TheraScreen®

molecular diagnostics
for personalised medicine

TheraScreen: K-RAS Mutation Kit:

For the Detection of 7 Mutations in the K-RAS Gene

Instructions for Use

Product Codes:

Kit Size	Product Code
20 Reactions	KR-41

20 Reactions: Product Code: KR-41

Instructions Version: DU004a

Date of Revision: March 2012

Store at -18°C to -25°C

1071097

IMPORTANT: Please note DxS is now QIAGEN Manchester Ltd. In addition, the kit will be distributed by QIAGEN starting January 2012. For QIAGEN contact details, please refer to Section 12 of this Instructions for Use.

Contents

⚠ **IMPORTANT: Read these instructions carefully and become familiar with all components of the K-RAS Kit prior to use.**

1. Intended Use / Indications for Use

Intended Use

The TheraScreen:® K-RAS Mutation Kit (K-RAS Kit) is an *in vitro* diagnostic test intended for the detection of seven somatic mutations in the K-RAS oncogene and will provide a qualitative assessment of mutation status.
The K-RAS Kit is to be used by trained personnel in a professional laboratory environment with DNA samples extracted from paraffin-embedded colorectal tissue.

Indications for Use

The results of the K-RAS test are intended to aid the clinician in identifying colorectal cancer patients who may not benefit from anti-epidermal growth factor receptor (EGFR) therapy, such as panitumumab or cetuximab.

The K-RAS Kit is not intended to screen for or to diagnose colorectal cancer. It is intended as an adjunct to other relevant prognostic factors used to select suitable patients for treatment with anti-EGFR therapies, based on the patient's mutation status. The patient's mutation status will be considered by a clinician, alongside other disease factors to make a therapy decision. No treatment decision for cancer patients should be based on K-RAS mutation status alone.

2. Summary and Explanation of the Test

Mutations in the K-RAS oncogene are frequently found in human cancers [1-4]. The presence of these mutations correlates with a lack of response to certain EGFR inhibitor cancer therapies, in metastatic colorectal cancer patients [5-10][14-21].

Detection of the seven mutations in the K-RAS gene is possible in a background of wild-type genomic DNA in a real time PCR assay based on Scorpions® technology. This method is highly selective. Providing there are enough copies of DNA (it is necessary to have approximately 2-3ng of DNA), detection of approximately 1% of mutant in a background of wild-type genomic DNA is possible.

The K-RAS Kit will detect seven K-RAS mutations in codons 12 and 13 of the K-RAS oncogene, as shown in Table 1.

Table 1: K-RAS Mutations Detected by the K-RAS Kit

Mutation	Base Change	Cosmic ID
GLY12ALA	(GGT>GCT)	522
GLY12ASP	(GGT>GAT)	521
GLY12ARG	(GGT>CGT)	518
GLY12CYS	(GGT>TGT)	516
GLY12SER	(GGT>AGT)	517
GLY12VAL	(GGT>GTT)	520
GLY13ASP	(GGC>GAC)	532

3. Technological Principles

The K-RAS kit combines two technologies, ARMS® and Scorpions [11, 12, 13], to detect mutations in real-time PCR assays.

ARMS

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. Specific mutated sequences can be selectively amplified, even in samples where the majority of the sequences do not carry the mutation as:

- When the primer is fully matched, the amplification proceeds with full efficiency.
- When the 3'-base is mismatched, no efficient amplification occurs.

Scorpions

Detection of amplification is performed using Scorpions. Scorpions are bi-functional molecules containing a PCR primer covalently linked to a probe. The fluorophore in this probe interacts with a quencher, also incorporated into the probe, 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.

Data Analysis: ΔCt Method

Scorpions real-time assays use the number of PCR cycles necessary to detect a fluorescent signal above a background signal, 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).

Sample ΔCt values are calculated as the difference between the mutation assay Ct and control assay Ct from the same sample.

Samples are classed as mutation positive if they give a ΔCt less than the 1% ΔCt value for the assay. Above this value, the sample may contain less than 1% mutation (beyond the limit of the assays), or the sample is mutation negative.

When using ARMS primers some inefficient priming may occur giving a very late background Ct from DNA not containing the mutation. All ΔCt values calculated from background amplification will be greater than the 1% ΔCt values and the sample will be classed mutation negative.

Kit Format

Eight assays are supplied in the K-RAS Kit.

- One control assay.
- Seven mutations assays, one for each mutation detected.

All reaction mixes contain an exogenous control assay monitored by the JOE dye detector. This controls for the presence of inhibitors which may lead to false negative results.

Control assay (Scorpions assay).

The control assay, labelled with FAM, is used to assess the total DNA in a sample. The control assay amplifies a region of exon 4 of the K-RAS gene.

The primers and probe have been designed to avoid any known K-RAS polymorphisms.

Mutation assays (Scorpions assay).

Each mutation assay, labelled with FAM, contains one Scorpion plus one ARMS primer, for discrimination between the wild-type DNA and the mutant DNA detected by a real-time PCR assay.

Each assay also contains an exogenous control, labelled with JOE, to control for the presence of inhibitors that may lead to false negative results.

