The San Diego Conference
New Technologies for Molecular Diagnostics
November 8–10, 2001

Poster Session Abstracts

1

Newborn Screening of Galactokinase Deficiency by Arms in Bulgarian Roma Population. Ivo Kremensky,[1] Richard Gitzelmann,[2] Alexey Savov,[1] Luba Kalaydjieva[3] and Maria Markova[4] [1]*Laboratory of Molecular Pathology, Medical University, Sofia, Bulgria;* [2]*Division of Metabolic and Molecular Paediatrics, University Children's Hospital, Zurich, Switzerland;* [3]*Centre for Human Genetics, Edith Cowan University Joondalup Campus, Perth, Australia;* [4]*Clinical Laboratory and Immunology, Medical University, Sofia, Bulgaria.*

Galactokinase deficiency is one of the three inborn errors of metabolism leading to hypergalactosemia. The mode of inheritance is autosomal recessive. The development of early cataracts in homozygous affected infants is fully preventable through early diagnosis by newborn screening and treatment with a galactose–restricted diet. Although the disease is generally considered to be rare there are observations that it is common among Roma (Gypsies). The traditional newborn screening has low efficiency as regards the timely detection of classical galactosemia. The molecular defect leading to the disorder among the Roma is a substitution of threonine for proline at amino acid position 28 (P28T) of the galactokinase enzyme protein. The aim of this study is to introduce a simple, highly sensitive and specific assay for P28T detection. We have examined 4 set of allele specific primers for direct PCR amplification from dried blood spots punched from the Gutrie cards, without prior DNA extraction. After amplification the samples were separated by agarose gel electrophoresis. Out of 1100 unrelated individuals, randomly chosen all over the country we identified 7 heterozygous for P28T individuals. The mutation was confirmed by allele specific restriction. Testing for the mutation will be conducted on newborns of Romani ethnicity only, which according to the observations of the screening laboratory over the last few years are about 7000 –8000 annually.

2

HFE S65C Mutation Analysis in Hereditary Hemochromatosis. Marla Thomas, Jeanette Underhill, Domnita Crisan, and Jeanne Carr, *Clinical Pathology, William Beaumont Hospital, Royal Oak, MI.*

Hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism leading to iron overload in parenchymal organs. It has been associated with two missense mutations: C282Y mutation, accounting for 80-90% of HH chromosomes, and H63D mutation, associated with a milder form of the disease representing 40-70% of non-C282Y HH chromosomes. Recently a S65C missense substitution was reported to be associated with a mild form of hemochromatosis. The purpose of this study was to evaluate the association of HFE S65C mutation and hereditary hemochromatosis. Genotyping for the S65C mutation was performed in 548 patients for whom HH genotype was requested for evaluation of possible HH or due to a family history. The genotypes of these patients are represented in Table 1. The S65C heterozygous mutation was seen in 15 (2.7%) of the patients. Seven of these were heterozygotes for S65C mutation only, three were compound heterozygotes for S65C and C282Y, and five were compound heterozygotes for S65C and H63D. Mild iron overload and increased transferrin saturation were seen in five of these 15 patients (Table 2). One was a compound heterozygous for C282Y and S65C, two were compound heterozygotes for H63D and S65C and two were heterozygous for S65C only. However definite hereditary hemochromatosis was not diagnosed in any of these patients. Four out of these five patients were alcoholics, which may account for mild iron overload in this group. In our study the S65C heterozygous mutation alone or in combination with C282Y or H63D was not associated with significant iron overload and tissue damage diagnostic of hereditary hemocromatosis.

Table 1. Genotype frequencies defined by C282Y, H63D and S65C mutations.

C282Y	H63D	S65C	Frequency
+/+	−/−	−/−	13.5% (74)
−/−	+/+	−/−	2.6% (14)
+/−	+/−	−/−	4.6% (25)
+/−	−/−	−/−	15.2% (83)
−/−	+/−	−/−	18.4% (101)
−/−	−/−	−/−	43% (236)
−/−	−/−	+/−	1.3% (7)
+/−	−/−	+/−	0.5% (3)
−/−	+/−	+/−	0.9% (5)

Table 2. Demographics and iron study of patients with S65C mutation.

