January 2020

ipsogen® MPL W515L/K MutaScreen Handbook

For research use only. Not for use in diagnostic procedures.

For use with Rotor-Gene® Q 5plex HRM, ABI PRISM® 7000, 7300, 7700 and 7900HT SDS, Applied Biosystems® 7500 Real-Time PCR System, LightCycler® 480 and 2.0 instruments

676413

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Contents

Intended Use

The *ipsogen* MPL W515L/K Muta*Screen* Kit is for research use only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed of recombinant DNA experiments, or to other applicable guidelines.

Principle of the Procedure

Two probes are used in a multiplexed assay as part of an allelic discrimination experiment. One is a perfect match to the allele 1 sequence (e.g., the wild-type allele) while the other one is a perfect match to the allele 2 (e.g., the allele with a mutation). Each probe is labeled with a distinctive fluorescent dye at its 5' end such as FAM™ or VIC® forming a reporter. The probe also contains a non-fluorescent quencher at the 3' end. The inclusion of a minor grove binder (MGB™) in the probe sequence permits the use of shorter probes with greater stability and thereby a more accurate allelic discrimination.

During the extension phase of the PCR, the perfectly matched probe is cleaved by the 5'→3' exonuclease activity of *Thermus aquaticus* (*Taq*) polymerase. This separates the reporter dye from the quencher and thus releases detectable fluorescence. If the probe is not a perfect match, it will be displaced rather than cleaved by the *Taq* polymerase and no reporter dye will be released. The fluorescence signal (VIC or FAM) generated is collected at the end of the PCR and immediately indicates the presence of the targeted sequence(s) in the sample (wild-type allele, mutated allele, or both). This negates the need for long and laborious post-

PCR steps, which may also increase the contamination risk. The *ipsogen* MPL W515L/K Muta*Screen* Kit uses this technology as illustrated in Figure 1.

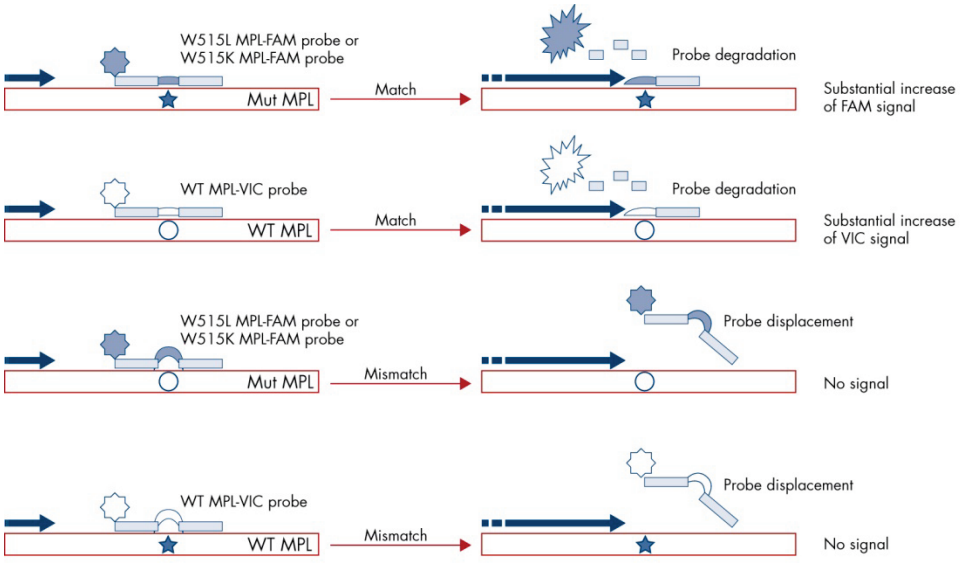


Figure 1. Reaction principle of the *ipsogen* MPL W515L/K Muta*Screen* Kit allelic discrimination assay.

Materials Provided

Kit contents

***ipsogen* MPL W515L/K Muta*Screen* Kit**		**(24)**
Catalog no.		**676413**
Number of reactions		**24**
W515L positive control	PC-515L	30 µl
W515K positive control	PC-515K	30 µl
MPL negative control	NC-MPL	60 µl
MPL W515L cut-off sample containing 1.5% W515L/wild-type allele mix	COS-515L	30 µl
MPL W515K cut-off sample containing 1.5% W515K/wild-type allele mix	COS-515K	30 µl
Primers and Probe Mix PPM-MPL W515L*	PPM-MPL W515L 10x	145 µl
Primers and Probe Mix PPM-MPL-W515K†	PPM-MPL W515K 10x	145 µl

* Mix of specific reverse and forward primers for the MPL gene plus a specific W515L FAM probe and a wild-type VIC probe.

† Mix of specific reverse and forward primers for the MPL gene plus a specific W515K FAM probe and a wild-type VIC probe.

Note: Vortex and briefly centrifuge the tubes before use.

Materials Required but Not Provided

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Reagents

- Nuclease-free, PCR-grade water
- Buffer and *Taq* DNA polymerase: The recommended reagents are TaqMan® Universal PCR Master Mix (Master Mix PCR 2x) (Thermo Fisher Scientific Inc., cat. no. 4304437) and LightCycler TaqMan Master (Master Mix PCR 5x) (Roche, cat. no. 04535286001)

Consumables

- Nuclease-free, aerosol-resistant sterile PCR pipet tips with hydrophobic filters
- 0.5 ml or 0.2 ml nuclease-free PCR tubes
- 0.1 ml strip tubes and caps, if using the Rotor-Gene Q 5plex HRM instrument
- Ice

Equipment

- Microtiter pipets dedicated for PCR (1–10 µl; 10–100 µl; 100–1000 µl)
- Benchtop centrifuge* with rotor for 0.2 ml/0.5 ml reaction tubes (capable of attaining 13,000 to 14,000 rpm)

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Real-time PCR instrument:* Rotor-Gene Q 5plex HRM; ABI PRISM 7000, 7300, 7700, or 7900HT SDS; Applied Biosystems 7500 Real-Time PCR System, LightCycler 480 or 2.0; and associated specific material

Warnings and Precautions

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN® kit and kit component.

Discard sample and assay waste according to your local safety regulations.

General precautions

Use of quantitative PCR (qPCR) requires good laboratory practices, including maintenance of equipment, that are dedicated to molecular biology and are compliant with applicable regulations and relevant standards.

This kit is intended for research use. Reagents and instructions supplied in this kit have been tested for optimal performance. Further dilution of the reagents or alteration of incubation times and temperatures may result in erroneous or discordant data. PPM-MPL reagents may be altered if exposed to light. All reagents are formulated specifically for use with this kit. For optimal performance of the kit, no substitutions should be made.

Use extreme caution to prevent:

- DNase contamination, which might cause degradation of the template DNA
- DNA or PCR carryover contamination resulting in false positive signal

We therefore recommend the following:

- Use nuclease-free labware (e.g., pipets, pipet tips, reaction vials) and wear gloves when performing the assay.
- Use fresh aerosol-resistant pipet tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- Prepare pre-PCR master mix with dedicated material (pipets, tips, etc.) in a dedicated area where no DNA matrixes (cDNA, DNA, plasmid) are introduced. Add template in a separate zone (preferably in a separate room) with specific material (pipets, tips, etc.).

Reagent Storage and Handling

The kits are shipped on dry ice and must be stored at –30 to –15°C upon receipt.

- Minimize exposure to light of the primers and probe mixes (PPM-MPL tubes).
- Gently mix and centrifuge the tubes before opening.
- Store all kit components in original containers.

These storage conditions apply to both opened and unopened components. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

Expiration dates for each reagent are indicated on the individual component labels. Under correct storage conditions, the product will maintain performance until the expiration date printed on the label.

There are no obvious signs to indicate instability of this product. However, positive and negative controls should be run simultaneously with unknown specimens.

Procedure

Sample DNA preparation

Genomic DNA should be obtained either from whole blood, purified blood lymphocytes of whole blood, polynuclear cells, or granulocytes. The same cellular fraction and DNA extraction method should be used for all samples to allow results to be compared. DNA extraction should be performed by any commercial or home brew method.

