## PRODUCT NAME

Product Name: PNAClamp™ Mutation Detection Kit
Brand Name: PNAClamp™ BRAF Mutation Detection Kit

## INTENDED USE

The PNAClamp™ BRAF Mutation Detection Kit detects the V600 somatic mutations in Valine 600 position of human in BRAF oncogene (Table 1).
The kit is to be used by trained laboratory professionals, within a laboratory environment, using (for example) DNA extracted from formalin-fixed paraffin-embedded samples of colorectal tissue, thyroid cancer biopsies, and thyroid fine-needle aspiration biopsy (FNAB).
The kit is for *in vitro* diagnostic use.

Please read the instructions carefully prior to use.

The PNAClamp™ BRAF Mutation Detection Kit is a CE marked diagnostic device in accordance with the European Union *in vitro* Diagnostic Medical Device Directive 98/79/EC.
It is MFDS approved for clinical use in Korea.

**Table 1. BRAF mutations detected by this kit**

| No. | Reagent | Exon | Amino Acid Change | Nucleotide change | Cosmic No. |
|---|---|---|---|---|---|
| 1 | BRAF PNA mix #2 | 15 | p.V600E | c.1799T>A | 476 |
| | | | p.V600E2 | c.1799_1800delinsAA | 475 |
| | | | p.V600D | c.1799_1800 TG>AT | 477 |
| | | | p.V600K | c.1798_1799 GT>AA | 473 |
| | | | p.V600R | c.1798_1799 GT>AG | 474 |
| | | | p.V600A | c.1799T>C | 18443 |

* Cosmic Numbers are taken from 'The Catalogue of Somatic Mutations in Cancer'.
(http://cancer.sanger.ac.uk/cosmic)

## PRINCIPLE AND OVERVIEW

The PNAClamp™ BRAF Mutation Detection Kit is based on peptide nucleic acid (PNA)-mediated real-time PCR clamping technology. PNA is a synthetic DNA analog in which the phosphodiester backbone is replaced by a peptide-like repeat formed by (2-aminoethyl)-glycine units.

PNA-mediated real-time PCR clamping relies on the following two unique properties of PNA probes. First, PNA will hybridize to its complementary DNA target sequence only if the sequence is in complete match. Since PNA/DNA duplexes are more thermodynamically stable than the corresponding DNA-DNA duplexes, even with a single mismatch, PNA will not bind to complementary DNA strand, unlike DNA. Second, PNA oligomers are not recognized by DNA polymerases and will not be utilized as primers in subsequence real-time PCR. Instead, it serves as a sequence-selective clamp that prevents amplification during subsequent PCR.

When there is a mutation in target gene and therefore a mismatch is present, the DNA/PNA duplex is destabilized, allowing strand elongation from a bound DNA oligomer which serves as a PCR primer. The outcome is the positive reaction in real-time PCR from the samples harboring mutant allele, while amplification of the wild-type gene is suppressed.

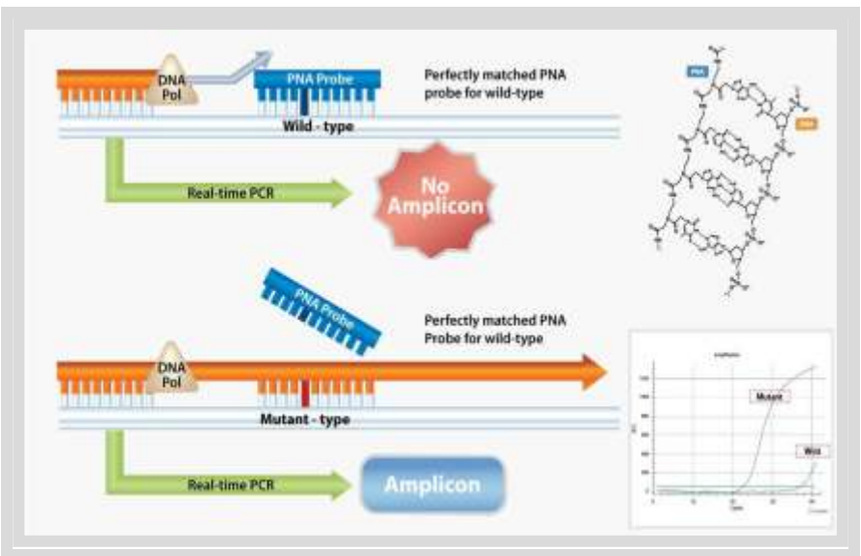



**Figure 1. Principle of the PNAClamp™ BRAF Mutation Detection Kit**

The kit can rapidly detect BRAF mutation (within 2 h) with high sensitivity even with a small amount of DNA (10~25 ng). The detection limit of the kit, when the mutated gene is mixed with wild-type background, is less than 1%.

