



**Instructions for Use:
Bloodhound™ MPN Reagents
RUO**

Please read these Instructions for Use thoroughly before you use this product.

Keep these Instructions for Use for future reference.

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Manufacturer

Manufacturer		Precipio, Inc. 4 Science Park, New Haven, CT 06511 , USA Phone: 1-203-787-7888
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Bloodhound™ MPN Reagents RUO

The Research Use Only (RUO) reagents are supplied as ready to use. This Instructions for Use is available as a download on the Precipio website, <https://www.precipiodx.com/resources/ifus/>.

Intended Use

For professional use only. Precipio's Bloodhound™ offerings are Research Use Only (RUO) assays that detect clinically actionable somatic mutations in targets of interest related to myeloproliferative disorders and haematological cancers. The reagents are designed to be used in a clinical diagnostic laboratory by suitably trained personnel testing DNA extracted from whole blood or bone marrow using a PCR-based method with accompanying Bloodhound™ analysis software. Samples are run in replicate analysis to confirm the presence of a mutation; however, if the exact mutation sequence is required, a sequencing method such as Sanger Sequencing is needed. In addition, if inconsistent results are observed, Sanger Sequencing or another comparable mutation detection technology can be used to confirm the exact mutation type.

Indications for Use

Bloodhound™ MPN Reagents RUO enhance mutation detection relevant to Myeloproliferative Disorders (MPD) or Myelodysplastic Syndrome (MDS). The Bloodhound™ MPN Reagents indicate the presence of a potential mutation within a sample but does not confirm the identity of the mutation. To confirm the precise mutation detected further analysis is required.

Although the results of analysis with this Bloodhound™ MPN Reagents will indicate the mutation status of the sample, results obtained using the Bloodhound™ MPN Reagents should not be used in making clinical decisions.

Biological Reference of MPN Mutations

The Bloodhound™ MPN Reagents have been designed to PCR amplify the relevant regions in *MPL*, *CALR*, and *JAK2* Exons 12, 13, & 14 for subsequent analysis.

Background of the genes chosen for this panel:

Myeloproliferative neoplasms (MPNs) arise from issues stemming from the bone marrow which lead to abnormally high numbers of certain blood cell types in the blood. Classic MPNs include disorders such as polycythemia vera (PV; Red blood cells), essential thrombocythemia (ET; Platelets), and primary myelofibrosis (PMF; Fibers and blasts).¹ Specific variants in the *JAK2*, *CALR*, and *MPL* genes (see above) are useful biomarkers for these diseases as they can play a role in either disease diagnosis or provide information regarding disease prognosis.²⁻⁶ These essentially mutually exclusive variants occur in a relatively high frequency, as 98% of sample with PV and 50-65% of samples with ET or PMF exhibit mutations in *JAK2*. Variants in *CALR* and *MPL* are observed in approximately 20-25% or 5-7% of both ET and PMF samples respectively, with only 10-15% of these samples exhibiting triple-negative morphology.¹ Positive controls have been updated to 2% as recommended by the updated World Health Organization (WHO- V5.0) guidelines for *CALR*, *MPL*, *JAK2* Exon 12 and 13, and 0.5% for *JAK2* Exon 14.⁶

Instructions for Use

The mutations covered in this reagent set are as follows:

MPL Exon 10:	CALR Exon 9:
c.1544G>T; p.W515L	c.1099_1150del; p.L367fs*46
c.1543_1544TG>AA; p.W515K	JAK2 Exon 14:
JAK2 Exon 12:	c.1849G>T; p.V617F
c.1611_1616delTCACAA; p.F537_K539delinsL	
c.1624_1629delAATGAA; p.N542_E543del	
c.1615_1616delAAinsTT; p.K539L	
JAK2 Exon 13:	
c.1711G>A; p.G571S	

The Bloodhound™ MPN Reagents provide quantitative results. The reagents provide a means to detect lower-level mutations in samples for relevant mutations in *MPL*, *CALR*, and *JAK2* as related to myeloproliferative neoplasms.

Principles of Procedure

Quantitative polymerase chain reaction (qPCR) is a technique in which the amount of PCR product can be determined during repeated cycles of DNA denaturation, annealing, and extension. This well-established method has been utilized to detect and quantify mutations in genes for diagnostic purposes where lower Ct values are associated with an increased initial copy number of the target. Controls enable quantitative results to be obtained and are included to ensure amplification. Amplification with this assay is based on chemistries that enhance mutation detection, therefore any variant present in the PCR product may differentiate from the Wild-Type (WT) DNA via Ct difference. The assay is highly sensitive, therefore factors such as pipetting errors, cytosine deamination, and polymerase errors may give rise to false positives.

