Molecular Diagnostics for Myeloproliferative Neoplasms

Noah Brown, MD
April 16, 2015
Outline

1. Brief MPN Introduction
2. When/How Should Molecular Testing be Performed?
3. JAK2 V617F Mutation Testing
4. JAK2 Exon 12 Mutation Testing
5. MPL Mutation Testing
6. CALR Mutation Testing
7. Take Home Points
Normal Blood Cell Maturation

Several MPN that capitulate each terminally differentiated myeloid cell

Adapted from: Gary Gilliland, Dana-Farber Cancer Inst.
Myeloproliferative Neoplasms

Characterized by:
- Enhanced proliferation/survival
- Normal differentiation

Systemic mastocytosis
Polycythemia vera
Essential thrombocythemia
Chronic eosinophilic leukemia
Chronic myeloid leukemia
Chronic myelomonocytic leukemia
Primary myelofibrosis
MPN in 2005

- Systemic mastocytosis: $KIT\ D816V$
- Polycythemia vera: ?
- Essential thrombocythemia: ?
- Chronic eosinophilic leukemia: $FIP1L1-PDGFR\_A$
- Chronic myeloid leukemia: $BCR-ABL$
- Chronic myelomonocytic leukemia: $TEL-PDGFRB$
- Primary myelofibrosis: ?

Adapted from: Gary Gilliland, Dana-Farber Cancer Inst.
Mutations in Tyrosine Kinases Underlie Many MPNs

Hypothesis: PV, ET and PMF are caused by mutations that activate an unidentified tyrosine kinase(s)

Experimental Design: Sequence DNA from MPN patients for all tyrosine kinases

Adapted from: Gary Gilliland, Dana-Farber Cancer Inst.
**Harvard MPN Study**

**IRB approved Internet Protocol**

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics</th>
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<tbody>
<tr>
<td><strong>Characteristic</strong></td>
</tr>
<tr>
<td>Total, n</td>
</tr>
<tr>
<td>Male/female, n</td>
</tr>
<tr>
<td>Median age, years (range)</td>
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<tr>
<td>Median age at diagnosis, years (range)</td>
</tr>
<tr>
<td>Race, n (%)</td>
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<tr>
<td>Caucasian</td>
</tr>
<tr>
<td>Hispanic</td>
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<tr>
<td>Asian</td>
</tr>
<tr>
<td>Black/African-American</td>
</tr>
<tr>
<td>Multiracial or other</td>
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<td>Self-reported diagnosis, n (%)</td>
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<tr>
<td>PV</td>
</tr>
<tr>
<td>ET</td>
</tr>
<tr>
<td>MMM</td>
</tr>
<tr>
<td>MPD, NOS</td>
</tr>
</tbody>
</table>

Levine R et al., Cancer Cell 7:387, 2005
“High-throughput” DNA sequencing platform

Robotic amplification of DNA encoding 90 tyrosine kinase genes
  - Targeted sequencing of kinase activation loops and autoinhibitory domains

Robotic Sanger Sequencing
  ~325 DNA sequencing reactions per patient
  ~325 X 325 patients = ~125,000 sequencing reactions

~125,000 sequencing runs X 500 bp/run = 62 million base pairs

Computer-Assisted DNA sequence analysis

Adapted from: Gary Gilliland, Dana-Farber Cancer Inst.
G → T substitution in JAK2 results in V617F

Adapted from: Gary Gilliland, Dana-Farber Cancer Inst.

PV: 74%
ET: 32%
PMF: 35%

Detection by Sanger Sequencing
G → T substitution in JAK2 results in V617F

Wildtype

MPN patient

Baxter...Green, *Lancet* 2005
James...Vainchencker, *Nature* 2005
Levine...Tefferi...Gilliland, *Cancer Cell* 2005

Adapted from: Gary Gilliland, Dana-Farber Cancer Inst.
MPN in 2007

- Systemic mastocytosis: **KIT D816V**
- Polycythemia vera: **JAK2 V617F**
- Essential thrombocythemia: **JAK2 V617F**
- Chronic eosinophilic leukemia: **FIP1L1-PDGFRA**
- Chronic myeloid leukemia: **BCR-ABL**
- Chronic myelomonocytic leukemia: **TEL-PDGFRB**
- Primary myelofibrosis: **JAK2 V617F**

Adapted from: Gary Gilliland, Dana-Farber Cancer Inst.
JAK2 is a non-receptor tyrosine kinase. It transduces signals downstream of various cytokine receptors. It is a switch that tells blood cells to grow.

Adapted from: Gary Gilliland, Dana-Farber Cancer Inst.
The V617F mutation leads to constitutive JAK2 activation

- Loss of JH2 domain-mediated autoinhibition
JAK2 V617F Prevalence is Higher With Sensitive Detection Methods

<table>
<thead>
<tr>
<th></th>
<th>JAK2 V617F</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV</td>
<td>96%</td>
</tr>
<tr>
<td>ET</td>
<td>55%</td>
</tr>
<tr>
<td>PMF</td>
<td>65%</td>
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</tbody>
</table>

~ 40% of ET and PMF patients are JAK2 V617F negative
What about JAK2 V617F-negative patients?

New discoveries in 2006: MPL mutations (ET and PMF)

What about JAK2 V617F-negative patients?

