



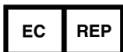
AmoyDx® *KRAS/NRAS/BRAF* Mutations Detection Kit

Instructions for Use

REF	8.01.0033	6 tests/kit	For Stratagene Mx3000P™, ABI7500
REF	8.01.0034	6 tests/kit	For LightCycler480
REF	8.01.0035	6 tests/kit	For SLAN-96S



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Background

KRAS, *NRAS* and *BRAF* gene are the key molecules in the downstream signaling pathway of epidermal growth factor receptor (EGFR), which transduce signals from membrane-bound receptors via multiple downstream effector pathways and thereby affect fundamental cellular processes, including proliferation, apoptosis, and differentiation. In total, activating *KRAS* and *NRAS* mutations occur in 20~50% and 1~6% of colorectal cancers respectively, mainly in exons 2, 3 or 4. And the most common *BRAF* mutations are V600 mutations in exon 15, which occur in 8~15% of colorectal cancers.

Studies have shown the mutation status of the *KRAS*, *NRAS* and *BRAF* gene is relevant to the primary drug resistance of colorectal cancers treated with anti-EGFR monoclonal antibodies. Patients with wild-type *KRAS* and *NRAS* gene could benefit from Erbitux (Cetuximab) or Vectibix (Panitumumab), whereas, the patients with mutant *KRAS* or *NRAS* gene show poor response to this treatment. And *BRAF* gene mutations predict poor prognosis of such treatments. As a result, *KRAS*, *NRAS* and *BRAF* mutations detection supplies evidence for targeted clinical therapy of tumor patients, which decreases cost and time of treatment.

Intended Use

The AmoyDx® *KRAS/NRAS/BRAF* Mutations Detection Kit is a real-time PCR assay for qualitative detection of 17 *KRAS* mutations (exons 2, 3 and 4), 13 *NRAS* mutations (exons 2, 3 and 4) and *BRAF* V600E mutation (exon 15) in human genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue. The kit is intended to assess *KRAS/NRAS/BRAF* mutation status in colorectal cancer patient.

The kit is for *in vitro* diagnostic use, and intended to be used by trained professionals in a laboratory environment.

Principles of the Procedure

The kit adopts Amplification Refractory Mutation System (ARMS) and real-time PCR technologies, which comprises specific primers and probes to detect *KRAS*, *NRAS* and *BRAF* mutations in human DNA samples. The *KRAS*, *NRAS* and *BRAF* mutant sequences are amplified by the mutant-specific primers, and detected by the fluorescence-labeled probes.

The kit is composed of 12 reaction mixes (KNB Reaction Mix 1~11 and KNB External Control Reaction Mix), Positive Control and Enzyme Mix.

- 1) The **KNB Reaction Mix 1~11** includes a mutation detection system and an internal control system. The mutation detection system contains primers and FAM-labeled probes specific for designated *KRAS/NRAS/BRAF* mutations, which is used to detect the mutation status of *KRAS*, *NRAS* and *BRAF* gene. The internal control system contains primers and HEX-labeled probe for a region of conserved segment in *RAS* gene, which is used to detect the presence of inhibitors and monitor the accuracy of the experimental operation.
- 2) The **KNB External Control Reaction Mix** contains primers and FAM-labeled probe for a region of genomic DNA adjacent to *KRAS*, *NRAS* and *BRAF* gene, which is used to assess the quality of DNA.
- 3) The **KNB Positive Control (PC)** contains recombinant gene with *KRAS* mutations, *NRAS* mutations and *BRAF* mutations.
- 4) The **KNB Enzyme Mix** contains 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).

Table 1 Kit Contents

Content	Main Ingredients	Quantity
KNB Reaction Mix	12-tube Strip*	8 strips
KNB Enzyme Mix	Taq DNA Polymerase, Uracil-N-Glycosylase	40µL
KNB Positive Control	Plasmid DNA	250 µL

*Each strip (12-tube) includes the following contents for testing one sample or one control (Table 2).

