ARUP Institute for Clinical and Experimental Pathology® presents our 8th Annual Research Colloquium

8th Annual RESEARCH COLLOQUIUM

Join us
May 30, 2008
9:00 a.m. - 4:00 p.m.
ARUP Matsen Conference Room

Presenters

Presenters from ARUP Institute for Clinical and Experimental Pathology® include:

Rebecca Margraf
Bingfang Yue
Denice Smith
Mike Pyne
Genevieve Pont-Kingdon
Mike Seipp
Philippe Szankasi
Orly Ardon
Mark Kushnir
Alison Millson
Joann Cloud
Bob Chou
Keith Simmon
Brian Anderson
Marcy Lloyd
Mark Herrmann
Tanya Sandrock
Lyndsey Rector
Kristy Damjanovich
Aiping Liu
David Crockett
Tom Martins
Jeff Stevenson
Nancy Augustine
Sergey Preobrazhensky
AGENDA

8th Annual
Research Colloquium

ARUP Institute for Clinical and Experimental Pathology®

Friday, May 30, 2008

Presentations
ARUP Matsen Conference Center

The order of presentations was determined by a random drawing.

9:00 AM ..........Welcome by Harry R. Hill, MD
9:12 AM .....................................Rebecca Margraf
9:24 AM .................................... Bingfang Yue
9:36 AM .................................... Denice Smith
9:48 AM .................................... Mike Pyne
10:00 AM .................. Genevieve Pont-Kingdon
10:12 AM ................................... Mike Seipp

10:24 AM ......................... BREAK

10:45 AM ................................ Philippe Szankasi
10:57 AM ................................ Orly Ardon
11:09 AM ................................ Mark Kushner
11:21 AM ................................ Alison Millson
11:33 AM ................................ Joann Cloud
11:45 AM ................................ Bob Chou
11:57 AM ................................ Keith Simmon

12:09 PM ......................... LUNCH

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

1:00 PM ........................................ Brian Anderson
1:12 PM ........................................ Marcy Lloyd
1:24 PM ........................................ Mark Hermann
1:36 PM ....................................... Tanya Sandrock
1:48 PM ...................................... Lyndsey Rector
2:00 PM ..................................... Kristy Damjanovich
2:12 PM ..................................... Aiping Liu

2:24 PM ......................... BREAK

3:00 PM ..................................... David Crockett
3:12 PM ....................................... Tom Martins
3:24 PM ...................................... Jeff Stevenson
3:36 PM ....................................... Nancy Augustine
3:48 PM ..................................... Sergey Preobrazhensky

4:30 PM ..........Group Photo at Waterfall
(Judges, R&D Personnel, Medical Directors)
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Multiple endocrine neoplasia type 2 RET proto-oncogene sequence variation database

Margraf R, Crockett D, Franckowiak P, Seamons R, Calderon F, Wittwer CT, Mao R

Objectives:

1) Learn the clinical differences between the MEN2 subtypes: MEN2A, MEN2B, and FMTC.

2) Understand the categories of RET sequence changes documented in the database.

3) Describe the layout and utility of the MEN2 RET database.

Introduction: The Multiple endocrine neoplasia type 2 (MEN2) is an inherited, autosomal dominant disorder which is caused by deleterious mutations within the RET proto-oncogene. MEN2 RET mutations are mainly heterozygous, missense sequence changes found within exons 8, 10, 11, and 13-16. Our group has developed a publicly available MEN2 RET database to aid in RET genotype/MEN2 phenotype correlation.

Aim: The aim of the MEN2 RET database is to record all sequence variations within the RET proto-oncogene relevant to the MEN2 syndromes, as well as any associated clinical information and the most informative literature references. Cataloguing the reported genotype/phenotype correlations may assist physicians in medical management of MEN2 patients.

Methods: The MEN2 RET database was created using the Human Genome Variation Society (HGVS) and HUGO recommendations for sequence variation nomenclature and database content. Information sources for the current MEN2 RET database entries were published reports on MEN2 or RET sequence variation found within PubMed and on the World Wide Web. The database’s MEN2 phenotype definitions were derived from the International RET Mutation Consortium guidelines for classification of MEN2 disease phenotypes.

Results: This searchable database contains 132 RET sequence variation entries as of April 2008. Each database entry contains these descriptive fields: location within the RET gene, codon number, codon change, protein change, genotype, pathogenicity classification, MEN2 phenotype, youngest age of MTC onset, first literature reference, and comments. The comments for each entry may contain more information on MEN2 clinical features, complex genotypes, or list additional literature references. The database website also contains links to selected MEN2 literature reviews, gene and protein information, and RET reference sequences. Novel RET sequence variation as well as new clinical information for already catalogued sequence variation can be submitted through the database website.

Conclusions: The MEN2 RET database (http://www.arup.utah.edu/database/MEN2/MEN2_welcome.php) will serve as a clinical resource for MEN2 disease, a reference for genotype/phenotype correlations, and a repository for MEN2-associated RET sequence variation. To maintain the accuracy and utility of the database, quarterly content updates will be performed using new RET sequence variation information from literature reports, database submissions from collaborators, and routine clinical testing performed at ARUP.
Rapid analysis of bile acids in serum by LC-MS/MS

Yue B, Roberts WL, Rockwood AL

**Introduction:** Bile acids play several vital roles in normal physiology including cholesterol excretion, intestinal uptake of lipid and fat-soluble vitamins, and the hepatic generation of the bile flow. Conditions that interfere with bile acid synthesis, cellular metabolism, bile excretion, intestinal absorption, or removal from the plasma can alter bile acid homeostasis and lead to various hepatobiliary diseases. Quantitative analysis of different species of bile acids has become an important tool for the diagnosis and follow-up of liver and intestinal disorders and other diseases affecting bile acids metabolism. However, the presence of various conjugated forms (glucose, taurine, sulfate, glycosides, etc.) at various positions on the different bile acid species makes the differentiated quantitative analysis a challenging task.

**Method:** We targeted four species of bile acids (Cholic acid, CA; Chenodeoxycholic acid, CDC; Deoxycholic acid, DCA; Ursodeoxycholic acid, UDC) in free, glycine-conjugated, and taurine-conjugated forms, that account for >95% of the bile acid pool in serum. Deuterated free and glycine-conjugated forms were used as internal standards (IS). Serum samples (50 µL) were mixed with 10µL IS working solution (4 µM) and 200 µL methanol. The mixture was vortexed for 5 min and centrifuged at 14,000g and 4 °C for 10 min. Supernatant (50 µL) was transferred into 96-well plate containing 400µL solvent (80/20 v/v water/methanol, 50 mM hydrochloric acid). 10 µL of the processed sample was quantified by on-line SPE-LC-MS/MS method in negative ion MRM mode.

**Preliminary Data:** The mean (SD) recovery by standard addition into serum was 100.8% (10.3%) for individual analyte and 100.8% (7.7%) for subtotal (differentiated species) and total concentrations. The linearity was verified as 0.1 (LLOQ) to 25.0 µM (ULOQ) for each individual analyte. The mean (SD) of within-run, between-run, and total CV for 12 individual analytes were 3.8% (0.8%), 6.7% (3.6%) and 7.8% (3.4%) for low QC and 2.7% (0.6%), 4.6% (1.4%) and 5.3% (1.5%) for high QC. The mean (SD) of within-run, between-run, and total %CV for subtotal and total were 4.4% (0.9%), 6.2% (1.8%) and 7.6% (1.9%) for low QC and 2.3% (0.3%), 3.6% (0.4%) and 4.3% (0.4%) for high QC.

