EGFR PCR Kit Handbook

Version 1

For the detection of 28 mutations in the Epidermal Growth Factor Receptor (EGFR) gene.

REF

871101 25 Reactions
871105 100 Reactions

HB

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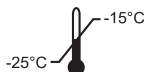

Store at -15°C to -25°C

For Research Use Only. Not for Use in Diagnostic Procedures.

Sample & Assay Technologies

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IMPORTANT: Read these instructions carefully and become familiar with all components of the EGFR PCR Kit prior to use.

Background

The use of the EGFR PCR Kit allows the detection of the most prevalent somatic mutations in the EGFR gene, which are common in human cancers. The excellent sensitivity of the kit permits detection of low-level mutations in the sample, ensuring that false negatives are minimised.

This kit enables the detection of the following mutations against a background of wild-type genomic DNA in a real-time PCR assay based on Scorpions® technology (see Appendix 1 for details of mutations).

- 19 Deletions in exon 19 (detects the presence of any of 19 deletions but does not distinguish between them)
- L858R
- L861Q
- G719X (detects the presence of G719S, G719A or G719C but does not distinguish between them)
- S768I
- 3 insertions in exon 20 (detects the presence of any of 3 insertions but does not distinguish between them).

The methods used in this kit are highly selective and, depending on the total amount of DNA present, can detect 1% of mutant in a background of wild-type genomic DNA. The assays have a limit of detection of 10 copies or below. These selectivity and detection limits are superior to technologies such as dye terminator sequencing.

Introduction

The EGFR PCR Kit is intended for the detection of somatic mutations in the EGFR gene. The kit is for use on DNA samples and will provide a qualitative assessment of mutation status.

Kit Contents

There are two EGFR PCR Kit sizes available. The kit contains sufficient reagents for 25 or 100 reactions plus additional reagents for sample assessment. The reaction mixes contain standard PCR buffer, deoxyribonucleotide triphosphates, magnesium chloride, oligonucleotides and sterile water.

Table 1: Kit Contents

The T790M Reaction Mix (formerly tube number 2) is no longer included in the EGFR PCR Kit.

Reagents Supplied	25 Reactions Volume	100 Reactions Volume	Tube
Control Reaction Mix	1056 µl	4224 µl	1
Deletions Reaction Mix	528 µl	2112 µl	3
L858R Reaction Mix	528 µl	2112 µl	4
L861Q Reaction Mix	528 µl	2112 µl	5
G719X Reaction Mix	528 µl	2112 µl	6
S768I Reaction Mix	528 µl	2112 µl	7
Insertions Reaction Mix	528 µl	2112 µl	8
Mixed Standard	150 µl	500 µl	9
Taq DNA polymerase	179 µl	702 µl	10

Equipment and Reagents Not Supplied with the Kit

The user will require the following equipment and consumables:

Block-based (96-well format) Real-time PCR instrument capable of collecting data for the following dyes or their equivalents:

FAM Ex495 Em520
HEX Ex535 Em556

IMPORTANT: The real-time PCR instrument must be calibrated according to its instructions for use before running the EGFR PCR Kit. The instrument should be maintained as per the manufacturer's instructions.

NOTE: Stratagene MX3000P® QPCR System and MX3005P® QPCR System instruments have been validated for use with the EGFR PCR Kit. If another instrument is used, its performance with the EGFR PCR Kit must be validated by the user.

0.2 ml DNAse-free PCR tubes or plates.
Sterile tubes for preparing master mixes.
Dedicated pipettes for PCR mix preparation.
Dedicated pipettes for dispensing of DNA template.
Nuclease-free water.

Symbols

Refer to information given in the handbook

REF Catalog number

Temperature limitations

Legal manufacturer

Important note

Distributor details

Shipping Conditions

The contents of the EGFR PCR Kit are shipped on dry ice and should still be frozen on arrival. If the EGFR PCR Kit is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain a packing note, instructions for use or the reagents, please contact your local QIAGEN Office; see Manufacturer and Distributor Details.

Storage Conditions

All the contents of this kit should be stored immediately upon receipt at -15°C to -25°C in the dark in a constant temperature freezer. Avoid unnecessary freeze-thawing of the contents of the kit. Do not use a reagent after 6 freeze-thaw cycles.

Stability

The contents of the kit are stable until the expiry date when stored under the recommended conditions and in the original packaging. Do not use the kit after the stated expiry date.