Table 2 Information for 12-tube strip

Tube No.	Reagent	Main Ingredients	Quantity	Fluorescent Signal
①	KNB Reaction Mix 1	Primers, Probes, Mg ²⁺ , dNTPs,	35 μL	FAM, HEX/VIC
②	KNB Reaction Mix 2	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
③	KNB Reaction Mix 3	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
④	KNB Reaction Mix 4	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
⑤	KNB Reaction Mix 5	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
⑥	KNB Reaction Mix 6	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
⑦	KNB Reaction Mix 7	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
⑧	KNB Reaction Mix 8	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
⑨	KNB Reaction Mix 9	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
⑩	KNB Reaction Mix 10	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
⑪	KNB Reaction Mix 11	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM, HEX/VIC
⑫	KNB External Control Reaction Mix	Primers, Probes, Mg ²⁺ , dNTPs	35 μL	FAM

Note:

Distinguish Tube ⑫ from Tube ① according to the hole position at the strip edge, described as follows.



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°C and protected from light.

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

Additional Reagents and Equipment Required but Not Supplied

- 1) Compatible real-time PCR instruments:
Stratagene Mx3000P™, ABI7500, LightCycler480 or SLAN-96S.
- 2) DNA extraction kit. We recommend to use AmoyDx DNA extraction kit (AmoyDx® FFPE DNA Kit) for paraffin embedded tissue specimens.
- 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) Adjustable pipettors and filtered pipette tips for handling DNA.
- 9) Tube racks.
- 10) Disposable powder-free gloves.
- 11) Sterile, nuclease-free water.
- 12) 1×TE buffer (pH 8.0).

Precautions and Handling Requirements

For *in vitro* diagnostic use.

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.
- 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.

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 μ L.
- For Stratagene Mx3000P™, if there is a low net fluorescence signal (dR) but a high background signal (R), please reduce the signal gain setting of instrument properly.
- For ABI instrument, please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.
- For LightCycler480 instrument, please use the Roche 480 adaptor, available from BIOplastics, Cat No. B79480. It's essential to place a 12-tube strip on the first row (Row A) of the adaptor.
- 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 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 fluorescence calibration once a year.

Assay Procedure

1. DNA Extraction

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

Tumor samples are not homogeneous, they may also contain non-tumor tissue. Data from different tissue sections of the same tumor may be inconsistent. DNA from non-tumor tissue may not contain detectable with *KRAS/NRAS/BRAF* mutations. It's better to use tumor tissue

samples with more than 30% tumor cells.

The OD₂₆₀/OD₂₈₀ value of extracted DNA should be between 1.8 ~ 2.0 (measured using the spectrophotometer, the NanoDrop 1000 /2000 spectrophotometer is recommended).

The amount of extracted DNA from FFPE tissue used for PCR amplification is shown in Table 3.

Table 3 Recommended 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	2.5~3 ng/μL	11.75~14.1 ng

Note:

- The FFPE tissue should be handled and stored properly. 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°C for no more than 6 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 **KNB Positive Control (PC)** and **KNB Enzyme Mix** out of the kit from the freezer, and other reagents remained in freezer at -20±5°C.
- 2) Thaw **KNB PC** at room temperature. When the reagent is completely thawed, mix the reagent thoroughly by vortexing and centrifuge for 5~10 seconds to collect all liquid at the bottom of the tube.
- 3) Centrifuge **KNB Enzyme Mix** for 5~10 seconds prior to use.
- 4) Take out the sample DNA (see Table 3 for DNA concentration) and nuclease-free water for No Template Control (NTC).
- 5) Prepare NTC mixture: pipet 65.8 μL nuclease-free water (NTC) and 4.2 μL KNB Enzyme Mix into one centrifuge tube. Mix thoroughly by vortexing, and centrifuge for 5~10 seconds.
- 6) Prepare sample DNA mixture: pipet 65.8 μL each sample DNA and 4.2 μL KNB Enzyme Mix into one centrifuge tube. Mix thoroughly by vortexing, and centrifuge for 5~10 seconds.
- 7) Prepare PC mixture: pipet 65.8 μL KNB Positive control and 4.2 μL KNB Enzyme Mix into one centrifuge tube. Mix thoroughly by vortexing, and centrifuge for 5~10 seconds.

Note:

- Each run must contain one PC and one NTC.
 - 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.
- 8) Take out the **KNB Reaction Mix** strips and centrifuge the strips if there are any reagent droplets in the caps of the PCR tubes. Then gently uncover the caps prior to use.
 - 9) Add 5 μL of prepared NTC mixture to each PCR tube of the NTC strip, and cap the PCR tubes.
 - 10) Add 5 μL of each prepared sample DNA mixture to each PCR tube of the sample strip, and cap the PCR tubes.
 - 11) Add 5 μL of prepared PC mixture to each PCR tube of the PC strip, and cap the PCR tubes.
 - 12) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.
 - 13) Place the PCR tubes into the real-time PCR instrument. A recommended plate layout is shown in Table 4.