Mutation	Age, Mean/Med	Sex M/F	IRON μg/dL	FERRITIN ng/mL Mean/Med	Transferrin % SAT Mean/Med
S65C/WT (n=7)	60/61	4/3	75/76	685/699	44/36
C282Y/S65C (n=3)	50/63	0/3	162/154	197/96	48/49
H63D/S65C (n=5)	61/57	4/1	156/140	1000/599	66/65

3

Fragmentation of P3′-N5′ Phosphoramidate-containing DNA for High-Throughput MALDI-TOF Analysis of Genomic Sequence Polymorphisms. M.F. Denissenko, M.S. Shchepinov, K.J. Smylie, R.J. Wörl, A.L. Leppin, C.R. Cantor, and C.P. Rodi, *Sequenom, Inc. 3595 John Hopkins Court, San Diego 92121, CA.*

Upon the completion of the first draft of the Human Genome, rapid, automated methods of SNP analysis are now in even greater demand for applications in pharmacogenomics, target validation and identification of drug leads, as well as in agriculture, forensics, and environmental work. MALDI-TOF mass-spectrometry (MS) is the method of choice for high-throughput genotyping due to its speed, analytical accuracy and potential to automate. The aim of the present study was developing a novel MS-based procedure for analysis of sequence variations in targets exceeding a hundred nucleotides in length. We used chemical and enzymatic approaches to generate polynucleotide fragments containing acid-labile internucleotide P3′-N5′ phosphoramidate bonds, either in a surface-bound form or in solution. The primer extension reaction utilizing 5′-amino-5′-deoxynucleoside 5′-triphosphates generates polynucleotides that can be fragmented into short, easy-to-analyze pieces simply by being premixed with the acidic matrix typically used for MALDI-TOF mass-spectrometry of nucleic acids. Utilizing this approach, a polymorphic site in the human ADRB3 gene was interrogated. Primer extensions with phosphoramidate analogs of dNTPs allowed for unambiguous discrimination of all possible genotypes. This method has a great potential in high-throughput genotyping due to its simplicity, robustness and compatibility with MS. Complete sequence of the region under interrogation may be obtained by running forward and reverse polymerization reactions followed by fully automated MS analysis on a chip. The possibility of P-N bond incorporation into oligonucleotides attached to a solid support may suggest additional applications. These properties of the P-N bond-based cleavage of DNA are invaluable for high-throughput large-scale diagnostics of polymorphic sequences.

4

Detection of *Chlamydia trachomatis* by Isothermal Ramification Amplification Method. David Y. Zhang,[1] Wandi Zhang, Menashi Cohenford,[2] Brian Lentrichia,[2] Henji Li[1] and Jizu Yi,[1] [1]*Department of Pathology, Mount Sinai School of Medicine, New York, NY and* [2]*Cytyc Corporation, Boxborough, MA.*

Chlamydia trachomatis (CT) is the leading cause of sexually transmitted disease in the US. The recent introduction of liquid-based cytology has made possible the simultaneous screening of cervical intraepithelial lesions and

detection of CT in a single collection vial. In this study we determined if cytological fluid could support DNA-based amplification for the detection of CT. Two methods have been developed: ramification amplification (RAM) and real-time polymerase chain reaction (PCR) with molecular beacon. RAM is a novel, recently induced, isothermal DNA amplification that utilizes a circular probe for target detection and achieves exponential amplification through the mechanism of primer extension, strand displacement and ramification. RAM and real-time PCR were used to detect CT in specimens collected in Cytyc PreservCyt fluid. Our results show that RAM can detect as few as 10 CT elementary bodies in less then 2 hours, comparable to that of real time PCR. Thirty clinical specimens that have been previously tested by Abbott's Ligase chain reaction (LCx) were also tested, 15 were positive by PCR and 14 were positive by RAM. One specimen missed by RAM had an inadequate amount of residual cellular material. Our results show that nucleic acid amplification methods can serve to detect CT and presumably other sexual transmitted agents in cytological fluid and that the RAM assay could be an alternative method to PCR and LCx because of its simplicity and isothermal amplification.

5

Distribution of Hepatitis B Virus Genotypes in Singapore and Its Correlation with Different Mutations in the Core Promoter and Precore Regions. C. P. Ng and E. S. C. Koay, *Molecular Diagnosis Centre, Dept. of Laboratory Medicine, National University Hospital, Singapore.*

