







# Signature<sup>®</sup> BRAF Mutations Kit

## *Protocol*

|   |              |
|---|--------------|
| <i>For Research Use Only.<br/>Not for use in diagnostic procedures.</i>   |              |
| <b>REF</b>  | <b>46104</b> |
|    | <b>48</b>    |
|    |              |



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## Background Information

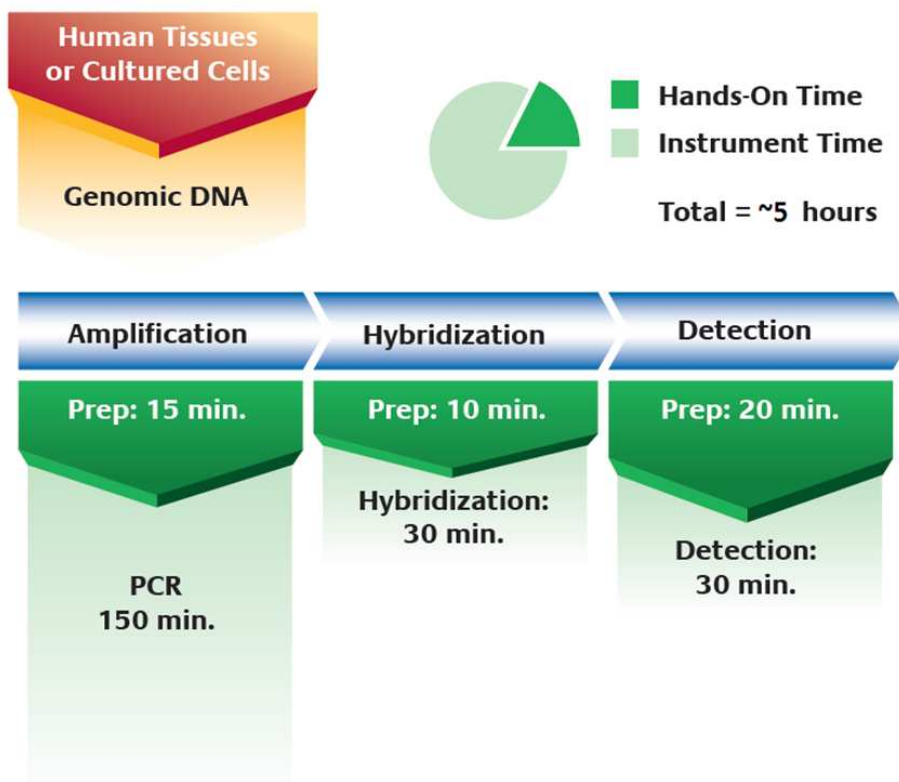
*BRAF* encodes a RAS-regulated serine/threonine kinase and member of the epidermal growth factor receptor (*EGFR*) signaling pathway that controls cell proliferation, differentiation and apoptosis<sup>1,2</sup>. Mutations in the *BRAF* oncogene are frequently found in human cancers, such as metastatic colorectal cancer (mCRC), non-small cell lung carcinoma (NSCLC), melanoma and papillary thyroid cancer (PTC)<sup>2,3</sup>. As several anti-cancer therapies directly target *EGFR*, mutations in downstream pathway members that constitutively activate the *EGFR* pathway, such as in *KRAS* or *BRAF*, can elicit drug resistance<sup>4-6</sup>. Drugs specifically targeting the oncogenic mutant *BRAF* kinase have also been developed<sup>2,7</sup>.

The Signature® *BRAF* Mutations Kit contains reagents for the rapid detection of the *BRAF* c.1799T>A mutation encoding the oncogenic V600E amino acid change that causes the constitutive activation of *BRAF* kinase activity. The qualitative test is performed using genomic DNA, multiplex polymerase chain reaction (PCR) and multiplex amplicon detection by liquid bead array cytometry<sup>7</sup>.