- DNA quantity is determined by measuring the absorbance at 260 nm.
- DNA quality should be assessed either by spectrophotometry or by gel electrophoresis.
- The ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280}) should be 1.7–1.9. Smaller ratios usually indicate contamination by protein or organic chemicals.

Electrophoretic analysis on a 0.8–1.0% agarose gel should allow visualization of the isolated DNA as a distinct band of about 20 kb. A slight smear is acceptable.

The DNA obtained in the extraction should be diluted to a concentration of 5 ng/µl in TE pH 8.0 buffer. The DNA sample can be stored at 4–8°C for a week or at –20°C if longer-term storage is required.

The qPCR is optimized for 25 ng of purified gDNA.

The quality of the assay is largely dependent on the quality of input DNA. We therefore recommend the QIAamp® DNA Blood Maxi Kit (cat. no. 51194).

Protocol: qPCR on Rotor-Gene Q 5plex HRM Instruments

Using this instrument, we recommend performing all measurements in duplicate, as indicated in Table 1 or Table 2.

Table 1. Number of reactions for a MPL W515L experiment using Rotor-Gene Q instruments with a 72-tube rotor with 0.1 ml strip tubes and caps

Samples	Reactions
With the PPM-MPL W515L/WT primers and probe mix	
n DNA samples	n x 2 reactions
3 controls	2 x 3 reactions (PC-515L, NC-MPL and COS-515L, each one tested in duplicate)
Water control	2 reactions

Table 2. Number of reactions for a MPL W515K experiment using Rotor-Gene Q instruments with a 72-tube rotor with 0.1 ml strip tubes and caps

Samples	Reactions
With the PPM-MPL W515K/WT primers and probe mix	
n DNA samples	n x 2 reactions
3 controls	2 x 3 reactions (PC-515K, NC-MPL and COS-515K, each one tested in duplicate)
Water control	2 reactions

Sample processing on Rotor-Gene Q instruments with 72-tube rotor with 0.1 ml strip tubes and caps

We recommend testing at least 24 genomic DNA samples in duplicate in one experiment, 10 samples in duplicate in two experiments, or five samples in duplicate in three experiments to optimize the use of the controls, and the primers and probe mixes. The rotor scheme in Figure 2 shows an example of such an experiment.

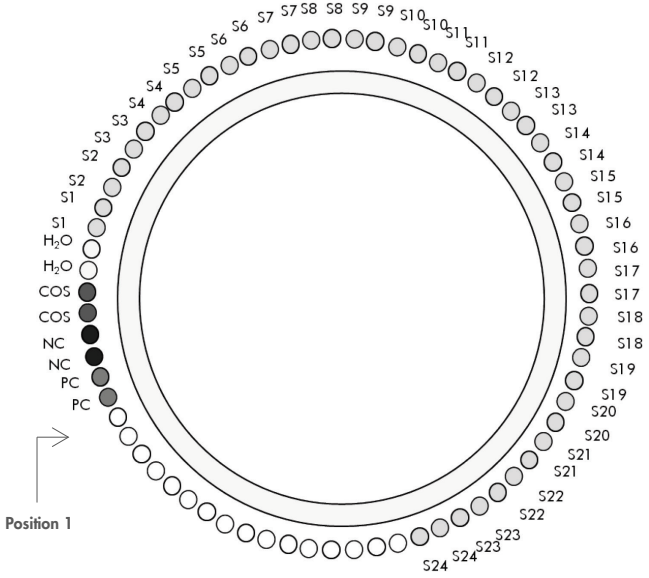


Figure 2. Suggested rotor setup for each experiment with the *ipsogen* MPL W515L/K Muta*Screen* Kit. **PC**: Positive control; **NC**: Negative control; **COS**: Cut-off sample (either 1.5% W5151L/wild-type allele mix or 1.5% W515K/wild-type allele mix); **H_2O**: Water control; **S**: Unknown DNA sample.

Important: Take care to always place the Positive Control in position 1 of the rotor. Otherwise, during the calibration step, the instrument will not perform calibration, and incorrect fluorescence data will be acquired. Fill all other positions with empty tubes.

qPCR on Rotor-Gene Q instruments with 72-tube rotor with 0.1 ml strip tubes and caps

Note: Perform all steps on ice.

Procedure

1. Thaw all necessary components and place them on ice.
2. Prepare the following qPCR mix according to the number of samples being processed.

   All concentrations are for the final volume of the reaction.

   Table 3 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 25 µl. A pre-mix can be prepared, according to the number of reactions, using the same primers and probe mix (either PPM-MPL W515L or PPM-MPL W515K). Extra volumes are included to compensate for pipetting error.

Table 3. Preparation of qPCR mix

Component	1 rxn (µl)	18+1 rxns (µl)	28+1 rxns (µl)	56+1 rxns (µl)	Final conc.
TaqMan Universal PCR Master Mix, 2x	12.5	237.5	362.5	712.5	1x
Primers and probe mix (PPM-MPL W515L or PPM-MPL W515K), 10x	2.5	47.5	72.5	142.5	1x
Nuclease-free, PCR-grade water	5.0	95.0	145.0	285	–
Sample (to be added at step 4)	5	5 each	5 each	5 each	–
Total volume	**25**	**25 each**	**25 each**	**25 each**	**–**

Conc.: concentration; rxn(s): reaction(s).

3. Dispense 20 µl of the qPCR pre-mix per tube.

4. Add 5 µl of the material to be tested (25 ng sample genomic DNA or controls) in the corresponding tube (total volume 25 µl).
5. Mix gently by pipetting up and down.
6. Place the tubes in the thermal cycler according to the manufacturer recommendations.
7. Program the Rotor-Gene Q instrument with the thermal cycling program as indicated in Table 4.

Table 4. Temperature profile

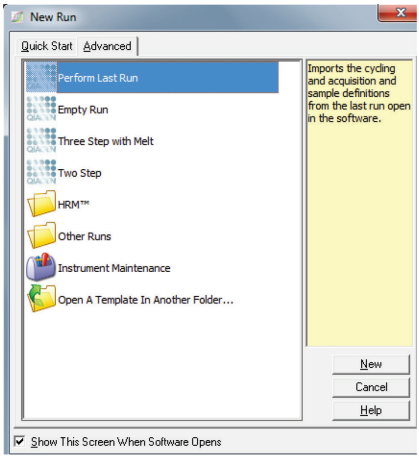
Parameter	
Mode of analysis	Quantitation
Hold 1	Temperature: 50°C Time: 2 min
Hold 2	Temperature: 95°C Time: 10 min
Cycling	50 times 92°C for 15 s 60°C for 1 min with acquisition of FAM fluorescence in channel Green and VIC fluorescence in channel Yellow: Single

8. Start the thermal cycling program, as indicated in Table 4.

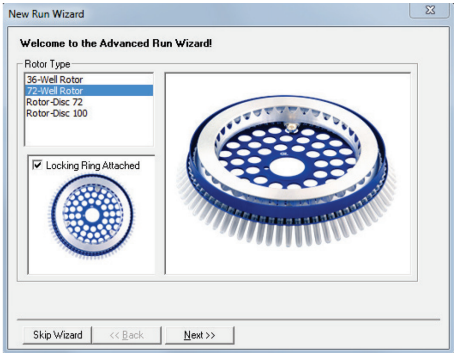
Detailed procedure for the Rotor-Gene Q instrument

Note: Instrument settings could slightly differ between instruments. Refer to your user manual for more details.

