GEN-PROBE

Elucigene

Elucigene® TRP Instructions for Use

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Elucigene kits are developed and manufactured by Gen-Probe Life Sciences Ltd. within quality systems accredited to ISO9001:2008 and ISO13485:2003.

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Elucigene® TRP (Thrombosis Risk Panel)

Catalogue Code: TH003B2 – 50 tests (with MTHFR)

Catalogue Code : TH002B2 – 50 tests (without MTHFR)

Note: For Cat No TH002B2 please disregard all references to MTHFR primers and analysis.

Intended Use

For the simultaneous *in vitro* qualitative detection of Factor V Leiden (R506Q), Prothrombin (Factor II 20210A) and methylenetetrahydrofolate reductase (MTHFR C677T) mutations in DNA extracted from whole blood (EDTA preserved) or dried bloodspot samples.

Summary and Explanation

Venous thrombosis causes an estimated 50,000 deaths/year in the US and has an incidence of 1 per 1000 annually (1). There are many reported risk factors that contribute to thrombosis, such as surgery, pregnancy, oral contraceptives and prolonged immobilisation (economy class syndrome) (2).

A genetic element also impacts the delicate equilibrium of the blood coagulation process. The biochemical coagulation pathway is complex and contains many factors that either enhance or inhibit the process, resulting in either defective (haemophilia) or excessive (thrombophilia) clotting. Of these factors, 3 have been identified as key and accounting for the majority of heritable thrombophilia cases (3)(4)(5). Mutations in the genes of these factors are useful indicators of increased risk of venous thrombosis. These are factor V Leiden (R506Q), factor II (Prothrombin 20210A) and MTHFR (677C>T). MTHFR (methylenetetrahydrofolate reductase) is essential for maintaining homocysteine levels, which impacts the blood clotting process.

Factor V Leiden is the most common inherited form of thrombophilia. Heterozygosity for factor V Leiden occurs in 3 to 8% of the general US and European populations (6). The frequency of homozygosity for the factor V Leiden mutation is approximately 1 in 5000, although the prevalence varies considerably in different populations. The risk of a venous thromboembolism is dependant upon both genetic and 'acquired' factors. Age is an important factor, increasing risk advances at a greater rate in individuals with a factor V Leiden mutation. An individual heterozygous for the factor V Leiden mutation is 7 fold more likely to develop a venous thromboembolism, and an individual homozygous for factor V Leiden has an 80 (eighty) fold increased risk (7). Other genetic risk factors (Prothrombin 20210A and MTHFR 677C) also contribute to increased risk, e.g. a factor V Leiden heterozygote has a 20 fold increased risk if also heterozygous for 20210A (8). In women who use oral contraceptives, risk of venous thrombosis is increased to 30 fold if heterozygous for factor V Leiden, or several hundred fold if homozygous for factor V (9).

Testing is generally targeted to patients under 50 years with venous thrombosis and patients or relatives with a family history of thrombolytic disease. Testing would additionally be warranted in relatives of individuals known to have factor V Leiden and women with recurrent pregnancy loss or severe preeclampsia or stillbirth. Knowledge of factor V Leiden status can influence management of a pregnancy or influence the decision making process regarding oral contraceptives (10). Once factor V Leiden has been identified, it is very informative to also test for the other thrombosis risk factors such as Prothrombin (factor II) and MTHFR. The benefits of identifying factor V Leiden mutations in patients with venous thrombosis is that asymptomatic family members can opt to determine if they are also at increased risk and therefore help to guide their anti-thrombolytic treatment during periods of

greatest risk, such as surgery, pregnancy, oral contraceptives, or long periods of immobilization, for examples long haul flight travel (11).

Principles of the Procedure

The method employed by the Elucigene TRP test is based on Amplification Refractory Mutation System (ARMS), an allele specific PCR amplification technology which can detect point mutations or small deletions in deoxyribonucleic acid (DNA)(12). The test comprises two complementary reaction mixes. The first reaction mix specifically amplifies all TRP alleles which are unaffected by the factor V Leiden (R506Q), factor II (Prothrombin 20210A) and MTHFR (677C>T) mutations, i.e. wildtype. By contrast, the second reaction mix contains primers which specifically amplify only the R506Q, 20210A and 677C>T mutant alleles. Each reaction mix also includes primers that amplify non factor V Leiden, Prothrombin and MTHFR DNA sequences as an assay internal amplification control to indicate a successful amplification. Amplified products (amplicons) of the two reactions are separated by electrophoresis on an agarose gel, presence or absence of bands on the gel indicate the status of factor V Leiden (R506Q), factor II (Prothrombin 20210A) and MTHFR (677C>T) alleles.

