INTENDED USE

The PNAClamp™ TERT Mutation Detection Kit is an in vitro diagnostic reagent for detecting 2 different TERT promoter mutations of formalin-fixed paraffin-embedded (FFPE) tissue fresh, tissues and biopsy tissues from patients with cancer such as the central nervous system, bladder, thyroid, skin, etc.

This kit is an amplified DNA test for the qualitative detection using peptide nucleic acid (PNA) probes and Ct analysis in a real-time PCR (polymerase chain reaction) system.

Table 1. Target genes detected by this kit

No.	Reagent	Nucleotide change	Cosmic No.
1	Non_C250 PNA mix	c.1-146C>T	1716559
2	C250 PNA mix		
3	Non_C228 PNA mix	c.1-124C>T	1716558
4	C228 PNA mix		

* Cosmic Number is taken from 'The Catalogue of Somatic Mutations in Cancer'. (https://cancer.sanger.ac.uk/cosmic)

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

PRINCIPLE AND OVERVIEW

PNAClamp™ TERT 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.

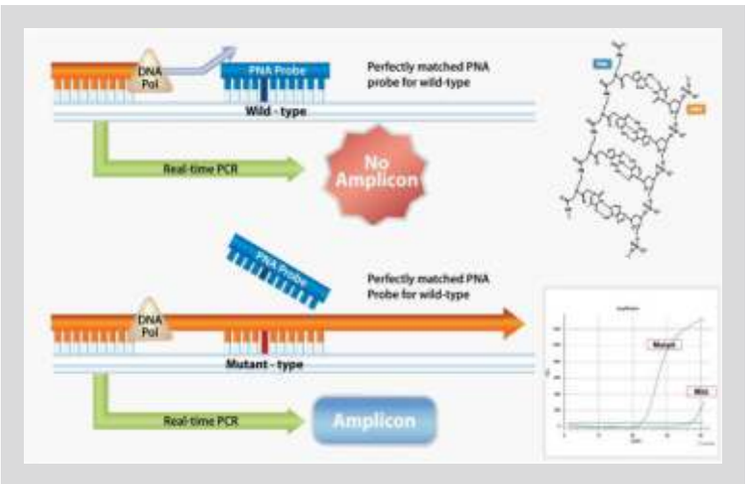


Figure 1. Principle of the PNAClamp™ TERT Mutation Detection Kit

The kit can rapidly detect TERT 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 2%.

EQUIPMENT AND MATERIALS SUPPLIED BY THE USER

- ✓ Reagents and equipment for DNA extraction
- ✓ Pipettes (capacity 10 µl, 20 µl, and 200 µl)
- ✓ Filter pipette tips
- ✓ Bench top microcentrifuge
- ✓ Vortex mixer
- ✓ Disposable gloves, powder-free

◆ **Only the following plastic consumables were validated. If you do not use the recommended plastic consumables, it may cause loss of performance and increase the chance of false result.**

Table 2. Compatible real time PCR instrument and plastic consumables

Company	Model	Consumables
Bio-Rad	CFX96	➢ Clear PCR plate (Bio-Rad, Catalog No. BRMLL-9601) ➢ Adhesive seals (Bio-Rad, Catalog No. MSB-1001)

WARNINGS AND PRECUATIONS

- ✓ **Please read carefully this instruction and become familiar with all components of the kit prior to use.**
- ✓ **PNAClamp™ TERT Mutation Detection Kit is for *in vitro* diagnostic use.**
- ✓ This kit should be used by trained laboratory professionals.
- ✓ All experiments should be performed under proper clean conditions in order to prevent contamination. It is recommended that a user has separate, dedicated pipettes and filter pipette tips to add DNA template and prepare PCR 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, please minimize exposure time of PCR reagents at room temperature for the optimal amplification.
- ✓ Dissolve reagents completely and mix them thoroughly by vortex.
- ✓ Tubes should be briefly centrifuged before use.
- ✓ Tubes containing PNA probe should be protected from prolonged exposure to light.
- ✓ Use only recommended instrument and consumables only. If not, it may cause loss of performance and increase the chance of false result.
- ✓ Additional validation testing by a user may necessary when non-recommended instrument is used.
- ✓ Do not use incorrect volume of reagent or target DNA; it may cause loss of performance and increase the chance of false result.
- ✓ Do not interchange or mix reagents from different lots or other manufacture's product.
- ✓ Do not re-use any remaining reagents after PCR amplification is completed**.**
- ✓ Do not use the reagents after their expiration date.