## SAFETY INFORMATION

Material Safety Data Sheet (MSDS) is available upon request.

## EQUIPMENT AND MATERIALS SUPPLIED BY THE USER

- ✓ Reagents and equipment for DNA extraction
- ✓ 0.2 ml DNase-free PCR tubes or plates
- ✓ Pipettes
- ✓ A real-time PCR instrument fitted with a detector enabling evaluation of SYBR Green dye

**Table 2. List of compatible real time PCR instruments**

| Company | Model |
|---|---|
| Bio-Rad | CFX 96 |
| Roche | Light cycler 480 II |
| ABI | ABI 7500 |
| ABI | ABI 7900 |
| ABI | StepOnePlus |
| Qiagen | Rotor-Gene Q |
| ABI | QuantStudio 5 |

**For other instruments, minor optimization might be necessary.**

## WARNINGS AND PRECUATIONS

- ✓ **Please read the instruction carefully and become familiar with all components of the kit prior to use.**
- ✓ **PNAClamp™ BRAF Mutation Detection Kit is for *in vitro* diagnostic use.**
- ✓ All experiments should be performed under proper sterile conditions with aseptic techniques. It recommended that users have separate, dedicated pipettes and filter pipette tips to add DNA template and during the preparation of reagents.
- ✓ Always wear powder-free gloves when you handle the kit.
- ✓ To avoid repeated freezing and thawing, aliquot all reagents into appropriate volumes and store frozen until use. Thaw appropriate volumes of reagents before each experiment.
- ✓ All experimental procedures should be performed at room temperature. However, exposing BRAF PNA 2X premix at room temperature should be minimized for the optimal amplification.
- ✓ Dissolve reagents completely and mix them thoroughly by vortex.
- ✓ The BRAF PNA 2X premix solution contains fluorescence dye and should be kept dark.
- ✓ If DNA has been extracted from a paraffin block, additional purification steps may be required.
- ✓ PCR tubes should be weakly centrifuged before use.
- ✓ Using non-recommended volume for reagent not only result in loss of performance but also increase the chance of false result.
- ✓ Using non-recommended volume and concentration for target DNA sample not only result in loss of performance but also increase the change of false result.
- ✓ Do not exchange and mix up different lots or other manufacture's product.
- ✓ Upon using instruments, use only recommended consumables only. If not, instruments will not be usable or false result may prominent.
- ✓ Additional validation testing by user may necessary when using non-recommended instruments.
- ✓ Do not re-use any remaining reagents after PCR amplification is completed.
- ✓ Do not use the reagents beyond the expiry date.

## STORAGE CONDITION AND STABILITY

The PNAClamp™ BRAF Mutation Detection Kit is shipped on ice package and must still be frozen on arrival. If the kit is not frozen on arrival please contacts PANAGENE Inc. or the local distributor.

The PNAClamp™ BRAF Mutation Detection Kit should be stored immediately upon receipt at -15℃ to -20℃. When stored under the recommended storage conditions in the package, the kit is stable until the labeled expiration date.

After opening the kit, shelf-life is 3 months.

## KIT CONTENTS

Store at -15℃ to -20℃

| No. | Name of component | Description | Volume | Cap label |
|---|---|---|---|---|
| 1 | Non PNA mix #1 | Primers only | 180 µl | BRAF 1 |
| 2 | BRAF PNA mix #2 | V600 PNA and primers | 180 µl | BRAF 2 |
| 3 | BRAF PNA 2X premix | PCR reaction premix | 1,250 µl | BRAF 2X premix |
| 4 | Clamping control | Wild-type DNA | 400 µl | BRAF Control |

* Each kit contains enough material to test 50 DNA samples for all mutations.