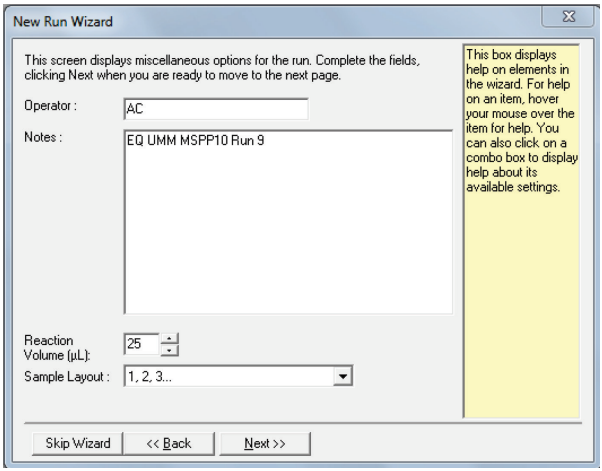
1. Start the Rotor-Gene software. In the New Run pop-up window, select the Advanced tab and click **New**.



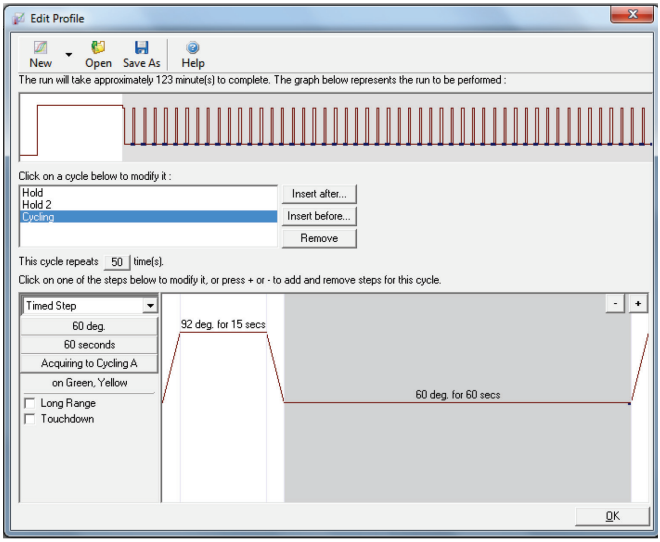
2. In the New Run Wizard dialog box, select **72-Well Rotor** from the drop-down list. Check the **Locking Ring Attached** box, then click **Next**.



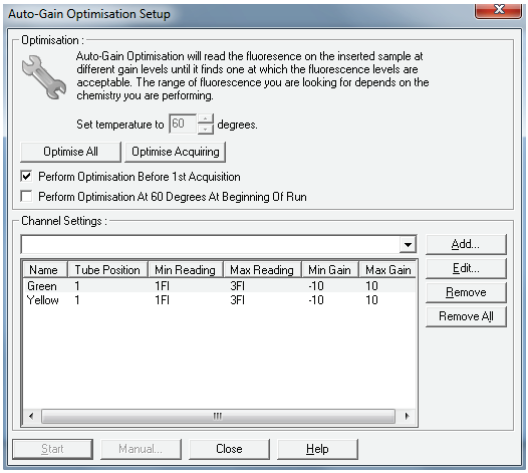
3. On the next window, set the reaction volume field to **25 µl** and click **Next**.



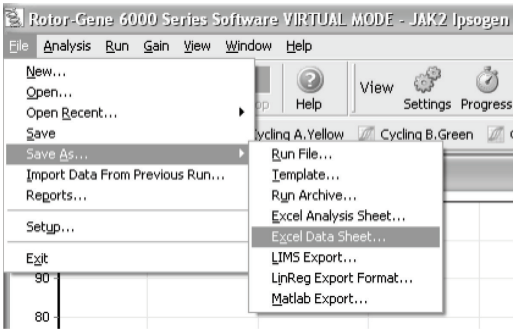
4. In the next window, click the **Edit Profile** to create the PCR program according to Table 4.



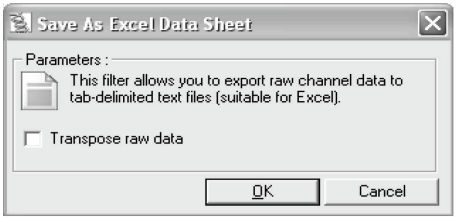
5. Click **Gain Optimisation** in the New Run Wizard dialog box to open the Auto-Gain Optimisation Setup dialog box. Set the range for the Green and Yellow channels from **1 Fl** in the Min Reading column to **3 Fl** in the Max Reading column and the acceptable Gain range from **–10** to **10**.



6. Check the **Perform Optimisation Before 1st Acquisition** box, then click **Close** to close the Auto-Gain Optimisation Setup dialog box.
7. Start the thermal cycling program.
8. After the cycling program has ended, click **Analysis** then double-click **Cycling A. Green**.
9. Select **Dynamic Tube** and **Slope Correct**.
10. Repeat steps 8–9 for **Cycling A. Yellow**.
11. To export the data, navigate to **File** > **Save As** > **Excel Data Sheet**.

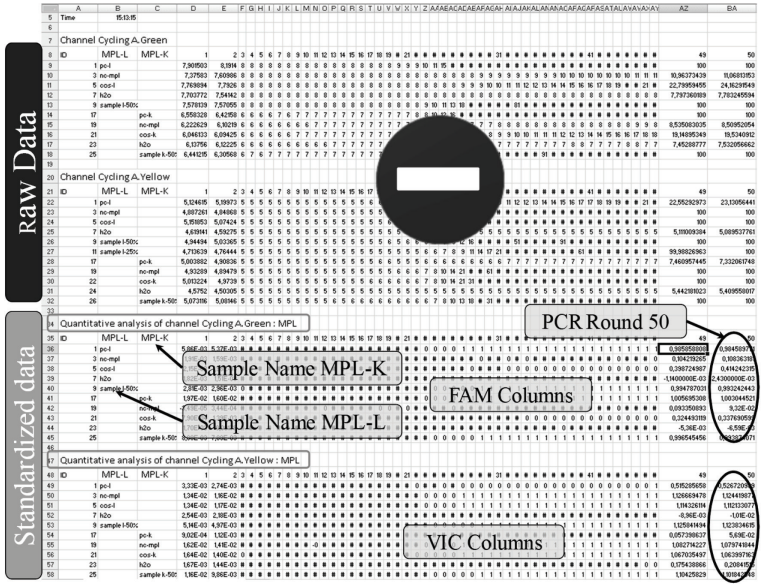


12. After adding a name for the datasheet, a pop-up window appears as follows. Click **OK** to leave the option disabled.



13. Open the .csv file using Excel®.

    An example of the output obtained is as follows:



The file contains both raw data and standardized data, but only the standardized should be considered. These data are given in the "Quantitative analysis of channel Cycling A Green" and "Quantitative analysis of channel Cycling A Yellow" sections of the table. The data that need to be interpreted are values acquired at the 50th PCR cycle, as shown by the circle in the above figure.

Protocol: qPCR on ABI PRISM 7000, 7300, 7700 and 7900HT SDS Instruments, the Applied Biosystems 7500 Real-Time PCR System, and the LightCycler 480

Using 96-well-plate qPCR equipment, we recommend performing all measurements in duplicate, as indicated in Table 5 or Table 6.

Table 5. Number of reactions for a MPL W515L experiment using 96 well-plate qPCR equipment

Samples	Reactions
With the PPM-MPL W515L/WT primers and probe mix	
n DNA samples	n x 2 reactions
3 controls	2 x 3 reactions (PC-515L, NC-MPL and COS-515L, each one tested in duplicate)
Water control	2 reactions

Table 6. Number of reactions for a MPL W515K experiment using 96 well-plate qPCR equipment

Samples	Reactions
With the PPM-MPL W515K/WT primers and probe mix	
n DNA samples	n x 2 reactions
3 controls	2 x 3 reactions (PC-515K, NC-MPL and COS-515K, each one tested in duplicate)
Water control	2 reactions

Sample processing on ABI PRISM 7000, 7300, 7700, and 7900 SDS instruments, the Applied Biosystems 7500 Real-Time PCR System, and the LightCycler 480

We recommend testing at least 24 genomic DNA samples in duplicate in one experiment, 10 samples in duplicate in two experiments, or five samples in duplicate in three experiments to optimize the use of the controls, and the primers and probe mixes. The plate scheme in Figure 3 shows an example of such an experiment.