Mutations in the basic core promoter (BCP) and precore (PreC) regions have been shown to arise spontaneously during seroconversion from hepatitis B e antigen (HBeAg) to anti-HBe or during interferon therapy. The clinical significance of such mutations was demonstrated when fulminant hepatitis was shown to be induced from primary infection with HBV mutants that do not express HBeAg. The aim of this study was to shed light on the prevalence of such mutants in Singaporean Chinese patients (n=100) who are hepatitis B virus (HBV)-positive and to investigate any possible correlation with their HBV genotype status. The HBV genotypes of these 100 patients, admitted into the National University Hospital, Singapore for HBV-related problems were determined by a nested-PCR method which could detect all 6 genotypes (A to F). Our results showed a predominance (95%) of viral genotypes B and C. This was in accordance with the known geographic distribution of HBV genotypes. Mutations in the BCP and PreC regions were analyzed by sequencing a PCR-amplified fragment encompassing part of the core promoter and precore regions. The mutations studied included the G to A mutation at nt 1896 in the PreC region (A1896) that converts codon 28 for tryptophan to a termination codon, and the double mutations in the BCP region that are associated with reduced synthesis of HBeAg [A to T mutation at nt 1762 (T1762) and G to A mutation at nt 1764 (A1764)]. Our preliminary data indicated that although the incidence of A1896 mutation was comparable between genotypes B and C, the double mutation in the BCP occurred significantly more frequently in genotype C than B.

6

Magnetic Bead Solid-Phase cDNA Synthesis for Microarray Gene Expression Profiling. Serena Pringle, Elsebeth Hoff, Kirsten Lycke, Marie Bosnes, and Dag Lillehaug, *Molecular Systems R&D, Dynal Biotech ASA, Oslo, Norway.*

DNA hybridization arrays are powerful tools in gene expression profiling, allowing investigators to examine changes in the expression levels of thousands of genes simultaneously. Success in this technique is dependent on many factors but the isolation of high quality, non-degraded mRNA is essential for target probe preparation. Traditional methods of synthesizing labeled cDNA target involve isolation of total RNA followed by mRNA enrichment before reverse transcription. These procedures are time consuming and require large quantities of starting material since sample loss occurs at each purification step. Here we describe a fast and simple method for cDNA target probe synthesis by isolating poly A+ RNA directly from cells and tissues. Oligo (dU)25VN covalently coupled to magnetic beads acts as a capture probe for the poly A tail of mRNA. The Oligo (dU) also functions as a primer for reverse transcription therefore cDNA is synthesized directly onto the magnetic beads using standard techniques. All commonly used DNA labels can be incorporated into the cDNA strand during the reverse transcription step. Purification and washing steps are fast and simple due to the paramagnetic properties of the beads, while sample loss is minimal. The labeled single strand cDNA is released from the magnetic bead by a 15-min incubation at 37°C with uracyl-DNA-n- glycosylase (UNG) followed by 10 min at 95°C. The labelled cDNA is then ready for microarray hybridization. Up to 98% of the labelled cDNA is released from the beads.

7

PCR-ICS (PCR-Immunochromatography System): A Novel Detection Method for PCR Amplicon. Yasumasa Mitani and Takanori Oka, *Institute for Medical Research, Wakunaga Pharmaceutical Co., Ltd., Hiroshima, 739-1195, Japan.*

The demand for rapid, reliable, and easy methods to detect a specific sequence has increased, thus we developed a novel method for detecting a target gene and named the method PCR-ICS (PCR-Immunochromatography system). This method is based on immunochromatography, which detects molecules with both biotin and dinitrophenyl (DNP) labels. This detection method is composed of three steps: i) specific amplification of a target gene by PCR with DNP-labeled primers, ii) hybridization of amplified DNA with a biotin-labeled oligonucleotide probe and iii) immunochromatographic detection of hybridized DNA. In this method, PCR is performed with target specific DNP-labeled primers. After PCR, a biotin-labeled oligonucleotide probe, with a complementary sequence to the amplified region is added to the reaction mixture. This reaction mixture is first heat denatured and then kept at 65°C in a thermal cycler allowing hybridization between the PCR product and the biotin labeled probe. This mixture is subsequently applied to an immunochromatographic strip equipped with anti-DNP antibody coated colloidal gold pad and a streptavidin-immobilized line for the detection of the complex between the PCR product and the colloidal gold. After development on the immunochromatographic strip, a red line derived from colloidal gold is observed at the streptavidin-immobilized zone when the complex is formed. In this system, we successfully detected the verocytotoxin gene of verocytotoxin-producing *Escherichia coli* (VTEC) with 10 CFU per tube. The total assay time, including PCR, is less than two hours (1.5 hours for PCR and 15 minutes for immunochromatographic detection). This method has potential for application to the simultaneous detection of multiple targets. Since this method is rapid, sensitive, and does not require any special equipment, besides a thermal cycler, it can be useful not only for routine genetic tests but also for the on-site detection of infectious agents.