## PROCEDURES

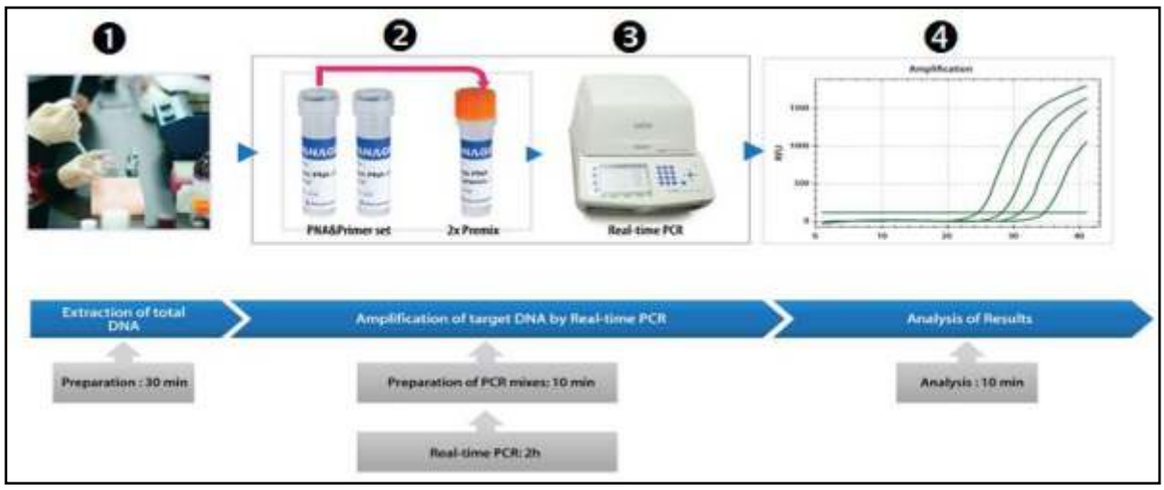



**Figure 2. Workflow of the PNAClamp™ BRAF Mutation Detection Kit**

### 1. DNA preparation

Specimen collection and DNA extraction reagents are not included in the kit so they should be provided by the user.

1) Paraffin embedded tissues, biopsy tissues or thyroid fine needle aspiration biopsy(FNAB) can be used as specimens.
2) Specimen transport: Use standard pathology methodology to ensure specimen quality.
3) For DNA extraction Kit is recommended below.

| Model | Company | Catalog number |
|---|---|---|
| High Pure PCR Template Preparation Kit | Roche Diagnostics | 11796828001 |
| QIAamp DNA FFPE Tissue Kit | Qiagen | 56404 |
| QIAamp DNA Mini Kit | Qiagen | 51304 |
| Maxwell® 16 FFPE Plus LEV DNA Purification Kit | Promega | AS1135 |

4) Extracted DNA can be stored at 4℃ for up to 24 hours, or at -20℃ for long term storage.

## 2. Preparation of the Real-Time PCR Mixture

**Table 3. Set up reaction mixture per on reaction.**

| Components | Volume |
|---|---|
| BRAF PNA 2X Premix (#3) | 10 µl |
| Each PNA mix (#1~#2) | 3 µl |
| Extracted DNA (10 ng total) or Clamping control (#4) | 7 µl |
| Total volume | 20 µl |

1) Prepare 2 PCR tubes for one set of DNA samples to be tested. Label them as S1 and S2. Prepare another set of 2 tubes for Clamping control (wild-type DNA) and label them as C1 and C2.
2) Add 10 µl of BRAF PNA 2X Premix (#3 from the kit) to each tube.
3) For each PCR tube, add 3 µl of corresponding PNA mix from #1~2 from the kit. For example, S1 and C1 tubes will have #1 Non PNA mix #1, S2 and C2 tubes will have #2 BRAF PNA mix #2.
4) For S1~S2 PCR tubes, add 7 µl of prepared DNA sample (10~25 ng total) to each tube to yield 20 µl final volume.
5) For C1~C2 PCR tubes, add 7 µl of Clamping control (#4 from the kit).
6) If you have more than one DNA sample to be tested, prepare one set of Clamping control for the entire experiment. In such case, it is recommended to prepare a master mix containing 2X Premix and each PNA mix for all the samples and to aliquot 13 µl to each PCR tube.
7) When all reagents are loaded, tightly close/seal the PCR tube or 96 well plate. Otherwise, any remaining reagents may evaporate.