The present method correlated well with an enzymatic method for total bile acids, y (enzymatic method) = 1.06 (1.02-1.09) + 0.90 (-0.57-2.36), s = 6.1, r = 0.99, 95% CI, Deming regression. When compared to a GC-MS method that employs hydrolysis and solvolysis to recover different kinds of conjugates including sulfated and glycosided forms, the correlation were excellent for CA, fair for CDC and DCA, poor for UDC. The reference intervals for each species and total concentration were determined with nonparametric methods as CA < 1.9 µM, CDC <3.4 µM, DCA <2.5 µM, UDC < 1.0 µM and total <7.0 µM (95% CI).

**Novel Aspect:** Chromatographic co-elution of different forms of one bile acid species led to rapid analysis of 12 analytes in 7 min in an assay validated for clinical use.
Fishing to detect urinary and other cancers: do imaging systems help?

Smith GD, Bentz J

Objectives:
1) To gain insight into the potential and limitations of microscopic imaging systems for cancer interpretation.
2) To learn how imaging systems can aid in the interpretation of UroVysion FISH.
3) To learn how imaging can be used for Location Guided or Target FISH.

Introduction: Urinary neoplasms are associated with a variety of chromosomal aberrations, including amplifications of chromosomes, 3, 7 and 17, and the deletion of 9p21. The Vysis UroVysionTM kit detects these aberrations by fluorescence in situ hybridization (FISH). Because the manual interpretation and documentation of UroVysion FISH is time consuming, we investigated image analysis systems designed to streamline the UroVysion FISH screening process. This study evaluated the BioView Duet imaging system as an aid to the interpretation of UroVysion FISH cases, comparing it to manual review. We additionally evaluated the BioView feature, Target FISH.

Materials and Methods: 135 consecutive UroVysion FISH cases that were received in the ARUP laboratory were screened manually as well as with the aid of a BioView Duet imaging system. Manual and BioView-aided interpretations were compared with respect to accuracy, between-run precision, and the time required to perform it.

For Target FISH, BioView was used to capture target cell images from Pap stained slides, which were then destained using acidic alcohol, before carrying out UroVysion FISH. The BioView instrument was used to relocate the original target cells, and FISH signals were evaluated.

Results: Sixty percent of the 135 cases could be interpreted as clearly positive or negative, with urine specimens showing 98.5% concordance, and non-urine specimens showing 100% concordance, between the manual and BioView-aided interpretations. Two cases judged to be BioView-positive, but manual-negative, were resolved to be positive, and thus represent manual screen false negative cases. Thirteen percent of the scanned cases displayed tetrasomy/tetraploidy or trisomy, and the remaining 27 percent of the slides were unsatisfactory for BioView interpretation because of scant cellularity or excessive clumping. The total time required for pathologist evaluation was 4 min/case with the aid of BioView, compared to 30 min/case for a manual interpretation.

For Target FISH, it was important to fully destain the slides before FISH was carried out, and some cell loss occurred during the procedure. Brightfield scans did not demonstrate the same quality as fluorescent scans, with non-cellular material and inflammatory cells often included as targets. The ability of the instrument to relocate previously scanned target cells was excellent, with target cells in near-perfect registration or located within the field of view.

Conclusions: In this study, the BioView-aided interpretations were at least equivalent to manual interpretations, with 2 manual review false negatives cases being detected with the aid of the BioView instrument. To work optimally, cell distribution on the slide must be of high quality. The images generated are of excellent quality for archiving. The system permits interactive review of abnormal cells, as well as the ability to evaluate the same cells for brightfield cytology followed by UroVysion FISH (Target FISH).
The effect of sequence variants on quantification of HIV-1 RNA

Pyne M, Hillyard D

Objectives:
1) Describe the increasing genetic diversity of HIV-1, including groups, subtypes, and recombinants and their increasing prevalence in North America.
2) Describe the impact of HIV-1 diversity on HIV-1 viral load test performance.
3) Outline approaches to identify HIV-1 polymorphic variants and their impact on test development and result interpretation.

Introduction: Several recent publications have described the increasing genetic diversity of HIV-1. Non-B subtypes and circulating recombinant forms (CRFs) have typically been linked to underquantification by viral load assays. These variants are more common in Europe and Africa, but have recently increased in prevalence in North America. However, diversity in any HIV-1 type has the potential to affect the performance of viral load assays. The recently FDA-approved Roche COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test (CAP/CTM) with docking station was evaluated for analytical sensitivity, reproducibility, linearity, and correlation to the Roche AMPLICOR HIV-1 MONITOR Test, Version 1.5 (Amplicor). Additionally, an HIV-1 group M subtype panel was evaluated by the CAP/CTM, Amplicor, and Bayer Versant HIV-1 bDNA 3.0 assays (Versant).

Materials and Methods: All assays were performed as recommended by the manufacturers. Dilutions of WHO 2nd International Standard material and Viral Quality Assurance material (Rush University Medical Center – VQA) were used to determine the limit of detection of the CAP/CTM assay by probit analysis. Six high-titer samples were serially diluted, aliquoted, and tested to determine inter- and intra-assay reproducibility and linearity. Aliquots of the Acrometrix ValiQuant RNA Quantification Panel members were tested in triplicate on each of three days. Archived samples that were previously measured with the Amplicor assay were analyzed using the CAP/CTM assay. The members of the BBI HIV RNA Genotype Performance Panel PRD202 were diluted three-fold and tested in triplicate in the CAP/CTM, Amplicor, and Versant assays.

Results: Probit analysis predicted a limit of detection of 23.79 copies/mL (95% confidence interval [CI]: 20.11 – 27.46 copies/mL) using WHO reference material and 30.83 copies/mL (95% CI: 26.37 – 35.29 copies/mL) using VQA material. Intra- and inter-assay linearity and reproducibility were good over the range of the assay tested (~ 1.7 – 6.7 log10 copies/mL). All assays were able to detect and quantitate all members of the Group M subtype panel with good reproducibility. On average, the Amplicor assay results were 0.008 log10 copies/mL higher than the CAP/CTM assay. Ninety-six percent (96%) of samples differed by less than 0.5 log10 copies/mL and ninety-eight percent (98%) of samples differed by less than 1.0 log10 copies/mL. One sample was highly discrepant (1.7 log10 copies/mL in CAP/CTM versus 4.4 log10 copies/mL in Amplicor). Repeat measurement of this sample in Amplicor confirmed the high result, but insufficient sample was available for retesting by CAP/CTM. Roche sequenced this sample and determined that it was subtype B. Alignment of this sequence against the sequences of the assay primers and probe revealed a mismatch near the 3’ end of a primer and additional mismatches in the probe region.