## Test Principle

The Signature® *BRAF* Mutations Kit combines multiplex PCR with multiplex fluorescent bead-array detection (Figure 1). Positive and negative controls are included with the kit to assess the validity of each run (Table 1). The entire procedure, not including DNA extraction, can be completed in approximately five hours. The test is designed to simultaneously amplify and detect the *BRAF* c.1799T>A mutation and an endogenous internal control in a single well. After the multiplex PCR, the identity of labeled PCR products is determined by hybridization-based fluorescent bead array using the Luminex® 100™ IS or 200™ system (Luminex Corporation, Austin, TX, USA). Briefly, a specific oligonucleotide (the capture probe) with sequence complementary to that of the target is covalently coupled to the surface of a bead possessing a unique spectral identity. Hybridization occurs between the capture probe and amplified DNA targets from the multiplex PCR. Following hybridization, a reporter molecule is introduced and the fluorescence is measured by flow cytometry. Samples are automatically injected into and read by the high-throughput Luminex® analyzer. The median fluorescence intensity (MFI) is calculated for each spectrally distinct bead to detect targets hybridized to the bead-bound capture probe. The raw MFI signals are exported in a Luminex Output file that is subsequently analyzed for the presence or absence of the internal control target and *BRAF* mutation.

**Figure 1. Overview of the Signature® *BRAF* Mutations Kit Principle and Workflow**



## Kit Components

### Reagents Supplied with this Kit

**Table 1. Signature® BRAF Mutations Kit Components (Catalog No. 46104)**

| Product No.  | Description                                   | Volume  | Storage Temp |
|--------------|---|---------|--------------|
| <b>46102</b> | <b>Signature® BRAF Mutations Amp Reagents</b> |         |              |
| 145158       | Signature® Mutations Diluent                  | 1.0 mL  | -15 to -30°C |
| 145159       | Signature® Mutations PCR Mix I                | 480 µL  | -15 to -30°C |
| 145249       | Signature® BRAF Mutations PCR Mix II          | 120 µL  | -15 to -30°C |
| 145237       | Signature® BRAF Mutations Primers             | 336 µL  | -15 to -30°C |
| 145239       | Signature® BRAF Pos Control                   | 10 µL   | -15 to -30°C |
| 145240       | Signature® BRAF Neg Control                   | 10 µL   | -15 to -30°C |
| <b>46103</b> | <b>Signature® BRAF Mutations Hyb Reagents</b> |         |              |
| 145238       | Signature® BRAF Mutations Bead Mix            | 2.16 mL | 2 to 8°C     |
| 145165       | Signature® Mutations Hyb Buffer               | 6.0 mL  | 2 to 8°C     |
| 145166       | Signature® Mutations Conjugate                | 24 µL   | 2 to 8°C     |

### Handling and Storage

- The kit reagents should be stored in the dark at the recommended temperature immediately upon receipt.
- Store the Signature® Amplification Reagents and Controls in a non-frost-free freezer and avoid unnecessary freeze/thaw cycles.
- Avoid exposure of the Signature® Mutations Bead Mix and Conjugate to light, as these reagents are photosensitive.
- All amplification components should be briefly vortexed and centrifuged prior to use.
- The Signature® Mutations Conjugate should NOT be vortexed, but should be centrifuged briefly prior to use.

### Number of Reactions

- The provided kit reagents are sufficient for up to 48 gene-specific multiplexed PCR reactions and multiplexed hybridization reactions.
- The reagents have been designed and verified for use for up to three freeze-thaw cycles.

### Reagent Stability

- The product will maintain performance through the expiration date printed on the label when stored under the specified conditions.

### Reagents Required but not Provided

- AmpliTaq Gold® DNA polymerase (Hot-start modified) (5 U/µL) (Applied Biosystems, Catalog No. 4338856).

### Other Components Supplied with this Kit

- A product insert card is included in each box and contains directions on how to access an electronic version of this protocol.