Specimen Material

Human genomic DNA extracted from fresh, frozen or paraffin embedded tissue. DNA must be extracted from material before use. Following extraction, DNA samples should be stored at -20°C.

Technical Assistance

For technical questions relating to our products, please contact QIAGEN Technical Service in your country, or your local QIAGEN distributor. Visit www.qiagen.com for details.

Safety Information

Caution: All chemicals and biological material should be considered as potentially hazardous. Specimens are potentially infectious and should be treated accordingly.

This kit should be used only by those persons who have been trained in the appropriate laboratory techniques. When working with the components of this kit always wear a suitable lab coat, disposable gloves and safety glasses.

After use, kit components should be disposed of into clinical waste.

Warnings and Precautions

- **To ensure optimal activity and performance Scorpions primers (as with all fluorescently labelled molecules) should always be protected from light to avoid photo bleaching.**
- **Use extreme caution to prevent contamination of PCR reactions with synthetic control material. It is recommended that separate, dedicated pipettes be used for setting up reaction mixes and adding DNA template. The preparation and dispensing of reaction mixes should be carried out in a separate area to the addition of template. Tubes should never be opened after a PCR reaction.**
- **Reagents for this kit have been diluted optimally. Further dilution of the reagents is not recommended and may result in a loss of performance.**
- **Use of less than 25 µl reaction volumes is not recommended and will increase the risk of false negatives.**
- **All reagents in this kit are formulated specifically for use with the stated tests. No substitutions should be made to the kit if optimal performance is to be maintained.**
- **The kit contains human genomic DNA in the Mixed Standard. This should be treated with the same caution as clinical samples.**
- **Note that tumour samples are non-homogeneous and data from a sample of tumour may not be concordant with data from other sections of the same tumour. Tumour samples can also contain non-tumour tissue. DNA from this tissue would not be expected to contain the EGFR mutations detected by this kit.**
- **Each assay included in the kit has its own unique characteristics. Calculation of the result must be made with reference to the correct assay parameters (see Table 4 under Assay Performance Characteristics).**
- **All assays in the kit produce small PCR product sizes. However, the kit will not work on heavily fragmented DNA.**
- **The assays contain an exogenous control reaction in addition to the reaction of interest (see Technological Principles section). If both assays have failed the data must be discarded, as there may be inhibitors present, which could lead to false negative results. Diluting the sample can reduce the effect of inhibitors but it should be noted that this would also dilute down the DNA.**

- **Only use the *Taq* polymerase (*Taq*) that is provided in the kit, do not mix and match from other kits of the same or any other type.**
- **Do not substitute the *Taq* for *Taq* from another supplier.**
- **Only thaw the reagents required for each run, do not thaw the whole kit each time to minimise the amount of freeze-thaw cycles.**
- **Care should be taken when using *Taq* polymerase due to the viscosity, as an erroneous pipetting technique can lead to an incorrect volume of *Taq* being added to the reaction mix.**
- **Due to the viscosity of the *Taq*, care should be taken when pipetting. Allow the *Taq* to come to room temperature before using, then pipette by placing the pipette tip just under the surface of the *Taq*, to minimise the risk of coating the tip in excess *Taq*.**
- **Spin the *Taq* vial each time before use to ensure all the *Taq* is collected at the bottom of the vial.**
- **DO NOT vortex the *Taq*, or any reaction mixes that contain *Taq*, as this may cause inactivation of the enzyme.**
- **DO NOT ingest any of the contents of the EGFR PCR Kit. If you do, seek medical attention immediately.**

Technological Principles

This kit combines two technologies, ARMS® and Scorpions [1-3], to detect mutations in real-time PCR reactions.

Allele specific amplification is achieved by ARMS. *Taq* DNA polymerase is extremely effective at distinguishing between a match and a mismatch at the 3′-end of a PCR primer. When the primer is fully matched, the amplification proceeds with full efficiency.

When the 3′-base is mismatched, no efficient amplification occurs. In this way specific mutated sequences can be selectively amplified, even in samples where the majority of the sequences do not carry the mutation.

Scorpions are bi-functional molecules containing a PCR primer covalently linked to a probe. The fluorophore in the probe interacts with a quencher, which reduces fluorescence. During a PCR reaction, when the probe binds to the amplicon, the fluorophore and quencher become separated. This leads to an increase in fluorescence from the reaction tube.

By coupling Scorpions and ARMS, a sensitive and specific technique for detecting the EGFR mutations against a background of genomic DNA has been developed.