4. Reagents

This kit contains sufficient reagents to carry out 20 reactions for each assay.

- 20 reactions: Product Code: KR-41

It also contains additional reagents for mixed standard reactions, no template controls and follow-up testing. The reaction mixes contain standard PCR buffer, deoxyribonucleotide triphosphates, magnesium chloride, oligonucleotides and sterile water.

K-RAS Kit Contents

Reagents Supplied	Volume	Tube
Control Reaction Mix	1300 µl	1
12ALA Reaction Mix	650 µl	2
12ASP Reaction Mix	650 µl	3
12ARG Reaction Mix	650 µl	4
12CYS Reaction Mix	650 µl	5
12SER Reaction Mix	650 µl	6
12VAL Reaction Mix	650 µl	7
13ASP Reaction Mix	650 µl	8
Mixed standard	300 µl	9
Taq DNA polymerase	60 µl	10

Equipment and Reagents Not Supplied With Kit

The user will require the following equipment and consumables:

- Real-time PCR Machine, capable of cycling as defined in Section 8, K-RAS Mutation Detection Protocol.
- 0.2 ml DNAse-free PCR tubes, strips or plates.
- Sterile tubes for preparing master mixes.
- Dedicated pipettes for PCR mix preparation.
- Dedicated pipettes for dispensing of DNA template.
- Sterile, nuclease-free H_2O.
- DNA Extraction Kit

WARNINGS AND PRECAUTIONS

- For *In Vitro* Diagnostic Use.
- The TheraScreen: K-RAS Kit is not intended to screen for or to diagnose any type of cancer or colorectal cancer. It is intended to be used as an adjunct to other prognostic factors currently used to select patients who would not benefit from anti-EGFR cancer therapy.
- Therapy for cancer patients should not be based on K-RAS gene mutation status alone. A clinician should consider the mutation status of the patient alongside other disease factors.
- It is necessary to have approximately 2-3ng of DNA present in the real-time PCR assay to detect 1% mutation in a background of wild-type DNA.

- Note that tumour samples are non-homogeneous and data from a sample of tumour may not be concordant with other sections from the same tumour. Tumour samples may also contain non-tumour tissue. DNA from non-tumour tissue would not be expected to contain the K-RAS mutations detected by the K-RAS Kit.

- All assays in the K-RAS Kit give short PCR products. However, the K-RAS Kit will not work in heavily fragmented DNA. Extra control reaction mix is supplied for assessing quality and quantity of the DNA, if required.

- The concentration of DNA required to detect 1% mutation is based on PCR quantification and may differ from quantification based on Optical Density readings. Additional control reaction mix is supplied to allow assessment of the sample before running the K-RAS Kit, if required.

- Reagents for the K-RAS Kit have been diluted optimally. Further dilution of the reagent is not recommended and will result in a loss of performance. Use of less than 25µl reaction volumes is not recommended and will increase the risk of false negatives.

- All reagents in the K-RAS Kit are formulated specifically for use with the stated tests. No substitutions should be made to the K-RAS Kit or reagents if optimal performance is to be maintained.

- To ensure optimal activity and performance Scorpions primers (as with all fluorescently labelled molecules) should 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.

- Each assay included in the K-RAS Kit has its own characteristics. Calculation of the result must be made with reference to the correct assay parameters, (see Table 5: Mixed standard ΔCt values and Sample 1% ΔCt values).

- If a sample gives a positive result where the mutation Ct is greater than 38 the assay must be repeated in triplicate and all replicates must be positive for the sample to be classed as mutation positive.

- 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 that could lead to false negative results. Diluting the sample may reduce the effect of inhibitors but it should be noted that this would also dilute down the DNA.

- General laboratory precautions should be used, including but not limited to:
  a) Do not pipette by mouth
  b) Do not smoke, eat or drink in areas where specimens or kit reagents are being handled
  c) Wash hands after performing the test

- Care should be taken when using *Taq* polymerase (*Taq*) due to the viscosity, as an erroneous pipetting technique can lead to an incorrect volume of *Taq* being added to the

reaction mix. Please read and use the following guidelines for good laboratory techniques when using the TheraScreen: K-RAS Mutation Kit:

a) For each kit, only use the *Taq* that is provided in that kit, do not mix and match from other kits of the same or any other type.

b) Do not substitute the *Taq* for *Taq* from another supplier.

c) Only thaw the reagents required for each run, do not thaw the whole kit each time to minimise the amount of freeze/thaw cycles.

d) 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 the tip getting coated in excess *Taq*.

e) 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.

Safety Information

Caution: All chemicals and biological material should be considered as potentially hazardous. Specimens are potentially infectious and should be treated accordingly.
This K-RAS Kit should be used only by those persons who have been trained in the appropriate laboratory techniques. When working with the components of this K-RAS Kit always wear a suitable lab coat, disposable gloves and safety glasses. After use, K-RAS Kit components should be disposed of as clinical waste.

5. Storage, Stability and Shipping Conditions

Storage

All the contents of this K-RAS Kit should be stored immediately upon receipt at -18°C to -25°C, in the dark, in a constant temperature freezer. Avoid unnecessary freeze thawing of the contents of the K-RAS Kit.

