ARUP Institute for Clinical and Experimental Pathology™ presents our 6th Annual Colloquium

Presenters from ARUP Institute of Clinical and Experimental Pathology™ include:

Leslie Rowe  Tom Martins
Melinda Procter  Cecily Vaughn
Keith Simmon  Troy Jaskowski
Tanya Sandrock  Philippe Szankasi
Mark Herrmann  Jeff Stevenson
Michael Liew  June Pounder
Alan Erickson  Joann Cloud
Sam Page  Carlynn Willmore-Payne
Rick Roberts  Bob Chou
Sergey Preobrazhensky  David Crockett
Jonathan Schumacher  Mike Seipp
Jun Lu  Weston Hymas
Scott Reading  Genevieve Pont-Kingdon
Eric Konnick  Fernanda Calderon

At the forefront of innovative Research and Development

6th Annual Colloquium | June 9, 2006
**AGENDA**

**6th Annual Research Colloquium**

**ARUP Institute for Clinical and Experimental Pathology™**

**Friday, June 9, 2006**

---

### Presentations
**ARUP Matsen Conference Center**

*The order of presentations was determined by a random drawing.*

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:30 a.m.</td>
<td>Welcome by Harry R. Hill, MD</td>
</tr>
<tr>
<td>8:40 a.m.</td>
<td>Leslie Rowe</td>
</tr>
<tr>
<td>8:52 a.m.</td>
<td>Melinda Procter</td>
</tr>
<tr>
<td>9:04 a.m.</td>
<td>Keith Simmon</td>
</tr>
<tr>
<td>9:16 a.m.</td>
<td>Tanya Sandrock</td>
</tr>
<tr>
<td>9:28 a.m.</td>
<td>Mark Herrmann</td>
</tr>
<tr>
<td>9:40 a.m.</td>
<td>Michael Liew</td>
</tr>
<tr>
<td>9:52 a.m.</td>
<td>Alan Erickson</td>
</tr>
<tr>
<td>10:04 a.m.</td>
<td>Sam Page</td>
</tr>
<tr>
<td>10:16 a.m.</td>
<td>BREAK</td>
</tr>
<tr>
<td>10:30 a.m.</td>
<td>Rick Roberts</td>
</tr>
<tr>
<td>10:42 a.m.</td>
<td>Sergey Preobrazhensky</td>
</tr>
<tr>
<td>10:54 a.m.</td>
<td>Jonathan Schumacher</td>
</tr>
<tr>
<td>11:06 a.m.</td>
<td>Jun Lu</td>
</tr>
<tr>
<td>11:18 a.m.</td>
<td>Scott Reading</td>
</tr>
<tr>
<td>11:30 a.m.</td>
<td>Eric Konnick</td>
</tr>
<tr>
<td>11:42 a.m.</td>
<td>Lis Schwarz</td>
</tr>
<tr>
<td>11:54 a.m.</td>
<td>Maria Erali</td>
</tr>
</tbody>
</table>

### Reception and Awards Ceremony
**Marriott University Park Hotel**

*5:00 p.m. | Social Hour (drinks and hors d’oeuvres)*
*5:30 p.m. | Awards Presentation*

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:06 p.m.</td>
<td>LUNCH</td>
</tr>
<tr>
<td>1:00 p.m.</td>
<td>Fernanda Calderon</td>
</tr>
<tr>
<td>1:12 p.m.</td>
<td>Tom Martins</td>
</tr>
<tr>
<td>1:24 p.m.</td>
<td>Cecily Vaughn</td>
</tr>
<tr>
<td>1:36 p.m.</td>
<td>Troy Jaskowski</td>
</tr>
<tr>
<td>1:48 p.m.</td>
<td>Philippe Szankasi</td>
</tr>
<tr>
<td>2:00 p.m.</td>
<td>Jeff Stevenson</td>
</tr>
<tr>
<td>2:12 p.m.</td>
<td>June Pounder</td>
</tr>
<tr>
<td>2:24 p.m.</td>
<td>Joann Cloud</td>
</tr>
<tr>
<td>2:36 p.m.</td>
<td>BREAK</td>
</tr>
<tr>
<td>3:00 p.m.</td>
<td>Carlynn Willmore-Payne</td>
</tr>
<tr>
<td>3:12 p.m.</td>
<td>Bob Chou</td>
</tr>
<tr>
<td>3:24 p.m.</td>
<td>David Crockett</td>
</tr>
<tr>
<td>3:36 p.m.</td>
<td>Mike Seipp</td>
</tr>
<tr>
<td>3:48 p.m.</td>
<td>Weston Hymas</td>
</tr>
<tr>
<td>4:00 p.m.</td>
<td>Genevieve Pont-Kingdon</td>
</tr>
</tbody>
</table>
Table of Contents

Agenda ...................................................................................................................................................................................2

Unusual Secondary Chromosomal Change in Pediatric Acute Lymphoblastic Leukemia with Cryptic t(12;21): A Case Report (Rowe) ..............................................................................................................................................................5

Molecular Diagnosis of Prader-Willi Syndrome and Angelman Syndrome by Methylation-Specific Melting Analysis and Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (Procter) ...........................................................................................................................................................................6

Identification of Anaerobic Bacteria by 16S rRNA Gene Sequencing with SmartGene and Microseq Software and Databases (Simmon)................................................................................................................................................................................7

Detection of Thyroid Stimulating Immunoglobulins using Enzyme Fragment Complementation (Sandrock) ..............................................................................................................................................................................8

High Resolution Detection of Paroxysmal Nocturnal Hemoglobinuria Type Cells (Herrmann) ................................................................................................................................................................................9

Quantitative Heteroduplex Analysis and Optimization of DNA Mixtures for All SNPs by High-Resolution Melting (Liew) ....................................................................................................................................................................................10

Evaluation of an Enzyme-Linked Binding Protein Assay for Hyaluronic Acid and Concentrations in Hepatitis C Infected Patients (Erickson) ..................................................................................................................................................................................................11

Sequencing the Reverse Transcriptase Domain of the Hepatitis B Virus (HBV) Polymerase Gene to Determine Genotype (Page) ................................................................................................................................................................................................12

Removal of Macroprolactin using Protein G Agarose (Roberts) ................................................................................................................................................................................................13

Flow Cytometric Detection of ZAP-70 Expression in CLL Cells (Preobrazhensky) ...................................................................................................................................................................................................14

Detection of the D816V Mutation in Systemic Mastocytosis by Allele-Specific PCR (Schumacher) ................................................................................................................................................................................................15

Measurement of Total Coenzyme Q10 Using HPLC with Electrochemical Detection (Lu) ................................................................................................................................................................................................16

Detection of Acquired JAK2 V617F Mutation in Myeloproliferative Disorders by Fluorescence Melting Curve Analysis (Reading) ................................................................................................................................................................................................17

*Bordetella pertussis* and *Bordetella parapertussis* Detection Using PCR and Capillary Electrophoresis (Konnick) ................................................................................................................................................................................................18

Increased Sensitivity of Newborn Screening for CAH by UPLC-MS/MS (Schwarz) ....................................................................................................................................................................................................19

Human Papillomavirus Genotyping Using the AutoGenomics INFINITI™ Microarray Analyzer (Erali) ................................................................................................................................................................................................20

Laboratory Diagnosis of Galactosemia: Combination of Enzyme Activity, Allele-specific PCR and Full Gene Analysis (Calderon) ................................................................................................................................................................................................21

Analysis of Antibody Responses to Specific and Non-Specific Streptococcal and Tissue Antigens in Patients with Acute Rheumatic Fever (Martins) ................................................................................................................................................................................................22

Molecular Detection of FMS-like Tyrosine Kinase 3 Mutations in Acute Myeloid Leukemia (Vaughn) ................................................................................................................................................................................................23
## Table of Contents

Detection of IgG Autoantibody Against F-Actin in Patients Suspected of Having Autoimmune or Acute Viral Hepatitis (Jaskowski) .......................................................... 24

Characterization of SCF<sup>FBXO45</sup> a Novel Ubiquitin Ligase Involved in Follicular Lymphoma Transformation (Szankasi) ......................................................................................................................... 25

Quantitation of EBV Virus by Real Time PCR (Stevenson) ......................................................................................................................... 26

Identification of Nonsporulating Molds and Dermatophytes by Sequencing Internal Transcribed Spacer Regions with SmartGene Software and Database (Pounder) ........................................................................................................... 27

Evaluation of an Enzyme Immunoassay for the Quantitative Detection of *Histoplasma* Antigen in Human Urine Samples (Cloud) .......................................................................................................................... 28

The L576P C-kit Mutation as a Predominant Activating Mutation in Non-GiST Tumors (Willmore-Payne) ................................................. 29

Unlabeled Oligonucleotide Probes Modified with Locked Nucleic Acids (LNA) for Improved Mismatch Discrimination in Genotyping by Melting Analysis (Chou) ........................................................................................................... 30

Fusion Mapping by Tandem Mass Spectrometry: A Proteomic Approach for the Identification of Translocation Partners Encoding Chimeric Fusion Proteins (Crockett) ............................................................................................................................ 31

Improved High Resolution Genotyping by Incorporation of Melting Temperature Standards (Seipp) ............................................................... 32

A Real Time RT-PCR Assay for Enterovirus Detection Using Hybridization Probes and a Non-Competitive Internal Control (Hymas) .......................................................................................................................... 33

Use of Single “Loop-Out” Probes for Multiplex Genotyping (Pont-Kingdon) .......................................................................................... 34
Unusual Secondary Chromosomal Change in Pediatric Acute Lymphoblastic Leukemia with Cryptic t(12;21): A Case Report

Sarah T. South, Leslie R. Rowe, Emily Aston, Bo Hong, Mahmoud Issa, Elizabeth Drozd-Borysiuk, Anna Hooker, Jia Xu, Arthur B. Brothman, and Zhong Chen

