



Precipio, Inc.

TaqMan SNP Genotyping Protocol

ICE COLD-PCR Product Analysis with TaqMan SNP
Genotyping Assays using the ABI 7900HT System

Table of Contents

TaqMan SNP Genotyping Guidelines (ABI 7900HT System)	2
Technical Support/Questions	8

Copyright © 2018 by Precipio, Inc.

All rights reserved. This Instruction manual or any portion thereof may not be reproduced in any form, or transmitted outside of the recipient's organization in any form by any means — electronic, mechanical, photocopy, recording, or otherwise — or used in any manner whatsoever without the express written permission of the Company.

TaqMan SNP Genotyping Guidelines (ABI 7900HT System)

NOTE: Please follow the TaqMan SNP Genotyping Assays User Guide for PCR Setup and Analysis.

IMPORTANT! To avoid cross contamination, set up TaqMan SNP Genotyping reactions in an area or room that is separated from DNA extraction and the designated pre-PCR areas. Good laboratory practices, especially cleaning the bench and pipettes after reaction setup, are also required to avoid contamination.

IMPORTANT! The following procedures are optional but highly recommended prior to PCR setup:

- Turn on UV light inside hood or a UV crosslinker.
- Prior to preparing Master Mixes, UV crosslink all empty Master Mix tubes. Also UV crosslink 1.7 mL tubes containing appropriate volume of Molecular Biology Grade Water needed for Master Mix preparation. These tubes should be UV irradiated for 10 min.

Make sure all work areas are prepared for analysis of low level mutations. This includes correct use of the PCR Workstation, dedicated pipettes, tips, 10% bleach solution and/or DNA Away™ solutions.

IMPORTANT! TaqMan SNP Genotyping assays need to be diluted to 20X in Low TE prior to performing the real-time PCR assay.

1. Preparation of Template DNA for TaqMan SNP Genotyping Analysis
 - a. Make a 1:200 dilution by adding 199 μL water into the wells of a clean, labelled 96-well plate. Add 1 μL of ICE COLD-PCR product from the ICE COLD-PCR plate to the water in the corresponding wells. Maintain the original plate layout. Cap the wells and mix well by vortexing for ~10 sec. Spin down briefly.
2. TaqMan SNP genotyping after ICE COLD-PCR
 - a. Remove the TaqMan SNP Genotyping Assay from freezer, thaw on ice, vortex each for 10 sec, and spin the tubes briefly.
 - b. Remove the 2X TaqMan Master Mix from refrigerator, mix by inverting the tube and briefly spin it down. Keep it on ice.

NOTE: Keep TaqMan reagents in the dark until ready to use. Add them last to reaction mixtures. Once added, keep the plate in the dark until the plate is disposed of following run on the ABI 7900HT. Minimize freeze-thaw cycles.

- c. Prepare reaction mix for each target, using **Table 1** as a guide.

Table 1: Master Mix calculations for each TaqMan assay.

Components	1X Reaction*
Molecular Biology Grade Water (μL)	6.25
TaqMan Master Mix, 2X (μL)	12.50
TaqMan SNP Genotyping Assay Mix, 20X (μL)	1.25
Total Volume Reaction Mix (μL)	20.00
Volume DNA added to reaction	5.00

**Multiply volumes in this table by the number of samples being tested.*

NOTE: A Master Mix volume slightly greater than this calculation will be required to allow for losses

during pipetting.

- d. Mix the reaction mix tube by pulse vortexing on low and spin the tube briefly.
- a. Aliquot 20 μ L of the reaction mix to the relevant wells of a MicroAmp Optical 96-Well Reaction plate. See **Table 2** for an example plate layout for 24 samples with controls (real plate layout dependent on original ICE COLD-PCR layout).

Table 2: Example template for running **24** samples with controls.

	1	2	3	4
A	SMP01	SMP09	SMP17	WT
B	SMP02	SMP10	SMP18	CTRL1_1per
C	SMP03	SMP11	SMP19	CTRL2_1per ^a
D	SMP04	SMP12	SMP20	CTRL3_1per ^a
E	SMP05	SMP13	SMP21	
F	SMP06	SMP14	SMP22	
G	SMP07	SMP15	SMP23	NTC1
H	SMP08	SMP16	SMP24	NTC2*

^a As supplied in the specific ICE COLD-PCR assay

* NTC2 is a reagent control for the HRM assay. NTC1 is carried over from the ICE COLD-PCR amplification.

