Outline of Talk

• Setting up a Lab (Molecular Hematopathology)
  – Space
  – Personnel

• Instrumentation

• Assay Development, Validation and Implementation
  – Laboratory Development phase
  – Clinical Validation Phase
  – Test Implementation
Centralized or Decentralized
Molecular Diagnostics at Yale

• Yale New Haven Hospital
  – Laboratory Medicine
    • Microbiology- David Peaper, MD, PhD
    • Molecular Diagnostics- Greg Howe, PhD, ABMG, DABCC
    • Virology- Marie Landry, MD

• Yale University School of Medicine
  – Anatomic Pathology
    • Molecular Diagnostics- Jeff Sklar, MD, PhD; Pei Hui, MD, PhD
    • Tumor Profiling- Jeff Sklar MD, PhD; Zenta Walther, MD, PhD

  – Genetics/Genomics Center
    • Clinical Molecular Genetics- Allen Bale, MD
    • Clinical Cytogenetics- Peining Li, PhD, ABMG
Yale New Haven Hospital, Department of Laboratory Medicine, 55 Park St, New Haven, CT
Staff Training and Competency

- All technologists are certified as Medical Laboratory Technologists by ASCP.

- Limited molecular diagnostics experience when hired

- Molecular diagnostics is the ever expanding menu based on new research and increasing volume.

- PhD director, five technologists and one PhD development scientist; support staff.
Basic Instruments In The Molecular Lab

- Automated DNA extraction
- Amplification (PCR)
- Capillary electrophoresis (DNA sequencing/ fragment analysis)
- Gel electrophoresis
- Real-time PCR quantitation
Next Generation Sequencing
Ion Torrent PGM

316 chip has >6 million wells
Laboratory and Hospital Information Systems

- Soft Computer Systems (soft-lab; soft-flow)
- Bar codes on samples
- Hospital computers
- Epic electronic record software in hospital
Clinical Neoplastic Hematopathology Tests

- Chronic myeloproliferative diseases
  - Chronic myeloid leukemia- BCR-ABL (9;22) (Quant RT-PCR); Kinase (sequencing)
  - Polycythemia vera- Jak2 (V617F) (Quant); JAK2 exon 12, 13 mutations
  - Essential thrombocytopenia- MPL mutation analysis
  - Eosinophilia- FiP1L1-PDGRFA; PDGRFB and FGRF1 translocations
  - Systemic mastocytosis- c-kit (D816V) (Quant)
- Myelodysplastic/myeloproliferative diseases
- Myelodysplastic syndromes
- Acute myeloid leukemia
  - Translocation screen (15;17) (Quant), (8;21) (Quant), inv16, (9;22)
  - Normal karyotype- FLT3, NPM1, CEBPA, Kit mutations; tumor profiling
- Precursor B-and T- neoplasms (Acute lymphoblastic leukemia)
  - Translocation screen (12;21), (4;11), (1;19), (9;22) (FISH or RT-PCR)
  - B and T cell gene rearrangement
- Mature B-cell neoplasms
  - B cell gene rearrangement
  - Chronic lymphocytic leukemia- IgV hypermutation (Sequencing)
  - Mantle cell leukemia- (11;14) bcl-1 diagnostics
  - Follicular lymphoma- (14;18) bcl-2 (Quant)
- Mature T-cell and NK- cell neoplasms
  - T cell gene rearrangement; EBV (in situ hybridization)
- Hodgkin lymphoma- Epstein Barr Virus (EBV)
- Immunodeficiency associated lymphoproliferative disorders
  - Epstein Barr Virus (Quant)
- Histiocytic and dendritic cell neoplasms
Neoplastic Hematopathology Commercial Molecular Kits/Probes Used in Clinical Labs

• FDA approved
  – Abbott, B-Cell Chronic Lymphocytic Leukemia (B-CLL), CEP12 DNA Probe pharmDx Kit, FISH on FFPE
  – Abbott, Chromosome 8 Enumeration (CML, AML, MPD, MDS), CEP8 DNA Probe Kit, FISH

• ASR
  – Abbott, Numerous translocations probes, FISH
  – DakoCytomation, Numerous translocation probes, FISH
  – Asuragen, Leukemia translocation Panel, RT-PCR