Seven assays are supplied. The Control assay, labelled with FAM, is used to assess the total DNA in a sample. This Scorpions assay amplifies a region of exon 2 of the EGFR gene. The primers and probe have been designed to avoid any known EGFR polymorphisms.

The mutation assays are also labelled with FAM. They contain one Scorpion plus one or more ARMS primers for discrimination between the wild-type DNA and the mutant DNA detected by that assay. All assays also contain a Scorpions assay for an exogenous Control labelled with HEX. This controls for the presence of inhibitors, which may lead to false negative results.

Scorpions real-time assays use the number of PCR cycles necessary to detect a signal above a background as a measure of the target molecules present at the beginning of the reaction. The threshold at which the signal is detected above background fluorescence is called the Cycle threshold (Ct). When using ARMS primers some inefficient priming may still occur, giving a very late background Ct, from DNA not containing the mutation. Sample ΔCt values are calculated as the difference between the mutation Ct and control Ct. If this difference is less than the supplied 1% cut-off point, the sample is classed as positive. The greater the ΔCt (closer to the cut-off) the less mutation the sample contains. Beyond the cut-off point the sample is classed as mutation negative or beyond the limits of the test.

The limits of the tests depend on the total level of DNA in a sample. Each test sees 5-10 copies of mutation. This equates to approximately 1% if 1000 copies are present. However, if only 100 copies are present, 1% is only 1 copy of the mutation. Stochastic variation at such a low copy number means that, if run in triplicate, 1-2 of the repeats may not amplify. If the mutation gives a very late Ct but is before the cut-off point it is recommended that, if possible, more DNA is used.

Similarly, if the mutation is at very low copy number even though there is a high level of total DNA (i.e. a low percentage of mutation), increasing the amount of DNA will increase the mutation copy number and improve the reproducibility of the test. Additional Control reaction mix is supplied to allow assessment of DNA before running the kit, if required. It is important to use PCR quantification of DNA as this may differ from other methods, such as OD readings, due to DNA fragmentation.

EGFR Mutation Detection Protocol

- **The user should read these instructions carefully and become familiar with all components of the kit prior to use.**

To use the EGFR PCR Kit efficiently samples must be batched into groups of 10 (to fill one 96 well plate). Smaller batch sizes will mean that fewer samples can be tested with the EGFR PCR Kit.

Extra Control assay mix is supplied with the EGFR PCR Kit to be used to assess the total DNA in a sample. It is recommended that this sample assessment is carried out prior to testing the sample with the full EGFR PCR Kit. This will prevent the wastage of EGFR PCR Kit reagents on samples which are too concentrated and need diluting or which do not contain sufficient amplifiable material for the test to function. This ensures that optimal dilution of the DNA can be performed and kit reagents are not wasted on samples containing little or no amplifiable DNA (control Ct of 35 or above).

Testing of Samples – Experimental Design and Method

For each sample, the control and mutation assays should be analysed on the same PCR run, to avoid run-to-run variations in threshold settings. The Mixed Standard must be analysed on all PCR runs, along with no template controls. Note that a total volume of 35 µl of sample is required to run all eight assays once.

1. Thaw the 7 reaction mixes and DNA standard. In the 100-reaction kit the reagents are split into 4 tubes. Only thaw 1 tube of each, unless more reagent is required, to avoid freeze-thawing. Mix each solution, once thawed, to avoid localized concentrations of salts. Prepare enough working solution for the samples, the standard and no template controls (control and mutation mixes) plus an excess of 2 reactions per mix as shown in Table 2. The volumes given for each reaction mix have been optimised and validated. Changing volumes of any reagent will result in a loss of performance. Do not store user-prepared mixes; use immediately.
2. Add 20 µl of Control reaction mix to each of the control reaction wells. Add 20 µl of each of the mutation reaction mixes to the relevant reaction wells. A recommended plate layout is given in Appendix 2.
3. Add 5 µl of test sample, standard or nuclease free water (for the no template controls) to the control and mutation reactions. Approximately 1.5-3.0 ng are required if 1% mutant DNA is to be detected. DNA quantification is based on PCR analysis. This should give a control Ct of approximately 26. Reducing the DNA below this level will reduce the apparent selectivity of the test.
4. Spin the PCR plate/tubes briefly to collect the reagents at the bottom of the wells, taking care to maintain the correct orientation of the plate or set(s) of tubes.
5. Seal the PCR plate/tubes, ensuring that all caps or seals are firmly in place, and load them into the real-time PCR instrument in the correct orientation (normally A1 in the top left hand corner of the block). Any loss of volume, through evaporation during the PCR, will adversely affect the data. Note that real-time PCR machines must be serviced, calibrated and used under conditions according to manufacturer's recommendations. Use of the kit on an un-calibrated machine may affect the performance of the kit.
6. Carry out real-time PCR using the cycling conditions described in Table 3. Note that this kit does not contain ROX passive reference. It is important to deselect passive reference or set the passive reference dye to "None".

