Primerdesign™ Ltd

BRAF V600E mutation

mutation detection by quantitative allele specific amplification (quasa)
BRAF (V600E)

50 tests

GENESIG
Kits by Primerdesign

For general laboratory and research use only

Contents

Introduction

BRAF is a serine/threonine kinase that is part of cellular signaling pathways, including the MAP kinase signaling network, and is involved in cell division and differentiation. More than 30 mutations of the *BRAF* gene associated with human cancers have been identified. In 90% of the cases, thymine is substituted with adenine at nucleotide 1799. This leads to an amino acid substitution at codon 600 whereby Valine (V) is replaced by Glutamic acid (E) in the activation segment. This mutation has been widely observed in papillary thyroid carcinoma, colorectal cancer, melanoma and non-small-cell lung cancer. A large number of BRAF mutations have been described, most of which occur in the glycine-rich P loop of the N lobe and the activation segment and flanking regions. These mutations influence the activation segment, whereby BRAF becomes constitutively active. Depending on the type of mutation the kinase activity towards MEK may also vary and conformational changes can lead to activation of Wild type C-RAF.

Acquired mutations in this gene have been found in many cancers, including non-Hodgkin lymphoma, colorectal cancer, malignant melanoma, papillary thyroid carcinoma, non-small cell lung carcinoma, hairy cell Leukemia and adenocarcinoma of lung.

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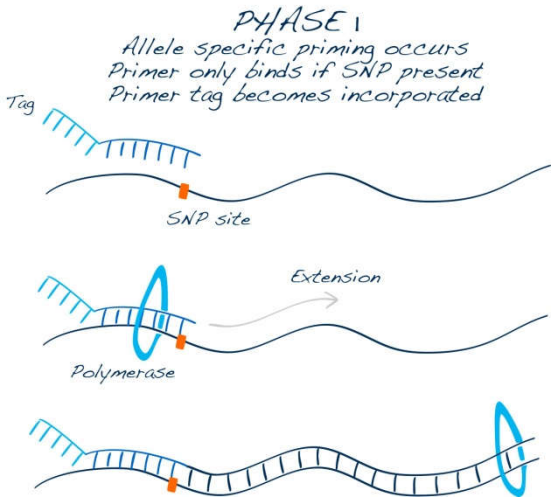
Principles of the test

quasa (quantitative allele specific amplification)

quasa is a method for sensitive detection of mutations that may be present in clinical samples at low levels. quasa utilizes Primerdesign's own method based on 'allele specific PCR', using **modified primers** and hydrolysis probe, **modified cycling conditions** and a **modified master mix**. In this method the 3' terminal base of the mutation detecting primer is sited to bind to the mutant base. In the samples where the mutation is present, efficient amplification results in detection of the mutant sequence whereas PCR from this primer on Wild type samples is blocked due to the mismatch.

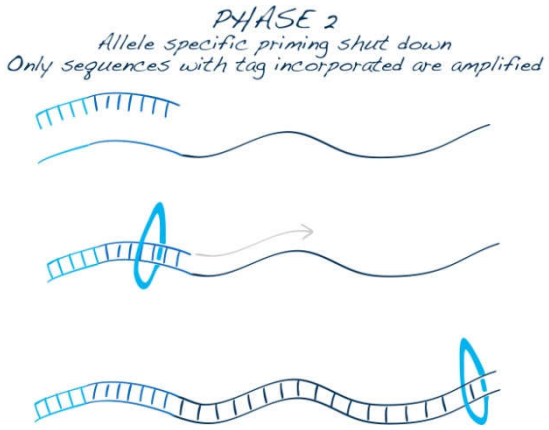
Modified primers

quasa primers are designed such that the 3' terminal base overlies the mutation site. Thus, Wild type primers confer 100% specificity with the Wild type sequence but have a single base mismatch with the mutated sequences (The converse is true of the mutant specific primers). This is typical of allele specific PCR and relies on the principle that the single base mismatch will prevent the Wild type primers successfully priming on the mutated template. However, this principle alone is often insufficient to confer specificity and false positive amplification is common. Thus, quasa primers are further modified to multiply the level of specificity possible. Firstly, the primers are designed to have a low melting Tm such that a single base mismatch will be as disruptive as possible. quasa primers also contain a sequence independent 'tag' at the 5' end. The tag is incorporated in to the amplicon during the first round of PCR and is thus present in the amplicon for subsequent cycles. This means that the tagged primers will prime preferentially on this template and thus drive amplification of the correct sequence in subsequent cycles. The quasa method requires no other primers, clamping primers, modified bases or blocking probes to achieve the extraordinary sensitivity of the kit.



Modified cycling conditions

The quasa protocol uses a two-stage cycling strategy. The first ten cycles of PCR use a very low annealing temperate of 50°C. This allows the low Tm primers to prime successfully whilst conferring the highest possible levels of specificity. After the first ten cycles the annealing temperate is switched to 60°C. Thus, allele specific priming is effectively blocked and priming only occurs where the tagged primers have been incorporated. This also drives highly efficient amplification and probe cleavage and thus confers the sensitivity of the method. It is essential that the correct cycling parameters are used to ensure both sensitive and selective detection for each primer mix on its cognate target sequence.