STORAGE CONDITION AND STABILITY

The PNAClamp™ TERT Mutation Detection Kit is shipped on ice packages and must still be frozen on arrival. If the kit is not frozen on arrival please contacts PANAGENE or the local distributor (see back cover).

The PNAClamp™ TERT Mutation Detection Kit should be stored immediately upon receipt below -20℃. When stored under the recommended storage conditions, the kit is stable until the labeled expiration date.

After open the kit, reagents can be stored in their original packaging below -20℃ for 90 days or until the expiration date, whichever comes first.

KIT CONTENTS

◆ A total of 25 samples can be tested using a kit.

Table 3. Reagents provided in the PNAClamp™ TERT Mutation Detection Kit

No.	Name of content	Description	Volume	Label & color of cap
1	Non_C250 PNA mix	Primers only	400 µl	**Non_C250**
2	C250 PNA mix	C250 PNA and primers	400 µl	**C250**
3	Non_C228 PNA mix	Primers only	400 µl	**Non_C228**
4	C228 PNA mix	C228 PNA and primers	400 µl	**C228**
5	Clamping control	Negative Control	200 µl	**CC**
6	Positive control	Positive Control	200 µl	**PC**

PROCEDURES

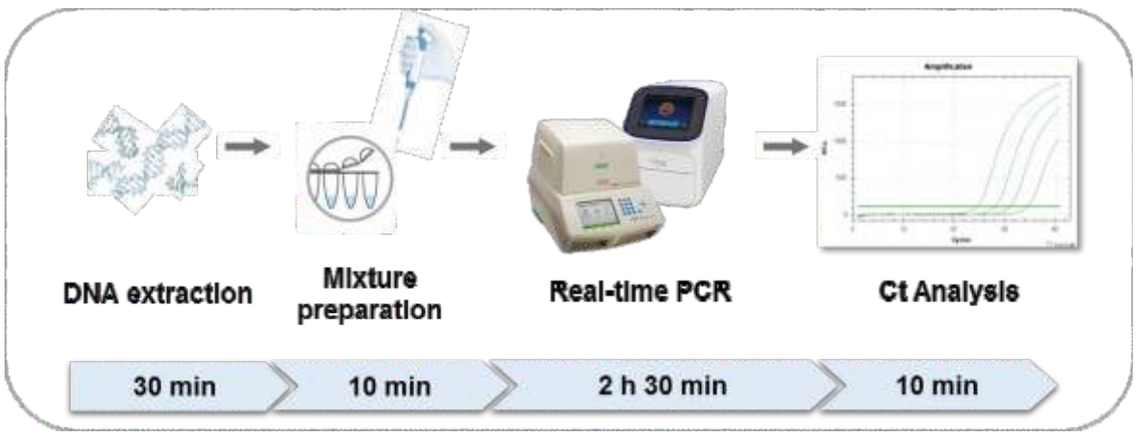


Figure 2. Workflow of the PNAClamp™ TERT Mutation Detection Kit

1. Sample Preparation and Storage

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 (FFPE) tissues, fresh tissues and biopsy tissues can be used as specimens.
2) Specimen transport: Use standard pathology methods to ensure specimen quality.
3) For DNA extraction, it is recommended to use the following DNA extraction kits in Table 4.

Table 4. The list of recommended DNA isolation kit

Type	Model	Company
FFPE tissue	QIAamp DNA FFPE Tissue Kit	QIAGEN (Germany)
	Maxwell® 16 FFPE LEV DNA Purification Kit	Promega Corporation (USA)
	High Pure PCR Template Preparation Kit	Roche Diagnostics GmbH (Germany)
Fresh tissues and biopsy tissues	QIAamp DNA Mini Kit	QIAGEN (Germany)

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

2. Preparation of the Real-Time PCR Mixture

Table 5. Set up reaction mixture per one reaction

Reagent	Volume*
Each PNA mix (#1~#4)	15 µl
Extracted DNA, Clamping control (CC), or Positive control (PC)	5 µl
Total volume	20 µl

* Prepare one extra volume for each component to compensate pipette error.