Stability

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

The contents of the K-RAS kit may be freeze-thawed up to 8 times without an adverse effect on assay performance. DO NOT freeze-thaw the K-RAS Kit reagents more than 8 times.

Shipping Conditions

The contents of the K-RAS Kit are shipped on dry ice and should still be frozen on arrival. If the K-RAS Kit is not frozen on arrival, the outer packaging has been opened during transit, the shipment does not contain a packing note, instructions for use or the reagents please contact QIAGEN Technical Services; see Section 12.

6. Instrument

Refer to the instrument user manual for complete instructions on set-up, calibration, and use of the real-time PCR instrument.

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

NOTE: The ABI 7500 instrument has been validated for use with the K-RAS kit. If another instrument is used, its performance with the K-RAS Kit must be validated before reporting patient results.

7. Specimens

Specimen material must be human genomic DNA, extracted from formalin-fixed paraffin embedded colorectal tumour samples.

It is necessary to have approximately 2-3ng of DNA present in the real time PCR assay, to detect 1% mutation in a background of wild type DNA.

To achieve 2-3 ng of DNA, 5-10 μm sections from 3-5 pre-cut, adjacent slides should be used. It is sufficient to scrape all the tissue material from all of the slides and use that total tissue volume for DNA extraction.

Specimen Collection and Preparation

1. Tissue Specimen: Formalin fixed paraffin embedded tissue, at least three pre-cut adjacent slides, at 5-10μm sections (one H&E stained, two unstained). The H&E stained slide enables pathologist review of the sample, to ensure that the material contains tumour cells, and should be reviewed before undertaking K-RAS testing to estimate the area of tissue in the sample and the percentage of tumour content. The unstained slides are used for K-RAS testing.

2. Specimen Transport: Standard pathology methodology to ensure specimen quality. Typically, transport at ambient conditions is sufficient to preserve specimen quality. "Ambient conditions" are considered to be temperatures between 15-30°C. If extremes of temperature are anticipated, insulate the specimen appropriately and be sure to utilise overnight transport if shipping is required.

3. Sample Extraction Process: Pre-treat the sample as specified below, and then extract DNA from the sample using any commercially available DNA extraction kit. If the DNA extraction kit includes a pre-treatment step, then you may skip this step and go directly to the DNA extraction step.

    a. Pre-treatment:

    - First remove any visibly excess paraffin from the slide by scraping with a sterile scalpel.
    - Remove the tissue sample from the slide by scraping the tissue sample into a sterile microcentrifuge tube, using a fresh sterile scalpel for each slide scraped.
    - Add 1 mL xylene to the microtube, close the tube, and vortex vigorously for 10 sec. (The xylene removes the paraffin from the tissue sample)
    - Centrifuge the sample in the microtube at full speed for 2 min. at room temperature.
    - Carefully remove the supernatant by pipette. Do not disturb the pellet.
    - Add 1mL 96-100% ethanol to the pellet, close the tube, and mix by vortexing. (The ethanol removes any residual xylene.)
    - Centrifuge the sample in the microtube at full speed for 2 min. at room temperature.

- Carefully remove the supernatant by pipette. Do not disturb the pellet.
- Open the tube and incubate at room temperature (15–25°C) or up to 37°C for 10 min or until all residual ethanol has evaporated

b. DNA extraction: follow the instructions for use for the DNA extraction kit used. QIAGEN has validated the QIAamp FFPE extraction kit for use with the K-RAS Kit, and also recommends the QIAamp Mini Kit for acceptable DNA extraction. NOTE: it is important that the sample be fully digested before K-RAS analysis. QIAGEN recommends 48 hours of Proteinase K digestion to ensure complete sample digestion.

4. Determination of DNA concentration, yield, and purity: DNA yields are determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7–1.9.
   - Absorbance readings at 260 nm should lie between 0.1 and 1.0 to be accurate.
   - Sample dilution should be adjusted accordingly. Use elution buffer or water (as appropriate) to dilute samples and to calibrate the spectrophotometer.
   - Measure the absorbance at 260 and 280 nm, or scan absorbance from 220–320 nm (a scan will show if there are other factors affecting absorbance at 260 nm). Both DNA and RNA are measured with a spectrophotometer. To measure only DNA, a fluorometer must be used.

5. Extracted DNA storage: Store at 4°C (up to 24 hour), or at -20°C (long term), prior to analysis. Use a tightly capped, hydrophobic, plastic tube for storage, preferably with a rubber gasket to prevent evaporation. Polyallomer and specially designed polypropylene tubes have been shown to be acceptable storage tubes for DNA samples. (CLSI Approved Guideline MM13-A.)[22]

Accepting / rejecting human genomic DNA sample

The control assay supplied with the K-RAS Kit is used to assess the total DNA in a sample. The control assay amplifies a region of exon 4 of the K-RAS gene.

To assess the total DNA in a sample, extract the DNA as specified above and run the K-RAS control assay on a microplate using the extracted sample. Be sure to reserve enough sample to run the full K-RAS kit later. See Section 8, K-RAS Mutation Detection Protocol for instructions on running the K-RAS test. Use only the K-RAS control reaction mix in the sample wells; do not use the K-RAS mutation reaction mixes. See Appendix I for suggested layout.