• RUO
  – Gene rearrangement InVivoScribe (CE), Patent
  – JAK2 InVivoScribe, Ipsogen, Invitrogen, Patent
  – BCR/ABL Quantitative Roche, Ipsogen (CE), Cepheid
  – FLT3 InVivoScribe, Patent
  – BCL2 Roche, InVivoScribe (CE)
  – Various translocations Ipsogen (some CE), Hemovision
  – Somatic hypermutation InVivoScribe
  – NPM1 Ipsogen, Asuragen, Patent
# Myeloproliferative Disorders

<table>
<thead>
<tr>
<th>HSC</th>
<th>Myeloid progenitor</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Mast cell</th>
<th>Systemic mastocytosis</th>
<th>Activating mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells</td>
<td>Polycythaemia vera</td>
<td>JAK2V617F, JAK2 Exon 12</td>
</tr>
<tr>
<td>Platelets</td>
<td>Essential thrombocythaemia</td>
<td>JAK2V617F, MPLW515L/K</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Chronic eosinophilic leukemia</td>
<td>FIP1L1–PDGFRA</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Chronic myeloid leukaemia</td>
<td>BCR-ABL</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Chronic myelomonocytic leukaemia, Primary myelofibrosis</td>
<td>TEL–PDGFRB, BCR–PDGFR, TEL–JAK2, other fusion TKs, JAK2V617F, MPLW515L/K</td>
</tr>
</tbody>
</table>

*Nature Reviews Cancer (2007) 7; 673*
Chronic Myeloid Leukemia (CML)

- CML is 15-20% of the cases of leukemia
- Patients 50-60 yrs old
- Leukocyte levels can reach 50,000-200,000/µl
- Chronic phase: 2-4 years; <5% blasts in marrow
- Accelerated phase: weeks to months; 10-19% blasts in PB
- Blast phase: days to weeks; 20% blasts in PB
t(9;22) BCR-ABL Translocation

Normal chromosome 9

Normal chromosome 22

Chromosomes break

Changed chromosome 9

Changed chromosome 22 (Philadelphia chromosome)

Normal BCR-ABL

Positive BCR-ABL

FISH

National Cancer Institute
Chronic Myeloid Leukemia (CML)

- 90% of patients with CML have a Philadelphia (Ph) chromosome and 100% have BCR-ABL translocation.
  - 20% of adults with acute lymphoblastic leukemia (ALL)
  - 3-4% of children with acute lymphoblastic leukemia (ALL)
  - Rarely in AML

- Three protein forms:
  - p210 (major breakpoint) found in almost all CML patients with BCR-ABL translocation.
  - p190 (minor breakpoint); found in 50% ALL-adults and 80% ALL-childhood that are (BCR-ABL+).
  - p230- rare (neutrophilic maturation)
Imatinib Mesylate

First member of a new class of chemotherapy agents, which acts by inhibiting a particular tyrosine kinase. Gleevec is the trade name.
Molecular Diagnostics Testing

Evolution of Molecular Tests

- HIV and Chronic Myeloid Leukemia (CML)
  - Qualitative testing for diagnosis.
  - With the introduction of therapy, there is a need for quantitative monitoring.
  - Resistance to drugs – need for DNA sequencing to test for resistance in order to change therapy.

- Cystic Fibrosis
  - Single target testing to multiplex testing

- Tumor Profiling
  - Multiple gene testing
Laboratory Test Development Phase
Initial Attempts To Get Assay To Work

• Maximize chances to be successful.
  – Experienced technologist or careful oversight
  – Known positive material
  – New quality reagents
    • Primers and probes- double check sequence
  – Use successful concentrations and temperatures, if possible
  – Functioning instruments

• Optimize each step of the assay

• Preanalytical variables
  – Specimen type and stability
  – Master mix stability
  – Interfering substances
Laboratory Test Development Phase
Control Material- In House/ASRs

• Known positive and negative material
  – Cancer types in blood and tissue blocks
  – Cell lines (K562)
  – Normal clinical specimen type (e.g. blood, BM, tissue) that can be used as a matrix for spiking in abnormal cells.
  – Known positive specimens tested by another assay or another lab.