Traceability of Bloodhound™ MPN Reagents Controls

The “Wild-Type Control” and “Mutant Control” sequences were constructed by synthesis and cloning for *CALR* Exon 9, *JAK2* Exons 12, 13, & 14, and *MPL* Exon 10 using the NCBI Reference Sequences:

NC_000001.11 *MPL* Exon 10
 NC_000019.10 *CALR* Exon 9
 NC_000009.12 *JAK2* Exons 12, 13 & 14

A restriction enzyme site was added 5' and 3' of each cloning region in order to linearize the plasmid which reduces the possibility of contamination. DNA sequence was confirmed using Sanger sequencing with the primers supplied in this reagent set.

The standardization plate is a set of digitally verified controls that allows the software to adjust for the absolute quantification variations from one machine to another. The standardization plate contains ready to run assays which include the following mutation percentages:

JAK2 Exon 14: WT, 0.5%, 1%, 2%, 5%, 10%, 20%, 50%, 90%
JAK2 exon 12, *JAK2* exon 13, *CALR*, and *MPL*: WT, 1%, 2%, 5%, 10%, 20%, 50%, 90%

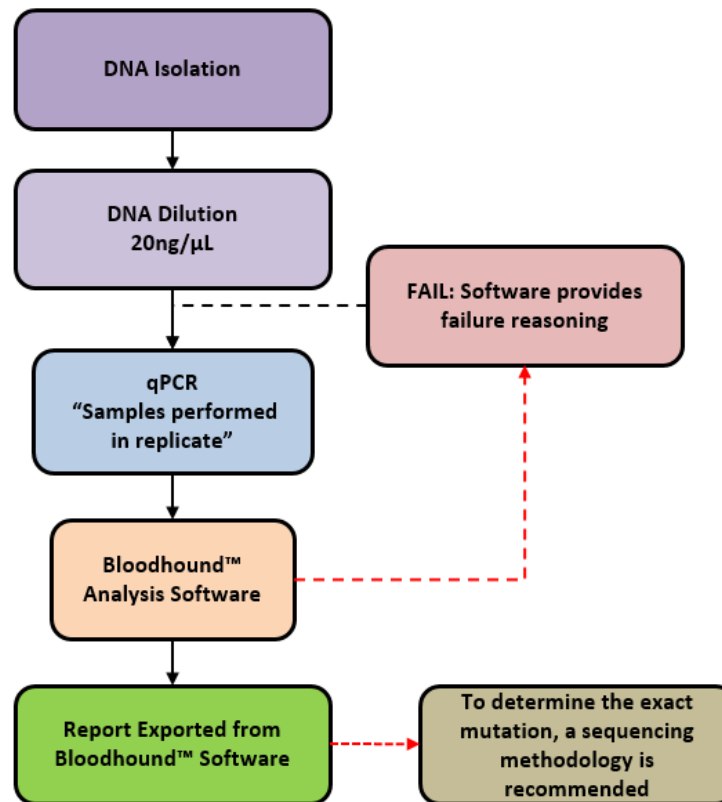
Bloodhound™ Reagent Set Preparation

All reagents supplied with the Bloodhound™ reagent set are ready to use. The plates will need to be thawed and spun in a microcentrifuge before use; check details in [Bloodhound™ Preparation and Procedure](#) section below.

Overview of Samples using Bloodhound™ Reagents

In general, processing of samples should be carried out from start to finish as described in these Instructions for Use. If processing of a sample has to be stopped before completion of all steps, the DNA should be stored (see [Primary Sample Collection, Handling and Storage](#) section below). The analysis of The Bloodhound™ Reagents should only be used in the context of the workflow indicated below.

Figure 1: Bloodhound™ Reagents Workflow



Explanation to Figure 1:

1. Isolate the DNA from peripheral blood or bone marrow using standard laboratory procedures.
2. Dilute DNA to 20 ng/μL.
3. Perform Bloodhound™ qPCR.
4. See Bloodhound™ Analysis Software IFU for further analysis information.
 - a. In the event of a sample failure or plate failure, repeat sample testing.

Components and Storage Conditions

Each Bloodhound™ MPN plated reagent set contains plated reactions for 4 or 8 samples in duplicate and includes a Wild-Type, Mutant, and No Template control (NTC).

Plated reagents should be stored in the dark between -18 °C and -25 °C in a constant temperature freezer until use. Note the expiry date of each reagent set received. Do not use after the expiry date has elapsed.

Safety Information, Warnings, and Precautions

1. None of the reagents present a hazard to health in the quantities supplied. The SDS is available on our website, <https://www.precipiodx.com>.
2. There are no substances of animal or human origin that present a risk of infection in the supplied reagents.
3. These reagents should be used only by those persons who have been trained in the appropriate laboratory techniques.
4. Biological sample handling:
 - a. All samples to be treated as hazardous and infectious, when handling whole blood or bone marrow samples always wear a suitable lab coat, disposable gloves, and protective glasses. Use a biohazard container for DNA extraction waste.
 - b. Biological waste during the DNA extraction process should be disposed in accordance with national/local regulations relating to biohazard waste disposal.
 - c. Liquid waste must be considered infectious and be handled and discarded according to national/local regulations relating to biohazard waste disposal.
5. Sample or Reagent Spillage
 - a. Avoid contact with skin and eyes.
 - b. Wear appropriate protective clothing, gloves, facemask and glasses.
 - c. Absorb the material using gauze.
 - d. Clean the surface with 10% (v/v) sodium hypochlorite (10% bleach) and leave the surface wet for 30 min to one hour.
 - e. Wash thoroughly with water. Place the waste in suitable container and dispose of in accordance with national/local regulations relating to Bio-hazard waste disposal.
 - i. If the spilt liquid contains infectious agents, clean the affected area first with laboratory detergent and water followed with 1% (v/v) sodium hypochlorite.