Modified master mix

Even greater levels of specificity and sensitivity are conferred by the specialized master mix used with quasa. Enzyme, Salt, dNTP and $MgCl_2$ levels are all highly optimised. The annealing temperatures of the primer and probe have been carefully calibrated and any change in the reaction buffer can significantly alter the performance of the assay. For this reason, Primerdesign can only guarantee accurate results when Precision quasa A Master Mix is used.

Quantitative analysis

The kit employs a two-reaction protocol. The user runs one reaction to detect the level of Wild type sequence present and at the same time another reaction to detect the level of mutant sequence present. By comparing the detection levels obtained by both primer/probe sets, the proportion of sequences that contain the mutation can be measured and expressed as a percentage relative to the Wild type sequences. When using the kit for a quantitative test each sample must be run in duplicate in order to get accurate results. The kit is sensitive down to detection levels of 0.1% of mutant DNA but at these levels the copy number of mutant DNA will always be very low and close to the limits of PCR detection. When working close to the limits of detection PCR becomes less accurate and it is therefore necessary to take the average of two points to ensure that a reliable result is calculated.

Qualitative uses of the kit

In some clinical scenarios it is sufficient to know if a mutation is present or absent and the exact proportion of the mutant is not of diagnostic or therapeutic value. In these circumstances, the sample can be tested using a single test for each of the Wild type and mutant primer/probe mixes.

Positive template containing 1% mutant sequence

The kit contains a positive control which contains a blend of both Wild type and mutant sequences at a known copy number. The ratio of Wild type to mutant template is 100:1 which is typical of some biological samples which can contain mutant sequences at a very low level. The positive control therefore provides a template for both primer and probe mixes and the quantification cycle (Cq) data from this control is used in the quantitative analysis.

Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. In this instance the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources of contamination should first be explored and removed.

Kit contents

- **BRAF V600E specific primer/probe mix (50 reactions BROWN)**
  FAM labelled
- **BRAF WT specific primer/probe mix (50 reactions BROWN)**
  FAM labelled
- **Precision® quasa A Master Mix (2x 0.5ml ORANGE)**
- **Positive control template (BRAF V600E 1% mutant) (RED)**
- **Template preparation buffer (YELLOW)**
  For resuspension of positive control template
- **RNase/DNase free water (WHITE)**

Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
  Must be able to read fluorescence through the FAM channel
- **Pipettes and tips**
- **Vortex and centrifuge**
- **Thin walled 0.2ml PCR reaction tubes**

Kit storage

This kit is should be transported frozen and stored at -20ºC on arrival. Freeze/thawing cycles should be kept to a minimum once re-suspended. Under these conditions reagents are stable for six months from date of re-suspension.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration and DNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the test sample with RNase/DNase free water.

Licensing agreement and limitations of use

PCR is covered by several patents owned by Hoffman-Roche Inc and Hoffman-LaRoche, Ltd. Purchase of Primerdesign kits does not include or provide license with respect to any patents owned by Hoffman-La Roche or others.

Primerdesign™ satisfaction guarantee

Primerdesign takes pride in the quality of all our products. Should this product fail to perform satisfactorily when used according to the protocols in this manual, Primerdesign will replace the item free of charge.

Quality control

As part of our ISO9001 and ISO13485 quality assurance system, all Primerdesign products are monitored to ensure the highest levels of performance and reliability.

Bench-side protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps. The positive control template is a significant contamination risk and should therefore be pipetted after negative control and sample wells.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Resuspend the primer/probe mixes in the DNase/RNase free water supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component – resuspend in water	Volume
Pre-PCR box	
BRAF V600E Genotyping primer/probe mixes (**BROWN**)	60µl
BRAF WT Genotyping primer/probe mixes (**BROWN**)	60µl

3. **Resuspend the positive control template in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component – resuspend in template preparation buffer	Volume
Post-PCR heat sealed envelope	
Positive control template (**RED**)*	500µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

4. **Prepare DNA detection mix for each of the SNP and Wild type primer mixes**
   Prepare sufficient reactions for all samples and extra reactions to include wells for the positive control and a 'no template control'.

Component	e.g. 1 reaction	e.g. 10 reactions
2X Precision quasa A Master Mix	10µl	100µl
Genotyping primer/probe mix (**BROWN**)	1µl	10µl
RNase/DNase free water (**WHITE**)	4µl	40µl
Final volume	**15µl**	**150µl**

5. Dispense 15µl of each master mix according to your plate lay out

Sample plate layout for 5 samples for Qualitative analysis

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	+ve ctrl	NTC
BRAF V600E	●	●	●	●	●	●	●
BRAF Wild type	●	●	●	●	●	●	●

Sample plate layout for 5 samples for Quantitative analysis

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	+ve ctrl	NTC
BRAF V600E	●	●	●	●	●	●	●
Duplicate wells	●	●	●	●	●	●	●
BRAF Wild type	●	●	●	●	●	●	●
Duplicate wells	●	●	●	●	●	●	●

6. Dispense 5µl of extracted DNA sample according to your plate set up

To obtain a strong signal whilst minimizing the addition of possible PCR inhibitors from the extraction, the recommended concentration of DNA is 5ng/µl (25ng in total). For highest detection sensitivity, more template can be added but the concentration should not exceed 10ng/µl (50ng in total). For the 'No Template Control' substitute 5µl of RNase/DNase free water.