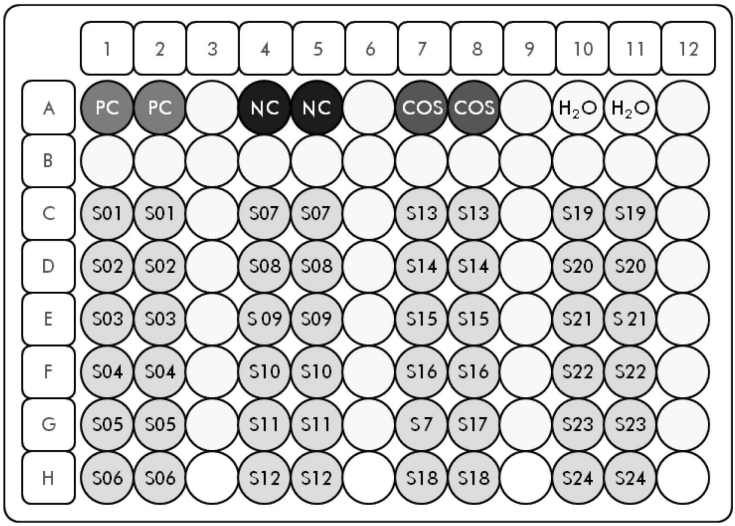


Figure 3. Suggested plate setup for one experiment with the *ipsogen* MPL W515L/K Muta*Screen* Kit. PC: Positive control; **NC**: Negative control; **COS**: Cut-off sample; **H_2O**: Water control; **S**: DNA sample.

qPCR on ABI PRISM 7000, 7300, 7700 and 7900 SDS instruments, the Applied Biosystems 7500 Real-Time PCR System, and the LightCycler 480

Note: Perform all steps on ice.

Procedure

1. Thaw all necessary components and place them on ice.
2. Prepare the following qPCR mix according to the number of samples being processed. If using 96-well-plate qPCR equipment, we recommend performing all measurements in duplicate.

   Note: All concentrations are for the final volume of the reaction.

   Table 7 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 25 µl. A pre-mix can be prepared, according to the number of reactions, using the same primers and probe mix (either PPM-MPL W515L or PPM-MPL W515K). Extra volumes are included to compensate for pipetting error.

Table 7. Preparation of qPCR mix

Component	1 rxn (µl)	18+1 rxns (µl)	28+1 rxns (µl)	56+1 rxns (µl)	Final conc.
TaqMan Universal PCR Master Mix, 2x	12.5	237.5	362.5	712.5	1x
Primers and probe mix (PPM-MPL W515L or PPM-MPL W515K), 10x	2.5	47.5	72.5	142.5	1x
Nuclease-free, PCR-grade water	5.0	95.0	145.0	285	–
Sample (to be added at step 4)	5	5 each	5 each	5 each	–
Total volume	**25**	**25 each**	**25 each**	**25 each**	**–**

Conc.: concentration; rxn(s): reaction(s).

3. Dispense 20 µl of the qPCR pre-mix per well.
4. Add 5 µl of the material to be tested (25 ng sample genomic DNA or controls) in the corresponding well (total volume 25 µl).
5. Mix gently by pipetting up and down.
6. Close the plate and briefly centrifuge (300 x *g* for approximately 10 s).
7. Load the plate in the thermal cycler apparatus according to the manufacturer's recommendations.
8. Program the thermal cycler with the thermal cycling program as specified in Table 8 or 9 depending on the instrument being used.

Detailed procedure for the ABI PRISM 7000, 7300, 7700 and 7900HT SDS instruments, and the Applied Biosystems 7500 Real-Time PCR System

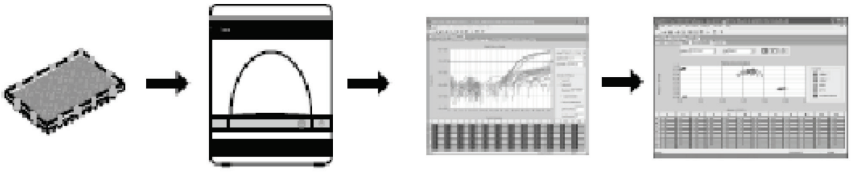
The thermal cycling program is shown in Table 8.

Table 8. Temperature profile for ABI PRISM 7000, 7300, 7700 and 7900HT SDS instruments, and the Applied Biosystems 7500 Real-Time PCR System

Parameters	
Mode of analysis	Standard Curve – Absolute Quantitation
Hold	Temperature: 50°C Time: 2 min
Hold 2	Temperature: 95°C Time: 10 min
Cycling	50 times 92°C for 15 s 60°C for 1 min with acquisition of FAM and VIC fluorescence; quencher: MGB

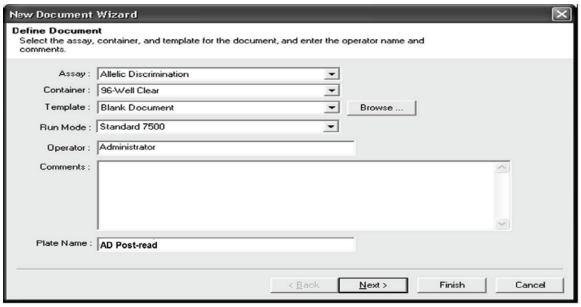
Detailed procedure for the ABI PRISM 7000, 7300, 7700 and 7900HT SDS, and the Applied Biosystems 7500 Real-Time PCR System instrument setting of post-read run analysis

- Users who are familiar with the use of the function “allelic discrimination” should go directly to step 6.
- Instrument setting could slightly differ between instruments. Refer to your user manual for more details.

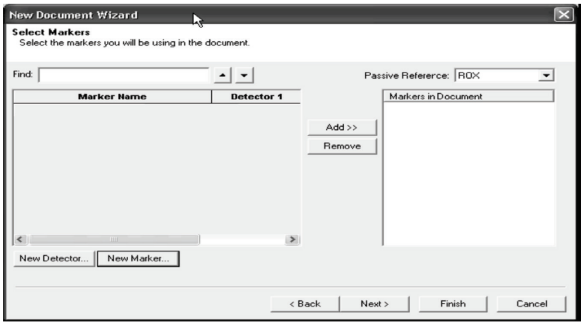


Reaction plate System Amplification run Post-read run

1. Navigate to **Start** > **Programs.**
2. Select “File” and then “New”.
3. In the new document wizard:
   - 3a. From the Assay drop-down list, select **Allelic Discrimination**.
   - 3b. Accept the default settings for the **Container** and **Template** fields (96-well clear and blank document).
   - 3c. In the **Plate Name** field, enter **AD post-read**.



4. Click **Next** to access the "Select Markers" page. If the marker list on this page contains a marker suitable for your application, skip to step 6.



5. If the marker list does not contain a marker suitable for your application, as above, follow these steps to create detectors and marker:

   5a. Click **New Detector**.

   5b. In the New Detector dialog box, enter **Allele A** in the **Name** field.

   5c. Leave the **Reporter Dye** field set to **FAM**.

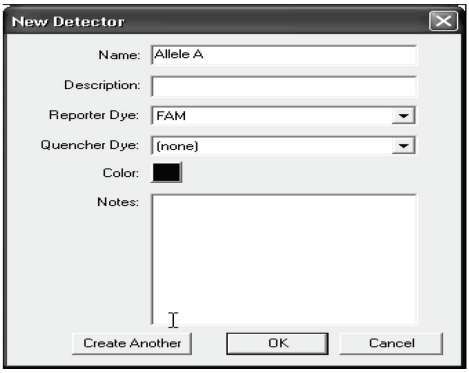
   5d. Select a color you prefer, and then click **OK**.

   5e. Click **Create Another**.

   5f. In the **Name** field, enter **Allele B**.

   5g. Select **VIC** in the Reporter Dye field.

5h. Select a color you prefer, then click **OK**.