Materials Required but not Provided

Reagents:

1. MeltDoctor™ HRM Calibration Plate, Fast 96-well (Thermo Fisher Scientific Catalog Number 4425618)
2. TE buffer (Fisher Healthcare Catalog Number AAJ75793AE)
3. Nuclease free water (Fisher Healthcare Catalog Number AM9932)
4. DNA Extraction Kit and associated reagents

Consumables/Supplies:

1. MicroAmp™ Optical Adhesive Film (Fisher Healthcare Catalog Number 4360954)
2. Pipettes
3. Pipette tips
4. Vortex
5. 1.5 mL Microcentrifuge tubes
6. Microfuge capable of holding 1.5 mL tubes
7. Microfuge capable of holding 96-well plates

Instrument:

1. QuantStudio™ 3 Real Time PCR System with the 96-well, 0.1 mL block or equivalent.

NOTE: This assay has been optimized using the QuantStudio™ 3 Real-Time PCR System (96-well, 0.1 mL). Alternative platforms may require further optimization per the platform specifications.

Primary Sample Collection, Handling and Storage

Sample type:

- This assay can be utilized with peripheral blood and bone marrow.

DNA Extraction:

- The quality of the assay and the consistency of HRM is highly dependent on the quality of the analyte. All samples and controls should be extracted with the same high quality DNA extraction method and protocol to avoid any variations due to reagents/buffers. Each extraction should have an A_{260}/A_{280} ratio of 1.8 - 2.0 and an A_{260}/A_{230} ratio of 2.0 - 2.2 and must be diluted to the same starting concentration (20 ng/ μ L) with the same dilution buffer. Ensure sample-to-sample uniformity. Lastly, any additional control DNA to be analyzed should be of the same quality and quantity as the sample DNA.

DNA Storage:

- Extracted DNA can be stored at 4 °C for short term storage and use (up to 4 weeks) or at -20 °C for long term storage (up to 3 years).

Procedure for Running the Standardization Plate

NOTE: An initial 4-sample plate (the qualification plate) will be performed using known negative samples as an initial check for instrument specifics.

The standardization plate is used to determine instrument specific fluorescence values, based on known digitally verified controls. The standardization plate accounts for instrument variables such as light bulb brightness, instrument age, and heat block distribution. The plate is provided ready to use, and it is a one-time use plate.

NOTE: Any major machine maintenance including, but not limited to, replacement of the bulb, replacement of filters, and replacement of the heat block requires the instrument to be re-calibrated.

1. Thaw the standardization plate. Thaw the plate for a minimum of 20 minutes.
2. Once thawed, centrifuge for approximately 1-2 min (500-2000 rpm) to collect the liquid in the bottom of the plate.
3. Ensure the seal is properly secured on the plate.
4. Place the plate in the qPCR instrument, ensuring the plate is properly orientated.
5. Perform the run method on the qPCR instrument according to Section 1.5 ([Table 1](#)).

Bloodhound™ Preparation and Procedure

NOTE: For background information and general HRM workflows, refer to the Manual, Applied Biosystems High-Resolution Melt Experiment Getting Started Guide (Publication Number MAN0014394)

NOTE: The QuantStudio™ is factory calibrated. Initial Dye calibrations expire after two years. The HRM calibration must be performed prior to the first HRM assay. The field technician will not complete the MeltDoctor™ HRM calibration plate. This plate must be run on the instrument prior to running Bloodhound™ - see section below.

1.1. Recommendations Before You Start:

1.1.1. The following procedures are optional but highly recommended prior to PCR setup:

- 1.1.1.1. Turn on UV light inside hood or a UV crosslinker (15 min).
- 1.1.1.2. UV crosslink empty 1.5 mL tubes and sufficient TE buffer for DNA dilution (10 min).
- 1.1.1.3. Make sure all work areas are prepared for analysis of low-level mutations. This includes correct use of the PCR Workstation, dedicated pipettes, tips, 75% ETOH solution and/or DNA Away™ solutions.

1.2. DNA Dilutions:

1.2.1. Prepare the calculations to dilute all extracted sample DNA to 20 ng/μL in TE buffer.

OPTIONAL: Use the provided excel spreadsheet to prepare the working concentration of each sample.