Table 4 Recommended PCR Plate Layout

Well	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1
B	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2
C	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3
D	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4
E	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5

F	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6	Sample 6
G	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
H	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

14) Setup the PCR Protocol using the cycling conditions described in Table 5.

Table 5 Cycling Parameters

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

15) Start the PCR run immediately.

16) When the PCR run is finished, analyze the data according to the “Results Interpretation” procedures.

3. Results Interpretation

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

- 1) For NTC: The FAM Ct values of Tubes ①~⑪ should be ≥ 31 . If not, the data is *INVALID*. The sample should be retested.
- 2) For Positive Control: The FAM Ct values of Tubes ①~⑫ and HEX/VIC Ct values of Tubes ①~⑪ should be < 20 . If not, the data is *INVALID*. The sample should be retested.
- 3) For the internal control assay in Tubes ①~⑪ for each sample: The HEX/VIC Ct values of Tubes ①~⑪ should be < 31 . If not, check the FAM signals of Tubes ①~⑪ :
 - 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.
- 4) For the external control assay in Tube ⑫ for each sample:
 - a) The FAM Ct value should be between 15~21.
 - b) If the FAM Ct value is < 15 , this indicates the DNA is overloaded. The DNA amount should be reduced and retested. But if the FAM Ct values of Tubes ①~⑪ are in Negative Ct range (see Table 6), the sample is determined as negative.
 - c) If the FAM Ct value is > 21 , 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 ①~⑪ is < 26 , the sample is determined as positive.

Analyze the mutation assay for each sample:

- 1) Check the mutant FAM Ct values of Tubes ①~⑪ for each sample.
- 2) Check the mutant FAM Ct values of Tubes ①~⑪ according to Table 6:
 - a) If any FAM Ct value of Tube ①~⑪ is < 26 , the sample is determined as positive (*KRAS/NRAS/BRAF* mutations detected).
 - b) If any FAM Ct value of Tube ①~⑪ is in Acceptable Ct range, calculate the ΔCt value for each mutation showing positive amplification.
 - i. $\Delta Ct \text{ value} = \text{Mutant FAM Ct value} - \text{External control FAM Ct value}$.
 - ii. If the ΔCt value is $< \text{cut-off } \Delta Ct \text{ value}$, the sample is determined as positive (Mutation detected).
 - iii. If the ΔCt value is $\geq \text{cut-off } \Delta Ct \text{ value}$, 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 ①~⑪ 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).

Table 6 Results Determination

Tube No.	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	⑪	Results
Optimal Ct range	Ct <26	Ct <26	Ct <26	Ct <26	Ct <26	Ct <26	Ct <26	Ct <26	Ct <26	Ct <26	Ct <26	Positive
Acceptable Ct range	26≤Ct <28	26≤Ct <29	26≤Ct <29	26≤Ct <29	26≤Ct <29	26≤Ct <28	26≤Ct <29	26≤Ct <29	26≤Ct <28	26≤Ct <29	26≤Ct <29	Interpret the results according to the ΔCt value.
Cut-off ΔCt value	9	9	9	10	9	9	10	10	9	9	10	
Negative Ct range	Ct ≥28	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥29	Ct ≥28	Ct ≥29	Ct ≥29	Ct ≥28	Ct ≥29	Ct ≥29	Negative or under the LOD*

* LOD: limit of detection

Performance Characteristics

The performance characteristics of this kit were validated on Stratagene Mx3000P™, ABI7500, LightCycler480 and SLAN-96S.

1) Analytical Sensitivity:

- a) For Stratagene Mx3000P™, the kit allows detection of 1% mutant DNA in a background of 99% normal DNA at 10 ng sample DNA amount.
- b) For other PCR instruments, the kit allows detection of 1% mutant DNA in a background of 99% normal DNA at 10 ng sample DNA amount. Except: the sensitivity of KRAS-M16, KRAS-M18, NRAS-M1, NRAS-M2 and NRAS-M12 mutations are 2% at 10 ng sample DNA amount, the sensitivity of KRAS-M20, KRAS-M22 and KRAS-M23 mutations are 5% at 10 ng sample DNA amount.