Conclusions: The “sample in, result out” CAP/CTM assay is highly sensitive and exhibits excellent reproducibility over a wide dynamic range. With archived samples, the CAP/CTM assay correlated extremely well with the Amplicor assay, with negligible differences between assays. However, one discrepant sample contained several primer and probe mismatches that drastically affected the assay’s performance. Further study is warranted to determine the frequency of polymorphisms that affect the assay.
Molecular testing for adult type alport syndrome

Pont-Kingdon G, Sumner K, Gedge, Miller C, Denison J, Gregory M, Lyon E

**Objectives:**

1) Describe the function of type IV collagen.

2) Describe Alport syndrome.

3) Describe design and characteristics of molecular test assay

Alport syndrome (AS) is a progressive renal disease with cochlear and ocular involvement due to defects in one of the alpha chain of type IV collagen. The majority of AS cases are X-linked (XLAS) and due to mutations in COL4A5 that encodes the alpha 5 collagen IV chain. Although the disease may appear early in life and progress to end stage renal disease (ESRD) in young adults, in some families ESRD occurs in middle age. Few of the more than three hundred mutations described in COL4A5 are associated with adult type XLAS, but the families may be very large. We developed a molecular assay identifying the three most common adult type XLAS mutations in the US; C1564S, L1649R, and R1677Q. The test was validated on samples previously determined to contain one or none of these mutations. The test’s clinical specificity and sensitivity are estimated to be higher than 99% and 90% respectively. Analytical specificity and sensitivity are above 99%. This test may be useful for presymptomatic and carrier testing in families with one of the mutations and in the diagnosis of unexplained hematuria or chronic kidney disease.
Thrombophilia quadruplex assay on the LS-32 instrument

Seipp M, Voelkerding KV, Wittwer CT

Objectives:
1) To provide a basic explanation of high resolution melting analysis.
2) To explain the LS-32 machine and its use in amplification and high resolution melting analysis.
3) To demonstrate the application of the LS-32 on the thrombophilia quadruplex.

Background: High resolution (HR) melting analysis is a simple closed-tube method for genotyping which utilizes the dsDNA binding dye LCGreen-Plus for detection. Multiplex HR melting analysis was demonstrated previously on the HR-1 instrument for the four most common mutations associated with thrombophilia; F5 (Factor V Leiden), F2 (Prothrombin), and both MTHFR 1298 and 677. The utility of this assay was limited, because of the amount of technologist time needed to run the HR-1. This current study was designed to convert the thrombophilia quadruplex assay to the LS-32 which has automated melting analysis.

Methods: The LS-32 is a new instrument with the capability of amplifying and melting 32 samples without any further intervention. Previously, this assay required individual capillaries transferred by hand to the HR-1 instrument for HR melting analysis. Unlike the single sample method, the capillaries are loaded into the LS-32 and amplification, HR melting and analysis are all done without any contact with the capillaries.

Results: With the single sample method, proper assignment of genotype on the HR-1 instrument required resolution for mutation detection only attained using a 0.1 °C/s temperature ramp rate during HR melting analysis. Using the LS-32, similar resolution has been observed with a 0.3 °C/s ramp rate. The overall analysis time on the LS-32, including amplification and HR melting, was shortened from over 8 hours to ~2 hours.

Discussion: Conversion of the thrombophilia quadruplex assay to the LS-32 has decreased the amount of time involved in the assay without affecting the overall resolution of the melting analysis. The sample analysis has been simplified as well due to the fact that there is no hands-on transfer between the LightCycler and the HR-1 instruments.
Analysis of immunoglobulin heavy chain variable region genes in chronic lymphocytic leukemia

Szankasi P, Bahler DW

**Objectives:**

1) Understand the two major prognostic subgroups of chronic lymphocytic leukemia.

2) Understand the function of somatic hypermutation in B cell activation.

3) Understand the method for determining the mutation status of immunoglobulin genes in leukemic cells.

**Background:** Chronic lymphocytic leukemia (CLL) is a B-cell neoplasm and the most common leukemia in the US and Europe. The clinical course of CLL is variable with approximately one half of cases being very indolent while in the other half the disease behaves in a much more aggressive fashion. The clinical course of CLL has been shown to correlate with somatic mutations that may be present in the expressed immunoglobulin (Ig) heavy chain variable region gene segments ($V_H$). In addition, the expression of particular $V_H$ gene segments has also been associated with prognosis in CLL regardless of the variable region mutation status.

**Methods:** We developed a test for sequence analysis of the $V_H$ genes expressed by CLL leukemic cells. PCR amplification from whole blood-derived cDNA enriches for Ig sequences because of their strong expression. Multiple PCR reactions using variable region family-specific $V_H$ leader region forward primers along with a consensus joining region reverse primer allow for the amplification of even low level clonal Ig sequences. Derived DNA sequences are compared against a database of germline $V_H$ sequences to determine the mutation status and identity of the $V_H$ region.

**Results:** We were able to determine the identity and mutation status of the $V_H$ genes in over 100 CLL cases. In approximately 5% of the cases no clonal Ig sequences could be identified. Dilution experiments showed that data could be obtained from CLL clones as small as 50% of total B cells. Several cases expressing the $V_H^{3-21}$ gene segment were identified.

**Conclusion:** This test allows for the unequivocal determination of the mutation status of $V_H$ genes in CLL which can provide important prognostic and clinically useful information. In addition, our test can easily identify cases that use the $V_H^{3-21}$ gene segment which as been associated with poor prognosis regardless of mutational status.

1) Understand the two major prognostic subgroups of chronic lymphocytic leukemia.

2) Understand the function of somatic hypermutation in B cell activation.

3) Understand the method for determining the mutation status of immunoglobulin genes in leukemic cells.
Creatine transporter deficiency: a cause of X-linked mental retardation

Ardon O, Di San Filippo C, Longo N

Objectives:

1) Define disorders caused by defective synthesis/transport of creatine.
2) Treatment of creatine deficiency syndromes.
3) Testing for creatine transporter defects.

Creatine is important for the utilization of energy derived from ATP at sites of high energy utilization (muscle, brain, heart). In humans, creatine is either synthesized in the kidney and liver, or obtained from the diet. Conservation and inter-organ transfer of creatine requires specific membrane transporters. The creatine transporter gene SLC6A8 (MIM 300036) encodes for creatine transporter 1 (CT1 or CRTR), maps to Xq28 and is expressed in most tissues, with highest levels in skeletal muscle and kidney. Other genes, SLC6A10, SLC6A17 and SLCA18, encode for CT2 and other transporters with unclear substrate specificity. A defect in the CT1 creatine transporter, encoded by SLC6A8, causes brain creatine deficiency (OMIM 300352), an X-linked disorder characterized by mental retardation, seizures, mid-facial hypoplasia and short stature in hemizygous males. Females can have mild cognitive impairment with behavior and learning problems. Defects in the CT1 transporter were found in 1% of males with mental retardation.

