ELUCIGENE™ FH20

Instructions For Use

The Amplification Refractory Mutation System (ARMS™) is claimed in European Patent No. 0332435, US patent No. 5595890 and corresponding world-wide patents.

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ELUCIGENE kits are developed and manufactured by Tepnel Molecular Diagnostics within quality systems accredited to ISO9001;2000 and ISO13485 (EN46001).

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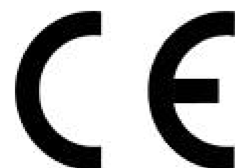

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ELUCIGENE FH20

Catalogue No. - FH020B2 – 50 tests

Intended Use

For the simultaneous *in vitro* qualitative detection of the following mutations associated with Familial Hypercholesterolaemia in human Low-Density Lipoprotein Receptor (LDLR) gene (P664L, L458P, R329X, E207X, D200G, E80K, IVS3+1G>A, D461H, ?G197, fs206, Q363X, W66G, V408M, D206E, C656R, K290RfsX20, C163Y and D461N), in human Apo B gene (R3500Q) and in human PCSK9 gene (D374Y) in human whole blood (EDTA).

Principles of the procedure

The method employed by the ELUCIGENE FH20 kit uses ARMS™ allele specific amplification technology, which detects point mutations, insertions or deletions in deoxyribonucleic acid (DNA)[(1)]. The principle of ARMS™ is that oligonucleotides with a 3' mismatched residue will not function as Polymerase Chain Reaction (PCR) primers under specified conditions. Selection of appropriate oligonucleotides allows specific mutant or normal DNA sequences to be amplified and detected.

Warnings and Precautions

1. For professional *in vitro* diagnostic use.

2. The Normal DNA Control provided in this kit has been independently tested and found to be negative for Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV) 1 and 2.

3. Care should be taken when handling material of human origin. All samples should be considered potentially infectious. No test method can offer complete assurance that HBV, HCV, HIV or other infectious agents are absent.

4. Licences for the *in vitro* diagnostic analysis of gene mutations detected by these reagents may be required and are the responsibility of the reagent purchaser.

5. Handling of samples and test components, their use, storage and disposal should be in accordance with the procedures defined by the appropriate national biohazard safety guideline or regulation.

6. Store all components below –20°C. Discard 3 months after opening unless sub – aliquoted

Nomenclature

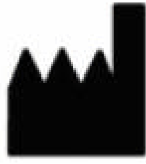
HGVS recommended nomenclature of the sequence variants is listed on Table 1

Table 1

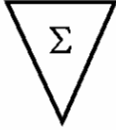
Traditional	HGVS Nomenclature Nucleotide (protein)
E80K	c.301G>A (p.Glu101Lys)
IVS3+1G>A	c.313+1G>A
ΔG197	c.654_656delTGG (p.Gly218del)
D200G	c.662A>G (p.Asp221Gly)
fs206	c.680_681delAC (p.Asp227GlyfsX12)
E207X	c.682G>T (p.Glu228X)
R329X	c.1048C>T (p.Arg350X)
Q363X	c.1150C>T (p.Gln384X)
L458P	c.1436T>C (p.Leu479Pro)
D461H	c.1444G>C (p.Asp482His)
P664L	c.2054C>T (p.Pro685Leu)
C163Y	c.551G>A (p.Cys184Tyr)
D461N	c.1444G>A (p.Asp482Asn)
W66G	c.259T>G (p.Trp87Gly)
V408M	c.1285G>A (p.Val429Met)
D206E	c.681C>G (p.Asp227Glu)
C656R	c.2029T>C (p.Cys677Arg)
K290RfsX20	c.932_933delAA (p.Lys311ArgfsX20)
R3500Q (APOB)	c.1058G>A (p.Arg3527Gln)
D374Y (PCSK9)	c.1120G>T (p.Asp374Tyr)

Symbols used on labels

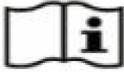
The symbols used on all labels and packaging conform to the harmonised standard EN980



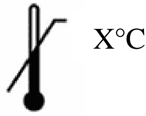
Manufacturer



Number of tests



See Instructions for Use



Store below temperature shown

Use before date shown

Catalogue code

Lot or batch number

Materials Provided

Sufficient materials for 50 tests are provided

1. 2 x 450µL vial of Primer Mix A (colour coded orange) containing primers to detect the following mutations associated with Familial Hypercholesterolaemia in the human Low-Density Lipoprotein Receptor (LDLR) and PCSK9 genes: (D374Y, P664L, L458P, R329X, E207X, D200G & E80K) and deoxynucleotide triphosphates in buffer (FH020TA).

