CE IVD

# AmoyDx® *NRAS* Mutation Detection Kit

**Detection of 16 mutations in *NRAS* codons 12, 13, 59, 61, 117 and 146**

Instruction for Use

| | | | |
|---|---|---|---|
| REF 8.01.25001X024H | 24 tests | For Stratagene Mx3000P™, ABI7500, LightCycler480, Bio-Rad CFX96, Rotor-Gene Q/6000 (72 wells), SLAN-96S | |

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## Background

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. *RAS* family members are frequently found in their mutated, oncogenic forms in human tumors. In total, activating mutations in the *NRAS* genes occur in 13~25% of cutaneous melanomas, 1~6% of colorectal cancer and 1% of lung cancer, mainly in exons 2, 3 or 4. The mutation status of *NRAS* gene is relevant to drug resistance of colorectal cancers treated with anti-*EGFR* monoclonal antibodies. As a result, *NRAS* mutations detection supplies evidence for targeted clinical therapy of tumor patients, decreases cost and time of treatment.

## Intended Use

The AmoyDx® *NRAS* Mutation Detection Kit is a real-time PCR assay for qualitative detection of 16 somatic mutations in codons 12, 13, 59, 61, 117 and 146 of *NRAS* gene in human genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue. The kit is intended to be used to assess *NRAS* mutation status in colorectal cancer patients.

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) technology which 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 eight reaction mixes (*NRAS* Reaction Mix 1~7 and *NRAS* External Control Reaction Mix), sufficient Enzyme Mix and Positive Control.

1) The ***NRAS* Reaction Mix 1~7** includes a mutation detection system and an internal control system. The mutation detection system includes primers and FAM-labeled probes specific for designated *NRAS* mutations, which is used to detect the *NRAS* mutation status. The internal control system contains primers and HEX-labeled probe for a region of a region of genomic DNA adjacent to the *NRAS* gene, which is used to detect the presence of inhibitors and confirm the validity of each experiment.
2) The ***NRAS* External Control Reaction Mix** contains primers and FAM-labeled probe for a region of genomic DNA adjacent to the *NRAS* gene, which is used to assess the quality of DNA.
3) The ***NRAS* Positive Control** contains a recombinant gene with *NRAS* mutations.
4) The ***NRAS* 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 | ***NRAS* Reaction Mix 1** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 μL/tube ×1 | FAM, HEX/VIC |
| ② | ***NRAS* Reaction Mix 2** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 μL/tube ×1 | FAM, HEX/VIC |
| ③ | ***NRAS* Reaction Mix 3** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 μL/tube ×1 | FAM, HEX/VIC |
| ④ | ***NRAS* Reaction Mix 4** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 μL/tube ×1 | FAM, HEX/VIC |
| ⑤ | ***NRAS* Reaction Mix 5** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 μL/tube ×1 | FAM, HEX/VIC |
| ⑥ | ***NRAS* Reaction Mix 6** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 μL/tube ×1 | FAM, HEX/VIC |
| ⑦ | ***NRAS* Reaction Mix 7** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 μL/tube ×1 | FAM, HEX/VIC |
| ⑧ | ***NRAS* External Control Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 1100 μL/tube ×1 | FAM |
| / | ***NRAS* Enzyme Mix** | Taq DNA Polymerase, Uracil-N-Glycosylase | 85 μL/tube ×1 | / |
| / | ***NRAS* Positive Control** | Plasmid DNA | 500 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 recommend maximum freeze-thaw cycle is five cycles.

## Additional Reagents and Equipment Required but Not Supplied

1) Compatible PCR instruments:
   Stratagene Mx3000P™, ABI7500, LightCycler480, Bio-Rad CFX96, Rotor-Gene Q/6000 (72 wells), or SLAN-96S.
2) DNA extraction kit. We recommend use of DNA extraction kit (AmoyDx® FFPE DNA Kit, Cat No.: 8.02.23501X036G).
3) Spectrophotometer for measuring DNA concentration.
4) Mini centrifuge with rotor for centrifuge tubes.
5) Mini centrifuge with rotor for PCR tubes.
6) Nuclease-free centrifuge tubes.
7) Nuclease-free PCR tubs and caps.
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, and 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 skin, eyes and mucous membranes contact 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.
- 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.
- Using 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 μL
- 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 ABI instruments please set up as follows: Reporter Dye: FAM, VIC; Quencher Dye: TAMRA; Passive Reference: NONE.
- For LightCycler480 I instrument, it's necessary to conduct fluorescence calibration prior to use. If there is fluorescence crossover on LightCycler480 II instrument, fluorescence calibration is also required. To run the assays on a LightCycler machine, please use the Roche 480 adaptor, available from BIOplastics, Cat No. B79480.
- 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 real-time PCR instrument operator's manual for detailed instructions.
- We recommend that all PCR instruments in use 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. 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 *NRAS* mutations. It's better to use tumor tissue samples with more than 30% tumor cells.