2) Specificity:

Specificity of the kit was established by testing negative reference controls, the tests gave positive results and negative concordance rate was 100%.

3) Accuracy:

Accuracy of the kit was established by testing positive reference controls, the tests gave negative results and positive concordance rate was 100%.

4) Precision:

Precision of the kit was established by performing a certain mutant positive reference control for 10 repeats; all the controls can be detected.

5) Interfering substances:

Two common potential interfering substances hemoglobin and triglyceride were evaluated. It is confirmed that the potential maximum concentrations: 2 g/L hemoglobin and 37 mmol/L triglyceride would not interfere with the test result.

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 17 KRAS mutations and 13 NRAS mutations and BRAF V600E mutation 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 KRAS/NRAS/BRAF mutation.
- 7) Samples with negative result (No mutation detected) may harbor KRAS/NRAS/BRAF mutations not detected by this assay.

References

- 1) Douillard JY, Oliner KS, Siena S, et al. 2013. Panitumumab-FOLFOX4 Treatment and RAS Mutations in Colorectal Cancer. The New England Journal of Medicine, 369 (11):1023-1034.
- 2) Di M, Pietrantonio F, Perrone F, et al. 2013. Lack of KRAS, NRAS, BRAF and TP53 mutations improves outcome of elderly metastatic colorectal cancer patients treated with cetuximab, oxaliplatin and UFT. Targeted Oncology, 1-8.

- 3) Rook WD, Claes Bart, Bernasconi D, et al. 2010. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol*, 11: 753-762.

Symbols

	Authorized Representative in the European Community		In Vitro Diagnostic Medical Device
	Manufacturer		Catalogue Number
	Batch Code		Use By
	Contains Sufficient for <n> Tests		Temperature Limitation
	Consult Instructions For Use		Keep Dry
	This Way Up		Fragile, Handle With Care
	Keep Away from Sunlight		

Appendix

KRAS, NRAS and BRAF Mutations Detected by the Kit

Tube No.	Reagent Supplied	Exon	Mutation	Base change	Cosmic ID	Name
①	KNB Reaction Mix 1	KRAS 2	G12S	34G>A	517	KRAS-M4
			G12D	35G>A	521	KRAS-M1
			G12C	34G>T	516	KRAS-M6
②	KNB Reaction Mix 2	KRAS 2	G12R	34G>C	518	KRAS-M5
			G12V	35G>T	520	KRAS-M3
			G12A	35G>C	522	KRAS-M2
			G13C	37G>T	527	KRAS-M14
③	KNB Reaction Mix 3	KRAS 2	G13D	38G>A	532	KRAS-M7
			Q61L	182A>T	553	KRAS-M15
④	KNB Reaction Mix 4	KRAS 3	Q61R	182A>G	552	KRAS-M16
			Q61H	183A>C	554	KRAS-M17
			Q61H	183A>T	555	KRAS-M18
			K117N	351A>C	19940	KRAS-M20
⑤	KNB Reaction Mix 5	KRAS 4	K117N	351A>T	28519	KRAS-M21
			A146T	436G>A	19404	KRAS-M22
			A146V	437C>T	19900	KRAS-M23
⑥	KNB Reaction Mix 6	NRAS 2	A146P	436G>C	19905	KRAS-M24
			G12D	35G>A	564	NRAS-M3
⑦	KNB Reaction Mix 7	NRAS 2	G12S	34G>A	563	NRAS-M10
			G13D	38G>A	573	NRAS-M4
⑧	KNB Reaction Mix 8	NRAS 2	G13R	37G>C	569	NRAS-M6
			G12C	34G>T	562	NRAS-M7
			G12V	35G>T	566	NRAS-M9
			G12A	35G>C	565	NRAS-M11
			G13V	38G>T	574	NRAS-M14
⑨	KNB Reaction Mix 9	NRAS 3	Q61R	182A>G	584	NRAS-M1
			Q61K	181C>A	580	NRAS-M2
			Q61L	182A>T	583	NRAS-M5
⑩	KNB Reaction Mix 10	NRAS 4	Q61H	183A>C	586	NRAS-M8
			A146T	436G>A	27174	NRAS-M12
⑪	KNB Reaction Mix 11	BRAF 15	V600E	1799T>A	476	BRAF-M1