- e. To appropriate wells, add 5 μ L of diluted ICE COLD-PCR product from Step 1a or water (NTC2). See **Table 6** for plate layout. Pipette-mix to ensure sample is well mixed into reaction mixture.
 - f. Place MicroAmp Optical Film over plate and seal. Spin the plate briefly to eliminate any air bubbles from the solution.
 - g. Use the MicroAmp Optical Film Compression Pad over the reaction plate while on the 7900HT instrument. Remember the reaction mix is light sensitive so keep the plate in the dark until loaded onto ABI 7900HT.
3. Set up of the ABI 7900HT to perform an Allelic Discrimination Pre-Read Run.

IMPORTANT! AD_Setup-Table file and AQ_Setup-Table file are available for download at www.precipiodx.com to facilitate .sds file setup on ABI 7900HT System. It is recommended to use the files and track the user sample names with relevant setup table file sample names.

- a. System Set Up
 - i. Power on the 7900HT instrument.
 - ii. Power on the computer.
 - iii. Launch the SDS 2.4 software from the icon on the desktop
- b. If AD_Setup-Table file and AQ_Setup-Table file are to be used:
 - i. Make a copy of the downloaded Setup-Table file. Open the copied file in Microsoft Excel. On the "Sample List" sheet, fill in or copy/paste the sample names in the highlighted "Assigned Sample Name" column. Check the resulted plate layout on the right to make sure it is as

expected. Save the "AD_Setup-Table" sheet and "AQ_Setup-Table" sheet separately as Text (Tab delimited, .txt) files. Use these .txt files as AD_Setup-Table file and AQ_Setup-Table later in Step 3 and Step 4, respectively.

- c. If this is the first time to run the assays, create new markers and new detectors.
 - i. To create a new detector, select **Tools**, click on **Detector Manager**. The Detector Manager window opens. Click on **New...** Then type in the name, choose a reporter, the type of quencher, and then select a color to represent the detector (optional). Next, click **Ok**. Click **Done**.
 - ii. To create a new marker, select **Tools**, click on **Marker Manager**. The Marker Manager window opens. Click on **New...** Enter marker name. Leave the Assay ID field blank. Click on the **color** box and choose a color to represent the marker (optional). Next, click on **Allele X Browse** button to open the Detector Manager. Click on the relevant detector. Make Allele X the Wild-Type detector and Allele Y the mutant detector to make sample comparison easier. Next, click on the **Base** drop down list and choose the base that represents allele X and allele Y. Then click **Ok**. Click **Done**.
- d. Open a new plate document, select **File, New Plate Wizard**.
- e. Click on **Allelic Discrimination (AD)** and then click on **Next**.
- f. Choose the plate type that is being used (i.e. 96-Well Clear Plate). Then click on **Blank Document**. If the AD_Setup-Table file is used to set up the AD program and the reaction plate layout is strictly followed **Table 2**, click on **Setup File**, and then **Browse** button to select the AD Setup Table file. Click on **Next**.

NOTE: If the AD_Setup-Table file is used to set up the program, then all samples, markers and detectors have been input into the program, so click **Next** and go to Step 3m.

- g. Enter the samples names by double-clicking in the sample field to activate the cursor. Then type in the sample name. After typing in the name, press **Enter** and a new row will appear. Make sure to press **Enter** after the very last sample row. If a same set of samples was used in an existing plate, click **Existing Plate** to import the existing sample names.
- h. Click on **Marker Manager** and select all necessary markers for the assay(s) and then click on **Copy to Plate Document**. Select **OK** in the pop-up window, then click **Done**.
- i. Once the marker has been added to the plate, click on **Next**.
- j. Assign samples and markers to the appropriate wells. From the plate layout drop-down list, select **Individual Wells**.
- k. Select the well(s) that contain the first sample and then select the **Add Samples and Markers tab**. Then select the appropriate sample and marker that corresponds to that well. In the Task field drop-down list, click on NTC for the NTC1 and NTC2 and Unknown for the controls and samples.
- l. Repeat for all samples.
- m. Select the **Well Inspector** tab to check that all the wells are correctly assigned. Click on **Finish**.
- n. Export the setup table of the file by clicking on **File > Export**. From the Export drop-down list, select Setup table. Then click Save.
- o. **IMPORTANT!** Save the new plate document by clicking on **File > Save**. Save the document as the plate label + _AD (for allelic discrimination) making sure it is a SDS 7900HT Document (*.sds) file. Then click **Save**.
- p. Performing the Pre-Read Run
 - i. Click on **Instrument > Plate Read > Connect > Open/Close**. Then load the plate onto the

instrument tray with the compression pad on top of the plate. Then click on **Open/Close** again followed by **Pre Read**. After the pre-read is finished, click ok. Click **File > Save** to save the data.

