PRODUCT NAME

Product Name: PNAClamp™ Mutation Detection Kit
Brand Name: PNAClamp™ IDH2 Mutation Detection Kit

INTENDED USE

The PNAClamp™ IDH2 Mutation Detection Kit is an *in vitro* diagnostic test intended for the detection of the somatic mutations in the IDH2 oncogene and will provide a qualitative assessment of mutation status. The PNAClamp™ IDH2 Mutation Detection 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 glioma tissue, glioma biopsies.
The kit is for *in vitro* diagnostic use.

Please read the instructions carefully prior to use.

The PNAClamp™ IDH2 Mutation Detection Kit is a CE marked diagnostic device in accordance with the European Union *in vitro* Diagnostic Medical Device Directive 98/79/EC.

Table 1.IDH2 mutations detected by this kit

No.	Reagent	Exon	Amino Acid Change	Nucleotide change	Cosmic No.
1	R140 PNA mix	4	p.I139F	c.415A>T	227366
			p.R140W	c.418C>T	41877
			p.R140Q	c.419G>A	41590
			p.R140L	c.419G>T	41875
2	R172 PNA mix	4	p.G171D	c.512G>A	86960
			p.R172G	c.514A>G	33731
			p.R172W	c.514A>T	34039
			p.R172K	c.515G>A	33733
			p.R172M	c.515G>T	33732
			p.R172S	c.516G>C	133672
			p.R172S	c.516G>T	34090

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

PRINCIPLE AND OVERVIE W

The PNAClamp™ IDH2 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.

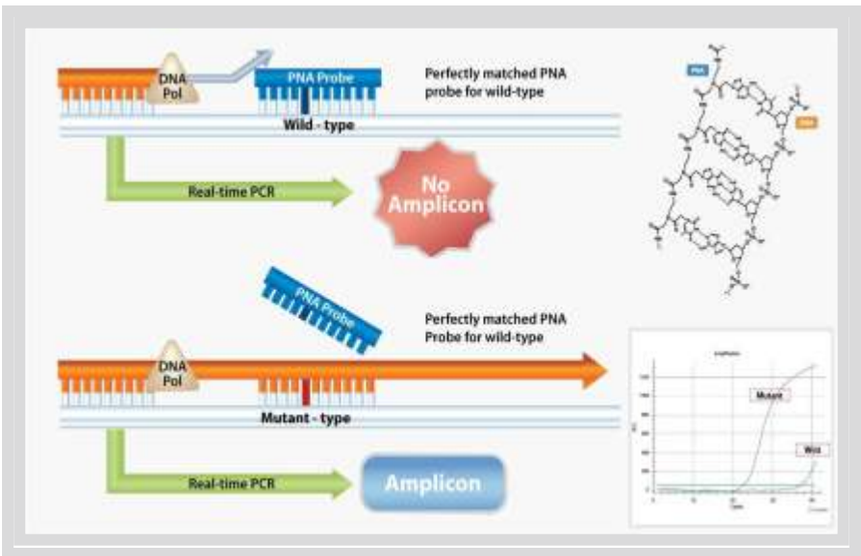


Figure 1. Principle of the PNAClamp™ IDH2 Mutation Detection Kit

The kit can rapidly detect IDH2 mutation (within 2 h) with high sensitivity even with a small amount of DNA (10 ng). The detection limit of the kit, when the mutated gene is mixed with wild-type background, is less than 1%.

WARNINGS AND PRECUATIONS

Please read the instruction carefully and become familiar with all components of the kit prior to use.

PNAClamp™ IDH2 Mutation Detection Kits 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 IDH2 PNA 2X premix at room temperature should be minimized for the optimal amplification.

Dissolve reagents completely and mix them thoroughly by vortex.

The IDH2 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™ IDH2 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™ IDH2 Mutation Detection Kit should be stored immediately upon receipt at -15°C to -20°C. 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°C to -20°C

No.	Name ofcomponent	Description	Volume	Cap label
1	Non PNA mix	Primers only	90 1	IDH2 1
2	R140 PNA mix	R140 PNA and primers	90 1	IDH2 2
3	R172 PNA mix	R172 PNA and primers	90 1	IDH2 3
4	IDH2 PNA 2X premix	PCR reaction premix	900 1	IDH2 2X premix
5	Clamping control	Wild-type DNA	200 1	IDH2 Control

* Each kit contains enough material to test 25 DNA samples for all mutations.