## Consumables & Equipment Required but not Provided

- General laboratory equipment and workspace to perform PCR
- Multi-channel Pipettors (if available), capable of pipetting 2-5 µL and 50-100 µL
- 96-Well PCR Plates for amplification (AB-Gene, Catalog No. AB-0900 or equivalent)
- PCR Plate Seals (AB-Gene, Catalog No. AB-0558 or equivalent)
- Thermal Cycler (ABI GeneAmp® PCR System 9700, ABI Veriti™ (run in 9700 emulation mode))
- Thermowell Plates for hybridization (Costar Corning Inc, Catalog No. 6509 or equivalent)
- Hybridization Plate Seals (MJ-Research, Catalog No. MSA-5001 or equivalent)
- Hybridization Compression Pad
- V-channel boats (Labcor Products Inc, Catalog No. 730-001 or equivalent)
- Centrifuge capable of spinning 96-well plates at ~400 x *g* and ~1200 x *g*
- Sonicator (Cole-Parmer, Model No. 08849-00 or equivalent)
- Luminex® 100™ IS or 200™ Total System (version 2.2, 2.3, or xPONENT® software. Asuragen Catalog No. 48007 for Luminex 200™ only)

## Optional Components Not Provided

- PCR Compression Pad (Applied Biosystems, Catalog No. 4312639)
- Uracil-N Glycosylase (HK™-UNG) (Epicentre. Catalog No. HU59100 or equivalent), 1 U/µL and subsequently diluted to 0.1 U/µL with UNG dilution buffer.

## **Warnings and Precautions**

- Use proper personal protective equipment. Wear appropriate protective eyeglasses, protective gloves, and protective clothing when working with these materials.
- Follow Universal Precautions in compliance with OSHA 1910:1030, CLSI M29, or other applicable guidance when handling human samples.
- DNase contamination can cause degradation of the genomic DNA samples and PCR products. Use nuclease-free labware, filter pipette tips and nuclease-free tubes and reaction vessels.
- Do not pool components from different reagent lots. Do not use reagents after the labeled expiration date.
- PCR carry-over contamination can result in false positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
- Prior to use, ensure that all instruments are calibrated according to the manufacturer's instructions.
- Prior to use, ensure that Luminex® 100™ IS or 200™ System is calibrated, maintained, and primed according to the manufacturer's instructions. Confirm that the probe height and probe alignment for the V-bottom plates have been adjusted properly (see Sample Probe Height section in Luminex® 100™ IS or 200™ manual).



**Caution:** Tetramethylammonium Chloride (TMAC), contained within the Signature® Mutations Hyb Buffer (Product No. 145165) and Bead Mix (Product No. 145238), is known to be toxic when swallowed. If swallowed, seek medical advice immediately. TMAC is also known to be irritating to eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice immediately. MSDS documents are available upon request.

## Pre-Analytical Considerations

### Nucleic Acid Preparation

Because optimal test sensitivity is dependent on the DNA quality and mass input, a validated DNA isolation method yielding greater than 2 ng/ $\mu$ L and OD260/280 ratios between 1.70 and 2.10 is recommended. Thus the purified genomic DNA should be evaluated for concentration (OD260) and purity (OD260/280) prior to use and yields should be sufficient to allow normalization and subsequent incorporation of 5 - 20 ng DNA input in up to 4.5  $\mu$ L per 25  $\mu$ L PCR.

## Signature® BRAF Mutations Kit Protocol

### Test Workflow

The workflow should proceed in a unidirectional manner starting with a dedicated pre-amplification area and moving to a segregated post-amplification area. Amplified product should remain in the post-amplification area to minimize risk of amplicon contamination.

### Step 1: DNA Amplification

**Note:** If UNG (optional) is not used, replace it with 0.5  $\mu$ L of Signature® Mutations Diluent per reaction in the Master Mix

1. Thaw, vortex and pulse spin PCR Mix I, PCR Mix II, Primers, Controls and DNA samples (and Diluent as needed).
2. Flick to mix and pulse spin enzyme(s).
3. Prepare a DNA Amplification Master Mix for the total number of samples to be tested (including the controls) with appropriate overage (Table 2). Add components in the order listed. Vortex briefly to mix and pulse spin.

