Precipio, Inc.

Instructions for Use

EGFR Exon 20 Mutation Analysis using ICE COLD-PCR with Real-Time PCR Detection

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Reagent Preparation

All reagents supplied with this Kit are ready to use. Some components will need to be thawed, vortexed and/ or spun in a microcentrifuge before use; check details in Assay Procedure below. Reagents do need to be combined to produce Master Mixes and reaction mixtures; full details are given in the Procedure below.

Kit Components and Storage Conditions

The EGFR Exon 20 Kit contains the components to perform ICE COLD-PCR amplification with mutation enrichment as well as real time PCR detection for 26 total samples with controls. Recommended storage conditions are listed in Table 1.

Table 1: Kit components with recommended storage conditions.

Reagent, ICE COLD-PCR	Lid Color	Color of Label	Volume Total (µL)	Storage (°C)
Pre-Amplification Primers/RS-Oligo	Clear	Blue	160	-20
Pre-Amplification 2X Polymerase Master Mix	Clear	Green	800	-20
Wild Type Control	Clear	White	15	-20
1% T790M Control	Clear	Red	15	-20
1% C797S TA Control	Clear	Red	15	-20
1% C797S GC Control	Clear	Red	15	-20
Reagent, TaqMan	**Lid Color**	**Color of Label**	**Volume Total (µL)**	**Storage (°C)**
Taqman Assay - C797S G>C	Blue	White	40	-20
Taqman Assay - C797S T>A	Yellow	White	40	-20
Taqman Assay - T790M	Orange	White	40	-20
2X GTXpress Master Mix	Green	White	1200	4

The kit contains enough reagents to perform 32 total analyses. For optimal usage of the kit, it is suggested that 26 samples are run/batch along with 1 set of controls. In addition, running with this batch size further improves the ability for automated real-time calling for the mutations detected in this kit.

Notes for this assay

- The ICE COLD-PCR assay has been optimized using the **Bio-Rad C1000** thermal cycler. In addition, the **ABI Veriti** thermal cycler has been tested and provided comparable results.
- The real-time PCR assays have been optimized using the **ABI 7900HT** instrument. It is expected that the **ABI 7500** instrument would give comparable results.

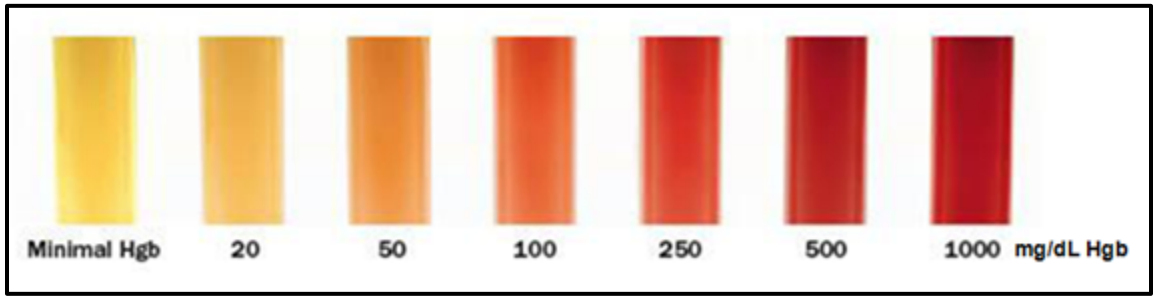
Plasma DNA Extraction Protocol (NOT INCLUDED IN KIT)

IMPORTANT! Use dedicated area/room for DNA extraction to avoid contamination from post-PCR products.

This protocol is used for purifying circulating DNA from plasma/serum using the QIAamp® Circulating Nucleic Acid Kit from Qiagen and was adapted from the QIAamp® Circulating Nucleic Acid Handbook.

1. Prepare buffers/reagents according to the Kit User Manual.
2. Starting From blood samples (Cell-Free DNA BCT Streck Tubes)
   a. Gently invert sample 10 times.
   b. Centrifuge samples at room temperature at 1600 x g (RCF) for 20 min.
   c. Transfer the top plasma layer carefully without disturbing the buffy coat into a clean, labeled centrifuge tube/vial.
   d. Centrifuge samples at room temperature at 16000 x g (RCF) for 10 min to remove residual cells.
   e. Determine the grade of hemolysis for the sample. If the sample has greater than 100 mg/dL Hgb, it may affect the downstream PCR amplification. See Figure 1 below for hemolysis grading.