New discoveries in 2007: JAK2 exon 12 mutations (PV)

Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms


Somatic CALR Mutations in Myeloproliferative Neoplasms with Nonmutated JAK2

Clinical Implications – CALR Mutations

- ET
  - Higher PLT count, Lower Hgb
  - Lower risk of thrombosis

- PMF:
  - Longer survival

What about JAK2 V617F-negative patients?

New discoveries in 2013: CALR mutations (ET and PMF)
CALR Mutation Frequency

Polycythemia Vera | Essential Thrombocythemia | Primary Myelofibrosis

- **Nonmutated JAK2, MPL, and CALR**
  - **JAK2 mutation**
  - **MPL mutation**
  - **CALR mutation**

**CALR Freq (wild-type JAK2 & MPL):**

- Polycythemia Vera: 60-70%
- Essential Thrombocythemia: 70-80%
- Primary Myelofibrosis: 70-80%

What about JAK2 V617F-negative patients?

New discoveries in 2006 – 2013:

- 2006: MPL mutations (ET and PMF)
- 2007: JAK2 exon12 mutations (PV)
- 2013: CALR mutations (ET and PMF)

<table>
<thead>
<tr>
<th></th>
<th>Polycythemia Vera</th>
<th>Essential Thrombocythemia</th>
<th>Primary Myelofibrosis</th>
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</thead>
<tbody>
<tr>
<td>JAK2 V617F</td>
<td>96%</td>
<td>55%</td>
<td>65%</td>
</tr>
<tr>
<td>JAK2 Exon 12</td>
<td>3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CALR</td>
<td></td>
<td>25%</td>
<td>30%</td>
</tr>
<tr>
<td>MPL</td>
<td></td>
<td>3%</td>
<td>10%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>99%</td>
<td>83%</td>
<td>95%</td>
</tr>
<tr>
<td>Major criteria</td>
<td>Polycythemia vera&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Essential thrombocythemia&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Primary myelofibrosis&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------------------</td>
<td>----------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Hgb &gt; 18.5 g/dL (men), &gt; 16.5 g/dL (women), or&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Platelet count ≥ 450 × 10&lt;sup&gt;9&lt;/sup&gt;/L</td>
<td>1 Megakaryocyte proliferation and atypia&lt;sup&gt;c&lt;/sup&gt; accompanied by either reticulin and/or collagen fibrosis, or&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Presence of JAK2V617F or JAK2 exon 12 mutation</td>
<td>2 Megakaryocyte proliferation with large and mature morphology</td>
<td>2 Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 Not meeting WHO criteria for CML, PV, PMF, MDS, or other myeloid neoplasm</td>
<td>3 Demonstration of JAK2V617F or other clonal marker or no evidence of reactive marrow fibrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 Demonstration of JAK2V617F or other clonal marker or no evidence of reactive thrombocytosis</td>
<td></td>
</tr>
<tr>
<td>Minor criteria</td>
<td>BM trilineage myeloproliferation</td>
<td>1 Leukoerythroblastosis</td>
<td>1 Leukoerythroblastosis</td>
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<tr>
<td></td>
<td>2 Subnormal serum Epo level</td>
<td>2 Increased serum LDH level</td>
<td>2 Increased serum LDH level</td>
</tr>
<tr>
<td></td>
<td>3 EEC growth</td>
<td>3 Anemia</td>
<td>3 Anemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 Palpable splenomegaly</td>
<td>4 Palpable splenomegaly</td>
</tr>
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</table>
2014 Proposed Revision for WHO Diagnostic Criteria for PV, ET and PMF

<table>
<thead>
<tr>
<th>Polycythemia vera (PV)(^a)</th>
<th>Essential thrombocytemia (ET)(^b)</th>
<th>Primary myelofibrosis (PMF)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major criteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Hemoglobin &gt; 16.5 g/dl (men) &gt; 16 g/dl (women) or hematocrit &gt; 49% (men) &gt; 48% (women)</td>
<td>Platelet count (\geq 450 \times 10^9/\text{l})</td>
<td>Megakaryocyte proliferation and atypia(^d), accompanied by either reticulin and/or collagen fibrosis or(^e)</td>
</tr>
<tr>
<td>2. BM trilineage myeloproliferation with pleomorphic megakaryocytes</td>
<td>Megakaryocyte proliferation with large and mature morphology</td>
<td>Not meeting WHO criteria for CML, PV, ET, MDS or other myeloid neoplasm</td>
</tr>
<tr>
<td>3. <strong>Presence of JAK2 mutation</strong></td>
<td>Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm</td>
<td><strong>Presence of JAK2, CALR or MPL mutation</strong></td>
</tr>
<tr>
<td>4.</td>
<td><strong>Presence of JAK2, CALR or MPL mutation</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Minor criteria</strong></th>
<th>Presence of a clonal marker (e.g. abnormal karyotype) or absence of evidence for reactive thrombocytosis</th>
<th>Presence of a clonal marker (e.g. abnormal karyotype) or absence of evidence for reactive bone marrow fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Subnormal serum erythropoietin level</td>
<td>Presence of anemia or palpable splenomegaly</td>
<td>Presence of leukoerythroblastosis(^f) or increased lactate dehydrogenase(^i)</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
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</table>

When Should Mutation Testing be Performed?