PROCEDURES

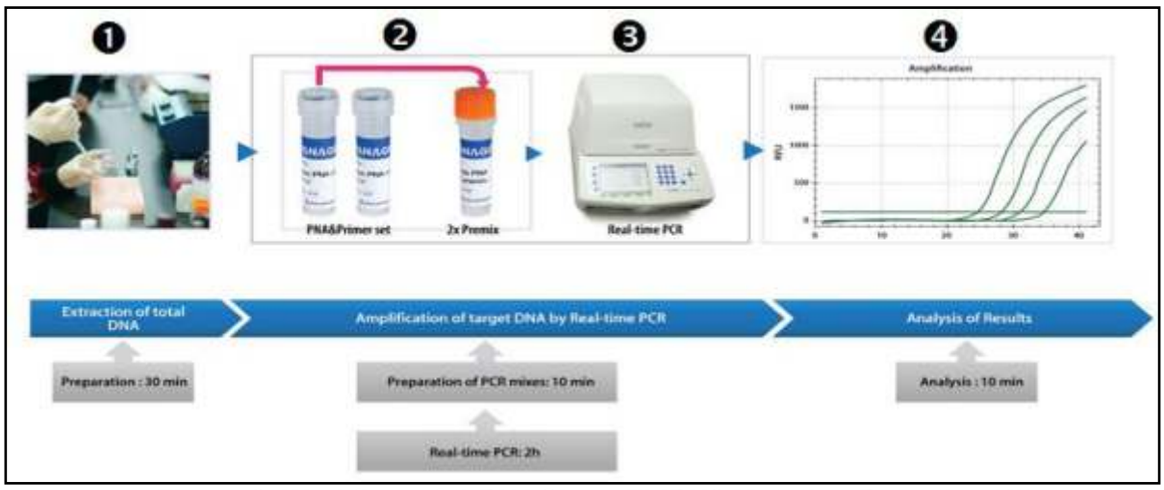


Figure 2. Workflow of the PNAClamp™ IDH2 Mutation Detection Kit

1. DNA preparation

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

1) Formalin-fixed paraffin -embedded samples of glioma tissue, glioma biopsies 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°C for up to 24 hours, or at -20°C for long term storage.

2. Preparation of the Real-Time PCR Mixture

Table 3. Set up reaction mixture per one reaction.

Components	Volume
IDH2 PNA 2X premix (#4)	10 µl
Each PNA mix (#1~#3)	3 µl
Extracted DNA (10 ng total) or Clamping control (#5)	7 µl
Total volume	20 µl

1) Prepare 3 PCR tubes for one set of DNA samples to be tested. Label them as S1, S2 and S3. Prepare another set of 3 tubes for Clamping control (wild-type DNA) and label them as C1, C2 and C3.
2) Add 10 µl of IDH2 PNA 2X premix (#4 from the kit) to each tube.
3) For each PCR tube, add 3 µl of corresponding PNA mix from #1~3 from the kit. For example, S1 and C1 tubes will have #1 Non PNA mix #1, S2 and C2 tubes will have #2 R140 PNA mix #2, and S3 and C3 tubes will have #3 R172 PNA mix #3.
4) For S1~S3 PCR tubes, add 7 µl of prepared DNA sample (10 ng total) to each tube to yield 20 µl final volume.
5) For C1~C3 PCR tubes, add 7 µl of Clamping control (#5 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°C	5 min
FOUR-STEP CYCLING (40 CYCLES)**		
Denaturation	94°C	30 sec
PNA clamping	70°C	20 sec
Annealing	63°C	30 sec
Extension*	72°C	30 sec

* Set up the detection for reading SYBR Green at 72°C.
** 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~C3) 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
⊖ Non PNA mix (C1)	23≤ X ≤ 29

Assay	Acceptable ΔCt-1* range
⊜ R140 PNA mix (C2)	< 2
⊛ R172 PNA mix (C3)	< 2

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

2) DNA samples

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

i. Ct value of Non PNA mix (S1) should be 23~34.

