ADPS™

BRAF Mutation Test kit (RUO)

Detection of 4 mutations in codon 600
Instruction for Use

GENECAST

GENECAST, Co., Ltd.
T-10009 Garden Five Techno, 66 Chungmin-ro, Songpa-gu, Seoul, South Korea ZIP 05838

Tel: +82 2 2157 3150 | Fax: +82 2 2157 3152
E-mail: sales@genecast.com
Website: http://www.genecast.com

Background

The *BRAF* gene encodes a protein called B-Raf which is serine/threonine kinase and also known as proto-oncogene. B-Raf acts on the RAS/MAPK signaling pathway, which controls several crucial cell functions including cell growth, proliferation, cell movement and apoptosis (self-destruction of cells). The most frequent somatic mutation in the BRAF gene is V600E, where the 600th amino acid valine be changed to glutamic acid, and rarely V600D are found in codon 600. Vemurafenib and dabrafenib which are inhibitors of these mutations in BRAF have been approved by the US Food and Drug Administration (FDA). These drugs showed a high rate of rapid response that had not previously been seen in patients with melanoma. The response rates of vemurafenib and dabrafenib were from 48% to 59% in phase Ⅱ and Ⅲ trials. This mutation has been widely observed in papillary thyroid cancer (PTC), colorectal cancer (CRC), melanoma and non-small cell lung cancer (NSCLC). BRAF mutations occur in ~50% of melanoma, ~30% of ovarian cancer, ~ 10% CRC and ~10% prostate cancer. In particular, V600E mutation is detected with ~40% of PTC and considered as a prognostic marker for thyroid cancer.

However, our understanding of an individual patient's cancer is often limited by tumor accessibility because of the high risk and invasive nature of current tissue biopsy procedures. "Liquid biopsy", the analysis of circulating tumor DNA (ctDNA), offers a new source of cancer-derived materials that may reflect the status of the disease better and thereby contribute to more personalized treatment. In addition, it is critical to develop more sensitive technologies for detection of mutation.

The ADPS^{TM} BRAF Mutation Test kit (RUO) is a qualitative PCR assay designed to detect V600X substitution mutations including V600E.a, V600E.aa, V600D.at and V600D.ac in codon 600. This kit can detect up to 0.001% of the BRAF mutation in tumor tissues or peripheral blood samples and enables qualitative detection.

Intended Use

The ADPS^{TM} BRAF Mutation Test kit (RUO) detects somatic mutations in codon 600 of the BRAF gene. The kit is should only be used by experienced lab personnel in a lab environment. DNA extracted from formalin-fixed paraffin-embedded samples of tissue biopsy or from liquid biopsy could be used.

The ADPS^{TM} BRAF Mutation Test kit (RUO) is a real-time PCR assay for qualitative detection of 4 somatic mutations in codon 600 of *BRAF* gene in human genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue or circulating DNA extracted from plasma sample. The kit is for Research Use Only (RUO) and intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

The kit adopts Allele-specific PCR (AS-PCR) and real-time PCR technologies, which comprises specific primers and fluorescent probes to detect *BRAF* mutations in human plasma DNA samples. During the nucleic acid amplification, the targeted mutant DNA is matched with the bases at 3' end of the primer, amplified selectively and efficiently, then the mutant amplicon is detected by fluorescent probes labeled with FAM. While the wild-type DNA cannot be matched with specific primers, there is no amplification occurs.

The kit is composed of ***BRAF* Master Mixture, ADPS^{TM} Enzyme, *BRAF* Positive Control and nuclease-free water**.

