myT® BRAF

qPCR Primers for Detection of Human BRAF V600E/K

For Research Use Only

Version 1112

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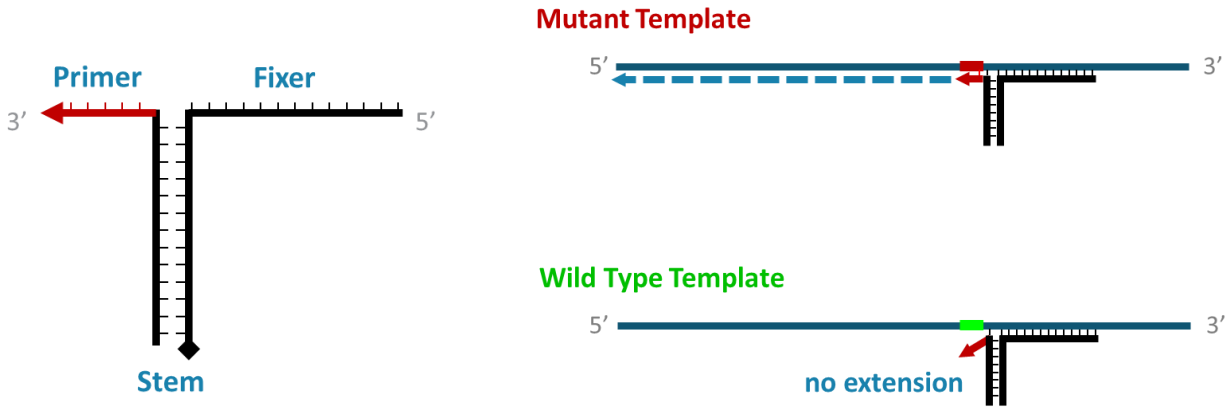
CFX96™Real Time PCR Detection System is a trademark and product of Bio-Rad Laboratories, Inc.

LightCycler® 480 Real-Time PCR System is a registered trademark and product of Roche Applied Science

myT® Primer Technology

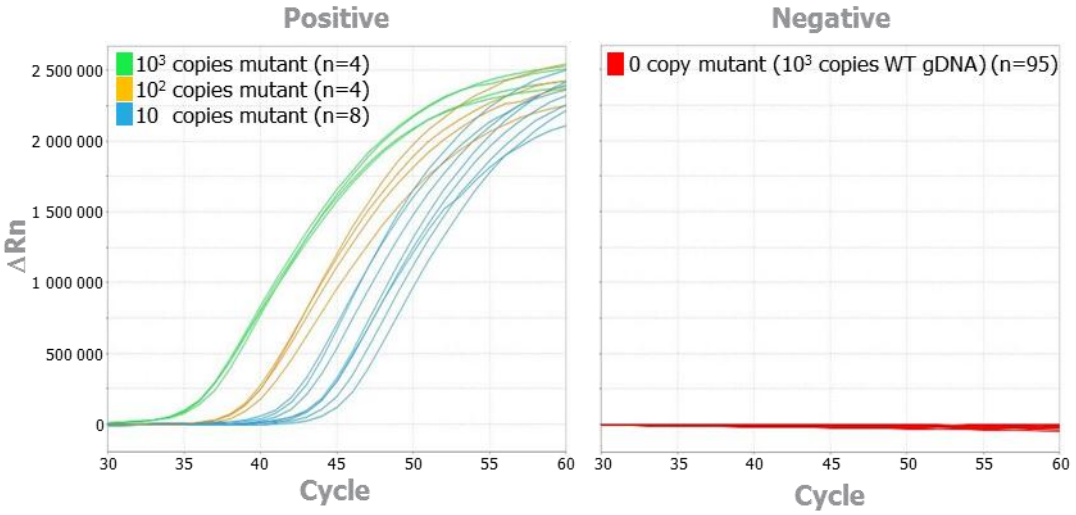
myT Primers have unique structural and thermodynamic properties that make them highly sensitive to mismatch discrimination. myT Primers are comprised of Primer and Fixer oligonucleotides with three functional domains: the long Fixer domain provides a high level of specificity for genomic DNA templates, the Primer domain is highly sensitive to single base mutations due to its very short length, and the double stranded stem links the Fixer and Primer domains.

When a mutant-specific myT Primer is combined with a reverse primer and hydrolysis probe, myT Primers can detect 1% mutant BRAF V600E or K present in a background of 10^3 wild-type genomic DNA copies without non-specific amplification from wild-type; either a positive or negative amplification signal is generated and a delta Ct method to distinguish specific from non-specific amplification is not required (see data on page 4).