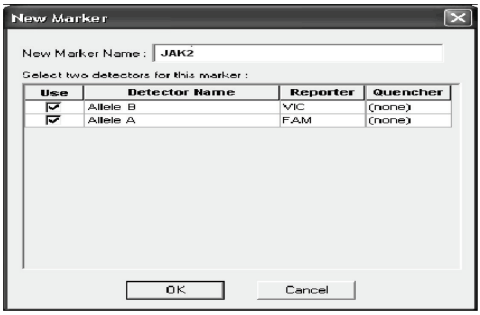
6. In the select markers window:

6a. Click **New Marker**.

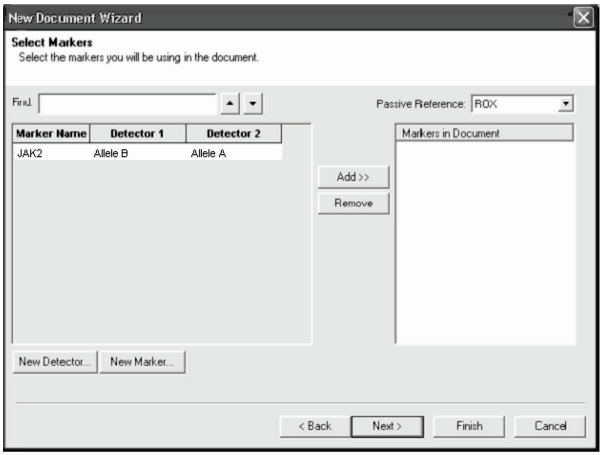
6b. In the New Marker dialog box, enter **MPL** in the New Marker Name field.

6c. In the Detector Name field, select the **Allele A** and **Allele B** detectors you created in the step 5.

6d. Click **OK**.



7. In the Select Markers window, select either the **MPL** marker you created in step 5 or a suitable marker, then click **Add**.

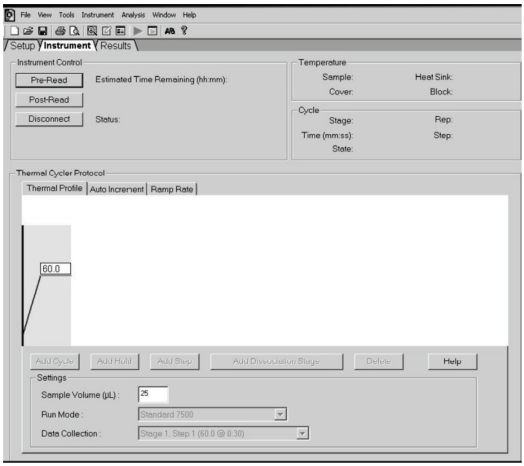


Note: To remove a marker, select it, and then click **Remove**.

8. Click **Next**.
9. In the Setup Sample Plate page, select the marker for wells. Click-drag to select wells that contain samples.
10. Click **Finish**.
11. Select the Instrument tab and set the value in the Sample Volume field to **25 µL**.
12. Click **File** > **Save** > **Save** to retain the name you assigned when you created the plate document.
13. Load the reaction plate into the instrument.
14. Start the post-read run.
15. Click **Post-Read**.

    The instrument will perform a run of 1 cycle of 60 s at 60°C.

    Note: During this post-read run, the instrument collects FAM and VIC fluorescence in each well.



To export the results, click **File** then **Export**.Click **Results**.

Analyze export file. An example of the output is shown below:

Comments:							VIC Fluorescence	FAM Fluorescence			
SDS v1.2											
Well	Sample Name	Marker	Task	Passive Ref	Allele X	Allele Y	Allele X Rn	Allele Y Rn	Call	Quality Value	Method
A1	PC-WK	VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM	9.367	29.565	Undetermined	100.00	Manual Call
A2	PC-WK	VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM	9.733	29.988	Undetermined	100.00	Manual Call
A3		VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM			Undetermined	100.00	Manual Call
A4	NC-MPL	VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM	14.476	4.686	Undetermined	100.00	Manual Call
A5	NC-MPL	VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM	14.034	4.596	Undetermined	100.00	Manual Call
A6		VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM			Undetermined	100.00	Manual Call
A7	RS-WK	VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM	15.488	7.79	Undetermined	100.00	Manual Call
A8	RS-WK	VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM	14.267	7.422	Undetermined	100.00	Manual Call
A9		VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM			Undetermined	100.00	Manual Call
A10	H2O	VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM	0.18	0.168	Undetermined	100.00	Manual Call
A11	H2O	VIC	Unknown		MPL 515K-VIC	MPL 515 K-FAM	0.177	0.165	Undetermined	100.00	Manual Call

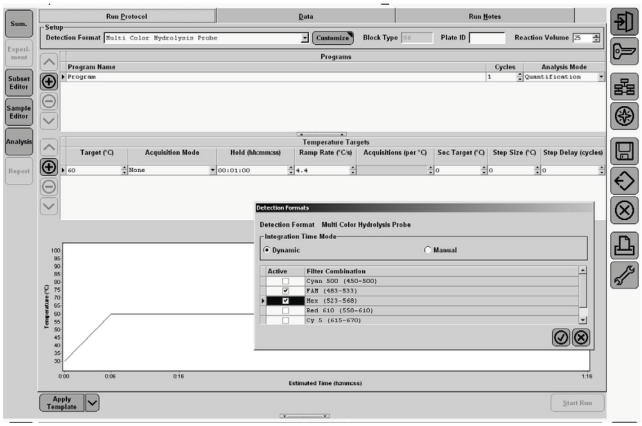
Detailed procedure for the LightCycler 480 instrument

1. On the home page, click **New Experiment**.
2. Depending on the instrument used (LightCycler 480 I or II), perform either step 2a for the LightCycler 480 I or step 2b for the LightCycler 480 II.

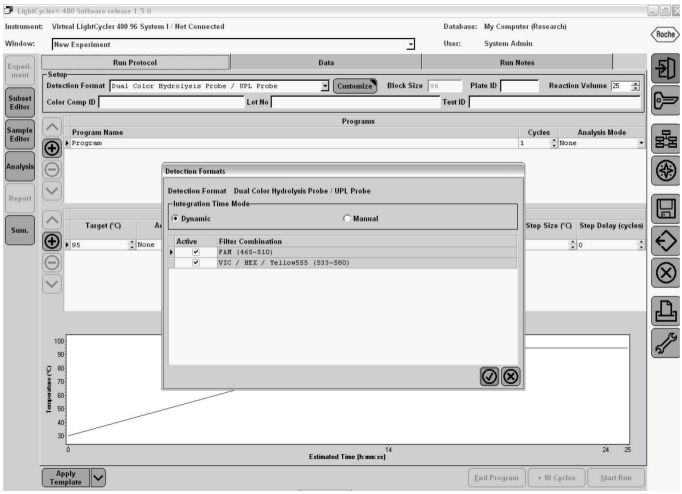
   2a. For the LightCycler 480 I: Select **Multi Color Hydrolysis Probe** in the **Detection Format** field, then click **Customize**. Verify that the channels **FAM (483-533)** and **VIC/Hex (523-568)** are selected in the **Detection Format** field.

   2b. For the LightCycler 480 II: Select **Dual Color Hydrolysis Probe** in the **Detection Format** field, then click **Customize**. Verify that the channels **FAM (465-510)** and **VIC/Hex (533-580)** are selected in the **Detection Format** field.

   A screenshot from the LightCycler 480 I instrument is shown below.



A screenshot from the LightCycler 480 II instrument is shown below.



Note: When describing the plate set up on the instrument, click **Endpt Geno** and in step 1, select **Workflow** section.

