# AmoyDx® *EGFR* 29 Mutations Detection Kit

Instructions for Use

For Research Use Only

REF 8.01.0053 24 tests/kit For Rotor-Gene Q/ 6000 (72 wells)

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## Background

The epidermal growth factor receptor (EGFR) plays a central role in transmitting signals that promote cell growth and proliferation. Due to its association with malignancies, *EGFR* has become the target of an expanding class of anticancer therapies, such as gefitinib, erlotinib and afatinib, which are tyrosine kinase inhibitors (TKIs). The TKIs target the *EGFR* tyrosine kinase domain. These drugs work best on non-small cell lung cancer (NSCLC) patients whose cancer is driven by abnormal *EGFR* signaling. Lung cancer patients who experienced rapid, durable, complete or partial responses to TKI therapy have been found to harbor somatic mutations in *EGFR* gene. NSCLC patients with sensitizing *EGFR* mutations treated with TKI therapy have shown longer progression-free survival and higher response rate, compared with conventional chemotherapy. Resistance to TKI therapy, either in the primary tumor or acquired after TKI treatment, is associated with *EGFR* T790M mutation. Therefore, assessment of *EGFR* mutation status facilitates personalized treatment to lung cancer patients.

## Intended Use

The AmoyDx® *EGFR* 29 Mutations Detection Kit is a real-time PCR assay for qualitative detection of 29 somatic mutations in exons 18, 19, 20 and 21 of *EGFR* gene in human genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue, or circulating DNA extracted from plasma/serum. The kit is intended to be used to assess *EGFR* mutation status in NSCLC patients and aid in identifying patients who may response to the treatment with *EGFR*-TKI.

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 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.

The kit is composed of nine Reaction Mixes, *EGFR* Enzyme Mix and *EGFR* Positive Control.

1) The **Reaction Mix in Tubes** ①~⑧ include mutation detection and internal control systems. The mutation detection system includes primers and FAM-labeled probes specific for designated *EGFR* mutations, which is used to detect the *EGFR* mutation status. The internal control system contains primers and HEX-labeled probe for a region of genomic DNA without known mutations and polymorphism, to detect the presence of inhibitors and confirm the validity of each experiment.
2) The **External Control Reaction Mix** contains primers and FAM-labeled probe for a region of genomic DNA without known mutations and polymorphism, which is used to assess the quality of DNA.
3) The ***EGFR* Positive Control** contains a recombinant gene with *EGFR* mutations.
4) The **EGFR 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

| Tube No. | Content | Main Ingredients | Quantity | Fluorescent signal |
|---|---|---|---|---|
| ① | **19-Del Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 μL/tube ×1 | FAM, HEX |
| ② | **L858R Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 μL/tube ×1 | FAM, HEX |
| ③ | **T790M Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 μL/tube ×1 | FAM, HEX |
| ④ | **Insertions Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 μL/tube ×1 | FAM, HEX |
| ⑤ | **G719A/G719C Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 μL/tube ×1 | FAM, HEX |
| ⑥ | **G719S Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 μL/tube ×1 | FAM, HEX |
| ⑦ | **S768I Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 μL/tube ×1 | FAM, HEX |
| ⑧ | **L861Q Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 μL/tube ×1 | FAM, HEX |

| | | | | |
|---|---|---|---|---|
| ⑨ | **External Control Reaction Mix** | Primers, Probes, $Mg^{2+}$, dNTPs | 700 µL/tube ×1 | FAM |
| ⑩ | ***EGFR* Positive Control 2** | Plasmid DNA | 500 µL/tube ×1 | / |
| ⑪ | ***EGFR* Enzyme Mix** | Taq DNA Polymerase, Uracil-N-Glycosylase | 80 µL/tube ×1 | / |

## 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℃ 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 Real-time PCR instrument:
   Rotor-Gene Q/6000 (72 wells).
2) DNA extraction kit. We recommend use of AmoyDx® FFPE DNA Kit for FFPE tissues, AmoyDx® Circulating DNA kit for plasma/serum sample.
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 tubes 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, 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.
- 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 25 μL.
- Prior to the operation, please set up the PCR program by the following steps: ①select "**Gain Optimization**", the "**Auto Gain Optimization Setup**" window will open (see Figure 1); ②Click "**Perform Calibration Before 1st Acquisition**" and "**Optimize Acquiring**" (see Figure 2). ③Click "**OK**", then click "**Close**" to continue (see Figure 3).