The $OD_{260}/OD_{280}$ 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 differs according to different storage time (see Table 2).

Table 2 Recommend 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, 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 ***NRAS* Reaction Mix 1~7**, ***NRAS* External Control Reaction Mix** and ***NRAS* Positive Control** at room temperature. When the reagents completely thawed, invert the tube for 10 times and briefly centrifuge to collect all liquid at the bottom of the tube.
2) Briefly centrifuge ***NRAS* Enzyme Mix** prior to use.
3) Prepare sufficient *NRAS* Master Mix 1~8 containing *NRAS* Enzyme Mix and each *NRAS* Reaction Mix (*NRAS* Reaction Mix 1~7 or

*NRAS* External Control Reaction Mix, respectively) in separate sterile centrifuge tube according to the ratio in Table 3. Mix each *NRAS* Master Mix thoroughly by gently pipetting up and down more than 10 times, and centrifuge briefly.

Table 3 *NRAS* Master Mix

| Content | Volume per test |
|---|---|
| **Reaction Mix** | 35 μL |
| ***NRAS* Enzyme Mix** | 0.3 μL |
| **Total volume** | **35.3 μL** |

***Note:***

- *Every PCR run must contain one PC (Positive control) and one NTC (No template control).*
- *Do not vortex enzyme mix or any mixture with enzyme mix.*
- *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 8 PCR tubes for NTC: Dispense 35.3 μL of *NRAS* Master Mix 1~8 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 8 PCR tubes for each sample: Dispense 35.3 μL of *NRAS* Master Mix 1~8 to each PCR tube respectively. Then add 4.7 μL of sample DNA to each PCR tube, and cap the PCR tubes.
7) Prepare 8 PCR tubes for PC: Dispense 35.3 μL of *NRAS* Master Mix 1~8 to each PCR tube respectively. Then add 4.7 μL of *NRAS* 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 | … | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|
| ① | Sample1 | Sample2 | Sample3 | … | Sample8 | Sample9 | Sample10 | PC | NTC |
| ② | Sample1 | Sample2 | Sample3 | … | Sample8 | Sample9 | Sample10 | PC | NTC |
| ③ | Sample1 | Sample2 | Sample3 | … | Sample8 | Sample9 | Sample10 | PC | NTC |
| ④ | Sample1 | Sample2 | Sample3 | … | Sample8 | Sample9 | Sample10 | PC | NTC |
| ⑤ | Sample1 | Sample2 | Sample3 | … | Sample8 | Sample9 | Sample10 | PC | NTC |
| ⑥ | Sample1 | Sample2 | Sample3 | … | Sample8 | Sample9 | Sample10 | PC | NTC |
| ⑦ | Sample1 | Sample2 | Sample3 | … | Sample8 | Sample9 | Sample10 | PC | NTC |
| ⑧ | Sample1 | Sample2 | Sample3 | … | Sample8 | Sample9 | Sample10 | PC | 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℃ | 5min | / |
| 2 | 15 | 95℃ | 25s | / |
| | | 64℃ | 20s | / |
| | | 72℃ | 20s | / |
| 3 | 31 | 93℃ | 25s | / |
| | | 60℃ | 35s | FAM and HEX/VIC |
| | | 72℃ | 20s | / |

11) Start the PCR run immediately.
12) When the PCR run 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 ①~⑦ should be ≥31. If not, the data is *INVALID*. The sample should be retested.
2) For Positive Control: The FAM Ct values of Tubes ①~⑧ and HEX/VIC Ct values of Tubes ①~⑦ should be < 20. If not, the data is *INVALID*. The sample should be retested.
3) For the internal control assay in Tubes ①~⑦ for each sample: The HEX/VIC Ct values of Tubes ①~⑦ should be < 31. If not, check the FAM signals of Tubes ①~⑦ :
   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 ⑧ 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 ①~⑦ 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 ①~⑦ is < 26, the sample is determined as positive.