**Table 2. Signature® BRAF Mutations DNA Amplification Master Mix Setup**

| Master Mix Component                                       | Volume                        |
|--|-------------------------------|
| Signature® Mutations PCR Mix I                             | 10 $\mu$ L                    |
| Signature® BRAF Mutations PCR Mix II                       | 2.5 $\mu$ L                   |
| Signature® BRAF Mutations Primers                          | 7 $\mu$ L                     |
| AmpliTaq Gold (5 U/ $\mu$ L)                               | 0.5 $\mu$ L                   |
| UNG (0.1 U/ $\mu$ L) (Optional)<br>or replace with Diluent | 0.5 $\mu$ L                   |
| <b>Total Volume per Well</b>                               | <b>20.5 <math>\mu</math>L</b> |

4. Add 20.5  $\mu$ L of Amplification Master Mix to each well of the PCR plate.
5. Add up to 4.5  $\mu$ L of sample DNA (5 to 20 ng total input) to individual sample wells, supplementing with Diluent as necessary, to achieve a final reaction volume of 25  $\mu$ L.
6. Add 2  $\mu$ L of Signature® BRAF Mutations Control to the corresponding positive and negative control wells supplementing with Diluent as necessary to achieve a final reaction volume of 25  $\mu$ L.
7. Mix at least five times with gentle pipetting (avoid forming bubbles in wells).
8. Cover wells with a PCR plate seal.
9. Spin the plate for 5 seconds after reaching  $\sim 400 \times g$  to remove any bubbles and collect all liquid to the bottom of each well.
10. Transfer plate to the thermal cycler. Run the following program:
  - 37°C 15 minutes
  - 95°C 10 minutes
  - 45 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds
  - Hold at 4°C

**Note:** Thermal cyclers vary widely in sample ramp rate, block ramp rate and default ramp rate settings. The Signature® BRAF Mutations Kit has been optimized on the ABI GeneAmp® PCR System 9700 and ABI Veriti™ (run in 9700 emulation mode), which exhibit sample ramp rates of approximately 1.5°C/second. Caution should be exercised with the use of alternative thermal cycler instruments. Thermal cyclers using a sample ramp rate other than 1.5°C/second must be validated by the user for use with the Signature® BRAF Mutations Kit.

11. After the program has completed, remove the PCR plate from the thermal cycler and spin the plate for up to 5 seconds after reaching  $\sim 400 \times g$  to collect all liquid to the bottom of each well.

**Note:** The amplified product can be temporarily stored at 2 to 8°C in the dark for up to 48 hours (with UNG) or 72 hours (without UNG) prior to proceeding with the Hybridization Step. Signal to noise ratios are compromised by extended storage at 2 to 8°C.

## Step 2: Hybridization

**Note:** Before setting-up the hybridization reaction, ensure that the Signature® Mutations Bead Mix and Hyb Buffer are equilibrated to room temperature (18 to 25°C).

1. Thoroughly vortex and sonicate (15 sec each) the Signature® BRAF Mutations Bead Mix **immediately before use**.
2. Add 45 µL of Bead Mix at 18 to 25°C to each well of a **new** hybridization plate.
3. Carefully remove the seal from the PCR plate in Step 1.11 (avoid cross-contamination between the wells) and transfer 5 µL of well-mixed amplified product to the corresponding wells of the hybridization plate **using a multi-channel pipettor as available**.
4. Mix at least five times with gentle pipetting (avoid forming bubbles in wells).
5. Cover the hybridization plate with a hybridization plate seal.
6. Transfer the hybridization plate to the thermal cycler. Run the following program:
  - 95°C for 5 minutes
  - 52°C for 25 minutes
  - 52°C hold for 10 minutes (the plate **MUST** be removed within this 10 minute period)

**Note:** During the hybridization step (Step 2.6), complete steps 3.1 through 3.3.

## Step 3: Detection

1. Prepare the Luminex instrument:
  - i. Verify that the Luminex instrument is calibrated.
  - ii. Ensure that the lasers are warmed up.
  - iii. Ensure that the heated XY plate (and associated brass block) is set to 52°C (same temperature as for hybridization) using the Luminex software.
  - iv. Load the Luminex Bead Setting Template for the appropriate test panel (Table 3) and add the appropriate sample names to the batch template. The bead setting template should contain the following steps: Warmup, Sanitize, Prime, Prime, Wash, Wash, Acquire.