- Primary indication is a diagnostic aid to “prove” a proliferation is neoplastic

- Can not help subclassify MPNs since mutations are not MPN specific
  - Exception: JAK2 exon 12 (PV)

- Molecular diagnostics for has become standard of care for diagnostic workup of any suspected MPN.

  **May Include:**
  - JAK2 V617F
  - JAK2 exon 12
  - MPL
  - CALR
  - BCR-ABL
When Should Mutation Testing be Performed (cont)?

- JAK2 V617F is usually ordered first with a suspected MPN diagnosis

- If JAK2 V617F negative, then may add:
  - JAK2 Exon 12 (if suspected PV)
  - CALR, then MPL (if suspected ET / PMF)

- Significance of a JAK2, CALR, or MPL mutation:
  - “Confirms” that a proliferation is clonal (neoplastic)
  - Will, in general, exclude the possibility of a secondary or reactive condition

- Repeat testing is sometimes indicated:
  - Change in clinical status
  - Negative result and concerned about low-level mutation
  - Trials may require testing at time of enrollment
One Algorithm for Work-up of Suspected MPN

Diagnosis of myeloproliferative neoplasm

- Polycythemic vera (Suspected)
  - WHO criteria satisfied (JAK2 V617F or other JAK2 mutations)
    - STOP (No need for CALR or MPL mutation screening)

- Essential thrombocytopenia
  - Primary myelofibrosis
  - Pre-fibrotic myelofibrosis (Suspected)
    - JAK2 V617F+
      - STOP
    - CALR+
      - STOP
    - MPL+
      - STOP
    - ‘Triple-negative’ Consider screening for other clonal markers if feasible

How Should Mutation Testing be Performed?

Blood vs Bone Marrow
- Concern that blood testing could miss the affected BM precursor cells since RBC’s and platelets are not nucleated in PB
- With sensitive assays, no difference has been found

All Cells, Purified Neutrophils, or Purified CD34+ Cells
- Purification may increase sensitivity
- With sensitive assays, fine to use total nucleated cells (all cells)
- Purification adds unnecessary cost and labor

Mutation Testing in DNA or RNA?
- Testing RNA could add sensitivity
  - More copies/cell
- With sensitive assays, no difference has been found
- DNA-based testing more practical
JAK2 V617F Mutation Test

Mutation-Specific PCR

Detection by TaqMan Chemistry (Real-Time PCR)
JAK2 c.1849G>T, p.V617F Mutation

617
Y G V C V C G D
tat gga gta tgt gtc tgt gga gac

↓
Y G V C F C G D
tat gga gta tgt ttc tgt gga gac
Mutated allele levels vary widely among MPN patients

- Sensitive assays can detect JAK2 V617F at 0.1% in MPNs
- Very low JAK2 V617F levels (<0.1%) can be detected in “healthy patients”
- Mutation testing requires highly sensitive assays
- Clinically relevant sensitivity: 0.1% - 100% mutant allele

Vannucchi AM et al., *Leukemia*. 2008 Jul;22(7):1299
JAK2 V617F in Healthy Population

- 5/198 had statistically significant V617F mutation levels
- Highest level: 0.059%
- Below analytic sensitivity of most assays
- 0.1% - 100% is the clinically relevant test range

# JAK2 V617F Mutation Testing Methods

<table>
<thead>
<tr>
<th></th>
<th>Mutation Scanning</th>
<th>Targeted Mutation Detection</th>
<th>Analytic Sensitivity (% mutant allele)</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanger Sequencing</td>
<td>X</td>
<td></td>
<td>10-15%</td>
<td>Insufficient sensitivity</td>
</tr>
<tr>
<td>High Resolution Melting</td>
<td>X</td>
<td></td>
<td>10%</td>
<td>Insufficient sensitivity</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>X</td>
<td></td>
<td>5-10%</td>
<td>Insufficient sensitivity</td>
</tr>
<tr>
<td>Restriction Digest</td>
<td></td>
<td>X</td>
<td>2%</td>
<td>Insufficient sensitivity</td>
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<tr>
<td><strong>Allele-Specific PCR</strong></td>
<td></td>
<td>X</td>
<td><strong>0.1%</strong></td>
<td>Might detect + in healthy subjects</td>
</tr>
</tbody>
</table>
Allele-Specific Real-Time PCR

Quencher

Probe

REPORTER

Mut-Spec Primer

Denature
JAK2 V617F Mutation-Specific Assay

Tube 1

T Mutant Allele-Specific PCR Primer

TACTGTGGGAGACGAGAGTAAGTAAA

\[\ldots\text{ATGGAGTATGTGTCTTGGAGACGAGAGTAAGTAAA}^{\text{ACTA}}\ldots\]

V617F

JAK2 exon 14

Fwd  Probe  T-mut Rev

G>T V617F Substitution
JAK2 Control Assay

Tube 2

G Wild-Type Allele-Specific Primer

GACTGTGGAGACGAGAGTAAGTAAA

...ATGGAGTATGTGTCTGTGGAGACGAGATAGTAAAAACTA...

