



Precipio, Inc.

Instructions for Use

**Heme Panel Screening for Myeloproliferative Disorders using High Resolution Melting as a Pre-Screening Tool –
RESEARCH USE ONLY**

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Manufacturer

This Kit was manufactured by Precipio, Inc. at 8813 F Street, Omaha, NE 68127, USA.
USA Telephone 1-203-787-7888

Intended Use

For professional use only. Precipio's Heme Panel offerings are *in vitro* diagnostic assays that screen for clinically actionable somatic mutations in targets of interest related to myeloproliferative disorders. This Research Use Only (RUO) kit is specifically designed to screen for mutations using High Resolution Melt Analysis in JAK2, MPL and CALR genes that are of clinical relevance (see below). This kit is designed to be used in a clinical diagnostic laboratory by suitably trained personnel testing DNA extracted from whole blood or bone marrow samples using a PCR-based method, High Resolution Melt (HRM) Analysis, and Sanger Sequencing.

1. MPL Exon 10:
 - a. c.1544G>T; W515L
 - b. c.1543_1544TG>AA; p. W515K
2. CALR Exon 9:
 - a. c.1092_1143del; p.L367fs*46
 - b. c.1154_1155insTTGCC; p.K385fs*47
3. JAK2 Exon 14:
 - a. c.1849G>T; p.V617F
4. JAK2 Exon 12 deletions:
 - a. c.1611_1616delTCACAA; p.F537_K539delinsL
 - b. c.1624_1629delAATGAA; p. N542_E543del
 - c. c.1615_1616delAAinsTT; p.K539L

This Kit is supplied as a single box containing the components indicated below.

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 Heme Panel 1 RUO Kit contains the components, including Wild-Type controls to perform PCR amplification followed by High Resolution Melt Analysis for 24 total samples.

NOTE: Research Use Only Kits contain enough reagents for one mutation positive control reaction for each amplicon for either batch size (not supplied in Kit).

Recommended storage conditions are listed in **Table 1**.

Table 1: Kit components with recommended storage conditions.

Reagent	Lid Color	Color of Label	Number of Tubes	Volume Total (μL)	Storage (°C)
MPL Exon 10 Primer Mix	Orange 	White	1	360	-20
CALR Exon 9 Primer Mix	Green 	White	1	360	-20
JAK2 Exon 12 Primer Mix	Yellow 	White	1	360	-20
JAK2 Exon 14 Primer Mix	Blue 	White	1	360	-20
2X HRM Master Mix	Black 	White	2	900	-20
Wild-Type Control (Cell Line)	Violet 	White	1	66	-20

Primary Sample Collection, Handling and Storage

This kit can be used with the following:

1. DNA extracted from whole blood or bone marrow samples
2. Cell lines

Required Reagents, Not Supplied

1. MeltDoctor™ HRM Calibration Plate, Fast 96-well, Thermo Fisher Scientific, Catalog Number 4425618

Required Materials, Not Supplied

1. MicroAmp™ Optical Adhesive Film
2. MicroAmp Optical 96-Well Reaction Plate
3. Pipettors and pipette tips
4. Vortexer
5. Centrifuge
6. Microcentrifuge tubes

Notes for this Assay

- This assay has been optimized using the ABI 7500 Fast Real-Time PCR System, other platforms might require further optimization per the platform specifications.
- Due to heterogeneity associated with tumors, blood or bone marrow samples may contain normal cells as well as Wild-Type and mutant tumor cells.
- The mutation detection range for each of the variants listed above was determined to be ~10 – 100% in our laboratory when coupling HRM to Sanger Sequencing; however, sample processing differences and instrument calibration/variations may affect overall range.
- Only the DNA polymerase supplied with this kit should be used as indicated for the HRM assay.

Principles of High Resolution Melting

High Resolution Melting (HRM) is a screening technique which allows the user to determine if the PCR fragment contains a variant or not based upon the dissociation of double-stranded DNA in the presence of an intercalating dye as the temperature increases. As the temperature increases, changes in the fluorescence due to the amplicon dissociation are monitored real-time. It is expected that fragments containing a variant will have slightly different fluorescence profiles than that of Wild-Type DNA which allows for the entire amplicon to be screened for mutations.

Since this is a scanning technique and does not identify an exact mutation, any variant present in the PCR product may differentiate from the Wild-Type DNA, thus leading to a mutation positive result. Because HRM is a highly sensitive assay, factors such as pipetting errors, cytosine deamination and polymerase errors may give rise to these false positives. Any positive HRM result must be confirmed by a sequencing methodology.