1) Prepare each PNA mix (#1 ~ #4) after thaw, vortex and spin down at room temperature.
2) Prepare test sample (extracted DNA) and control samples (CC and PC).
3) Prepare PCR plate. Label them as A1 if it is necessary.
4) Load 15 µl of each PNA mix (#1 ~ #4) into PCR plate.
   For example, A1 well will contain Non_C250 PNA mix, A2 well will contain C250 PNA mix, A3 well will contain Non_C228 PNA mix, and A4 well will contain C228 PNA mix.
5) Add 5 µl of prepared test sample into each well of PCR plate to yield a total 20 µl of final volume.
6) One set of CC and PC for each PNA mix should be included in each run. Add 5 µl of CC or PC into each well of PCR plate to yield a total 20 µl of final volume.
7) Immediately seal the PCR plate tightly and spin down. Otherwise, the PCR mixture can be evaporated and the result of the test may not accurate.

3. Real-time PCR reaction

1) Place the prepared the PCR plate on the block of a real-time PCR instrument.
2) Please set the PCR protocol according to following Table 6.

Table 6. Real-time PCR protocol for PNAClamp™ TERT Mutation Detection Kit

ONE CYCLE		
Pre-denaturation	95℃	15 min
FOUR-STEP CYCLING (40 CYCLES)		
Denaturation	95℃	30 sec
PNA clamping	80℃	20 sec
	76℃	20 sec
	72℃	20 sec
Annealing	68℃	30 sec
Extension*	72℃	30 sec

3) Select the fluorescent dye (SYBR Green) for all reaction wells (*).

4. PCR result and data analysis

1) Set the baseline threshold as 1/20 of the maximum RFU for CFX96.
2) Calculate ΔCt-1 values and ΔCt-2 values for each sample. If Ct values are displayed as N/A (not applicable), then set Ct value as 40 for further calculation.

*ΔCt-1 = [Standard Ct] - [PNA mix Ct], Standard Ct is 33 for C250 and 35 for C228.

**ΔCt-2 = [PNA mix Ct] - [Non_PNA mix Ct]

Assess the results 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	
		Non_C250 PNA mix & C250 PNA mix	Non_C228 PNA mix & C228 PNA mix
2≤ ΔCt-1	ΔCt-2 ≤8	C250 mutant	C228 mutant
	8< ΔCt-2	Wild	Wild
0< ΔCt-1 <2	ΔCt-2 ≤6	C250 mutant	C228 mutant
	6< ΔCt-2	Wild	Wild
ΔCt-1 ≤0	All value	Wild	Wild

3) Ct value of Non_PNA mix 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 as shown in Table 8.

Table 8. Acceptability of Samples

Ct value for Non_PNA mix	Acceptability		Descriptions and recommendations
22< Ct <30	Valid	Optimal	The amplification and the amount of DNA sample are optimal
30≤ Ct <35		Acceptable	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.
Ct ≤22	Invalid		Possibility of false positive is high. Repeat the PCR reaction with a lower amount of DNA.
Ct ≥35			The amplification was failed. Check DNA amount and purity. New DNA prep might be required.

4) The Ct for Clamping control (CC) and Positive control (PC) must fall into the ranges that given in Table 7 and Table 8. The assay must be repeated if the values are not in these recommended ranges.

Table 9. Acceptance criteria of CC and PC

Reagent	Clamping control	Positive control
Non_C250 PNA mix	Wild	C250 mutant
C250 PNA mix		
Non_C228 PNA mix	Wild	C228 mutant
C228 PNA mix		

5) Interpretation of results

Test results are interpreted as shown in Table 10.

