AmoyDx® *TERT*/*HRAS* Mutations Detection Kit

Instructions for Use

For Research Use Only

REF	8.01.0087	24 tests/kit	For ABI7500, LightCycler480 II
REF	8.01.0088	24 tests/kit	For Stratagene Mx3000P™, SLAN-96S

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Background

Telomerase reverse transcriptase (*TERT*) is a gene that encodes an enzymatic protein that possesses reverse transcriptase activity. *TERT* gene promoter mutations lead to constitutive activation and expression, which resulting in replication and proliferation of cancer cells. Mutations in the TERT promoter are found in multiple cancers, approximately 70% of melanomas, 80–90% of glioblastoma multiforme, and up to 30% of thyroid cancers. In papillary thyroid carcinomas, the co-presence of mutations in the *TERT* promoter region and *BRAF* are associated with significantly more aggressive disease and shorter survival.

The *RAS* proto-oncogenes (*HRAS*, *KRAS* and *NRAS*) encode a family of GDP/GTP-regulated switches that convey extracellular signals to regulate the growth and survival properties of cells. GTP-bound RAS transmits its signal through downstream EGFR signaling pathways, for example the RAF→MEK→ERK and PI3K→AKT cascades. *HRAS* mutations are found in a wide variety of solid tumors, approximately 4% of thyroid cancer.

Intended Use

The AmoyDx® *TERT/HRAS* Mutations Detection Kit is a real-time PCR assay for qualitative detection of two *TERT* promoter mutations and six *HRAS* mutations in human genomic DNA extracted from tumor tissue. The kit is intended to be used to assess *TERT/HRAS* mutation status in thyroid cancer patients.

The kit is for research use only, and intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

The kit comprises specific primers and fluorescent probes to detect gene mutations in real-time PCR assay. 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.

This kit is composed of three reaction mixes (TH Reaction Mix 1~2 and TH External Control Reaction Mix), sufficient Enzyme Mix and Positive Control.

1) The **TH Reaction Mix 1~2** includes a mutation detection system and an internal control system. The mutation detection system includes primers and FAM-labeled probes specific for designated *TERT/HRAS* mutations, which is used to detect the *TERT/HRAS* mutation status. The internal control system contains primers and HEX-labeled probe for a conserved region of genomic DNA, which is used to detect the presence of inhibitors and confirm the validity of each experiment.
2) The **TH External Control Reaction Mix** contains primers and FAM-labeled probe for a conserved region of genomic DNA, which is used to assess the quality of DNA.
3) The **TH Positive Control** contains a recombinant gene with *TERT* and *HRAS* mutations.
4) The **TH Enzyme Mix** contains the Taq DNA polymerase for PCR amplification and uracil-N-glycosylase which works at room temperature to prevent PCR amplicon carryover contamination.

Kit Contents

This kit contains the following materials:

Table 1 Kit Contents

Tube No.	Content	Main Ingredients	Quantity	Fluorescent Signal
①	**TH Reaction Mix 1**	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM, HEX/VIC
②	**TH Reaction Mix 2**	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM, HEX/VIC
③	**TH External Control Reaction Mix**	Primers, Probes, Mg^{2+}, dNTPs	1100 μL/tube ×1	FAM, HEX/VIC
/	**TH Enzyme Mix**	Taq DNA Polymerase, Uracil-N-Glycosylase	35 μL/tube ×1	/
/	**TH Positive Control**	Plasmid DNA, wild-type genomic DNA	250 μL/tube ×1	/

Storage and Stability

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

The shelf-life of the kit is twelve months. The maximal number of freeze-thaw cycle is five.

Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments:
   ABI7500, LightCycler480 II, Stratagene Mx3000P™, or SLAN-96S.
2) DNA extraction kit. We recommend use of AmoyDx® FFPE DNA Kit for FFPE tissues, AmoyDx® Tissue DNA kit for fresh or frozen tumor tissue.
3) Spectrophotometer for measuring DNA concentration.
4) Mini centrifuge with rotor for centrifuge tubes.
5) Mini centrifuge with rotor for PCR tubes.
6) Vortexer.
7) Nuclease-free centrifuge tubes.
8) Nuclease-free PCR tubs and caps.
9) Adjustable pipettors and filtered pipette tips for handling DNA.
10) Tube racks.
11) Disposable powder-free gloves.
12) Sterile, nuclease-free water.
13) 1×TE buffer (pH 8.0).

Precautions and Handling Requirements

Precautions

- Please read the instruction carefully and become familiar with all components of the kit prior to use. 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.
- As all the chemicals have potential hazard, only trained professionals can use this kit. Please wear suitable lab coat and disposable gloves while handling the reagents.
- Avoid contact of the skin, eyes, and mucous membranes 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 results.
- 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.
- Use 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.