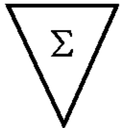
Warnings and Precautions

1. For In Vitro Diagnostic Use.
2. The DNA Control supplied with this kit is of human origin and has been independently tested using a PCR based assay and found to be negative for Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus 1 (HIV1).
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 1 or other infectious agents are absent. 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.
4. Store all components below -20°C. Discard 3 months after opening unless sub-aliquotted.
5. In line with current good laboratory practice, laboratories should process their own internal QC samples of known genotype in each assay, so that the validity of the procedure can be assessed.

Symbols used on labels

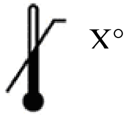
The symbols used on all labels and packaging conform to the harmonised standard EN 980

Manufacturer



Number of tests

See Instructions for Use



Store below temperature shown

Use before date shown

Catalogue code

Lot or batch number

Materials Provided

Storage of the reagents should be in an area free from contaminating DNA or PCR product.

All reagents are supplied ready for use. Store unopened and opened reagents at -20°C. Opened reagents can be stored for up to 3 months.

Sufficient materials for 50 tests are provided:

1. 2 vials x 450µL Primer Mix A (TA) containing primers specific for amplification of alleles unaffected by the Factor V, Factor II or MTHFR mutations, control primers and deoxynucleotide triphosphates in buffer (TH003TA, TH002TA (without MTHFR)).
2. 2 vials x 450µL Primer Mix B (TB) containing primers specific for amplification of alleles affected by the Factor V, Factor II or MTHFR mutations, control primers and deoxynucleotide triphosphates in buffer (TH003TB, TH002TB (without MTHFR)).
3. 1 vial x 600µL of Loading Dye (LD). (CR000TR)
4. 1 vial x 200µL of Dilution Buffer (DB). (CR000TV)
5. 1 vial x 50µL of DNA Control (DC), normal for the factor V Leiden R506Q, prothrombin 20210A mutations and homozygous for the MTHFR 677C allele variant. (CR002TX)

Materials Required but not Provided

Laboratory consumables - gloves; screw-capped microfuge tubes; pipette tips; thin-walled 0.2mL or 0.5mL PCR vials (use of two differently coloured vials will aid primer mix identification).

DNA Preparation - Good quality sterile deionised water; sodium chloride (NaCl); ethylenediaminetetra-acetic acid (EDTA) disodium salt; sodium hydroxide pellets (NaOH); 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-base) crystallised; hydrochloric acid 36% sp.gr.1.18 (HCl); ammonium chloride (NH_4Cl).

PCR Amplification - Sigma light white mineral oil*; good quality sterile distilled water; AmpliTaq Gold (Applied Biosystems).

Electrophoresis - Gel electrophoresis materials including NuSieve® 3:1 Agarose (Lonza); 50 Base-Pair Ladder (GE Healthcare); Ethidium bromide.

* For amplification carried out in 0.5ml PCR vials or thermal cyclers without heated lids

Equipment Required

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

DNA Preparation - Heating block (heating to 100°C).

Amplification - Thermal cycler to accommodate 0.5mL or 0.2mL vials (with a temperature accuracy of +/-1°C between 33°C and 100°C and static temperature uniformity of +/-1°C), heated lid optional.

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

Sample Collection and Storage

Whole blood (EDTA) or bloodspots 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(9). 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.