Swift also offers myT BRAF-Ultra Primers for ultra-low copy detection of V600E/K from genomic DNA. myT BRAF-Ultra can detect a single mutant BRAF copy in > 10^4 wild-type BRAF copies. Regardless of the sensitivity level of mutant BRAF detection needed, myT Primer reagents offer increased specificity, which provides higher confidence in test performance.

myT BRAF Performance



Positive amplification plot. qPCR reactions containing mutant genomic DNA at the specified quantity in a background of 10^3 wild-type genomic DNA resulted in BRAF V600E-specific amplification: 50% mutant content (green; n = 4 replicates), 10% mutant content (yellow; n = 4 replicates) and 1% mutant content (blue; n = 8 replicates). Assay performed on an ABI 7500.

Negative amplification plot. qPCR reactions containing 10^3 wild-type genomic DNA (no mutant DNA) resulted in no amplification (red) in 95 separate reaction wells. Assay performed on an ABI 7500.

Conclusion. These results demonstrate mismatch discrimination with very high specificity. The result is clear and unambiguous, eliminating the need for ΔCt analysis to distinguish specific from non-specific amplification. This high confidence, "Yes/No" clarity is a feature that is exclusive to myT Primer reagents.

Protocol

The myT BRAF package provides sufficient reagents to perform a total of 60 reactions to assess BRAF mutations V600E/K using the ABI 7500, Roche LightCycler 480 or Bio-Rad CFX96 Real Time PCR Systems.

Mutation detection with myT BRAF consists of two steps:

1. Locus-specific qPCR
   - A non-allele-specific qPCR is performed to assess total (mutant + wild-type) amplifiable BRAF for each sample
   - This determines the quantity of DNA to be used in step 2 for each sample
   - Reagents for 30 reactions, including controls, are included

2. Allele-specific qPCR
   - A mutant allele-specific BRAF qPCR is then performed to assess presence of V600E or V600K
   - This assay DOES distinguish between wild-type and mutant but DOES NOT distinguish between the two mutant alleles
   - Results are reported as positive or negative for mutant BRAF for each sample with a sensitivity limit of 1% (10 mutant copies in 10^3 wild-type copies)
   - Reagents for 30 reactions, including controls, are included

Reagents Included

Reagent Mixes	Volume	Description
BRAF Locus-specific Primers	198 µl	Non-allele-specific myT BRAF primers
BRAF Allele-specific Primers	198 µl	V600E/K Allele-specific myT BRAF primers
Nuclease-free Buffer	1 ml	For DNA sample dilution and NTC* reactions

***NTC = no-template control**

Shipped in a separate box:

BRAF DNA Standard	50 µl	BRAF V600E control DNA (200 copies/µl)

- Store all reagents at -20° C upon arrival
- To avoid cross-contamination, store the myT Primers box separately from the DNA Standard box
- Refreeze unused myT Primers and DNA Standard at -20° C
- For best performance, limit freeze-thaw cycles to 4

Reagents not included

Reagents	Scale	Recommended Vendor
qPCR Master Mix (2X)	200 reactions	Maxima Probe qPCR Master Mix (2X) ThermoFisher/Fermentas catalog # K0261
Dual-Labeled Probe	Mini / 0.5 nmol	IDT PrimeTime Dual-Labeled Probe Free of charge – voucher provided

Note: *myT BRAF has been optimized for use with the above reagents. Reagents from other vendors may be substituted, but substitutions may result in reduced assay performance or require the user to modify assay conditions to achieve maximal performance.*

Probe sequence

5'- /56-FAM/CAC CTC AGA TAT ATT TCT TCA TGA AGA CCT CAC AG/3IaBkFQ/ -3'

- When ordering this probe, please include an internal Zen quencher
- Details on how to redeem the free of charge voucher for the dual-labeled probe from IDT were sent with your order acknowledgement. If you have any questions, please contact Swift Technical Support at 734.330.2568 or technicalsupport@swiftbiosci.com.

Instructions for re-suspension of probe

- Spin lyophilized probe to collect contents
- Resuspend in 167 µl of Nuclease-free Buffer provided
- Distribute 72 µl each to the Locus-specific and Allele-specific myT Primer stocks
- The final volume for each stock will be 198 + 72 = 270 µl
- Probes are light-sensitive. Avoid prolonged exposure to light once probe has been added.

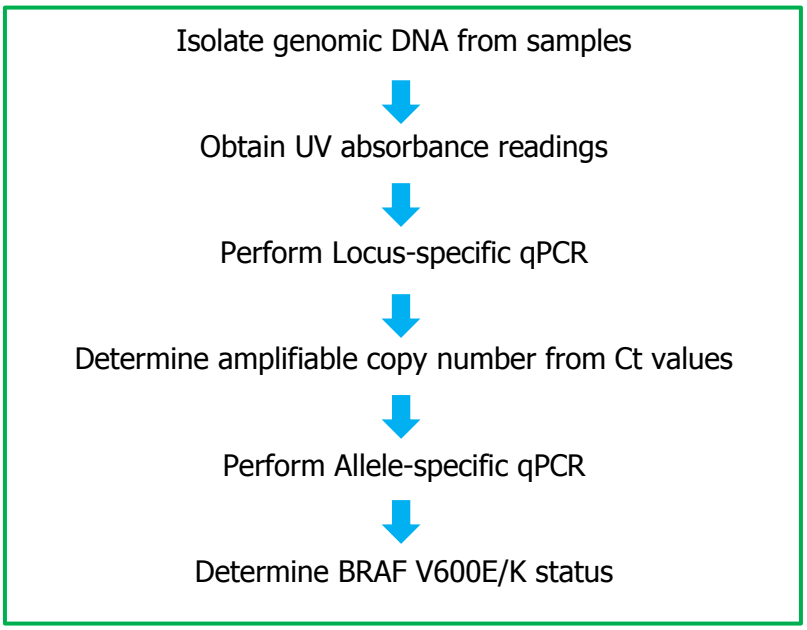
96-well plates are not supplied, but the following have been tested using myT BRAF:

- ABI 7500: Order from Applied Biosystems/Life Technologies
  - 96-well optical reaction plates cat. no. 4306737
  - MicroAmp optical Adhesive Film cat. no. 4311971
- LightCycler 480: Order from Roche Applied Science
  - LightCycler 480 Multiwell Plate 96, white with sealing foils cat no. 04729692001
- CFX96: Order from Bio-Rad
  - Multi-plate Low-profile unskirted 96-well plates, natural cat no. MLL-9601
  - Microseal Film "B" cat no. MSB1001

Notes Regarding DNA Samples

- For high quality DNA derived from cell lines or fresh-frozen clinical samples, UV absorbance readings correlate well with amplifiable content.
- For DNA derived from formalin-fixed paraffin embedded samples (FFPE), UV absorbance readings determine the DNA concentration but do NOT accurately determine amplifiable content due to DNA damage from fixation.
- It is recommended to obtain UV absorbance readings for each sample in order to determine the amount of DNA to use in the Locus-specific qPCR (Step 1).
- It is recommended to use ~5 ng of high quality DNA or a range of 10 – 50 ng of FFPE DNA for the Locus-specific qPCR.
- In the case of heavily damaged samples, >50 ng DNA can be placed into a reaction, but inhibition of PCR is likely to occur. Similarly, it is not recommended to place greater than 20% volume of DNA per 25 µl reaction as PCR inhibitors are present in FFPE samples.
- This assay has been tested using DNA isolated by the Qiagen QIAamp DNA FFPE Tissue Kit with RNase treatment (not included in this kit). Since RNA co-purifies with DNA, RNase treatment provides more accurate DNA quantification based on UV absorbance reading.
- To avoid cross-contamination that could lead to false positive results:
    - Change gloves frequently
    - Use aerosol-resistant pipette tips
    - Use pipettes dedicated for template and non-template containing reagents
    - Maintain separate work areas for template and non-template containing reagents
    - Routinely decontaminate work areas with 10% bleach and/or UV light
    - Never open PCR reaction wells that resulted in allele-specific amplification

myT BRAF Workflow



The contents provided are sufficient to perform 60 reactions consisting of 30 Locus-specific and 30 Allele-specific reactions. This enables testing of up to 28 samples when including a positive control and NTC if performed as a single qPCR run. If testing is split into multiple batches, total samples tested will be less since a positive control and NTC are required for each run. For example, if testing is batched into 3 qPCR runs, the total number of samples analyzed will be 24 (8 per run) where 3 positive control and 3 NTC reactions are also run. A 10% excess volume is included to compensate for pipetting loss.

Step 1: Locus-specific BRAF qPCR

Thaw reagents completely at room temperature. Once thawed, invert repeatedly or gently vortex and briefly centrifuge to collect contents. To avoid cross-contamination, always briefly centrifuge DNA Standard and DNA samples prior to opening caps. Also, gently mix reactions containing 2X qPCR Master Mix to avoid formation of bubbles that can interfere with fluorescence detection.

Each reaction contains:

BRAF Locus-specific Primers + Probe*	7.5 µl
2X qPCR Master Mix	12.5 µl
DNA Template	5 µl
Total Volume	**25 µl**

*Remember to add 72 µl of resuspended probe to the myT Primer tube before initial use

1. Make a cocktail with BRAF Locus-specific primers and 2X qPCR Master Mix in the amount needed for the number of reactions to be run plus up to 5% extra volume to compensate for pipetting loss (maximum = 28 samples plus 2 control wells).
2. Invert tube with the cocktail repeatedly to mix reagents and briefly centrifuge to collect contents.
3. Dispense 20 µl cocktail into each reaction well.
4. Add 5 µl sample DNA corresponding to 5 ng high quality DNA or 10 to 50 ng of FFPE DNA. If necessary, use Nuclease-free Buffer (provided) to dilute samples.
5. Include a "no template control" (NTC) by adding 5 µl Nuclease-free Buffer to one reaction well.
6. Include a 10^3 copy positive control by adding 5 µl BRAF DNA Standard to one reaction well.
7. Seal plate and briefly centrifuge at 1000-2000 RPM for 15 seconds to collect contents.
8. Load plate into the selected thermocycler and follow run instructions (for details, see Appendix for ABI 7500, Roche LightCycler 480, or Bio-Rad CFX96 instructions).

