INSTRUCTIONS FOR USE FOR THE BRAF P.VAL600GLU KIT

TABLE OF CONTENTS

Instructions for use for the BRAF p.Val600Glu kit 1

1. Introduction 4

1.1 Labelling 4

1.2 Areas of use 4

1.3 Tested item 4

1.4 Values determined 4

1.5 Instruments and equipment 4

1.6 Standards and reference materials 4

1.7 Conditions of the environment 5

2. Procedure 6

2.1 Flow chart of the method 6

2.2 Pre-analysis 7

2.3 Pre-analysis check 7

2.4 Procedure itself 7

2.5 Check during the analysis 8

2.6 Methods of recording results 9

2.7 Safety precautions 9

3. Evaluation 10

3.1 Evaluating algorithm 10

3.2 Criteria for rejection 10

3.3 Recorded data, analysis, presentation 10

3.4 Uncertainty of measurement 10

1. INTRODUCTION

1.1 LABELLING

Real-time examination of molecular genetic changes in DNA by the PCR method with fluorescent measurement of amplification in two fluorescent channels.

1.2 AREAS OF USE

Any human DNA of sufficient quality (non-degraded and unmodified chemically) in which mutation of the gene *BRAF* can be expected and the examination of which is of diagnostic, prognostic, or predictive significance (e.g. colorectal carcinoma and melanoma).

1.3 TESTED ITEM

Gene *BRAF* in human DNA, codon 600.

1.4 VALUES DETERMINED

BRAF

The test is intended for the detection of point mutation of p.Val600Glu with at least one-percent proportion in wildtype DNA.

1.5 INSTRUMENTS AND EQUIPMENT

For cutting the blocks and DNA preparation: microtome, heat block, fume chamber, vortex, centrifuge, Nanodrop spectrophotometer, adjustable pipettes, and plastic consumables

For amplification: thermocycler with real-time measurement of fluorescence LightCycler 480 II or Cobas, vortex, mini-centrifuge, centrifuge for microtitration plates and PCR strips, cooler rack, adjustable pipettes, pipette box, and plastic consumables

1.6 STANDARDS AND REFERENCE MATERIALS

Positive control: A sample with known*BRAF* mutation status which is included in the typing kit, or any other DNA with at least 1% of mutated *BRAF* p.Val600Glu. Negative control: A sample without*BRAF* mutation (any DNA isolated from blood of a healthy person).

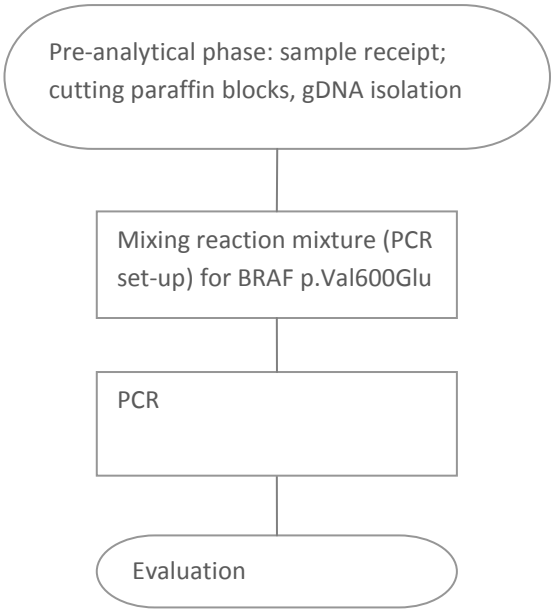
1.7 Conditions of the environment

There are no special requirements for working environment other than adhering to common molecular biological laboratory conditions (taking anti-contamination measures, including physiological separation of prePCR and PCR/postPCR area).

Paraffin slices and cytological sections are maintained in a folder at room temperature or in a freezer. Freshly frozen tissues or tissues in RNAlater are best stored at -70 to -80°C (tissues in RNA-later can also be stored at room temperature). Isolated and amplified DNAs are stored in labelled tubes at -10 to -20°C. Biological material must be handled with protective gloves. For the cycler, temperature and moisture conditions in the laboratory specified by the manufacturer must be met.

2. PROCEDURE

2.1 FLOW CHART OF THE METHOD



2.2 Pre-analysis

It is not included in the test.

2.3 Pre-analysis check

Check whether all the required equipment and reagents (and sample data) are available. Check whether you have sufficient amount of DNA (based on spectrophotometric/ fluorescent estimate of concentration and purity or according to the results from the previous PCR testing).