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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 40 μL
- For ABI7500, please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.
- For LightCycler480 II instrument, please conduct fluorescence calibration prior to use if there is fluorescence crossover. To run the assays on a LightCycler machine, please use the Roche 480 adaptor, available from BIOplastics, Cat No. B79480.
- For Stratagene Mx3000P™, if there's low net fluorescence signal (dR) but high background signal (R), please reduce the signal gain setting of instrument properly.
- For SLAN-96S, please set up as follows: Probe mode: FAM, VIC. During the result analysis, open the "Preference" window, in "Chart Options" section, select "Selected Wells" for "Y-Axis Scaling Auto-adjust By" and "Absolute Fluorescence Value Normalization" for "Amplification Curve".
- Refer to the operation manual of the real-time PCR instrument for detailed instructions.
- We recommend that for all PCR instruments in use a fluorescence calibration should be conducted once a year.

Assay Procedure

1. DNA Extraction

The specimen material must be human genomic DNA extracted from tumor tissue. 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 *TERT*/*HRAS* mutations.

The OD_{260}/OD_{280} value of extracted DNA should be between 1.5 ~ 2.2 (measured using the spectrophotometer, the NanoDrop 1000 /2000 spectrophotometer is recommended).

For fresh, frozen tissue, the recommended DNA amount in each reaction mix is 1.88 ~ 4.7 ng (concentration 0.4~1 ng/μL).

For FFPE tissue, the amount of extracted DNA for PCR amplification differs according to different storage time (see Table 2).

Table 2 Recommend FFPE DNA concentration

Tissue	Storage time	DNA concentration	DNA amount per reaction
FFPE tissue	≤ 3 months	1.5 ng/μL	7.05 ng
	> 3 months & ≤ 1 year	2 ng/μL	9.4 ng
	> 1 year & ≤ 3 years	2~3 ng/μL	9.4~14.1 ng

Note:

- *The FFPE tissue should be handled and stored properly, and the storage time should preferably be less than 3 years.*
- *The extracted DNA should be used immediately, if not, it should be stored at -20±5℃ for no more than 6 months.*
- *Before detection, dilute the extracted tissue DNA with 1×TE buffer (pH 8.0) to proper concentration. We recommend using at least 5 μL DNA for 10 times dilution, to ensure the accuracy of final concentration.*

2. Mutation Detection

1) Thaw **TH Reaction Mix 1~2**, **TH External Control Reaction Mix** and **TH Positive Control** at room temperature. When the reagents are completely thawed, mix each reagent thoroughly by vortexing and centrifuge for 5~10 seconds to collect all liquid at the bottom of the tube.
2) Centrifuge **TH Enzyme Mix** for 5~10 seconds prior to use.

3) Prepare sufficient TH Master Mix 1~3 containing TH Enzyme Mix and each TH Reaction Mix (TH Reaction Mix 1~2 or TH External Control Reaction Mix, respectively) in separate sterile centrifuge tube according to the ratio in Table 3. Mix TH Master Mix thoroughly by vortexing, and centrifuge for 5~10 seconds.

Table 3 TH Master Mix

Content	Volume per test
Reaction Mix	35 μL
TH Enzyme Mix	0.3 μL
Total volume	**35.3 μL**

Note:

- *Every PCR run must contain one PC and one NTC (No template 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.*

4) Take out the sample DNA (see Table 2 for DNA concentration) and nuclease-free water for NTC.

5) Prepare three PCR tubes for NTC: Dispense 35.3 μL of TH Master Mix 1~3 to each PCR tube respectively. Then add 4.7 μL of nuclease-free water to each PCR tube, and cap the PCR tubes.

6) Prepare three PCR tubes for each sample: Dispense 35.3 μL of TH Master Mix 1~3 to each PCR tube respectively. Then add 4.7 μL of sample DNA to each PCR tube, and cap the PCR tubes.

7) Prepare three PCR tubes for PC: Dispense 35.3 μL of TH Master Mix 1~3 to each PCR tube respectively. Then add 4.7 μL of TH Positive Control to each PCR tube, and cap the PCR tubes.

8) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.

9) Place the PCR tubes into the real-time PCR instrument. A recommended plate layout is shown in Table 4.

Table 4 Recommended Plate Layout

Tube	①	②	③	①	②	③
A	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9
B	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10
C	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11
D	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12
E	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13
F	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14
G	Sample 7	Sample 7	Sample 7	PC	PC	PC
H	Sample 8	Sample 8	Sample 8	NTC	NTC	NTC

10) Setup the PCR Protocol using the cycling parameters in Table 5.

Table 5 Cycling Parameters

Stage	Cycles	Temperature	Time	Data collection
1	1	95℃	5 min	/
2	15	95℃	25 s	/
		64℃	20 s	/
		72℃	20 s	/
3	31	93℃	25 s	/
		60℃	35 s	FAM and HEX/VIC
		72℃	20 s	/

11) Start the PCR run immediately.

