CPAL
Central **P**ennsylvania **A**lliance **L**aboratory

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Thrombophilia Risk Test
- New Assay, New Platform -

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Ordering Information and Suggested Codes:

CPAL Test Name	Test Code	LOINC Code	CPT Code (s)
Factor V Leiden	7000250	21667-1	81241
Factor II (Prothrombin)	7000270	24475-6	81240
Methylenetetrahydrofolate reductase (MTHFR): C677T, A1298C	7000290	28005-7 (C677T), 28060-2 (A1298C)	81291
Thrombophilia Risk Test (TRT) Panel: Factor V, Prothrombin, MTHFR 677 & 1298	7000300	21667-1, 24475-6, 21709-1	81241, 81240, 81291

Testing Method: DNA Extraction; PCR; Solid-phase electrochemical detection (gene chip)

Specimen Requirements:

- Peripheral blood samples collected in tubes containing EDTA as the anticoagulant (3 mL). Minimum volume accepted: 1 mL.
- Store and ship samples at 2°C to 8°C, stable up to 5 days refrigerated.

Effective Date: Testing offered beginning on August 20, 2018.

Performed: Monday-Wednesday-Friday, dayshift

Background:

Thrombosis is one of the most common types of blood coagulation disorders, affecting 1 in 1000 individuals with a fatality rate of 1-2%. Inherited Thrombosis is associated with congenital predisposing risk factors such as Factor II and Factor V proteins involved in the blood coagulation enzyme activity cascade and the Methylenetetrahydrofolate Reductase (MTHFR) that converts homocysteine to methionine as part of the pathway that converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The Factor II and Factor V mutations are present in ~2% and 5% of individuals with N. European ancestry respectively, but at much lower levels in other populations. The MTHFR C677T and A1298C mutations are present in ~40-50% of those with N. European ancestry, but the frequency varies in other ethnic groups. Inherited Thrombosis is characterized by increased risk of deep vein thrombosis, ectopic pregnancy, pulmonary embolism, myocardial infarction, cardiovascular disease, and other complications related to abnormal blood coagulation. Detection and genotyping of the Factor II (Prothrombin) G20210A mutation and the Factor V Leiden G1691A mutation aids in the evaluation of patients with suspected thrombophilia. The detection and genotyping of methylenetetrahydrofolate reductase (MTHFR), a key regulatory enzyme in folate and homocysteine metabolism, has been found to be beneficial as well.

The GenMark eSensor® Thrombophilia Risk Test is an in vitro diagnostic for the detection and genotyping of Factor II (Prothrombin) G20210A, Factor V (Factor V Leiden) G1691A, and MTHFR (human 5,10 methylenetetrahydrofolate reductase gene) C677T and A1298C mutations in patients with suspected Thrombophilia from isolated genomic DNA obtained from whole blood samples. eSensor® Technology uses a solid-phase electrochemical method for determining the genotyping status of a defined panel of mutations. The genotype of each polymorphism is determined by voltammetry, which generates specific electrical signals from ferrocene-labeled, allele-specific signal probes.

Validation Data:

Reproducibility Studies:

Between-run and within-run reproducibility were evaluated at two levels for each test, Not Detected (Wildtype) and Detected (Homozygous). For between-run reproducibility, seven replicates of the wildtype sample and seven replicates of the homozygous mutant sample were performed in a five-day study. Within-run reproducibility was performed by testing the same two samples used for the between-run reproducibility study. The wildtype sample was tested five times each within the same run, and the homozygous mutant sample was tested five times each within the same run. All between-run and within-run reproducibility samples were 100% concordant.

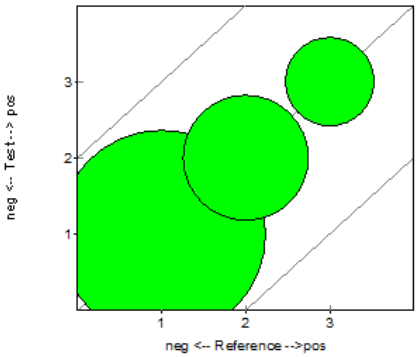
Method Comparison and Accuracy Studies:

The objective of the method comparison studies was to demonstrate that the eSensor® Thrombophilia Risk Test will perform as expected based on result comparison, ensuring that peripheral blood samples received by CPAL will achieve acceptable results. To accomplish this, a total of 68 peripheral blood were tested that were a combination of wildtype, heterozygous, and homozygous results for Factor V, Factor II/Prothrombin, MTHFR C677T, and MTHFR A1298C.