The diagnosis of brain creatine deficiency is suspected based on abnormal brain MRI findings (that are however nonspecific) and reduced brain creatine concentration (measured noninvasively by magnetic resonance spectroscopy). Laboratory testing indicates normal plasma levels of creatine and the creatine precursor guanidinoacetate, but increased urinary creatine/creatinine ratio, which however is not diagnostic. The diagnosis is confirmed by measuring creatine transport in human fibroblasts or by DNA testing. We have established a new method to measure creatine transport in fibroblasts. This method relies on the uptake of [14C]-creatine uptake into the cells and normalization for cell proteins and intracellular volume. Fibroblasts from affected male patients had less than 5% of the transport measured in control cells. There is currently no causal therapy for brain creatine deficiency. We are using cells obtained from these patients to identify creatine analogs capable of entering the cell independently of the creatine transporter that could cross the blood-brain barrier and restore brain energy levels. Such analogs may prevent mental retardation in patients diagnosed early in life because of a positive family history.
Steroid profiles in ovarian follicular fluid from healthy women and from women after ovarian stimulation

Kushnir M, Naessen T, Rockwood A, Bergquist J

Objectives:

1) Use of high sensitivity methods for measurement of steroids in small samples.

2) Biosynthesis of steroids in ovaries.

3) Comparison of steroid concentrations between ovarian follicles and blood.

Information on concentrations of steroids in ovarian follicular fluid (FF) from healthy women is limited because of absence in past of sensitive and specific methods for simultaneous quantitation of multiple steroids in small volumes of FF. The objective of the study was to compare steroid profiles in FF during the early follicular phase of the menstrual cycle in regularly menstruating women and in women after ovarian stimulation for in vitro fertilization. Using LC-MS/MS methods, 15 steroids were analyzed in 40 µL-aliquots of individual FF samples from 21 healthy women and 5 women after ovarian stimulation. In healthy women, androgens were the most abundant class of steroids with androstenedione being the major constituent. Compared to serum, concentrations of 17 hydroxyprogesterone, total androgens and estrogens in FF were 200 to 1000 fold greater. Compared with healthy women, women undergoing ovarian stimulation had significantly higher concentrations of estradiol, pregnenolone, 17 hydroxyprogesterone and cortisol and significantly higher ratios of estradiol/estrone, estradiol/testosterone and cortisol/cortisone. Concentrations of testosterone, androstenedione and estradiol determined by LC-MS/MS methods were significantly lower than those previously reported in literature in studies that were using immunoassays.

Obtained data provide the first mass spectrometry-based values for multiple steroids concentration in the ovarian FF during early follicular phase of the cycle and after ovarian stimulation. Accurate analysis of multiple steroids in minute FF volumes will be useful for better understanding of the normal ovarian physiology, anovulation and effects of ovarian stimulation regimens used in IVF treatments.
Haptoglobin genotyping and cardiovascular risk in subjects with diabetes mellitus

Millson A, Horne BD, Anderson JL, Carlquist JF, Roberts WL, Lyon E

Haptoglobin (Hp) is a serum protein with many functions. The best known is as an antioxidant, binding hemoglobin released during red cell hemolysis, thus reducing kidney damage. Hp is composed of 4 polypeptide chains, 2 alpha and 2 beta. The alpha chain has two common alleles, Hp1 and Hp2, the Hp2 allele resulting from a duplication of Hp1. The beta chain is identical in all Hp types. The biochemical properties of the haptoglobin molecule vary depending on which alleles are present (Hp1-1, Hp1-2 or Hp2-2). Haptoglobin genotype has been shown to be an independent risk factor in individuals with diabetes mellitus for coronary artery disease (CAD). We evaluated a series of 3,137 subjects enrolled in the Intermountain Heart Collaborative Study Registry to assess possible association of haptoglobin genotype and CAD. About 70% of the study group had severe CAD and 60% showed abnormal glucose metabolism. 705 out of 3,137 subjects were diabetic. All subjects had >3 years of clinical follow-up. Haptoglobin genotyping was performed on the LightCycler™ using two polymerase chain reactions, one for the Hp1 and one for the Hp2 allele, followed by fluorescent monitoring using hybridization probes. One of the study objectives was to assess risk of atherosclerotic complications in the diabetic subjects. Our primary endpoint was angiographic CAD. Our secondary endpoints were death due to myocardial infarction (MI), all-cause death and MI. The genotype frequencies were similar between the diabetics and non-diabetics. We found our primary endpoint of angiographic CAD to be significant, \( p=0.013 \) with the \( p\)-trend = 0.003, in only the diabetic subjects with the Hp1-1 genotype. Our findings are similar to the Framingham Offspring cohort study (2004) yet contradict other studies that found diabetics subjects with the Hp2-2 genotype at increased risk for CAD.

Objectives:

1) Introduce the basic physiological role of haptoglobin.

2) Describe the haptoglobin assay design and result interpretation.

3) Discuss the design and outcomes of the collaborative ARUP/Intermountain Health Care haptoglobin genotype and cardiovascular risk study.
Validation of a serum histoplasma antigen assay

Cloud JL, Bauman SK, Pelfrey JM, Ludwig KG, Ashwood ER

Objectives:

1) The serum histoplasma antigen assay is a valid assay at ARUP.

2) Human urine and serum samples contain antigenic differences for histoplasma.

3) The ARUP histoplasma antigen detection assay performs very similar to the MiraVista histoplasma antigen detection assay.

A validated polyclonal sandwich enzyme immunoassay (EIA) for the detection and quantitation of histoplasma antigens in human urine has successfully been in use at ARUP Laboratories. The performance evaluation of the assay resulted in good precision, both within-run and between-run. The assay is linear when testing dilutions of the highest calibrator, but non-linear when testing dilutions of a positive human serum sample. The assay, as performed on urine samples, is invalid with untreated human sera. Serum pre-treatment with pronase has been shown to work well in reducing interference from proteins and blocking epitopes by immune complexes. Pronase-treating urine samples, however, results in lower level of detection and cannot be used. To validate pronase-treated serum samples in the Histoplasma Antigen EIA, we tested 71 samples that were previously tested by MiraVista Diagnostics (MVD) to determine percent agreement in positive and negative samples. Thirty-seven of the samples were compared with the MVD second generation assay while 34 samples were compared with the current MVD third generation assay. The second generation assay comparison resulted in an R^2 of 0.5957 while the improved third generation comparison resulted in an R^2 of 0.7514. After excluding samples testing in the equivocal range, 24 of 28 (86%) MVD positive serum samples were in agreement and 28 of 28 (100%) MVD negative serum samples were in agreement. We conclude the serum histoplasma antigen test performs well and is valid in our hands. Our studies further show that antigenic differences between urine and serum sample matrices are present.
Comparative sequence analysis by sanger sequencing and Roche/454 GS FLX System: CFTR gene as a model

Chou LS, Voelkerding KV, Lyon E

Objectives:

1) What is the NGS (next generation sequencing)?

2) What are the available platforms?

3) What are the applications that the NGS can achieve?

Background: The recent introduction of instruments capable of generating millions of DNA sequence reads in a single run is rapidly changing the field of genetic testing, providing the ability to analyze multiple genes at one time. The main applications include: de novo sequencing, re-sequencing, and amplicon metagenomics. These systems include: Roche 454 GS FLX sequencer, Illumina genome analyzer (GAII), and ABI SOLiD sequencer. While the implementation of these next generation sequencers (NGS) in a clinical diagnostic laboratory remains in the future, the goal of the current study was to determine the feasibility of NGS (in particular the 454 system) and compare its data quality with the current gold standard Sanger sequencing.