Cycling Temperature	Cycling Time	Cycles
95°C	10 minutes	1 cycle
95°C	14 seconds	**45 cycles**
62°C	1 minute*	

*with FAM read; disable any reads for passive reference dyes such as ROX

Determination of amplifiable copy number for the allele-specific assay

1. The control DNA Standard has 10^3 amplifiable BRAF copies per 5 µl and should have a Ct value as specified in the table below depending on the thermocycler used. 10^3 is the recommended amplifiable copy number to place in the Allele-specific assay. Limiting the assay to 10^3 amplifiable copies reduces the likelihood of PCR inhibition and detection of low-level cross contamination that can be present in FFPE samples.

Thermocycler	Expected Average Ct value for Locus-specific 10^3 copies
ABI 7500	27.3
LC 480	28.7
CFX96	27.7

2. If samples have a Ct value less than the control DNA well, dilute with Nuclease-free Buffer to 10^3 amplifiable copies per 5 µl, assuming that a 2-fold dilution will increase the Ct value by 1.

   LC 480 Example: If a Ct of 26.7 is obtained, 28.7 – 26.7 = 2 Ct, so dilute sample 4-fold

3. If samples have a Ct value greater than the control DNA well, add up to 10^3 amplifiable copies per 5 µl, assuming that a two-fold increase in DNA will decrease the Ct value by 1. Do not exceed 20% DNA per reaction volume, as PCR inhibitors are present in FFPE preparations.

   ABI 7500 Example: If a Ct of 29.3 is obtained, 29.3 – 27.3 = 2, so add 4-fold more DNA, if possible

4. If samples have insufficient amplifiable copy number, 1% sensitivity is not likely to be achieved as 1% represents 10 mutant copies in 10^3 total copies. Based on Poisson distribution, copy number less than 10 is not detected at 100% frequency in a single well reaction.

5. If the Locus-specific Ct value is >35, the amplifiable copy number is too low to proceed.

6. Regarding the NTC, either no amplification or an occasional Ct $\geq$38 may be obtained. If the NTC or DNA Standard (positive control) fails, contact technical service.

Step 2: Allele-specific BRAF V600E/K qPCR

Thaw reagents completely at room temperature. Once thawed, invert repeatedly or gently vortex and briefly centrifuge to collect contents. To avoid cross-contamination, always briefly centrifuge DNA Standard and DNA samples prior to opening caps. Also, gently mix reactions containing 2X Master Mix to avoid formation of bubbles that can interfere with fluorescence detection.

Each reaction contains:

BRAF Allele-specific Primers + Probe*	7.5 µl
2X qPCR Master Mix	12.5 µl
DNA Template	5 µl
Total Volume	**25 µl**

*Remember to add 72 µl of resuspended probe to the myT Primer tube before initial use

1. Make a cocktail with BRAF Allele-specific Primers and 2X qPCR Master Mix in the amount needed for the number of reactions to be run plus up to 5% extra volume to compensate for pipetting loss (maximum = 28 samples plus 2 control wells).
2. Invert cocktail repeatedly to mix reagents and briefly centrifuge to collect contents.
3. Dispense 20 µl cocktail into each reaction well.
4. Add 5 µl sample DNA that corresponds to 10^3 amplifiable copies (as determined from the Locus-specific qPCR in Step 1, above).
5. Include a "no template control" (NTC) by adding 5 µl Nuclease-free Buffer to one reaction well.
6. Include a 10^3 copy positive control by adding 5 µl DNA Standard to one reaction well.
7. Seal plate and briefly centrifuge 1000-2000 RPM for 15 seconds to collect contents.
8. Load plate into the selected thermocycler and follow run instructions (for details, see Appendix for ABI 7500, Roche LightCycler 480, or Bio-Rad CFX96 instructions).

Cycling Temperature	Cycling Time	Cycles
95°C	10 minutes	1 cycle
95°C	14 seconds	**60 cycles**
62°C	1 minute*	

*with FAM read; disable any reads for passive reference dyes such as ROX

Determination of BRAF V600E/K status for each sample

- For the Allele-specific myT BRAF qPCR, either a positive or negative amplification signal will be obtained.
- If 10^3 amplifiable copies are analyzed, a 1% sensitivity limit which represents 10 mutant copies in 10^3 wild-type copies can be achieved.
- If only 10^2 amplifiable copies are analyzed, a reduced 10% sensitivity limit can be achieved which represents 10 mutant copies in 10^2 wild-type copies.
- The cut-off Ct value for detection of 10 mutant copies for the three thermocyclers tested are in the table below.