      Figure 1. Hemolysis chart for centrifuged plasma.



   f. Determine the extraction volume to be used (1-5 mL) for the individual sample(s). Ideally, 3-4 mL is the preferred sample volume for extraction. If 3-4 mL of plasma is not available, recover as much as possible without disturbing the cell pellet
   g. Depending on the extraction volume determined, transfer 1-5 mL of plasma to a clean, labeled centrifuge tube without disturbing the cell pellet. Discard tube containing any residual cell pellets. Freeze any unused plasma at -80°C for storage, as appropriate.

3. DNA Isolation

IMPORTANT! Round sample volume up to the nearest milliliter to determine reagent volumes, not to exceed 5 mL

a. Table 2 shows the expected final lysate volume expected with the QIAgen extraction kit:

Table 2. Final lysate volume for differing amounts of input plasma.

Volume Plasma	1 mL	2 mL	3 mL	4 mL	5 mL
Final Lysate Volume	3.7 mL	7.4 mL	11.1 mL	14.8 mL	18.5 mL

b. Add 100 µL Qiagen Proteinase K (PK) per mL of plasma extracted to each sample.

c. Prepare a master mix consisting of 795 µL Buffer ACL and 5 µL Carrier RNA (at 0.2 µg /µL) per mL of plasma extracted (1 mL= 1 reaction). When calculating for the total reactions, add 1 extra reaction per every 10 reactions to account for pipette loss. Invert the master mix 10 times to combine all reagents.

d. Add 800 µL of the Buffer ACL/Carrier RNA master mix per mL of plasma extracted and pulse-vortex for 30 sec.

e. Incubate at 60°C for 1 hour in a water bath.

f. During the incubation, prepare the QIAvac 24 Plus Vacuum Manifold and vacuum pump for samples.

g. Immediately following incubation, add 1.8 mL of Buffer ACB per mL of plasma extracted to the lysate and pulse-vortex for 15-30 sec.

NOTE: Add Buffer ACB within 2 min after removing samples from water bath to avoid clogging of the filter.

h. Incubate on ice for 5 min.

i. Transfer the lysate-Buffer ACB mixture into the tube extender by carefully pouring into the sterile, labeled column extender. Turn on vacuum pump and let all the lysate be drawn through the column. Close the VacValves of the finished samples if the flow rates differ significantly between samples. Turn off the vacuum and release the pressure to 0 mbar. Carefully remove and discard the tube extender.

Alternatively: Transfer 700 µL lysate to the spin column and centrifuge 1 min at 6000 x g (RCF). Discard collection tube and place spin column in a clean collection tube. Repeat until all lysate has passed through the column.

j. Add 600 µL of Buffer ACW1 to QIAamp Mini Column. Leaving the column lid open, turn on the vacuum pump until all the liquid has been drawn through the column. Turn off the vacuum pump

and release the pressure to 0 mbar.

Alternatively: Centrifuge 1 min at 6000 x g (RCF). Discard collection tube and place spin column in a clean collection tube.

k. Add 750 µL of Buffer ACW2 to QIAamp Mini Column. Leaving the column lid open, turn on the vacuum pump until all the liquid has been drawn through the column. Turn off the vacuum pump and release the pressure to 0 mbar.

Alternatively: Centrifuge 1 min at 6000 x g (RCF). Discard collection tube and place spin column in a clean collection tube.

l. Add 750 µL of 100% Ethanol to QIAamp Mini Column. Leaving the column lid open, turn on the vacuum pump until all the liquid has been drawn through the column. Turn off the vacuum pump and release the pressure to 0 mbar.

Alternatively: Centrifuge 3 min at 6000 x g (RCF).

m. Close the column lid and place the QIAamp Mini Column into a clean collection tube.
n. Centrifuge for 1 min at 20,000 x g (RCF). Discard collection tube and place column into a clean collection tube.
o. Open column lid and incubate at 56°C for 10 min on a heat block to dry the membrane.
p. Place the QIAamp Mini column into a clean, labeled 1.5mL elution tube and discard the collection tube.
q. Add 30 µL of Buffer AVE to the center of the QIAamp Mini Column membrane and incubate at room temperature for 5 min.
r. Centrifuge for 1 min at 20,000 x g (RCF).