Materials and Methods: In this pilot study, we selected CFTR as the targeted gene using 16 de-identified clinical samples. CFTR has 27 exons and some regions of interest containing complicated sequence structure such as the polymorphic (TG)m(T)n tract in the intron 8/exon 9 boundary. We compared the Roche 454 GS FLX sequencer with the Sanger sequencing (by ABI BigDye Terminator Cycle Sequencing) data, in the aspect of call rate accuracy, as well as the data analysis and management.

Results and Conclusions: Disregarding the primer coverage, in general we found 1 discrepancy of 688 amplicons sequenced by 454, when compared with Sanger sequencing data. This particular exon has been repeated by Sanger sequencing but not 454, without resolving the discrepancy. The heterozygous deletion (delF508, 3 base deletion), as well as compound heterozygous mutations, were detected accurately by 454. Although the (TG) m(T)n tract was difficult to analyze initially (by automatic software calls), this complex region was re-analyzed manually and the number of TG and T repeats were able to be determined. In conclusions, the next generation sequencers are capable of providing genome-wide sequence readouts in a single run, as an end-point toward applications such as mutation or SNP discovery. However, before implementing this new technology in clinical applications, parameters that need consideration include the work flow (process automation), data analysis (especially the short tandem repeats when using a pyrosequencing-based 454 GS FLX sequencer), and data management (LIMS, hardware, software).
A new member of the *mycobacterium chelonae / abscessus* complex found in North Eastern United States


**Objectives:**
1) Illustrate the current problems with *M. chelonae / abscessus* complex taxonomy.
2) Illustrate the strengths and limitations of multi-locus sequencing as the primary arbiter of taxonomy.
3) Illustrate how we plan to use next-generation sequencing technology to resolve issues above.

**Background:** The *Mycobacterium chelonae/abscessus* complex (MCAC) consist of five validated species (*M. chelonae, M. abscessus, M. immunogenum, M. bolletii,* and *M. massiliense*). Often clinical labs do not speciate members of this complex because of similar biochemical profiles and identical 16S rRNA gene sequence over the first 500bp for 4 of 5 species. However, speciation does provide useful information for antibiotic administration. Treatment of MCAC is limited to a few agents, tobramycin is the preferred for *M. chelonae* and amikacin for *M. abscessus* and unlike the other members *M. chelonae* is resistant to cefoxitin.

**Method:** From February 2005 to July 2007 ARUP Laboratories identified 159 isolates as *M. chelonae* using a published real-time PCR assay targeting the internal transcribed spacer (ITS) between rRNA genes. Susceptibilities were performed on the 159 isolates and 5 were found to have atypical results to cefoxitin, having either sensitive or intermediate results ranging from 16 to 64 µg/ml. Sequencing of the 16S rRNA, *sodA, hsp65*, ITS, and *rpoB* DNA targets and have been performed to confirm identification of the 5 isolates. Phylogenetic analysis was preformed with all DNA targets to determine taxonomic relationship.

**Results:** Phylogenetic analysis confirmed the identification of 1 of the 5 isolates with atypical cefoxitin results as *M. chelonae*. The remaining 4 isolates were found on a separate branch indicating a new member within MCAC. Interestingly, we did not observe phylogenetic concordance with all DNA targets. The new member was most closely related to *M. chelonae* when comparing 16S rRNA and ITS sequences, however, *sodA* and *hsp65* targets showed the isolates to be more related to *M. immunogenum*. Two unique genotypes were noted and preserved with all gene targets, with the exception of 16s rRNA gene, which were identical. All 4 isolates were isolated from patients in the North Eastern United States (3 Pennsylvania (2007), 1 in New York (2005)).

**Discussion:** We identify a new member of the MCAC, which to date has only been found in the North Eastern United States and of the recently isolated organisms (2007-2008) have only been isolated from a single institution. After notifying the institution of the unique characteristics of this novel MCAC member, we were able characterized and identified 8 new isolates from patients. In total we have 12 isolates, 11 from a single institution. Given that the majority of isolates were identified from a single institution, we assume these isolates originated from a single reservoir, this is most interesting since we observed two distinct genotypes within this novel species. The characteristics of this isolate make it an ideal model organism for resolving confusing taxonomy of the *M. chelonae / abscessus* group, we plan to do this by sequencing the genomes of all validated species and our novel isolates.
An assessment of the ability of *mycobacterium tuberculosis* serology to detect active infection

Anderson B, Litwin C

**Objectives:**

1) Know the significance of differentiating between active and latent TB infection.

2) Know the advantages and disadvantages of TST and the QuantiFERON® TB tests for the diagnosis of latent TB infection.

3) Know the advantages and disadvantages of TB antibody testing for the diagnosis of active TB infection.

*Mycobacterium tuberculosis* (*Mtb*) remains a major disease throughout the world, with approximately 9 million new cases and about 2 million deaths occurring each year. It is estimated that one-third of the world’s population is infected. An essential component of *Mtb* control efforts is to identify and treat individuals with active, infectious cases. Current test procedures are inadequate to accurately detect and identify active *Mtb* infection. The ability to differentiate between latent and active *Mtb* infection is critical in patient care and containment of the disease. The addition of serology testing to conventional microbiologic methods offers increased sensitivity in detecting active disease when a patient presents with a positive tuberculin skin test (TST) or QuantiFERON®-TB (QFT) result. We evaluated three commercially available ELISA assays, InBios Active TbDetect™ IgG ELISA, IBL *Mycobacterium tuberculosis* IgG ELISA, and Anda Biologicals TB ELISA, for their ability to detect antibodies to *M. tuberculosis* and differentiate active from latent infection. Nineteen serum samples from culture positive or Amplified Direct Detection positive patients and 88 serum samples from *Mtb* low-risk patients were evaluated in this study. Seventy-eight serum samples from QFT positive and/or TST positive patients were also tested for the presence of IgG antibodies. We evaluated each ELISA assay for agreement, sensitivity, and specificity compared to culture or Amplified Direct Detection and *Mtb* low-risk patients. InBios Active TbDetect™ had an agreement of 95.3%, a sensitivity of 78.9%, and a specificity of 98.9%. The IBL *Mtb* ELISA had an agreement of 83.2%, a sensitivity of 5.3%, and a specificity of 100.0%. The agreement, sensitivity, and specificity of Anda Biologicals TB ELISA were 73.4%, 78.9%, and 72.0% respectively. The percent positivity for InBios Active TbDetect™ ELISA, IBL *Mtb* ELISA, and Anda Biologicals TB ELISA compared to TST and/or QFT positive samples were 5.1%, 0.0%, and 30.8% respectively. In conclusion, InBios Active TbDetect™ IgG ELISA is far superior to the other ELISA assays in accurately detecting active *Mtb* disease.
Newborn screening second tier test for the detection of methylmalonic and propionic acidemias

Lloyd M, Pasquali M

Objectives:

1) Discuss the Supplemental Newborn Screening program by tandem mass spectrometry (MS/MS).

2) Review performance metrics for newborn screening.

3) Discuss the use of second tier tests to improve the sensitivity and specificity of newborn screening.