Rejection of sample: Control assay Ct $\geq$ 35. This means that only a few amplifiable copies of DNA are present in the sample and mutations are only likely to be seen if most copies are mutated. Therefore, there is not enough DNA in the sample to obtain a reliable result from the K-RAS Kit.

8. K-RAS Mutation Detection Protocol

❖ **Read these instructions carefully and become familiar with all components of the K-RAS Kit prior to use.**

If required, the control assay can be used to assess the optimal dilution of the sample DNA. This prevents waste of K-RAS Kit reagents on samples containing little or no amplifiable DNA. Additional control reaction mix is supplied for this purpose. See Section 7 and Section 9 for information on accepting / rejecting specimens based on the Control assay results.

Testing of Extracted DNA Samples

For each DNA sample the control and mutation assays must be analysed on the same PCR run to avoid run-to-run variations in threshold settings. It is recommended that the mixed standard be analysed on all PCR runs, along with no template controls (i.e. water blank).

1. Thaw the reaction mixes and mixed standard from the K-RAS Kit. Mix each solution by inverting 10 times, once thawed, to avoid localised concentrations of salts. Prepare sufficient mixes for the DNA samples, the mixed standard and no template controls, plus an excess of 2 reactions per mix as shown in Table 3.

Table 3: Test Reaction Set up: volumes presented are for one reaction

Assay	Master Mixes	
	Reaction Mix (µl)	*Taq* (µl)
Control	19.8	0.2
K-RAS assays	19.8	0.2

2. Add 20 µl of control reaction mix to each of the control reaction wells. Add 20 µl of the mutation reaction mix to each of the mutation reaction wells, (see Appendix II for suggested sample layout). Each DNA sample must be tested with both the control and mutation assays.

3. Add 5µl of test sample, mixed standard or water (for the no template controls) to the control and mutation reaction wells. It is recommended that no more than 20ng of amplifiable template be added to each reaction.

4. Spin the PCR plate briefly to collect the reagents at the bottom of the wells.

5. Seal the PCR plate/tubes and place them into the real time PCR instrument

6. Carry out real time PCR using the same cycling conditions as described in Table 4.

Table 4: Cycling Parameters

Temperature	Time	Cycles	Data Collection
Stage 1			
95°C	4 min	1	
Stage 2			
95°C	30 sec		
60°C	1 min	40	FAM, JOE

Sample Analysis

Factors requiring consideration:

1. Ensure that the passive reference is set to 'none' in the well inspector screen.

2. Check that each well gives a JOE signal from the exogenous control assay.
   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 amplified strongly, 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. These inhibitors could lead to false negative results.

3. **Using the ABI7500 instrument:** In the Amplification Plot tab select all the wells in use and select the FAM dye from the detector drop down menu. Use the automatic baseline setting and then set the threshold manually in the middle of the exponential phase, using the log scale for the Y axis, as described in the ABI 7500 user guide.

4. **If using another real time PCR instrument:** follow the instrument's instructions for use to plot the data.

5. Analyse the data and calculate the ΔCt value as follows
   [sample mutation assay Ct] – [sample control assay Ct] =ΔCt

9. Expected Values and Interpretation of Results

NOTE: The information presented here applies to the use of the ABI 7500 real time PCR instrument to run the assay and calculate results. If using a different real time PCR instrument, you must determine the Control Ct, Mixed Standard ΔCt, and 1% ΔCt values specific for that instrument.

1. Control Ct values:
   - ❖ The control Ct value must be ≥ 20 to avoid overloading the assay.
   - ❖ Control Ct ≤ 29: the K-RAS Kit is capable of detecting as little as 1% mutation in these samples.
   - ❖ In samples with a control Ct ≥ 29, the kit will not detect as low as 1% mutation, but will be able to detect higher level mutations.
   - ❖ Control Ct ≥ 35, only a few amplifiable copies of DNA are present in the sample and mutations are only likely to be seen if most copies are mutated. The sample should be rejected if control Ct is ≥ 35.

2. Mixed Standard ΔCt value

The mixed standard ΔCt value should be as given in Table 5 below, but variation of ±2 may occur due to different threshold settings on different instruments.

Table 5: Mixed Standard ΔCt values and Sample 1% ΔCt values

Assays	Mixed Standard ΔCt	Sample 1% ΔCt
12ALA	-0.1	6.5
12ASP	-0.65	8
12ARG	0.45	8
12CYS	-0.3	7
12SER	0.49	9
12VAL	0.44	6.5
13ASP	-1.26	9

3. Sample ΔCt value:
   - ❖ If the sample ΔCt is greater than the 1% value (as indicated in Table 5) the DNA sample is classed as mutation negative or below the limits of the K-RAS Kit.
   - ❖ If the sample ΔCt is less than the 1% value the DNA sample is classed mutation positive.
   - ❖ DNA samples that give a ΔCt close to the 1% value (i.e., that is between the 1% ΔCt value and the 1% ΔCt value minus one) should be repeated in triplicate and all replicates should be in the positive zone for the sample to be classed as mutation positive.

