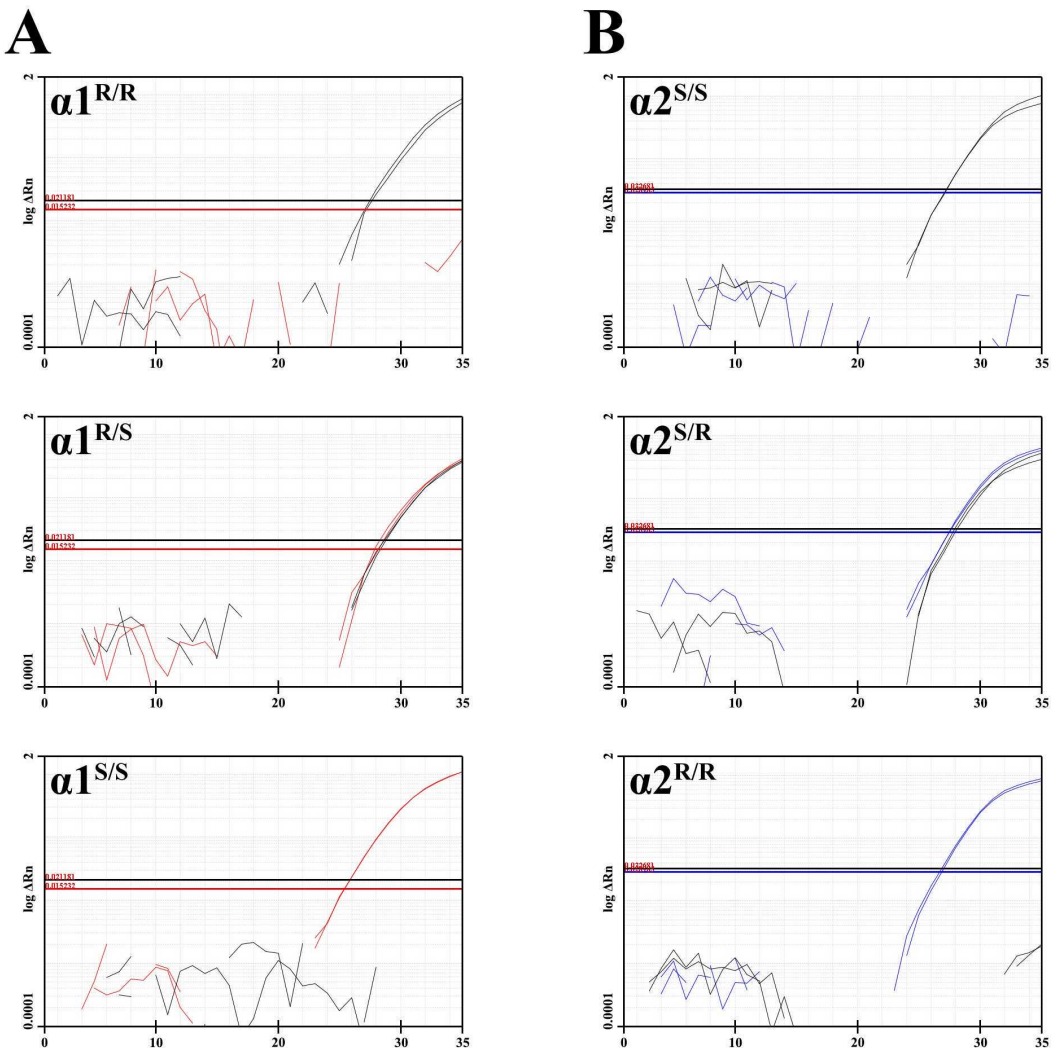
## Interpretation

19 Analysis of the PCR results begins by checking the outcome of the negative control wells. If all NTC wells 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.

20 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.



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