1) The contents in ***BRAF* Master Mixture** formed a mutation detection system and an internal control system. The mutation detection system includes primers and FAM-labeled probe specific for designated *BRAF* mutations. The internal control system contains the primers and Quasar 670-labeled probe for a BRAF region of genomic DNA without known mutations and polymorphism, to check the presence of inhibitors and monitor the accuracy of experimental operation.
2) The ***BRAF* Positive Control** contains recombinant plasmid DNA that carries fragments of the *BRAF* gene with V600E mutation.
3) The **ADPS^{TM} Enzyme** contains ADPS^{TM} DNA polymerase for PCR amplification

Kit Contents

This kit contains the following materials (see Table 1):

Table 1. Kit Contents (for 24 tests)

Contents	Main Ingredient	Quantity
BRAF Master Mixture	Primers, Probes, dNTPs, Primers/probe for IC	300 µL/tube ×1
ADPS™ Enzyme	ADPS™ DNA Polymerase	30 µL/tube ×1
BRAF Positive Control	Recombinant plasmid (*BRAF* V600E) ($3x10^4$ copies/5 µL)	75 µL/tube ×1
Nuclease-free Water	PCR grade water	150 µL/tube ×1

The *BRAF* Master Mixture contains all reagents except ADPS™ DNA polymerase that is supplied in a separate tube and has to be added before the assay. The detailed detection information is listed in Table 2.

Table 2. Detection Information

Reagent	Mutation detected	Fluorescent Signal	
		FAM	Quasar 670
BRAF Master Mixture	V600 (E.a, E.aa, D.at, D.ac)	V600	IC

*IC: Internal control for PCR **Alternative dye: Quasar 670 - CY5

Storage and Stability

The kit requires shipment on frozen ice packs. All contents of the kit should be stored immediately upon receipt at -20±5 ℃ and protected from light.

The shelf-life of the kit is 18 months, and 90 days of opened reagents for the kit. ***BRAF* Master Mixture** and reaction Master Mixtures should be protected from prolonged exposure to light. Reaction Master Mixtures must be stored at 2ºC to 8ºC in the dark. The prepared samples and controls must be added within 1 hour of preparation of the reaction Master Mixtures. Amplification must be started within 1 hour from the time that the processed samples and controls are added to the reaction Master Mixtures.

The recommend maximum freeze-thaw cycle is five cycles.

Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments:
   AB 7500/7500 FAST or CFX Connect™.
2) DNA Extraction kit. FFPE DNA Kit for FFPE tissues, Circulating DNA kit for plasma sample.
3) Spectrophotometer for measuring DNA concentration.
4) Mini centrifuge with rotor for centrifuge tubes.
5) Mini centrifuge with rotor for PCR tubes.
6) Nuclease-free centrifuge tubes.
7) Nuclease-free PCR tubes and caps.
8) Adjustable pipettors and filtered pipette tips for handling DNA.
9) Tube racks.
10) Disposable powder-free gloves.
11) Sterile, nuclease-free water.

Precautions and Handling Requirements

For research use only.

Precautions

- DO NOT use for *in-vitro* Diagnostic (IVD).
- Please read the instruction carefully and become familiar with all components of the kit prior to use, and strictly follow the instruction during operation.
- Please check the compatible real-time PCR instruments prior to use.
- DO NOT use the kit or any kit component after their expiry date.
- DO NOT use any other reagents from different lots in the tests.
- DO NOT use any other reagent in the other test kits.

Safety Information

- Handle all specimens and components of the kit as potentially infectious material using safe laboratory procedures.
- Only trained professionals can use this kit. Please wear suitable lab coat and disposable gloves while handling the reagents.
- Avoid skin, eyes and mucous membranes contact with the chemicals. In case of contact, flush with water immediately.
- DO NOT pipet by mouth.

Decontamination and Disposal

- The kit contains positive control; strictly distinguish the positive control from other reagents to avoid contamination which may cause false positive.
- PCR amplification is extremely sensitive to cross-contamination. The flow of tubes, racks, pipets and other materials used should be from pre-amplification to post-amplification, and never backwards.
- Gloves should be worn and changed frequently when handling samples and reagents to prevent contamination.
- Using separate, dedicated pipettes and filtered pipette tips when handling samples and reagents to prevent exogenous DNA contamination to the reagents.
- Please pack the post-amplification tubes with two disposable gloves and discard properly. DO NOT open the post-amplification PCR tubes.
- All disposable materials are for one time use. DO NOT reuse
- The unused reagents, used kit, and waste must be disposed of properly.