JAK2 exon 14

Fwd

Probe

G-wt Rev

JAK2 exon 14
PCR Amplification Plot – G Wild-Type Allele

Threshold

Cycle Threshold (CT)
PCR Amplification Plot – T Mutant Allele

Fluorescence

PCR Cycle

50% Mutant Control

1% Mutant Control

Pos/Neg Cutoff
<table>
<thead>
<tr>
<th>Sample</th>
<th>CT-G</th>
<th>CT-Mean G</th>
<th>Std. Dev. CT G</th>
<th>CT-T</th>
<th>CT-Mean T</th>
<th>Std. Dev. CT T</th>
<th>% T Frequency</th>
<th>Interpretation</th>
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<tr>
<td>50% G/T CAL</td>
<td>27</td>
<td></td>
<td>0.0608</td>
<td>27.9205</td>
<td>0.0266</td>
<td>47.52%</td>
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<td>50% G/T CAL</td>
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<td>26.957</td>
<td>0.0608</td>
<td>27.8828</td>
<td>27.90165</td>
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<td>NEG QC</td>
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<td>OK</td>
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<td>NEG QC</td>
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<td>27.2225</td>
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<td>08-05257</td>
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<td>0.00101</td>
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<td>08-05306</td>
<td>26.6684</td>
<td>0.0172</td>
<td>31.8178</td>
<td>0.129</td>
<td></td>
<td>4.88%</td>
<td>POSITIVE</td>
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<td>08-05306</td>
<td>26.6927</td>
<td>26.68055</td>
<td>0.0172</td>
<td>32</td>
<td>31.9089</td>
<td>0.129</td>
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<td>08-05318</td>
<td>26.5894</td>
<td>0.0444</td>
<td>Undetermined</td>
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<td></td>
<td></td>
<td>NEGATIVE</td>
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<td>26.5266</td>
<td>26.558</td>
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<tr>
<td>1% T Control</td>
<td>25.5329</td>
<td>0.0577</td>
<td>33.8474</td>
<td>0.0383</td>
<td></td>
<td>0.87%</td>
<td>OK</td>
<td></td>
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<tr>
<td>1% T Control</td>
<td>25.4514</td>
<td>25.49215</td>
<td>0.0577</td>
<td>33.9016</td>
<td>33.8745</td>
<td>0.0383</td>
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</tbody>
</table>
JAK2 V617F Test Analytic Sensitivity is 0.1% Mutant Allele
- Clinically relevant range 0.1% - 100%
JAK2 V617F Testing – The UM Experience

August 2007 – February 2012

Test Result

<table>
<thead>
<tr>
<th>Test Result</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>3450</td>
<td>(83.3%)</td>
</tr>
<tr>
<td>Positive</td>
<td>693</td>
<td>(16.7%)</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>1</td>
<td>(0.03%)</td>
</tr>
</tbody>
</table>

4144 Total

Pie chart showing the distribution of test results with a majority of negative results.
JAK2 V617F Testing – The UM Experience

August 2007 – February 2012

**Specimen**

- Peripheral Blood: 3564 (86%)
- Bone Marrow: 580 (14%)

4144 Total
JAK2 V617F Testing – The UM Experience

August 2007 – February 2012

**Peripheral Blood**
- Negative: 3013 (84.5%)
- Positive: 551 (15.5%)

3564 Total

**Bone Marrow**
- Negative: 437 (75.5%)
- Positive: 142 (24.5%)

579 Total
JAK2 V617F Mutant-Allele Burden

Range: 0.028 - 99.1%
Mean: 26.2%
Median: 18.2%
JAK2 V617F Testing – The UM Experience

JAK2 V617F Allele Burden – Blood vs. BM

<table>
<thead>
<tr>
<th>% Mutant V617F Allele</th>
<th># Cases</th>
</tr>
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<tbody>
<tr>
<td>&lt;0.1</td>
<td>5</td>
</tr>
<tr>
<td>0.1-1</td>
<td>4</td>
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<tr>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>5-10</td>
<td>0</td>
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<tr>
<td>10-15</td>
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<td>85-90</td>
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<tr>
<td>90-95</td>
<td>0</td>
</tr>
<tr>
<td>95-100</td>
<td>6</td>
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</table>

Blood

Range: 0.03 - 99.1%
Mean: 26.7%
Median: 17.7%

BM

Range: 0.28 - 96.0%
Mean: 24.2%
Median: 18.5%
JAK2 Exon 12 Mutations in PV
JAK2 Exon 12 Mutations in Polycythemia vera

- > 20 different mutations described
- Cause constitutive JAK2 activation
- Occur in patients with a diagnosis of PV or idiopathic erythrocytosis
- Associated (65% cases) with isolated erythrocytosis (w/o abnormal megakaryocytes)
- Similar clinical outcome as patients with V617F mutation
- Do **not** occur in ET and PMF
## JAK2 Exon 12 Mutations in Polycythemia vera