8

Use of Polymorphic DNA Sequences as Internal Markers for the Positive Identification of Biological Samples: The Barcode of the Future? A. Haliassos,[1,2] A. Siorfane,[1] N. Drakoulis,[2] [1]*Central Laboratories, Metropolitan Hospital, N. Faliro, Greece and* [2]*Molecular Diagnostics and Biotechnology S.A., Athens, Greece.*

During routine validation of results on our L.I.S. system we where presented with a normal lipoprotein alpha [lp(a)] result (15mg/dL, ref. range: 10-35mg/dl) which was flagged for delta-check variation with a previous sample of the same patient. Indeed, before a year, the same patient had an lp(a) result of 45mg/dL and we know that this parameter, with great genetic predisposition, is not changing during the aging process and is not influenced by any known therapy. We promptly requested a new sample and the measured lp(a) was 43mg/dL, well within the normal variation and the method imprecision. As our hospital uses bar-coded at the bedside primary tubes with serum separating gel (Vacutainer SST, Becton-Dickinson, Plymouth, UK), which are directly analysed for most common parameters [including lp(a)] on automated instruments using the barcode for positive identification of the sample, we assumed that misidentification of the tube happened during the labelling of the first sample by the phlebotomist. We tried to prove our assumption using blood group identification of these two samples, but this was not possible because the blood groups on the two tubes where found identical. As phlebotomists denied our hypothesis, we tried to use DNA typing technique. We extracted the DNA from the two tubes using a suitable method *(1)*. Using our records we found that our patient was enrolled in a research protocol and archived DNA from a previous sample was available. We tested this DNA alongside with the DNA extracted from the above-mentioned two samples for HLA Dqa genotype using the AmpliType Dqa1 Kit (Applied BioSystems Inc., Foster City, CA). The genotype of the archived DNA and of the DNA extracted from the second tube was 1.1/1.2, while the genotype of DNA extracted from the first tube was 1.2/2 and this proved our hypothesis. We hope that in the recent future there will be a possibility of low cost analysis and detection of highly polymorphic DNA sequences probably using microchips, and this will lead to the use of these sequences as internal markers for the positive identification of biological samples. Privacy issues related to the use of such a unique identifying sequence can be surpassed by storing these personal data in a smart card and not in any database, while the transmission of this information to an authorized and approved by the patient information system using public key cryptography (that means that only the system which was trusted by the patient can receive and process the information) can prevent any leak of sensitive information during the transfer and storage of the data. Furthermore, the use of specialized software permits the destruction without any chance of recovery of these data from the hospital database after a pre-approved by the patient-cardholder time period. Both these techniques are already commercially used for the exchange and the storage of sensitive e-mails. We can then expect to find embedded sensors for such DNA sequences in analytical instruments along with classic barcode readers used only for the determination of the sequence of the episode (order) on a given patient, using barcodes containing only time and date information and thus not so important is they are exchanged during the collection of the samples.

References

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9

Stabilization of Gene Expression Profiles in Post-Phlebotomy Whole Blood Using the PAXgene^{TM} Blood RNA System. L. Rainen,[1] C. Ballas,[1] U. Oelmueller,[2] S. Jurgensen,[3] R. Wyrich,[2] J. Schram,[3] M. Walenciak,[1] C. Herd-

man,[3] M. Paumen,[2] N. Nicholls,[4] T. Koga,[4] J. Goodrich,[4] J. Vanderbeek,[4] B. Bankaitis-Davis,[4] V. Tryon,[4] [1]*PreAnalytiX (CH) c/o BD, Franklin Lakes, NJ;* [2]*PreAnalytiX (CH)c/o QIAGEN GmbH, Hilden, Germany;* [3]*BD Technologies, RTP, NC;* [4]*Source Precision Medicine, Boulder, CO.*

A major impediment to accurate analysis of gene expression in whole blood is the post-phlebotomy change in cellular transcript patterns. Nuclease degradation of RNA as well as non-specific gene induction is triggered minutes after blood collection and continues during sample transport and processing. We have developed an evacuated blood collection tube containing a stabilizing additive and a chemically linked companion sample processing system (PAXgene) that: 1) stabilizes cellular RNA in whole blood and, 2) purifies, via spin-column technology, high quality total RNA. We compared gene expression profiles of room temperature stored PAXgene and unpreserved (EDTA) whole blood using quantitative PCR (ABI Prism 7700 TaqMan™) assays for 36 gene transcripts (Source Precision Profile™ for Inflammation) optimized for precision (repeatability: C.V. $<2\%$). RNA was purified from PAXgene and unpreserved EDTA whole blood at the time of phlebotomy (t_o) and 4, 8, 24, 72 and 120 hours post-phlebotomy. Eleven of 36 transcripts were undetectable in either PAXgene or EDTA blood. mRNA expression levels were unchanged in 3 of 25 measurable transcripts in both donors in EDTA blood; the relative amount of each of the remaining 22 transcripts changed by up to 10^4-fold. By contrast, 23 of 25 measurable transcripts were stable in PAXgene blood in one or both donors for 5 days at ambient temperature. The %CV of the PAXgene Blood RNA System for three donors (8 tubes/donor) were 7-24% for total RNA yield and 1% for C_t values in a GAPDH RT PCR assay. The ability to stabilize and purify high quality RNA using the PAXgene Blood RNA System, coupled with Source Precision Medicine's optimized quantitative PCR, enables routine use of gene expression analysis in the evaluation of clinical samples.