Background: Newborn screening by tandem mass spectrometry (MS/MS) can detect a number of metabolic disorders before symptoms appear, allowing early intervention to reduce mortality and morbidity associated with these disorders. One of the pitfalls of the system is the number of false positives that can be detected. Propionylcarnitine (C3-carnitine) is one of the analytes responsible for the highest number of false positives. C3-carnitine is a marker for propionic acidemia, methylmalonic acidemia, and multiple carboxylase deficiency, although the most common causes for its elevation are diet, medications, or prematurity. To reduce the number of false positives due to elevated C3-carnitine, we have developed a second tier test geared to the detection of secondary metabolites specific for propionic acidemia and methylmalonic acidemia (methylmalonic acid, methylcitric acid, and 3-hydroxypropionic acid).

Methods: Methylmalonic, methylcitric, and 3-hydroxypropionic acids were extracted from blood spotted on filter paper with solution containing deuterated internal standard. The extract was dried, reconstituted with water, and analyzed by LC-MS/MS using a Waters Quattro Premier tandem mass spectrometer and Acquity UPLC system as liquid manager. The separation of the analytes was obtained using a Phenyl Acquity column. The analytical performance was evaluated using filter paper bloodspots spiked with known concentration of the analyte.

Results: The limit of detection for methylmalonic, methylcitric, and 3-hydroxypropionic acids was 0.75µM, 1µM, and 65µM respectively. De-identified normal bloodspots were analyzed with this method to evaluate normal values. In all of these samples the concentration of the three analytes was below the limit of quantitation. We have also analyzed bloodspots and plasma spotted on filter paper from patients with methylmalonic acidemia and propionic acidemia. All the samples studied were correctly identified. We have also analyzed de-identified bloodspots from normal infants with elevated C3-carnitine due to diet/dietary supplements. In all of these samples, the concentrations of methylmalonic, methylcitric, and 3-hydroxypropionic acids were within the normal range.

Conclusions: The addition of a second tier test for methylmalonic, methylcitric, and 3-hydroxypropionic acids to newborn screening is an effective way to reduce the false positive results due to elevated C3-carnitine and greatly increase the positive predictive value of the test.
An automated workcell for specimen tube disposal

Herrmann M, Behr KC, Last E, Mejia J, Kildee H, Semrow T

**Objectives:**

1) To understand the basics of ARUP's automation initiative.

2) To understand the advantages/disadvantages of home brew automation vs. corporate automation.

3) What are the current issues facing full automation of specimen tube disposal.

ARUP receives on average over 23,000 specimens a day for laboratory testing. The residual samples are stored in trays with a capacity of 450 tubes at -20 °C for up to a year. Previously, the disposal of these samples required a minimum of 1 FTE for the manual disposal of up to 39,000 samples a day. We report the development of an automated work cell for the disposal of these samples. The work cell has reduced the FTE requirement 6 fold to 0.18 and has minimized potential biohazardous exposures.
Detection of thyroid stimulating immunoglobulins using enzyme fragment complementation

Sandrock T, Richardson K, Terry A, Gillespie G, Martin J, Erdogan E, Meikle AW

Objectives:
1) Know the molecular mechanism of Graves’ disease.
2) Describe how the method “enzyme fragment complementation” was adapted to detect thyroid stimulated immunoglobulin.
3) Describe the future benefits of automation of the assay.

Background: In Graves’ disease autoantibodies activate thyroid stimulating hormone receptor (TSHR), leading to an increase in intracellular cAMP and thyroid hormone, thereby causing hyperthyroidism. We developed a non-radioactive assay to measure thyroid stimulating immunoglobulins.

Methods: We have adapted enzyme fragment complementation (EFC, DiscoveRx, Freemont CA) to measure cyclic adenosine monophosphate (cAMP) levels in a bioassay using a stable transfected Chinese hamster ovary (CHO) cell line producing the human TSH receptor. Four hundred and twenty-five specimens submitted for thyroid stimulating immunoglobulin (TSI) testing were assayed using a traditional radio-immune assay (RIA-cAMP) and the non-radioactive EFC-cAMP method.

Results: The correlation for all 425 samples was R=0.92, slope 1.048, intercept = -2.2 by Demings regression. In addition, 46 serum samples from healthy individuals were tested using the EFC-cAMP method (mean 94.4%, median 94.5%, and SD 6.8%). The normal samples ranged from 80% to 106% of the control. Five of six samples from clinically diagnosed Graves’ patients provided by KRONUS, Inc. were positive (370%, 382%, 371%, 262%, 316%); one was indeterminate (115%). For correlation, three samples were assayed in our laboratory and a second reference laboratory: (100%, 106%); (446%, 278%); and (149%, 180%); respectively.

Conclusions: This EFC-cAMP assay is a non-radioactive surrogate for RIA-cAMP for studying antibody-mediated rise in cAMP concentrations in response to activation of the G-coupled thyroid stimulating hormone receptor.
13Q deletions in MM and CLL: Further evidence that the critical region does not include RB1

Rector L, Rowe L, South S, Lamb A, Brothman A

Objectives:
1) To understand the importance of FISH in MM and CLL.
2) To answer our major question: does the 13q critical region include RB1?
3) What role will this retrospective study play in the clinical lab?

The importance of obtaining cytogenetic data beyond the chromosomal level has been reported many times. By using fluorescence in situ hybridization (FISH) in interphase cells, one is often able to better identify pathogenetic and prognostically significant genetic findings in hematologic malignancies. This is especially true for malignancies in which the abnormal cells do not tend to grow well in culture, such as multiple myeloma (MM) and chronic lymphocytic leukemia (CLL). The deletion of the RB1 gene is a common anomaly associated with both MM and CLL and our panels include the 13q14 region, with RB1 and D13S319 used in MM, and RB1 and D13S25 in CLL. However, studies suggest that the important gene in the 13q14 region is located distal to RB1. We performed a 3 year retrospective analysis of patients with CLL or MM and a deletion either of RB1, D13S319, or D13S25 and noted the status of the other genes of this group. In 90 patients with a RB1 deletion, either D13S319 or D13S25 was also deleted. Furthermore, when RB1 was present on both homologues, D13S319 (MM) was deleted in one case, and D13S25 (CLL) was deleted in 37 cases. Our data support the concept of removing RB1 from FISH panels for MM and CLL, thus simplifying the assay. Other reports in the literature suggest that D13S319 is close to a critical region for these disorders, indicating that this may be the ultimate probe of choice.
Role of noncoding region mutations of ACVRL1 and ENG in the pathogenesis of Hereditary Hemorrhagic Telangiectasia

Damjanovich K, Escobar H, McDonald J, Gedge F, Chou LS, Bayrak-Toydemir P

Objectives:
1) Describe the background information and unknowns regarding Hereditary Hemorrhagic Telangiectasia (HHT).
2) Describe the sequencing results from non-coding regions of the HHT genes in order to explain the disease in patients.
3) Understand the role of regulatory and intronic regions in gene function.

Hereditary Hemorrhagic Telangiectasia (HHT) is a vascular dysplasia characterized by arteriovenous malformations and telangiectasia. The majority of the HHT patients have mutations in the coding regions of activin A receptor type II-like (ACVRL1) and endoglin (ENG) genes. However, approximately 20% of the HHT patients do not have mutations in the coding regions of either gene that can be detected by sequencing or deletion/duplication testing by multiplex ligation probe amplification (MLPA). For proper counseling and to prevent complications of the disease, it is important to identify mutations in these patients.