3. Run the PCR program, which is shown in Table 9.

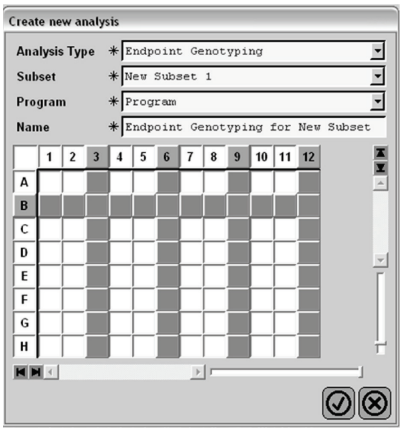
Table 9. Temperature profile for the LightCycler 480 instrument

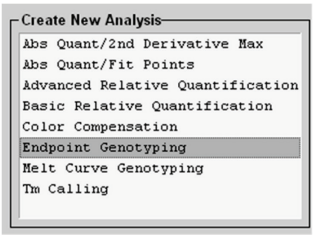
Parameters	
Hold 1	Temperature: 50°C Time: 2 min
Hold 2	Temperature: 95°C Time: 10 min
Cycling	50 times 92°C for 15 s 60°C for 1 min with acquisition of FAM fluorescence corresponding to 483–533 nm and VIC fluorescence corresponding to 523–568 nm for LC480 version 01 and acquisition of FAM fluorescence corresponding to 465–510 nm and VIC fluorescence corresponding to 533–580 nm for LC480 version 02
Hold 3	Temperature: 60°C Time: 1 min with acquisition of FAM fluorescence corresponding to 483–533 nm and VIC fluorescence corresponding to 523–568 nm for LC480 version 01 and acquisition of FAM fluorescence corresponding to 465–510 nm and VIC fluorescence corresponding to 533–580 nm for LC480 version 02

Detailed procedure for LightCycler 480 instrument setting of end point analysis

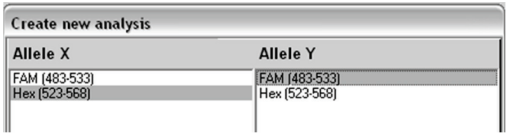
Users who are familiar with the use of the function "Allelic Discrimination" can go directly to step 6.

1. At the end of PCR, click **Analysis**.
2. In the Create New Analysis dialog box, select **Endpoint Genotyping** in the **Analysis Type** field, then select the subset to analyze in the **Subset** field.

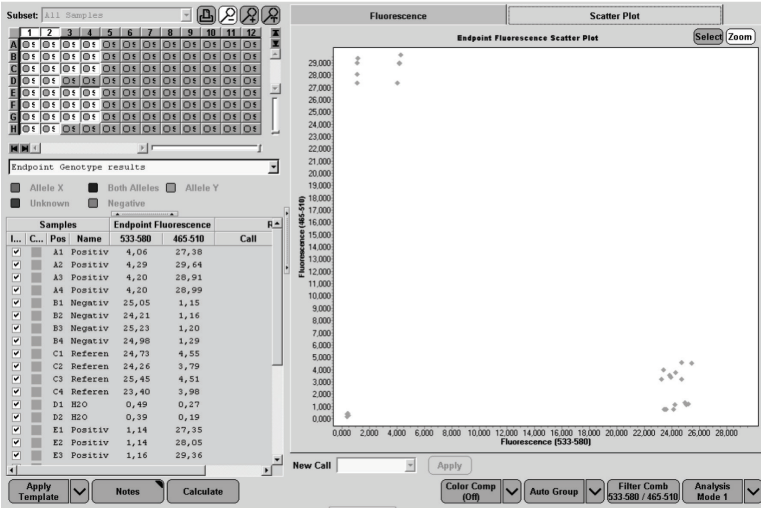




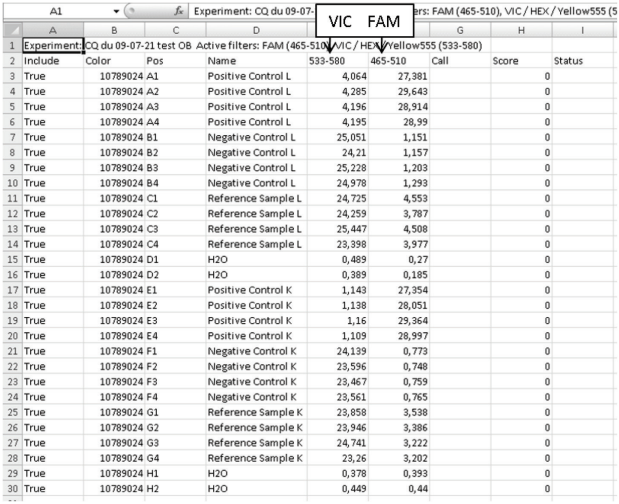
3. In the next window, select **VIC/HEX** fluorescence for the X allele column and **FAM** fluorescence for the Y allele column.



The following window appears. In this window, the upper left segment shows the plate set up while the bottom left shows the fluorescence results for each sample. On the right of the screen, a scatter plot is shown that gives the allelic discrimination based on FAM and VIC fluorescence measured at the 50th PCR cycle.



4. To export data, right-click thesamples results template, then select **Export Table**. The file will be saved in a text (*.txt) format.
5. To view and analyze results, open the file using Excel.



A1 | Experiment: CQ du 09-07- ... ers: FAM (465-510), VIC / HEX / Yellow555 (5

VIC FAM

	A	B	C	D	E	F	G	H	I
1	Experiment:	CQ du 09-07-21 test OB Active filters: FAM (465-510), VIC / HEX / Yellow555 (533-580)							
2	Include	Color	Pos	Name	533-580	465-510	Call	Score	Status
3	True	10789024	A1	Positive Control L	4,064	27,381		0	
4	True	10789024	A2	Positive Control L	4,285	29,643		0	
5	True	10789024	A3	Positive Control L	4,196	28,914		0	
6	True	10789024	A4	Positive Control L	4,195	28,99		0	
7	True	10789024	B1	Negative Control L	25,051	1,151		0	
8	True	10789024	B2	Negative Control L	24,21	1,157		0	
9	True	10789024	B3	Negative Control L	25,228	1,203		0	
10	True	10789024	B4	Negative Control L	24,978	1,293		0	
11	True	10789024	C1	Reference Sample L	24,725	4,553		0	
12	True	10789024	C2	Reference Sample L	24,259	3,787		0	
13	True	10789024	C3	Reference Sample L	25,447	4,508		0	
14	True	10789024	C4	Reference Sample L	23,398	3,977		0	
15	True	10789024	D1	H2O	0,489	0,27		0	
16	True	10789024	D2	H2O	0,389	0,185		0	
17	True	10789024	E1	Positive Control K	1,143	27,354		0	
18	True	10789024	E2	Positive Control K	1,138	28,051		0	
19	True	10789024	E3	Positive Control K	1,16	29,364		0	
20	True	10789024	E4	Positive Control K	1,109	28,997		0	
21	True	10789024	F1	Negative Control K	24,139	0,773		0	
22	True	10789024	F2	Negative Control K	23,596	0,748		0	
23	True	10789024	F3	Negative Control K	23,467	0,759		0	
24	True	10789024	F4	Negative Control K	23,561	0,765		0	
25	True	10789024	G1	Reference Sample K	23,858	3,538		0	
26	True	10789024	G2	Reference Sample K	23,946	3,386		0	
27	True	10789024	G3	Reference Sample K	24,741	3,222		0	
28	True	10789024	G4	Reference Sample K	23,26	3,202		0	
29	True	10789024	H1	H2O	0,378	0,393		0	
30	True	10789024	H2	H2O	0,449	0,44		0	

Protocol: qPCR on LightCycler 2.0 Instruments

Using capillary instruments, we recommend measuring samples and controls in duplicate, as indicated in Table 10 or Table 11.

Table 10. Number of reactions for a MPL W515L experiment using LightCycler 2.0 instruments

Samples	Reactions
With the PPM-MPL W515L/WT primers and probe mix	
n DNA samples	n x 2 reactions
Plasmid controls	2 x 3 reactions (PC-515L, NC-MPL and COS-515L, each one tested in duplicate)
Water control	2 reactions

Table 11. Number of reactions for a MPL W515K experiment using LightCycler 2.0 instruments

Samples	Reactions
With the PPM-MPL W515K/WT primers and probe mix	
n DNA samples	n x 2 reactions
controls	2 x 3 reactions (PC-515K, NC-MPL and COS-515K, each one tested in duplicate)
Water control	2 reactions

Sample processing on LightCycler 2.0 instrument

We recommend testing at least 12 DNA samples in the same experiment to optimize the use of the controls, and primers and probe mixes. The capillary scheme in Figure 4 shows an example of an experiment.