Cleaning

- After the experiment, wipe down the work area, spray down the pipettes and equipment with 75% ethanol or 10% hypochlorous acid solution.

Instrument Setup

- Setup the reaction volume as 20 μL.
- For AB 7500 and AB7500 FAST, please set up as follows: Reporter Dye: FAM, CY5; Quencher Dye: NFQ-MGB; Passive Reference dye: ROX.
- Refer to the real-time PCR instrument operator's manual for detailed instructions.
- We recommend that all PCR instruments in use should be conducted fluorescence calibration once a year.

Assay Procedure

1. DNA Extraction

The specimen material must be human genomic DNA extracted from FFPE tissue or circulating DNA extracted from the plasma sample. DNA extraction reagents are not included in the kit. Before DNA extraction, it's essential to use standard pathology methodology to ensure tumor sample quality. Carry out the DNA extraction according to the instructions of DNA extraction kit.

Tumor samples are non-homogeneous, may also contain non-tumor tissue. Data from different tissue sections of the same tumor may be inconsistent. DNA from non-tumor tissue would not be detected with *BRAF* mutations. It's better to use tumor tissue samples with more than 30% tumor cells.

The OD_{260}/OD_{280} value of extracted DNA from FFPE tissue should be between 1.7 ~ 2.1 (measured using the spectrophotometer). The storage conditions for extracted DNA from tissue and plasma used for PCR amplification is shown in Table 3. And the circulating DNA isolated from plasma should be used directly without dilution.

Table 3. Recommended Conditions for Sample and Extracted DNA

	≤ -70°C	**-15°C to -25°C**	**2°C to 8°C**	**15°C to 30°C**
FFPE	≤ 3 years			
Plasma	Up to 12 months		Up to 3 days	
Extracted DNA		Up to 2 freeze thaws over 60 days	Up to 21 days	Up to 7 days

Note:

- *The FFPE tissue should be handled and stored properly, and the storage time should preferably be less than 3 years.*
- *The plasma sample should be derived from EDTA anti-coagulated peripheral whole blood. The recommended volume of whole blood is no less than 10 mL.*
- *The extracted DNA should be used immediately, if not, it should be stored at -20±5℃ for no more than 3 months.*
- *Before detection, dilute the extracted tissue DNA with 1×TE buffer (pH 8.0) to designated concentration. We recommend using at least 5 μL DNA for 10 times dilution, to ensure the validity of final concentration.*

2. Mutation Detection

1) Take the *BRAF* Master Mixture, *BRAF* Positive Control, Nuclease-free water and ADPS™ Enzyme out of the kit from the freezer.
2) Thaw the *BRAF* Master Mixture, *BRAF* Positive Control, and Nuclease-free water at room temperature. When the reagents are completely thawed, vortex each tubes for 3 sec and centrifuge briefly to collect all liquid at the bottom of the tube.
3) Gently tap the ADPS™ **Enzyme** and centrifuge briefly prior to use.
4) Prepare sufficient ***BRAF* Reaction Mixture** containing ADPS™ Enzyme, BRAF Master Mixture in separate sterile centrifuge tube respectively according to the ratio in Table 4-1 to 4-3. Mix Reaction Master Mix thoroughly by vortexing for 3 sec and centrifuge briefly.

Table 4-1. *BRAF* Reaction Master Mixture for Mutation Detection

Content	Volume per test (μL)
BRAF Master Mixture	10.0
ADPS™ Enzyme	0.5
Sample	2.0~9.5
Nulclease-free water	0~7.5
Total volume	20.0

Table 4-2. *BRAF Reaction Master* Mixture for Positive control

Content	Volume per test (μL)
BRAF Master Mixture	10.0
ADPS™ Enzyme	0.5
Positive control	5.0
Nulclease-free water	4.5
Total volume	20.0

Note:

- *Prepare a positive control experiment at the very end to prevent cross-contamination.*
- *Every PCR run must contain at least one PC (Positive control).*
- *The prepared mixtures should be used immediately, avoid prolonged storage.*
- *Due to the viscosity of the enzyme mix, pipet slowly to ensure all mix is completely dispensed from the tip.*
- *Pipet enzyme mix by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.*