4. Perform a Standard Curve Amplification Run.
 - a. Open a new plate document select **File > New Plate Wizard**.
 - b. Click on **Standard Curve (AQ)** and then click on **Next**.
 - c. Select the plate type that is being used (i.e. 96-Well Clear Plate) and then click on **Setup File**. Click **Browse** button to select a Setup Table file. Click on **Next**.

NOTE: If the **AQ_Setup-Table** file is selected here, click on **Next** and skip to Step 4h. If the **Setup Table** file saved in Step 3n is selected, go to next step.

- d. All relevant samples and detectors have been imported in this window. Click on **Next**.
- e. Samples have been automatically assigned to the wells. Now assign markers to the wells. From the plate layout drop-down list, ensure **Individual Wells** is selected.
- f. In the **Add Samples and Detectors** tab, choose the appropriate detectors that pertain to the selected well(s). In the Task field drop-down list, click on NTC for the Non-Template controls and Unknown for the controls and samples.
- g. Repeat as necessary until all wells have been assigned appropriate detectors and tasks.
- h. Select the **Well Inspector** tab to check that all the wells are correctly assigned. Click on **Finish**.
- i. **IMPORTANT!** Save the new plate document by clicking on **File > Save**. Save the document using appropriate identifying information, making sure it is a SDS 7900HT Document (*.sds) file. Then click **Save**.
- j. Performing an Amplification Run:
 - i. In the Standard Curve (AQ) plate document that was created, click **Setup**.
 - ii. Verify that the Passive Reference setting is **ROX** dye.
 - iii. Select the **Instrument > Thermal Cycler** tabs.
 - iv. Select **Standard** mode.
 - v. Enter 25 µL as the sample volume, if 96-well reaction plate is used.
 - vi. Select the **Thermal Profile** tab.
 - vii. The following thermal cycler conditions should be used (**Table 3**).

Table 3: Thermal cycler program to be used for the TaqMan assay.

	Temperature	Time	Cycles
Polymerase Activation	95 °C	20 sec	1
PCR Amplification	95 °C	3 sec	40
	Ta* °C	20 sec	

* Specific targets may require a different annealing temperature.

- viii. Select **Real-Time** tab.
- ix. Select **Connect to Instrument**.
- x. Select **Start Run**.
- xi. Once the reaction is completed, select **File > Save** and close the plate document.
- k. Performing the Post-Read

- i. Select **File > Open**. Find the previously saved Allelic discrimination (AD) plate document and open it.
- ii. Click on **Instrument > Plate Read > Connect > Post Read**.
- iii. After the run, the status values and buttons are grayed-out, the Analysis button is enabled, and a message indicates whether or not the run was successful. When the run is complete click on **Open/Close** to eject the reaction plate.
- iv. Select **File > Close** to save the raw data; or, go directly to the next step to analyze the run.

5. Data Analysis using TaqMan Genotyper Software (V. 1.3)

NOTE: This software is available for download via ThermoFisher Scientific at:

<https://www.thermofisher.com/us/en/home/technical-resources/software-downloads/TaqMan-genotyper-software.html>

a. Create a study

- i. Click **Create Study** in the home window of the software.
 1. Enter the study properties
 - a. In the Properties dialog box, enter a study name, select an instrument type, then select “Endpoint+pre-PCR read” as the genotyping experiment type

OPTIONAL: Enter a description of the study and/or enter comments.