<table>
<thead>
<tr>
<th>535</th>
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<th>543</th>
<th>547</th>
<th>Cosmic Database</th>
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<td>M</td>
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<td>F</td>
<td>H</td>
<td>K</td>
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<td>V</td>
<td>F</td>
<td><strong>H</strong></td>
<td><strong>L</strong></td>
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<td>I</td>
<td><strong>H</strong></td>
<td><strong>E</strong></td>
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<td>V</td>
<td>F</td>
<td><strong>-</strong></td>
<td><strong>F</strong></td>
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<td>V</td>
<td>F</td>
<td><strong>-</strong></td>
<td><strong>-</strong></td>
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<td>V</td>
<td>F</td>
<td><strong>H</strong></td>
<td><strong>K</strong></td>
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<tr>
<td>M</td>
<td>V</td>
<td>F</td>
<td><strong>H</strong></td>
<td><strong>K</strong></td>
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<td><strong>H</strong></td>
<td><strong>K</strong></td>
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<td><strong>K</strong></td>
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<td>V</td>
<td>F</td>
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<td><strong>K</strong></td>
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<td>F</td>
<td><strong>H</strong></td>
<td><strong>K</strong></td>
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</tbody>
</table>

Modified and updated from:
# JAK2 Exon 12 Mutations in Polycythemia vera

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Various Duplications</td>
<td>6</td>
</tr>
<tr>
<td>K539L Substitutions (18)</td>
<td></td>
</tr>
<tr>
<td>H538_K539delinsQL</td>
<td>6</td>
</tr>
<tr>
<td>H538_L540delinsDLS</td>
<td>1</td>
</tr>
<tr>
<td>K539L</td>
<td>11</td>
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<tr>
<td>Various Substitutions</td>
<td>4</td>
</tr>
<tr>
<td>F537_K539delinsL</td>
<td>12</td>
</tr>
<tr>
<td>H538_K539delinsF</td>
<td>1</td>
</tr>
<tr>
<td>H538_K539delinsI</td>
<td>1</td>
</tr>
<tr>
<td>H538_K539delinsL</td>
<td>9</td>
</tr>
<tr>
<td>H538_K539del</td>
<td>1</td>
</tr>
<tr>
<td>I540_E543delinsMK</td>
<td>7</td>
</tr>
<tr>
<td>I540_N542delinsS</td>
<td>1</td>
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<tr>
<td>R541_E543delinsK</td>
<td>13</td>
</tr>
<tr>
<td>N542_E543del</td>
<td>47</td>
</tr>
<tr>
<td>E543_D544del</td>
<td>24</td>
</tr>
<tr>
<td>D544_L545del</td>
<td>1</td>
</tr>
<tr>
<td>Other Substitutions (4)</td>
<td></td>
</tr>
<tr>
<td>Deletions (117)</td>
<td></td>
</tr>
<tr>
<td>Various Duplications</td>
<td>6</td>
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</tbody>
</table>
### JAK2 Exon 12 Mutation Testing Methods

Gold-standard not yet established

<table>
<thead>
<tr>
<th></th>
<th>Mutation Scanning</th>
<th>Targeted Mutation Detection</th>
<th>Analytic Sensitivity (% mutation)</th>
<th>Minimum Required MPN Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sanger Sequencing</strong></td>
<td>X</td>
<td></td>
<td>10-15%</td>
<td>30%</td>
</tr>
<tr>
<td><strong>High Resolution Melting</strong></td>
<td>X</td>
<td></td>
<td>10%</td>
<td>20%</td>
</tr>
<tr>
<td><strong>Pyrosequencing</strong></td>
<td>X</td>
<td></td>
<td>5-10%</td>
<td>10-20%</td>
</tr>
<tr>
<td><strong>Fragment Analysis</strong></td>
<td></td>
<td>X</td>
<td>2%</td>
<td>4%</td>
</tr>
<tr>
<td><strong>Allele-Specific PCR</strong></td>
<td></td>
<td>X</td>
<td>0.01 - 1%</td>
<td>2%</td>
</tr>
</tbody>
</table>
Novel Test to Detect JAK2 Exon 12 Mutations

Multiplexed fragment analysis / AS-PCR test to detect JAK2 exon 12:

- Deletions & duplications
- K539L substitutions

141/145 (97%)

Compared performance of fragment analysis to:
- Direct sequencing
- High resolution melting

Fragment Analysis Assay for JAK2 Exon 12 Mutations

Length Mutation Assay

281 bp

K539L Allele-Specific Assay

131 bp

- Fwd
- Rev

Exon 12

Deletions & Duplications

K539L Substitutions
Fragment Analysis for JAK2 Exon 12 Mutations

Case 4
N542_E543del
Fragment Analysis for JAK2 Exon 12 Mutations

Case 1
K539L

Case 7
V536_L546dup
Fragment Analysis Achieves a Sensitivity of 1% Mutation

- **K539L**
  - Sensitivity: 5%

- **Deletion**
  - Sensitivity: 2%

- **Duplication**
  - Sensitivity: 1%
  - Sensitivity: 0.5%
JAK2 Exon 12 Mutation Testing at UM

Fragment Analysis Test

March 2010 – December 2014

3387  Negative

27  Positive (0.8%)

5  Inadequate Sample

3419

3/3 Positive cases:

- JAK2 V617F-negative polycythemia
- Exon 12 mutation confirmed a diagnosis of PV
## JAK2 Exon 12 Mutations Identified at UM