- 1.2.1.1. Record each sample name, DNA concentration, and associated A_{260/280} and A_{260/230} ratios.
- 1.2.1.2. Each dilution calculations will populate automatically.
 - 1.2.1.2.1. If the original sample is < 20 ng/μL, re-extract the sample. For best performance, a consistent DNA input is required.
 - 1.2.1.2.2. For pipetting convenience, prepare a final dilution volume of at least 40 μL, provided enough DNA is available. The total volume may be adjusted as needed.

NOTE: It is not recommended to pipet less than 1 μL of stock DNA.
NOTE: The sensitivity of this assay is highly dependent on the input DNA; accuracy in the addition is of importance.

- 1.2.2. Print the sheet containing the plate layout and dilution calculation sheet.
- 1.2.3. Label individual 1.5 mL tubes with sample name or case number.
- 1.2.4. Add the required volume of TE buffer to the 1.5 mL tubes.
 - 1.2.4.1. Prior to adding the required volume of stock DNA to the TE buffer, ensure the sample has been adequately vortexed and spun down.
 - 1.2.4.2. Close the tubes, vortex, and centrifuge for 5 sec.

NOTE: Please make sure you are using different tips while adding stock DNA to 1.5 mL tubes.

1.2.5. Measure all dilutions to ensure the concentration is 20 ng/μL. All dilutions should not be more than +/- 1 μL from each other. If there are any outliers, a new dilution must be created and re-measured.

1.3. **Bloodhound™ MPN Layout Setup:**

1.3.1. The Bloodhound™ MPN Plated Reagents are arranged for PCR and analysis of 4-Samples and 8-Samples in duplicate.

Figure 2: Bloodhound™ MPN Plate layouts for 4 samples per plate (A) and 8 samples per plate (B).

A

	MPL_sample 1	MPL_sample 1	CALR_sample 1	CALR_sample 1	JAK12_sample 1	JAK12_sample 1	JAK13_sample 1	JAK13_sample 1	JAK14_sample 1	JAK14_sample 1	
	MPL_sample 2	MPL_sample 2	CALR_sample 2	CALR_sample 2	JAK12_sample 2	JAK12_sample 2	JAK13_sample 2	JAK13_sample 2	JAK14_sample 2	JAK14_sample 2	
	MPL_sample 3	MPL_sample 3	CALR_sample 3	CALR_sample 3	JAK12_sample 3	JAK12_sample 3	JAK13_sample 3	JAK13_sample 3	JAK14_sample 3	JAK14_sample 3	
	MPL_sample 4	MPL_sample 4	CALR_sample 4	CALR_sample 4	JAK12_sample 4	JAK12_sample 4	JAK13_sample 4	JAK13_sample 4	JAK14_sample 4	JAK14_sample 4	
		MPL_MUT		CALR_MUT		JAK12_MUT		JAK13_MUT		JAK14_MUT	
		MPL_WT		CALR_WT		JAK12_WT		JAK13_WT		JAK14_WT	
		MPL_NTC		CALR_NTC		JAK12_NTC		JAK13_NTC		JAK14_NTC	

B

MPL_SAMPLE 1	MPL_SAMPLE 1	CALR_SAMPLE 1	CALR_SAMPLE 1	JAK12_SAMPLE 1	JAK12_SAMPLE 1	JAK13_SAMPLE 1	JAK13_SAMPLE 1	JAK14_SAMPLE 1	JAK14_SAMPLE 1	MPL_MUT	MPL_WT
MPL_SAMPLE 2	MPL_SAMPLE 2	CALR_SAMPLE 2	CALR_SAMPLE 2	JAK12_SAMPLE 2	JAK12_SAMPLE 2	JAK13_SAMPLE 2	JAK13_SAMPLE 2	JAK14_SAMPLE 2	JAK14_SAMPLE 2	CALR_MUT	CALR_WT
MPL_SAMPLE 3	MPL_SAMPLE 3	CALR_SAMPLE 3	CALR_SAMPLE 3	JAK12_SAMPLE 3	JAK12_SAMPLE 3	JAK13_SAMPLE 3	JAK13_SAMPLE 3	JAK14_SAMPLE 3	JAK14_SAMPLE 3	JAK12_MUT	JAK12_WT
MPL_SAMPLE 4	MPL_SAMPLE 4	CALR_SAMPLE 4	CALR_SAMPLE 4	JAK12_SAMPLE 4	JAK12_SAMPLE 4	JAK13_SAMPLE 4	JAK13_SAMPLE 4	JAK14_SAMPLE 4	JAK14_SAMPLE 4	JAK13_MUT	JAK13_WT
MPL_SAMPLE 5	MPL_SAMPLE 5	CALR_SAMPLE 5	CALR_SAMPLE 5	JAK12_SAMPLE 5	JAK12_SAMPLE 5	JAK13_SAMPLE 5	JAK13_SAMPLE 5	JAK14_SAMPLE 5	JAK14_SAMPLE 5	JAK14_MUT	JAK14_WT
MPL_SAMPLE 6	MPL_SAMPLE 6	CALR_SAMPLE 6	CALR_SAMPLE 6	JAK12_SAMPLE 6	JAK12_SAMPLE 6	JAK13_SAMPLE 6	JAK13_SAMPLE 6	JAK14_SAMPLE 6	JAK14_SAMPLE 6	MPL_NTC	CALR_NTC
MPL_SAMPLE 7	MPL_SAMPLE 7	CALR_SAMPLE 7	CALR_SAMPLE 7	JAK12_SAMPLE 7	JAK12_SAMPLE 7	JAK13_SAMPLE 7	JAK13_SAMPLE 7	JAK14_SAMPLE 7	JAK14_SAMPLE 7	JAK12_NTC	JAK13_NTC
MPL_SAMPLE 8	MPL_SAMPLE 8	CALR_SAMPLE 8	CALR_SAMPLE 8	JAK12_SAMPLE 8	JAK12_SAMPLE 8	JAK13_SAMPLE 8	JAK13_SAMPLE 8	JAK14_SAMPLE 8	JAK14_SAMPLE 8	JAK14_NTC	