Table 2: Test Reaction Set-up (Volumes given are for 1 Reaction)

Assay		Master Mixes	
	Reaction Mix (μl)	*Taq* (μl)	Water (μl)
Control	16	0.2	3.8
Deletions	16	0.6	3.4
L858R	16	0.6	3.4
L861Q	16	0.8	3.2
G719X	16	0.6	3.4
S768I	16	0.8	3.2
Insertions	16	0.8	3.2

Table 3: Cycling Parameters

Temperature	Time	Cycles	Data Collection
Stage 1			
95°C	10 minutes	1 cycle	
Stage 2			
95°C	30 seconds		
61°C	1 minute	40 cycles	FAM, HEX

Sample Analysis

For interpretation on the real-time PCR software, where possible use linear scale on the y-axis, manual baseline and manual threshold. Please note this will vary between real-time PCR platforms, for further information please contact Technical Support.

NOTE: Stratagene MX3000P® QPCR System and MX3005P® QPCR System instruments have been validated for use with the EGFR PCR Kit. If another instrument is used, its performance with the EGFR PCR Kit must be validated by the user.

1. Ensure that the passive reference is set to ‘none’ in the well inspector screen.
2. Check that each well gives a HEX signal from the exogenous control assay. There are 3 possible outcomes:
   a) If the exogenous control assay gives a positive result continue with the analysis.
   b) If the exogenous control assay has failed but the FAM reaction has worked well, continue with the analysis as the FAM reaction has out-competed the exogenous control reaction.

c) If both the FAM and exogenous control reactions have failed, the data must be discarded as there may be inhibitors present, which could lead to false negative results. Diluting the sample can reduce the effect of inhibitors but it should be noted that this would also dilute down the DNA.

3. Obtain Ct values for the control and mutation assays by observing the FAM amplification plots under a linear scale, ensuring that the passive reference is deselected.

4. Calculate the ΔCt value as follows, ensuring that the mutation and control Ct values are from the same sample.

$$[\text{mutation Ct}] - [\text{control Ct}] = \Delta\text{Ct}$$

5. Plate Controls (Mixed Standard and NTC):

   a) Assess the NTC Ct values to ensure that there is no contamination giving a positive amplification in the FAM channel (Ct less than 40 indicates contamination) or a failed exogenous control reaction in the HEX channel (no Ct indicates a failed exogenous control).

   b) Mixed Standard ΔCt values.

      - The Mixed Standard ΔCt values should be as given in Table 4, but variations of ±2 may occur due to different threshold settings on different instruments.

   c) Data should not be used if either of these Plate Controls fail.

6. The control Ct value must be above 20 to avoid overloading the assay.

7. Compare the ΔCt value for the sample with the cut-off point for the assay in question (Table 4), ensuring that the correct cut-off point is applied to each assay. The cut-off point is the point above which a positive signal could be due to background signal of the ARMS primer on wild-type DNA. If the sample ΔCt is greater than the cut-off point, it is classed as negative or below the limits of detection of the kit. If the sample value is lower than the cut-off point, the sample is classed as positive for a mutation detected by that assay. Samples should be repeated in triplicate for a mutation assay and the control assay if they give a ΔCt value close to the cut-off point. All replicates should fall in the positive zone to be classed as mutation positive.

8. For positive results that give a late Ct (above 35) it is possible that the stochastic variation will cause a number of the replicates to fail even if the sample contains the mutation. It is recommended that more DNA be used where possible.

Assay Performance Characteristics

The mutation assay performance characteristics are given in Table 4.

The positive control for each assay is a Mixed Standard containing 6 synthetic constructs (one positive control for each mutation assay), in 2 ng/µl of genomic DNA. The Mixed Standard ΔCt values, given in Table 4, should be used to ascertain that the assays are working correctly. Some deviations from these values are expected due to differences in threshold settings between runs.