10

Simultaneous Detection of Nucleic Acids and Proteins in Biological Samples via Target Labeling by ULS®. Rob van Gijlswijk, Inge Peekel, Judith Bloem, Sandor Snoeijers, Edger Talman, and Rob Heetebrij. *Kreatech Biotechnology BV, Vlierweg 20, 1032 LG Amsterdam, The Netherlands.*

The Universal Linkage System (ULS) has been proven to be the only chemical labeling technology that equals or outperforms conventional enzymatic nucleic acid labeling procedures. The potential of this chemical labeling technology to label and detect both nucleic acid and proteins in complex mixtures like blood, milk and cell lysates was the objective of this study. The ULS, a platinum based labeling reagent containing haptens, fluorophores or enzymes as reporting moieties, reacts predominantly with nitrogen (purine bases, imidazoles, lysines) and sulfur (methionine and cysteine) donor groups within biomolecules. The labeling of these biological relevant molecules is hardly affected by pH variations and salt concentration. We used ULS for the labeling of different proteins in mixtures such as whole serum and cell culture supernatants. We show that all immunoglobulins can be labeled and detected *via* the ULS in whole sera or ammonium precipitates thereof as well as human milk while retaining their immuno-reactivity. Other labeling methods such as maleimides or N-hydroxysuccinimide esters could not be used to label (ammonium sulfate precipitates of) cell culture supernatants. In order to study the labeling and detection of nucleic acids within a native serum sample, whole serum was spiked with a pUC 1.77 DNA probe (for visualization of human satellite III DNA region in chromosome 1) and subsequently labeled by Fluorescein-ULS. Without any purification the whole mixture was split in two and analyzed in a FISH assay on human metaphases for detection of the nucleic acid component and in an immunological assay (ELISA) for detection of immunoglobulin fractions. Both types of biological components could be easily detected. These findings show that the ULS technology is an attractive and unique research instrument for establishing the presence and concentration of biological relevant molecules in non-manipulated biological samples. This will be demonstrated with a range of examples, including 1. Labeling and immunological detection of IgG,IgA,IgM,IgD and IgE in serum by DNP-ULS 2. Labeling of culture supernatant of *Mycobacterium tuberculosis* by Fluorescein-ULS and detection of anti-TB antibodies 3. Labeling of lysed Jurkat cells and detection of mRNA on microarrays.

11

TaqMan Probe Assay for the Detection of West Nile Virus. Jay Ji, Xiuli Chen and Mark Manak, *BBI Biotech Research Lab, 217 Perry Parkway, Gaithersburg, MD 20877.*

With the rapid emergence of West Nile Virus (WNV) in the US, a sensitive, reliable, easy to use nucleic acid based test is needed to accurately screen and confirm actual infection in various biological fluids and tissues. BBI Biotech has developed a RT-PCR-based closed-tube fluorescent assay for the detection of WNV suitable for high throughput viral monitoring and diagnosis. WNV RNA purified from biological samples including mosquito pools, bird, animal and human tissue, CSF or blood specimens is amplified with RT-PCR and quantitated with TaqMan probe specific for WNV. An internal RNA control is designed to simultaneously monitor nucleic acid recovery and RT-PCR inhibitors using the same WNV amplification primer and differentiated with an internal control specific probe labeled with a different fluorophore. Sample preparation was optimized with the Qiagen manual extraction procedure which is adaptable to an automated Qiagen BioRobot for screening and quantifying large numbers of samples within a 4-hour period. Serial dilutions of positive samples along with negative specimens were used to establish the linear quantitative range and an appropriate cut-off level for positives. This method is currently being validated with actual clinical samples and is expected to provide a sensitive and robust screening method for high throughput WNV monitoring and diagnostics.