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	23< Ct <30	The amplification and the amount of DNA sample are optimal.
Acceptable	30≤ Ct <34	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 ≤23	Possibility of false positive is high. Repeat the PCR reaction with a lower amount of DNA.
	34≤ 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 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 7500	ABI 7900	ABI StepOne Plus	ABI Quant Studio 5	Rotor-Gene Q
R140 PNA mix	35	35.5	35	35	35.5	36	35
R172 PNA mix	35	35	35	35	35	35	35

(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 ≤9	IDH2 mutant
	9< ΔCt-2	Wild
0< ΔCt-1 <2	ΔCt-2 ≤4	IDH2 mutant
	4< ΔCt-2	Wild
ΔCt-1 ≤0	All value	Wild

(5) Assess the result along with the result for each IDH2 PNA mix as given in Table 8.

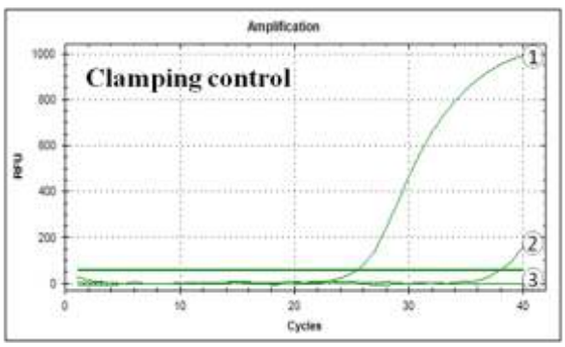
Table 8. Final assessment of the result

R140 PNA mix(S2)	R172 PNA mix(S3)	Results
Wild	Wild	Wild
Mutant	Wild	Codon 140 mutant
Wild	Mutant	Codon 172 mutant
Mutant	Mutant	Codon 140 and 172 mutant

EXAMPLES OF ANALYSIS

1. Using Bio-Rad CFX96

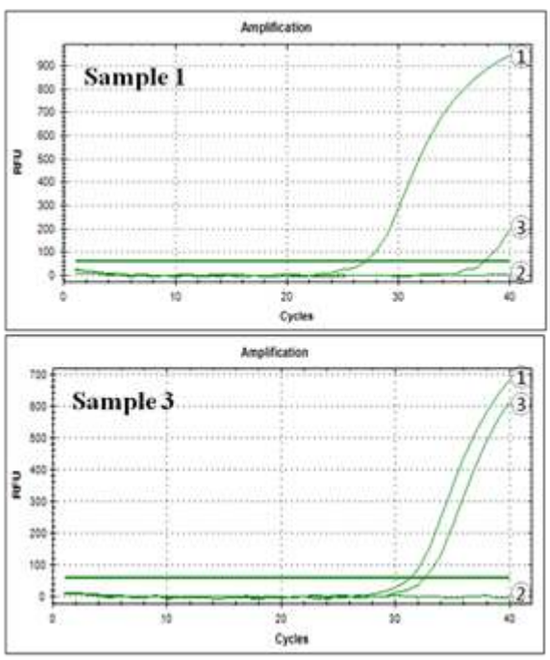
1) Profile of Clamping control

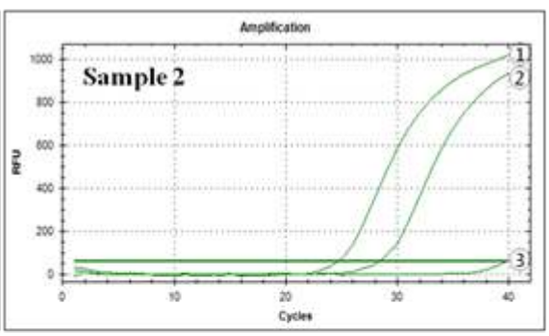


Assay	Clamping control Ct	Accep. range	Result
⊖ Non PNA mix (C1)	25.47	23≤ X ≤ 29	Acceptable

Assay	Clamping control Ct	ΔCt-1	Accep. ΔCt-1 range	Result
⊜ R140 PNA mix (C2)	38.01	-3.01	<2	Acceptable
⊛ R172 PNA mix (C2)	38.00	-4.00	<2	Acceptable