NB. If you would like to add a larger volume of DNA (up to 9µl) simply reduce the volume of water at step 4. The final reaction volume is 20µl.

7. Dispense Positive control template.

Add 5µl of diluted positive control template to the positive control wells. The positive control well will therefore automatically contain 100,000 copies of WT DNA and 1,000 copies of mutant DNA. The final reaction volume is 20µl.

qPCR amplification protocol

Recommended
The following cycling conditions must be used to ensure both sensitive and efficient detection of mutant sequences:

	Step	Time	Temp
	qPCR Enzyme Activation	2 min	95°C
x10 cycles	Denaturation	10s	95°C
	Annealing	3s	**50°C**
	Extension	15s	72°C
x40 cycles	Denaturation	10s	95°C
	Annealing *	30s	**60°C**
	Extension	15s	72°C

*Fluorogenic data should be collected through the **FAM** channel.

Interpretation of results

The percentage of SNP present in the sample is calculated using the delta Cq method. The proportions of SNP and WT in the sample are corrected by reference to a positive control standard where the SNP is present at a known proportion of 1%.

Sample data and calculations

The calculation is performed in two stages. Firstly, the delta Cq values are used to calculate relative detection levels between the biological sample and the 1% control for both the wild type and the mutant. These relative amounts are then converted into a percentage.

Equations

BRAF WT Relative Amount = [2^ - (BRAF WT Sample - BRAF WT Control)] *Control Proportion of WT

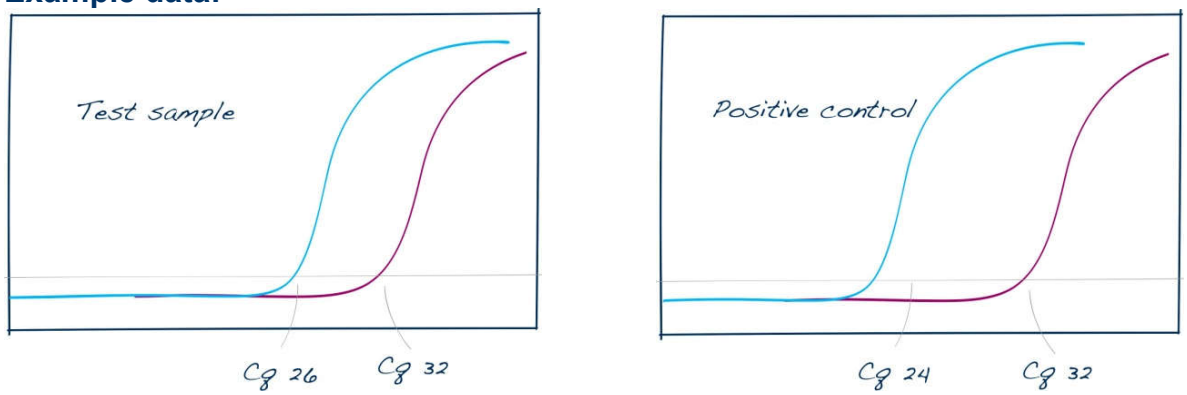
BRAF V600E Relative Amount = [(2^ - (BRAF V600E Sample - BRAF V600E Control)] *Control Proportion of SNP

BRAF V600E Percentage conversion

$$= \frac{\text{BRAF V600E Relative Amount}}{\text{BRAF WT Relative Amount + BRAF V600E Relative Amount}} * 100$$

1. **Calculate percentages for samples and control data**

Example data:



Test sample		**Positive control**	
BRAF Wild type Sample	Cq = 26	BRAF V600E Sample	Cq = 32
BRAF Wild type Control	Cq = 24	BRAF V600E Control	Cq = 32
Delta Cq calculation= 2^ - (26-24) = 0.25		Delta Cq calculation = 2^ - (32-32) = 1	
Ratio calibration	= 0.25 *100 = **25**	Ratio calibration	= 1 *1 = **1**

2. Calculate the Percentages

From the example set of data the wild type to mutant ratio is 25:1, when calibrated against the 1% positive control. Calculating the percentage of either the wild type or the mutant can be calculated from this ratio. For the mutant, the percentage conversion is as follows

BRAF V600E Percentage conversion

$$= \frac{1}{25 + 1} * 100$$

$$= \mathbf{3.85\%}$$

Interpretation: “3.85% of the sample DNA is mutated in background of 96.15% wild type.”

The kit is sensitive down to detection levels of 0.1%. Results that report detection at a lower proportion than 0.1% should be considered as negative.