❖ DNA samples with a mutation Ct ≥ 38 should also be repeated in triplicate and all replicates should be in the positive zone for the sample to be classed as mutation positive.

10. Limitations of the Test

It is necessary to have approximately 2-3ng of DNA present in a real-time PCR assay to detect 1% mutation in a background of wild-type DNA. This gives a control Ct of approximately 29. The assays will not be able to detect 1% mutation in samples with control Ct values of greater than 29.

The concentration of DNA required to detect 1% mutation is based on PCR quantification and may differ from quantification based on Optical Density readings. Additional control reaction mix is supplied to allow assessment of the sample Cts before running the kit, if required.

If a sample gives a mutation positive result where the mutation Ct ≥ 38 the assay must be repeated in triplicate and all replicates must be positive for the sample to be classed as mutation positive.

Some cross-talk may occur between mutation reactions. For example, if a high level 12ALA mutation is seen some of the other mutation reactions will also give a positive result. This is due to the ARMS primers detecting other mutations within a few bases of one another. On synthetic control material the cross-talk forms a readable pattern that allows the true positive to be determined from several signals (see Table 6).

Table 6: Crosstalk Pattern from Synthetic Control Material

Yes indicates the true signal. Numbers indicate the approximate number of cycles after the true signal, that a cross-talk signal may be seen. Beyond 9 cycles values have not been entered as these will be in the negative zone.

Positive Sample	12ALA Signal	12ASP Signal	12ARG Signal	12CYS Signal	12SER Signal	12VAL Signal	13ASP Signal
12ALA	Yes	9	-	6	3	6	-
12ASP	9	Yes	-	-	-	-	-
12ARG	-	-	Yes	-	-	-	-
12CYS	-	7	4	Yes	-	-	-
12SER	9	6	9	-	Yes	-	8
12VAL	4	-	-	-	-	Yes	-
13ASP	-	-	-	-	-	-	Yes

Note: The cross-talk pattern may be different on DNA samples, e.g. paraffin embedded DNA samples. The K-RAS Kit is designed to detect one mutation in a DNA sample. If a second mutation assay gives a positive result this is likely to be cross-talk.
Although rare, double mutants have been observed, if the pattern does not fit the cross-talk pattern, further investigation may be required.

11. Assay Performance Characteristics

The TheraScreen: K-RAS Mutation Kit has been characterized for batch to batch precision, accuracy, linearity, limit of detection, breakthrough, cross-talk, cycling tolerance, and *Taq* tolerance. In addition, studies were performed to establish the 1% cut-off and the mixed standard ΔCt values.

Performance characterization studies were carried out on 3 batches of kits. Each test protocol utilized four different mixed mutation-positive templates as the samples, and the mixed standard was used as an additional test sample. The standard mutation detection protocol as outlined in this package insert was used to test the samples. The studies were run on the ABI 7500 real-time PCR instrument.

1% Cut-off Validation: The detection of 1% mutation in background genomic material was determined over a range of three different concentrations of genomic wild-type DNA to assign a cut-off ΔCt value for each mutation assay.

For each assay, 1% dilutions were tested in triplicate, for each kit batch, on 3 separate runs, for both the control assay and mutation assay. The mixed standard was used on each plate to ensure that the assays were performing within the specified criteria.

The 1% ΔCt values were calculated from averaged Ct values over the replicates within a run and batch. The cut-off ΔCt values were set as the average value (to the nearest 0.5) over all batches, runs and concentrations, for concentrations where all replicates gave a Ct value. The results of this testing are summarized in Table 7 below.

Table 7: 1% Cut-Off Values Validation Results

Assay	Avg	1% ΔCt Values
12ALA	6.68	6.5
12ASP	8.2	8
12ARG	8.16	8
12CYS	6.94	7
12SER	8.83	9
12VAL	7.67	7.5*
13ASP	8.89	9

* The 1% cut-off for 12VAL was changed to 6.5 based on the Breakthrough study. See discussion below.

Mixed Standard ΔCt Values Validation:

Each kit batch was tested in at least triplicate on a minimum of 3 plates. With a minimum of 3 control Cts and 3 test Cts, at least 9 ΔCts were determined for each run. A statistical analysis of variation was performed to ensure that variation within and between batches and runs was acceptable.

In addition, a series of 50 runs, incorporating at least three batches of mixed standard, was performed over the course of 6 weeks. This large data series was used to determine the mean ΔCt for each assay and the variation about that mean.

The expected ΔCt values for the mixed standard are shown in Table 8 below.

Table 8: Mixed Standard ΔCt Values Validation Results

Assay	Mixed Standard ΔCt
12ALA	-0.1
12ASP	-0.65
12ARG	0.45
12CYS	-0.3
12SER	0.49
12VAL	0.44
13ASP	-1.26

Batch to Batch Precision Validation: Experiments were performed to establish the precision performance of each assay within a day, between days, between operators, and between instruments.