<table>
<thead>
<tr>
<th>Case</th>
<th>Mutation</th>
<th>Mutation (cDNA)</th>
<th>Level</th>
<th>Fragment Analysis</th>
<th>Direct Sequencing</th>
<th>HRM</th>
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<tbody>
<tr>
<td>1</td>
<td>R541_E543delinsK</td>
<td>1622_1627del</td>
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<td>Pos</td>
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<tr>
<td>2</td>
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<td>1627_1632del</td>
<td>15%</td>
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<tr>
<td>3</td>
<td>N542_E543del</td>
<td>1624_1629del</td>
<td>6%</td>
<td>Pos</td>
<td>Pos/Neg</td>
<td>Pos/Neg</td>
</tr>
<tr>
<td>4</td>
<td>N542_E543del</td>
<td>1624_1629del</td>
<td>20%</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>5</td>
<td>F537_K539delinsL</td>
<td>1611_1616del</td>
<td>23%</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
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<tr>
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<td>E543_D544del</td>
<td>1627_1632del</td>
<td>13%</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
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<tr>
<td>7</td>
<td>I540_D544delinsRNG</td>
<td>1619_1632delinsGAAATGGA</td>
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<td>8</td>
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<td>Pos/Neg</td>
<td>Pos/Neg</td>
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<td>9</td>
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<td>6%</td>
<td>Pos</td>
<td>Pos/Neg</td>
<td>Pos/Neg</td>
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<tr>
<td>10</td>
<td>H538_K539delinsL</td>
<td>1613_1616delinsT</td>
<td>17%</td>
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<tr>
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<td>1613_1618delinsTTG</td>
<td>23%</td>
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<td>13</td>
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<td>16</td>
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<td>1608_1640dup</td>
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</tbody>
</table>

Fragment Analysis, Direct Sequencing, and HRM all represent viable assays for JAK2 Exon 12 mutation testing.
UM Clinical Case #3

JAK2 exon 12 mutations can occur at low level
  - Sanger Sequencing may have insufficient sensitivity

N542_E543del
6 bp deletion
HRM May Also Have Insufficient Sensitivity for JAK2 Exon 12 Mutations
MPL Mutations in ET and PMF
MPL Mutations in PMF and ET

- 6 different mutations reported to date
- All located in MPL exon 10 (juxtamembrane domain) at W515 and S505
- Cause constitutive receptor activation and cytokine-independent growth \textit{in-vitro}
- Mutation frequency:
  - PMF 10%
  - ET 3%
  - PV 0%
- Mutations frequently identified at high allelic burden (>50%) (biallelic mutation); reported range 5-95% mutant allele
- MPL-positive PMF patients present with more severe anemia than JAK2 V617F positive patients
- Familial ET cases associated with germline MPL S505N mutation
MPL Mutations in PMF and ET

- Cosmic Mutation Database search
- Recurrent mutations (3 or more instances)
- 314 cases with 6 different mutations

```
CTCAGCGGCGTCTGCTGCTGCTGAGG
TGGCAG
```

```
505 515
L S A V L G L L L L R W Q
```

```
CTCAGCGGCGTCTGCTGCTGCTGAGG
A S505N (2%) T W515L (67%)
AA W515K (26%)
GC W515A (3%)
A W515R (1%)
C W515R (1%)
```
## MPL Mutation Testing Methods

Gold-standard not yet established

<table>
<thead>
<tr>
<th>Method</th>
<th>Mutation Scanning</th>
<th>Targeted Mutation Detection</th>
<th>Analytic Sensitivity (% mutation)</th>
<th>Minimum Required MPN Cells</th>
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</thead>
<tbody>
<tr>
<td>Sanger Sequencing</td>
<td>X</td>
<td></td>
<td>15%</td>
<td>30%</td>
</tr>
<tr>
<td>High Resolution Melting</td>
<td>X</td>
<td></td>
<td>10%</td>
<td>20%</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>X</td>
<td></td>
<td>5-10%</td>
<td>10-20%</td>
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<td><strong>Allele-Specific PCR Fragment Analysis</strong></td>
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<td>1-2.5%</td>
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<td><strong>Allele-Specific PCR Real-Time</strong></td>
<td>X</td>
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<td>0.1 - 1%</td>
<td>0.2 - 2%</td>
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</table>
Novel Test to Detect MPL Mutations

- Multiplexed allele-specific PCR-based strategy to detect:

  Frequency
  - W515L  (67%)
  - W515K  (26%)
  - W515A  (3%)
  - S505N  (2%)

  98% of reported MPL mutations

- Compared assay performance to direct sequencing

Allele-Specific PCR for MPL Mutation Detection

TUBE 1

Control PCR Product
211 bp

S505N Allele-Specific Assay
94 bp

W515L Allele-Specific Assay
124 bp

MPL exon 10

S505N
Fwd

W515L
Rev

S505N Substitutions

W515 Substitutions

TUBE 1
Allele-Specific PCR for MPL Mutation Detection

MPL exon 10

Control PCR Product
211 bp

W515A Allele-Specific Assay
126 bp

W515K Allele-Specific Assay
122 bp

W515A

W515K

Fwd
Rev

MPL exon 10

S505 Substitutions
W515 Substitutions

TUBE 2
Allele-Specific PCR for MPL Mutation Detection

Case 6 – Negative

Tube 1

Tube 2
Allele-Specific PCR for MPL Mutation Detection

Case 5 – W515L positive

Tube 1

Tube 2
Allele-Specific PCR for MPL Mutation Detection

Case 3 – W515K positive
Analytic Sensitivity of Multiplexed Allele-Specific PCR

2.5% Mutant Allele

<table>
<thead>
<tr>
<th>Allele</th>
<th>10%</th>
<th>5%</th>
<th>2.5%</th>
<th>Neg</th>
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<tbody>
<tr>
<td>S505N</td>
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<td></td>
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</tr>
<tr>
<td>W515L</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>W515A</td>
<td></td>
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<tr>
<td>W515K</td>
<td>10%</td>
<td>5%</td>
<td>2.5%</td>
<td>Neg</td>
</tr>
</tbody>
</table>
MPL Mutation Testing – The UM Experience