1.3.2. Bloodhound™ MPN Plate Layout Templates (xlsx) are available in the Precipio Cloud storage. The template file can facilitate plate organization and can generate QuantStudio™ plate setup files. Please make sure you pick the correct template for the correct SKU/sample number.

1.3.2.1. Enter the sample names in the “Layout Maker” section.” The 96-well plate view and the QuantStudio™ Template will automatically update.

1.3.2.2. Click to the save button on the upper right hand corner.

1.3.2.2.1. A .txt file will be automatically saved in the Templates folder located inside the POL folder.

1.4. **Preparation of Plate and Addition of DNA:**

1.4.1. Remove the plate from the freezer and thaw the plate in its packaging.

1.4.1.1. Thaw the plate for a minimum of 20 min at room temperature, protected from light.

1.4.2. Remove the plate from the packaging.

1.4.3. Centrifuge the plate for 2 min at 500 to 2000 rpm.

1.4.3.1. Confirm that the liquid in each well is at the bottom of the well and the volume is consistent well to well. If not, centrifuge the plate again for another 2 minutes.

1.4.3.2. Keep the bottom of the plate clean. Fluids or other debris on the bottom of the plate may dirty the sample block and cause abnormal fluorescent signals.

1.4.4. Ensure that the plate orientation is correct and that well A1 is the top left well.

1.4.5. Being very careful, **SLOWLY** remove the plate septa from the Bloodhound™ MPN Plate.

1.4.6. To appropriate wells, add 2 µL of diluted DNA sample. Ensure to adequately vortex and spin down all DNA dilutions prior to use.

1.4.6.1. Pipette-mix to ensure sample is well mixed into reaction mixture.

1.4.6.2. Controls are already added to the appropriate wells (Wild-Type, Mutant and No Template Controls).

DO NOT ADD any DNA or water/low TE to these wells.

1.4.7. Place MicroAmp Optical Film over plate and seal. Ensure the plate is appropriately sealed on all edges by pressing firmly across the top of the plate and around the outside edges with the plate sealing tool.

1.4.8. Centrifuge the plate for 1 min to eliminate any air bubbles from the solution. Verify the optical film is adequately sealed, and verify the well volumes before placing the plate on the instrument.

1.5. **Amplification and Melt Curve/Dissociation on QuantStudio™ 3:**

1.5.1. Open the QuantStudio™ v1.5.1 software.

1.5.1.1. Create a new experiment > From Template (see **Figure 3 & 4**).

1.5.1.1.1. Select FILE, NEW EXPERIMENT, FROM TEMPLATE then browse to the “Bloodhound™ QuantStudio™ 3 Template.edt” file from the POL folder (see **Figure 3 & 4**).


1.5.1.1.2. This .edt file provided by Precipio has the thermal cycler profile already programmed under Experimental Method. See **Figure 5** for a screenshot of the entire thermal cycler program.

1.5.1.1.2.1. Do not change or alter any of the parameters of the thermal cycler program.

1.5.1.1.3. See **Table 1** for the thermal cycler protocol.

1.5.1.2. Input the experiment name under the properties tab.

1.5.1.2.1. Recommended to format as DD_MM_YY_PlateBarcode_tech initials

- 1.5.1.2.2. Confirm the following run options:
 - 1.5.1.2.2.1. Instrument type: QuantStudio™ 3 System
 - 1.5.1.2.2.2. Block type: 96-Well 0.1-mL Block
 - 1.5.1.2.2.3. Experiment type: Standard Curve
 - 1.5.1.2.2.4. Chemistry: Other
 - 1.5.1.2.2.5. Run mode: Standard
- 1.5.1.3. Click **Plate** in the **top row to open Assign Targets and Samples** tab.
- 1.5.1.4. Select **File -> Import Plate Setup...** to import the HRM Plate Layout Excel file made in earlier step. (See [Figure 6](#)). The samples, plate layout, and target(s) are now assigned.
- 1.5.1.5. Confirm all sample wells have been assigned a sample name and target. If there are empty wells, you can confirm there is no sample name or Target assigned to the well.
 - 1.5.1.5.1. Click Advanced Setup. In the 96-well Plate Layout view, select the wells you would like to exclude from analysis and then uncheck the sample name and target from the advanced setup pane on the left side.
- 1.5.1.6. Touch  to eject the instrument drawer.
 - 1.5.1.6.1. Place the plate in the drawer so well A1 of the plate is in the top left corner.
- 1.5.1.7. Click on **START RUN** followed by the specific instrument serial number to start the run (see [Figure 7](#)).
- 1.5.1.8. The software will ask you to save the run. Please save the run in your designated Bloodhound™ POL folder (i.e. \Egnyte\shared\HemeScreen POL\YOUR SITE).
- 1.5.1.9. The run automatically starts after saving the file.