12

Detection of Single Nucleotide Polymorphisms in Seven Genes Associated with Neuropathy in Diabetic Patients. Brian Dukek, Sherri Longenbach-Huber, Dane Mathiesen, Youvraj Sohni, and Dennis O'Kane, *Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN 55901*

Single nucleotide polymorphisms (SNP's) in multiple genes have been associated with increased frequencies of neuropathy in diabetic patients. In scenarios where more than one SNP can lead to disease it is not adequate to screen only a small proportion of known SNP's. A positive result indicates disease susceptibility, but a negative result shows only that the SNP being screened is not present. The larger the set of SNP's screened the greater the odds of correctly identifying a patient with susceptibility for diabetic neuropathy. In order to screen large numbers of SNP's it is important to use a method that is easy to develop and validate, and has high throughput. We have developed a panel of diabetic neuropathy related assays using an electronically addressed microarray (Nanogen, Inc.). The seven genes screened include aldose reductase, angiotensinogen, apolipoprotein E (ApoE4), beta3-adrenoreceptor, cholesteryl ester transfer protein (CETP), pronatriodilatin, and receptor for advanced glycation end products (RAGE). After initial amplification using a biotinylated primer, the samples were desalted, denatured, and electronically addressed to a 99-pad streptavidin coated microarray. Cy5 and Cy3 labeled polymorphic and normal allelic probes were allowed to hybridize and then discriminated by thermal stringency. The array sites were analyzed using laser-excited two-color fluorescence detection. A 5:1 fluorescence ratio indicated a homozygous result (normal or polymorphic), while a fluorescence ratio of 3:1 or less indicated a heterozygous result. Signals between 5:1 and 3:1 were considered no calls. Two hundred forty-two samples were analyzed from individuals of the Rochester Diabetic Neuropathy Study. Either bi-directional DNA sequencing or a combination of uni-directional DNA sequencing and restriction fragment length polymorphism (RFLP) detection confirmed heterozygous samples. Concordance was 100% between the microarray, DNA sequencing, and RFLP. Several patient samples can be simultaneously analyzed on the microarrays at a single time thereby increasing genotyping throughput.

13

Rapid Isolation of Nucleic Acids by MagaPure$_{EX}$™. D. Nargessi,[1] H. Doan,[1] P. Nguyen,[2] W. McMillan,[2] and J. Ching,[2] [1]*Cortex Biochem Inc., San Leandro, CA;* [2]*Cepheid, Sunnyvale, CA.*

A magnetizable particles-based technology for the isolation of nucleic acids from various sources has been developed, which is simple, fast and cost-effective, does not require an organic solvent, and is readily adaptable to automation. In one example, lysed sample is briefly incubated with the magnetizable particles (MagaPure$_{EX}$ Reagent), followed by two washes with a single Wash Buffer. The DNA is then eluted and is ready for subsequent downstream applications such as PCR, sequencing or blotting procedures. High yield of PCR quality DNA is isolated from various sources including whole blood, buffy coat, cultured cells, plant, and animal tissues. Similarly, high quality plasmid DNA is isolated from bacterial cell lysates. Typically $>85\%$ of highly purified DNA is effectively eluted in a single elution. Greater than 95% of the M13 DNA spiked at various levels into serum is recovered after processing with MagaPure$_{EX}$ DNA. Moreover, adaptability of the technique to automation makes it ideal for high throughput screening (HTS) platforms. Further application of the MagaPure$_{EX}$ technology to the isolation of RNA is also demonstrated. The newly developed MagaPure$_{EX}$ Nucleic Acid Isolation technique overcomes the limitations of the conventional isolation procedures including the use of organic solvents, centrifugation, column separation, vacuum filtration, and overall laborious and lengthy procedures.

14

Microfluidic Chip-based Method for Genotyping Microsatellites, VNTRs, and Insertion/Deletion polymorphisms. Youvraj R. Sohni,[1] James P. Burke,[2] Peter J. Dyck,[3] and Dennis O'Kane,[1] *Departments of* [1]*Laboratory Medicine and Pathology,* [2]*Health Sciences Research, and* [3]*Neurology, Mayo Clinic, Rochester, MN 55905.*

Introduction. A generalized integrated microfluidic chip-based method was developed to genotype microsatellites, variable number tandem repeats (VNTRs) and insertion/deletion polymorphisms. Three types of polymorphisms associated with diabetic complications were analyzed: i. a highly polymorphic pentanucleotide repeat region within the 5′-upstream promoter region of the human inducible nitric oxide synthase gene (iNOS5); ii. a biallelic VNTR region within endothelial nitric oxide gene (eNOS27); iii. a 287 bp insertion