12) When the PCR run is finished, analyze the data according to the "Results Interpretation" procedures.

3. Results Interpretation

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

1) For NTC: The FAM Ct values of Tubes ①~② should be ≥31. If not, the data is *INVALID*. The sample should be retested.

2) For Positive Control: The FAM Ct values of Tubes ①~③ and HEX/VIC Ct values of Tubes ①~② should be < 20. If not, the data is *INVALID*. The sample should be retested.

3) For the external control assay in Tube ③ for each sample:

   a) For FFPE tissue sample, the external control FAM Ct value should be between 15~21. For fresh or frozen tissue sample, the external control FAM Ct value should be between 13~19.

   b) If the external control FAM Ct value is <15 (for FFPE tissue sample) or <13 (for fresh or frozen tissue sample), this indicates the DNA is overloaded. The DNA amount should be reduced and retested. But if the FAM Ct values of Tubes ①~② are in Negative Ct range (see Table 6), the sample is determined as negative.

   c) If the external control FAM Ct value is >21 (for FFPE tissue sample) or > 19 (for fresh or frozen tissue sample), this indicates the DNA degradation or the presence of PCR inhibitors, or any error in experimental operation. The sample should be retested with increased or re-extracted DNA. But if any FAM Ct value of tubes ①~② is < 26, the sample is determined as positive.

4) For the internal control assay in Tubes ①~② for each sample: The HEX/VIC Ct values of Tubes ①~② should be < 31. If not, check the FAM signals of Tubes ①~②:

   a) If mutant FAM Ct value is < 31, continue with the analysis.

   b) If mutant FAM Ct value is ≥ 31, the data is *INVALID*. The sample should be retested.

Analyze the mutation assay for each sample:

5) Record the mutant FAM Ct values of Tubes ①~② for each sample.

6) Check the mutant FAM Ct values of Tubes ①~② according to Table 6:

Table 6 Results Determination

Tube No.	①	②	Results
Optimal Ct range	Ct <26	Ct <26	Positive.
Acceptable Ct range	26≤Ct <28	26≤Ct <29	Interpret the results according to the ΔCt value.
Cut-off ΔCt value	9	9	
Negative Ct range	Ct≥28	Ct ≥29	Negative or under the LOD*.

* LOD: limit of detection

   a) If any FAM Ct value of Tube ①~② is < 26, the sample is determined as positive (Mutation detected).

   b) If any FAM Ct value of Tube ①~② is in Acceptable Ct range, calculate the ΔCt value for each mutation showing positive amplification.

      i. **ΔCt value = Mutant FAM Ct value – External control FAM Ct value**.

      ii. If the ΔCt value is less than the corresponding cut-off ΔCt value, the sample is determined as positive (Mutation detected).

      iii. If the ΔCt value is equal or more than the corresponding cut-off ΔCt value, the sample is determined as negative (No mutation detected) or under the LOD of the kit.

   c) If all the FAM Ct values of Tubes ①~② are in Negative Ct range or there is no amplification, the sample is determined as negative (No mutation detected) or under the LOD of the kit.

Limitations

1) The kit is to be used only by personnel specially trained with PCR techniques.

2) The results can be used to assist clinical diagnosis, combining with other clinical and laboratory findings.

3) The kit has been validated for use with FFPE tumor tissue DNA.

4) The kit can only detect the two *TERT* mutations and six *HRAS* mutations listed in the appendix.

5) Reliable results are dependent on proper sample processing, transport, and storage.

6) The sample containing degraded DNA may affect the ability of the test to detect *TERT/HRAS* mutation.

7) Samples with negative result (No mutation detected) may harbor *TERT/HRAS* mutations not detected by this assay.

Symbols

	Manufacturer		Catalogue Number
	Batch Code		Use-by Date
	Contains Sufficient for <n> Tests		Temperature Limit
	Consult Instructions For Use		Keep Dry
	This Way Up		Fragile, Handle With Care
	Keep Away from Sunlight		

Appendix

***TERT/HRAS* Mutations Detected by the Kit**

Tube No.	Reagent Supplied	Region	Mutation	Base Change	Cosmic ID	Name
①	TH Reaction Mix 1	*TERT* Promoter region	C228T	1-124C>T	1716558	TERT-M1
			C250T	1-146C>T	1716559	TERT-M2
②	TH Reaction Mix 2	*HRAS* Exon 3	Q61R	182A>G	499	HRAS-M5
			Q61K	181C>A	496	HRAS-M6
			Q61P	182A>C	500	HRAS-M7
			Q61L	182A>T	498	HRAS-M8
			Q61H	183G>C	503	HRAS-M9
			Q61H	183G>T	502	HRAS-M10