NOTE: Before clicking start run, make sure your instrument is connected to your laptop. You should see your instrument serial number in the drop-down menu.

NOTE: QuantStudio™ automatically saves the run file on the instrument, if you failed to save the run on the computer you can always retrieve .eds file from the QuantStudio™.

TIP: At this stage PCR/HRM products can be stored at less than or equal to -20 °C for up to one week.

Figure 3: Create a new experiment from template.

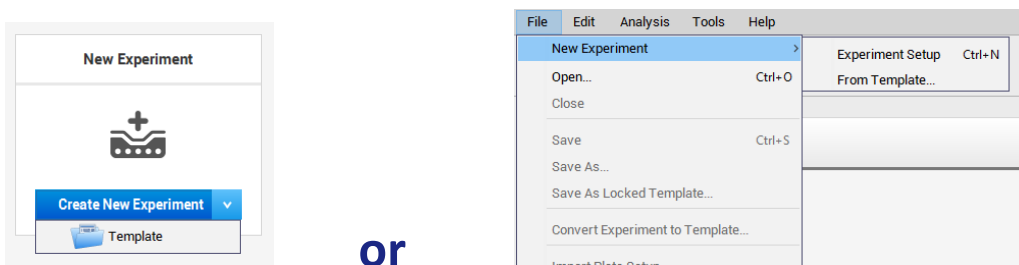
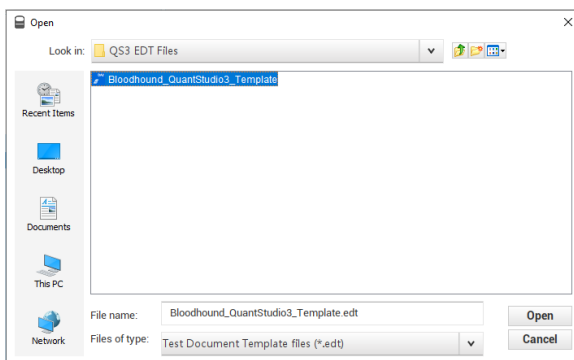


Figure 4: Bloodhound™ QuantStudio™ Template.



Instructions for Use

Figure 5: HRM thermal cycler conditions.

Make sure reaction volume is 20 μL (blue arrow), data collection points are selected in step 2 of PCR stage and step 3 of Melt curve stage (black arrows). Make sure PCR stage has 40 cycles (red arrow) entered and “continuous” is checked (green arrow).