5) Prepare two PCR tubes for each sample: Dispense 10.0 μL of *BRAF* Reaction Mixture to each PCR tube respectively. Then add 2.0~9.5 μL of each sample DNA to each sample tube and cap the PCR tubes.
6) Prepare one PCR tube for PC: Dispense 10.0 μL of *BRAF* Reaction Mixture to one PCR tube. Then add PC (5 μL) to sample tube and cap the PCR tube.
7) Add nuclease-free water to all PCR tubes upto 20.0 μL.
8) Briefly centrifuge the PCR strips to collect all liquid at the bottom of each PCR tube.
9) Place the PCR strip tubes into the real-time PCR instrument. A recommended plate layout is shown in Table 5.

Table 5. PCR Plate Layout (Example)

Well	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC										
B	Positive Control											
C	Sample 1	Sample 1										
D	Sample 2	Sample 2										
E	Sample 3	Sample 3										
F	Sample 4	Sample 4										
G	Sample 5	Sample 5										
H	Sample 6	Sample 6										

10) Setup the PCR Protocol using the cycling parameters in Table 6.

Table 6. Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	5 min	-
2	50	95℃	10 sec	-
		60℃	30 sec	**FAM/Quasar670** (CY5)
		72℃	10 sec	-

11) Run the PCR run immediately.

12) When the PCR operation finished, analyze the data according to the "Results Interpretation" procedures

3. Results Interpretation

Before mutation data analysis, the following items should be checked:

1) Set the threshold to 10% of the maximum fluorescence value of the positive control(V600E).
2) For WT gDNA: FAM signal of the samples should be no amplification or Ct value should be ≥ 48. If not, the data is *INVALID*. The sample should be retested.
3) For Positive control: FAM Ct value range of the samples should be 26 <Ct < 28. If not, the data is *INVALID*. The sample should be retested.
4) For the internal control assay for each sample: Ct values of the samples should be 22 <Ct < 39. (Based on copy number of total *BRAF* gene). If not, this indicates insufficient DNA or presence of PCR inhibitors. The sample should be retested with increased or re-extracted DNA.

Analyze the mutation assay for each sample:

1) Record the FAM/Quasar670 Ct values of the samples.
2) Calculate the ΔCt value for each well:

   ΔCt value = Mutant Ct value (FAM) – Internal control Ct value (Quasar 670)
3) Result interpretation for each tube according to the Cut-off ΔCt value in Table 7.
   a) If the ΔCt value is< the Cut-off ΔCt value, the sample is determined as positive.
   b) If there are no FAM signal and Ct values of internal control is 22 <Ct < 39, the sample is determined as negative.
   c) If the ΔCt value is ≥ the Cut-off ΔCt value, the sample is determined as negative or under the LOD (limit of Detection) of the kit.
   d) Two or more *BRAF* mutations may be detected for a sample.

Table 7. Result Determination (Example)

Sample	Ct value		ΔCt value	Determination
	FAM	Quasar 670		
#1	30.0	23.5	6.5	Positive
#2	49.0	28.0	21.0	Negative
#3	38.0	32.0	6.0	Positive
#4	No signal	30.0	-	Negative

Performance Characteristics

The performance characteristics of this kit were validated on Stratagene Mx3000P™, ABI7500 and SLAN-96S.

1. Limit of Detection

The Low Limit of Detection (LLOD) of the kit for each mutation is shown in Table 8.

Table 8. LLOD for each BRAF mutation

Codon	Mutation	Base Change	COSMIC ID	LOD (copy)	Detection sensitivity (%)
600	V600E.a	1799T>A	476	3	0.0001
	V600E.aa	1799_1800TG>AA	475	3	0.0001
	V600D.at	1799_1800TG>AT	477	3	0.0001
	V600D.ac	1799_1800TG>AC	308550	3	0.0001

2. Cross-reactivity

The cross reaction among the mutant sequences targeted by this kit, the cross reaction with other homologous mutant nucleotide sequence, the cross reaction with wild-type genomic DNA (DNA concentrations are 30~50 ng/reaction), and the cross reaction with non-human gene (the DNA was extracted from *Staphylococcus aureus and Staphylococcus epidermidis* which were common microorganism causing skin infection) were evaluated, the results shown no cross reactions.

