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

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Calreticulin Mutation Analysis by DNA Sequencing
- Panel Offering Update -

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Ordering Information and Suggested Codes:

CPAL Test Name	Lab Nexus Test Mneumonic	Tests Included	LOINC Code	CPT Code (s)
Calreticulin	CALR	Calreticulin	77174-1	81219
Extended MPN Panel	EXTMPN	JAK2, Exon 12, Calreticulin, MPL	48726-4, 63421-2, 77174-1, 56142-3	81270, 81403, 81219, 81402
JAK2 Reflex CALR	JAK2RCALR	JAK2, Calreticulin	48726-4, 77174-1	81270, 81219
JAK2 Relfex CALR, MPL	JAK2RCALRMPL	JAK2, Calreticulin, MPL	48726-4, 77174-1, 56142-3	81270, 81219. 81402
JAK2 Reflex MPL, CALR	JAK2RMPLCALR	JAK2, MPL, Calreticulin	48726-4, 56142-3, 77174-1	81270, 81402, 81219
BCR-ABL FISH Reflex Extended MPN	BCRFISHREMPN	BCR-ABL FISH, JAK2, Exon 12, Calreticulin, MPL	51867-0, 48726-4, 63421-2, 77174-1, 56142-3	88374, 81270, 81403, 81219, 81402

Testing Method: DNA Extraction; PCR; Sanger DNA Sequencing

Specimen Requirements:

- Peripheral blood or bone marrow 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 Tuesday, May 29th, 2018.

Performed: Monday through Friday, dayshift

Background:

Myeloproliferative neoplasms (MPNs) are clonal disorders that arise from hematopoietic progenitor cells and are characterized by the proliferation of one or more myeloid cell lineages within the bone marrow. Philadelphia chromosome (BCR-ABL) negative MPNs include Polycythemia vera (PV), Essential thrombocythemia (ET), and Primary myelofibrosis (PMF). Approximately 50-60% of the patients with ET or PMF, negative for BCR-ABL, carry the Janus kinase 2 (JAK2) mutation JAK2 V617F and 5-10% carry the thrombopoietin receptor gene (MPL) mutation (MPL 515K/L). It has recently been discovered that patients affected by these neoplasms who show no mutation in either JAK2 or MPL may exhibit a mutation within exon 9 of the Calreticulin gene (CALR) found on chromosome 19. This mutation appears to be mutually exclusive from JAK2 and MPL mutations. Recent studies have found CALR mutations are commonly seen in patients expressing wild-type JAK2/MPL ET (67-71%) and PMF (56-88%). The most commonly seen CALR mutations are separated into two types: Type 1 is a 52 base-pair (bp) deletion (p.L367fs*46, most commonly seen beginning at bp 1092-1099) and Type 2 is a 5 bp insertion (p.K385fs*47 most commonly seen at c.1154_1155insTTGTC), though other mutations can also be identified.

The CPAL Calreticulin assay is intended to detect mutations within a specific region of CALR Exon 9 where the majority of mutations occur. Genomic DNA is extracted from peripheral blood or bone marrow using EDTA as the anticoagulant. Specific DNA amplification of a portion of the CALR Exon 9 region is performed through Polymerase Chain Reaction (PCR) using specific primers. Di-deoxy chain terminating (Sanger) methods are used to evaluate the DNA sequence for mutations in this region using the Applied Biosystems 3130 Genetic Analyzer. Data obtained from the sequencer is analyzed by comparing the obtained sequence to a known portion of Exon 9 (NCBI Reference Sequence: NG_029662.1: Homo sapiens calreticulin (CALR), RefSeqGene (LRG_828) on chromosome 19).

Validation Data:

Reproducibility Studies:

Between-run and within-run reproducibility were evaluated at two levels, Not Detected and Detected (Type 2, c.1154_1155insTTGTC). For between-run reproducibility, twenty replicates of each sample were performed in a twenty-day study with single measurement of each sample on a run each day. For within run reproducibility, the same two samples were used, and each sample was tested twenty times each within the same run. Ten aliquots of each of the two pools were tested on a single run. All between-run and within-run reproducibility samples were 100% concordant.

Method Comparison and Accuracy Studies:

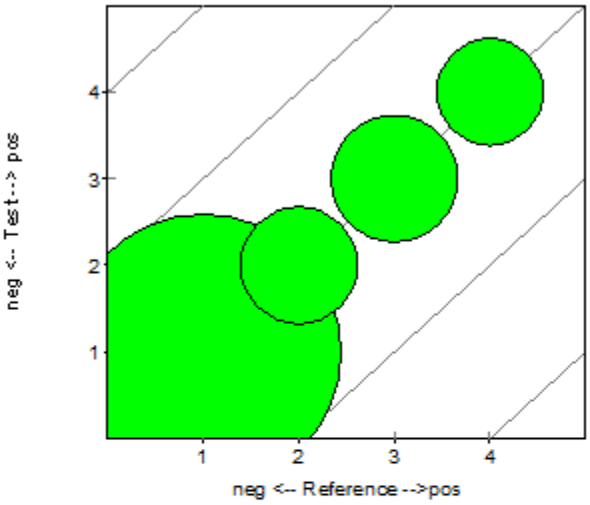
The objective of the method comparison study was to demonstrate that the Calreticulin assay is capable of performing as expected based on result comparison, ensuring that peripheral blood and bone marrow samples received by CPAL will achieve acceptable results. To accomplish this, a total of 51 peripheral blood and bone marrow samples were tested, 33 Mutation Not Detected and 18 Mutation Detected.