**Table 3. Signature® BRAF Mutations Bead Maps (A) and Run Template Parameter Settings (B)**

| (A)                       |                   |      |         |                    | (B)           |         |
|---------------------------|-------------------|------|---------|--------------------|---------------|---------|
| Panel                     | Target Name       | Unit | Bead ID | Minimum Bead Count | Parameter     | Setting |
| Signature® BRAF Mutations | c.1799T>A (V600E) | MFI  | 19      | 50                 | Sample Volume | 50      |
|                           | EC                | MFI  | 45      | 50                 | Timeout       | 25      |
|                           |                   |      |         |                    | XY Heater     | 52      |
|                           |                   |      |         |                    | DD Gate Low   | 7500    |
|                           |                   |      |         |                    | DD Gate High  | 19000   |

**Note:** Please contact your local distributor for additional assistance setting up Bead Maps and/or templates.

2. Prepare sufficient Reporter Solution for the total number of samples, including overage as necessary (Table 4).

**Table 4. Reporter Solution Master Mix Setup**

| Reporter Solution Master Mix Component | Volume       |
|--|--------------|
| Signature® Mutations Hyb Buffer        | 49.5 µL      |
| Signature® Mutations Conjugate         | 0.5 µL       |
| <b>Total Volume per Well</b>           | <b>50 µL</b> |

**Note:** *Do not vortex the Signature® Mutations Conjugate. The Reporter Solution may be mixed by pipetting, swirling, and/or vortexing. Store Reporter Solution in the dark at room temperature (18 to 25°C) until use.*

3. Dispense the required volume of Signature® Mutations Hyb Buffer at 18 to 25°C into a V-channel reservoir or equivalent (75 µL for each well plus overage).
4. **During the 10 minute hold at 52°C (Step 2.6)**, remove the hybridization plate from the thermal cycler.
5. Remove the seal from the hybridization plate (avoid cross-contamination between the wells) and carefully add 75 µL of Hyb Buffer at 18 to 25°C to each well **using a multi-channel pipettor as available**. Mix at least ten times with gentle pipetting (avoid forming bubbles in wells).
6. Cover the plate with a new hybridization plate seal.
7. Spin the plate at 1,200 × g for 3 min to pellet the beads.
8. **Carefully** remove the seal of the hybridization plate (avoid cross-contamination between the wells) and aspirate 100 µL of the Hyb Buffer supernatant **from the top** of each well **using a multi-channel pipettor as available**.  
**Note:** *To avoid disturbing the bead pellets, do not contact the bottom of the wells with the pipette tips. Approximately 25 µL of the Hyb Buffer mixture should remain in each well after the aspiration step.*
9. Confirm that the Luminex XY plate has reached 52°C and that the lasers are warmed up (Step 3.1 (ii and iii))
10. Transfer the hybridization/detection plate to the XY plate in the Luminex instrument and retract the plate.
11. Allow the reactions to equilibrate in the Luminex heat block for **at least 2 but no longer than 5 minutes**.
12. Eject the XY plate and add 50 µL of the room temperature Reporter Solution from Step 3.2 to each well **using a multi-channel pipettor as available**. Mix at least five times with gentle pipetting.
13. Start the batch template on the Luminex, from Step 3.1 (iv).

After the run is complete, the output file can be viewed using Microsoft Excel (or equivalent software program) and results can be analyzed based on the Median Fluorescent Intensity (MFI) signals generated.

## Control Procedure

The Signature® BRAF Mutations Kit includes two controls designed to assess the amplification, hybridization and detection steps. Both controls must be included to validate each run. A “no template control”, for example 4.5 µL of Signature® Mutations Diluent, may also be included. The Signature® BRAF Pos Control (Product No. 145239; Table 1) contains DNA sequences corresponding to the c.1799T>A mutation and the endogenous control. The Signature® BRAF Neg Control (Product No. 145240; Table 1) contains only the endogenous control sequence. Use 2 µL per 25 µL amplification reaction and refer to “Recommended Data Interpretation” below for representative examples and data analysis.