Clonal cytogenetic changes can be either primary or secondary. Primary changes are generally responsible for the initial pathogenesis and have direct diagnostic significance. Secondary changes play an important role in disease progression and are associated with the prognosis of a disease. We report a case of pediatric acute precursor B-lymphoblastic leukemia (pre B-ALL) with an unexpected 5q deletion secondary to an underlying cryptic primary chromosomal abnormality. A 4-year-old Caucasian female was diagnosed with pre B-ALL. Cytogenetic analysis of bone marrow revealed two distinct cell lines, one showing del(5)(q13q31) as the sole clonal abnormality in 6/20 (30%) of metaphase cells and the remaining 14/20 (70%) metaphase cells showing a normal female complement. Fluorescent in-situ hybridization (FISH), using the EGR1 probe (Vysis) specific to 5q31, confirmed the loss of this locus in 102/200 (51%) of interphase nuclei. Deletions of 5q have been frequently identified in myeloid disorders. Due to the diagnosis of pre B-ALL in this patient, additional FISH studies were performed. The presence of a cryptic t(12;21)(p13;q22) was identified in 192/200 (96%) interphase cells analyzed. The TEL-AML1 fusion gene, created by the t(12;21)(p13;q22), occurs in approximately 25-30% of pediatric ALL cases and is associated with a favorable prognosis. Although t(12;21) can be difficult to identify by conventional cytogenetic techniques, it can be visualized using FISH or reverse transcriptase-polymerase chain reaction (RT-PCR). Due to the high percentage of cells positive for t(12;21) versus del(5q) in interphase FISH (96% versus 51%, respectively), t(12;21) likely represents the primary clonal abnormality and the del(5q) a secondary abnormality. In cases of ALL, known but cryptic abnormalities should always be considered when either the chromosomes are normal or the identified cytogenetic abnormality is unexpected. Additional studies are needed to determine the possible relationship between 5q deletion and t(12;21) in pediatric ALL.

Objectives:
1) Describe the role of primary versus secondary clonal cytogenetic changes
2) Discuss the relationship of t(12;21) to acute lymphocytic leukemia
3) Discuss the importance of identifying cryptic abnormalities when the identified cytogenetic abnormality is unexpected
Molecular Diagnosis of Prader-Willi Syndrome and Angelman Syndrome by Methylation-Specific Melting Analysis and Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

Melinda Procter, Lan-Szu Chou, Wei Tang, Mohamed Jama, and Rong Mao

**Objectives:**

1) To be able to identify Prader-Willi Syndrome (PWS) and Angelman Syndrome (AS)

2) To understand the genetic mechanism behind imprinting disorders using Prader-Willi Syndrome and Angelman Syndrome as examples

3) To understand the use of MLPA to detect PWS and AS

**Background:** Approximately 99% of Prader-Willi syndrome (PWS) and 80% of Angelman syndrome (AS) cases have either deletions at a common region in chromosome 15q11.2-q13, uniparental disomy for chromosomes 15 (UPD15), or imprinting center defects affecting gene expression in this region. The resulting clinical phenotype (PWS or AS) in each class of mutation depends upon the parent of origin. Both disorders are characterized at the molecular level by abnormal methylation of imprinted regions at 15q11.2-q13. Other rare chromosome 15 rearrangements and a few smaller atypical deletions associated with abnormal methylation patterns, also present symptoms overlapping with either PWS or AS.

**Methods:** We designed a methylation-specific melting analysis (MS-MA) for a rapid screening of PWS/AS, and evaluated methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) for diagnosis of PWS/AS associated with deletions, UPD15 or rare duplications. Forty-nine previously genotyped samples were tested by MS-MA. We also tested 26 MS-MA genotyped samples and one additional sample with rare duplication of chromosome region 15q11-q12.

**Results:** Genotyping results of PWS/AS by MS-MA and by MS-MLPA were fully concordant. In addition, MS-MLPA was superior in detecting deletions/rare duplications, possible UPD15 or imprinting center defects, which were usually determined by the laborious FISH method or by chromosomal segregation analysis for the parental-origin using STR makers.

**Conclusions:** MS-MA as appears to be an efficient primary method to diagnose PWS/AS, and use of the quantitative MS-MLPA method provides detailed information about deletions, rare duplications, and possibly UPDs.
Identification of Anaerobic Bacteria by 16S rRNA Gene Sequencing with SmartGene and Microseq Software and Databases

K.E. Simmon, S. Mirrett, L.B. Reller, and C.A. Petti

Objectives:

1) Illustrate the pros and cons of commercially available 16S rRNA gene databases

2) Demonstrate how ARUP is tailoring SmartGene software to meet the needs of a Clinical Reference Lab

3) Illustrate how 16S rRNA sequence has impacted the identification of anaerobic organisms

Background: Anaerobic microorganisms are significant bloodstream pathogens, and rapid, accurate diagnosis is important for patient management. Identification of anaerobes from positive blood cultures by conventional methods (CM) is time-consuming, requires technical expertise, and often is presumptive. Partial sequencing of the 16S rRNA gene is faster, but its accuracy is limited by reference databases. We compared the accuracy of two 16S rRNA databases for the identification of clinically important anaerobic microorganisms from patients with bloodstream infection.

Methods: Anaerobic microorganisms recovered from blood cultures between Jan 2000 and Dec 2004 at Duke University Hospital and identified by CM were retrospectively reviewed. Frozen isolates were sent to ARUP for DNA extraction, amplification, and partial sequencing of the 16S rRNA gene (27F, 515R) by a standard protocol. Sequences were analyzed by SeqScape (Applied Biosystems) and compared with both the SmartGene and Microseq (Applied Biosystems) databases. Sequence-based identifications were compared to CM and arbitrarily defined by percent similarity: species > 99%, genus > 95%, and inconclusive < 95%.

Results: Partial sequencing has been performed on 299 isolates. SmartGene identified 225 (75%) to species and 62 (21%) to genus with 12 (4%) being inconclusive. Microseq identified 202 (67%) to species and 51 (17%) to genus with 46 (15%) being inconclusive. Fifteen species were not included in the Microseq database, which affected the results of 25 isolates. Using SmartGene as the gold standard, CM correctly identified 178 (60%) and 78 (26%) isolates to species and genus, respectively.

Conclusion: Partial sequencing of the 16S rRNA gene is more rapid and accurate than CM for identification of anaerobes. Compared with Microseq, the SmartGene database contains a larger number of comparative sequences, provides greater microbial diversity, and enables more accurate and complete identification of common and emerging anaerobic bacteria. SmartGene also allows users to customize its software and to easily create an internal database.
Detection of Thyroid Stimulating Immunoglobulins using Enzyme Fragment Complementation

Tanya Sandrock, Alan Terry, Jeff Martin, and Wayne Meikle

Objectives:

1) To introduce GPCR signaling pathways

2) To discuss Graves disease

3) To describe enzyme-fragment complementation and how the technology is used to detect thyroid stimulating immunoglobulins

Thyroid hormone is made in response to thyroid stimulating hormone (TSH). When thyroid hormone levels are low, TSH made in the pituitary stimulates thyroid stimulating hormone receptor, TSHR, a G-protein coupled receptor (GPCR). Activation results in a cascade leading to increased intracellular cAMP levels and ultimately increased thyroid hormone production. In Graves’ disease, auto-antibodies activate TSHR even in the absence of TSH leading to an increase in intracellular cAMP, thyroid hormone, and hyperthyroidism. We have adapted enzyme fragment complementation (EFC, DiscoveRx, Fremont CA) to measure cyclic adenosine monophosphate (cAMP) levels in a bioassay using Chinese hamster ovary (CHO) cell line producing the TSH receptor. Four hundred and twenty-five specimens submitted for thyroid stimulating immunoglobulins (TSI) testing were assayed using a traditional radio-immune assay (RIA-cAMP) and the non-radioactive EFC-cAMP method. There was a strong correlation between the RIA-cAMP and EFC-cAMP methods (Deming regression slope of 1.048 (1.008-1.087), intercept -2.2 (-8.3 to 3.8), 95% confidence interval are shown in parentheses). Forty-six normal individuals tested using the EFC-cAMP method ranged from 80-106% of the normal control with a mean of 94% and a Std. dev. of 6.8%. The advantages of EFC-cAMP over the RIA assay are the following: The TSI EFC-cAMP method is 1) non-radioactive, 2) automated, 3) less labor intensive, and 4) measures internal cAMP.
High Resolution Detection of Paroxysmal Nocturnal Hemoglobinuria Type Cells

M.J. Herrmann, C.J. Parker and C.T. Wittwer

Objectives:

1) Discuss what is Paroxysmal Nocturnal Hemoglobinuria (PNH)

2) Discuss the significance of small population PNH cells in bone marrow failures

3) Discuss the limitations of current PNH detection methods

Paroxysmal nocturnal hemoglobinuria (PNH) is a nonmalignant clonal stem cell disorder resulting from somatic mutation in the enzyme PIGA. The disabling of PIGA results in stem cells deficient in glycosyl phosphatidylinositol (GPI) anchored proteins. GPI proteins anchor complement regulatory proteins CD55 and CD59 to the external cell wall, their absence results in premature cell destruction. Clinical symptoms are intermittent hemoglobinuria, elevated plasma hemoglobin, and anemia. PNH has also been identified with bone marrow failures such as aplastic anemia and myelodysplastic syndrome. However, bone marrow failures can have PNH cells yet have no PNH symptoms. Recent studies have shown that patients with small populations of PNH cells in combination with aplastic anemia or refractory anemia –MDS, respond better to immunosuppressive therapy. Current methods of testing for PNH by flow cytometry, resolve PNH cell populations greater then 1%. We have been investigating a ultra sensitive method that can resolve PNH cells to 0.001%. We demonstrate correlation between positive PNH samples and normal samples. We also demonstrate linearity of detection for erythrocytes down to 0.02% and granulocytes down to 0.01%.
Quantitative heteroduplex analysis and optimization of DNA mixtures for all SNPs by high-resolution melting

Robert A. Palais, Michael A. Liew, and Carl T. Wittwer

Objectives:

1) To explain heteroduplex formation

2) To explain how DNA heteroduplexes can be detected by high resolution melting

3) To explain how quantification of DNA heteroduplexes can be used to resolve DNA homoduplexes that have identical melting temperatures