It is known that the noncoding regions of genes have regulatory functions. We hypothesized that sequence alterations in these regions could have role in disease pathogenesis through affecting the recognition or binding efficiency of transcription factors, splice site enhancers or depressors. We had 16 clinically affected HHT patients in whom we were unable to find the causative mutations in the coding regions of ACVRL1 and ENG. In order to test our hypothesis, we first confirmed the causative gene is in one of the known HHT loci. We had access to samples from family members, to run locus specific linkage analysis, in 3 of these 16 patients, two of which suggested linkage to ACVRL1 locus and other to the ENG locus. This prompted us to screen for the non-coding regions of the HHT genes in our cohort of 16 patients. Based on studies in mice and evolutionary conservation analysis, we selected regulatory regions of the 5’ and 3’ untranslated regions (UTRs) of both genes, and sequenced about 9 kb 5’UTR region of the ACVRL1 and two 600 bp segments, up to 8 kb upstream of the ENG. In addition, we sequenced the introns of both genes (11.6 kb for ACVRL1 and 7.9 kb for ENG), except for the 4 large introns of ENG, for which we used a mutation screening protocol based on heteroduplex formation followed by targeted sequencing approximately.

Approximately 40% of ACVRL1 variants and 60% of ENG variants were previously reported as polymorphisms. Currently we are analyzing the unreported variants (20 ACVRL1 and 30 ENG variants). For every unreported sequence variant found, we sequenced the available family members to determine the segregation with the disease. Then we analyzed the effects of these variants on evolutionary conserved regions and transcription factor binding sites by using rVista, GeneACT, and some other web based programs. Some of the interesting findings of our study include an ACVRL1 variant (c.-273-2774T>C) at 5’UTR region, that creates a SP1 transcription binding site; another ACVRL1 variant (c.-6+825A>T) at intron 1 which destroys a TATA binding site. SP1 is one of the twelve transcription factors shown to regulate endothelial cell specific genes. These and some of the other variants we found are candidates for future studies to explore their disease causing potential.
Urinary excretion of glutaryl carnitine

Liu A, Pasquali M

**Objectives:**

1) Review Glutaric acidemia type I.

2) Review current diagnostic methods for Glutaric acidemia type I.

3) Discuss the use of urinary measurement of glutaryl carnitine for the diagnosis of Glutaric acidemia type I.

**Objective:** Glutaric acidemia Type I (GA-I) is a metabolic disorder that can result in progressive neurodegeneration. The diagnosis relies on increased excretion of glutaric and 3-hydroxyglutaric acids in urine and increased glutaryl carnitine in plasma. Recently, urine glutaryl carnitine has been proposed as a marker for this condition. Here we report a quantitative study of the urinary excretion of glutaryl carnitine in GA-I patients as compared to normal controls and patients with other metabolic disorders.

**Methods:** We defined the reference range for urine glutaryl carnitine in 200 normal urine controls (collected according to a protocol approved by the IRB of the University of Utah) and then analyzed 25 urine samples (de-identified) collected from classical GA-I patients and low excretors (patients who have minimal or no elevation of glutaric acid in urine) at diagnosis and after treatment. Patients with GA-II (glutaric acidemia type II), GA-III (glutaric acidemia type III) and MCAD (Medium Chain Acyl-CoA Dehydrogenase) deficiency were also studied to determine the assay specificity. Aliquots of urine containing 0.01 umole of creatinine were extracted with acidified (formic acid 0.05%) acetonitrile, and derivatized as butyl esters before analysis by UPLC/MS/MS on Waters Acquity UPLC™/Quattro Premier system.

**Results:** Normal controls excreted in urine ≤ 0.3 mmol/mole creat of glutaryl carnitine. Patients with classic GA-I had a dramatic increase in the excretion of glutaryl carnitine (up to 86.9 mmol/mole creat in the samples studied) that decreased with appropriate dietary treatment. Nevertheless, the concentration of glutaryl carnitine remained > 0.4 mmol/mole creat even during optimal treatment. Small to moderate elevations of urinary glutaryl carnitine were always detected in GA-I low excretors. We did not observe any elevation of glutaryl carnitine in patients with GA-III and MCAD deficiency, while patients with GA-II might have elevated glutaryl carnitine.

**Conclusions:** Our study indicates that quantitative measurement of urinary glutaryl carnitine can aid in the diagnosis of glutaric acidemia type I, in conjunction with standard urine organic acids and plasma acylcarnitine analysis.
Definition of HLA Class I motifs by large scale sequencing of peptides eluted from HLA molecules

Crockett D, Escobar H, Reyes-Vargas E, Jensen P, Delgado J

Objective: Our goal was to develop robust methodologies to catalogue hundreds of peptides presented by a given HLA molecule of interest. Identifying the preferred amino acid sequence of HLA presented peptides can then be used to determine HLA class I motifs and specificity.

Methods: An HLA class I null cell line (K562) was transduced with a retroviral construct carrying the HLA-B*3501 gene sequence. Sepharose affinity columns were used to capture HLA-B*3501 molecules from the transfected cell lysate. Bound peptides were separated from denatured HLA molecules by ultracentrifugation. Peptide pools were fractioned by HPLC and analyzed using the Agilent 6510 quadrupole time-of-flight mass spectrometer (QTOF) and ChipCube nanospray source. The acquired MS/MS spectra were searched against the Swiss Prot human database to identify the HLA bound peptide sequence information. The transfection, enrichment, and LC-MS/MS analysis was then repeated for additional HLA-B35 subtypes (B*3502, B*3503, B*3504, B*3506, and B*3508).

Results: LC-MS/MS analysis identified more than 1000 peptides in transfected cell lysate from six HLA B35 subtypes. This peptide catalogue represents the largest data set of HLA presented peptides published to date. This large scale sampling of peptides allowed HLA motif definition by amino acid frequency and position across the six different HLA-B35 molecules.

Conclusions: Discovery of HLA motif specificity by large scale peptide sequencing allowed validation of reported HLA motifs and elucidation of novel HLA peptide motifs. This approach allows for precise characterization and differentiation of peptide motifs among HLA molecules – even when differing by a single amino acid position. This methodology will be the basis for the discovery of CTL epitopes useful for immunotherapy of infectious diseases and tumors.
Development of a multiplexed fluorescent immunoassay for the quantitation of antibody responses to four *Neisseria meningitidis* serogroups

Martins TB, Jaskowski T, Tebo A and Hill HR

**Objectives:**

1) Appreciate the worldwide significance of Meningococcal disease.

2) Describe the multi-analyte assay developed for determining antibody responses to four *Neisseria meningitidis* serogroups.

3) Present results of the assays performance characteristics as well as applications in evaluating immune status and vaccine response.

*Neisseria meningitidis* is a gram-negative diplococcal bacterium causing disease worldwide with a fatality rate of 5-10%. Five serogroups, A, B, C, Y and W135 are responsible for virtually all cases of the disease in humans.