## Software Procedure

The Luminex software processes the flow cytometry data for each run and generates an “output.csv” file that is typically stored in the “Batch” folder on the system hard drive. No additional software is required as the “comma separated value” format is compatible with any standard spreadsheet software and the raw data can be reviewed and interpreted directly from the output file.

## Recommended Data Interpretation

The Signature® BRAF Mutations Kit is a qualitative test. An Endogenous Control (EC) is included to facilitate the assessment of individual sample DNA performance. An EC value  $\geq 2,500$  MFI indicates the presence of acceptable amplifiable DNA template. A sample is considered positive if the c.1799T>A probe signal is  $\geq 500$  MFI, independently of the EC signal value. Results for test samples should be analyzed and interpreted only if all the controls included in the run generate valid results. Potential test results and re-test recommendations are summarized in Table 5.

**Table 5. Summary of Test Interpretation**

| Run   | Sample              | Result   | Interpretation         | Re-Test Recommendation   |
|---|---------------------|--|------------------------|--|
| <b>Invalid</b><br>(One or more control results are invalid) | No Template control | Any signal $\geq 500$ MFI                            | Invalid control result | Re-run plate from Step 1   |
|   | Positive control    | c.1799T>A $< 500$ MFI <b>or</b> EC $< 2,500$ MFI     | Invalid control result | Re-run plate from Step 1   |
|   | Negative control    | c.1799T>A $\geq 500$ MFI <b>or</b> EC $< 2,500$ MFI  | Invalid control result | Re-run plate from Step 1   |
|   | Any control         | Low bead count for any bead                          | Invalid control result | Re-run plate from Step 2   |
| <b>Valid</b><br>(All control results are valid)             | Tested sample       | c.1799T>A $< 500$ MFI <b>and</b> EC $< 2,500$ MFI    | Invalid sample result  | Re-run sample from Step 1 or re-extract DNA from same or new specimen at laboratory discretion |
|   |                     | Low bead count for any bead                          | Invalid sample result  | Re-run sample from Step 2  |
|   |                     | c.1799T>A $\geq 500$ MFI                             | Mutation detected      | N/A  |
|   |                     | c.1799T>A $< 500$ MFI <b>and</b> EC $\geq 2,500$ MFI | Mutation not detected  | N/A  |

**Note:** Cut offs can be affected by many variables including but not limited to the purity and amount of DNA, the fraction of amplifiable DNA within each DNA preparation and instrument variation. The use of non-standard or non-validated DNA extraction methods or instruments, or disregarding or modifying the guidelines and parameters defined in this document (e.g., DNA input per amplification or cycling conditions) may require that the end user establish and validate appropriate cut offs using the altered conditions.










## Notice to Purchaser

1. ***This Product is intended for research use only. It is not intended for diagnostic use.***
2. *This Product may not be resold, modified for resale, or used to manufacture commercial products without the written approval of Asuragen.*
3. *Signature® is a registered trademark of Asuragen, Inc.*
4. *This Product is covered by US Patent No. 7,378,233 and other patents pending.*
5. *You, the customer, acquire the right under Luminex Corporation's patent rights, if any, to use this Product or any portion of the Product, including without limitation the microsphere beads contained herein, only with Luminex Corporation's laser based fluorescent analytical test instrumentation marketed under the name Luminex® 100™ IS or 200™ System.*
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## Appendix A: Glossary of Symbols

| Symbol  | Description                       |
|---|-----------------------------------|
|  | Catalog number                    |
|  | Batch code                        |
|  | Contains sufficient for <n> tests |
|  | Consult instructions before use   |
|  | Temperature limitation            |
|  | Use by                            |
|  | Manufactured by                   |
|  | Harmful                           |
|  | Toxic                             |

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