High resolution melting analysis of small amplicons is a rapid closed tube method for genotyping homozygous and heterozygous single nucleotide polymorphisms (SNPs). Homozygous polymorphisms pose more of a challenge than the heterozygous polymorphisms, because their melting curves have similar shapes so they are distinguished primarily by Tm, and a small percentage of SNPs produce Tm differences too small to detect. The example used in this study is the SNP found in the hemochromatosis gene (HFE) 187C>G. To genotype these difficult to distinguish homozygous SNPs it was determined that by “spiking” in a certain amount of wild type DNA into samples it would be possible to resolve the 3 genotypes. In order to determine the exact percentage of wild type DNA to spike in to achieve maximal resolution of the genotypes, a mathematical model was generated, and experiments were implemented to see how well these correlated. DNA samples of known HFE 187C>G genotype were extracted and quantified for PCR. Different ratios of the known genotypes and wild type genotype were mixed together, then amplified and analyzed by high resolution melt analysis or temperature gradient capillary electrophoresis (TGCE). It was determined that having wild type DNA in at a concentration of 1/7 of the total provided the optimal separation of genotypes. The experimental results were also in excellent agreement with the mathematical model. Using this method aids with these difficult to distinguish homozygous SNPs and reduces the amount of post-PCR handling to minimize the chance of contamination.
Evaluation of an Enzyme-Linked Binding Protein Assay for Hyaluronic Acid and Concentrations in Hepatitis C Infected Patients

J. Alan Erickson and Edward R. Ashwood

Objectives:

1) Understand the general structure and role of hyaluronic acid

2) Recognize the advantages of hyaluronic acid over liver biopsy for assessing cirrhosis and fibrosis

3) Evaluate serum/plasma hyaluronic acid levels in healthy subjects versus individuals with hepatitis and other liver diseases

Serological hyaluronic acid (HA) has been proposed as a noninvasive alternative to liver biopsy for assessing the extent of liver fibrosis. Liver biopsy correctly identifies hepatic disease in about 65 to 75% of cases, being strongly dependent on the length of the biopsy obtained. Furthermore, hepatic fibrosis is often not distributed homogeneously throughout the liver. In contrast, studies suggest HA to have better sensitivity and specificity with areas under receiver operating curves of 0.86 and 0.92, and negative predictive values of 93% and 99% for fibrosis and cirrhosis respectively. Moreover, the non-uniform distribution of hepatic fibrotic tissue does not affect HA results. In this study, we evaluated and validated a commercially available enzyme-linked binding protein assay for measuring HA. We also investigated serum/plasma HA concentrations in individuals having elevated levels of hepatitis C virus (HCV) RNA, hepatitis B virus (HBV) DNA, positive for both hepatitis B virus surface antigen (HBsAg) and core IgM antibody (anti-HBc), hepatitis A IgM (anti-HAV) positive, with Alpha-1-antitrypsin (A1a) Z phenotype and end stage liver disease. Subjects suffering rheumatoid arthritis (elevated cyclic citrullinated peptide IgG) and healthy elderly individuals (80 – 90 years) were also investigated.

The HA assay (Corgenix Inc., Westminster, CO) is a spectrophotometric sandwich protein binding assay in microplate format. The test utilizes a highly specific HA binding protein (HABP) coated to the microwell surface to capture HA. An enzyme conjugated version of HABP is subsequently used to detect the HA in the sample. The assay uses six calibrators with results expressed as ng HA/mL.

Serum and/or plasma samples were stored and assayed according to the kit manufacturer’s instructions. The assay’s limit of detection was 8 ng/mL resulting in an analytical measurement range of 8 – 800 ng/mL. A linearity study generated a slope of 1.008, intercept of 13.64 and R2 of 0.998 (n = 7). The within-run precision at three levels (n = 8) was determined to be 29 ± 0.8, 138 ± 3.4 and 546 ± 6.7 ng/mL with CVs of 2.7, 2.5 and 1.2% respectively. Between-run precision at three levels (n = 7) resulted in values of 44 ± 3.9, 87 ± 7.6 and 459 ± 29.3 ng/mL generating CVs of 8.8, 8.5 and 6.4% respectively. A correlation study using samples previously assayed at Corgenix produced a slope, intercept and R2 of 0.957, –0.56 and 0.993 respectively as analyzed by Deming Regression (n = 22, range 29 – 866 ng/mL). Utilizing donations from 122 healthy individuals, an upper 97.5% reference limit of 54 ng/mL was established. HA was found stable for 24 hours at room temperature, and a minimum of two weeks at 4 °C.

Deidentified serum or plasma samples from patients with HCV infection were assayed for HA. Of 68 specimens, 47% were found to have HA greater than the established 54 ng/mL reference interval. HA concentration did not correlate with HCV RNA level. HA was also elevated (n = 20 per group) in 45% of the HBV DNA samples (chronic hepatitis B), 80% of the HBsAg/anti-HBc positive samples (acute hepatitis B), 65% of the anti-HAV positive samples (acute hepatitis A), 40% of those with A1a Z phenotypes and 100% with end stage liver disease. In addition, 24% with rheumatoid arthritis and 60% of the elderly were elevated in HA. However, the elevated concentrations measured in these groups were generally several times lower than those of the previous groupings.

Conclusion: The Corgenix HA test kit has shown acceptable performance characteristics for quantifying HA. Although the stages of liver fibrosis for the subjects in this study were unavailable, the large percentage having elevated HA supports previous studies suggesting the possible use of HA in assessing liver fibrosis and/or cirrhosis in lieu of liver biopsy.
Sequencing the Reverse Transcriptase Domain of the Hepatitis B Virus (HBV) Polymerase Gene to Determine Genotype

Sam Page and David R. Hillyard

Hepatitis B Virus (HBV) is a small, hepatotrophic DNA virus that chronically infects an estimated 350 million individuals worldwide. Diseases caused by HBV include liver cirrhosis and hepatocellular carcinoma (HCC). Eight HBV genotypes (A-H) have been identified (that infect humans), each having diverged by more than approximately 8% in total genome sequence or 4.1% in surface/polymerase gene sequence. Although type A virus accounts for most chronic infections in North America, an increasing number of non-A HBV infections have been documented. Treatment for chronic HBV infection includes interferon alpha, nucleoside analogs and an evolving array of new drugs. Each treatment has limited therapeutic effectiveness and can cause serious side effects. In addition, nucleoside analog treatment can cause the emergence of resistant viral populations. Recent studies have documented an improved outcome for HBV type A infected patients treated with pegylated interferon compared to lamivudine. This provides a rationale for clinical HBV genotype testing.

We developed a sensitive sequencing assay that analyzes a 941 bp region of the reverse transcriptase (RT) domain of the HBV polymerase gene. This region is amplified by PCR, purified using Ampure magnetic beads (Agencourt), sequenced bi-directionally using 4 sequencing primers and detected by capillary electrophoresis using an ABI Prism 3730 DNA Analyzer (Applied Biosystems). Sequences are compared to a library containing known RT sequences (genotypes A-G) for similarity. Samples with viral loads as low as 200 IU/mL (measured by the Roche COBAS TaqManTM HBV Test) can be reproducibly amplified and genotyped.

Accurate HBV genotyping does not require sequencing of the entire 941 bp amplicon. Therefore, one approach to clinical genotype testing is to sequence only a portion of the amplicon. However, the full 941 bp amplicon also contains domains that encode principle drug resistance and immune escape mutations. Therefore, the efficient generation of the full amplicon, even from low copy number samples, provides a general template for genotyping, as well as drug resistance and escape mutant analysis.

**Objectives:**

1) To explain the role of viral genotyping, resistance testing and escape mutant identification as they relate to the treatment of a Hepatitis B infection

2) To describe the design of a sequencing assay that is capable of HBV genotyping, resistance testing and escape mutant identification

3) To describe the performance of the HBV sequencing assay and findings from the scanning of random HBV DNA positive clinical samples
Elevated prolactin concentrations have been attributed to the presence of macroprolactin, a complex between prolactin and other macromolecules, typically immunoglobulins. Macroprolactin lacks biological activity but can lead to additional diagnostic studies in a search for a prolactin-secreting adenoma. Our objective was to evaluate a rapid method to determine the true prolactin concentration using Protein G to remove immunoglobulin-containing macroprolactin. We evaluated this approach using 398 samples with ADVIA Centaur prolactin concentrations >30 ng/mL. Protein-G agarose was washed with one volume of normal saline and the supernatant removed. An equal volume of serum was added to the agarose, mixed for ten minutes, centrifuged, and the supernatant assayed for prolactin. The serum IgG concentration of a sample was reduced from 1120 mg/dL initially to < 7 mg/dL after Protein-G treatment. We compared the prolactin result on the ADVIA Centaur® before and after Protein-G treatment. Deming regression gave a slope of 1.712 (r=0.99). We multiplied the Protein-G supernatant result by 1.712 to correct for dilution (referred to as Protein-G Corrected). Samples with adequate volume had prolactin determined on the Roche E170 (no Protein-G treatment). Deming regression of this data gave E170 = 1.75 x Protein-G Corrected – 1.4, r=0.973, n=393. We calculated the percent recovery for the neat ADVIA Centaur and E170 result compared to the Protein-G corrected result. Percent recoveries greater than 149% for the Centaur and 242% for the E170 were considered positive for the presence of macroprolactin. Our positivity rates for macroprolactin were 2.5% and 5.1% for the ADVIA Centaur and E170, respectively. Results for all macroprolactins detected by the ADVIA Centaur method are shown in the Table. We conclude that Protein-G treatment is useful for removal of macroprolactin from samples. Nearly all samples that had macroprolactin by the ADVIA Centaur method also had macroprolactin detected by the E170 method.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Centaur Neat (ng/mL)</th>
<th>E 170 Neat (ng/mL)</th>
<th>Protein G Corrected (ng/mL)</th>
<th>Centaur Recovery (%)</th>
<th>E170 Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>33</td>
<td>42</td>
<td>19</td>
<td>175</td>
<td>223</td>
</tr>
<tr>
<td>17</td>
<td>31</td>
<td>39</td>
<td>19</td>
<td>165</td>
<td>207</td>
</tr>
<tr>
<td>31</td>
<td>55</td>
<td>117</td>
<td>34</td>
<td>161</td>
<td>342</td>
</tr>
<tr>
<td>113</td>
<td>39</td>
<td>124</td>
<td>10</td>
<td>380</td>
<td>1207</td>
</tr>
<tr>
<td>141</td>
<td>49</td>
<td>427</td>
<td>33</td>
<td>151</td>
<td>1313</td>
</tr>
<tr>
<td>143</td>
<td>36</td>
<td>90</td>
<td>24</td>
<td>150</td>
<td>376</td>
</tr>
<tr>
<td>146</td>
<td>39</td>
<td>72</td>
<td>26</td>
<td>152</td>
<td>380</td>
</tr>
<tr>
<td>151</td>
<td>58</td>
<td>105</td>
<td>38</td>
<td>154</td>
<td>279</td>
</tr>
<tr>
<td>198</td>
<td>42</td>
<td>73</td>
<td>27</td>
<td>153</td>
<td>267</td>
</tr>
<tr>
<td>231</td>
<td>38</td>
<td>361</td>
<td>22</td>
<td>171</td>
<td>1622</td>
</tr>
</tbody>
</table>
Flow Cytometric Detection of ZAP-70 Expression in CLL Cells