## 3. Real-Time PCR reaction

Perform real-time PCR using the cycling conditions described below

| ONE CYCLE | | |
|---|---|---|
| Pre-denaturation | 94℃ | 5 min |
| **FOUR-STEP CYCLING (40 CYCLES)** | | |
| Denaturation | 94℃ | 30 sec |
| PNA clamping | 70℃ | 20 sec |
| Annealing | 63℃ | 30 sec |
| Extension* | 72℃ | 30 sec |

* Set up the detection for reading SYBR Green at 72℃.
** If you use Light Cycler 480 II, Please set up 45 cycles for four-step cycling.

## 4. Assessment

*** Refer to the specialized instrument user guide by Panagene for detail analysis method.**

### 1) Clamping control (wild-type DNA control)

(1) Determine Ct value from each PCR reaction. The cycle number at which a signal is detected above background fluorescence is termed as the cycle threshold (Ct).

(2) The Ct values of the Clamping control (tube C1~C2) should fall in the range given in Table 4. The assay should be repeated if the values are not in recommended range.

**Table 4. The acceptable Ct ranges of Clamping control**

| Assay | Acceptable Ct range |
|---|---|
| ① Non PNA mix #1 (C1) | ≤ 30 |

| Assay | Acceptable ΔCt-1* range |
|---|---|
| ② BRAF PNA mix #2 (C2) | < 2 |

*ΔCt-1 = [Standard Ct] – [Sample Ct or Clamping control Ct]

### 2) DNA samples

(1) Determine Ct values of each sample (S1 and S2).

i. Ct value of Non PNA mix (S1) should be 22~35.

ii. Ct value of Non PNA mix (S1) can serve as an internal control to indicate the purity and the concentration of DNA. Thus, the validity of the test can be decided by the Ct value of Non PNA mix (S1) as shown in Table 5.

**Table 5. The acceptability of samples**

| Acceptability | Ct value of Non PNA mix(S1) | Descriptions and recommendations |
|---|---|---|
| Optimal | 22< Ct <30 | The amplification and the amount of DNA sample are optimal. |
| Acceptable | 30≤ Ct <35 | The target gene was amplified with low efficiency. For more reliable result, it is suggested that repeat PCR reaction with a higher amount of DNA. |
| Invalid | Ct ≤22 | Possibility of false positive is high. Repeat the PCR reaction with a lower amount of DNA. |
| | 35≤ Ct | The amplification was failed. Check DNA amount and purity. New DNA prep might be required. |

(2) Calculate the ΔCt-1 values by subtracting the sample Ct values (or Clamping control Ct value) from the Standard Ct values. If the Ct of DNA samples is displayed as NA (not applicable), then set Ct value as 38 for further calculation.

*ΔCt-1 = [Standard Ct] – [Sample Ct (S2) or Clamping control Ct]

**Table 6. The value of Standard Ct**

| Standard Ct | Instrument | | | | | | |
|---|---|---|---|---|---|---|---|
| | Bio-Rad CFX96 | Roche LC480 | ABI 7900 | ABI 7500 | ABI StepOnePlus | Rotor-Gene Q | QuantStudio 5 |
| BRAF PNA mix | 35 | 35 | 35 | 35 | 35 | 35 | 35.5 |

(3) Calculate ΔCt-2 [Ct value of sample subtracted by Ct value of Non PNA mix].

**ΔCt-2 = [Sample Ct (S2)] - [Non PNA mix Ct (S1)]

(4) Assess the result along with the values of ΔCt-1 and ΔCt-2 as given in Table 7.

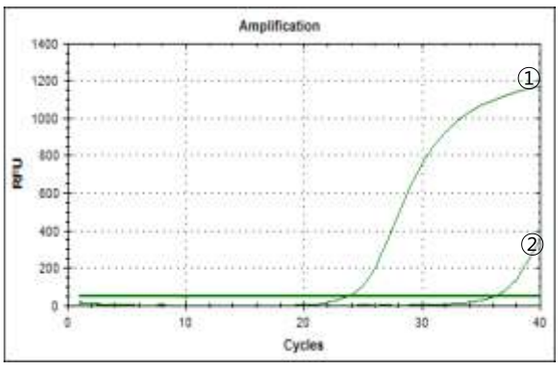
**Table 7. Assessment of the result**

| ΔCt-1 | ΔCt-2 | Assessment |
|---|---|---|
| 2≤ ΔCt-1 | ΔCt-2 ≤8 | V600 Mutant |
| | 8< ΔCt-2 | Wild |
| 0< ΔCt-1 <2 | ΔCt-2 ≤3 | V600 Mutant |
| | 3< ΔCt-2 | Wild |
| ΔCt-1 ≤0 | All value | Wild |