Thermocycler	10 Copy Ct Cut-off
ABI 7500	> 47
LightCycler 480	> 50
CFX96	> 48

- If a Ct value is obtained that exceeds the cut-off, it is scored as negative or below the limit of detection for this assay.
- Occasionally when amplifiable copy number is limiting, a Ct value near the cut-off will be obtained. In this case, the assay can be repeated to confirm a positive amplification signal.
- If positive, the Allele-specific Ct value will be dependent on the percent tumor cell content and the tumor heterogeneity of the sample from which the DNA was derived. Low percentage tumor cell samples will have limited sensitivity.

Appendix

Roche LightCycler® 480 - Run protocol

1. Turn on the LightCycler®
2. Open LightCycler® software
3. Click on "New Experiment from Template"
4. Select "Monocolor Hydrolysis Probe/UPL Probe"

Setup

1. Detection format "Monocolor Hydrolysis Probe/UPL Probe"
2. Click on "Customize", select "Dynamic" and "FAM 465-510"
3. Block Size "96"
4. Reaction Volume "25µl"
5. Set Programs and Temperature targets as follows:

A. Pre-incubation

Programs

Program Name	Cycles	Analysis Mode
Pre-incubation	1	None

Temperature Targets †

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)
95°C	None	00:10:00	4.4

B. Amplification

Programs

Program Name	Cycles	Analysis Mode
Amplification	45 or 60*	Quantification

Temperature Targets †

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)
95	None	00:00:14	4.4
62	Single	00:01:00	2.2

***45 cycles of amplification are required for Locus-specific reactions and 60 cycles for Allele-specific reactions**

C. Cooling

Programs

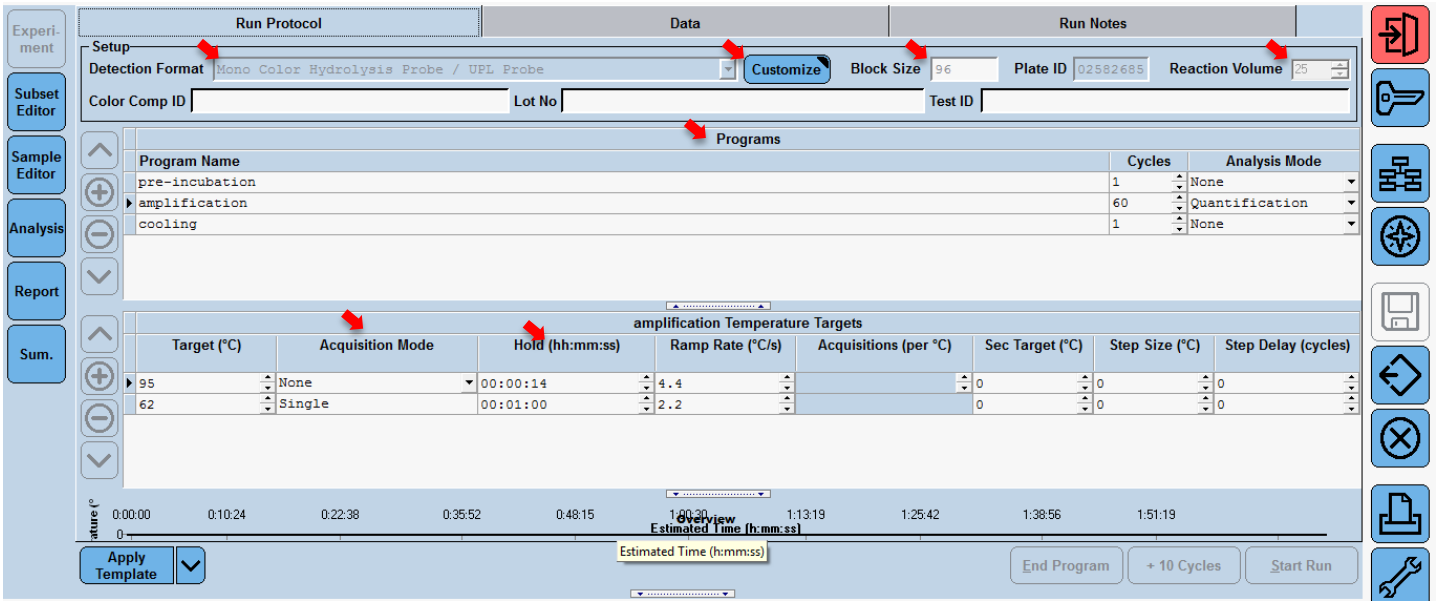
Program Name	Cycles	Analysis Mode
Cooling	1	None

Temperature Targets †

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)
40	None	00:00:30	2.2

†For all Temperature Targets:

Acquisition (per °C): not used
Sec Target (°C), Step Size (°C), Step Delay (cycles): "0"



