rhAmp™ SNP Genotyping

Genotyping with rhAmp SNP Assays and rhAmp reagent mixes

Introduction

rhAmp SNP Assays provide a precise and reliable PCR-based genotyping solution. Improved precision over existing methods is achieved by using a novel, dual-enzyme mismatch recognition system in conjunction with modified DNA-RNA hybrid primers. RNase H2 enzyme enables target primer activation, which is followed by extension using a mutant Taq DNA polymerase that results in enhanced mismatch recognition [1]. The rhAmp SNP Genotyping System includes the rhAmp Genotyping Master Mix and the universal reporter–based rhAmp Reporter Mix to provide a complete genotyping solution. Components of the system are listed in Table 1.

Table 1. Required materials.

Product	Size	Ordering information (Cat #)
rhAmp SNP Assay	100 rxn (XS)	Order at: www.idtdna.com/rhAmp-Genotyping
	750 rxn (S)	
	2000 rxn (M)	
	6000 rxn (L)	
rhAmp Genotyping Master Mix	0.5 mL (1 X 0.5 mL)	1076014
	5 mL (1 X 5 mL)	1076015
	10 mL (2 X 5 mL)	1076016
	25 mL (5 X 5 mL)	1076017
	50 mL (1 X 50 mL)	1076018
rhAmp Reporter Mix Only for instruments that do not require a reference dye	25 µL*	1076025
	250 µL†	1076026
	500 µL‡	1076027
	1250 µL§	1076028
	2500 µL‖	1076029
rhAmp Reporter Mix with Reference Only for instruments that require a reference dye	25 µL*	1076020
	250 µL†	1076021
	500 µL‡	1076022
	1250 µL§	1076023
	2500 µL‖	1076024
Nuclease-Free Water	300 mL	11-05-01-14
IDTE, pH 7.5 (1X TE Solution)	10 X 2 mL	11-01-02-02

* For use with 0.5 mL rhAmp Genotyping Master Mix, Cat # 1076014.
† For use with 5 mL rhAmp Genotyping Master Mix, Cat # 1076015.
‡ For use with 10 mL rhAmp Genotyping Master Mix, Cat # 1076016.
§ For use with 25 mL rhAmp Genotyping Master Mix, Cat # 1076017.
‖ For use with 50 mL rhAmp Genotyping Master Mix, Cat # 1076018.

Storage

Upon receipt, store:

- rhAmp SNP Assays at –20°C for up to 2 years.
- rhAmp Genotyping Master Mix at –20°C for up to 1 year.
- rhAmp Reporter Mix at –20°C for up to 1 year protected from light.

Alternatively, store:

- rhAmp SNP Assays and Genotyping Master Mix at 4°C for up to 2 weeks.
- rhAmp Reporter Mix at 4°C for up to 2 weeks protected from light.

Genotyping is supported on commonly used real-time instruments and fluorescence identification platforms by availability of the rhAmp Reporter Mix with or without reference dye. To aid in selecting the appropriate reporter mix, the reference dye requirements for commonly used instruments are displayed in Table 2.

Table 2. Reference dye requirements for various PCR systems*.

PCR system	Reference dye required	
	Yes	No
7900HT Fast Real-Time PCR System (Thermo Fisher Scientific)	X	
StepOne™ and StepOnePlus™ Real-Time PCR Systems (Thermo Fisher Scientific)	X	
Mx3005P® and Mx4000P™ qPCR Systems (Agilent)	X	
7500 Real-Time PCR System (Thermo Fisher Scientific)	X	
Viia™ 7 Real-Time PCR System (Thermo Fisher Scientific)	X	
QuantStudio™ Flex Systems (Thermo Fisher Scientific)	X	
Biomark™ HD (Fluidigm)	X	
CFX, iQ™, and Opticon™ Real-Time PCR Detection Systems (Bio-Rad)		X
LightCycler® Real-Time PCR Systems (Roche)		X

* For instruments not listed, please check with the manufacturer.

Protocol

A. Prepare sample DNA

1. Dilute each purified genomic DNA sample with IDTE, pH 7.5, to a concentration of 1.5–5 ng/µL.

   Notes:

   - Maintaining a consistent DNA concentration across samples is recommended to obtain tight clusters.
   - For sample DNA concentrations outside of the recommended range, adjust sample and water volumes in the final reaction to deliver 3–10 ng per reaction.
   - To store sample plates in a dried format, dispense 3–10 ng of sample per well and dry down.

2. (Optional) Dilute control templates (e.g., gBlocks™ Gene Fragments) for the reference allele and alternate allele to 500 copies/µL in IDTE, pH 7.5. For the heterozygous control, pool an equal volume of the controls for each allele.

B. Combine Master Mix and Reporter Mix

1. Calculate the total volume of combined Master Mix and Reporter Mix needed, based on the desired reaction volume and total number of reactions in the experiment, including controls (e.g., no-template controls, positive controls) and 2–5 additional reactions to account for pipetting error.