/deletion polymorphism within angiotensin-converting enzyme gene (ACE/ID). **Methods**. Following separate PCRs for iNOS5, eNOS27 and ACE/ID, amplicons were analyzed on an Agilent 2100 Bioanalyzer using DNA 500 LabChip® kit. Gel-dye mix, markers, a size ladder, and 1 μL of test sample were loaded on a chip for fluorescent detection of DNA fragments following electrokinetic migration through microchannels. Markers bracketed the sizing range and served to align ladder and samples. Genotypes were assigned based on fragment sizes determined from a calibration curve. **Results.** For iNOS5 (n=230), the number of pentanucleotide repeats varied between 7 and 13 with 95 (41%) of the amplicons homozygous and 135 (59%) heterozygous for the repeats. For eNOS27, 173 (75%) amplicons were homozygous for the 4b allele containing five 27-bp repeats with none of the amplicons homozygous for 4a allele containing four repeats and 57 (25%) were heterozygous. For ACE/ID, 47 (20%) amplicons were homozygous for the insertion, 65 (28%) were homozygous for the deletion and 118 (51%) were heterozygous. **Conclusion**. The microfluidic chip-based system described here outperforms conventional technologies and can be used for both large- and small-scale genotyping studies of microsatellites, VNTRs and insertion/deletion polymorphisms. The method is rapid, accurate, and sensitive, and consumes small sample and reagent volumes. DNA fragments resolve clearly with a clean banding pattern. This technology improves genotyping throughput, generating high quality data.

15

M235T and T174M Variants of Angiotensinogen Gene, Coronary Heart Disease, and Hypertension in Indian Population. T.F. Ashavaid, K.K. Shalia, K.G. Nair, and J.J. Dalal, *P.D. Hinduja National Hospital & Medical Research Centre, Mumbai, India.*

Genes encoding components of renin angiotensin system have been implicated with increased risk of cardiovascular disease. M235T and T174M variants of the angiotensinogen; primary substrate of this system were initially reported to increase the risk of hypertension and subsequently of coronary heart disease (CHD). In the present study, we have examined the association of these two polymorphisms with hypertension and CHD and their synergistic interaction with D allele of the ACE gene. We studied 131 healthy individuals, 141 angiographically verified CHD patients and 159 hypertensive subjects. Within the control group the frequencies of the M235M, M235T and T235T variants were 8.30%, 30.53% and 61.16% respectively while that of T174T, T174M and M174M genotypes were 77.86%, 20.60% and 1.53% respectively. There was no significant difference in the distribution of the M235T and T174M variants between the two-test groups and control group. The risk was not restricted to subjects carrying D allele of ACE gene. Association was also not seen when analysis was carried out in-patients when sub-grouped according to the extent of the severity of the disease. M235T and T174M variants do not contribute to the increased risk of CHD or hypertension in Indian population.

16

Ultrarapid Detection of Gene Expression in MRSA and *M. tuberculosis* by Real-Time Fluorescence Monitoring of Isothermal RNA Amplification with INAF DNA Probe. Kiyoshi Yasukawa and Takahiko Ishiguro, *Tokyo Research Laboratories, Tosoh Corporation, Ayase-shi, Kanagawa 252-1123, Japan.*

Homogeneous detection and isothermal sequence amplification are key technologies for large-scale screenings and fully automation in upcoming clinical settings. We established a new method to realize ultra rapid and direct detection of pathogenic target RNA sequences in highly sensitivity, which relies on real-time monitoring of isothermal RNA amplification by a novel fluorescent DNA probe, named 'INAF probe'. We demonstrated the homogeneous detection of gene expression of *mecA* in methicillin resistant *Staphylococcus* and *Pab*, protein antigen b in *Mycobacterium tuberculosis*. The mRNA was amplified isothermally (at 41°C) by the cooperative two-enzyme feed-back reaction (named 'TRC reaction'): *in vitro* transcription by T7 RNA polymerase and the conversion of the product, RNA, into the promoter bearing double stranded DNA by AMV RTase. The reaction was carried out in the presence of oxazole yellow - linked INAF probe, which binds to the RNA produced in the reaction to give rise to the fluorescence enhancement. The fluorescence intensity (ex: 470 nm, em: 520 nm) was monitored in the course of the reaction. Even in case of the starting mRNA as low as 10 copies, the fluorescence intensity was markedly enhanced in 10 minute for *mecA* and 12 minute for *Pab*. The increase of the fluorescence intensity depended on starting RNA copies ranging from 10 to 10^8 copies, which enabled quantification of the target RNA in a sample in highly accuracy without any post-amplification analysis.