Sergey N. Preobrazhensky and David W. Bahler

Objectives:
1) Review clinical significance of flow cytometric procedure for detection of ZAP-70 in CLL cells
2) Understand complicated issues in detection of ZAP-70 with flow cytometry
3) Describe potential ways to overcome these issues

Background: Chronic lymphocytic leukemia (CLL) represents the most common type of leukemia among adult leukemias. The clinical prognosis of patients with CLL is variable but has been shown to correlate strongly with somatic mutations in the gene encoding immunoglobulin heavy chain variable region (IgVH) of CLL B-cells. Non-mutant IgVH phenotype is associated with more aggressive type of the disease. However, at the present time the procedure for detection of IgVH mutations is not readily available for clinical screening of CLL patients. Recently, it was shown that levels of zeta-associated protein of 70 KD (ZAP-70) are elevated in B-cells of CLL patients with non-mutant IgVH and are associated with more aggressive type of disease. ZAP-70 level can be measured with flow cytometry, making it potentially an excellent prognostic marker. Nevertheless, due to low staining intensities with available commercial reagents, initial attempts to develop a procedure for clinical testing of ZAP-70 expression in CLL cells were facing significant challenges.

Objectives: The objective of this study was to optimize a flow cytometric procedure for quantitating ZAP-70 in CLL cells. Particularly, to compare various monoclonal antibodies, cell permeabilization procedures and different approaches for establishing negative cutoff settings.

Methods: CLL cells were identified in lyzed peripheral blood samples by staining of cell surface antigens with anti-CD5 and anti-CD19 monoclonal antibodies. ZAP-70 level in CLL cells was evaluated by measuring the intensity of cell fluorescence after the cells were fixed, permeabilized and stained with fluorochrome conjugated anti-ZAP-70 monoclonal antibodies. Normal B and T-cells from peripheral blood of healthy donors were used as negative and positive controls, respectively. Anti-ZAP-70 antibodies from three different suppliers were compared along with four different cell permeabilization procedures. Negative threshold values were determined using two different approaches. One using ZAP-70 stained normal peripheral blood B-cells, and the other using isotype control stained CLL cells. Three different isotype control antibodies (Mouse IgG, anti-CD13 and anti-BrdU) were evaluated. Percentage of ZAP-70 positive cells was determined in 42 CLL cases, using both negative cutoff threshold settings.

Results: The best resolution between specific and non-specific anti-ZAP-70 binding determined from multiple titrations studies were obtained with an Alexa Fluor 488-anti-ZAP-70 antibody (clone 1E7.2) and saponin permeabilization procedure. The best performing isotype control tested was an Alexa Fluor 488-anti-CD13. Comparing ZAP-70 CLL results using normal peripheral blood B-cells, and the isotype control stained CLL cells cutoff methods indicated a strong positive correlation between the percentage of ZAP-70-positive. However, intra-assay precision was better with isotype cutoff than with a normal B-cell cutoff (CV=16% and 40%, respectively). The higher level of precision can possibly explain better resolution between positive and negative CLL samples, observed with isotype cutoff settings.

Conclusions: The results of these studies suggest that optimization of antibody concentrations and permeabilization procedures are needed to adequately measure ZAP-70 levels in CLL cells. Moreover, use of a properly tested isotype control antibody has several advantages over using ZAP-70 normal B-cells in setting negative cutoff levels by increasing the reliability and precision of the procedure.
Detection of the D816V Mutation in Systemic Mastocytosis by Allele-Specific PCR

Jonathan A. Schumacher, Kojo S.J. Elenitoba-Johnson, and Megan S. Lim

Objectives:

1) To describe the most frequent genetic aberration in mastocytosis
2) To explain what is meant by allele-specific PCR
3) To tell the sensitivity of the D816V assay

Systemic mast cell disease (SMCD) is a clonal disorder involving mast cell proliferation with infiltration of cutaneous and extracutaneous organs including bone marrow, gastrointestinal tract, liver, and spleen. Recent studies have shown that mutations of the c-kit proto-oncogene may cause the majority of SMCD cases. One of the most common mutations found in SMCD is the D816V mutation of c-kit, which causes ligand-independent activation of the tyrosine kinase II domain of the protein, resulting in mast cell proliferation and transformation. Here we describe an allele-specific PCR assay designed to detect the D816V mutation in SMCD from routine paraffin-embedded tissue. Our assay was designed to amplify wild type c-kit as a control in one tube and D816V with a mutant-specific forward primer in a second tube. A positive control was developed by site-directed mutagenesis of a D816V-negative cell line, EOL-1. In addition, nine patient samples that were previously diagnosed with SMCD by morphologic and immunophenotypic studies and 25 non-SMCD patient samples expressing BCR-ABL, inv(16), or a mutation in the Jak2 gene were assessed. We were able to detect D816V down to 0.5% tumor cells. The results of our studies show high specificity, sensitivity, and reproducibility for detection of the D816V mutation from routinely fixed paraffin-embedded bone marrow biopsy samples. Our data demonstrate the clinical applications of allele-specific PCR to detect the D816V in SMCD.
Measurement of Total Coenzyme Q10 Using HPLC with Electrochemical Detection

Jun Lu and Elizabeth L. Frank

**Objectives:**

1) Discuss what is coenzyme Q10

2) Discuss why did coenzyme Q10 get its fame

3) Discuss how the assay was validated

**Background:** Coenzyme Q10 (CoQ10, ubiquinone) is one of a group of lipid-soluble benzoquinone involved in mitochondrial electron transport. CoQ10 also functions as an intracellular antioxidant. Due to its role in energy metabolism, supplemental CoQ10 has been proposed for the treatment of a variety of disorders. Research supporting the efficacy of CoQ10 shows the most promise for its use in neurodegenerative disorders such as Parkinson’s disease and some encephalomyopathies. Study results in other areas, including use of CoQ10 in the treatment of cardiovascular disease and diabetes, are inconclusive. The value of CoQ10 supplementation with statin use has not been clearly established.

**Methodology:** The concentration of coenzyme Q10 is measured in plasma or serum by coulometric detection following isolation of the compound by reverse phase high performance liquid chromatography (HPLC). The method is calibrated using a five-point curve with an internal standard, coenzyme Q9. Samples are prepared for analysis by protein precipitation using 1-propanol.

**Results:** The data were plotted as the ratios of standard CoQ10 vs. internal standard CoQ9 over CoQ10 concentration range from 0.05 mg/L to 4.00 mg/L. Excellent linear relationships were observed using both peak-height and peak-area measurements. The regression equations for six replicates were: y = 8.19x + 0.13 (r² = 1) by peak height, and y = 10.24x + 0.13 (r² = 1) by peak area. The limit of quantification was 0.0075 mg/L. Imprecision was measured by analyzing spiked samples in triplicate for five days. Within run reproducibility at 0.520, 0.689, and 1.97 mg/L was 2.3, 5.1, and 1.3 %CV, respectively; total reproducibility was 10.1, 7.2, and 2.4 %CV, respectively, for the same concentrations. Patient specimens analyzed previously by a reference laboratory were tested using the new method. The linear regression equation for correlation of 32 specimens was y = 0.98x – 0.03 (r=0.83). A published reference interval of 0.4 mg/L-1.6 mg/L was confirmed by testing plasma samples from self-reported healthy adults (n = 38, age range 21 – 69 years).

**Conclusion:** We have validated a method for the determination of total coenzyme Q10 concentration using HPLC-EC measurement. The advantages of this robust method include high sensitivity, accuracy, and consistency. The clinical laboratories also will benefit from the speed of this test.
Detection of Acquired JAK2 V617F Mutation in Myeloproliferative Disorders by Fluorescence Melting Curve Analysis

Scott Reading

Objectives:

1) Introduce the JAK2 V617F detection assay
2) Describe the 3 protocols developed to detect the mutation
3) Illustrate the sensitivity of the detection protocols

The genetic lesion underlying the pathogenesis of chronic myeloproliferative disorders (MPD) has been identified in the Janus kinase 2 (JAK2) gene. A point mutation in codon 617 causes a valine to phenylalanine substitution in the JH2 autoinhibitory region of the protein, resulting in constitutive activation of the tyrosine kinase. The high prevalence of this conserved mutation in the CMPD makes it an excellent candidate as a diagnostic molecular marker. We report here the development and validation of a single oligonucleotide probe-based PCR approach using fluorescence melting curve analysis for point mutation detection in DNA derived from unfractionated peripheral blood samples. Using this assay and serial dilutions of an erythroleukemia cell line harboring the homozygous JAK2 V617F mutation, we successfully detected the mutation within a background of wild-type sequences at a sensitivity of 2.5%. Our novel fluorescence probe-based assay was compared with allele-specific PCR-gel assay and sequencing. Using the single probe assay on 38 peripheral blood DNA samples submitted with a presumptive diagnosis of MPD, we identified 10/38 samples as wild type and 28/38 samples as having the JAK2 V617F mutation. Additionally, the probe-based assay detected a previously unreported T to C base substitution at nucleotide 2342 (JAK2, codon 616) that was not detected by an allele-specific PCR assay. The single fluorescent probe-based assay, described herein, is a rapid, homogeneous, and robust method for the detection of the JAK2 V617F mutation with favorable performance characteristics that make it advantageous for clinical diagnosis.
**Bordetella pertussis and Bordetella parapertussis**
Detection Using PCR and Capillary Electrophoresis

Eric Q. Konnick and David R. Hillyard

---

**Objectives:**

1) Describe the principles of fragment analysis (FA) detection of PCR products

2) List 3 advantages and disadvantages of FA in the context of infectious disease testing

3) Explain 2 solutions to problems presented by FA

Whooping cough is a major worldwide problem that is responsible for significant morbidity and mortality. The main etiologic agent implicated in whooping cough is *Bordetella pertussis* although *Bordetella parapertussis* has also been implicated in disease. While effective vaccines and treatment exist for *B. pertussis*, the incidence of disease has been observed to be increasing recently. Detection of *B. pertussis* and *B. parapertussis* by molecular methods has become commonplace and are routinely used to diagnose *Bordetella* infections.