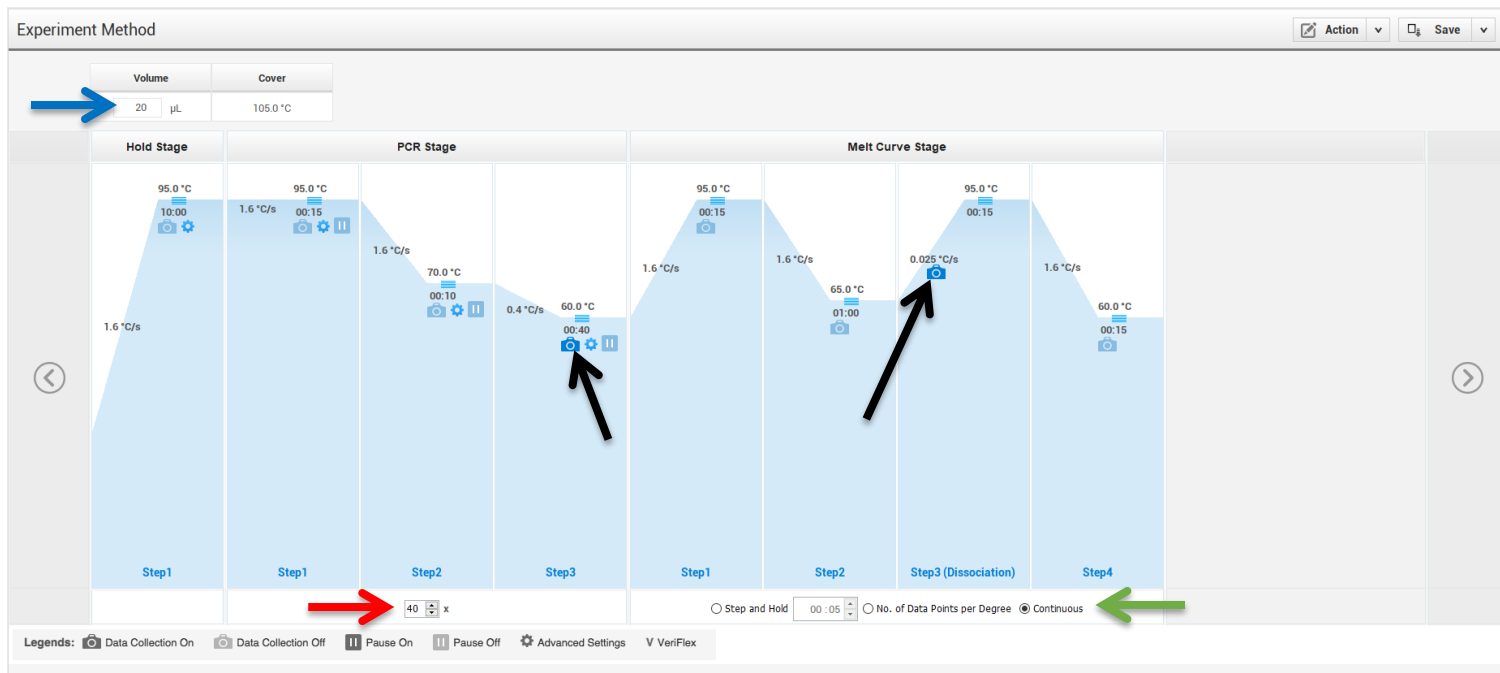
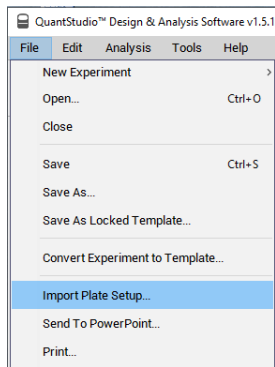
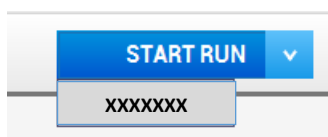


Table 1: Thermal cycling and melt curve program for the HRM reaction.

Stage	Step	Temp	Time	Ramp rate
Holding	Enzyme activation	95°C	10 min	1.6 °C/Sec
	Denature	95°C	15 sec	1.6 °C/Sec
	Probe Annealing	70°C	10 sec	1.6 °C/Sec
Cycling (40 cycles)	Primer Annealing, Extension (Data Capture on)	60°C	40 sec	0.4 °C/Sec
	Denature	95°C	15 sec	1.6 °C/Sec
Melt curve/Dissociation	Anneal	65°C	1 min	1.6 °C/Sec
	High resolution melting (Data Capture on)	95°C	15 sec	0.025 °C/Sec
	Anneal	60°C	15 sec	1.6 °C/Sec

Figure 6: Import Plate Layout files.

Figure 7: Starting the run.


Manual Review of Quality Control Amplification Data

NOTE: This guide is written specific to the QuantStudio™ 3 Real-Time PCR System. If you are using a similar system, please refer to that system's user guide for guidance.

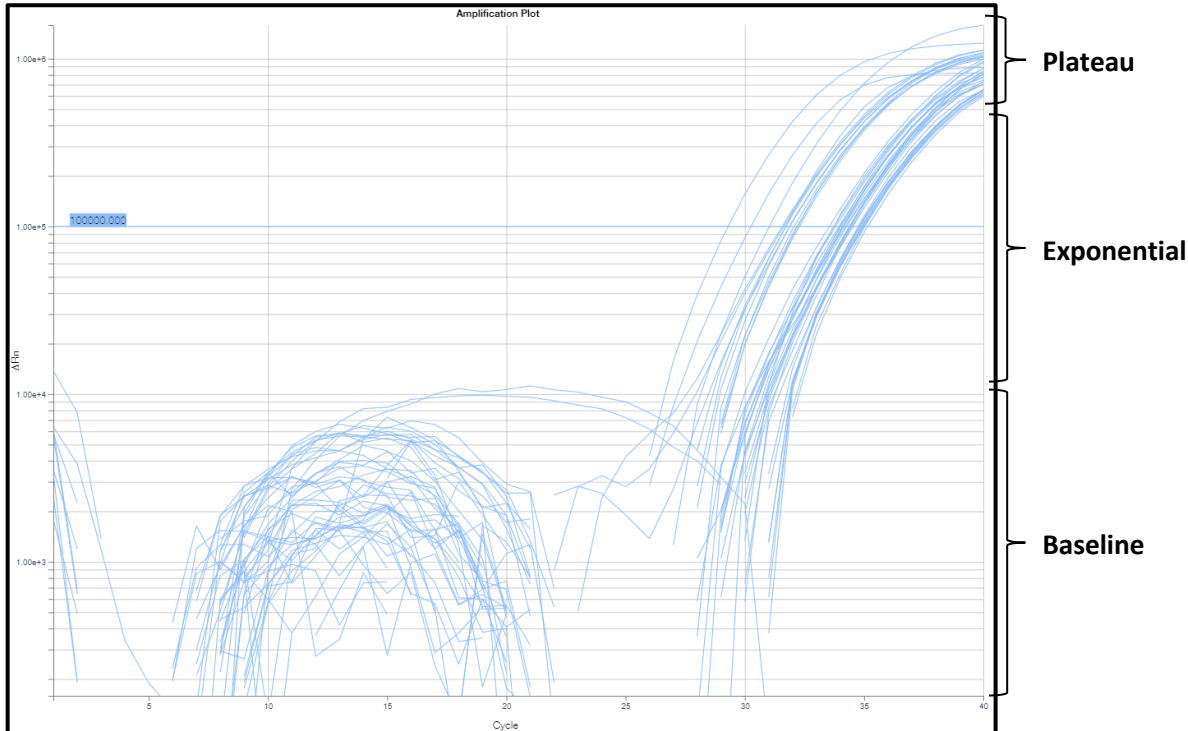
NOTE: Bloodhound™ Analysis software examines pass/fail criteria and performs mutation calling.