The cut-off ΔCt has been determined over a range of DNA concentrations up to 50 ng per PCR.

The theoretical limit of selectivity has been based on the assumption that the amplifications are 100% efficient. The addition of greater than 50 ng of DNA to a PCR has not been tested. All assays have been shown to detect 1% mutant DNA.

The limit of detection has been based on the number of copies that can be reproducibly detected. For all assays, except the insertions, this value is 5 copies. The insertion assay can detect 5 copies but this is not reproducible for 2 of the insertions. Ten copies can be detected reproducibly for all mutations detected by this assay.

The kit has been shown to be 100% accurate at 3 times the limit of detection. Precision has been assessed at 3 times the limit of detection.

Sample data analysis has shown that testing using this kit and sequencing are concordant for high-level mutations. Cloning and sequencing have confirmed low-level mutations not detected by standard sequencing.

Table 4: Assay Performance Characteristics

Assays	Mixed Standard ΔCt	Cut-off ΔCt	Theoretical Limit of Selectivity	Limit of Detection (copies)	ng of DNA Required to Detect 1%
Deletions	0	12	0.02%	5	1.5
L858R	3.5	12	0.4%	5	1.5
L861Q	2.4	12	0.16%	5	1.5
G719X	3.2	12	0.16%	5	1.5
S768I	2	12	0.16%	5	1.5
Insertions	1.5	12	0.16%	10	3

Manufacturer and Distributor Details

QIAGEN Manchester Ltd, Skelton House,
Lloyd Street North, Manchester, M15 6SH, UK

Distributed by

Please visit www.qiagen.com for details of your local QIAGEN distributor.

Instructions for Use Revision History

New version.

References

1. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C et al. (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS) Nucleic Acids Res. 17 (7): 2503-16.
2. Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., Little, S. (1999). Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotech 17: 804-807.
3. Thelwell, N., Millington, S., Solinas, A., Booth, J. and Brown, T. (2000). Mode of Action and Application of Scorpion Primers to Mutation Detection. Nucleic Acids Research 28(19): 3752-3761.

Appendix 1 – Mutation Details

Cosmic IDs are taken from the Catalogue of Somatic Mutations in Cancer
http://www.sanger.ac.uk/genetics/CGP/cosmic/

Mutation	Exon	Base Change	Cosmic ID
L858R	21	2573T>G	6224
L861Q	21	2582T>A	6213
S768I	20	2303G>T	6241
G719A	18	2156G>C	6239
G719S	18	2155G>A	6252
G719C	18	2155G>T	6253
Insertions	20	2307_2308ins9	12376
		2319_2320insCAC	12377
		2310_2311insGGT	12378
Deletions	19	2235_2249del15	6223
		2235_2252>AAT (complex)	13551
		2236_2253del18	12728
		2237_2251del15	12678
		2237_2254del18	12367
		2237_2255>T (complex)	12384
		2236_2250del15	6225
		2238_2255del18	6220
		2238_2248>GC (complex)	12422
		2238_2252>GCA (complex)	12419
		2239_2247del9	6218
		2239_2253del15	6254
		2239_2256del18	6255
		2239_2248TTAAGAGAAG>C (complex)	12382
		2239_2258>CA (complex)	12387
		2240_2251del12	6210
		2240_2257del18	12370
		2240_2254del15	12369
		2239_2251>C (complex)	12383

Appendix 2 – Suggested PCR Plate Layout

Suggested PCR plate layout for 10 samples with no replicates.

						96 well layout						
Assay	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A Control	Mixed Standard	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
B Deletions	Mixed Standard	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
C L858R	Mixed Standard	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
D L861Q	Mixed Standard	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
E G719X	Mixed Standard	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
F S786I	Mixed Standard	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
G Insertions	Mixed Standard	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10

Notes To The Purchaser

Neither this product, the EGFR PCR Kit, nor any of its components can be resold or otherwise transferred or modified for resale without the written approval of QIAGEN.

This product is optimized for use in the Polymerase Chain Reaction ("PCR") covered by European and other patents owned by Hoffmann-La Roche Inc, and Roche Molecular Systems Ltd ("Roche"). No license under these patents to use the PCR process is conveyed expressly or by implication to the purchaser by the purchase of this product.

This product is licensed for use with Scorpions in the research field. Scorpions is covered by patents US 6326145 and EP 1088102.

"Scorpions®" is a registered trademark of QIAGEN.

"ARMS®" is a registered trademark owned by AstraZeneca Limited.

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