2.4 Procedure itself

The reagents should only be exposed to light for the necessary time, otherwise they should be protected from light. Use DNA with the concentration of 20 to 200 ng/µl. Number of samples to be tested in one run is determined by needs of laboratory and by free tube positions in the cycler. Overview for 13 tested samples:

1 positive control – DNA with mutation

2 negative control – DNA without mutation

3 non-template control – water instead of DNA, or preferably an isolate from paraffin with no bioptic sample

4 sample 1

5 sample 2

6 sample 3

7 sample 4

8 sample 5

9 sample 6

10 sample 7

11 sample 8

12 sample 9

13 sample 10

14 sample 11

15 sample 12

16 sample 13

Mix 3.4 µl Taq + 157 µl H_2O

Pipette sixteen times 8.5 µl into tubes holding a dehydrated mixture

Mix by pipetting with a tip up and down (you can use the same tip)

Add 0.5 µl of DNA, according to the schedule

Cap the tubes

Vortex the rack briefly

Decant

Apply BRAF program

95°C 15´

(95°C 15´´/65°C 50´´/72°C 20´´)*50

37°C 1´´

2.5 QC DURING THE ANALYSIS

The person responsible for the evaluation of samples, checks continually the process of the examination by taking the following steps:

DNA amplification control

Based on spectrophotometric/fluorimetric data or based on the results of testing PCR, you can stop the examination with the result "cannot be evaluated".

Internal control

In each tube, there is an independent primer pair fluorescently labelled for the JOE/HEX channel. This primer pair has the globin gene as a template which is constitutively present in every human being. This control has to be positive if amplifiable human DNA is present in the sample.

Negative control of isolation and negative control of PCR reaction

DNA isolation procedure and the PCR itself is applied to pure paraffin (without the presence of DNA). If this control is positive, it is necessary to repeat the analysis using a new paraffin slice and at the same time to distinguish whether possible contamination occurred during the preparation of DNA from the sample or during the PCR set-up - in PCR reagents (add the "no template" control with water to PCR set-up, instead of DNA).

Positive control

DNA with proven mutation of *BRAF* p.Val600Glu.

External control

In cooperation with other workplaces and organisers of inter-laboratory proficiency testing, anonymised samples are examined and then the established mutation and its location is compared.

Problems during PCR

Problem	Probably caused by	Possible solution
No amplicon after PCR, late upslope of the amplification curve	Poor quality of DNA, error during isolation, error during PCR set-up	Repeat isolation from the same sample, or perform isolation from another sample.
Amplification in a negative control (water instead of DNA, pure paraffin instead of FFPE)	Contamination during the template set-up or PCR set-up	Looking for the source of contamination: replacement of cheap reaction components, cleaning the desk top with a DNA destroying product, or cleaning pipettes and repeating the whole process of PCR adding no-template control (water instead of DNA), alternatively repeating from the isolation from pure paraffin

2.6 Methods of recording results

Archiving all protocols in the operations log, saving the results on computer using the Roche LC480 and Cobas software, printing result protocols from software Roche and Cobas.

Entering visual data readings in a form, pasting in the operations log.

2.7 Safety precautions

Hazardous substances: biological material

Any biological material is treated as potentially infectious. During work, it is necessary to use appropriate protection (disposable gloves). In addition, general principles of occupational safety in molecular biology laboratory apply.

3. EVALUATION

3.1 EVALUATING ALGORITHM

After PCR, perform evaluation by the command Abs Quant/Fit points. Follow HEX/JOE (filter comb 533 to 580) and FAM (filter comb 465-510) signal.

- If the signal HEX/JOE in the negative control NTC Ct is lower than 38, or if in the negative control (standard, non-mutated DNA) the signal (Ct FAM – Ct HEX/JOE)<6, similar to the positive control DNA with mutation, the run is contaminated and invalid.

- If the value Ct in the channel HEX/JOE is higher than 38, or if it is zero, the sample cannot be analysed.

- If Ct HEX/JOE is lower than 38 and Ct FAM is higher than Ct HEX by more than 9 cycles, mutation of BRAF p.Val600Glu is not proven.

- If Ct HEX/JOE is lower than 38 and Ct FAM is higher than Ct HEX by more than 6, but less than 9 cycles, the examination has to be repeated. When obtaining the same or a more evidential result, it is to be concluded that mutation of BRAF p.Val600Glu has been proven.

- If Ct HEX/JOE is lower than 38 and Ct FAM is lower than Ct HEX/JOE, or if Ct HEX/JOE is lower than 38 and Ct FAM is higher by less than 6 cycles, mutation of BRAF p.Val600Glu has been proven.

3.2 CRITERIA FOR REJECTION

Reject positive results for the entire run, if there is a positive signal even for the no-template control. The result is to be considered as "uninterpretable" if the signal for the particular sample is missing in both the FAM and JOE/HEX channels.

3.3 RECORDED DATA, ANALYSIS, AND PRESENTATION

Recording is performed using the company software. With the software, analyse raw data of the measured fluorescence and compare the obtained sequences with standard non-mutated sequences.

3.4 UNCERTAINTY OF MEASUREMENT

The method for *BRAF* is able to detect mutation even with 1% proportion of mutation in the sample, but due to the possibility of quantitative distortion, analysis of each positive result with delta Ct between 6 and 9 cycles is to be repeated using a new microtome slice or the same DNA.