Primerdesign™ Ltd

EGFR T790M mutation

mutation detection by quantitative allele specific amplification (quasa)
EGFR (T790M)

50 tests

GENESIG
Kits by Primerdesign

For general laboratory and research use only

Contents

Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane protein receptor for members of the epidermal growth factor family. Binding of growth factors to the receptor activates the protein kinase domain in the intracellular region of the receptor. The protein kinase phosphorylates a number of intracellular substrates that activate signalling pathways responsible for DNA synthesis and cell proliferation.

A single nucleotide change at position 2615 of cytosine to thymine, results in the amino acid substitution of threonine for methionine at codon 790. The mutation occurs within exon 20, which encodes part of the kinase domain of the receptor.

The T790M mutation is associated with lung cancer, in particular non-small cell lung cancer.

Notices and disclaimers

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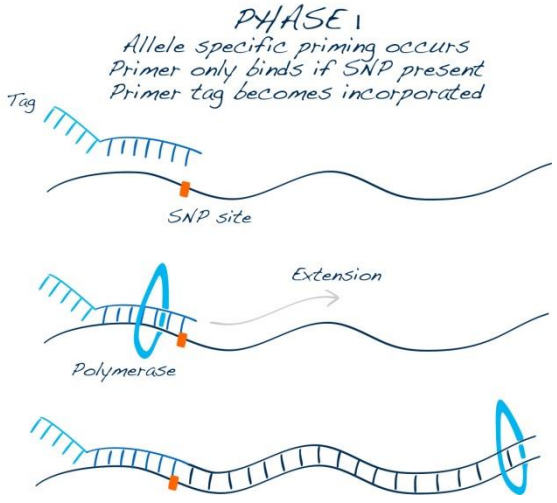
Principles of the test

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.

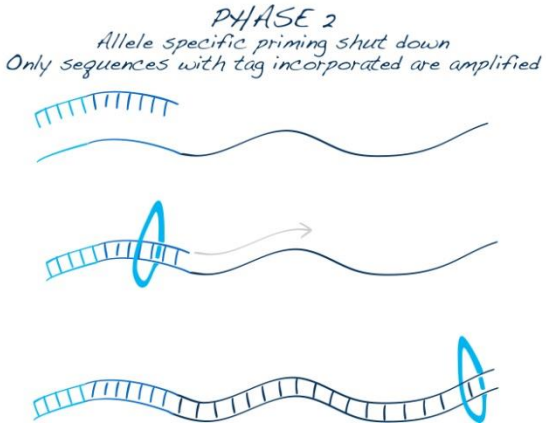
Modified primers

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 low annealing temperate of 50°C. This allows the low Tm primers to prime successfully whilst conferring the highest possible level 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 B 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

- **EGFR-T790M specific primer/probe mix (50 reactions BROWN)**
  FAM labelled
- **EGFR WT specific primer/probe mix (50 reactions BROWN)**
  FAM labelled
- **Precision® quasa B Master Mix (2x 0.6ml ORANGE)**
- **Positive control template (EGFR-T790M 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 0.2 ml PCR reaction tubes**

Kit storage

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

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 mix 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	
EGFR T790M Genotyping primer/probe mixes (**BROWN**)	60µl
EGFR 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 B 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 layout

Sample plate layout for 5 samples for Qualitative analysis

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	+ve ctrl	NTC
EGFR-T790M	●	●	●	●	●	●	●
EGFR Wild Type	●	●	●	●	●	●	●

Sample plate layout for 5 samples for Quantitative analysis

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	+ve ctrl	NTC
EGFR-T790M	●	●	●	●	●	●	●
Duplicate wells	●	●	●	●	●	●	●
EGFR 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 Annealing Extension		10s 3s 15s	95°C **50°C** 72°C
x40 cycles	Denaturation Annealing * Extension		10s 30s 15s	95°C **60°C** 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

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

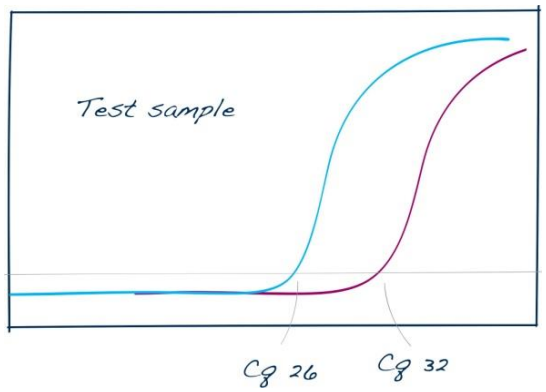
EGFR T790M Relative Amount = [(2^ - (EGFR T790M Sample - EGFR T790M Control)] *Control Proportion of SNP

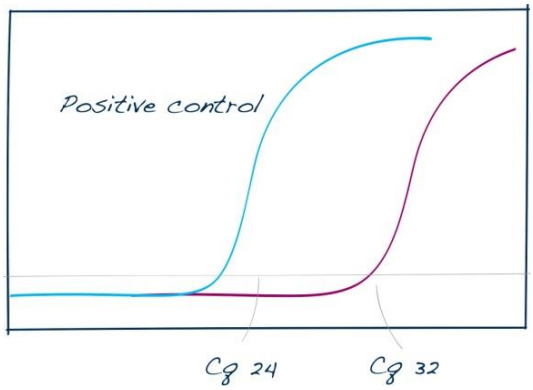
EGFR T790M Percentage conversion

$$= \frac{\text{EGFR T790M Relative Amount}}{\text{EGFR WT Relative Amount + EGFR T790M Relative Amount}} * 100$$

1. Calculate percentages for samples and control data

Example data:





Test sample		**Positive control**	
EGFR Wild type Sample	Cq = 26	EGFR T790M Sample	Cq = 32
EGFR Wild type Control	Cq = 24	EGFR T790M 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

EGFR T790M Percentage conversion

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

= **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.