Other considerations:

1. It is recommended that all samples and controls are extracted with the same protocol to avoid any variations due to reagents/buffers.
2. All DNA samples should be diluted to the same starting concentration (10 ng/μL) with the same dilution buffer.
3. Ensure sample to sample uniformity.
4. Any additional control DNA to be analyzed should be of the same quality and quantity as the sample DNA.
5. Limit of Detection of Sanger sequencing is ~10% depending on the quality of the sequencing (cleanup method, array lifetime, etc.).
6. Guidance for HRM data analysis is given in this IFU; however, due to instrument-to-instrument variation,

calibration variation, as well as other factors, data analysis parameters for a given data set will need to be determined by the end-user.

Amplification Protocol

NOTE: For the standard HRM protocol, refer to [Applied Biosystems High Resolution Melting Getting Started Guide](#) (Part Number 4393102, Rev. D).

NOTE: Calibrate the 7500 Fast system according to standard protocol ([Chapter 1](#)) of the [Applied Biosystems High Resolution Melting Getting Started Guide](#) for the MeltDoctor™ HRM Dye if this is the first time performing HRM experiments.

NOTE: Please review [Chapter 4](#) (Perform a HRM Mutation Scanning Experiment) of the [Applied Biosystems High Resolution Melting Getting Started Guide](#) for a general overview of the HRM protocol.

IMPORTANT! To avoid cross contamination, set up HRM reactions in 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.
1. Remove 2X HRM Master Mix, controls and Primer Mixes from freezer and thaw on ice.
 2. Once thawed, vortex controls and Primer Mixes ~3 - 5 sec to mix thoroughly. Briefly centrifuge 5 sec to ensure no liquid remains on tube lids and place on ice.
 3. Mix the 2X HRM Master Mix by inverting the tube and briefly spin it down. Keep it on ice.

NOTE: Keep 2X HRM Master Mix in the dark until ready to use. Once added, keep the plate in the dark until the plate is disposed of following run on the ABI 7500 Fast system and sequence conformation. Minimize freeze-thaw cycles.

4. **Example experiment layout:** Follow **Table 2** for the proposed plate layout.

NOTE: Additional controls may be added in place of test samples.

Table 2A: Proposed experimental layout for **21** samples in one batch.

	MPL Exon 10			CALR Exon 9			JAK2 Exon 12			JAK2 Exon 14		
	1	2	3	4	5	6	7	8	9	10	11	12
A	SMP01	SMP09	SMP17	SMP01	SMP09	SMP17	SMP01	SMP09	SMP17	SMP01	SMP09	SMP17
B	SMP02	SMP10	SMP18	SMP02	SMP10	SMP18	SMP02	SMP10	SMP18	SMP02	SMP10	SMP18
C	SMP03	SMP11	SMP19	SMP03	SMP11	SMP19	SMP03	SMP11	SMP19	SMP03	SMP11	SMP19
D	SMP04	SMP12	SMP20	SMP04	SMP12	SMP20	SMP04	SMP12	SMP20	SMP04	SMP12	SMP20
E	SMP05	SMP13	SMP21	SMP05	SMP13	SMP21	SMP05	SMP13	SMP21	SMP05	SMP13	SMP21
F	SMP06	SMP14	WT	SMP06	SMP14	WT	SMP06	SMP14	WT	SMP06	SMP14	WT
G	SMP07	SMP15	MUT*	SMP07	SMP15	MUT*	SMP07	SMP15	MUT*	SMP07	SMP15	MUT*
H	SMP08	SMP16	NTC	SMP08	SMP16	NTC	SMP08	SMP16	NTC	SMP08	SMP16	NTC

Table 2B: Proposed experimental layout for **6** samples in one batch.

	MPL Exon 10		CALR Exon 9		JAK2 Exon 12		JAK2 Exon 14	
	1	2	3	4	5	6	7	8
A	SMP01		SMP01		SMP01		SMP01	
B	SMP02		SMP02		SMP02		SMP02	
C	SMP03		SMP03		SMP03		SMP03	
D	SMP04		SMP04		SMP04		SMP04	
E	SMP05		SMP05		SMP05		SMP05	
F	SMP06		SMP06		SMP06		SMP06	
G	WT		WT		WT		WT	
H	MUT*	NTC	MUT*	NTC	MUT*	NTC	MUT*	NTC

5. Use **Table 3** as a guide for calculating and preparing a Master Mix for each of the 4 targets using a DNA volume of 2 μ L at 10 ng/ μ L.