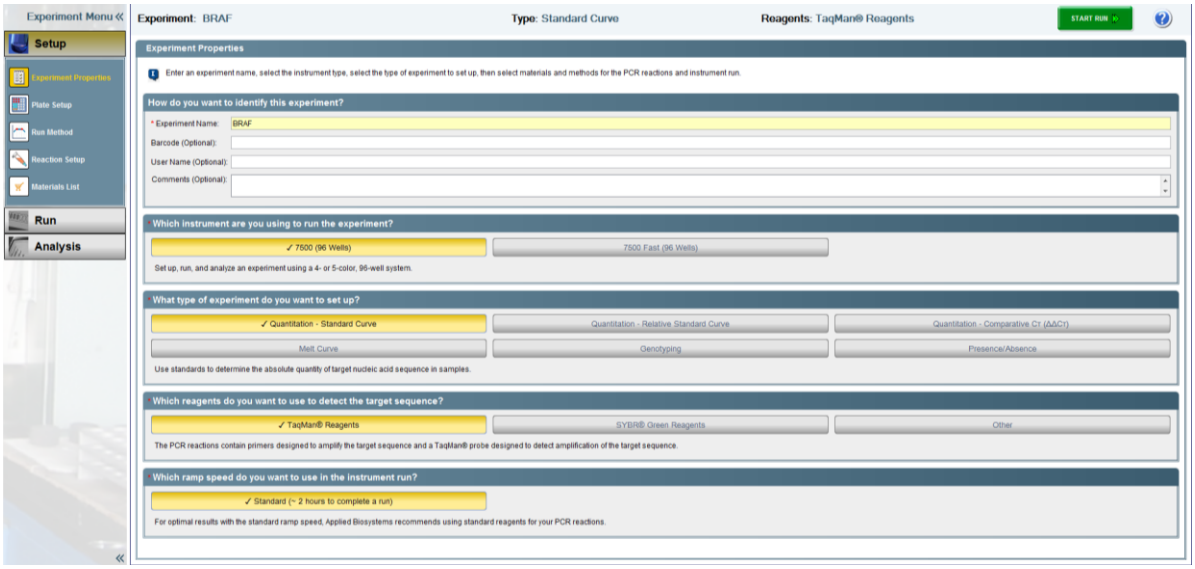
6. Open the door, insert your plate, and close the door
7. Click on "Start Run" at the bottom right corner of the screen

Life Technologies ABI 7500 - Run protocol

1. Turn on the ABI 7500
2. Open ABI7500 software on your computer
3. Select "Advanced Setup"

Setup – Experiment properties

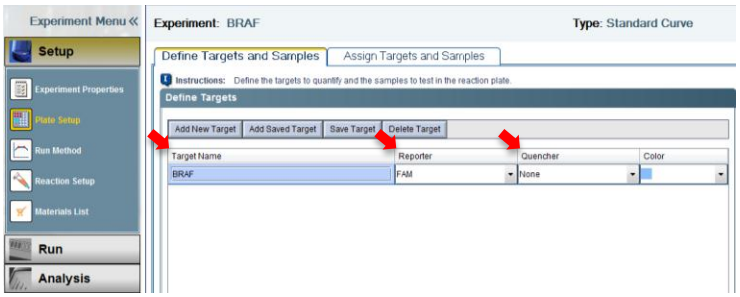
1. Name your experiment
2. Select:
   "7500 (96 Wells)"
   "Quantitation – Standard Curve"
   "TaqMan® Reagents"
   "Standard (~ 2 hours to complete a run)"



Setup – Plate Setup

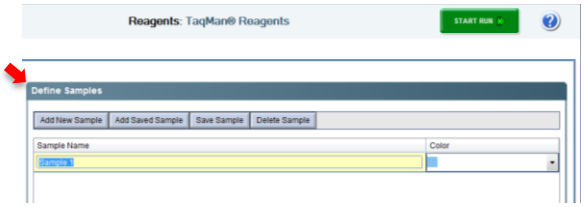
1. Define Targets and Samples (*Define Targets)*

Target Name	Reporter	Quencher	Color
BRAF	FAM	None	your choice



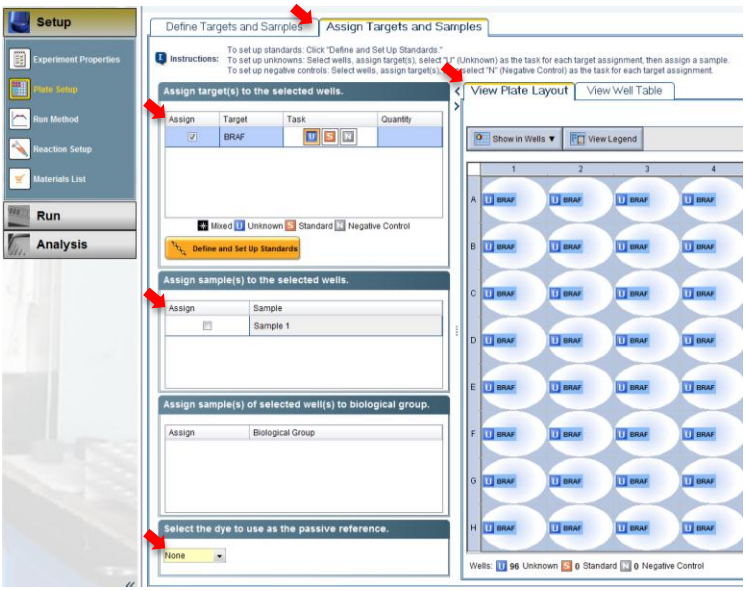
2. Define Targets and Samples (*Define Samples)*