2. 2 x 450µL vial of Primer Mix B (colour coded pink) containing primers to detect the following mutations associated with Familial Hypercholesterolaemia in the Apo B and LDLR genes (R3500Q, IVS3+1G>A, D461H, dG197, fs206 and Q363X) and deoxynucleotide triphosphates in buffer (FH020TB).

3. 2 x 450µL vial of Primer Mix C (colour coded green) containing primers to detect the following mutations associated with Familial Hypercholesterolaemia in the LDLR gene (W66G, V408M, D206E, C656R, K290RfsX20, C163Y, D461N,) and deoxynucleotide triphosphates in buffer (FH020TC).

4. 1 vial x 200µL of CR000TV Dilution Buffer (DB)

5. 1 vial x 600µL of CR000TR Loading Dye (LD)

6. 1 x 50µL vial of FH020TX DNA Control (DC), normal for the mutations detected by ELUCIGENE FH20.

Materials Required but not Provided

General

Laboratory consumables – gloves; screw-capped microfuge tubes; pipette tips; thin-walled 0.2mL PCR vials (use of three differently coloured vials will aid primer mix identification).

DNA Extraction

DNA Preparation - QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Cat No 51104) or equivalent kit.

PCR Amplification - AmpliTaq Gold® (Applied Biosystems, Cat No N8080160); good quality sterile distilled water.

Gel Electrophoresis - NuSieve® 3:1 Agarose (Lonza, Cat No 50090) and 50 Base-Pair Ladder (GE Healthcare, Cat No 27-4005-01), ethidium bromide.

Equipment Required

General

Laboratory equipment – precision pipettes (2 sets: 1 for pre-amplification and 1 for post-amplification handling:- preferably positive displacement pipettes); protective clothing; vortex mixer; microfuge; balance.

PCR Amplification - Thermal cycler to accommodate 0.2ml vials with a temperature accuracy of +/-1°C between 33°C and 100°C and static temperature uniformity of +/-1°C with heated lid.

Electrophoresis - Horizontal submarine gel tank; power pack; microwave; waterbath to cool agarose; UV transilluminator; photographic system.

Sample Collection and Storage

Whole blood (EDTA) samples should be used.

Sample collection devices have on occasion been reported to be detrimental to the integrity of certain analytes and could interfere with some method technologies[2]. It is recommended that each user ensure that the chosen device is used according to the manufacturer's instructions and both sample collection devices and alternative DNA preparation methods are compatible with this test.

Blood samples should be stored at -20°C prior to preparation of DNA. Avoid repeated freezing and thawing.

DNA Preparation

The QIAamp® DNA Blood Mini Kit is recommended

Under optimal PCR conditions results are consistently obtained with DNA extracted using the QIAamp® DNA Blood Mini Kit. For less concentrated DNA samples a higher volume of water can be used for eluting. For more concentrated DNA samples a lower volume of water can be used for eluting.

It is recommended that alternative DNA extraction methods and sample types are thoroughly evaluated with the ELUCIGENE FH20 test prior to the results being used for diagnostic use

DNA Concentration

Under optimal PCR conditions results are consistently obtained at DNA concentrations between 5 and 50ng/5µL. Testing of DNA samples at concentrations <1ng/µL is not recommended.

Amplification Procedure

Note: To minimise the risk of contamination, steps 4 - 7 must be carried out in an area free from DNA. Steps should also be taken to avoid contamination with PCR product.

1. Program the thermal cycler for a single step cycle to activate the AmpliTaq Gold at 94°C for 20 minutes linked to an amplification cycling program of 30 seconds at 94°C (denaturation), 1 minute at 61°C (annealing) and 1 minute at 72°C (extension) for 35 cycles. This should be linked to a 30-minute time-delay file at 72°C (extension) on the final cycle.

2. A negative control must be included in each PCR run.

3. Thaw and centrifuge the Primer Mixes (TA,TB,TC), AmpliTaq Gold (not provided), Dilution Buffer (DB) and Loading Dye (LD) vials for 10 seconds at 12 000g, mix gently by vortexing and centrifuge the vials again for 10 seconds.

4. Prepare sufficient dilution of the AmpliTaq Gold with the Dilution Buffer and Loading Dye supplied in sterile distilled water for the number of samples and controls to be tested. For 10 samples or controls pipette 63µL sterile deionised water, 18µL Dilution Buffer, 90µL Loading Dye and 9µL AmpliTaq Gold into a microfuge tube. Mix the enzyme dilution thoroughly by gently pipetting up and down.

5. Prepare a reaction mix by adding the appropriate volume of Primer Mix to the enzyme dilution from step 4. For 10 samples or controls separately pipette 165µL of each Primer Mix (TA, TB and TC) into the 55µL of enzyme dilution prepared in step 4 into labelled tubes. Mix thoroughly by gently pipetting up and down.