3. Interference factor

12 common interference substances: endogenous Hemoglobin, Ferritin, Albumin and Triglyceride, exogenous pathogenic microorganism such as *Staphylococcus aureus and Staphylococcus epidermidis,* common anticoagulants such as Heparin sodium, Sodium citrate and EDTA were evaluated in this study. It is confirmed that the potential maximum concentrations: 2 g/L hemoglobin, 200 ng/mL Ferritin, 60 g/L Albumin, 0.645 mol/L Sodium citrate and 27 μmol/L EDTA would not interfere with the test result. While 150 U/mL Heparin sodium would inhibit the test performance. It is stated in DNA Extraction section in this Instructions to avoid using *heparin anticoagulant.*

4. Precision

3 precision controls: negative control, weak positive control (with 0.0001% mutant content) and strong positive control (with 10% mutant content) were used in the validation. 3 batches of the kits were tested with the precision controls by 2 operators twice a day for 20 days on different PCR instruments. The Ct values were calculated, the CV values were all within 5%.

Limitations

1) The kit is to be used only by personnel specially trained with PCR techniques.
2) The 0.0001 % of detection sensitivity is guaranteed only when input DNA is used with maximum of 3,000,000 copies.
3) The results can only be used for research use only.
4) The kit has been validated for use with circulating DNA extracted from plasma sample and human genomic DNA extracted from FFPE tissue.
5) Reliable results are dependent on proper specimen collection, processing, transport, and storage.
6) The sample containing degraded DNA may affect the ability of the test to detect *BRAF* mutation.

7) This kit can only assess the *BRAF* V600 mutation status and detect 4 *BRAF* mutations indicated above.

8) Samples with negative result (No mutation detected) may harbor another *BRAF* mutations not detected by this assay.

References

1) Kebebew, Electron, et al. The prevalence and prognostic value of BRAF mutation in thyroid cancer. *Annals of surgery*, 2007, 246(3): 466–471

2) Chapman, Paul B., et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *New England Journal of Medicine*, 2011, 364(26), 2507-2516

3) Eroglu, Zeynep; RIBAS, Antoni. Combination therapy with BRAF and MEK inhibitors for melanoma: latest evidence and place in therapy. *Therapeutic advances in medical oncology*, 2016, 8(1), 48-56

4) O'brien, Odharnaith, et al. BRAF V600 mutation detection in melanoma: a comparison of two laboratory testing methods. *Journal of clinical pathology*, 2017, 70(11), 935-940

5) Flaherty, Keith T., et al. Improved survival with MEK inhibition in BRAF-mutated melanoma. *New England Journal of Medicine*, 2012, 367(2), 107-114

6) Xing, Mingzhao, et al. Association between BRAF V600E mutation and mortality in patients with papillary thyroid cancer. *Jama*, 2013, 309(14), 1493-1501

7) ROSSI, Esther Diana, et al. Diagnostic and prognostic value of immunocytochemistry and BRAF mutation analysis on liquid-based biopsies of thyroid neoplasms suspicious for carcinoma. *European journal of endocrinology*, 2013, 168(6), 853-859

8) Gonzalez-Cao, Maria, et al. BRAF mutation analysis in circulating free tumor DNA of melanoma patients treated with BRAF inhibitors. *Melanoma research*, 2015, *25*(6), 486-495

9) Anguera, Geòrgia; MAJEM, Margarita. BRAF inhibitors in metastatic non-small cell lung cancer. *Journal of thoracic disease*, 2018, 10(2), 589

10) Long, Georgina V., et al. Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *New England Journal of Medicine*, 2014, 371(20), 1877-1888

11) Sosman, Jeffrey A., et al. Survival in BRAF V600–mutant advanced melanoma treated with vemurafenib. *New England Journal of Medicine*, 2012, 366(8), 707-714

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