January 2011 – December 2014

Negative 2025

Positive 95 (4.5%)

Inadequate Sample 18

2138 Total

74 W515L
11 W515K
8 S505N
1 W515A
1 W515L + S505N
Direct Sequencing Has Insufficient Analytic Sensitivity

MPL W515L positive UM cases

Direct Sequencing

Case 14

Case 9

Case 20

Case 47

Negative

Allele-Specific PCR
Direct Sequencing Has Insufficient Analytic Sensitivity

<table>
<thead>
<tr>
<th></th>
<th>Sanger</th>
<th>Level</th>
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<tr>
<td>Negative</td>
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<tr>
<td>Positive</td>
<td>58</td>
<td>(4.4%)</td>
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<tr>
<td>Indeterminate</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1380</td>
<td></td>
</tr>
</tbody>
</table>

41 Positive 15-100%
10 Pos/Neg 5-15%
7 Negative
CALR Mutations in ET and PMF
CALR Mutations in MPN

- >36 different deletion and insertion mutations
- All cluster in CALR exon 9

Genomic Position of the 36 Mutation Types Detected in CALR

CALR Mutations in MPN

Frequency of the 36 Mutation Types Detected in CALR

- Type 1 (53.0%)
- Type 2 (31.7%)
- Type 3 (1.7%)
- Type 4 (1.0%)
- Types 5–10 (0.7% each)
- Types 11–36 (0.3% each)

52-bp deletion

5-bp insertion

CALR Mutation Testing – Considerations

- All CALR mutations result in a **frame-shift**

- Germline polymorphisms (**in-frame deletions**) occur in healthy population
  - Klampfl et al., 3 and 18 bp deletions, 2 in 1107 (0.2%)
  - Nangalia et al., 9 and 12 bp deletions, frequency not reported
  - Our experience: 9 bp deletions, 6/590 (1.0%)

- CALR mutations can be **homozygous**

- More than one type of CALR mutation can be present
  - Clonal evolution

- Rarely other mutations may be present with CALR
CALR Mutation Detection by Fragment Analysis

Deletions and Insertions

Intron 8

52 bp Deletion

WT 265 bp

5 bp Insertion

Neg

Pos
Fragment Analysis Achieves a Sensitivity of 1% Mutation

Sanger sequencing likely has insufficient sensitivity
Distinguishing Length-Affecting Polymorphisms from Mutations

Gray analysis bins denote location of 3, 9, 12, 18bp deletion polymorphisms
Uncommon Length-Mutations are Verified by Sanger Sequencing Prior to Reporting

To rule out length-affecting polymorphism
Fragment sizing is accurate to +/- 0.5 bp

46 bp deletion?
CALR Mutation Testing – The UM Experience

March 2014 – December 2014

Negative 351
Positive 72 (17%)
Inadequate Sample 1

424 Total

39 deletion (52 bp)
21 insertion (5 bp)
3 deletion (1 bp)
2 deletion (46 bp)
2 deletion (34 bp)
1 deletion (61 bp)
1 deletion (49 bp)
1 deletion (31 bp)
1 deletion (19 bp)
1 deletion (7 bp)
Mutation testing is standard of care for diagnostic workup of any suspected MPN

JAK2 V617F is most useful first test for PV, ET, and PMF

Well established second order tests:
- JAK2 exon 12 mutations (suspected PV)
- CALR mutation (suspected PMF or ET)
- MPL mutation (suspected PMF or ET)

Presence of a mutation establishes a clonal (neoplastic) proliferation and rules out a secondary/reactive condition

Absence of JAK2 V617F and exon 12 mutations rules out PV

Absence of JAK2 V617F, CALR, and MPL does not rule out ET or PMF
- 15% ET and 5% PMF cases are triple negative
Allele-Specific Real-Time PCR
Advantages of the fragment analysis-based test:

• Detects nearly all JAK2 exon 12 mutations:
  Deletions
  Duplications
  K539L substitution mutations (AA>TT and AA>CT)

• Sensitive: 1-2%

• Robust

• Simple interpretation

• Single tube

Limitations:

• Won’t detect rare (non-K539L) substitution mutations (3%)

• Not a closed-tube assay like HRM
2008 WHO Classification of Myeloid Neoplasms

Myeloproliferative neoplasms (MPN)
- Chronic myelogenous leukemia (CML)
- Polycythemia vera (PV)
- Essential thrombocythemia (ET)
- Primary myelofibrosis (PMF)
- Chronic neutrophilic leukemia (CNL)
- Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
- Mast cell disease (MCD)
- MPN, unclassifiable

Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB, and FGFR1

MDS/MPN

Myelodysplastic syndromes (MDS)

Acute myeloid leukemia (AML)
MLabs Technical Bulletin

Myeloproliferative Neoplasms

JAK2 V617F MUTATION

Detection of the JAK2 c.1849G>T (V617F) mutation in myeloproliferative neoplasms: polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). This test is indicated for evaluation of patients with unexplained and sustained elevation of red blood cell or platelet counts, splenomegaly or bone marrow fibrosis of undetermined causation, and patients in whom a diagnosis of a chronic myeloproliferative disorder is a consideration. The JAK2 V617F mutation is detectable in approximately 95% PV, 55% ET, and 55% PMF patients. A negative result does not rule out a diagnosis of PV, ET, or PMF.