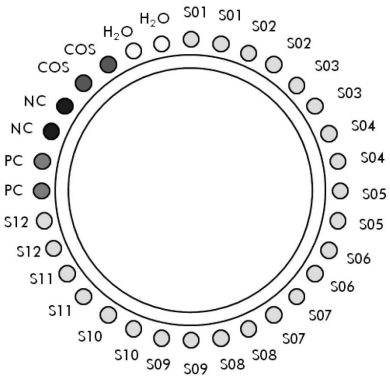


Figure 4. Suggested rotor setup for each experiment with the *ipsogen* MPL W515L/K Muta*Screen* Kit. PC: Positive control; **NC**: Negative control; **H_2O**: Water control; **S**: Unknown DNA sample to be analyzed.

qPCR on LightCycler 2.0 instruments

Note: Because of particular technological requirements, LightCycler 2.0 experiments must be performed using specific reagents. We recommend to use the LightCycler TaqMan Master and to follow the manufacturer's instructions to prepare the Master Mix 5x.

Note: Perform all steps on ice.

Procedure

1. Thaw all necessary components and place them on ice.
2. Prepare the following qPCR mix according to the number of samples being processed.

   Note: All concentrations are for the final volume of the reaction.

   Table 12 describes the pipetting scheme for the preparation of one reagent mix, calculated to achieve a final reaction volume of 20 µl. A pre-mix can be prepared, according to the number of reactions, using the same primer and probe mix (either PPM-MPL W515L or PPM-MPL W515K). Extra volumes are included to compensate for pipetting error.

Table 12. Preparation of qPCR mix

Component	1 rxn (µl)	32+1 rxns (µl)	Final conc.
LightCycler TaqMan Master Mix, 5x	4	132	1x
Primers and probe mix (PPM-MPL W515L or PPM-MPL W515K), 10x	2.0	66.0	1x
Nuclease-free, PCR-grade water	9	297.0	–
Sample (to be added at step 4)	5	5 each	–
Total volume	**20**	**20 each**	**–**

Conc.: concentration; rxn(s): reaction(s).

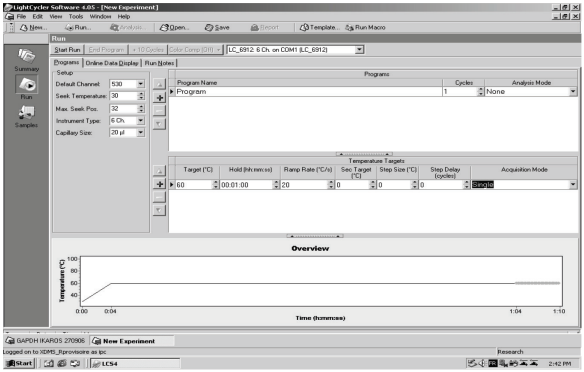
3. Dispense 15 µl of the qPCR pre-mix per capillary.
4. Add 5 µl of the material to be tested (25 ng sample genomic DNA) in the corresponding capillary (total volume 20 µl).
5. Mix gently by pipetting up and down.
6. Place the capillaries in the adapters provided with the instrument, and briefly centrifuge (700 x *g*, approximately 10 s).
7. Load the capillaries into the thermal cycler according to the manufacturer recommendations.
8. Program the LightCycler 2.0 instrument with the thermal cycling program as indicated in Table 13.

Table 13. Temperature profile

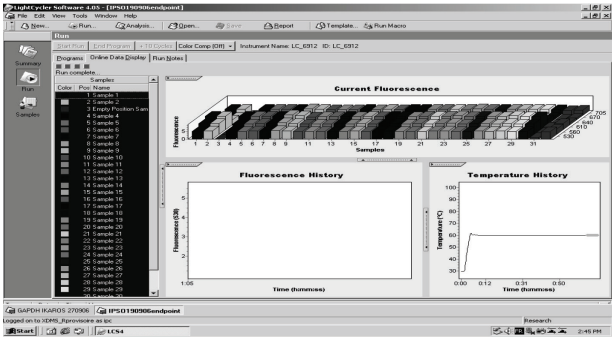
Parameters	
Mode of analysis	Quantitation
Hold 1	Temperature: 55°C Time: 2 min Ramp: 20
Hold 2	Temperature: 95°C Time: 10 min Ramp: 20
Cycling	50 times 92°C for 15 s; ramp: 20 60°C for 1 min; ramp: 20; with acquisition of FAM fluorescence: Single acquisition of VIC fluorescence: Single

Detailed procedure for the LightCycler 2.0 instrument setting of post-read run analysis

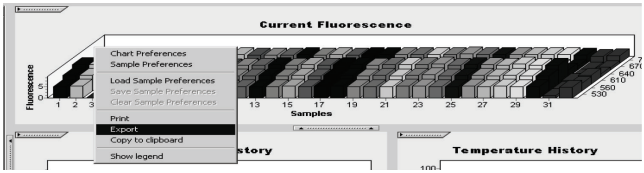
1. At the end of the amplification run, select a new LightCycler experiment.
2. Click **Post read** and run a PCR program of 1 cycle of 1 min at 60°C, a ramp of 20, and FAM and VIC single acquisition.



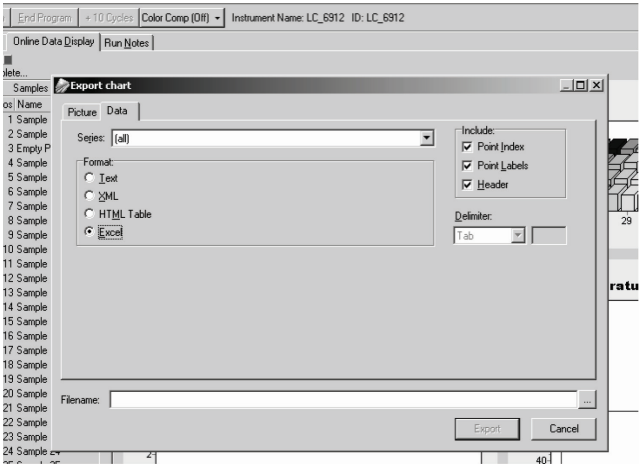
3. In the window Online Data Display, right-click near the **Current Data Fluorescent** graph as shown in the following screenshot.



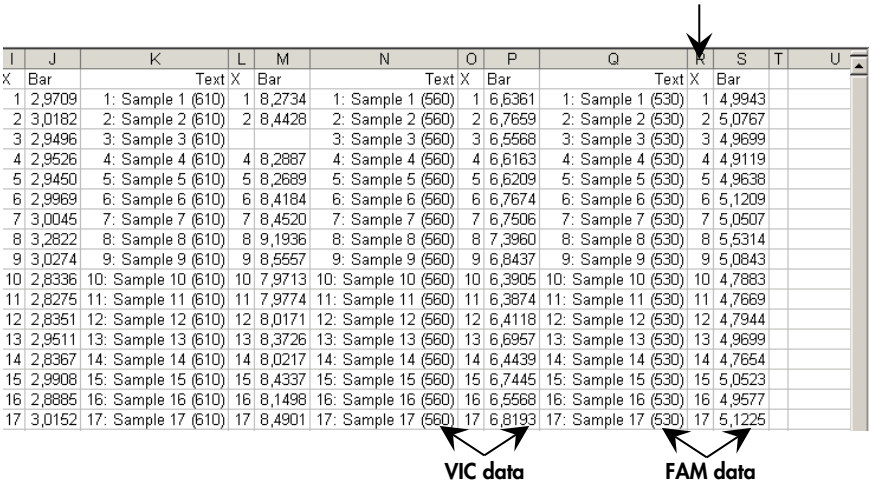
4. Select **Export**.