- ii. Import experiments into the study
 1. In the Workflow Menu pane, select **Experiments** to open the Experiments screen.
 2. Click **Import**, browse to and select the Allelic Discrimination experiment(s) of interest, then click **Import**.
- iii. Set the analysis settings
 1. In the toolbar, click **Analysis Settings** to open the Analysis Settings dialog box.
 2. Select the **Call Settings** tab, select **Autocalling** as the call method. Uncheck “Use Reference Panels for Autocalling”. Uncheck “Use Hardy-Weinberg for Analysis”.
 3. Select the **Control Identifiers** tab, select **Override Control Settings from Experiments**, then type in “NTC1, NTC2” for **NTC (for all Assays)** and “WT” for the **Positive Controls VIC/VIC**. A drop down list with sample names will be created when names are typed in. It can be used to select relevant controls.
 4. Click **QC Settings** tab. Uncheck “Assay Call Rate Low”. Uncheck “Experiment Call Rate Low”. Uncheck “Sample Call Rate Low”. These QC flags are not relevant to the study.
 5. Click **OK** to save the changes and close the dialog box.

b. View and analyze the data

- i. In the Workflow Menu pane, select **Analysis** to open the Results screen.
- ii. View the data in the scatter plot. In the **Assays** panel, select specific assay to be viewed. The blue and green dots indicate samples with mutations targeted by the assay. Red dots indicate samples that are Wild-Type at the nucleotide targeted by the assay.
- iii. View the data in the **Results** table. If needed, you can bookmark wells, omit wells, and/or enter comments about the wells.

NOTE: Since the TaqMan SNP Genotyping analysis is done on ICE COLD-PCR amplified DNA samples with enriched variant(s), the heterozygosity/homozygosity analysis is not an accurate representation of the original mutant frequency. Consider both VIC/FAM and FAM/FAM calls as a positive variant call.

- iv. Click **Save**.
- v. View the data in the Quality Control screen
 1. In the Workflow Menu pane, select **Quality Control** to open the Quality Control screen.
 2. Select the **Flag Summary** tab. Look for any flags that were generated by the analysis. **Table 4** shows a list of flag descriptions and recommended actions.
 3. Any wells with quality concerns can be bookmarked. To bookmark a sample, highlight the sample in the **Samples** panel, then highlight the relevant assay in the **Results** panel and click **Bookmarks > Set Bookmarks**. The bookmarks are now associated with the sample in other screens too.

Table 4: Flag descriptions and recommended actions when using the Genotyper Software.

QC Flag	Description	Action
Failed Control	A Failed Control flag can be raised for any data point that is identified as a control: NTC, or Positive Control. If the user-assigned control identifier for a data point is inconsistent with the call that would be assigned by the software algorithm to an Unknown with the same FAM™ and VIC® dye intensities, a flag is raised.	Review the flagged data point. Note: This may be an indication of wrongly assigned controls or cross-contamination of the controls, including NTC or Positive Control. Track this for further troubleshooting.
Genotype Quality Low	A Genotype Quality Low flag can be raised for any data point that is identified or tasked as an Unknown. If the quality value assigned by the software algorithm for a data point is below the threshold, a flag will be raised.	Review the flagged data point and determine if the data point is an outlier or located in acceptable coordinates. You can manually assign a call or modify the quality value threshold to include the data point.
Low ROX™ Intensity	A Low ROX™ Intensity flag can be raised for any data point. If the ROX™ dye intensity determined by the software for a data point is below the threshold, a flag will be raised.	No action should be taken for these data points. If the ROX dye intensity is below the default threshold, the data point does not meet the minimum conditions for assigning a call. This may be a reaction issue.
NTC FAM™ Intensity High	An NTC FAM™ Intensity High flag can be raised for any data point that is identified or tasked as an NTC. If the FAM™ dye signal intensity for a data point tasked as NTC is greater than the threshold, a flag will be raised.	Review the flagged data point and determine if the high signal is acceptable or not. Note: If the coordinates of the NTC data point are located next to Unknown data points, this could indicate experiment cross-contamination, or an amplifying NTC. Track this for further troubleshooting.
NTC VIC® Intensity High	An NTC VIC® Intensity High flag can be raised for any data point that is identified or tasked as an NTC. If the VIC® dye signal intensity for a data point tasked as NTC is greater than the threshold, a flag will be raised.	Review the flagged data point and determine if the high signal is acceptable or not. Note: If the coordinates of the NTC data point are located next to Unknown data points, this could indicate experiment cross-contamination, or an amplifying NTC. Track this for further troubleshooting.

Refer to the [TaqMan® Genotyper Software Getting Started Guide \(PN 4448637\)](#) for any other operations such as data export and transfer.

Technical Support/Questions

For any questions regarding the kit or IFU, please contact us at Techsupport@preciptdx.com or call 1-203-787-7888 ext. 509.