We have developed a novel multiplex fragment analysis (FA) method using short PCR products to simultaneously detect *B. pertussis*, *B. parapertussis*, and a synthetic, non-competitive internal control in clinical samples. Fluorescein-labeled primers were used to amplify organism-specific transposable elements present in multiple copies in *B. pertussis* and *B. parapertussis*. Fluorescently-labeled PCR products were detected using capillary electrophoresis performed on an ABI 3100 instrument and results were compared to several real-time PCR methods. Multiplexed FA as evaluated is highly sensitive and offers a cost-effective alternative to real-time PCR for high-throughput qualitative infectious disease testing.
**Increased Sensitivity of Newborn Screening for CAH by UPLC-MS/MS**

E. Schwarz, A. Liu, R. Guymon, L. Richardson, S. Parker, M. Pasquali

**Objectives:**
1) Review of Congenital Adrenal Hyperplasia (CAH)
2) Review of newborn screening for CAH
3) Application of tandem mass spectrometry for steroid profiling

**Background:** Congenital Adrenal Hyperplasia (CAH) is caused by inherited defects in steroid biosynthesis. The majority of cases are due to deficiency of 21-hydroxylase and result in the accumulation of 17-hydroxyprogesterone (17-OHP) and androstenedione with reduction of cortisol. The clinical presentation of CAH includes ambiguous genitalia, precocious puberty, and adrenal crisis, with the latter leading to life threatening salt wasting crisis. Currently, several states screen newborns for CAH measuring 17-OHP by immunoassay in blood spots. This test has a high false positives rate due to transient elevation of 17-OHP in newborns, illness, cross-reactivity of antibodies, and other causes. Measurement of 17-OHP and evaluation of steroid profile by LC-MS/MS can reduce the rate of false positives in newborns blood spots. UPLC (Ultra Performance Liquid Chromatography) is a new technology that combines very high pressure with reduced particle size in the chromatographic column, allowing rapid analysis and increased resolution. We have modified an existing LC-MS/MS method and adapted it to the UPLC-MS/MS system to measure 17-OHP, androstenedione, and cortisol from dried blood spots to identify newborn at risk for CAH.

**Method:** 17-OHP, androstenedione and cortisol were extracted from a 4.8-mm (3/16-inch) dried blood spot with 200 μL of 80% acetonitrile/water containing 0.4 ng of deuterated internal standards, d8-17α-hydroxyprogesterone and d7-androstenedione. The extracts were dried under Nitrogen and reconstituted with 50 μL of 10% acetone/ water. 14 μL of the reconstituted sample were injected in the Waters Acquity UPLC/Micromass Quattro Premier tandem mass spectrometer equipped with ESI source. The analysis was complete in less than three minutes using a reverse-phase chromatography column (ACQUITY UPLC BEH C18, 2.1 x 50mm, 1.7μm) at a flow rate of 0.6 ml/min, at 40°C. The analytes were well separated using a linear gradient elution from 60:40 to 30:70 (Water + 0.05% Formic Acid): Methanol. The data were collected in positive ion mode using Multiple Reaction Monitoring (MRM) scans for the following transitions: Cortisol, 363.3 > 121.10 and 363.30 > 97.00; androstenedione 287.5 > 97.10 and 287.5 > 109.10 and d7-Androstenedione 294.60 > 100.10; 17-OHP, 331.50 > 97.00 and 331.50 > 109.10; d8-17- OHP, 339.70 > 100.20. We evaluated the concentration of 17-OHP and the ratio (17-OHP+Androstenedione)/Cortisol. We have analyzed over 100 blood spots from normal full term and pre-term newborns; in addition we have analyzed several blood spots from infants with confirmed CAH to validate this method.

**Results:** The method was linear from 0 to 222 ng/ml serum with the limit of detection of 1.7ng/ml serum and the limit of quantitation of 4 ng/ml serum for all analytes. The analysis of our data indicated that the published cut-offs for 17-OHP of 12.5 ng/mL of serum and of 3.75 for the ratio (17-OHP+Androstenedione)/Cortisol were valid also in our population. We have used this method as second tier test for infants with elevated 17-OHP on the immunoassay based screening. With this approach we have reduced dramatically the number of false positives and we have been able to positively identify infants with CAH.

**Conclusions:** Evaluation of the steroid profile by UPLC-MS/MS can identify children with CAH and reduce the number of false positive results in newborn screening programs.
Human Papillomavirus Genotyping Using The AutoGenomics INFINITI™ Microarray Analyzer

Maria Erali, David C. Pattison, Michael Liew, Carl T. Wittwer and Joel S. Bentz

**Objectives:**

1) To review microarray technologies in the clinical lab

2) To understand the use of the AutoGenomics microarray for multiplex analysis of different HPV types

3) To describe the comparison between the Digene and AutoGenomics results

**Introduction:** Human Papillomavirus (HPV) infection is the primary risk factor for development of cervical cancer. There are over 100 HPV genotypes classified into high-risk types associated with invasive cervical cancer and low-risk types generally found in genital warts. Recent studies show that certain high-risk types are more strongly associated with cervical cancer (e.g. types 16 and 18), and suggest that identification of the specific genotypes may be a useful adjunct to the current methods of cytology and HPV DNA testing.

**Methods:** Cervicovaginal samples collected in liquid-based cytology media and submitted for HPV DNA testing using the Digene Hybrid Capture 2 High-Risk (HR) HPV DNA Test® were analyzed in the AutoGenomics INFINITI Analyzer. Fifty Hybrid Capture 2 HR-positive samples and 29 HR-negative samples were tested. DNA was extracted using the Qiagen QIAamp DNA Blood Mini Kit and PCR was performed with INFINITI reagents in an Applied Biosystems GeneAmp 9700 thermal cycler utilizing appropriate controls. PCR products were placed in the INFINITI Analyzer and all further processing for primer extension, hybridization, washing and detection was performed automatically on a BioFilm Chip™ microarray in the self-contained instrument.

**Results:** Comparison between the Hybrid Capture 2 and INFINITI assays showed agreement of 77%. Eighteen discrepant samples included 9 Hybrid Capture 2 positive samples, 7 of which were negative in the INFINITI assay and 2 that were not called due to failed amplification of the internal control. There were 9 Hybrid Capture 2 negative samples of which 7 were positive in the INFINITI assay and 2 were not called. All discrepant samples were repeated and sequencing was done to confirm the identification of HPV genotypes. HPV typing results for clinical samples run on different days were reproducible.

**Conclusions:** The AutoGenomics INFINITI microarray Analyzer provides a user friendly, automated, walk-away system for typing HPV from cervical specimens. The results from the INFINITI Analyzer were comparable to Digene Hybrid Capture 2 results and the HPV genotypes identified using the INFINITI assay were confirmed by sequencing.
Laboratory Diagnosis of Galactosemia: Combination of Enzyme Activity, Allele-specific PCR and Full Gene Analysis

Fernanda R.O. Calderon, Pam Dobrowolski, Irina Sinitsyna, Amit Phansalkar, Nichola Longo, Marzia Pasquali and Rong Mao

Objectives:

1) Discuss laboratory diagnosis of galactosemia

2) Understand the importance of full gene analysis to complement current genetic testing

3) Discuss novel mutations found in the GALT gene and the implementation of an online mutation database

Background: Classical galactosemia is an autosomal recessive disorder caused by deficiency of the enzyme galactose-1-phosphate uridylyltransferase (GALT), which is involved in the metabolism of galactose sugar. Our laboratory currently runs a biochemical assay to measure GALT activity and allele-specific PCR for a panel of the most common mutations in the GALT. However, over 180 mutations were reported in this gene up to date, and most of them are rare. In this study we designed a full gene assay to identify additional mutations in the GALT gene and resolve discordant results between enzyme and genotyping assays, thus increasing the sensitivity of classical galactosemia diagnosis.

Materials and Methods: A total of 32 samples were selected for full gene analysis (15 had discordant genotyping and enzyme results; 17 had low enzyme activity). The coding regions of the GALT gene were amplified via polymerase chain reaction (PCR) and further submitted to bidirectional capillary electrophoresis. Disease association was determined statistically, via conservation analysis, and impact on enzyme activity for all new mutations found.

Results: 9 secondary rare mutations, including 2 unreported variations were found in samples with discordant enzyme and genotype results. Furthermore, 5 new mutations were detected in the samples with low enzyme activities. Full gene analysis enabled concordance between current enzyme assay and genotype and further increased the clinical sensitivity of classical galactosemia diagnosis to 99% of all alleles tested.