Optional: If second elution is desired, remove the spin column from the elution tube and place into another clean, labeled 1.5 mL elution tube. Repeat steps III. 15. and 16. Then continue to step 18.

s. Remove the spin column from the elution tube and discard the spin column.
t. Perform KAPA hsDNA real time PCR, Qubit or equivalent to determine the concentration of dsDNA.
u. Store extracted DNA appropriately (4°C if using immediately; -20°C if using within 1 month).

ICE COLD-PCR Protocol

IMPORTANT! Use dedicated hood/room for ICE COLD-PCR reaction setup to avoid contamination from post-PCR products.

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.

1. Remove PCR Primer/RS-Oligo Mix and 2X Phusion Master Mix from freezer and thaw on ice.
2. Once thawed, vortex all kit components ~10 seconds to mix thoroughly. Briefly centrifuge ~10 seconds to ensure no liquid remains on tube lids and place on ice.
3. Experiment layout: Follow Table 3 for layout to simplify later steps of the procedure. The following 5 controls are required for each setup: **NTC1** (No Template Control #1) and the control DNAs provided by the kits, including **WT** (EGFR wild type), **T790M_1per** (1% EGFR-T790M), **C797S-TA_1per** (1% EGFR_C797S-TA) and **C797S-GC_1per** (1% EGFR_C797S-GC). Additional controls may be added in place of test samples, but keeping the naming system as it is will simplify later steps.

Table 3: Proposed experimental layout for the ICE COLD-PCR for 32 reactions (26 samples plus controls)

	1	2	3	4
A	SMP01	SMP09	SMP17	SMP25
B	SMP02	SMP10	SMP18	SMP26
C	SMP03	SMP11	SMP19	WT
D	SMP04	SMP12	SMP20	T790M_1per
E	SMP05	SMP13	SMP21	C797S-TA_1per
F	SMP06	SMP14	SMP22	C797S-GC_1per
G	SMP07	SMP15	SMP23	NTC1
H	SMP08	SMP16	SMP24	Leave Empty*

* This well will be used for No Template Control #2 (NTC2) in later steps.

4. Use Table 4 as a guide for calculating and preparing the ICE COLD-PCR Master Mix assuming a DNA volume of 15 µL at 3.3 ng/µL. Please note that less total DNA can be added to the reaction.
   a. If the extracted DNA concentration is > 3.3 ng/uL, dilute to a working concentration of 3.3 ng/µL and use 15 µL of the diluted DNA for analysis.
   b. If the extracted DNA concentration is ≤3.3 ng/µL, add 15 µL of extracted DNA for analysis.

Table 4: Calculations for Master Mix preparation

	1X reaction*
UV-treated Molecular Biology Grade Water (µL)	5.0
2X Phusion Master Mix (µL)	25.0
PCR/RS-Oligo Primer Mix (µL)	5.0
Total Volume PCR Master Mix for 1 reaction (µL)	35.0
Volume DNA added to reaction (µL)	15.0

**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.

NOTE: Amount of UV-treated Molecular Biology Grade Water and volume of DNA can be adjusted accordingly.

NOTE: We suggest **fifty** ng of DNA should be used for each PCR reaction in order to potentially detect ≥ 0.1% mutation present in the starting material (minimum 18 ng). The assay has been shown to be robust for DNA input amounts between 1 and 300 ng (using high quality DNA). Up to 20 µL DNA can be used in each PCR reaction with adjustment of volume of water used.

NOTE: If DNA contains contaminants (ethanol carryover, EDTA etc.), increasing DNA volume may decrease PCR efficiency.

5. Label a 1.7 mL-centrifuge tube for PCR Master Mix preparation.
6. Add required volume of molecular biology grade water to Master Mix tube.
7. Add required volume of 2X Phusion Master Mix to Master Mix tube.
8. Add required volume of PCR Primer/RS-Oligo Mix to Master Mix tube.
9. Cap Master Mix tube, vortex for ~10 sec, briefly centrifuge and store on ice until use.
10. Label a 96-well plate with appropriate sample information.
11. Pipette appropriate volume of ICE COLD-PCR Master Mix into applicable wells. If using a repeat pipettor, ensure that there is no spillage or splashing from well to well.
12. To appropriate wells, add required volume of each DNA sample, control, or water (NTC1). Use separate pipette tips for each sample and avoid cross-contamination of the samples by splashing. Securely cap all wells containing DNA samples, controls, and NTC1 with 8-cap strips.