The second component of this experiment is to demonstrate accuracy: to demonstrate that the eSensor® TRT assay results are concordant with results provided from another laboratory and results previously tested at CPAL. Since this is a qualitative assessment, concordance is the tool to measure testing accuracy against Western PA Hospital, who is performing the test the same way through the GenMark eSensor® TRT assay, and the Roche LightCycler 1.2 testing performed at CPAL. All samples in the method comparison study were 100% concordant, see figures below.

Figure 1: Method Comparison and Accuracy Statistical Analysis, Factor V

Ref Method WPA XT-8/LC 1.2 **Test Method GenMark XT-8**



Statistical Analysis
(Comparison of two Laboratory Methods)

Agreement 100.0% (94.7 to 100.0%)
95% confidence interval calculated by the "Score" method.

McNemar Test for Symmetry:
Test < Reference 0 (0.0%)
Test > Reference 0 (0.0%)
Symmetry test PASSES

Cohen's Kappa 100.0% (100.0 to 100.0%)
Kappa is the portion of agreement above what is expected by chance.
The rule of thumb is that Kappa > 75% indicates "high" agreement.
We would like to see VERY high agreement (close to 100%).

Statistical Summary

Test	Reference 1	2	3	Total
1	44	--	--	44
2	--	16	--	16
3	--	--	8	8
Total	44	16	8	68

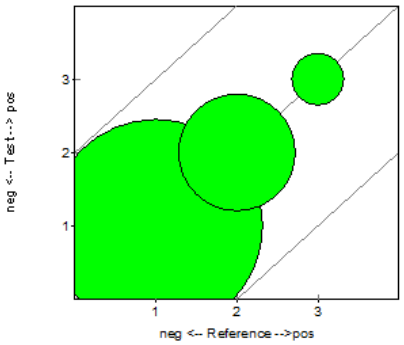
Number excluded or missing: 0

Legend

	Reference	Test
1	Wildtype (W)	Wildtype (W)
2	Heterozygous (H)	Heterozygous (H)
3	Homozygous (O)	Homozygous (O)

Figure 2: Method Comparison and Accuracy Statistical Analysis, Factor II/Prothrombin

Ref Method WPA XT-8/LC 1.2 **Test Method GenMark XT-8**



Statistical Analysis
(Comparison of two Laboratory Methods)

Agreement 100.0% (94.7 to 100.0%)
95% confidence interval calculated by the "Score" method.

McNemar Test for Symmetry:
Test < Reference 0 (0.0%)
Test > Reference 0 (0.0%)
Symmetry test PASSES

Cohen's Kappa 100.0% (100.0 to 100.0%)
Kappa is the portion of agreement above what is expected by chance.
The rule of thumb is that Kappa > 75% indicates "high" agreement.
We would like to see VERY high agreement (close to 100%).

Statistical Summary

Test	Reference 1	2	3	Total
1	50	--	--	50
2	--	15	--	15
3	--	--	3	3
Total	50	15	3	68

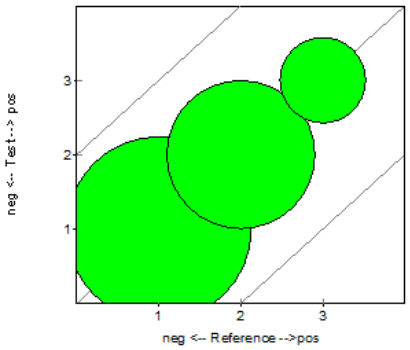
Number excluded or missing: 0

Legend

	Reference	Test
1	Wildtype (W)	Wildtype (W)
2	Heterozygous (H)	Heterozygous (H)
3	Homozygous (O)	Homozygous (O)

Figure 3: Method Comparison/Accuracy Statistical Analysis, MTHFR 677

Ref Method WPA XT-8/LC 1.2 **Test Method GenMark XT-8**



Statistical Analysis
(Comparison of two Laboratory Methods)

Agreement 100.0% (87.1 to 100.0%)
95% confidence interval calculated by the "Score" method.

McNemar Test for Symmetry:
Test < Reference 0 (0.0%)
Test > Reference 0 (0.0%)
Symmetry test PASSES

Cohen's Kappa 100.0% (100.0 to 100.0%)
Kappa is the portion of agreement above what is expected by chance.
The rule of thumb is that Kappa > 75% indicates "high" agreement.
We would like to see VERY high agreement (close to 100%).