Table 3: Calculations for Master Mix preparation for each of the targets:

Reagent:	1X reaction*
2X HRM Master Mix (μ L)	10
Primer Mix (μ L)	8
Total Volume PCR Master Mix for 1 reaction (μ L)	18
Volume DNA at 10 ng/ μ L added to reaction (μ L)	2

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

6. Label a 1.7 mL-centrifuge tube for PCR Master Mix preparation for each of the 4 targets.

7. Add required volume of 2X HRM Master Mix to **EACH** Master Mix tube (●).
8. Add Primer Mix:
 - a. Add required volume of MPL Exon 10 Primer Mix to designated Master Mix tube (●).
 - b. Add required volume of CALR Exon 9 Primer Mix to designated Master Mix tube (●).
 - c. Add required volume of JAK2 Exon 12 Primer Mix to designated Master Mix tube (●).
 - d. Add required volume of JAK2 Exon 14 Primer Mix to designated Master Mix tube (●).
9. Cap each Master Mix tube, vortex for ~3-5 sec, briefly centrifuge and store on ice until use.
10. Label a MicroAmp Optical 96-Well Reaction Plate with appropriate sample information.
11. Aliquot 18 µL of the reaction mix to the relevant wells of a MicroAmp Optical 96-Well Reaction Plate.
12. To appropriate wells, add 2 µL of each DNA sample, control(s), or water (NTC).
 - a. Use separate pipette tips for each sample and avoid cross-contamination of the samples by splashing.
 - b. Pipette-mix to ensure sample is well mixed into reaction mixture.

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

13. Place MicroAmp Optical Film over plate and seal. Spin the plate briefly to eliminate any air bubbles from the solution.

NOTE: If it is planned to run the reactions 24 hours after set up, store the plate at 4 °C. Bring the plate to room temperature before running the HRM program.

Amplification and Melting on the ABI 7500

IMPORTANT! HRM_template file is available for download at www.precipiodx.com to facilitate experiment file setup on 7500 Fast System. It is recommended to use the file and track the user sample names with relevant template file sample names. But this is not required.

1. If HRM_Template file is to be used:
 - a. Make a copy of the downloaded template file. Open the copied file in Microsoft Excel. On the "Plate Layout" sheet, fill in or copy/paste the sample names in the 96-well plate area. Check the resulted plate layout to make sure it is as expected. Save the "7500 Template" sheet separately as Text (Tab delimited, .txt) file. Use this .txt file as HRM_template later.
2. In the 7500 Software v2.3, create a new run file for the HRM plate by clicking **New** Experiment in the upper left hand corner. In the Experiment Properties Window, input the desired experiment name and select the following conditions:
 - a. Instrument: 7500 Fast (96 Wells)
 - b. Type of Experiment: Quantitation – Standard Curve
 - c. Reagents: Other
 - d. Ensure that the **Include Melt Curve** box is checked.
 - e. Ramp Speed: Standard (~2 hours to complete a run)
 - f. Leave other conditions as they are.
3. Click **Plate Setup** in the **Setup** pane on the left.
4. Select **File -> Import...** to import the HRM_template file made in earlier step. The relevant sample names and target name are imported now.

5. Select the **Assign Targets and Samples** tab. When importing samples and target from a template file, all empty wells are also assigned a name (0) and a target. It is optional to clear these wells as follows: In the 96-well Plate Layout view, highlight the empty wells and then uncheck the sample name (0) and target from the **Assign target(s) to the selected wells** pane and the Assign sample(s) to the selected wells pane on the left.
6. Click **Run Method** in the **Setup** pane on the left. Revise the run program according to the following chart (Table 4). Keep the reaction volume per well at 20 μ L.

Table 4: Amplification and melt thermal cycler profile.

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95 °C	10 min	100%
Cycling (40 cycles)	Denature	95 °C	15 sec	100%
	Anneal/extend	60 °C	1 min	100%
Melt curve/dissociation	Denature	95 °C	10 sec	100%
	Anneal	60 °C	1 min	100%
	High resolution melting	95 °C	15 sec	1%
	Anneal	60 °C	15 sec	100%

7. Check **Expert Mode** and then click **Select/View Filters...** button. Uncheck Filter - 2 through Filter - 5. Leave Filter – 1 checked only. Click **Ok**.
8. Save the experimental conditions by clicking **File -> Save**.
9. Load plate on instrument, and click **Run**.
10. Once the plate has finished running, analyze using the 7500 software and save.