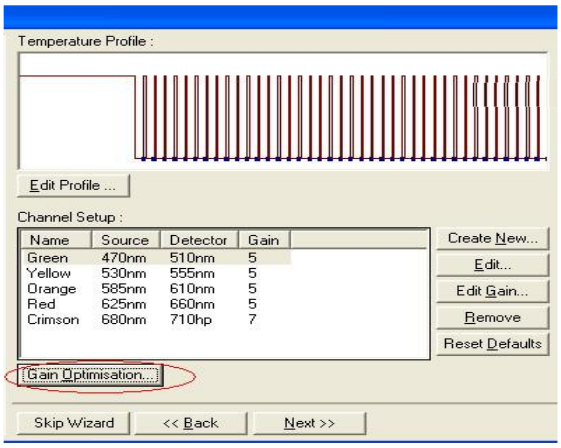


Figure 1

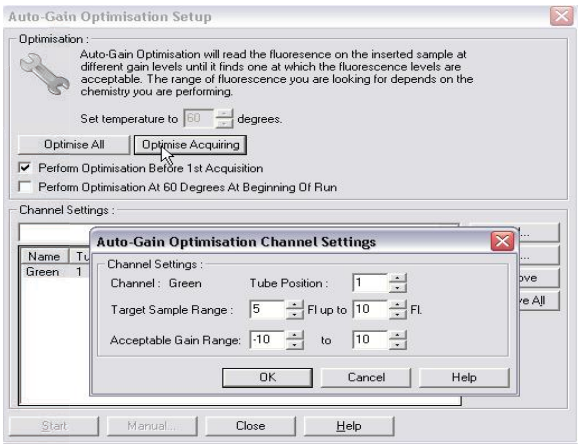


Figure 2

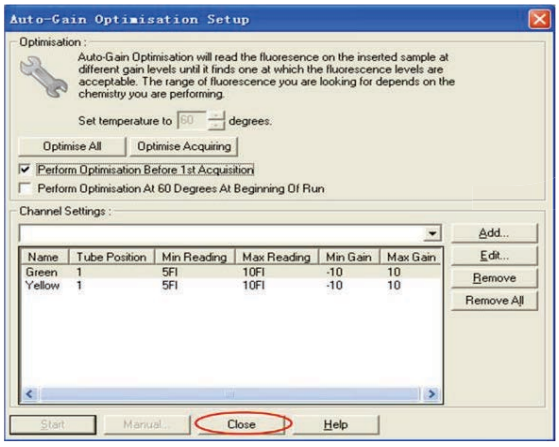


Figure 3

## Assay Procedure

### 1. DNA Extraction

The specimen material must be human genomic DNA extracted from FFPE tissue or plasma/serum samples. 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 *EGFR* mutations. It's better to use tumor tissue samples with more than 30% tumor cells.

The $OD_{260}/OD_{280}$ value of extracted DNA from FFPE tissue 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 2. And the circulating DNA isolated from plasma/serum should be used directly without dilution.

Table 2 Recommended DNA concentration

| Tissue | Storage time | DNA concentration | DNA amount per reaction |
|---|---|---|---|
| FFPE sample | ≤ 3 years | 2~3 ng/μL | 6~9 ng |

***Note:***

- *The FFPE tissue should be handled and stored properly, and the storage time should preferably be less than 3 years.*
- *The plasma/serum sample should be derived from EDTA anti-coagulated peripheral whole blood.*
- *The extracted DNA should be used immediately, if not, it should be stored at -20±5℃ for no more than 3 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 nine **Reaction Mix**, ***EGFR* Positive Control**, and ***EGFR* Enzyme Mix** out of the kit from the freezer
2) Thaw the **Reaction Mix** and ***EGFR* 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.
3) Centrifuge ***EGFR* Enzyme Mix** for 5~10 seconds prior to use.
4) Prepare sufficient *EGFR* Master Mix containing each Reaction Mix and *EGFR* Enzyme Mix respectively in separate sterile centrifuge tube according to the ratio in Table 3. Mix each *EGFR* Master Mix thoroughly by vortexing, and centrifuge for 5~10 seconds.