***Analyze the mutation assay for each sample:***

5) Record the mutant FAM Ct values of Tubes ①~⑦ for each sample.
6) Check the mutant FAM Ct values of Tubes ①~⑦ according to Table 6:

Table 6 Results Determination

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

* LOD: limit of detection

   a) If any FAM Ct value of Tube ①~⑦ is < 26, the sample is determined as positive (*NRAS* mutation detected).
   b) If any FAM Ct value of Tube ①~⑦ 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 ①~⑦ 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.

## Performance Characteristics

The performance characteristics of this kit were validated on Stratagene Mx3000P™, ABI7500, LightCycler480, Bio-Rad CFX96, Rotor-Gene Q/6000 (72 wells), and SLAN-96S.

1) Limit of Detection:

   The limit of detection was established by testing plasmids with 16 *NRAS* mutations. 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 *NRAS*-M1 mutation on Rotor-Gene Q/6000 (72 wells) is 2% at 10 ng sample DNA amount. The sensitivity of *NRAS*-M1, *NRAS*-M2 and *NRAS*-M12 on LightCycler480 and Bio-Rad CFX96 are 2% at 10 ng sample DNA amount.

2) Interference factor:

   4 common interference substances: hemoglobin, triglyceride, *mycobacterium tuberculosis* and *streptococcus pneumonia* that are easily present in FFPE samples, were evaluated in this study. It is confirmed that the potential maximum concentrations: 2 mg/mL hemoglobin, 37 mmol/L triglyceride $10^6$ CFU/mL *mycobacterium tuberculosis* and $10^6$ CFU/mL *streptococcus pneumonia* would not interfere with the test result.

3) Precision:

   3 precision controls: negative control, weak positive control (the mutant content is 5%) and strong positive control (the mutant content is 50%) were used in the validation. 3 batches of the kits were tested with the precision controls by 2 operators twice a day for 20 days on different PCR instruments. The Ct values were calculated, the CV values were all within 10%.

## 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 16 *NRAS* 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 *NRAS* mutation.
7) Samples with negative result (No mutation detected) may harbor *NRAS* mutations not detected by this assay.

## References

1) Brose MS, Volpe P, Feldman M, et al. BRAF and RAS Mutations in Human Lung Cancer and Melanoma. Cancer Res.2002,62:6997-7000.
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3) Kadoaki Ohashi, Lecia V, Sequist, et al. Characteristics of Lung Cancers Harboring NRAS Mutations.Clin Cancer Res.2013,19:2584–91.
4) Douillard JY,Oliner KS,Siena S,et al. Panitumumab-FOLFOX4 Treatment and RAS Mutations in Colorectal Cancer.The New England Journal of Medicine.2013,369 (11):1023-34.

## Symbols

| | | | |
|---|---|---|---|
| EC REP | Authorized Representative in the European Community | IVD | In Vitro Diagnostic Medical Device |
| | Manufacturer | REF | Catalogue Number |
| LOT | Batch Code | | Use By |
| Σ | Contains Sufficient for <n> Tests | | Temperature Limitation |
| | Consult Instructions For Use | | Keep Dry |
| | This Way Up | | Fragile, Handle With Care |

# Appendix

***NRAS* Mutations Detected by the Kit**

| Tube No. | Reagent Supplied | Exon | Mutation | Base Change | Cosmic ID | Name |
|---|---|---|---|---|---|---|
| ① | *NRAS* Reaction Mix 1 | *2* | G12D | 35G>A | 564 | NRAS-M3 |
| | | | G12S | 34G>A | 563 | NRAS-M10 |
| ② | *NRAS* Reaction Mix 2 | *2* | G13D | 38G>A | 573 | NRAS-M4 |
| ③ | *NRAS* Reaction Mix 3 | *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 |
| ④ | *NRAS* Reaction Mix *4* | *3* | A59D | 176C>A | 253327 | NRAS-M15 |
| ⑤ | *NRAS* Reaction Mix 5 | *3* | Q61R | 182A>G | 584 | NRAS-M1 |
| | | | Q61K | 181C>A | 580 | NRAS-M2 |
| | | | Q61L | 182A>T | 583 | NRAS-M5 |
| | | | Q61H | 183A>C | 586 | NRAS-M8 |
| ⑥ | *NRAS* Reaction Mix 6 | *4* | K117N | 351G>C | \ | NRAS-M16 |
| | | | K117N | 351G>T | \ | NRAS-M17 |
| ⑦ | *NRAS* Reaction Mix 7 | *4* | A146T | 436G>A | 27174 | NRAS-M12 |