Statistical Summary

Test	Reference 1	2	3	Total
1	14	--	--	14
2	--	9	--	9
3	--	--	3	3
Total	14	9	3	26

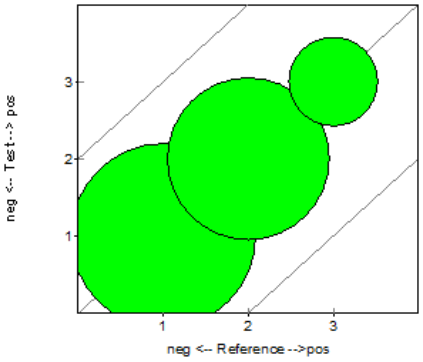
Number excluded or missing: 0

Legend

	Reference	Test
1	Wildtype (W)	Wildtype (W)
2	Heterozygous (H)	Heterozygous (H)
3	Homozygous (O)	Homozygous (O)

Figure 4: Method Comparison/Accuracy Statistical Analysis, MTHFR 1298

Ref Method WPA XT-8/LC 1.2 **Test Method GenMark XT-8**



Statistical Analysis
(Comparison of two Laboratory Methods)

Agreement 100.0% (87.1 to 100.0%)
95% confidence interval calculated by the "Score" method.

McNemar Test for Symmetry:
Test < Reference 0 (0.0%)
Test > Reference 0 (0.0%)
Symmetry test PASSES

Cohen's Kappa 100.0% (100.0 to 100.0%)
Kappa is the portion of agreement above what is expected by chance.
The rule of thumb is that Kappa > 75% indicates "high" agreement.
We would like to see VERY high agreement (close to 100%).

Statistical Summary

Test	Reference 1	2	3	Total
1	13	--	--	13
2	--	10	--	10
3	--	--	3	3
Total	13	10	3	26

Number excluded or missing: 0

Legend

	Reference	Test
1	Wildtype (W)	Wildtype (W)
2	Heterzygous (H)	Heterozygous (H)
3	Homozygous (O)	Homozygous (O)

Sensitivity and Specificity Studies:

Based on the same data used in the Method Comparison/Accuracy study, the assay was found to have a 100% sensitivity and 100% specificity. The Positive Predictive Value was found to be 100%, and the Negative Predictive Value 100%.

Interfering Substances:

According to a study of PCR-inhibitory components by Al-Soud and Radstrom in the *Journal of Clinical Microbiology*, several compounds in blood have been found to be PCR inhibitory, namely, heme, leukocyte DNA, and anti-coagulants. The inhibitory effect of heparin has been suggested on the basis of an interaction between heparin and DNA and heparin's ability to compete with target nucleic acid. The inhibition is not reversed by repeated ethanol precipitation.

The Qiagen Gentra Puregene procedure for extraction of whole blood and bone marrow samples removes contaminants and enzyme inhibitors such as proteins and divalent cations through a series of steps that include RBC lysis, cell lysis, and protein precipitation.

According to the GenMark eSensor® Thrombophilia Risk Test package insert, the following interfering substances were added separately to two whole blood samples at the following concentrations: heparin (3,000 U/L), cholesterol (250 mg/dL), bilirubin (30 mg/dL), and hemoglobin (~20g/dL). No effects were observed on yield of extracted DNA, multiplex amplification of target gene sequences, or genotyping of mutations by the eSensor® Thrombophilia Risk Test. No effect of elevated EDTA concentration due to a reduced volume blood draw was observed by adding a total of 5 times the normal amount of EDTA anticoagulant to a whole blood sample.

Limit of Detection (LOD) Study:

The LOD was validated during studies at GenMark. Two genomic DNA (gDNA) samples with different genotypes were serially diluted to 500, 100, 10, 1.0, and 0.1 ng per sample input and tested 20 times each. All replicates at $\geq$ 1.0 ng gave 100% final correct results. Thus, the limit of detection for the TRT assay was determined to be 1.0 ng of gDNA, with 10 to 500 ng gDNA as the recommended input of the test. See Table 5 below.

References:

GenMark Diagnostics, Inc. eSensor® Thrombophilia Risk Test Package Insert, Carlsbad CA 92008 Rev D, May 2017.

Maine Molecular Quality Controls, Inc (MMQCI). INTROL™ Thrombosis Genotype Panel, Saco ME 04072. G123 061416.01

Al-Soud, Waleed Abu and Radstrom, Peter. "Purification and Characterization of PCR-Inhibitory Components in Blood Cells." *Journal of Clinical Microbiology.* Feb. 2001; 39(2):485-493. Accessed 5/17/2018..