Review and Analysis of the HRM Melt Curve

1. Review the high-resolution melting data in 7500 software
 - a. Review the Amplification Plot for normal characteristics:
 - i. Fluorescence levels that exceed the amplification threshold between cycles 8 and 35
 - ii. An exponential increase in fluorescence.

NOTE: Document which wells are outliers with CT values that differ from replicates by more than 2. The outliers may produce erroneous HRM results.

- b. Verify that the Dissociation Curve/Melt Curve shows no unexpected T_m peaks.

NOTE: If the sequence you amplified contains more than 1 variant or a complex mutation, you may see more than a single T_m peak

NOTE: Unexpected peaks may indicate contamination, primer dimers, or nonspecific amplification

NOTE: The data may appear noisy because more data points are collected during a high resolution melt curve than during a standard melt curve.

IMPORTANT! Please refer to [Chapter 4 \(Review the high-resolution melting data\)](#) of the HRM Experiments User's Guide ([Using MeltDoctor™ HRM Reagents and High Resolution Melt Software](#))

v3.0*, Part Number 4457847, Rev. B) for complete data analysis workflow. However, suggestions are provided below.

* This is the latest version of the software manual available online. To see the release notes, please go to this site:

https://downloads.thermofisher.com/High_Resolution_Melt_Software_v3_1_Release_Notes_New.pdf

2. Analyzing melt curve file with High Resolution Melt Software v3.1.
 - a. Once the software is opened, click **Open** on the main window to import the saved *.eds run file saved in the previous step.

NOTE: If this is your first time creating an HRM experiment with the HRM software, select the default HRM calibration file at prompt.

- b. Analyze each amplicon/assay separately:
 - i. In the **Plate Layout Pane**, select specific samples to omit by highlighting the samples and then right-clicking to choose **Omit** if multiple targeted regions/amplicons are run on the same plate. This includes empty wells and NTC.
 - ii. To add samples, select specific samples to add by highlighting the samples and then right-clicking to choose **Include**.

NOTE: It is important to omit any of the samples that are not to be analyzed and to only include the samples that are to be analyzed.

- c. Click **Analyze** on the top right of the main window to show the melt plots in the High Resolution Melt Plots pane.
- d. Click **Analysis Settings** on the top right of the main window.

NOTE: Please use the analysis settings as guidance for the melt curve analysis.

- i. Select the check box for **Automatically set the pre- and post-melt regions**.
- ii. Click the **Apply Analysis Settings** button.
- e. Click the **Derivative Melt Curves** tab in the High Resolution Melt Plots pane to review the software's prediction of the pre-and post-melt regions.
 - i. Review and adjust as needed the pre- and post-melt regions to optimize your separation and variant calls. The pre- and post-melt regions should be as close as possible to the melting transition region if any adjustment is performed.
 1. The pre-melt Start and Stop temperature lines (green and red arrows on the left) should be approximately 0.2 to 0.5 °C apart from each other on the left side of the Derivative Melt Curve.
 2. The post-melt Start and Stop temperature lines (green and red arrows on the right) should be approximately 0.2 to 0.5 °C apart from each other on the right side of the Derivative Melt Curve.
 3. After any adjustments, **Analyze** must be selected again.

- f. Variant calls for each sample will be shown in the **Plate Layout view**. Any samples that were not assigned the same call as the Wild-Type control are considered mutation positive or some type of polymorphism.
 - g. Review the **Difference Plot**.
 - i. In the **High Resolution Melt Plots Pane**, select the **Difference Plot** tab.
 - ii. From the **Reference** dropdown list, select a control or any well as the reference, and then review variant clusters and outliers.
 - 1. Upon review of the samples, the variant call can be changed manually if desired.
- NOTE:** Try selecting different reference samples to find the optimal display of the clusters. Changing the reference will not affect variant calls.
- NOTE:** Further information can be found in the [Amplify and melt the DNA](#) and [Review the high-resolution melting data](#) sections in [Chapter 4. Perform an HRM Mutation Scanning Experiment](#) of the [Getting Started Guide](#).
- h. Export the result by clicking **Export** on the left of the main window.

Mutation Confirmation of HRM Positive Samples

- 1. It is recommended that any HRM mutation positive samples be confirmed by a secondary method such as Sanger Sequencing or Real-Time PCR.

Technical Support/Questions

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