Conclusions: This two-tiered testing approach: enzymatic analysis and genotyping; followed by full gene analysis of GALT when discordant results are observed can significantly improve our current classical galactosemia testing. Data generated in-house will be collected into a pedigree database for internal disease association studies, as well as, a public online database of genetic variations in GALT for both internal referencing and educational purposes.
Analysis of Antibody Responses to Specific and Non-specific Streptococcal and Tissue Antigens in Patients with Acute Rheumatic Fever

Thomas B. Martins

Objectives:

1) Present the theories on the pathogenesis of acute rheumatic fever and rheumatic heart disease

2) Describe the multi-analyte assay for determining non-specific and cross-reactive antibodies between streptococcal components and human tissues

3) Present the results of the multi-analyte assessment of these non-specific and cross-reactive antibodies in 49 confirmed rheumatic fever patients, 58 uncomplicated streptococcal pharyngitis cases and sex and age matched normal controls. Point out new cross-reactive antigens likely important in the pathogenesis of rheumatic heart disease.

Acute Rheumatic Fever (ARF) is an autoimmune disease occurring in individuals following group A streptococcal pharyngitis which is believed to be triggered, in part, by antibodies specific for components of group A streptococci that cross react with human tissues. We developed a multiplexed immunoassay for the simultaneous quantitation of antibodies to nine different streptococcal related antigens including streptolysin O (SLO), DNase B, Collagen I and IV, fibronectin, myosin, group A carbohydrate, M6 protein, and streptococcal C5a peptidase. This study included 49 diagnosed ARF patients, 58 culture positive pharyngitis patients, and age and sex matched normal controls with serum samples collected at initial disease onset, 4 weeks, 6 months, and 1 year. When antibody responses in the ARF patients were compared to those with uncomplicated pharyngitis, we found significant differences (p <0.05) for SLO, DNase B, collagen I, myosin, and M6 protein. Comparing ARF patients with rheumatic heart disease (RHD) to ARF patients without, we found significant differences in antibody responses to SLO, fibronectin and collagen I. Thus, we observed a significant increase in antibody responses to 6 of the 9 antigens when comparing the ARF and pharyngitis groups, but only antibody levels to M6 protein persisted past week 4. To our knowledge, this is the first reported study describing elevated antibodies to collagen I, a component of heart valves, in patients with RHD. For the other antigens, antibody concentrations were comparable with the age matched controls by 6 months. This implies either that these antibodies lead to tissue injury acutely, and then other mechanisms such as cellular immunity result in RHD or the antibodies have nothing to do with the pathogenesis of RHD.
Molecular Detection of FMS-like Tyrosine Kinase 3 Mutations in Acute Myeloid Leukemia

Cecily P. Vaughn and Kojo S. J. Elenitoba-Johnson

Oncogenic activation of tyrosine kinases is an important mechanism underlying the pathogenesis of malignant neoplasia. Identification of activating mutations in tyrosine kinase genes is particularly significant due to the increasing availability of small molecule inhibitors that selectively target the kinases as therapeutic options in the management of patients with cancer. Mutations in the tyrosine kinase gene FMS-like tyrosine kinase 3 (FLT3) are the most common somatic mutations in human acute myelofenoous leukemias (AML) and can be detected in up to 30% of cases. Significantly, the mutations define a subset of poor-prognosis AMLs. These mutations occur as internal tandem duplications (ITD) in the juxtamembrane domain (JMD) or as point mutations in codons D835 and I836 in the activation loop. Detection of these mutations is important in the diagnosis and treatment of AML. To this end, we have designed molecular assays to detect these FLT3 gene mutations. DNA extracted from AML patient samples was amplified using primers spanning both mutation regions. Following amplification, PCR products were analyzed by multiple methods, including high resolution melting analysis, direct sequencing, and fragment analysis by capillary electrophoresis. The analytical sensitivity of each method was assessed to determine the most sensitive detection method. Of 62 samples screened for mutations in the JMD, 13 contained ITDs. Additionally, 3 of 25 samples screened for activation loop mutations were also positive. The identities of all mutations initially identified by screening methods were confirmed by DNA sequencing. ITD mutations were detected at a dilution of 1:20 using fragment analysis, as compared to detection at 1:5 for high resolution melting analysis. For the detection of D835/I836 mutations, restriction digestion using the enzyme EcoRV which ablates the GATATC sequence in the wild-type sequence, followed by fragment analysis yielded the most sensitive results. Amplification, followed by restriction digestion (for D835/I836 amplicons), and multiplexed detection by fragment analysis provides a simple, sensitive method for the detection of both ITD mutations in the JMD and point mutations in the activation loop of the FLT3 gene. Detection of these mutations is important in the prognosis of AML and will be increasingly important with the continued development of tyrosine kinase-specific drug therapies.

Objectives:

1) Understand that protein kinases play a role in some types of cancer.

2) Know the two types of mutations that may be present in the FLT3 gene.

3) Learn which methods may be used to detect FLT3 gene mutations.
Autoimmune hepatitis (AIH) is a chronic, progressive, inflammatory liver disease of unknown etiology. Diagnosis is often difficult since there is no single diagnostic test for AIH, and presenting symptoms are quite variable. Diagnosis requires evaluation of clinical, laboratory, and histological findings as well as the exclusion of other causes of chronic hepatitis. Autoantibody against smooth muscle (SMA) and nuclear antigens (ANA) are the serological hallmarks of AIH Type 1, which represents about 80% of AIH cases. The SMA in patients with autoimmune liver disease is directed against the actin component of the cytoskeleton and is traditionally detected using indirect fluorescent antibody (IFA) techniques. Recently, an enzyme immunoassay (EIA) detecting IgG antibody against F-actin has become commercially available. Our objectives in this study were to compare the results obtain with an F-actin IgG EIA to those determined by SMA IgG IFA and to determine the prevalence of F-actin IgG in patients positive for acute viral hepatitis. Sera from 415 patients suspected of having autoimmune liver disease; 208 patients suspected of having acute viral hepatitis A (HAV), acute hepatitis B (HBV), or hepatitis C (HCV); and 100 healthy blood donors were included in the study. In patients suspected of having autoimmune liver disease, the F-actin EIA showed increasing degrees of positivity as SMA IFA titers increased; all sera having SMA IFA titers of ≥1:160 had positive F-actin EIA results. In contrast, there were several sera with negative (<1:20) or low (1:20 - 1:80) SMA IFA titers that demonstrated moderate to high levels of IgG antibody against F-actin. The prevalence of F-actin IgG in viral hepatitis positive sera (n=108) was 22.2% for HAV, 25.0% for HBV, and 25.0% for HCV, with the majority containing low levels (20 – 30 Units). Only 3 of 100 acute viral hepatitis negative sera were positive for F-actin IgG. One of 100 sera from healthy blood donors gave low positive results (26 Units) for F-actin. Of 100 SMA IFA negative (<1:20) sera assessed for acute viral hepatitis markers, 19% were reactive against at least one marker tested. We conclude that F-actin by EIA may be more sensitive than SMA by IFA in identifying patients with autoimmune liver disease, and that F-actin IgG is of low prevalence in the normal healthy population and is found in low levels in patients that are positive for acute viral hepatitis. In addition, patients suspected of having AIH that are SMA/F-actin negative should be evaluated for viral hepatitis since 19% of our SMA IFA negative sera had serologic test results that were consistent with viral hepatitis.
Characterization of SCF^{FBXO45} A Novel Ubiquitin Ligase Involved in Follicular Lymphoma Transformation

Philippe Szankasi, David K. Crockett, Zhaosheng Lin, Megan S. Lim and Kojo S. J. Elenitoba-Johnson

Follicular lymphoma (FL) is the most common form of low-grade non-Hodgkin lymphoma in the United States. A significant proportion of FL cases undergo conversion into an aggressive neoplasm with a rapid clinical course and increased mortality. The pathogenetic alterations underlying this transformation event are largely uncharacterized. Using a quantitative proteomic approach based on differential labeling by isotope-coded affinity tags and tandem mass spectrometry, we identified a novel F-box domain containing protein, FBXO45, that was overexpressed in a subset of matched primary samples of transformed follicular lymphoma as compared to the low-grade FL counterparts taken from the corresponding patients. We further established by quantitative real-time PCR that FBXO45 mRNA was highly expressed in transformed follicular lymphoma-derived cell lines carrying the t(14;18), but not in t(14;18)-negative cell lines. Using phenotypically purified lymphocyte subpopulations, we determined that germinal center B-cells exhibit 10-fold higher expression levels of FBXO45 than naïve B-cells. FBXO45 contains an F-box domain which suggests a role as an adaptor for ubiquitin ligases. To determine the interaction partners and potential targets of FBXO45, we have cloned the FBXO45 cDNA and expressed the recombinant protein fused to a variety of epitope tags. Coimmunoprecipitation followed by LC-MS/MS and western blotting demonstrate that FBXO45 exists in a complex with SKP1, a core component of ubiquitin ligases. This interaction was abolished when the F-box domain was deleted. FBXO45 also contains a SPRY domain which is postulated to be the binding domain for target proteins that are recruited for poly-ubiquitination and subsequent degradation by the 26S proteasome. As these target proteins have a very short half life once they are bound to the ubiquitin ligase, we employed a “pull-down strategy utilizing a mutant form of FBXO45 lacking the F-box domain. We hypothesized that expression of the mutant protein would act as a dominant negative allele and lead to a prolonged half life of target proteins. Indeed, co-immunoprecipitation experiments with the F-box deleted mutant of FBXO45 (SPRY-only mutant) reveals bands not observed with wild-type FBOXO45. The candidate proteins coimmunoprecipitating with the SPRY-only bait include proteins implicated in DNA damage repair and proteins with established tumor suppressor function among others. Interestingly, the expression of a number of these proteins was diminished by forced ponasterone-induced expression of the wild-type FBOXO45 protein in 293 human embryonic kidney cells suggesting that they may be targets of FBOXO45 mediated proteasomal degradation. In conclusion, our studies indicate FBXO45 is upregulated in FL transformation and is preferentially expressed in B-cells of germinal center origin. The protein binds to components of the SCF ubiquitin ligase and contains a SPRY interaction domain through which it may bind targets for degradation by the SCF-ubiquitin ligase proteasomal machinery. Our future studies include experiments to confirm the de facto targets of FBOXO45 and to establish its function as a ubiquitin ligase. Finally, we will determine the cellular effects of ablation of FBOXO45 by RNAi in transformed follicular lymphoma derived cells, providing some relevance as to its utility as a target for transformed FL therapy.