5. In the Export Chart dialog box, select **Excel** from the **Format** list.



6. In the **Filename** field, browse the location to export your result file to.
7. Click **Export**.
8. Analyze the export file. An example of the output is shown in the following screenshot.



I	J	K	L	M	N	O	P	Q	R	S	T	U
X	Bar	Text	X	Bar	Text	X	Bar	Text	X	Bar		
1	2,9709	1: Sample 1 (610)	1	8,2734	1: Sample 1 (560)	1	6,6361	1: Sample 1 (530)	1	4,9943		
2	3,0182	2: Sample 2 (610)	2	8,4428	2: Sample 2 (560)	2	6,7659	2: Sample 2 (530)	2	5,0767		
3	2,9496	3: Sample 3 (610)			3: Sample 3 (560)	3	6,5568	3: Sample 3 (530)	3	4,9699		
4	2,9526	4: Sample 4 (610)	4	8,2887	4: Sample 4 (560)	4	6,6163	4: Sample 4 (530)	4	4,9119		
5	2,9450	5: Sample 5 (610)	5	8,2689	5: Sample 5 (560)	5	6,6209	5: Sample 5 (530)	5	4,9638		
6	2,9969	6: Sample 6 (610)	6	8,4184	6: Sample 6 (560)	6	6,7674	6: Sample 6 (530)	6	5,1209		
7	3,0045	7: Sample 7 (610)	7	8,4520	7: Sample 7 (560)	7	6,7506	7: Sample 7 (530)	7	5,0507		
8	3,2822	8: Sample 8 (610)	8	9,1936	8: Sample 8 (560)	8	7,3960	8: Sample 8 (530)	8	5,5314		
9	3,0274	9: Sample 9 (610)	9	8,5557	9: Sample 9 (560)	9	6,8437	9: Sample 9 (530)	9	5,0843		
10	2,8336	10: Sample 10 (610)	10	7,9713	10: Sample 10 (560)	10	6,3905	10: Sample 10 (530)	10	4,7883		
11	2,8275	11: Sample 11 (610)	11	7,9774	11: Sample 11 (560)	11	6,3874	11: Sample 11 (530)	11	4,7669		
12	2,8351	12: Sample 12 (610)	12	8,0171	12: Sample 12 (560)	12	6,4118	12: Sample 12 (530)	12	4,7944		
13	2,9511	13: Sample 13 (610)	13	8,3726	13: Sample 13 (560)	13	6,6957	13: Sample 13 (530)	13	4,9699		
14	2,8367	14: Sample 14 (610)	14	8,0217	14: Sample 14 (560)	14	6,4439	14: Sample 14 (530)	14	4,7654		
15	2,9908	15: Sample 15 (610)	15	8,4337	15: Sample 15 (560)	15	6,7445	15: Sample 15 (530)	15	5,0523		
16	2,8885	16: Sample 16 (610)	16	8,1498	16: Sample 16 (560)	16	6,5568	16: Sample 16 (530)	16	4,9577		
17	3,0152	17: Sample 17 (610)	17	8,4901	17: Sample 17 (560)	17	6,8193	17: Sample 17 (530)	17	5,1225		

Results

FAM/VIC ratio calculation and genotyping

Regardless of the instrument used, an analysis export file will be created and this can be used to extract the data and process it as follows.

Procedure

1. For each test, calculate the FAM/VIC ratio.
2. If the fluorescence data are consistent between duplicates, calculate the mean ratio for each sample including the control samples.
3. Compare the mean ratio value obtained for each unknown sample (Ratio Sample) with the cut-off sample mean ratio (Ratio COS-WL– Ratio COS-WK, detection limit) as shown in Table 14.

   If the ratio of the sample is greater than or equal to the ratio for COS-WL or COS-WK, then the sample is either MPL W515L positive or MPL W515K depending on which probe mix was used.

   If the ratio of the sample is strictly less than the ratio for COS-WL or COS-WK, then either the MPL W515L mutation was not detected or the MPL W515K mutation was not detected depending on which probe mix was used.

Table 14. FAM/VIC ratio calculation and genotyping using MPL W515K as an example

Name	VIC	FAM	Ratio FAM/VIC	Mean ratio	Genotype
NC-MPL*	14.965 14.974	4.065 4.267	0.272 0.285	0.278	–
COS-WK*	14.661 13.938	6.023 5.782	0.411 0.415	0.413	–
PC-WK*	9.091 10.368	21.594 24.663	2.375 2.379	2.377	–
H_20*	0.195 0.225	0.258 0.304	1.323 1.351	X	NA
Sample 1*	14.429 14.058	5.571 5.382	0.386 0.383	0.384	Wild-type
Sample 2*	13.339 13.887	6.417 6.657	0.481 0.479	0.480	Mutant
Sample 3*	12.047 12.328	3.819 3.86	0.317 0.313	0.315	Wild-type
Sample 4*	12.141 12.145	3.362 3.493	0.277 0.288	0.282	Wild-type
Sample 5*	11.256 11.424	3.481 3.483	0.309 0.305	0.307	Wild-type
Sample 6*	12.643 11.847	3.459 3.365	0.274 0.284	0.279	Wild-type
Sample 7*	11.364 11.421	3.439 3.474	0.303 0.304	0.303	Wild-type

NA: not applicable.

* Assay performed in duplicate.

Graphical representation and quality control criteria

Figure 5 shows an example of the results allelic discrimination pattern that can be obtained.

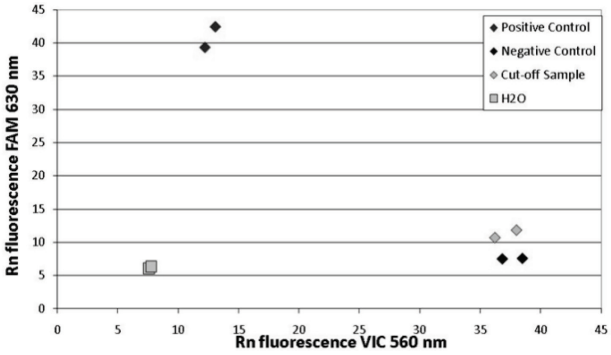


Figure 5. Allelic discrimination. Scatter plot with fluorescence data; VIC values are plotted on the x-axis, FAM values are plotted on the y-axis.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of *ipsogen* MPL W515L/K Muta*Screen* Kit is tested against predetermined specifications to ensure consistent product quality. Certificates of analysis are available on request at **www.qiagen.com/support/**.

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
 <N>	Contains reagents sufficient for <N> reactions
	Use by
	Catalog number
	Lot number
	Material number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Consult instructions for use

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Ordering Information

Product	Contents	Cat. no.
ipsogen MPL W515L/K Muta*Screen* Kit (24)	Kit for the detection of MPL W515L and W515K mutations in human genomic DNA.	676413
Rotor-Gene Q for outstanding performance in real-time PCR		
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included.	9001580

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Document Revision History

Date	Changes
January 2020	Corrected typo error throughout the document: “Standards” is replaced with “controls”; “to be quantified” is replaced with “to be tested”
	Corrected typo error in Table 3 from “RT mix” to “qPCR mix”
	Minor revision to Protocol: qPCR on Rotor-Gene Q 5plex HRM Instruments section to align Note description with Figure 2 layout
	Updated the Detailed procedure for Rotor-Gene Q instrument section to include additional steps in the procedure, add an analysis of parameters, and improve the data export process
	Removed Mode of analysis from Table 9
	Corrected typo error to qPCR on LightCycler 2.0 instrument and removed 1.2 from the section title of Sample processing on LightCycler 2.0 instruments
	Added VIC single acquisition in Table 13
	Added FAM and VIC single acquisition in Detailed procedure for the LightCycler 2.0 instrument setting of post-read analysis section
	Corrected title of Table 14 to FAM/VIC ratio calculation and genotyping using MPL W515K as an example

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