2) Profile of samples





⊖ Non PNA mix ⊜ R140 PNA mix ⊛ R172 PNA mix

Table 9. Example of sample Ct values

Assay \ Sample No	Sample 1 Ct	Sample 2 Ct	Sample 3 Ct	Standard Ct	**ΔCt-2	*ΔCt-1
⊖ Non PNA mix (S1)	27.08	24.66	31.29			
⊜ R140 PNA mix (S2)	38.00	28.38	38.00	35 (④)	⊜ - ⊖	④ - ⊜
⊛ R172 PNA mix (S3)	37.69	39.77	32.57	35 (⑤)	⊛ - ⊖	⑤ - ⊛

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

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

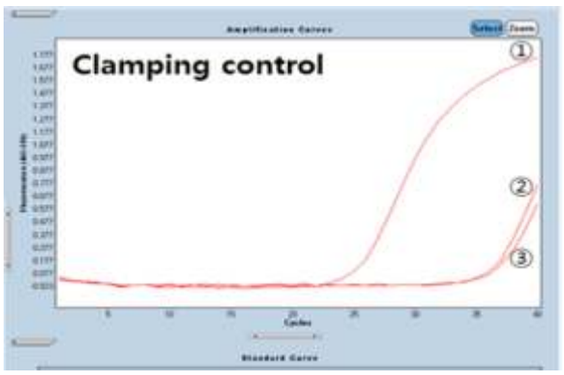
Table 10. Analysis of data

Assay \ Sample No.	Sample 1		Sample 2		Sample3	
	ΔCt-2	ΔCt-1	ΔCt-2	ΔCt-1	ΔCt-2	ΔCt-1
⊜ R140 PNA mix (S2)	10.92	-3.00	3.72	6.62	6.71	-3.00
⊛ R172 PNA mix (S3)	10.61	-2.69	15.11	-4.77	1.28	2.43
Results	Wild		R140 mutant		R172 mutant	

1. If ΔCt-1 is equal to or greater than 2 (▭),
   - ⊖ ΔCt-2 is greater than 9, the sample is assessed to be **wild**.
   - ⊜ ΔCt-2 is equal to or less than 9(▭), the sample is assessed to be **mutated**
2. If ΔCt-1 is greater than 0 and less than 2(▭),
   - ⊖ ΔCt-2 is greater than 4, the sample is assessed to be **wild**.
   - ⊜ ΔCt-2 is equal to or less than 4(▭), the sample is assessed to be **mutated**