For each day of testing, 6 plates were tested, 2 plates for each batch of kit reagents. Each sample on a plate was tested in duplicate. One plate for each batch was run each morning, and the 2nd plate was run each afternoon. Each plate contained all 8 assays in the kit. These data gave within run and within day precision results. Testing was performed on each of 5 days by the same operator on the same PCR instrument for a total of 30 runs (6 plates/day x 5 days) for the 3 batches of reaction mixtures, or a total of 10 runs/batch of reaction mixture. These data gave between-day precision results. A second operator also performed the same test protocol (2 plates/day/batch x 5 days) on the same ABI 7500 instrument, for between-operator results. The entire test protocol was also performed on a 2nd ABI 7500 instrument, for between-instrument results.

The samples used for the precision study were:
1. Mixed standard H (100% H) - the K-RAS mixed standard, which is used in the TheraScreen kits as a positive control.
2. Mixed standard L (100% L) – the K-RAS mixed standard diluted down into a final concentration of 0.6 ng/µl of genomic DNA.
3. 10% H – the "Mixed Standard H" diluted 1/10 into 2 ng/µl of genomic DNA.
4. 10% L – The "Mixed Standard L" diluted down 1/10 into a final concentration of 0.6 ng/µl genomic DNA.

A one-way ANOVA was used to assess the variation between replicates, batches of reagent, runs/day, day of testing, instrument, and operator. The null hypothesis was that the average mean values across categories of BATCH were equal; the p-value required to invalidate the null hypothesis was selected to be 0.01 or less. Total means, SD, and %CV were also calculated for the Ct values. All reagents performed acceptably. The data are summarized in Tables 9-1 to 9-8 below.

Table 9-1: Batch to Batch Precision Validation Results – 100%H samples - ANOVA

Assay	Avg Mean			p
	Batch 1	Batch 2	Batch 3	
12ALA	28.37	28.38	28.21	0.184
12ASP	29.72	29.80	29.74	0.722
12ARG	29.71	29.77	29.62	0.316
12CYS	28.49	28.66	28.48	0.116
12SER	29.01	29.24	28.98	0.252
12VAL	28.94	29.03	28.88	0.500
13ASP	30.67	30.96	30.89	0.103
Control	27.75	27.75	27.53	0.0488

Table 9-2: Batch to Batch Precision Results – 10%H samples – ANOVA

Assay	Avg Mean			p
	Batch 1	Batch 2	Batch 3	
12ALA	32.13	32.12	31.97	0.149
12ASP	33.25	33.30	33.21	0.697
12ARG	33.53	33.55	33.37	0.119
12CYS	32.32	32.46	32.29	0.145
12SER	33.33	33.68	33.46	0.025
12VAL	33.05	33.08	32.93	0.277
13ASP	34.79	35.04	34.87	0.107
Control	27.78	27.74	27.56	0.0579

Table 9-3: Batch to Batch Precision Results – 100%L samples – ANOVA

Assay	Avg Mean			p
	Batch 1	Batch 2	Batch 3	
12ALA	30.31	30.31	30.13	0.044
12ASP	31.52	31.59	31.53	0.780
12ARG	31.72	31.73	31.57	0.138
12CYS	30.56	30.73	30.52	0.079
12SER	31.43	31.76	31.58	0.032
12VAL	31.34	31.38	31.21	0.127
13ASP	32.83	33.08	33.06	0.143
Control	29.81	29.78	29.58	0.012

Table 9-4: Batch to Batch Precision Results – 10%L samples – ANOVA

Assay	Avg Mean			p
	Batch 1	Batch 2	Batch 3	
12ALA	33.71	33.87	33.64	0.158
12ASP	35.02	35.10	34.99	0.606
12ARG	35.23	35.33	35.19	0.539
12CYS	33.97	34.26	34.06	0.039
12SER	35.14	35.52	35.27	0.015
12VAL	34.83	34.98	34.81	0.288
13ASP	36.46	36.85	36.57	0.030
Control	29.52	29.37	29.24	0.021

Table 9-5: Batch to Batch Precision Results – 100%H samples – Total Precision

	Control	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
n	165	168	168	167	168	168	168	166
Mean	27.70829	28.31939	29.75351	29.70177	28.54065	29.07480	28.94969	30.79221
95% CI	27.63105	28.23306	29.66328	29.62144	28.46276	28.93973	28.84352	30.69715
SE	0.039115	0.043725	0.045704	0.040686	0.039452	0.068414	0.053779	0.048143
Variance	0.25245	0.32120	0.35092	0.27645	0.26148	0.78631	0.48589	0.38475
SD	0.50244	0.56674	0.59239	0.52578	0.51135	0.88674	0.69706	0.62028
CV	1.8%	2.0%	2.0%	1.8%	1.8%	3.0%	2.4%	2.0%

Table 9-6: Batch to Batch Precision Results – 10%H samples – Total Precision

	Control	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
n	168	168	168	168	168	167	168	166
Mean	27.69197	32.06099	33.25227	33.48605	32.35377	33.52165	33.02174	34.89994
95% CI	27.61346	31.98060	33.16715	33.41029	32.28101	33.40961	32.94472	34.80051
SE	0.039765	0.040717	0.043113	0.038376	0.036851	0.056746	0.039014	0.050361
Variance	0.26566	0.27852	0.31227	0.24742	0.22814	0.53777	0.25571	0.42101
SD	0.51542	0.52775	0.55881	0.49741	0.47764	0.73333	0.50567	0.64886
CV	1.9%	1.6%	1.7%	1.5%	1.5%	2.2%	1.5%	1.9%