Objectives:
1) Understand how genomic and proteomic approaches can help in the identification of prognostic markers and therapeutic targets in cancers
2) Understand the central role of the Ubiquitin/26S Proteasome machinery
3) Understand the rationale for determining protein-protein interactions
Quantitation of EBV Virus by Real Time PCR

Jeff Stevenson

Objectives:

1) Understand the rationale for quantitative EBV testing

2) Recognize assay improvements that result from the new probe chemistry

3) Identify sources of inter-lab variation in PCR results

Background: EBV is a member of the Herpesvirus family. Primary infection with EBV generally occurs during childhood and is widespread in the population, with approximately 90% of adults seropositive. The virus remains latent principally in B-lymphocytes, but can become reactivated. In transplant patients, this reactivation can lead to the syndrome of post-transplant lymphoproliferative disorder (PTLD). New intensive immunosuppressive regimens are considered a risk factor for PTLD. The use of quantitative real time PCR for EBV can provide a valuable tool for the clinician in diagnosing PTLD, as well as for monitoring the patient’s response to treatment. Developing an assay for absolute quantitation that is both accurate and reproducible presents a number of challenges, which include generating a reliable standard curve and stabilizing standard and control material. The key to reproducibility is using standards and controls that remain stable over time. Lyophilization is now being widely looked at as a practical solution to the problem of stabilizing reagents for real time viral load testing. We validated a quantitative real time PCR assay on the HT7900 Sequence Detection System (Applied Biosystems) to detect EBV in spinal fluid, serum, plasma, and whole blood. This assay uses a stored standard curve generated with lyophilized standards.

Methods: This assay is in an ASR format, targeting the viral BNRF1 gene with a primer and hybridization probe set from Nanogen/Epoch Biosciences. Dilutions of a plasmid containing the cloned amplicon are used as standards. With each new lot of Taq master mix or EBV ASR reagent, a new standard curve is generated using a lyophilized plasmid clone, and this curve is stored for quantitation of subsequent assays. Sequestration of reagent lots and the lyophilization of control material help maintain stability of assay performance over the period that a standard curve is being used. Prior to extraction, an exogenous plasmid internal control is added to the sample, providing the ability to detect PCR inhibition and extraction failures. To limit the risk of contamination, the assay is run in a 96-well, closed tube format, using UNG and dUTP in the master mix.

Results: Using log dilutions of the lyophilized standard, we generated a 6 log standard curve with a range of 390 to 39,000,000 copies per mL (10 to 1,000,000 copies per reaction). The 10 copies/reaction standard was routinely detected, consistent with an analytical sensitivity approaching the limits imposed by sampling error. The average slope of the standard curve was -3.28 (PCR efficiency approaching 100%) with an R2 value consistently >0.99. The melting temperature of the product is approximately 69°C. To determine the accuracy of the assay, we tested 6 serial log dilutions of a commercial, quantitated EBV DNA, ranging from 260 to 26,000,000 copies per reaction. The log difference between expected and observed values ranged from 0.0 to 0.2, with an average difference of less than 0.1 logs. The assay was also evaluated by comparing the results of this test with those obtained using a TaqMan assay performed at another reference lab. Our quantitative results were on average about 0.4 logs (2.5-fold) higher than those generated by the reference lab.

Conclusions: Quantitative real-time PCR is a reliable method for detecting primary EBV infection, EBV reactivation and monitoring therapy. Using a lyophilized plasmid standard, we are able to achieve a high degree of reproducibility in our standard curves. We propose that a significant source of the inter-lab variation observed in quantitative assays is the use of different calibration materials, which can vary in accuracy and stability.
Identification of Nonsporulating Molds and Dermatophytes by Sequencing Internal Transcribed Spacer Regions with SmartGene Software and Database

J. I. Pounder and C.A. Petti

**Objectives:**

1) To appreciate the utility and impact of DNA sequencing for the identification of fungi that are difficult to classify

2) To understand the selection of the internal transcribed spacers as a target for identification of fungi

3) To follow the process of species identification by ITS sequencing with SmartGene software

**Background:** Fungal infections are increasing, particularly among immunocompromised hosts, and a rapid, accurate diagnosis is essential for the initiation of targeted antifungal therapy. Identification of fungi from culture requires the presence of reproductive structures, and the absence of spores can increase the time to identification up to 21 days. Growth on special media for identification of fungi associated with skin, nails, and hair (dermatophytes) increases time to identification. We evaluated the utility of amplification and direct sequencing of internal transcribed regions ITS I and ITS II from cultures with nonsporulating molds (NSM) and dermatophytes.

**Methods:** 50 NSM and 27 dermatophytes from clinical isolates were randomly selected. After growth on potato dextrose agar, DNA was extracted from approximately 1 cm² mycelia with the IDI lysis kit. Using ITS1 and ITS4 primers, real-time PCR with SYBR green DNA binding dye followed by melting temperature analysis was performed on RotorGene 3000. Amplicons were sequenced using BigDye chemistry on ABI 3130. Sequences were identified using SmartGene software. Nucleotide sequences with match length of > 400 bp were analyzed. Sequence-based identifications were defined by percent similarity: species > 99%, genus > 93%, and inconclusive < 92%.

**Results:** For the NSM, 48 of 50 isolates had > 400 bp match length. Of this group, SmartGene identified 5 (10.4%) to genus only, 39 (81%) to species with 4 (8%) being inconclusive. 17 of 48 sequences shared similar nucleotide sequences with multiple species. Classification of molds was as follows: 25 plant or soil-associated, 8 well-recognized pathogens, and 11 potential/emerging pathogens. For the dermatophytes, 27 had ≥ 400 bp and ≥99% similarity. Identification of the dermatophytes was confounded by same teleomorph name used for more than one anamorphic species.

**Conclusions:** Sequencing the ITS regions identified 88% NSM that could not be identified by conventional methods. Moreover, a significant number of these molds were well-recognized or emerging pathogens for immunocompetent and immunocompromised hosts, and their identification potentially may have impacted patient management. For the dermatophytes, 100% were identified to species. SmartGene software was useful for sequence analysis, and a more thorough evaluation of this database is currently ongoing and expanding to include sporulating filamentous fungi and yeast.
The disease histoplasmosis occurs worldwide and is caused by the dimorphic fungus *Histoplasma capsulatum*. Traditional method of laboratory diagnosis of histoplasmosis relies on culture and isolation of the fungus, which takes 2-4 weeks for identification. An alternative to culture is serology. However, antibodies to *H. capsulatum* are only detected in approximately 77% of disseminated disease and long-term elevation of antibody titers makes it difficult to diagnose recurrent infections. Antigen detection tests have been performed and have proven to be appropriate for the diagnosis and monitoring of histoplasmosis. In this study we evaluated an antigen detection kit prepared with rabbit anti-*Histoplasma* antibodies to detect and quantitate *H. capsulatum* antigen in urine samples. Using a 4-parameter curve fit, the assay calibration ranges from 2 to 1000 EIA units. We compared results of 99 urine samples with those of a reference laboratory, half of which tested positive by that reference laboratory. Performance characteristics were further defined by studying the assay linearity, precision, sensitivity, and specificity. A 2 x 2 contingency table was prepared assuming the reference laboratory’s method as the gold standard. We achieved acceptable correlation with the reference laboratory (R²=0.717), obtaining results ranging from <2 (negative samples) to 132 EIA units by the new method. Compared to the reference laboratory, sensitivity was 71% and specificity was 98%. Most of the discrepant results were near to the assay cutoffs. Cross-reactivity occurred with culture filtrates of *Paracoccidiomyces brasiliensis*, *Coccidiomyces immitis*, and *Blastomyces dermatitidis*. No cross-reactivity was observed with a mixture of *Candida* species nor with *Aspergillus fumigatus*. Three levels of urine controls were prepared and tested with each run. Precision (CV) of the daily controls was 13.2, 6.6, and 4.3% for the high, medium, and low controls, respectively. We conclude the EIA assay for the detection and quantitation of *H. capsulatum* antigen in urine specimens is an acceptable and accurate assay which agrees well with the reference laboratory.

Objectives:

1) Define the disease caused by *Histoplasma capsulatum*

2) Review the diagnostic options for histoplasmosis

3) Describe the performance characteristics of the *Histoplasma* antigen EIA
The L576P C-kit Mutation as a Predominant Activating Mutation in Non-GIST Tumors

Carlynn Willmore-Payne, Joseph A. Holden and Lester J. Layfield

**Objectives:**

1) Discuss how c-kit mutations in non-GIST tumors differ from those present in GIST tumors

2) Discuss the use of sequencing electropherograms and c-kit FISH to determine the zygosity of the mutant allele

3) Discuss the potential therapeutic implications of the L576P mutation in melanoma and seminoma

**Background:** The successful treatment of GISTs (gastrointestinal stromal tumors) with the tyrosine kinase inhibitor Gleevec correlates with the presence of activating mutations in the c-kit transmembrane tyrosine kinase receptor. The majority of the mutations are located in exon 11 of the c-kit gene and consist of missense mutations or small in frame deletions or insertions. Since most c-kit mutant GISTs stain positive for c-kit (CD117) by IHC (immunohistochemistry), we tested for the presence of c-kit mutations in other tumor types that also stain positive for c-kit by IHC. The presence of c-kit mutations in non-GIST tumors may also indicate a patient population who could benefit from Gleevec.

**Design:** IHC was performed on 153 melanomas and 22 seminomas. We used high resolution amplicon melting, in conjunction with polymerase chain reaction (PCR), to screen for c-kit activating mutations c-kit IHC positive melanomas and seminomas. Exon 9, 11, 13, or 17 was amplified by PCR, and then subjected to HRMA (high-resolution melting analysis). An abnormal melting analysis indicated a genetic alteration in the form of an insertion, deletion, and/or a single base change. DNA sequencing was used to confirm abnormal results obtained by high resolution melting analysis. A c-kit/CEP4 FISH probe was developed to study cases in which there appeared to be an overabundance of mutant allele as determined from visual examination of the sequencing electropherogram.