Preparation of DNA from Whole Blood (EDTA) Samples

1. Pipette 80µL of each blood sample into a screw-capped microfuge tube.
2. Pipette 320µL of 170mM (9.09 g/L) NH_4Cl solution into each tube.
3. Mix for 20 minutes by gentle swirling and inversion. Avoid vigorous agitation and formation of foam.
4. Centrifuge each tube for 2 minutes at 12 000g until a cell pellet is formed.
5. Using a pipette remove and discard the supernatant liquid.
6. Pipette 300µL of 10mM (0.58g/L) NaCl/10mM (3.72g/L) EDTA into each tube and resuspend the cells by vortex mixing.
7. Centrifuge each tube for 1 minute at 12 000g until a cell pellet is formed.
8. Repeat steps 5 to 7 at least a further two times until all visible red coloration in the supernatant liquid has been removed.
9. Using a pipette remove and discard the supernatant liquid.
10. Pipette 200µL of 50mM (2g/L) NaOH solution into each tube and resuspend the cells by vortex mixing.
11. Incubate in a heated block at 100°C for 10 minutes.
12. Pipette 40µL of 1M (121.1g/L) Tris-base/HCl (pH 7.5) into each tube and vortex mix.
13. Add 1mL sterile deionised water to each microfuge tube to give a total DNA sample volume of 1.24mL.
14. Centrifuge each tube for 1 minute at 12 000g until a pellet of cell debris is formed. The DNA is contained within the supernatant liquid.

Preparation of DNA from Dried Bloodspots

1. Punch 2 x 3mm discs[b] from specimen card in to a 1.5mL screw-capped tube.

Spots should be punched from an area of card that is completely saturated with blood.

Punch several "clean" card spots to waste prior to each sample to prevent sample carryover.

2. Add 1mL of 10mM (0.58g/L) NaCl/10mM (3.72g/L) EDTA and mix on rotary mixer for 15 - 20 minutes. If rotary mixer not available then washes may need to be extended up to 30 minutes each.
3. Remove and discard wash solution.

4. Repeat steps 2 and 3 once more.
5. At this point most of the haem pigment should be eluted from the disc. A faint reddish brown colouring of the blood spots is not uncommon at this stage.
6. Microfuge briefly (3 seconds) to collect remaining wash solution at bottom of tube. Using a pipette, remove and discard as much wash solution as possible, without disturbing spots.
7. This brief microfuge step typically removes additional haem pigments from the blood spots.
8. Add 150μL of 50mM (2g/L) NaOH to each tube. Flick carefully with finger to mix.
9. Boil in hot block for 10 minutes. Hot block should be equilibrated at 100^0C.
10. Microfuge briefly to collect supernatant at bottom of tube.
11. Add 30μL of 1M (121.1g/L) Tris-base/HCl (pH 7.5) into each tube, to neutralise, and mix carefully.
12. Add 420μL of sterile Sigma water to each DNA sample to give a total volume of 600μL.
13. Mix samples well and microfuge to collect.
14. Transfer supernatant to a fresh labelled screw-capped tube.

Store extracted DNA at -20^0C.

[b] Total area of blood sample used should be at least equivalent to that of 2 x 3mm circular blood spots. For example, 1x 6mm card spots may be used if convenient.

The QIAmp DNA Blood Kit (Qiagen) has also been used for DNA preparation from whole liquid blood and has been found to produce reproducible and interpretable results.

The DNA preparation methods described above are recommended by Gen-Probe Life Sciences and have been demonstrated to produce consistent and reliable results. DNA prepared using other methods or from other sample types may not be optimal for the Elucigene TRP test and may produce sub-optimal results. The key criteria for alternative DNA preparation methods are DNA concentration and absence of PCR inhibitors.

It is recommended that alternative methods and sample types are thoroughly evaluated with the Elucigene TRP test prior to the results being used for diagnostic use. Testing of DNA samples at concentrations <10ng/5μL is not recommended. Under optimal PCR conditions results are consistently obtained at DNA concentrations between 10 and 100ng/5μL.

Note: - Due to varying quality and yield of DNA it may sometimes be necessary to dilute the final DNA solution a further 5 fold to ensure efficient amplification.

Test Protocol

Amplification Procedure

The figures given in Tables 1 and 2 can be increased proportionately for numbers of tests other than those specified. However, owing to the small volumes involved, Gen-Probe Life Sciences recommends that no fewer than 5 tests are prepared at one time.