Table 9-7: Batch to Batch Precision Results – 100%L samples – Total Precision

	Control	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
n	168	168	168	168	167	168	168	166
Mean	29.72715	30.25226	31.54978	31.67571	30.58367	31.62098	31.31043	32.93542
95% CI	29.65851	30.18394	31.46721	31.60315	30.51165	31.51270	31.24240	32.84847
SE	0.034765	0.034607	0.041823	0.036754	0.036476	0.054844	0.034457	0.044039
Variance	0.20305	0.20120	0.29386	0.22695	0.22219	0.50532	0.19946	0.32195
SD	0.45061	0.44855	0.54209	0.47639	0.47137	0.71086	0.44661	0.56741
CV	1.5%	1.5%	1.7%	1.5%	1.5%	2.2%	1.4%	1.7%

Table 9-8: Batch to Batch Precision Results – 10%L samples – Total Precision

	Control	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
n	168	166	166	166	166	164	166	164
Mean	29.37546	33.78964	35.04205	35.29890	34.14460	35.30912	34.92557	36.67824
95% CI	29.29591	33.71518	34.95603	35.22509	34.07390	35.20165	34.85697	36.57541
SE	0.040293	0.037710	0.043565	0.037381	0.035808	0.054429	0.034740	0.052078
Variance	0.27275	0.23606	0.31506	0.23196	0.21285	0.48586	0.20034	0.44479
SD	0.52225	0.48586	0.56130	0.48163	0.46136	0.69704	0.44759	0.66693
CV	1.8%	1.4%	1.6%	1.4%	1.4%	2.0%	1.3%	1.8%

Accuracy Validation: Accuracy was assessed using synthetic controls diluted into 2 levels of genomic DNA to give 3 different percentages of mutation covering both positive and negative mutation levels. Three (3) identical plates, each using all 3 validation reagent batches, were run for each mutation. The 3 mutation percentages used were 25%, 5% and 0.5%, in 2 different levels of DNA. (The 25% and 5% samples should give a mutation positive result (ΔCt below the 1% cut-off values); the 0.5% sample should give a mutation negative result (ΔCt above the 1% cut-off values)). The results are summarized in Table 10 below.

Table 10: Accuracy Validation Results

Assay	25%	5%	0.5%
12ALA	100%	100%	94%
12ASP	100%	100%	100%
12ARG	100%	100%	100%
12CYS	100%	100%	100%
12SER	100%	100%	100%
12VAL	100%	100%	100%
13ASP	100%	100%	100%

Linearity Validation: This study assessed the efficiency of each of the mutation assays across a range of mutant DNA concentrations in a background of wild type DNA and in water. Each mutation assay should have an efficiency of 90 - 110% (100% $\pm$ 10%).

One PCR plate containing all three batches of kits was run for each mutation assay. The 100% mutation samples were tested in 50 ng, 10 ng, 2 ng, and 0.4 ng DNA for each of the mutation assays. A 'breakthrough' control was also tested, where the background DNA was maintained at 10 ng/uL genomic DNA but the mutation was diluted in a 5 fold serial dilution. This kept the breakthrough level constant across all dilutions.
Standard curves were generated for each mutation assay, from the experimental data. The PCR efficiencies were calculated using the equation:

$$100((10^{-1/Slope})-1)$$

Overall efficiencies across reagent batches were also calculated.

Linearity was maintained acceptably over the range of 0.4 ng DNA to 50 ng DNA.

Limit of Detection Validation: These data were generated to determine if the assay could reproducibly detect 1% mutation in low concentrations of wild-type DNA.

Three PCR plates were run for each mutation assay; each plate contained all 3 reagent batches, and each mutation assay was run in replicates of 20 1% standards on each plate for a total of 60 replicates per assay. The 1% cut-off testing showed that 2 ng DNA should give a mutation positive result; if 2 ng DNA did not give a reproducible mutation positive result, then the testing was repeated at 3 ng of DNA and the overall failure rate (mutation assays without a Ct value) was required to be less than or equal to 10%. Table 11 below summarizes the Limit of Detection for each mutation assay.

Table 11: Limit of Detection Validation Results

Assay	Limit of DNA (ng per PCR)
12ALA	2
12ASP	3
12ARG	2
12CYS	2
12SER	3
12VAL	2
13ASP	3

Breakthrough Validation: Breakthrough is defined as the non-specific amplification of a wild-type DNA target present in a particular sample. This causes a measurable level of background noise which can be distinguished from a true positive signal. The breakthrough from wild-type DNA was assessed for each mutation assay. The breakthrough study ensured that the 1% cut-off values previously established were above the breakthrough level and would prevent reporting a false-positive result.

Three identical PCR plates were prepared and tested; each plate included all 3 validation batches of reagents. The Mixed Standard was used as the positive control. All other dilutions were negative for the mutation tested. In addition, extracted PET (paraffin-embedded tissue) samples were tested because these samples tend to show worse levels of breakthrough.