   Name your samples



3. Assign Targets and Samples

- Select each well containing a reaction in "View Plate Layout" and assign "Target BRAF"
- Assign your particular samples the same way
- Select the dye to use as a passive reference "None"

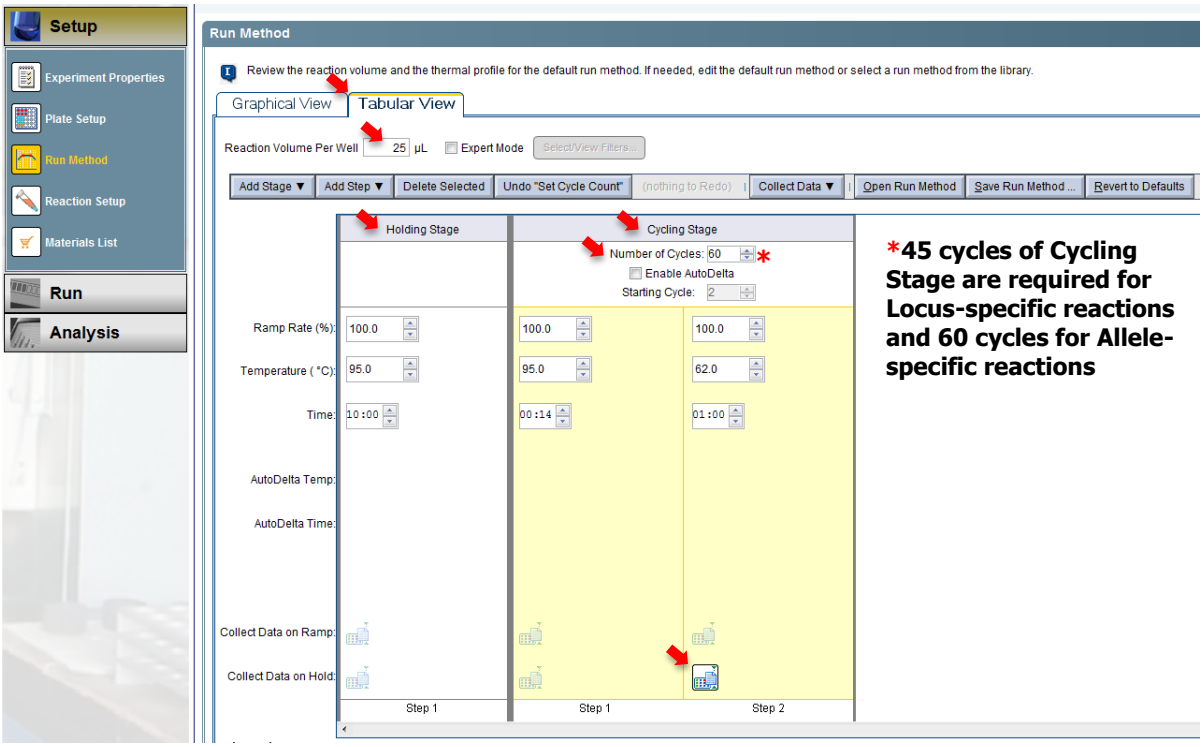


Setup - Run method

1. Select Tabular View
2. Reaction Volume Per Well "25µl"
3. Holding Stage (1 step):
   "95°C, 10 minutes, ramp rate 100%"
4. Cycling Stage (2 steps):
5. Number of Cycles: 45 or 60 cycles*

   "95°C, 14 seconds, ramp rate 100%"
   "62°C, 1 minute, ramp rate 100%" + "collect data on hold"

***45 cycles of Cycling Stage are required for Locus-specific reactions and 60 cycles for Allele-specific reactions**



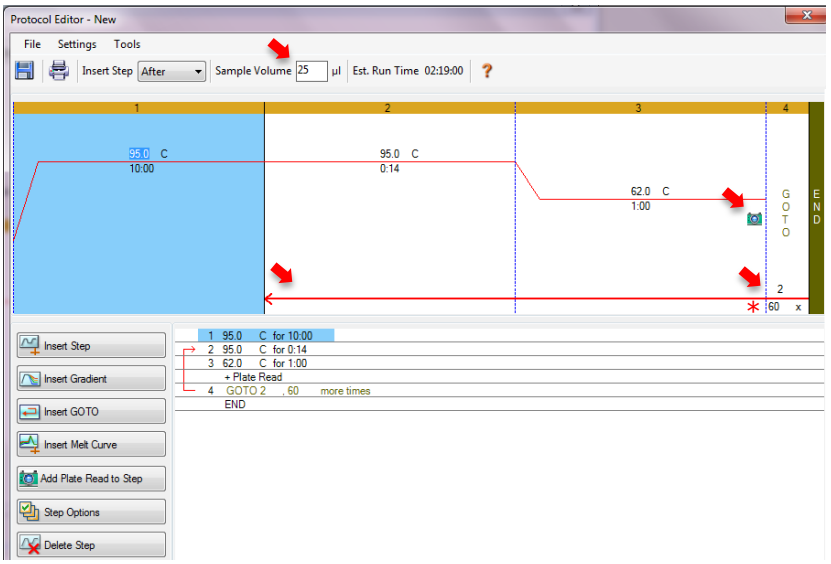
6. Open the door on the ABI 7500. Insert your plate. Close the door
7. Click on "START RUN" in the upper right corner of the screen