2. UsingRoche LC480

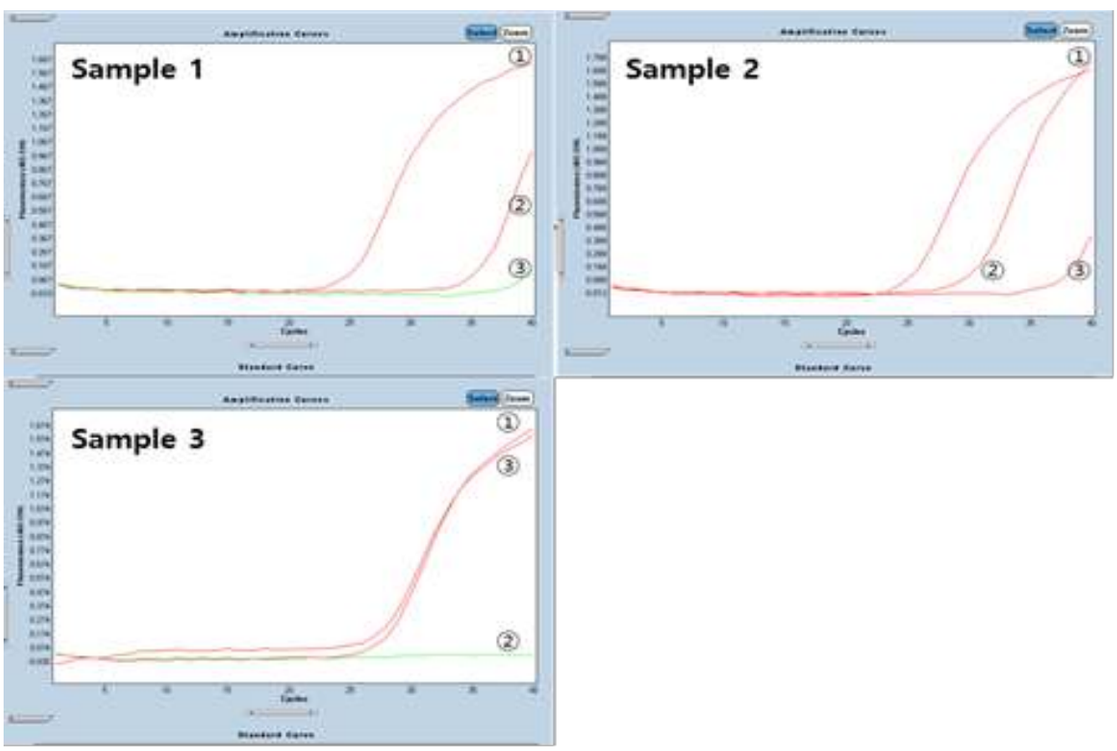
1) Profile ofClampingcontrol



Assay	Clamping control Ct	Accep. range	Result
⊖ Non PNA mix (C1)	26.21	23≤ X ≤ 29	Acceptable

Assay	Clamping control Ct	ΔCt-1	Accep. ΔCt-1 range	Result
⊜ R140 PNA mix (C2)	36.97	-1.47	<2	Acceptable
⊛ R172 PNA mix (C2)	38.50	-3.50	<2	Acceptable

2) Profileof samples



⊖ Non PNA mix ⊜ R140 PNA mix ⊛ R172 PNA mix

Table 11. Example of sample Ct values

Assay \ Sample No	Sample 1 Ct	Sample 2 Ct	Sample 3 Ct	Standard Ct	**ΔCt-2	*ΔCt-1
⊖ Non PNA mix (S1)	26.29	26.07	25.92			
⊜ R140 PNA mix (S2)	40.00	31.66	38.48	35.5(④)	⊜ - ⊖	④ - ⊜
⊛ R172 PNA mix (S3)	40.00	40.00	30.28	35(⑤)	⊛ - ⊖	⑤ - ⊖

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

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

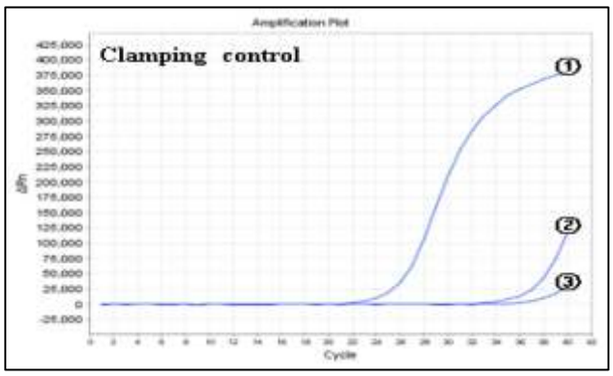
Table 12. Analysis of data

Assay \ Sample No.	Sample 1		Sample 2		Sample 3	
	ΔCt-2	ΔCt-1	ΔCt-2	ΔCt-1	ΔCt-2	ΔCt-1
⊜ R140 PNA mix (S2)	13.71	-4.50	5.59	3.84	12.56	-2.98
⊛ R172 PNA mix (S3)	13.71	-5.00	13.93	-5.00	4.36	4.72
Results	Wild		R140 mutant		R172 mutant	

1. If ΔCt-1 is equal to or greater than 2 (▭),
   - ⊖ ΔCt-2 is greater than 9, the sample is assessed to be **wild**.
   - ⊜ ΔCt-2 is equal to or less than 9(▭), the sample is assessed to be **mutated**
2. If △Ct-1 is greater than 0 and less than 2(▭),
   - ⊖ ΔCt-2 is greater than 4, the sample is assessed to be **wild**.
   - ⊜ ΔCt-2 is equal to or less than 4(▭), the sample is assessed to be **mutated**