1. Program the thermal cycler for a time-delay file to activate the AmpliTaq Gold at 94°C for 20 minutes linked to an amplification cycling program of 30 seconds at 94°C (denaturation), 2 minute at 58°C (annealing) and 1 minute at 72°C (extension) for 35 cycles. This should be linked to a 20-minute time-delay file at 72°C (extension) on the final cycle.

Note: Select ‘Block’ method option on the thermal cycler for PCR in 0.5ml vials.

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

Note: Steps 3 - 6 must be carried out in an area free from DNA

3. Referring to Table 1 prepare sufficient dilution of the AmpliTaq Gold in the Dilution Buffer and Loading Dye supplied and sterile distilled water for the number of samples and controls to be tested. **Mix thoroughly by gently pipetting up and down**.

Table 1. Dilution of AmpliTaq Gold

	Number of Tests Required			
	5	10	20	50
Volume of sterile distilled water (μL)	21	42	84	210
Volume of Loading Dye (μL)	30	60	120	300
Volume of Dilution Buffer (μL)	6	12	24	60
Volume of AmpliTaq Gold (μL)	3	6	12	30
Total Volume (μL)	60	120	240	600

4. Referring to Table 2, prepare the A and B reaction mixes. Remove the appropriate aliquot of Primer Mix A into a labelled microfuge tube. Repeat with Primer Mix B into a second labelled microfuge tube. Using separate pipette tips add the appropriate volume of the AmpliTaq Gold dilution (from step 3) to each microfuge tube. Mix gently by vortexing and centrifuge the vials for 10 seconds at 12 000g.

Table 2. Preparation of A and B Reaction Mixes

	Number of Tests Required							
	5		10		20		50	
	A	B	A	B	A	B	A	B
Volume of Primer Mix A (μL)	82.5		165		330		825	
Volume of Primer Mix B (μL)		82.5		165		330		825
Volume of Diluted Enzyme (μL)	27.5	27.5	55	55	110	110	275	275
Total Volume (μL)	110	110	220	220	440	440	1100	1100

5. Label one vial ‘A’ and one vial ‘B’ for each sample or if coloured vials are available use a different colour for each primer mix.

6. Pipette 20μL of the prepared A reaction mix into the bottom of each of the appropriate number of the PCR vials labelled ‘A’. Repeat with the B reaction mix into each of the appropriate number of the PCR vials labelled ‘B’.

7. Using separate pipette tips each time, add 5μL of test DNA sample into each of a vial A and B pair. Add one drop of Sigma light white mineral oil to cover the aqueous phase *. Re-cap firmly.

8. For the negative control add no DNA to a vial A and B pair. Add 1 drop of Sigma light white mineral oil to cover the aqueous phase *. Re-cap firmly.

9. Centrifuge the A and B vials for 10 seconds at 12 000g.

10. Place all vials firmly in the thermal cycler block. Initiate the 94°C time-delay file followed by the amplification cycling program.
11. Discard all the remaining unused AmpliTaq Gold dilution and prepared A and B reaction mixes.
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.

* For amplification carried out in 0.5mL PCR vials or thermal cyclers without heated lids.

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 from each of PCR vials should be loaded in adjacent well positions on the prepared agarose gel.
4. A 50 Base-Pair Ladder (GE Healthcare) was run adjacent to the samples as a molecular weight marker.
5. Electrophoresis was carried out at 5 to 6 V/cm between electrodes until the dye front had migrated 4cm from the loading wells towards the anode (1 to 1.5 hours).
6. After electrophoresis the gels were placed on a UV transilluminator at 260nm then visualised and photographed.