    NOTE: Addition of the kit controls last lessens the chance of contaminating any test sample wells.

13. Vortex (~1/2 speed) for 10 seconds. Centrifuge for ~20 seconds to ensure all solutions are collected at the bottom of wells or tubes. If not, repeat centrifugation.

Thermal Cycler Program for ICE COLD-PCR Enrichment

Use the thermal cycler protocol in Table 5 for ICE COLD-PCR Enrichment.

IMPORTANT! Ensure that the following ramp rates are used depending on the thermal cycler:

- BioRad C1000 Touch: 1.5°C/sec
- BioRad Tetrad II: default (3.0°C/sec)
- Applied Biosystems Veriti: 100%

Table 5: Thermal cycler conditions for ICE COLD-PCR enrichment.

	Temperature	Time	Cycles
Initial Denaturation	98 °C	2 min	1
Amplification	98 °C	10 sec	5
	63 °C	30 sec	
	72 °C	10 sec	
ICE COLD-PCR Amplification Step Up	98 °C	10 sec	5
	69 °C	30 sec	
	73 °C, +0.2 °C/cycle	30 sec	
	63 °C	30 sec	
	72 °C	10 sec	
Main ICE COLD-PCR Amplification	98 °C	10 sec	45
	69 °C	30 sec	
	74 °C	30 sec	
	63 °C	30 sec	
	72 °C	10 sec	
ICE COLD-PCR Amplification	98 °C	10 sec	5
	69 °C	30 sec	
	78 °C	30 sec	
	63 °C	30 sec	
	72 °C	10 sec	
Final Extension	72 °C	5 min	1
Hold	12 °C	∞	

NOTE: Samples can be stored at -20 °C post amplification.

Quality Control of ICE COLD-PCR Products (optional)

1. Analyze an aliquot of ICE COLD-PCR product alongside an aliquot of 100-bp DNA mass ladder on a 2% agarose gel to verify amplification of DNA.
2. Only a single band corresponding to the main ICE COLD-PCR product should be observed.

3. If multiple bands are present check quality of input DNA.
4. If no product is observed, ensure quality of input template DNA was sufficient.
5. No ICE COLD-PCR products should be visible in No Template Control sample.
    a. If DNA products of the expected product size are visible in No Template Control sample, contamination is likely and sample should not be taken to sequence analysis.

Real-Time PCR Step-by-Step Instructions using the ABI 7900HT System

IMPORTANT! To avoid cross contamination, set up real time PCR 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.

1. Preparation of Template DNA for Real Time PCR 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. Real Time PCR after ICE COLD-PCR
    a. Remove the three Taqman assays from freezer, thaw on ice, vortex each for 10 sec, spin the tubes briefly.
    b. Remove the 2X Taqman GTXpress Master Mix from fridge, mix by inverting the tube and briefly spin it down. Keep it on ice.

        NOTE: Keep Taqman assays in the dark until ready to use and 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 of the three targets, using Table 6 as a guide.

Table 6: Master Mix calculations for each Taqman assay.

	1X Reaction*
Molecular Biology Grade Water (µL)	6.25
2X Taqman GTXpress Master Mix (µL)	12.50
Assay Mix, 20X (µL)	1.25
Total Volume real time PCR Master Mix for 1 reaction (µL)	20.00
Volume DNA added to reaction	5.00

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

NOTE: See Table 7 for recommended plate layout. Following this layout will allow simplification of real time PCR program setup on ABI 7900HT later by applying AD_Setup-Table and AQ_Setup-Table files available for download at www.precipiodx.com. However, it is not required.
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.
e. Aliquot 20 µL of the reaction mix to the relevant wells of a MicroAmp Optical 96-well Reaction Plate. See Table 7 for plate layout.
f. To appropriate wells, add 5 µL of diluted ICE COLD-PCR product from Step 1a, or water (NTC2). See Table 7 for plate layout. Pipette-mix to ensure sample is well mixed into reaction mixture.
g. Place MicroAmp Optical Film over plate and seal. Spin the plate briefly to eliminate any air bubbles from the solution.
h. 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.