3. UsingABI 7500

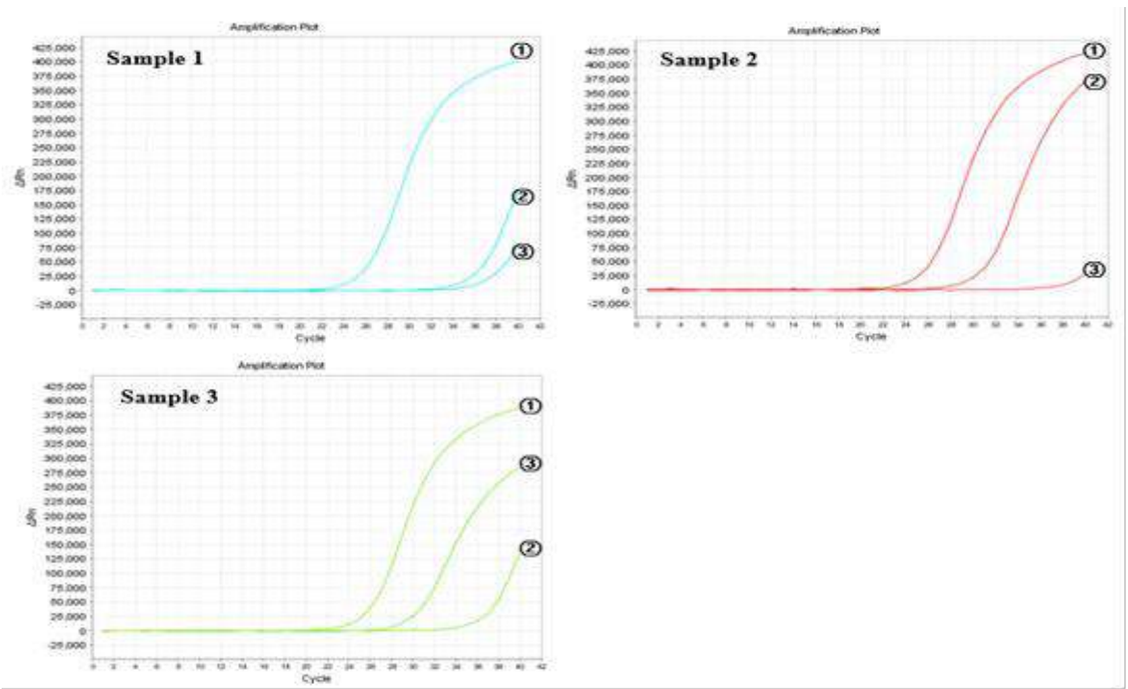
1) Profile ofClampingcontrol



Assay	Clamping control Ct	Accep. range	Result
⊖ Non PNA mix (C1)	25.99	23≤ X ≤ 29	Acceptable

Assay	Clamping control Ct	ΔCt-1	Accep. ΔCt-1 range	Result
⊜ R140 PNA mix (C2)	37.64	-2.64	<2	Acceptable
⊛ R172 PNA mix (C2)	38.00	-3.00	<2	Acceptable

2) Profileof samples



⊖ Non PNA mix ⊖ R140 PNA mix ⊛ R172 PNA mix

Table 13. Example of sample Ct values

Assay \ Sample No	Sample 1 Ct	Sample 2 Ct	Sample 3 Ct	Standard Ct	**ΔCt-2	*ΔCt-1
⊖ Non PNA mix (S1)	25.95	25.74	25.82			
⊜ R140 PNA mix (S2)	36.39	30.83	37.2	35(④)	⊜ - ⊖	④ - ⊜
⊛ R172 PNA mix (S3)	38.24	38.00	30.47	35(⑤)	⊛ - ⊖	⑤ - ⊖