Table 10. Interpretation of results

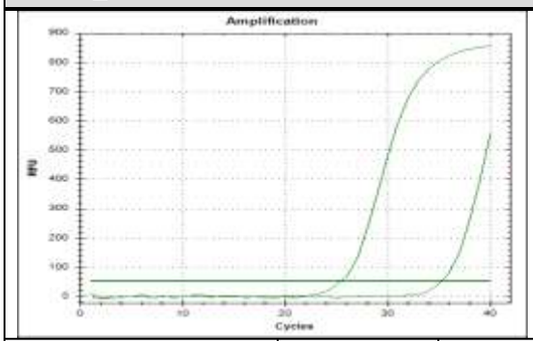
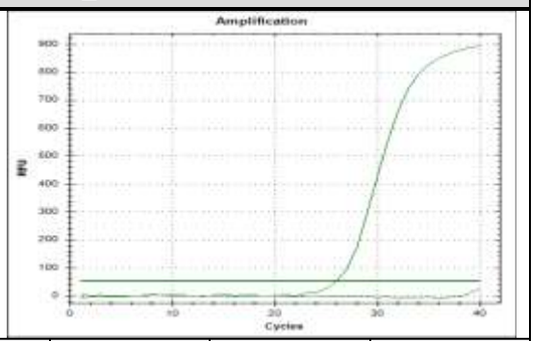
Non_C250 PNA mix & C250 PNA mix	**Non_C228 PNA mix & C228 PNA mix**	**Interpretation**
C250 mutant	C228 mutant	C250 and C228 mutant
C250 mutant	Wild	C250 mutant
C250 mutant	Invalid	Invalid*
Wild	C228 mutant	C228 mutant
Invalid	C228 mutant	Invalid*
Wild	Wild	Wild
Wild	Invalid	Invalid*
Invalid	Wild	Invalid*
Invalid	Invalid	Invalid*

* If the sample is invalid, please refer to the description and recommendations for the sample as given in Table 8.

EXAMPLES OF ANALYSIS

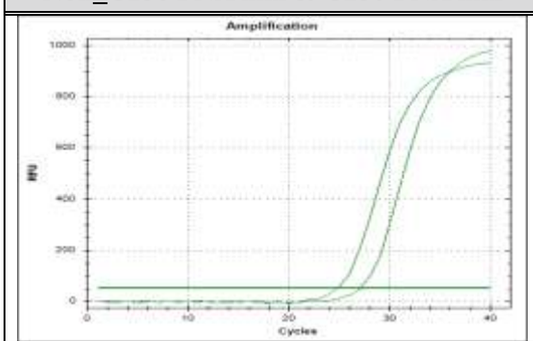
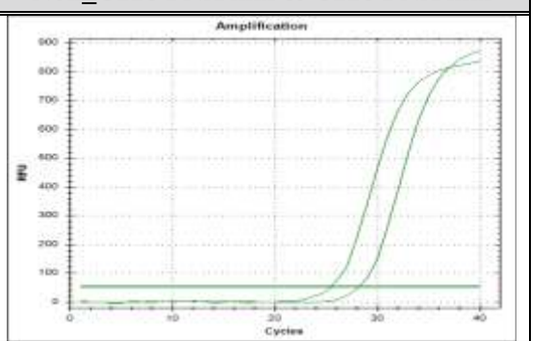
1. Using Bio-Rad CFX96

1) Clamping control (CC)

Non_C250 PNA mix & C250 PNA mix	Non_C228 PNA mix & C228 PNA mix
	

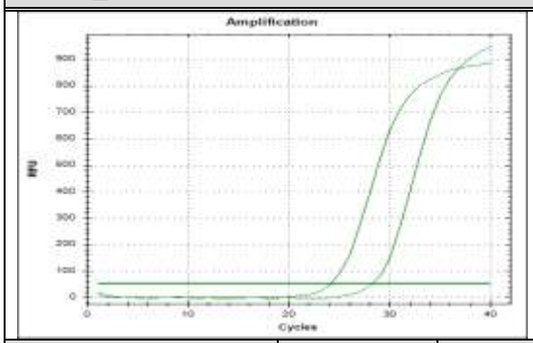
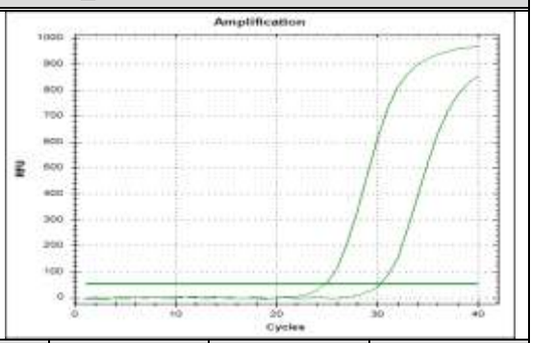
Reagent	Ct	ΔCt-1	ΔCt-2	Assessment	Result
Non_C250 PNA mix	25.46	-2.24	9.78	Wild	Acceptable
C250 PNA mix	35.24				
Non_C228 PNA mix	26.01	-5.00	13.99	Wild	
C228 PNA mix	N/A				