Table 3 Master Mix

| ***EGFR* Master Mix** | **Volume per test** | |
|---|---|---|
| | **Reaction Mix (μL)** | **Enzyme Mix (μL)** |
| 19-Del Master Mix | 22 | 0.2 |
| L858R Master Mix | 22 | 0.16 |
| T790M Master Mix | 22 | 0.2 |
| Insertions Master Mix | 22 | 0.16 |
| G719A/G719C Master Mix | 22 | 0.2 |
| G719S Master Mix | 22 | 0.2 |
| S768I Master Mix | 22 | 0.16 |
| L861Q Master Mix | 22 | 0.16 |
| External Control Master Mix | 22 | 0.16 |

***Note:***

- *Every PCR run must contain one PC (Positive control) and one NTC (No template control).*
- *The prepared master mix 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.*

5) Take out the sample DNA (see Table 2 for DNA concentration) and nuclease-free water for NTC.
6) Prepare 9 PCR tubes for NTC: Dispense 22 μL of each *EGFR* Master Mix to each PCR tube respectively, then add 3 μL NTC to each PCR tube, and cap the PCR tubes.
7) Prepare 9 PCR tubes for each sample: Dispense 22 μL of each *EGFR* Master Mix to each PCR tube respectively, then add 3 μL each

sample DNA to each PCR tube, and cap the PCR tubes.

8) Prepare 9 PCR tubes for PC: Dispense 22 μL of each *EGFR* Master Mix to each PCR tube respectively, then add 3 μL PC to each PCR tube of PC strip, and cap the PCR tubes.
9) Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube.
10) Place the PCR tubes into the real-time PCR instrument.
11) Setup the PCR Protocol using the cycling parameters in Table 4.

Table 4 Cycling Parameters

| Stage | Cycles | Temperature | Time | Data collection |
|---|---|---|---|---|
| 1 | 1 | 95℃ | 2.5min | / |
| 2 | 15 | 95℃ | 8s | / |
| | | 64℃ | 10s | / |
| | | 72℃ | 8s | / |
| 3 | 31 | 95℃ | 2s | / |
| | | 60℃ | 15s | Green / Yellow |
| | | 72℃ | 8s | / |

12) Start the PCR run immediately.
13) 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 ①~⑧ should be ≥31. If not, the data is *INVALID*. The sample should be retested.
2) For Positive Control: The FAM Ct values of Tubes ①~⑨ and HEX Ct values of Tubes ①~⑧ should be < 21. If not, the data is *INVALID*. The sample should be retested.
3) For the internal control assay in Tubes ①~⑧ for each sample: The HEX Ct values should be < 31. If not, check the mutant FAM signals in Tubes ①~⑧ :
   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 ⑨ for each sample:
   a) The FAM Ct value should be between 10~19.
   b) If the FAM Ct value is <10, this indicates the DNA is overloaded. The DNA amount should be reduced and retested. But if the FAM Ct values in tubes ①~⑧ are in Negative Ct range (see Table 5), the sample is determined as negative.
   c) If the FAM Ct value is >19, 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 the result in any of Tubes ①~⑧ is positive, the sample is determined as positive.

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

5) Record the FAM Ct value in Tubes ①~⑧ for each sample.
6) Check the mutant FAM Ct values in Tubes ①~⑧ according to Table 5:
   a) If all the FAM Ct values of Tubes ①~⑧ are in Negative Ct range or there is no amplification, the sample is determined as negative or under the LOD of the kit.
   b) If any FAM Ct value of Tubes ①~⑧ 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 < the Cut-off ΔCt value, the sample is determined as positive (Mutation detected).
      iii. If the ΔCt value is ≥ the Cut-off ΔCt value, the sample is determined as negative (No mutation detected) or under the LOD of the kit.

Table 5 Result Determination

| | 19-Del | L858R | T790M | Insertions | G719A/G719C | G719S | S768I | L861Q | Results |
|---|---|---|---|---|---|---|---|---|---|
| Acceptable Ct range | Ct <29 | Ct <29 | Ct <28 | Ct <29 | Ct <29 | Ct <28 | Ct <29 | Ct <29 | Positive: ΔCt < ΔCt Cut-off, Negative: ΔCt ≥ ΔCt Cut-off. |
| ΔCt Cut-off value | 13 | 14 | 12.5 | 13 | 13 | 13 | 12 | 12 | |
| Negative Ct range | 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

1) Analytical sensitivity:
   The kit allows detection of 1~2.5% mutant DNA in a background of 97.5~99% normal DNA at 10 ng sample DNA amount.
2) Positive concordance:
   29 positive controls with 29 *EGFR* mutations were tested by this kit. The test gave positive results and positive concordance rate was 100%.
3) Negative concordance:
   10 negative controls without the 29 *EGFR* mutations were tested by this kit, The test gave negative results and negative concordance rate was 100%
4) Specificity:
   The wild-type DNA tolerability study showed that the kit can tolerate 10 ng wild-type DNA without non-specificity.
5) Precision:
   Precision of the kit was established by performing the precision reference control for 10 repeats; the test gave positive results.