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

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

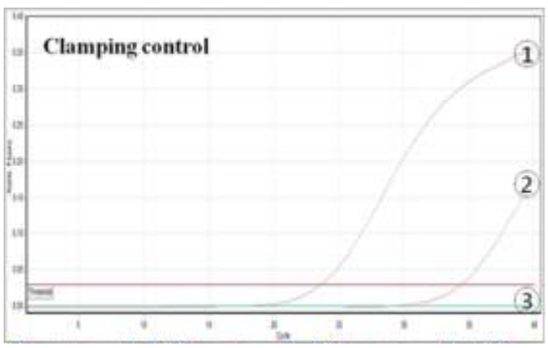
Table 14. Analysis of data

Assay \ Sample No.	Sample 1		Sample 2		Sample 3	
	ΔCt-2	ΔCt-1	ΔCt-2	ΔCt-1	ΔCt-2	ΔCt-1
⊜ R140 PNA mix (S2)	10.44	-1.39	5.09	4.17	11.38	-2.20
⊛ R172 PNA mix (S3)	12.29	-3.24	12.26	-3.00	4.65	4.53
Results	Wild		R140 mutant		R172 mutant	

1. If ΔCt-1 is equal to or greater than 2 (▭),
   - ⊖ ΔCt-2 is greater than 9, the sample is assessed to be **wild**.
   - ⊜ ΔCt-2 is equal to or less than 9(▭), the sample is assessed to be **mutated**
2. If ΔCt-1 is greater than 0 and less than 2(▭),
   - ⊖ ΔCt-2 is greater than 4, the sample is assessed to be **wild**.
   - ⊜ ΔCt-2 is equal to or less than 4(▭), the sample is assessed to be **mutated**

4. Using Rotor-Gene Q

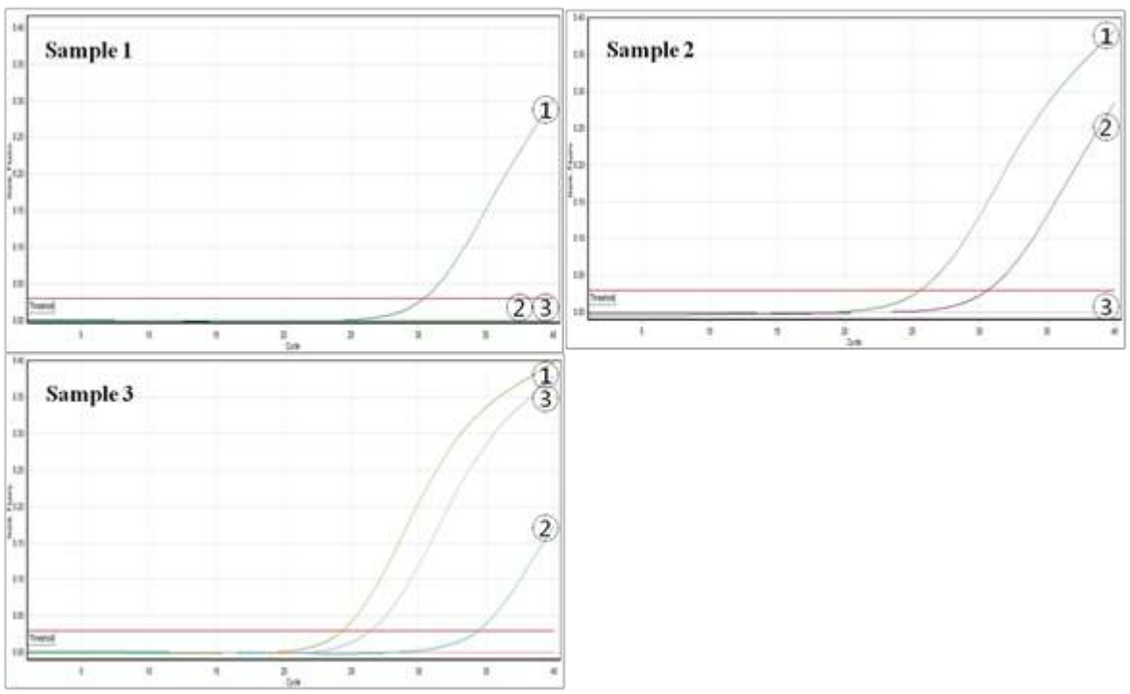
1) Profile of Clamping control



Assay	Clamping control Ct	Accep. range	Result
⊖ Non PNA mix (C1)	23.64	23≤ X ≤ 29	Acceptable