• Calibration materials for quantitative tests
  – Count number of cells
Laboratory Test Development Phase
Reagents

- In house developed reagents (e.g. master mixes)
  - Purchase quality materials
  - Label with content, concentration, date of preparation, expiration date
  - Important to aliquot and freeze master mixes, primers, probes
  - Important to test new reagents against old reagents
Analytical sensitivity
- Lowest amount target that can be detected
- Spiking BCR-ABL containing cell line (K562) to negative samples

Reference (normal) ranges
- Normal samples (< 45 cycles)
Laboratory Test Development Phase
Performance Characteristics

- Reportable ranges (linear dynamic range)
  - Highest and lowest limits of the assay that are reliable
  - BCR-ABL reportable range: 100% to 0.0001%
  - After introduction perform periodic Calibration Verification- testing throughout reportable range

- Analytical specificity
  - Confirm that the assay detects only the specific target
  - 82 patients with significant clinical presentations (leukocytosis, thrombocytosis, erythrocytosis, leukemia, PCV, R/O CML, MDS etc.) were tested.
  - Compared the original nested RT-PCR assay and newly developed BCR-ABL Screen tests. There were no discrepancies found for either assay.
Laboratory Test Development Phase
Performance Characteristics

- Precision - consistency of replicates
  - Same day and between days
  - Use reference material similar to intended patient sample.
  - Concentration close to limit of detection; several concentration points if quantitative
  - Patient samples were very reproducible.

![Graph showing reproducibility and logarithmic relative fold change for BCR-ABL p210. The equation is given as y = 1.0088x - 0.0573 with an R^2 of 0.9974.](image)
Laboratory Test Development Phase
Performance Characteristics

➢ Accuracy - return correct result compared to external standard
  • Commercial materials
  • Proficiency materials
  • Previously tested clinical material

➢ Needs to be carried out in a random and blinded fashion
  • Comparison to gold standard assay
  • Comparison to another lab’s results
  • Comparison to values in spiked clinical samples
Laboratory Test Development Phase
Performance Characteristics

Sample exchange program for Quantitative BCR-ABL p210

- 10 unknown samples, 7 spiked blood samples in RNAlater and 3 cDNA samples.
- Procedure required RNA purification, RNA quantification, breakpoint identification and calculation of % (log) reduction on positive samples.
- Twenty-nine laboratories across the country participated.
- Of the top 10 laboratories that were able to achieve both linear results and reasonable sensitivities, only one or two were able to achieve the expected result.
Laboratory #29 (Yale) was most consistent with the expected results and demonstrated the greatest sensitivity for the assay.
Clinical Validation

• Clinical Validation is to show that the assay results correlate with clinical disease.
  – Clinical sensitivity= \( \frac{TP}{TP+FN} \) \times 100
  – Clinical specificity = \( \frac{TN}{TN+FP} \) \times 100

• Compare with gold standard method, another test in the lab, or clinical data.

• For p210, 82 patients with a number of significant clinical presentations (leukocytosis, thrombocytosis, erythrocytosis, leukemia, PCV, R/O CML, MDS etc.) were tested comparing the original nested RT-PCR assay and this newly developed BCR-ABL Screen tests. Clinical sensitivity and specificity were both 100%

• 31 CML patients were tested by both the qualitative nested RT-PCR and the BCR-ABL Screen test. No discrepancies were found. 20 were positive for the p210 translocation and 13 were negative for both assays.
Laboratory Test Development Phase
Quality Controls

- Quality controls used in nucleic acid extraction and amplification
  - Water blank- contamination
  - Negative control- non-specific PCR products
  - Positive control: internal and external
  - Sensitivity control- lowest reportable limit of assay

- Other quality controls
  - No reverse transcriptase control- DNA contamination
  - Molecular weight markers
  - Standard curves
Quantitative RT-PCR Assay for BCR-ABL

1. For new patients the first test is a real-time multiplex RT-PCR screen that is able to pick up the 190, 210 and 230 translocation forms. Uses common ABL primer and unique primers for 190, 210, and 230 translocations.

2. If the sample has a Ct value of 45 then no more testing is done. If the result is less than 45 then the sample is positive and the nested RT-PCR is performed and the products run on a gel with controls.

3. If one of the specific translocations is identified then a quantitative RT-PCR assay is performed to determine the amount of BCR/ABL mRNA. An additional control gene is run, beta-2 microglobulin, as a control. And specific amounts of BCR/ABL positive control material is run for a standard curve.
Quantitation of BCR-ABL Translocation

p210 Translocation

- **BCR**: b1 | b2 | b3
- **ABL**: a2 | a3

Primer 1: Common Primer
Common Probe
Common Reverse Primer

p190 Translocation

- **BCR**: e1
- **ABL**: a2 | a3

Primer 2: Common Primer
Common Probe
Common Reverse Primer

p230 Translocation

- **BCR**: e19
- **ABL**: a2 | a3

Primer 3: Common Primer
Common Probe
Common Reverse Primer
Quantitative RT-PCR for BCR-ABL