JAK2 EXON 12 MUTATION

Mutations within exon 12 of the JAK2 gene occur in most cases of JAK2 V617F-mutation negative polycythemia vera. Testing for JAK2 exon 12 mutations may aid in the diagnosis of polycythemia vera, and is recommended in patients with JAK2 V617F-negative erythrocytosis. This test will qualitatively detect JAK2 exon 12 mutations in peripheral blood or bone marrow specimens with a sensitivity down to 2% mutant allele. This is a second order test and should be used only following a JAK2 V617F-negative result.

MPL MUTATION

MPL gene mutations occur in cases of primary myelofibrosis (PMF) and essential thrombocythemia (ET) at a frequency of ~10% and 3% respectively. Testing for MPL mutations may aid in the diagnosis of these myeloproliferative neoplasms. MPL mutations are usually found in cases that test negative for the JAK2 V617F mutation, although a small number of patients have been reported with both mutations. This test will qualitatively detect MPL mutations (W515L, W515K, W515A, and S505N) in peripheral blood or bone marrow with a sensitivity down to 5% mutant allele.

KIT D816V MUTATION

This test is used for qualitative detection of the KIT c.2447A>T (D816V) mutation found in most adults (>80%) with systemic mastocytosis. Detection of the KIT D816V mutation can aid in diagnosis of systemic mastocytosis and guide choice of therapy since it is associated with resistance to imatinib mesylate. The KIT D816V mutation also occurs in some cases of acute myelogenous leukemia and seminoma. This test is not intended to detect minimal residual disease.

MYELOPROLIFERATIVE NEOPLASMS (MPN) ALGORITHM

In keeping with our commitment to develop and offer the critical tests necessary for clinicians to care for their patients, the Department of Pathology (MLabs) at the University of Michigan utilizes the following mutation testing algorithm for suspected PV, ET, or PMF.

MLabs' MPN algorithm will:
- Correlate MPN mutation testing within a single source laboratory.
- Streamline efficiency of testing, decreasing turn around time.
- Provide highly accurate diagnostically relevant results.
- Improve cost efficiency.

As a leader in the rapidly evolving molecular diagnostic field, we hope that the following MPN Testing Algorithm will aid in your selection of the most informative and diagnostically relevant tests.

HOW TO SEND A SPECIMEN

For assistance 24 hours per day, 7 days per week, call MLabs at 800-862-7284 or visit our website at www.mlabs.umich.edu.

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Expertise Delivered Personally
Recommended Mutation Testing Algorithm for Suspected PV, ET, and PMF

Results of these tests must always be interpreted in the context of morphologic and other relevant data.
JAK2 and MPL Mutations Have Similar Biologic, But Different Phenotypic Effects

Direct Sequencing for JAK2 Exon 12 Mutations

Case 1
K539L

Case 6
R541_E543delinsK

Negative
Direct Sequencing Achieves Sensitivity of 10%

Case 1
K539L

Case 4
N542_E543del
Small-amplicon melting

126 bp

Exon 12
JAK2 Exon 12 HRM – Normalized Melt Curves

- Case 1: K539L
- Case 2: R541_E543delinsK, I540S
- Case 3: F537_K539delinsL
- Case 4: N542_E543del
JAK2 Exon 12 HRM – Difference Plot

Case 5  E543_D544del
Case 6  R541_E543delinsK
Case 7  V536_L546dup
JAK2 Exon 12 HRM Achieves a Sensitivity of 10%
Validation

• 36 MPL negative samples
  – 14 mutations
    • 1 case had two CALR mutations
  – 2 polymorphism (-3bp)
• 21 JAK2 positive samples (14 blood, 7 bone marrow)
  – 20 Negative
    – 1 case with a low level JAK2 (V6717F) mutation was positive for CALR mutation
• Results confirmed by Sanger sequencing
• 590 CF bloods screened
  – 6 polymorphism (-9bp)
  – Confirmed by Sanger sequencing
Reference Intervals/Cutoffs

- Determined by evaluating the results from the low positive control (5% mutation), the sensitivity dilutions, and the negative QC and negative validation samples
- Control peak: >8000
- Positive cutoff: >500
- Repeat: 200-500 (if repeated: weak positive)
Inter-Laboratory Comparison (ARUP)

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<th>U. Mich. CALR Result</th>
<th>ARUP CALR Result</th>
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Testing Strategy

• Direct reporting of type1 and type2 mutations
  – 3, 9, 12, 18 bp deletions (polymorphism) reported as negative
    • Plasmids for 12 and 18 bp deletions ordered

• Sanger sequencing of all other mutations for 1 year
  – Expected 1-2 cases/month