## EXAMPLES OF ANALYSIS

### 1. Using Bio-Rad CFX96

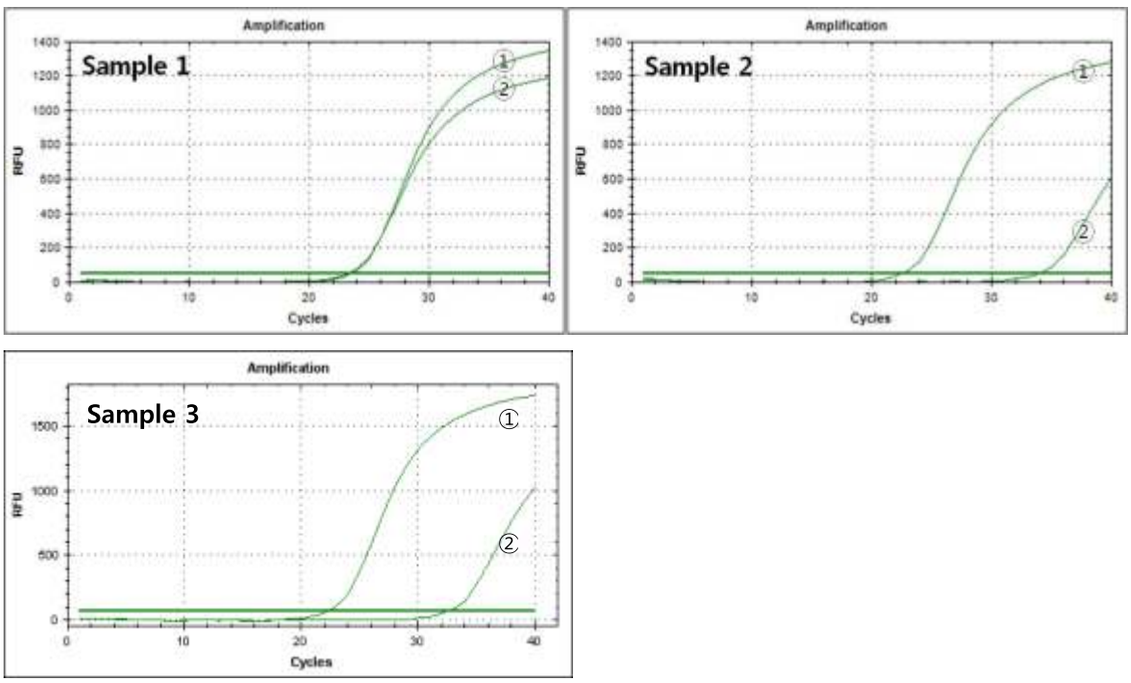
#### 1) Profile of Clamping control




| Assay | Clamping control Ct | Accep. range | Result |
|---|---|---|---|
| ① Non PNA mix #1 (C1) | 24.00 | ≤ 30 | Acceptable |

| Assay | Clamping control Ct | ΔCt-1 | Accep. ΔCt-1 range | Result |
|---|---|---|---|---|
| ② BRAF PNA mix #2 (C2) | 36.29 | -1.29 | < 2 | Acceptable |

#### 2) Profile of samples




① Non PNA mix #1 ② BRAF PNA mix #2

**Table 8. Example of sample Ct values**

| Assay \ Sample No. | Sample 1 Ct | Sample 2 Ct | Sample 3 Ct | Standard Ct | **ΔCt-2 | *ΔCt-1 |
|---|---|---|---|---|---|---|
| ① Non PNA mix #1(S1) | 23.66 | 22.66 | 22.34 | | | |
| ② BRAF PNA mix #2 (S2) | 23.37 | 34.20 | 32.55 | 35 (③) | ② - ① | ③ - ② |

*ΔCt-1 = [Standard Ct] – [Sample Ct or Clamping Control Ct]