2) Positive control (PC)

Non_C250 PNA mix & C250 PNA mix	Non_C228 PNA mix & C228 PNA mix
	

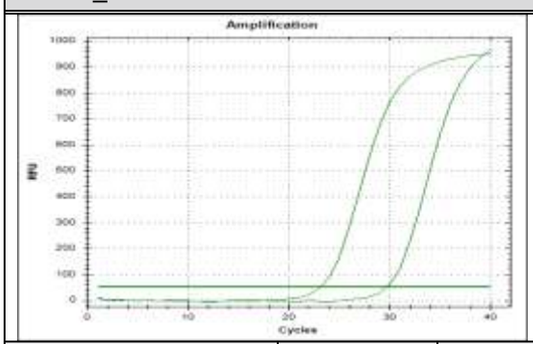
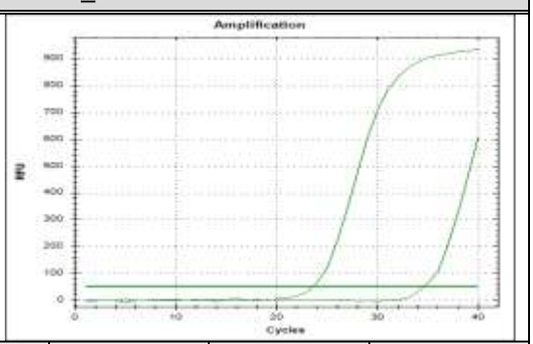
Reagent	Ct	ΔCt-1	ΔCt-2	Assessment	Result
Non_C250 PNA mix	24.82	5.89	2.29	C250 mutant	Acceptable
C250 PNA mix	27.11				
Non_C228 PNA mix	25.46	6.80	2.74	C228 mutant	
C228 PNA mix	28.20				

3) Sample 1

Non_C250 PNA mix & C250 PNA mix	Non_C228 PNA mix & C228 PNA mix
	

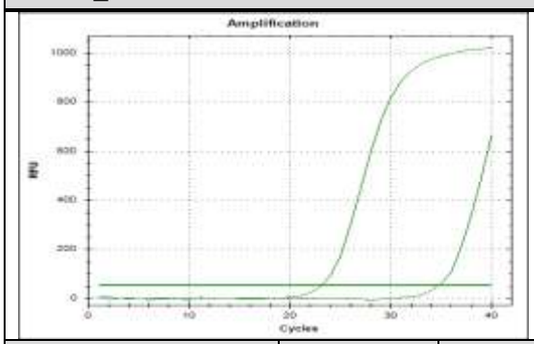
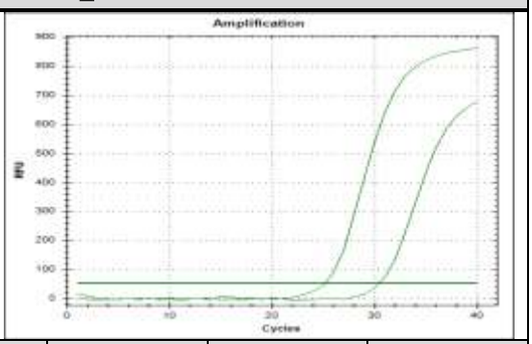
Reagent	Ct	ΔCt-1	ΔCt-2	Assessment	Result
Non_C250 PNA mix	24.18	4.75	4.07	C250 mutant	**C250 and C228 mutant**
C250 PNA mix	28.25				
Non_C228 PNA mix	24.80	4.71	5.49	C228 mutant	
C228 PNA mix	30.29				