The second component of this experiment is to demonstrate accuracy: to demonstrate that the Calreticulin assay interpretation is concordant with genomic DNA reference sequences provided from another laboratory. Since this is a qualitative assessment, concordance is the tool to measure CPAL testing accuracy against Quest Diagnostics and NeoGenomics Laboratories, who also perform the test using Sanger sequencing.

All samples in the method comparison study were 100% concordant.

Figure 1: Method Comparison and Accuracy Statistical Analysis

Ref Method Reference Lab **Test Method Genetic Analyzer**



Statistical Analysis
(Comparison of two Laboratory Methods)

Agreement 100.0% (93.0 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	4	Total
1	33	--	--	--	33
2	--	6	--	--	6
3	--	--	7	--	7
4	--	--	--	5	5
Total	33	6	7	5	51

Legend

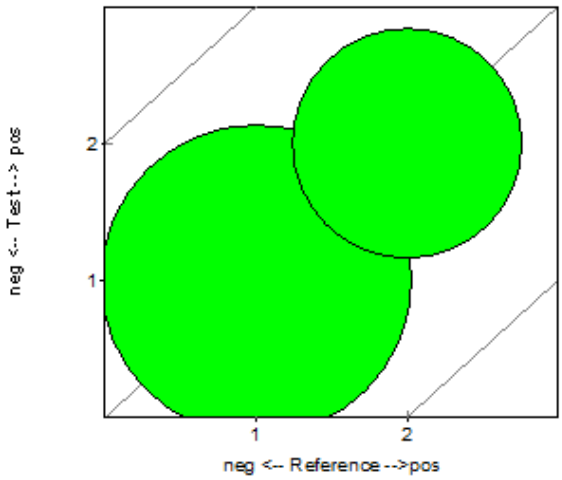
	Reference	Test
1	Negative (N)	Negative (N)
2	Type 1 (1)	Type 1 (1)
3	Type 2 (2)	Type 2 (2)
4	Other Mutation (O)	Other Mutation (O)

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%. See Figure 2 for data.

Figure 2. Sensitivity and Specificity Statistical Analysis

Ref Method Reference Lab **Test Method Genetic Analyzer**



Statistical Analysis
(Comparison of Test Method to a Gold Standard)

Agreement	100.0% (93.0 to 100.0%)
Sensitivity	100.0% (82.4 to 100.0%)
Specificity	100.0% (89.6 to 100.0%)

95% confidence interval calculated by the "Score" metho

Positive Agreement	100.0%
Negative Agreement	100.0%
Prevalence	35.3%
Predictive Value Positive	100.0%
Predictive Value Negative	100.0%

Prevalence estimated from experimental results.

Statistical Summary

	Negative Reference	Positive Reference	Total
Negative Test	33	--	33
Positive Test	--	18	18
Total	33	18	51

Legend

	Reference	Test
1	Negative (N)	Negative (N)
2	Positive (P)	Positive (P)

Limit of Detection (LOD) Study:

The LOD study was accomplished by performing a dilution experiment on a EDTA peripheral blood sample harboring a known CALR mutation at an established percent, with results obtained by another laboratory currently validated to perform the testing. This sample was also tested as part of the Method Comparison study. After initial studies of all levels, two levels were chosen to perform the full LOD study, 11% and 16.5%. The levels were tested 10 times each. Detection of approximately 20% to 25% is commonly cited as the LOD for Sanger Sequencing, although a lower percent mutant allele may be detected depending on the context of the targeted sequences. Based on these studies performed, the LOD for the assay is 10-20%, with possibility to detect at a lower percentage under certain conditions.

Limitations:

1. The results of this and all laboratory assays should be evaluated in conjunction with all clinical data.
2. This assay is sensitive and capable of detecting mutant alleles present at low levels under ideal conditions.
3. This test was developed and its performance characteristics determined by The Central Pennsylvania Alliance Laboratory, LLC. It has not been cleared or approved by the U.S. Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigational or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) as qualified to perform high-complexity clinical laboratory testing.

References:

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NCBI Reference Sequence: NG_029662.1: Homo sapiens calreticulin (CALR), RefSeqGene (LRG_828) on chromosome 19, Exon 9. Accessed 12/12/2017.
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