**ΔCt-2 = [Sample Ct] – [Non PNA mix Ct (S1)]

**Table 9. Analysis of data**

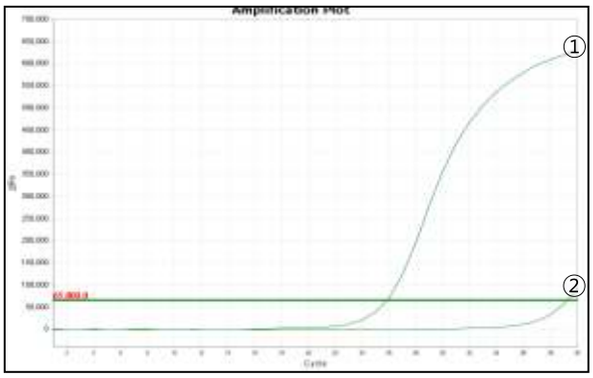
| Assay \ Sample No. | Sample 1 | | Sample 2 | | Sample 3 | |
|---|---|---|---|---|---|---|
| | ΔCt-2 | ΔCt-1 | ΔCt-2 | ΔCt-1 | ΔCt-2 | ΔCt-1 |
| ② BRAF PNA mix #2 (S2) | -0.29 | 11.63 | 11.54 | 0.8 | 10.22 | 2.45 |
| Results | V600 mutant | | Wild | | Wild | |

1. When ΔCt-1 is equal to or greater than 2 ( ▭ ).
   ① ΔCt-2 is greater than 8, the sample is assessed to be **wild**.
   ② ΔCt-2 is equal to less than 8 ( ▭ ), the sample is assessed to be **mutated**.

2. When ΔCt-1 is greater than 0 and less than 2( ▭ ),
   ① ΔCt-2 is greater than 3, the sample is assessed to be **wild**.
   ② ΔCt-2 is equal to or less than 3( ▭ ), the sample is assessed to be **mutated**.

## 2. Using ABI QuantStudio 5

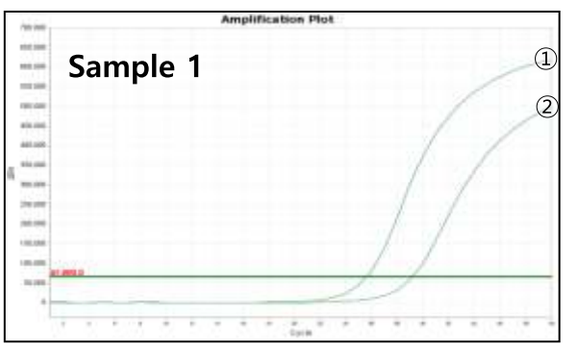
### 1) Profile of Clamping control

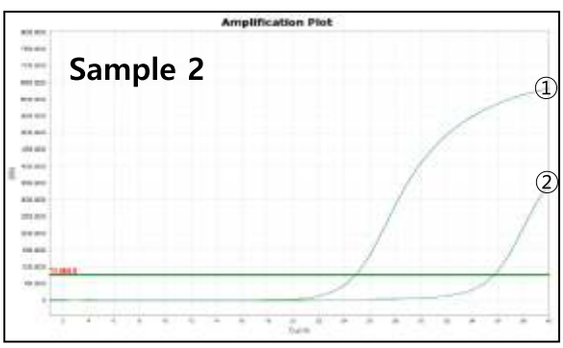


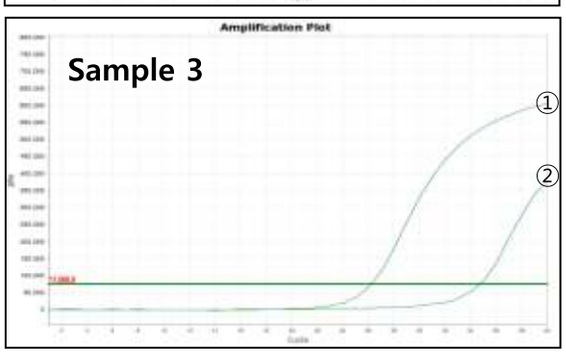

| Assay | Clamping control Ct | Accep. range | Result |
|---|---|---|---|
| ① Non PNA mix #1 (C1) | 23.62 | ≤ 30 | Acceptable |

| Assay | Clamping control Ct | ΔCt-1 | Accep. ΔCt-1 range | Result |
|---|---|---|---|---|
| ② BRAF PNA mix #2 (C2) | 36.78 | -1.28 | < 2 | Acceptable |