Assay	Clamping control Ct	ΔCt-1	Accep. ΔCt-1 range	Result
⊜ R140 PNA mix (C2)	34.43	-0.57	<2	Acceptable
⊛ R172 PNA mix (C2)	38.00	-3.00	<2	Acceptable

2) Profile of samples



⊖ Non PNA mix ⊜ R140 PNA mix ⊛ R172 PNA mix

Table 15. Example of sample Ct values

Assay \ Sample No	Sample 1 Ct	Sample 2 Ct	Sample 3 Ct	Standard Ct	**ΔCt-2	*ΔCt-1
⊖ Non PNA mix (S1)	30.44	25.63	24.35			
⊜ R140 PNA mix (S2)	38.00	30.57	34.41	35 (④)	⊜ - ⊖	④ - ⊜
⊛ R172 PNA mix (S3)	38.00	38.00	26.45	35 (⑤)	⊛ - ⊖	⑤ - ⊛

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

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

Table 16. Analysis of data

Assay \ Sample No.	Sample 1		Sample 2		Sample 3	
	ΔCt-2	ΔCt-1	ΔCt-2	ΔCt-1	ΔCt-2	ΔCt-1
⊜ R140 PNA mix (S2)	7.56	-3.00	4.94	4.43	10.06	0.59
⊛ R172 PNA mix (S3)	7.56	-3.00	12.37	-3.00	2.10	8.55
Results	Wild		R140 mutant		R172 mutant	

1. If ΔCt-1 is equal to or greater than 2 (▭),
   - ⊖ ΔCt-2 is greater than 9, the sample is assessed to be **wild**.
   - ⊜ ΔCt-2 is equal to or less than 9(▭), the sample is assessed to be **mutated**
2. If ΔCt-1 is greater than 0 and less than 2(▭),
   - ⊖ ΔCt-2 is greater than 4, the sample is assessed to be **wild**.
   - ⊜ ΔCt-2 is equal to or less than 4(▭), the sample is assessed to be **mutated**

QUALITY CONTROL

Each lot of **PNAClamp™ IDH2 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 IDH2 mutant clone DNA with the PNAClamp™ IDH2 Mutation Detection Kit.

The extracted DNA is measured as 10 ng. Each 10 ng DNAs were diluted to have 100, 5, 2 and 1% of different mutant ratio. Three tests were performed with these 4 conditions of DNAs for 3 different batches of the kit.

The results showed that 1% mutation was detected for all cases the mutant DNA 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 ng) extracted from wild-type cell line, HeLa. All the three tests showed wild-type locations. For the evaluation of the cross-reactivity by mutant location, the tests of IDH2 mutant cell line DNAs (10 ng) at 100, 5, 2 and 1% of different mutant ratio showed wild-type locations, except for each mutant location, and did not show cross-reactivity.

3. Reproducibility

Experiments were performed to evaluate the reproducibility of the standard IDH2 mutant clone DNA (10 ng) at 100, 5, 2 and 1% of different mutant ratio, for three batches, among three operators, and for five days. PNAClamp™ IDH2 Mutation Detection Kit had a correct call rate of 100%. All the results showed little variation, with CV ≤ 7%.

REFERENCES

1. Chotirat, S et al., Molecular alterations of isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) metabolic genes and additional genetic mutations in newly diagnosed acute myeloid leukemia patients. J Hematol Oncol. 5:5, 2012.
2. Yang, H et al., IDH1 and IDH2 mutations in tumorigenesis: mechanistic insights and clinical perspectives. Clin Cancer Res. 18 (20):5562-5571, 2012.
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4. Horbinski, C et al., Detection of IDH1 and IDH2 mutations by fluorescence melting curve analysis as a diagnostic tool for brain biopsies. J Mol Diagn. 12 (4):487-492, 2010.
5. Zou, Y et al., IDH1 and IDH2 mutations are frequent in Chinese patients with acute myeloid leukemia but rare in other types of hematological disorders. Biochem Biophys Res Commun. 402 (2):378-383, 2010.

EXPLANATION OF SYMBOL S 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

PANAGENE Inc.
54, Techno 10-ro, Yuseong-gu, Daejeon, 34027, Korea

EC REP **MT Promedt Consulting GmbH**
Altenhofstrasse 80, 66386 St. Ingbert, Germany