6. Pipette 20µL of each reaction mix into the bottom of the appropriate number of thin-walled PCR vials and re-cap.

Note: Coloured PCR vials should be used to distinguish reaction mixes of primers TA, TB and TC.

7. Label one of each coloured PCR vial for each sample or control.

8. Using separate pipette tips each time, add 5µL of test sample to each of the three coloured PCR vials and re-cap firmly. Do not add DNA to the PCR vial for the negative control, add 5uL of sterile deionised water..

9. Centrifuge the PCR vials for 10 seconds at 12 000g.

10. Place all vials firmly in the thermal cycler block. Initiate the 94°C single step cycle followed by the amplification cycling program.

11. Discard all the remaining unused AmpliTaq Gold dilution.

12. On completion of the amplification cycling program, the samples may be stored at room temperature overnight or at 2-8°C for up to 7 days before analysis by gel electrophoresis.

Gel Electrophoresis

It is recommended that each user ensure that the chosen equipment is used according to the manufacturer's instructions and is compatible with this test. In this context the key parameters are the gel matrix and comb (well former) dimensions. Results have been obtained using the following electrophoresis conditions:

1. PCR product was electrophoresed in a 3% NuSieve 3:1 agarose gel using tris-borate with ethidium bromide (TBE/EtBr) as running buffer. TBE/EtBr was prepared as 134mM (16.2g/L) Tris-base, 74.9mM (4.63g/L) boric acid, 2.55mM (0.95g/L) EDTA buffer with 0.1µg/mL ethidium bromide.

2. 3g of NuSieve 3:1 were dissolved in 100mL TBE/EtBr and poured into a 15 x 12cm horizontal gel tray with 1.5mm x 5mm well formers suspended 1mm above the base.

3. 15µL of the PCR product (with the Loading Dye added during the PCR set-up process) were loaded on a gel.

4. A 50 Base-Pair Ladder (GE Healthcare) at 1.5µg/15µL was prepared in the Loading Dye supplied (80µL distilled water / 10µL Loading Dye / 10µL 50 Base-Pair Ladder). Note that the mix may be orange in colour before gel loading. 15µL of this dilution was loaded on the gel and run adjacent to samples as a molecular weight marker.

5. Electrophoresis was carried out at 5 to 6 V/cm [(a)] between electrodes until the dye front had migrated 5cm from the loading wells toward the anode (1.5 to 2 hours).

6. After electrophoresis the gels were placed on a UV transilluminator at 260nm then visualised and photographed.

[a] Calculated using the distance in cm between the electrodes.

Interpretation of results

PCR products were observed as bands in the vial tracks of the gel.

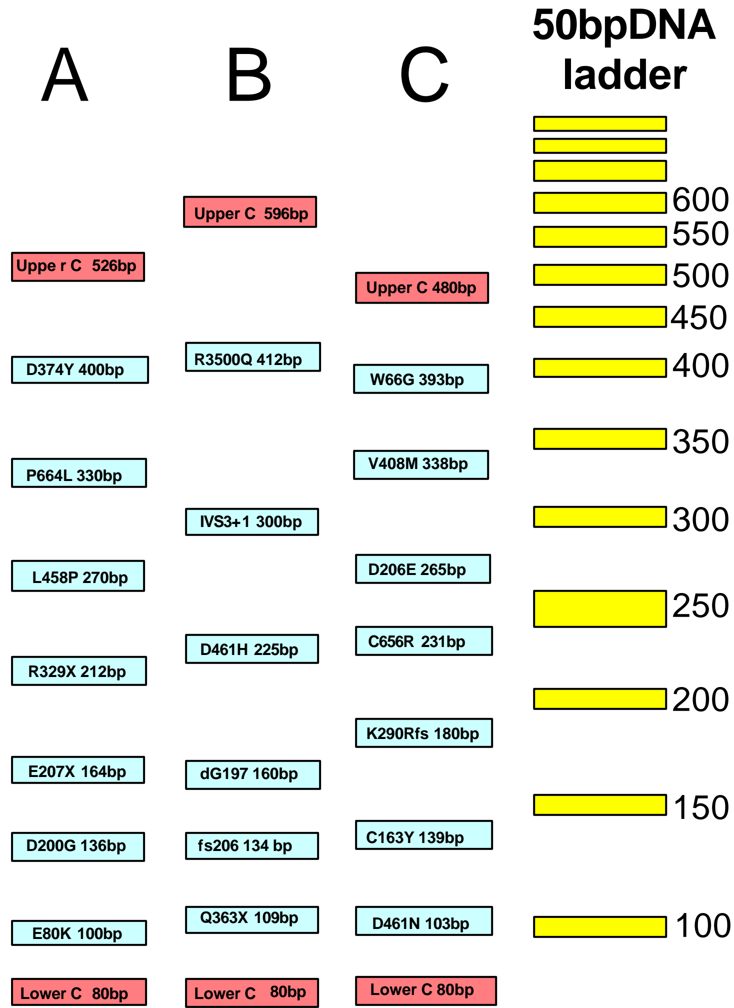
1. The upper & lower control bands must be **clearly** visible in all samples (see Figure 1).
2. All tracks should be free of excessive smearing and background fluorescence.
3. The position of the upper and lower control bands should indicate the correct molecular size (see Figure 1).
4. The negative control should show no bands within the area defined by the upper and lower control bands. A diagnostic band should not be interpreted if a similar band is also seen in the negative control for that PCR run as this is indicative of contamination with genomic DNA.