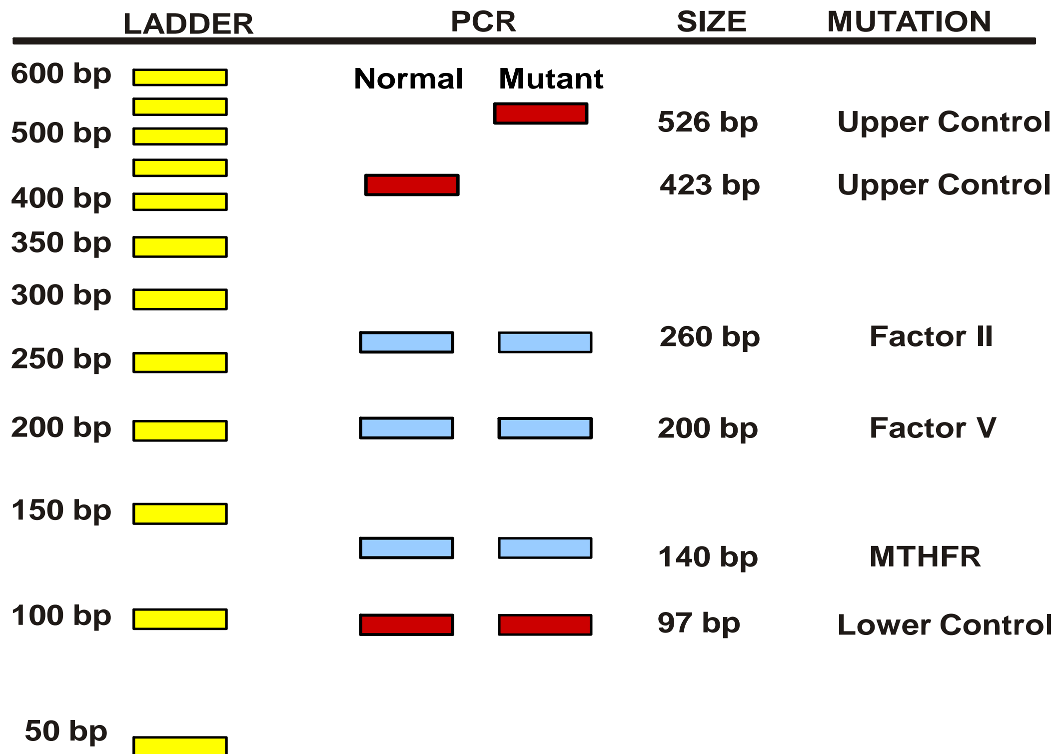
Interpretation of Results

1. The negative control must show no bands within the area defined by the upper and lower control bands (see Figure 1).
2. The upper and lower control bands must be clearly visible in all samples (see Figure 1).
3. The position of the upper and lower control bands should indicate the correct molecular size (see Figure 1).

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

Figure 1 shows diagrammatically the size, in base pairs, and relative location of the PCR products in a gel that is expected for a heterozygous Thrombosis genotype (carrying the Factor V, Factor II or MTHFR mutations) using the Thrombosis Risk test reagent.

Figure 1



Note: MTHFR alleles are not detected by Elucigene TRP Cat No TH002B2

Performance Characteristics

Thirty samples, previously genotyped using restriction enzyme digest analysis, were tested with the Elucigene TRP kit (TH003B2) in an in-house study using the recommended kit protocol. Twenty nine DNA samples amplified successfully using the Elucigene TRP kit. One DNA sample failed to amplify but gave an acceptable result on repeat testing. Twenty six of the samples tested positive for mutations detected by the Elucigene TRP kit, no mutations were detected in four samples. Of the 30 samples tested, 7 were positive for the factor V (R506Q) mutation, 10 were positive for the factor II (Prothrombin 20210A) mutation and 16 were positive for the MTHFR (677C>T) mutation. All results determined by the Elucigene TRP kit were confirmed by the original genotyping results.

Thirty matched whole blood and bloodspot samples taken from thirty individuals were tested with the Elucigene TRP kit (TH003B2) in an in-house study using the recommended kit protocol. Eighteen of the matched samples tested positive for mutations detected by the Elucigene TRP kit. The result obtained from each whole blood sample was concordant with that obtained using the bloodspot sample from the same individual.

Limitations of the Procedure

1. Results from this and other diagnostic assays should be interpreted in conjunction with other laboratory and clinical data available to the clinician.
2. The absence of the mutations detected by this kit is no guarantee that other mutations in the factor V Leiden, factor II and MTHFR genes are not present. Other mutations are possible and are not detected by this kit.

3. As with any genetic test, erroneous results may be obtained from blood samples that have been taken subsequent to a recent blood transfusion.

The user of these tests should emphasise these points when reporting results to the diagnosing clinician or genetic counsellor.

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