Final reaction volume	Volume of combined Master Mix and Reporter Mix per reaction
5 µL	2.65 µL
10 µL	5.3 µL

2. To a new tube or vial, combine rhAmp Genotyping Master Mix and rhAmp Reporter Mix at a 20:1 volume ratio. For example:

Component	Amount*
rhAmp Genotyping Master Mix (2X)	1 mL
rhAmp Reporter Mix (40X)	50 µL

* Scale volumes up or down, based on your needs (calculated in step B1).

Notes:

- Combined Master Mix and Reporter Mix may be stored, protected from light, at 4°C for up to 2 weeks or at –20°C for up to 6 months.
- For best results, always pipette components into a new tube or vial. Supplied vials contain volume overages, which will result in incorrect reporter concentration, if combined without measurement.

C. Prepare the SNP genotyping assay

1. To a new tube or vial, add the combined Master Mix and Reporter Mix, rhAmp SNP Assay, and water to form the SNP genotyping reaction mix as described in Table 3.

Table 3. Component amounts for a single rhAmp SNP Genotyping reaction.

Component	5 µL reaction volume*		10 µL reaction volume*	
	Liquid sample	Dried sample	Liquid sample	Dried sample
Combined Master Mix and Reporter Mix (from step B2)	2.65 µL	2.65 µL	5.3 µL	5.3 µL
rhAmp SNP Assay (20X)†	0.25 µL	0.25 µL	0.5 µL	0.5 µL
Nuclease-Free Water	0.10 µL	2.10 µL	2.2 µL	4.2 µL
Volume per well	3 µL	5 µL	8 µL	10 µL

* Scale up as needed for your experiment.

† Dilute larger scale (80X) rhAmp SNP Assays to 20X by mixing 1 part assay with 3 parts IDTE, pH 7.5.

2. Vortex, briefly centrifuge, and aliquot the SNP genotyping assay reaction mix (see Table 3, Volume per well) to the wells of your optical reaction plates.
3. For liquid samples and controls, add 2 µL of one of the following to the appropriate wells of the plates from step C2:
   - Sample DNA (from step A1): 3–10 ng of total DNA per reaction
   - Control template (from step A2): 1000 copies of gBlocks Gene Fragment controls per reaction
   - Nuclease-free water: for no-template control reactions
4. Seal all plates, vortex briefly, and spin down in a centrifuge.

D. Perform the PCR

Note: The PCR can be either run in a thermal cycler and transferred to an appropriate plate reader for end-point analysis, or run and analyzed in a real-time qPCR instrument.

1. Set up the thermal cycling conditions as shown in Table 4.

Table 4. Thermal cycling parameters for rhAmp SNP Genotyping.

Step	Temperature (°C)	Time (min:sec)	Cycles
Enzyme activation	95	10:00	1
Denaturation Annealing Extension	95 60 68	00:10 00:30 00:20	40
Heat inactivation (optional)*	99.9	15:00	1

* If the genotyping reactions will not be analyzed immediately following the PCR, use the heat inactivation step to preserve fluorescent signal for up to 48 hr at room temperature.

2. Load the reaction plate into the thermal cycler, and start the run.

E. Collect and analyze data

Follow the instructions for your instrument to collect data. A general overview of the procedure includes:

1. Create and set up an allelic discrimination plate-read file:
   a. Assign assay name to each group of wells containing one assay.
   b. Assign FAM to the reference allele, and assign VIC™ dye (Applied Biosystems) to the alternate allele.

      Note: The probe detecting the alternate allele contains Yakima Yellow® dye (Elitech Group), which has similar excitation and emission wavelengths to VIC dye and can be identified using the VIC channel without additional instrument calibration.

   c. Assign ROX as passive reference if rhAmp Reporter Mix with reference dye is used.
   d. Assign no-template control to appropriate wells.
2. Perform an allelic discrimination plate read.
3. Analyze the plate-read file.
4. Review the automatic allele calls.
5. Convert allele calls to genotypes.

F. Analyze data for triallelic assays

Genotypes for SNPs with 3 alleles can be obtained with a pair of biallelic rhAmp SNP Assays that each interrogate the reference allele and one alternate allele. To quickly identify the individual assays in the triallelic pair, the assay IDs incorporate the alternate allele in each tube. For example, assay ID rs72558186.A.1 tests for the A alternate allele, and rs72558186.C.1 tests for the C alternate allele.

The 2 assays used to assess the triallelic SNP are run separately, as described in this protocol. Use the following steps to analyze triallelic SNP assays:

1. Turn off the auto-call feature in the analysis software.
2. For each assay, call genotypes manually (Figure 1: blue, green, and orange circles), and assign samples exhibiting low or no amplification with an "undetermined" call (Figure 1: gray crosses).