2.1 Normal Characteristics of Bloodhound™ Amplification

- 2.1.1 To manually review the amplification data in QuantStudio™ Design & Analysis Software v1.5.1.
- 2.1.2 General QC metrics from the Real-Time PCR amplification are as follows; however, results may vary due to instrument to instrument variation, PCR setup, etc. See [Figure 8](#) for an example amplification plot.
 - 2.1.2.1 Genomic DNA typically amplifies between 26 - 38 cycles (Ct) when 40 ng of quality DNA is added to the PCR. An exponential increase in fluorescence is expected until the reaction reaches plateau.
 - 2.1.2.2 Plasmid control DNA typically amplifies between 26 - 38 cycles (Ct) when controls are used as directed. An exponential increase in fluorescence is expected until the reaction reaches plateau.
 - 2.1.2.3 NTC reactions typically have undetermined Ct values.

NOTE: Document which samples are outliers of the suggested QC metrics. The outliers may produce erroneous results

Figure 8: Example Amplification Plot.



Bloodhound™ MPN Analysis:

Guidance for Bloodhound™ data analysis is given in this manual; 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.

The analysis software can be accessed through this link: [BloodHound™ Software](#)

NOTE: Refer to the BloodHound™ Software User Manual for instructions on using the analysis software.

The analysis software utilizes the predicted Ct and the Tm values at a given threshold to determine (1) if a mutation is present and (2) relative quantification of the mutation if it is present. The analysis is based upon the amplification and coefficient of PCR.

- i. The software will determine the threshold for the particular sample based upon the amplification curve and coefficient of PCR for the given sample.
- ii. The software will then determine the estimated quantification of the mutation based upon the Ct value compared to the amplification and coefficient of PCR.

Prior to uploading the exported excel file into the BloodHound™ Analysis Software. The user must manually set the threshold for the amplicons in the QuantStudio™ Design & Analysis software.

1. Open the completed run file.
2. For all targets: MPL, CALR, JAK12, JAK13 and JAK14
 - a. Open the amplification curve settings.
 - b. Manually set the Ct threshold to 100,000.
 - c. Use Auto Baseline
3. Export file
 - a. Select Export
 - b. Save file as .xlsx or .CSV file format

- c. Select “Results”
- d. Save file to desired location
- e. Click export.

Troubleshooting












Plate Errors:

Plate sealing errors	Make sure the plate is sealed completely. Failure to properly seal each well may lead to evaporation or contaminate the sample block which will affect the analysis.
Air bubbles	Remove any air bubbles as possible by flicking or tapping on the wells before running the plate. Centrifuge the plate for 1 min or longer prior to each run.
Dilution errors	If the dilution measures greater or less than 20 ng/μL, repeat the dilution. If the problem persists, re-measure the stock solution to ensure accuracy of the dilution numbers.

Technical Errors:

Cross contamination	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.
NTC contamination	NTC amplification at Ct values < 34 or the Derivative Melt Curve Peaks align at the same melt temp with the Wild-Type or Mutation controls indicate contamination. Repeat the PCR run for samples and controls for that amplicon.

Explanation of Symbols and Warnings

			
Caution, consult accompanying documents	<i>In vitro</i> diagnostic medical device	Sterilized using aseptic processing techniques	Keep away from light
			
Use By: MM-YYYY	Catalog number	Manufacturer	Batch Code
			
Research Use Only	Consult instructions for use	Temperature Limitation	

Each manufactured lot of Bloodhound™ is performance-tested on positive control cell lines to ensure product performance

Each user must make an independent judgment on whether this product is suitable for use in applications conducted in their laboratory. Precipio does not guarantee the successful outcome of any testing based solely on the use of Bloodhound™. Precipio's contribution to these procedures is simply at the step of providing reagents for these procedures.

Literature References

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Technical Support/Questions

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