<table>
<thead>
<tr>
<th>Last Name</th>
<th>%K562</th>
<th>10</th>
<th>1.0</th>
<th>0.10</th>
<th>0.010</th>
<th>0.0010</th>
<th>0.0001</th>
<th>Normal</th>
<th>Reagent</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Name</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample Date</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accession/Flow #</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unit#</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Master Mix LOT #</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCR-ABL p210</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-B2micro</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio p210 / B2micro</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(log) %K562</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- $y = -0.2243x + 1.6553$
- $R^2 = 0.9986$

Patient- Monitor:
- PB

Patient- Diagnosis:
- PB

<table>
<thead>
<tr>
<th>PB</th>
<th>PB</th>
</tr>
</thead>
</table>

- 45 45
- 24.84 24.8

-6.04 -6.03 0.24 0.25
At 60 months, the patients who had a complete cytogenetic response and a reduction of at least 3 log in levels of BCR-ABL transcripts in bone marrow cells after 18 months of treatment had an estimated rate of survival without progression to the accelerated phase or blast crisis of CML of 100%.
Test Implementation

- Procedure Manual
  - Reviewed annually and signed by director

- Reports
  - Interpretation
  - Method
  - Language about test not being FDA approved, but developed and characterized locally.
  - Results figure

- Validation Documentation

- Computer interfacing
  - Test numbers
    - Cost and CPT code
  - Electronic reports
  - Interpretation comments
Test Implementation
Getting Paid (Reimbursement)

  – 81206; 81207; BCR-ABL 210 and 190

• New tests may not be recognized by insurance companies.

• Tests can be expensive.
  – What is Medicare reimbursement amount for your area?
  – Reimbursement may not cover costs, especially for low volume tests.

• Part B billing – only MDs, not PhDs.

Current Issues with BCR/ABL Monitoring

Average BCR/ABL level for Untreated CML Patients

Average (log) % K562

Derived Baseline = 0.4, n=31 patients

- MCR
- CCR
- MMR
- CMR

<4.0 is below background

Diagnosis

Treat with Gleevec
Current Issues with BCR/ABL Monitoring
International Standard

• Substantial variation in the real-time quantitative PCR methodologies used by different testing laboratories.

  – BCR-ABL Quantitative RT-PCR Proficiency Testing Results (MRD-A 2008)

<table>
<thead>
<tr>
<th># of Labs</th>
<th>Expected</th>
<th>Mean</th>
<th>S.D.</th>
<th>C.V.</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRD-2</td>
<td>51</td>
<td>2</td>
<td>1.07</td>
<td>0.66</td>
<td>-0.09</td>
<td>2.7</td>
</tr>
<tr>
<td>MRD-3</td>
<td>44</td>
<td>4</td>
<td>2.62</td>
<td>0.57</td>
<td>1.34</td>
<td>&gt;5.0</td>
</tr>
</tbody>
</table>

• Recommendation that 4 materials be established for quantitation of BCR-ABL translocation by real-time quantitative PCR was approved by the World Health Organization in November 2009.

• The reference material panel is comprised of 4 different dilution levels of freeze dried preparations of K562 cells diluted in HL60 cells.

• Limited resource availability restricted to manufacturers of secondary reference materials.

• Asuragen has developed secondary standards for clinical labs.
Validation of International Standard

A

ABL1

BCR-ABL1 e13a2

BCR-ABL1 e14a2

BCR

B

Assignment of mean IS % ratios to WHO primary standards by testing in multiple IS labs

Alignment of secondary Calibrators to mean IS % ratios of WHO standards

Alignment of local % ratios to mean WHO IS % ratios by testing secondary calibrators

CP = correction parameter
Current Issues with BCR/ABL Monitoring Resistance

- More than 60 mutations at different amino acid positions within the BCR-ABL kinase domain have been associated with clinical resistance.
- 2nd/3rd generation kinase inhibitors:
  - **Nilotinib**, engineered to bind more tightly to the BCR-ABL protein. 25-fold more potent than imatinib.
  - **Dasatinib**, structurally unrelated to imatinib, 325-fold more potent.
  - **Ponatinib**, effective against T315

Pathology 40; 231–246, 2008
Summary

• The hematopathology molecular diagnostics field has few FDA approved tests.

• Requires the development of lab developed tests (LDTs).

• Specific performance parameters are required by CAP to be obtained for a lab developed test.

• Medical technologists need to gain the experience to develop, maintain and perform LDTs.