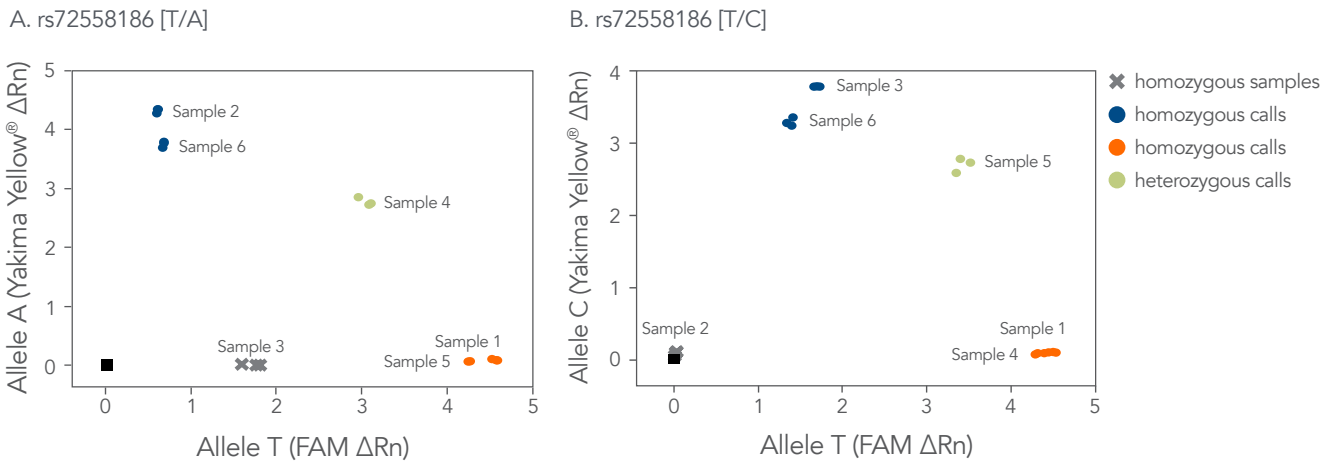

Figure 1. Allelic discrimination plots for rhAmp ADME SNP Assays targeting the triallelic SNP, rs72558186. The assay pair for rhAmp ADME SNP Assay targeting (**A**) SNP rs72558186 [T/A] and (**B**) SNP rs72558186 [T/C] was run as described in this protocol. gBlocks Gene Fragments (1000 copies/reaction) were used as sample template, with equal amounts of 2 different templates mixed together to mimic heterozygous samples. Reactions were performed on the QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific) with manual calling of sample genotypes. Gray crosses represent homozygous samples that were not amplified by the respective assays and were manually called "undetermined". Blue and orange circles represent homozygous calls, and green circles represent heterozygous calls. Refer to Table 5 for sample information.

3. Determine correct genotypes by comparing paired assay results for each sample (Table 5).

Table 5. How to determine genotypes of a triallelic SNP using 2 biallelic rhAmp Genotyping Assays*.

Sample	Observation	Manual genotyping calls		Sample genotype
		rhAmp assay for SNP rs72558186 [T/A]	rhAmp assay for SNP rs72558186 [T/C]	
Sample 1	Signal for the reference allele (T) only is identified in both assays	**Homozygous T/T**	**Homozygous T/T**	Homozygous T/T
Sample 2	Signal for the alternate allele (A) only is identified in one assay with little or no amplification in the other assay	**Homozygous A/A**	Undetermined	Homozygous A/A
Sample 3	Signal for the alternate allele (C) only is identified in one assay with little or no amplification in the other assay	Undetermined	**Homozygous C/C**	Homozygous C/C
Sample 4	Signal for the reference allele (T) is identified in both assays and one alternate allele (A) is identified	**Heterozygous T/A**	**Homozygous T/T**	Heterozygous T/A
Sample 5	Signal for the reference allele (T) is identified in both assays and one alternate allele (C) is identified	**Homozygous T/T**	**Heterozygous T/C**	Heterozygous T/C
Sample 6	Signal for the reference allele (T) is not identified, but signal for the alternate alleles (A and C) are identified	**Homozygous A/A**	**Homozygous C/C**	Heterozygous A/C

* These representative results use SNP ID rs72558186 [T/A/C] as an example. The colors used for the manual genotyping call text correspond to the colored data points in Figure 1.

References

1. Beltz K, Tsang D, Wang J, *et.al*. **A High-Performing and Cost-Effective SNP Genotyping Method Using rhPCR and Universal Reporters**. *Advances in Bioscience and Biotechnology*. 2018;09(09):497-512. doi:10.4236/abb.2018.99034

Revision history

Version	Date released	Description of changes
3	February 2023	Formatting updates
2	August 2017	Added instructions for option to dry samples in plates Clarified sample preparation and addition Clarified overview of read-file setup for the allelic-discrimination plate
1	June 2017	Original protocol

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