Table 7: Example template for running 26 samples with controls.

	Assay EGFR_T790M				Assay EGFR_C797S-TA				Assay EGFR_C797S-GC			
	1	2	3	4	5	6	7	8	9	10	11	12
A	SMP01	SMP09	SMP17	SMP25	SMP01	SMP09	SMP17	SMP25	SMP01	SMP09	SMP17	SMP25
B	SMP02	SMP10	SMP18	SMP26	SMP02	SMP10	SMP18	SMP26	SMP02	SMP10	SMP18	SMP26
C	SMP03	SMP11	SMP19	**WT**	SMP03	SMP11	SMP19	**WT**	SMP03	SMP11	SMP19	**WT**
D	SMP04	SMP12	SMP20	**T790M_1per**	SMP04	SMP12	SMP20	**T790M_1per**	SMP04	SMP12	SMP20	**T790M_1per**
E	SMP05	SMP13	SMP21	**C797S-TA_1per**	SMP05	SMP13	SMP21	**C797S-TA_1per**	SMP05	SMP13	SMP21	**C797S-TA_1per**
F	SMP06	SMP14	SMP22	**C797S-GC_1per**	SMP06	SMP14	SMP22	**C797S-GC_1per**	SMP06	SMP14	SMP22	**C797S-GC_1per**
G	SMP07	SMP15	SMP23	**NTC1**	SMP07	SMP15	SMP23	**NTC1**	SMP07	SMP15	SMP23	**NTC1**
H	SMP08	SMP16	SMP24	**NTC2**	SMP08	SMP16	SMP24	**NTC2**	SMP08	SMP16	SMP24	**NTC2**

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 listed in Table 8. **IMPORTANT!** Follow Table 8 exactly to simplify later steps. If these markers and detectors have previously been created, skip to step 3c.

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 (See Table 8 for detector information), choose a reporter, the type of quencher, and then select a color to represent the detector (optional). Next, click **Ok**. Repeat the above to create all 6 detectors in Table 8. 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 (See Table 8 for maker information). 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 (See Table 8). Next, click on the **Base** drop down list and choose the base that represents allele X and allele Y. Then click **Ok**. Repeat the above to create all 3 markers in Table 8. Click **Done**.

Table 8: Creating the markers and detectors for the EGFR Exon 20 real time PCR assay.

	Allele X Detector				Allele Y Detector			
Marker Name	**Allele/ Detector Name**	**Base Name**	**Reporter**	**Quencher**	**Allele/ Detector Name**	**Base Name**	**Reporter**	**Quencher**
EGFR_T790M	T790-WT	G*	VIC	Non Fluorescent	T790M	A*	FAM	Non Fluorescent
EGFR_C797S-TA	C797-WT-TA	T	VIC	Non Fluorescent	C797S-TA	A	FAM	Non Fluorescent
EGFR_C797S-GC	C797-WT-GC	G	VIC	Non Fluorescent	C797S-GC	C	FAM	Non Fluorescent

*: The G>A base change of Marker EGFR_T790M represents the nucleotide change in detection probes, which complements the C>T change in EGFR cDNA.

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 (eg. 96 Wells 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 strictly followed Table 7, 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 3n.

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 (eg. 96 Wells 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 3m 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 9).

Table 9: Thermal cycler program to be used for all Taqman assays.

	Temperature	Time	Cycles
Polymerase Activation	95 °C	20 sec	1
PCR Amplification	95 °C 63 °C	3 sec 20 sec	40

    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 real time PCR 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 **Flags Summary** tab. Look for any flags that were generated by the analysis. Table 10 shows a list of flag descriptions and recommended actions taken from *TaqMan® Genotyper Software Getting Started Guide* (PN 4448637).

Table 10: 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.