**Results:** Of the 153 melanomas evaluated, only 3 cases (2%) contained a mutation. Remarkably, DNA sequencing revealed that all 3 cases contained an L576P mutation. Of the 22 seminomas evaluated, 4 cases contained mutations in c-kit exon 17 (resistant to Gleevec) and 1 case (5%) contained a mutation in c-kit exon 11. Interestingly, the mutation in exon 11 of the seminoma was the same L576P mutation seen in the 3 melanoma cases. The c-kit mutant melanomas and seminomas all stained strongly positive for c-kit by IHC. Examination of the sequencing electropherograms in the L576P mutant tumors revealed that the tumor appeared to contain an excess of the mutant allele, suggesting that the mutation may not be heterozygous. C-kit FISH on the 3 melanomas showed slight amplification in one case and the other two cases were diploid and most likely homozygous. C-kit FISH in the L576P mutant seminoma showed polysomy for chromosome 4, in which excess of the mutant allele is most likely present.

**Conclusions:** There are well over 100 unique c-kit activating mutations in GISTs and the L576P mutation makes up less than 10%. The predominance of the L576P mutation in non-GIST tumors to the exclusion of all others is remarkable and suggests that these non-GIST patients may benefit from Gleevec treatment. Unlike c-kit mutations in GISTs, these tumors do not appear to be heterozygous for the mutant allele. It is then predicted that these tumors may have increased amount of mutant homodimer. This could result in a more aggressive tumor or a tumor that responds differently to drug treatment. Clinical trials are needed to correlate clinical response to Gleevec with the presence of c-kit mutations and gene copy number of the mutant allele.
Unlabeled Oligonucleotide Probes Modified with Locked Nucleic Acids (LNA) for Improved Mismatch Discrimination in Genotyping by Melting Analysis

Lan-Szu Chou, Cindy Meadows, Carl T. Wittwer and Elaine Lyon

With a frequency of 1 in 1000 bp, single nucleotide polymorphism(s) (SNPs) are used to study complex inherited diseases. A universal concern in genotyping techniques is that rare variants may interfere. Melting analysis addresses this concern and is a homogeneous and simple method for genotyping. For example, the HybProbe® format (a 2-probe/2-fluorophore system) detects unexpected variants under probes by Tm shifts different from that of the expected mutation. However, without close attention, minor shifts from the expected heterozygote Tm may be ignored, resulting in false-positive interpretations. Another option is melting analysis of amplicons using double-stranded DNA (dsDNA) dyes to screen for sequence alterations. This option costs less but may be prone to error unless high-resolution techniques are used. Extra processing steps such as adding urea to enhance melting resolution require opening the tubes, increasing the risk of cross-contamination.

In this study, we have used a recently introduced DNA dye, LCGreen®I, which is superior to SYBR Green I for detecting multiple products and allows closed-tube genotyping with unlabeled oligonucleotide probes. Although high-resolution melting is not an absolute requirement for unlabeled probe genotyping, conventional real-time instruments may not distinguish between multiple variants when the variant Tm is close to the targeted mutation Tm. To further increase mismatch discrimination on the LightCycler (Roche Diagnostics), we demonstrate the use of locked nucleic acids (LNA) in unlabeled probe genotyping, and study the effect of LNA position on mismatch discrimination using the Factor V Leiden (1691G>A) as a model system. As a result, mismatch destabilization is greater than conventional probes and different mismatches are often easier to discriminate. In conclusion, a highly specific genotyping assay that detects Factor V Leiden (1691G>A) and discriminates three additional rare variants close to the mutation site using a single unlabeled (LNA-modified) probe and the LightCycler was presented. This assay has the advantages of low-cost (no fluorescent labeling on probes), rapid, and high-throughout capable, which meets the criteria of a routine high-volume molecular test.

Objectives:

1) Understand molecular genotyping using melting analysis
2) Understand potential limitations of using FRET probes for melting analysis and the alternative of using unlabeled probes
3) Understand the advantages of incorporating locked nucleic acids (LNA) in unlabeled probes for improved mismatch discrimination
Chromosomal translocations are common cytogenetic aberrations in human cancers. Translocations often result in chimeric fusion proteins with oncogenic activities. Identification of translocation partners is important for understanding the molecular pathogenesis, and for the diagnosis of various tumors. We describe a mass spectrometry-based approach for the identification of fusion partners involved in chromosomal translocations, when one of the partners involved is known. This approach accurately identified the reported NPM-ALK fusion protein in an anaplastic large cell lymphoma (ALCL)-derived cell line carrying the t(2;5)(p23;q35), and the TPM3-ALK in a clinical biopsy of inflammatory myofibroblastic tumor (IMT) carrying the t(1;2)(q21;p23). This study shows for the first time, the ability of mass spectrometry to identify oncogenic chimeric proteins resulting from chromosomal rearrangements. This strategy is complementary to existing nucleic acid based methods and can be used for the identification of known and unknown translocation partners of chimeric fusion proteins involved in oncogenesis.

Objectives:

1) Explain importance of studying chromosomal translocations in cancer
2) Show feasibility of detecting unknown ALK translocation partners in cancer
3) List advantages of detecting translocations by LC-MS/MS
Improved High Resolution Genotyping by Incorporation of Melting Temperature Standards

M Seipp, M Liew, J Durtschi, J Williams, K Voelkerding and C Wittwer

**Background:** High resolution (HR) melting analysis is a closed-tube method for genotyping which utilizes the dsDNA binding dye LCGreen-Plus and does not require the use of fluorescently labeled primers or probes. Correct identification of sample genotype by HR melting requires standardization of reaction conditions to achieve reproducible, characteristic melting profiles. Basic studies of DNA duplex melting have shown that reaction conditions, and in particular ionic strength, can significantly affect $T_m$. The current study was undertaken to develop a method for correction of $T_m$ shifts in HR melting assays.

**Materials and Methods:** $T_m$ standards and custom software were designed and applied to a Methylene-Tetra-Hydrofolate Reductase (MTHFR) A1298C/C677T multiplex melting assay. The effect of solution chemistry differences on $T_m$ variance was investigated using a side-by-side comparison of three different DNA extraction methods. A blinded study comparing the MTHFR A1298C/C677T multiplex melting assay with $T_m$ standards to an in-house developed MTHFR FRET probe assay was conducted.

**Results:** Different extraction methods were shown to have significant effect on $T_m$ in HR melting analysis through a paired T test. Temperature correction can decrease variance of homozygous melting peaks by as much as 97%. Of the 37 blinded study samples, 35 were correctly genotyped without temperature correction. After temperature correction was applied, the MTHFR melting assay results were completely concordant with the MTHFR FRET probe assay.

**Discussion:** HR melting analysis resolution can be improved by the addition of $T_m$ standards. Amplicon melting assays with $T_m$ standards have genotype resolution similar to probe assays. The use of $T_m$ standards and custom software to correct for temperature shifts improved the genotyping results for a MTHFR A1298C/C677T multiplex HR melting assay.
Enteroviruses are single stranded RNA viruses that belong to the Picornaviridae family. They are the leading cause of aseptic meningitis in pediatric and adult populations and can be associated with severe disease such as myocarditis and encephalitis. RT-PCR has rapidly become the diagnostic methodology of choice due to its sensitivity and rapid turn-around-time allowing significant improvement in patient care and management. While most molecular assay target the conserved regions within the 5’ UTR described by Rotbart et al, we have developed a real time assay that utilizes primers and an Eclipse probe to amplify a 58 bp region of the 5’ UTR upstream of the traditional Rotbart amplicon. Armored RNA was added to the lysis buffer prior to extraction to monitor nucleic acid extraction and RT-PCR by amplification of a 78 bp region of the Armored RNA coat protein. RNA was extracted using the Qiagen BioRobot. We compared this real time assay to samples tested with the Chemicon PCR-EIA panenterovirus kit from the 2004 and 2005 enterovirus season. A total of 288 de-identified clinical samples were tested (145 positive and 143 negatives) as well as 27 different serotypes. Overall agreement between the two assays was 96%. Additional side by side testing will be done on samples from 2006 and the data will be presented.

Objectives:

1) Understand the clinical background and recognize the importance of rapid and accurate diagnosis of enterovirus

2) Understand the challenges in designing real time molecular diagnostic tests for enterovirus due to nucleotide polymorphisms

3) Describe the performance of a novel real time enterovirus assay in comparison to a non-real time RT-PCR enterovirus assay
Use of Single “Loop-Out” Probes for Multiplex Genotyping

Genevieve Pont-Kingdon, Kristy Damjanovich, Bob Chou, Mark Herrmann, Carl Wittwer and Elaine Lyon

**Objectives:**

1) Describe design and analysis of probes that span several mutations

2) Describe DNA software to predict spanning-probes behavior

3) Apply the technology to hemoglobinopathy causing mutations

“Loop-out” (lpo) probes are chimeric oligonucleotides that hybridize to two or more non-contiguous DNA sequences present in a PCR product template. The intervening template sequences, omitted in the probe, loop-out during binding. Lpo probes dissociate from the template as a unit allowing simultaneous characterization of several non-contiguous polymorphisms. We designed lpo-FRET probes previously to haplotype 3 SNPs present in a 101 nucleotides long fragment of the beta 2 adrenergic receptor gene. Here, we demonstrate the use of lpo-FRET probes and lpo-unlabelled probes that use a double strand DNA specific dye, to genotype simultaneously three SNPs of the beta globin gene. Our data demonstrate that lpo probes can be designed independently of the system of detection (FRET, double strand dye). The probes hybridize to the HbS (sickle cell anemia), HbE an HbC loci. While the HbC allele results from a mutation immediately adjacent to HbS, the HbE locus is 58 nucleotides away. One part of the lpo probe hybridizes to the HbS and HbC region while the other part hybridize to the HbE locus creating a 49 bp loop in the template. All three SNPs can be genotyped by one loop-out probe on a LightCycler or a HR1 instrument by characteristic melting temperatures. Our data also show that lpo-probes binding behaviors can be predicted using a commercially available software making experimental design possible.

The melting properties of lpo probes render possible the analysis of several polymorphisms at once, even when the mutations are separated by a distance too large to be interrogated using classic detection probes.