4) Sample 2

Non_C250 PNA mix & C250 PNA mix	Non_C228 PNA mix & C228 PNA mix
	

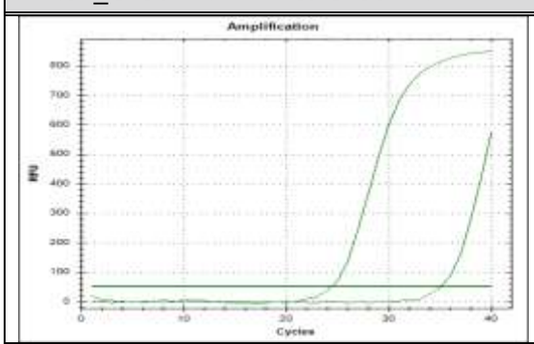
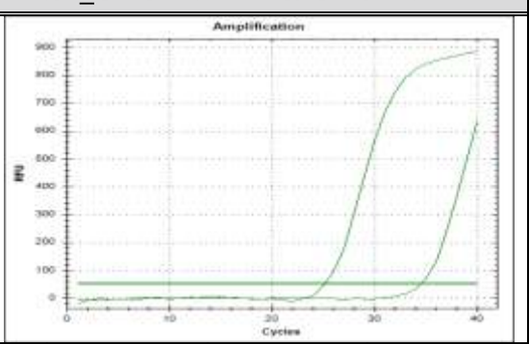
Reagent	Ct	ΔCt-1	ΔCt-2	Assessment	Result
Non_C250 PNA mix	23.12	3.33	6.55	C250 mutant	**C250 mutant**
C250 PNA mix	29.67				
Non_C228 PNA mix	23.65	1.68	11.03	Wild	
C228 PNA mix	34.68				

5) Sample 3

Non_C250 PNA mix & C250 PNA mix	Non_C228 PNA mix & C228 PNA mix
	

Reagent	Ct	ΔCt-1	ΔCt-2	Assessment	Result
Non_C250 PNA mix	23.12	-1.72	11.60	Wild	**C228 mutant**
C250 PNA mix	34.72				
Non_C228 PNA mix	25.14	4.58	5.28	C228 mutant	
C228 PNA mix	30.42				

6) Sample 4

Non_C250 PNA mix & C250 PNA mix	Non_C228 PNA mix & C228 PNA mix
	

Reagent	Ct	ΔCt-1	ΔCt-2	Assessment	Result
Non_C250 PNA mix	24.44	-2.14	10.70	Wild	**Wild**
C250 PNA mix	35.14				
Non_C228 PNA mix	25.16	1.54	9.38	Wild	
C228 PNA mix	34.54				

QUALITY CONTROL

Each lot of PNAClamp™ TERT 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 TERT promoter mutant samples with the PNAClamp™ TERT Mutation Detection Kit.

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

The results showed that 2% mutation was detected for all cases the mutant DNA samples 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 lines. All the three tests showed wild-type locations, and there was no cross reactivity.

3. Reproducibility

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

REFERENCES

1. Park, CK et al., Expression level of hTERT is regulated by somatic mutation and common single nucleotide polymorphism at promoter region in glioblastoma. Oncotarget, 5 (10):3399-3407, 2014.
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3. Liu, X et al., Highly prevalent TERT promoter mutations in aggressive thyroid cancers. Endocr Relat Cancer, 20 (4): 603-610, 2013.
4. Heidenreich, B et al., Telomerase reverse transcriptase promoter mutations in primary cutaneous melanoma. Nat Commun, 5:3401, 2014.
5. Melo, M et al., TERT promoter mutations are a major indicator of poor outcome in differentiated thyroid carcinomas. J Clin Endocrinol Metab.99 (5):E754-E765, 2014.

EXPLANATION OF SYMBOLS ON THE LABEL

IVD	*In Vitro* Diagnostic Medical Device		Manufacturer
LOT	Batch code	Σ	Contains Sufficient for <*n*> tests
REF	Catalogue number		Upper limit of storage temperature
EC REP	Authorized European representative		Use by
	Consult instructions for use	CE	This product fulfills the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices.

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

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