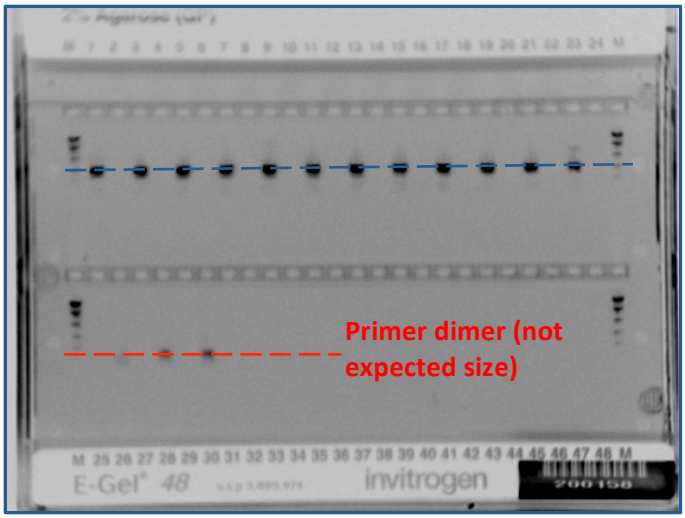
3. Any wells with quality concerns can be bookmarked. To bookmark a sample, highlight the sample in the **Samples** panel, then highlight the relevant assay in the **Results** panel and click **Bookmarks** > **Set Bookmarks**. The bookmarks are now associated with the sample in other screens too.

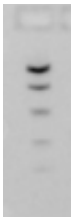
vi. Refer to the *TaqMan® Genotyper Software Getting Started Guide* (PN 4448637) for any other operations such as data export and transfer.

Example Data:

The following data was collected using the Bio-Rad C1000 for the ICE COLD-PCR enrichment and the ABI 7900HT for the real-time PCR analysis.

Figure 2. Gel electrophoresis QC of 3 separate lots of kit components. A single band at the expected EGFR Exon 20 amplicon size is shown for each of the sample ICE COLD-PCR amplification. The no template controls shows no amplification at the expected size of the EGFR Exon 20 amplicon.

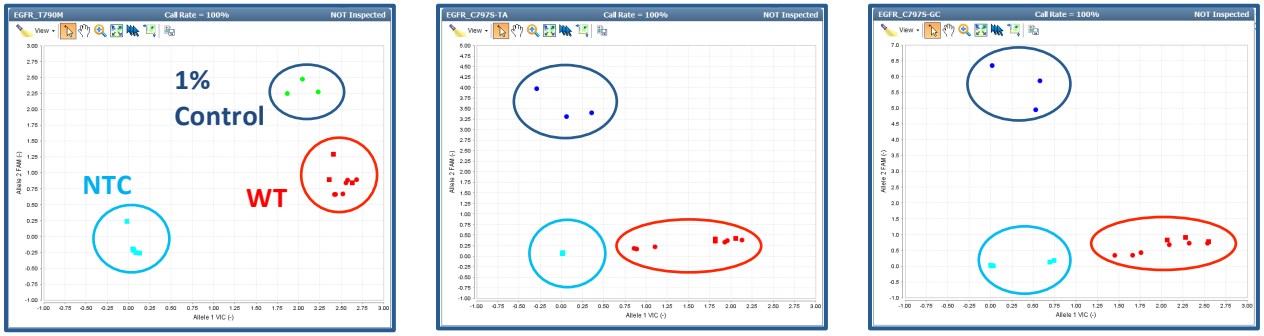




Band 1	2000 bp
Band 2	800 bp
Band 3	400 bp
Band 4	200 bp
Band 5	100 bp

Lane 1	Lot 1, 1% T790M Control
Lane 3	Lot 1, 1% C797S T>A Control
Lane 5	Lot 1, 1% C797S G>C Control
Lane 7	Lot 1, Wild Type Control
Lane 9	Lot 2, 1% T790M Control
Lane 11	Lot 2, 1% C797S T>A Control
Lane 13	Lot 2, 1% C797S G>C Control
Lane 15	Lot 2, Wild Type Control
Lane 17	Lot 3, 1% T790M Control
Lane 19	Lot 3, 1% C797S T>A Control
Lane 21	Lot 3, 1% C797S G>C Control
Lane 23	Lot 3, Wild Type Control
Lane 26	Lot 1, No Template Control
Lane 28	Lot 2, No Template Control
Lane 30	Lot 3, No Template Control

Figure 3: Real-time PCR results for the three assays, 3 separate lots. Automated calling was used to generate the results.



Technical Support/Questions:

For any questions regarding the kit or IFU, please contact us at Techsupport@precipiodx.com or call 1.203.787.788 ext. 509.