## 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 DNA extracted from FFPE tissue and plasma/serum samples.
4) The kit can only detect the 29 *EGFR* 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 *EGFR* mutation.
7) Samples with negative result (No mutation detected) may harbor *EGFR* mutations not detected by this assay.
8) Circulating DNA extracted from plasma or serum with negative results (No Mutation detected) may harbor *EGFR* mutation, which could be confirmed with matched tissue DNA detection.

## References

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2. Ressel R, Moran T, Queralt C, *et al*; Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med, 2009,361(10):958-67.
3. Mork Ts, Wu YL, Thongprasert S, *et al*; Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med, 2009,361(10):947-57.
4. Gazdar AF; Personalized medicine and inhibition of EGFR signaling in lung cancer. N Engl J Med, 2009, 361(10):1018-20.
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6. Kobayashi S, Boggon TJ, Dayaram T, *et al; EGFR* mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med, 2005, 352(8):786-92.
7. Yasuda H., S kobayshi, Costa, D. B, *et al. EGFR* exon 20 insertion mutations in non-small-cell lung cancer: preclinical data and clinical implications. Lancet Oncol，2012, 13(1): e23-31.
8. Kimura H, Suminoe M, Kasahara K, *et al*; Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). Br J Cancer, 2007, 97(6): 778-784.
9. Huang Z, Wang ZJ, Bai H, *et al*; The detection of EGFR mutation status in plasma is reproducible and can dynamically predict the efficacy of EGFR-TKI. Thoracic Cancer, 2012, 3(4): 334-340.

## Symbols

 Manufacturer

 Batch Code

 Contains Sufficient for <n> Tests

 Consult Instructions For Use

 This Way Up

 Keep Away from Sunlight

 Catalogue Number

 Use By

 Temperature Limitation

 Keep Dry

 Fragile, Handle With Care

## Appendix

***EGFR* Mutations Detected by this Kit**

| Tube No. | Reagent | Exon | Mutation | Base Change | Cosmic ID | LOD |
|---|---|---|---|---|---|---|
| ① | 19-Del Reaction Mix | 19 | E746_A750del (1) | 2235_2249del15 | 6223 | 1% |
| | | | E746_A750del (2) | 2236_2250del15 | 6225 | 1% |
| | | | L747_P753>S | 2240_2257del18 | 12370 | 1% |
| | | | E746_T751>I | 2235_2252>AAT(complex) | 13551 | 2% |
| | | | E746_T751del | 2236_2253del18 | 12728 | 1% |
| | | | E746_T751>A | 2237_2251del15 | 12678 | 1% |
| | | | E746_S752>A | 2237_2254del18 | 12367 | 1% |
| | | | E746_S752>V | 2237_2255>T(complex) | 12384 | 1% |
| | | | E746_S752>D | 2238_2255del18 | 6220 | 1% |
| | | | L747_A750>P | 2238_2248>GC(complex) | 12422 | 1% |
| | | | L747_T751>Q | 2238_2252>GCA(complex) | 12419 | 1% |
| | | | L747_E749del | 2239_2247del9 | 6218 | 1% |
| | | | L747_T751del | 2239_2253del15 | 6254 | 2% |
| | | | L747_S752del | 2239_2256del18 | 6255 | 1% |
| | | | L747_A750>P | 2239_2248TTAAGAGAAG>C(complex) | 12382 | 1% |
| | | | L747_P753>Q | 2239_2258>CA(complex) | 12387 | 1% |
| | | | L747_T751>S | 2240_2251del12 | 6210 | 1% |
| | | | L747_T751del | 2240_2254del15 | 12369 | 1% |
| | | | L747_T751>P | 2239_2251>C(complex) | 12383 | 1% |
| ② | L858R Reaction Mix | 21 | L858R | 2573T>G | 6224 | 1% |
| ③ | T790M Reaction Mix | 20 | T790M | 2369C>T | 6240 | 2.5% |
| ④ | Insertions Reaction Mix | 20 | H773_V774insH | 2319_2320insCAC | 12377 | 1% |
| | | | D770_N771insG | 2310_2311insGGT | 12378 | 1% |
| | | | V769_D770insASV | 2307_2308insGCCAGCGTG | 12376 | 1% |
| ⑤ | G719A/G719C Reaction Mix | 18 | G719A | 2156G>C | 6239 | 1% |
| | | | G719C | 2155G>T | 6253 | 1% |
| ⑥ | G719S Reaction Mix | 18 | G719S | 2155G>A | 6252 | 2.5% |
| ⑦ | S768I Reaction Mix | 20 | S768I | 2303G>T | 6241 | 1% |
| ⑧ | L861Q Reaction Mix | 21 | L861Q | 2582T>A | 6213 | 1% |