We have developed a multiplexed assay for the simultaneous quantitation of IgG antibody responses to the four most immunogenic (A, C, Y, and W-135) *Neisseria meningitidis* serogroups. A simple and less manipulative method was employed for conjugation of the capsular polysaccharide antigens to the microspheres. The multiplex assay compared well with traditional individual ELISA’s, but demonstrated greater than 1 log increase in dynamic range. Specificity studies of the multiplex assay showed greater than 95% homologous inhibition and less than 5% heterologous inhibition for all four serogroups. Intra and inter-assay CV’s were generally less than 10% and the limit of detection was <600 pg/ml. The multiplexed assay proved to be reproducible as well as specific and sensitive when compared to the standardized ELISA’s. Advantages included a greater dynamic range and simultaneous detection of antibody responses to the four serogroups contained in the tetravalent meningococcal polysaccharide vaccine.
Evaluation of an MGB real time PCR reagent from Nanogen for Herpes simplex virus detection and typing

Stevenson J, Hillyard D

Objectives:

1) Recognize the utility of HSV typing.

2) Become familiar with the challenges involved with real time PCR testing for HSV.

3) Understand the dual probe strategy used in this assay for accurate typing and sensitive detection.

Herpes simplex virus is the most common cause of acquired, sporadic encephalitis in the United States. Due to its specificity, sensitivity and potential for speed, PCR is now accepted as the diagnostic gold standard for the identification of HSV in cerebrospinal fluid. Besides sensitive target detection, this assay provides the ability to distinguish between HSV-1 and HSV-2. A variety of studies indicate that these subtypes display differences in mortality, severity of disease and reactivation frequencies. In spinal fluid, type 2 is typically associated with more serious outcomes in neonates. In the adult population, detection of type 2 in the CSF is associated with benign recurrent aseptic meningitis (Mollaret’s meningitis), while type 1 is the most common cause of Herpes simplex encephalitis, a life threatening disease.

We evaluated a prototype MGB HSV Typing assay from Nanogen that allows simultaneous detection of both HSV Type 1 and 2 viruses in a single sample. Type specific probes, labeled with different fluorescent dyes, are used to detect a common amplicon. Nanogen’s library of modified bases provides the ability to accommodate various sequence polymorphisms in the target sequence. Including primers and probe for detecting an internal control allows the user to monitor sample extraction and PCR inhibition. The post-PCR melt curve is used to identify the HSV type, as well as aiding in the detection of unknown variants. We evaluated this pre-release reagent for specificity, sensitivity and the ability to make the correct typing call.
Rapid phenotyping and genotyping of the CYBB gene associated with x-linked chronic granulomatous disease

Augustine NH, Hill HR, Pryor RJ, Reed G, Tebo A, Bender J, Pasi B, Chinen J, Hanson C, Wittwer CT

Objectives:

1) Describe the clinical features of chronic granulomatous disease (CGD) and the necessity of early diagnosis.

2) Illustrate the high resolution melting analysis and targeted sequencing method for gene scanning in the most common variety of CGD.

3) Summarize the mutations identified in both the classic and variant cases of CGD.

Chronic granulomatous disease (CGD), an inherited disorder of the phagocyte respiratory burst, results in an absence, or deficiency, in the production of oxygen dependent microbicidal agents. Consequently, phagocytes cannot kill a variety of microbes resulting in recurrent, severe infections along with granuloma at the site of infection. Among the four inherited forms of CGD, the X-linked variety due to mutations in the CYBB gene account for 65-70% of cases. Since early confirmatory diagnosis may lead to more appropriate antimicrobial and cytokine administration, we employed flow cytometry followed by high resolution melting analysis and targeted sequencing to rapidly identify, at the molecular level, eighteen patients with X-linked CGD. These included 14 classic cases with no increase in stimulated dihydrorhodamine (DHR) fluorescence, and 4 variant cases with intermediate DHR fluorescence. Mutations in these cases included 6 splice variants, 4 missense substitutions, 3 small frameshift deletions, 2 nonsense substitutions, and three large deletions of multiple exons. Eight were not listed in the CYBB database. In addition, 13 heterozygous female carriers of X-linked CGD (7 classic and 6 variant) were identified. Phenotype screening by flow cytometry, followed by high resolution melting and directed sequencing allowed rapid molecular diagnosis within 24 hours.
An improved flow cytometry staining procedure separates ZAP-70 positive from negative CLL cases

Preobrazhensky SN, Szankasi P, Bahler DW

**Background:** Measurement of zeta associated protein of 70 KD (ZAP-70) in chronic lymphocytic leukemia (CLL) cells has been proven valuable as a surrogate marker of the expressed immunoglobulin heavy chain (VH) mutational status and predicting the clinical aggressiveness of the disease. However, ZAP-70 is a dim, labile, cytoplasmic antigen and its detection with flow cytometric assays is technically difficult because of low staining intensities with the available commercial antibodies. It is currently thought that the distribution of ZAP-70 expression in CLL patients is continuous, and that negative and positive samples could be discriminated only by subjective criteria.

**Objectives:** Since imprecision in methods of ZAP-70 staining and data analysis can cause significant variation in the results and thus hinder the separation of positive and negative samples, we have tried to find out if improving of flow cytometric detection of ZAP-70 could allow better separation between positive and negative CLL samples.

**Methods:** ZAP-70 expression in normal peripheral blood lymphocytes and CLL cells was measured with an Alexa Fluor 488 conjugate of monoclonal anti-ZAP-70 antibody clone 1E7.2 using an optimized staining procedure (Preobrazhensky, Bahler; Cytometry 74B: 118, 2008). The percentage of ZAP-70 positive cells was determined using negative threshold gate markers set up with two different methods: using normal B cells stained with the anti-ZAP-70 antibody, as previously described by Rassenti et al (N Engl J Med; 2004; 351:893) or using CLL cells stained with an isotype control antibody. Alexa Fluor 488 mouse IgG1 and Alexa Fluor 488 anti-human CD13 (both from Caltag, Burlingame, CA) were used as isotype control antibodies. The mutational status of VH genes was determined by DNA sequence analysis of the heavy chain variable region expressed by the leukemia cells. DNA sequences were compared to the closest matching germline VH gene segment and a homology of less than 98% was used as the criterion for the occurrence of somatic hypermutation.

**Results:** Optimal concentration of isotype control antibody was selected by staining normal B and T cells with sequentially diluted antibodies. Significantly higher concentrations of mouse IgG1 isotype antibody compared to anti-CD13 antibody were required to avoid false positive staining of ZAP-70 negative B cells. Using an optimized concentration of anti-CD13 isotype antibody, we have measured the percentage of ZAP-70 positive cells in 60 clinical CLL samples. The results were compared to the percentage of ZAP-70 positive cells detected in the same samples with normal B cell negative threshold gate. Strong positive correlation (R = 0.94) between the results, obtained with different procedures, was found. With normal B cell negative threshold gate, distribution of samples was continuous, while with isotype gating samples were clearly divided in two separate groups with low (< 30%) and high (>60%) percentage of ZAP-70 positive cells. All samples in the group with low percentage of ZAP-70 cells had mutated IgVH genes, and 94% of the samples in the group with high level of ZAP-70 expression were unmutated. These results agree well with the current opinion that CLL cells with unmutated IgVH genes express higher levels of ZAP-70.

**Conclusions:** The results of this study indicate that our improved flow cytometric method of measuring ZAP-70 levels in CLL cells allows clear separation of ZAP-70 positive and negative CLL cases. Moreover, there is an extremely high degree of correlation of ZAP-70 expression levels with the mutational status of the VH genes expressed by the CLL cells.