If any of the above points are not observed the results should not be interpreted and a repeat test carried out.

5. PCR products from an individual carrying any of the D374Y, P664L, L458P, R329X, E207X, D200G & E80K mutations will be observed as bands in the vial A track and are identified by comparison of the position of each band with an adjacent marker track. The product band sizes in base pairs (bp) are shown in Figure 1. Only product bands of the correct size should be interpreted.
6. PCR products from an individual carrying any of the R3500Q, IVS3+1G>A, D461H, dG197, fs206 and Q363X mutations will be observed as bands in the vial B track and are identified by comparison of the position of each band with an adjacent marker track. The product band sizes in base pairs (bp) are shown in Figure 1. Only product bands of the correct size should be interpreted.
7. PCR products from an individual carrying any of the W66G, V408M, D206E, C656R, K290RfsX20, C136Y and D461N will be observed as bands in the vial C track and are identified by comparison of the position of each band with an adjacent marker track. The product band sizes in base pairs (bp) are shown in Figure 1. Only product bands of the correct size should be interpreted.

Figure 1 shows diagrammatically the size in base pairs and relative location of the PCR products in a gel for all of the tested mutations.

Figure1



Test Validation

Whole blood samples whose genotype is known were tested in an in-house study. DNA was prepared following the methods described in these Instructions for Use. All results were concordant with the known genotype obtained by alternative methods. Further testing using only the A and B primer mixes of ELUCIGENE FH20 (i.e. FH13), was carried out on 400 samples taken from patients attending UK lipid clinics. A mutation was correctly identified in 54 patients. A full screen using SSCP/dHPLC techniques identified a further 59 different mutations in 85 patients. FH13 correctly identified 38% of all detected mutations by the full screen, with no false positive or false negative results [3].

Cross Reactivity

Steps have been taken during test development to avoid interference of test function by the presence of other mutations in the LDLR, Apo B and PCSK9 genes.

Evaluation of known mutations and polymorphisms in the LDLR gene has highlighted the following effects on the ELUCIGENE FH20 results:

1. **The D206E primer will cross-react with FsD206 mutant DNA sequence and result in a visible 'diagnostic' band at the 265bp position on the gel from the C vial. Therefore a D206E mutant DNA sequence will result in only a visible diagnostic band from the C vial, whereas a FsD206 mutant DNA sequence will result in a visible diagnostic band from the B at position 134bp and a visible diagnostic band from the C vial at position 265bp.**

2. **The IVS3+1G>A primer will cross-react with 313+1delCG mutant DNA sequence. Therefore an individual carrying the 313+1delCG mutation will result in a visible diagnostic band at 300bp in the B mix.**

Limitations of the Procedure

1. The results obtained from this or any other diagnostic kit should be used and interpreted only in the context of the overall clinical picture. Tepnel Molecular Diagnostics is not responsible for any clinical decisions that are taken.

2. The absence of the mutations detected by this kit is no guarantee that other mutations in the LDLR, Apo B and PCSK9 genes are not present. Many other mutations are possible and are not detected by this kit.

3. Mutations vary in frequency between different populations.

The user of this kit should emphasise these points when reporting results to the diagnosing clinician/genetic counsellor.

References

1. Newton CR et al. Analysis of any point mutation in DNA. The Amplification Refractory Mutation System (ARMS). Nucleic Acid Res 17: 2503-2516 (1989).

2. Satsangi J et al. Effect of heparin on polymerase chain reaction. Lancet 343:1509-1510 (1994).

3. Taylor et al. Multiplex ARMS analysis to detect 13 common mutations in familial hypercholesterolaemia. Clinical Genetics 71: 561 – 568 (2007).