The results from cell lines DNAs and PET samples gave results that were all above the 1% cut-off values indicating that the 1% cut-off values were robust. Breakthrough was detected in the 12VAL assay for one PET sample, so the 1% ΔCt value for the 12VAL assay was changed from 7.5 to 6.5 to avoid the possibility of a false positive result. Table 12 below shows the current 1% ΔCt values, based on the breakthrough validation results.

Table 12: Breakthrough Validation Results

Assay	1% ΔCt values
12ALA	6.5
12ASP	8
12ARG	8
12CYS	7
12SER	9
12VAL	6.5
13ASP	9

Cross-talk Validation: Since all the mutations detected by the TheraScreen: K-RAS Mutation Kit occur within a 5 base pair region, it is expected that some cross-talk between primers would occur. This study determined the cross-talk pattern for the TheraScreen: K-RAS Mutation Kit.

Testing was performed on one of the validation batches, using synthetic mutation controls to establish how many cycles after a true positive a cross-talk signal could be detected. Each mutation reaction mix was run with six replicates of the seven individual 100% mutation standards. Two reaction mixes were tested per plate. Two replicates of the control reaction mix were also included on each plate. In addition, each cassette was assayed with the matched reaction mix and all other reaction mixes on the same run. This experimental layout is identical to the recommended layout for samples. It allowed easier interpretation of the cross-talk pattern expected if a positive sample is detected.

The cross-talk pattern was determined by calculating the difference between the real amplification Ct value from the matched cassette and the cross-talk signal from every other

primer. The "worst case scenario" of the 2 experimental designs was used as the cross-talk pattern for the TheraScreen: K-RAS Mutation Kit.

The expected cross-talk pattern is shown in Table 13 below. The cross-talk pattern has been shown to be consistent and will allow the user to read a pattern of amplification, where more than one assay gives a positive result, to obtain the correct assay result. The numbers in each cell indicate the approximate number of cycles after the true positive signal is detected that a cross-talk signal may be seen. Values above 9 cycles have not been included because these are classified as mutation negative results.

Table 13: Cross-talk Validation Results

Positive Sample	12ALA signal	12ASP signal	12ARG signal	12CYS signal	12SER signal	12VAL signal	13ASP signal
12ALA	Yes	9	-	6	3	6	-
12ASP	9	Yes	-	-	-	-	-
12ARG	-	-	Yes	-	-	-	-
12CYS	-	7	4	Yes	-	-	-
12SER	9	6	9	-	Yes	-	8
12VAL	4	-	-	-	-	Yes	-
13ASP	-	-	-	-	-	-	Yes

Cycling Tolerance Validation: These studies determined the tolerance of the mutation assays to variations in cycling temperature, at ± 2°C from the recommended temperature of 60°C. Since the annealing temperature is the most critical temperature that affects assay performance, the annealing temperature is the parameter that was varied.

Three identical PCR plates, each containing the same 3 kit reagent batches, were run at each annealing temperature. The Mixed Standard was tested, as was 50ng of cell line DNA. Since 60°C is the recommended annealing temperature, the plates were run at 58°C, 60°C, and 62°C. If the assays were robust enough to tolerate the experimental temperatures, the Mixed Standard ΔCt values should be within ±1.5 of the set values for the Mixed Standard.

All of the Mixed Standard ΔCt values performed acceptably at all 3 temperatures, showing that the assays are robust when run at temperatures 2°C higher or lower than optimal.

***Taq* Polymerase Tolerance Validation:** These studies determined the tolerance of the mutation assays to variations in the level of *Taq* polymerase, a potential source of user variation since this reagent is added by the user.

Three identical PCR plates, each containing all 3 reagent batches, were run for each mutation assay, with the *Taq* volume varied at +10% and -10% from the recommended amount. The recommended amount was also tested.

The Mixed Standard was tested, as were 1% dilutions of each of the Mutation Standards in 2 ng genomic DNA and in 0.6 ng genomic DNA, as well as 50 ng cell line DNA. The assay data were analyzed for mean, for standard deviation, and for coefficient of variation.

The results showed little variation (equivalent SD between assays, %CV <10%) in assay results when the *Taq* level was varied by ±10%, showing that the assays could tolerate as much as a 10% variation in *Taq* reagent levels.

12. Technical Assistance

For technical assistance and more information, please see our Technical Support Centre at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors available on the QIAGEN website on www.qiagen.com.

13. Manufacturer Details

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

This kit is now distributed by QIAGEN.

14. Date of Issuance of Last Revision

DU004a	March 2012	• Update to remove references to distributor Roche Diagnostics • Update to state DxS Ltd is now QIAGEN Manchester Ltd • Contact details changed from DxS and Roche to QIAGEN
DU004	June 2009	New Document

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Appendix I: Suggested Plate Layout – Assessment of Total DNA in a Sample

96 well layout												
Assay	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	STD	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10

Appendix II: Suggested Plate Layout – Extracted Sample Testing

96 well layout												
Assay	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A Control	STD	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
B 12ALA	STD	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
C 12ASP	STD	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
D 12ARG	STD	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
E 12CYS	STD	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
F 12SER	STD	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
G 12VAL	STD	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
H 13ASP	STD	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

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