Bio-Rad CFX96 - Run Protocol

1. Turn on CFX96
2. Open CFX96 software on your computer
3. Select "Create a new Run" "CFX96"

Run Setup – Protocol

1. Select "Create New…"
2. Sample Volume "25µl"
3. Edit protocol as followed:
   Step 1: 95°C, 10 minutes (1 cycle)
   Step 2: 95°C, 14 seconds
   Step 3: 62°C, 1 minute + read
   Step 2 and 3 are repeated 45 or 60 cycles*
4. Click on "OK", and save protocol as a template



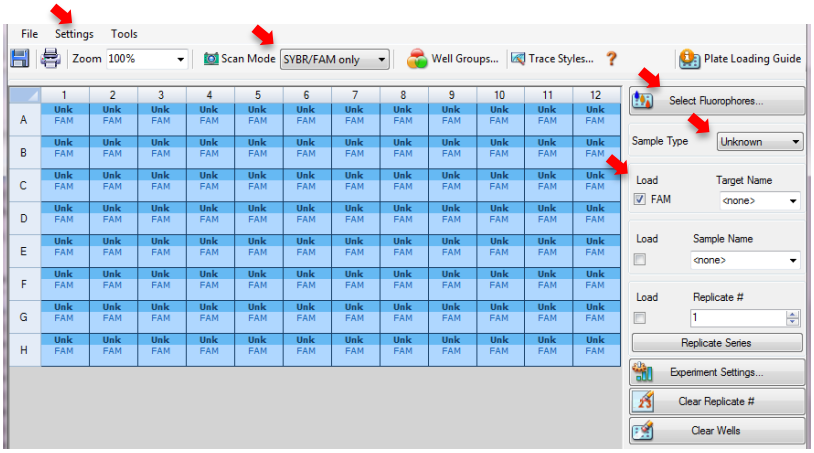
***45 cycles of steps 2 and 3 are required for Locus-specific reactions and 60 cycles for Allele-specific reactions**

***45 cycles of steps 2 and 3 are required for Locus-specific reactions and 60 cycles for Allele-specific reactions**

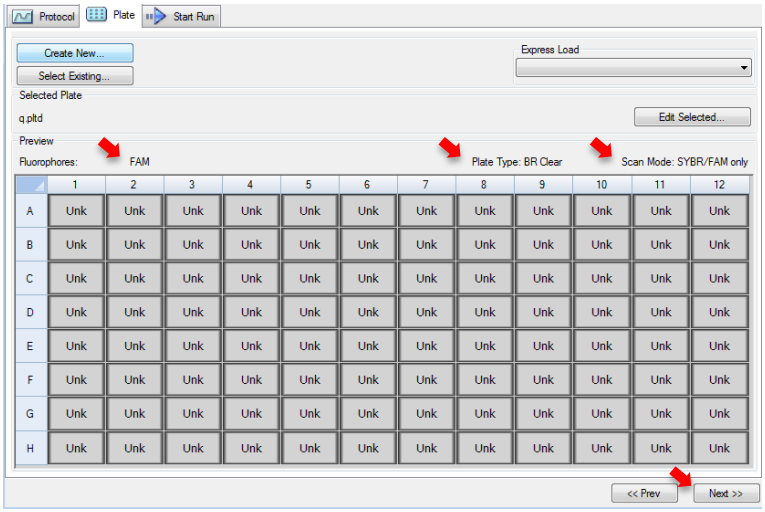
5. Review your protocol setup and Select: "Next >>"

Run Setup – Plate

1. Click on "Create new"
   Select the wells containing a reaction on the grid
2. Click on Settings:
   Select Plate Type (we suggest to use "BR clear" plates)
   Select Plate Size "96 wells"
3. Select Scan Mode " SYBR/FAM only"
4. Assign Select Fluorophores "FAM"
5. Assign your Sample Type for each well (Unknown, NTC, Positive control)
6. Select Load "FAM"
7. Click "OK" and save as template



8. Review your plate setup



9. Select: "Next >>"

Run Setup – Start Run

1. Open the CFX96 lid
2. Insert your plate in the CFX96
3. Close the lid
4. Select "Start run"
5. Save your experiment