### 2) Profile of samples




① Non PNA mix #1 ② BRAF PNA mix #2

**Table 10. Example of sample Ct values**

| Assay \ Sample No. | Sample 1 Ct | Sample 2 Ct | Sample 3 Ct | Standard Ct | **ΔCt-2 | *ΔCt-1 |
|---|---|---|---|---|---|---|
| ① Non PNA mix #1(S1) | 23.43 | 23.60 | 24.74 | | | |
| ② BRAF PNA mix #2 (S2) | 26.91 | 34.22 | 33.21 | 35.5 (③) | ② - ① | ③ - ② |

*ΔCt-1 = [Standard Ct] – [Sample Ct or Clamping Control Ct]

**ΔCt-2 = [Sample Ct] – [Non PNA mix Ct (S1)]

**Table 11. Analysis of data**

| Assay \ Sample No. | Sample 1 | | Sample 2 | | Sample 3 | |
|---|---|---|---|---|---|---|
| | ΔCt-2 | ΔCt-1 | ΔCt-2 | ΔCt-1 | ΔCt-2 | ΔCt-1 |
| ② BRAF PNA mix #2 (S2) | 3.48 | 8.59 | 10.62 | 1.28 | 8.47 | 2.29 |
| Results | V600 mutant | | Wild | | Wild | |

1. When ΔCt-1 is equal to or greater than 2 ( ).
   ① ΔCt-2 is greater than 8, the sample is assessed to be **wild**.
   ② ΔCt-2 is equal to less than 8 ( ), the sample is assessed to be **mutated**.

2. When ΔCt-1 is greater than 0 and less than 2( ),
   ① ΔCt-2 is greater than 3, the sample is assessed to be **wild**.
   ② ΔCt-2 is equal to or less than 3( ), the sample is assessed to be **mutated.**

## QUALITY CONTROL

Each lot of **PNAClamp™ BRAF Mutation Detection Kit** is tested against predetermined specifications to ensure consistent product quality in accordance with PANAGENE's ISO 9001 & 13485-Certified Quality Management System

## PERFORMANCE TEST

### 1. Analytical Sensitivity

The analytical sensitivity was determined by testing the standard BRAF mutant cell lines with the PNAClamp™ BRAF Mutation Detection Kit. The extracted DNA is measured as 10 ng and 25 ng.

Each 10 ng and 25 ng DNAs were diluted to have 100, 10 and 1% of different mutant ratio. Three tests were performed with these 3 conditions of DNAs for 3 different batches of the Kit.

The results showed that 1% mutation was detected for all cases the mutant DNA cell line concentrations.

### 2. Analytical Specificity

The analytical specificity was determined by testing the wild cell lines without mutant DNA. Three tests were performed on three batches of the kit using DNA (10, 25 and 50 ng) extracted from wild-type cell line HeLa. All the three tests showed wild-type locations, and there was no cross-reactivity.

### 3. Reproducibility

Experiments were performed to evaluate the reproducibility of seven standard cell line DNAs (10 and 25 ng) at 100, 10, 1 and 0% of different mutant ratio, for three batches, among three operators, and for three days. PNAClamp™ BRAF Mutation Detection Kit had a correct call rate of 100%. All the results showed little variation, with %CV<5%.

## REFERENCES

1. Jeong et al., Detection of BRAF(V600E) Mutations in Papillary Thyroid Carcinomas by Peptide Nucleic Acid Clamp Real-Time PCR: A Comparison with Direct Sequencing. Korean J Pathol 46(1):61-7, 2012.
2. Kwon et al., Frequency of KRAS, BRAF, and PIK3CA mutations in advanced colorectal cancers: Comparison of peptide nucleic acid-mediated PCR clamping and direct sequencing in formalin-fixed, paraffin-embedded tissue. Pathol Res Pract 207(12):762-8, 2011.
3. Shin et al., National Cancer Center, Korea. 2002 annual report of the Korea Central Cancer Registry: based on registered data from 139 hospitals. Cancer Res Treat. 36(2):103-14, 2004.
4. Kang et al., Prevalence, clinical and ultrasonographic characteristics of thyroid incidentalomas. Thyroid. 14(1):29-33, 2004.
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## EXPLANATION OF SYMBOLS ON THE LABEL

| | | | |
|---|---|---|---|
| LOT | Batch Code | | Use by (YYYY.MM.DD) |
| | Manufacturer | EC REP | EC Representative |
| IVD | In Vitro Diagnostic Medical Device | REF | Catalog number |
| | Temperature Limitation | CE | European conformity |

CE

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