17

Bacterial Expression of *in Vivo* Biotinylated Aequorin and Application to Hybridization Assays. M. E. Verhaegen[1] and T. K. Christopoulos,[2] [1]*University of Windsor, Windsor, ON, Canada and* [2]*University of Patra, Patra, Greece.*

We have constructed a plasmid suitable for bacterial expression of the *in vivo* biotinylated aequorin photoprotein. The biotin tag facilitated (a) the purification of aequorin from the crude cell extract and (b) the direct complexation of aequorin with streptavidin for utilization as a reporter molecule in the development of highly sensitive hybridization assays, thereby avoiding the need for chemical crosslinking. The plasmid contained the biotin-acceptor coding sequence from *Proprionibacterium shermanii* transcarboxylase fused in frame with the amino terminus of the apoaequorin-coding sequence. The birA gene, encoding biotin protein ligase (BPL), was amplified from *E. coli* genomic DNA and inserted downstream of the apoaequorin sequence. Thus, the plasmid overexpressed both a biotin acceptor peptide-apoaequorin fusion protein and the BPL, which post-translationally biotinylated the acceptor domain at a unique position. Functional aequorin was generated by incubating the crude cell lysate with coelenterazine, followed by isolation using a monomeric avidin column that allowed elution with free biotin under nondenaturing conditions. A single band was observed in SDS-PAGE, corresponding to the expected molecular mass of the fusion protein. A typical yield of biotinylated aequorin was 1.0-1.2 mg per liter of culture. The purified biotinylated aequorin was complexed with streptavidin and used as a reporter molecule in a hybridization assay. The assay entailed immobilization of an oligonucleotide probe on microtiter wells via a digoxigenin/ antidigoxigenin interaction followed by hybridization with a denatured DNA target labeled with biotin through polymerase chain reaction. The streptavidin-biotinylated aequorin complex was used to quantify the hybrids. Luminescence was measured in the presence of excess Ca^{2+}. The linearity of the hybridization assay extended from 78 amol of target DNA per well (with a S/B ratio of 2.1) to 40 fmol per well. Typical CVs obtained over the range of the assay were 6%.

18

RDB Flow Through Hybridization for HLA–DP, DR and DQ Genotyping Analysis. J. W. O Tam and S. S. Xie, *Department of Biochemistry, The University of Hong Kong, Hong Kong S.A.R.*

Our patented Direct Flow-through DNA Hybridization is the fastest annealing process that uses a very inexpensive device for accurate mutation detection, genotyping and fingerprinting analysis. We like to present here the data of analyzing the HLA loci of DP, DR and DQ beta sequences by ASO oligo-probes using the Flow-through format. DNA sequencing is considered the method of choice for accurate genotyping the HLA cluster. Unfortunately, because of the existence of highly homologous sequence of pseudgens that may be co-amplified during the PCR amplification process, accurate genotyping by DNA sequencing alone may prove more difficult and costly. Our preliminary results suggested that the ASO direct Flow-through Hybridization is a good alternative. Using one pair of PCR primer and 35 ASO oligo-probes, we can effectively classify the 83 DPB_1 alleles identified by the WHO four digit codes. Similarly, using one common PCR primer pair, and 18 ASO oligo-probes each, this simple hybridization protocol can identify the first 2 digit codes of the specific genotypes of the DR and DQ beta loci, enough to distinguish major classes of the HLA. However, when we use the same PCR primer pairs to perform the direct sequencing on the DR and DQ loci, un-interpretable sequencing data occurred frequently because of the co-amplification of pseudogen fragments. Hence, when we tried to confirm our ASO data (all samples were confirmed) we have to create many specific sets of PCR primers for which PCR amplification done in separate reactions. The positive amplicon(s) were then sequenced. This is why direct sequencing may prove to be costly. Although further classification of the DR, DQ subtypes requires additional oligo-probes using the Direct Flow-through method, the number of such oligo-probes are well within the capability of the present format. This alternative method for HLA typing is faster, simpler, requires no expensive equipment and therefore much less costly compared to DNA sequencing. (This investigation was supported by ITF grant of the Hong Kong Industry Department No. AF/204/99)

19

Tessera Array Technology: A Rapid, Specific Detection of *Mycobacterium tuberculosis* Using Nucleic Acid Probe. Yeasing Yang,[1] Polina Zaslavskaya,[1] Ramesh Bharadwaj,[1] Ken Fu,[1] Whei-Kuo Wu,[1] Nanibhushan Dattagupta,[1] I-shiou Huang,[2] Allen Huang,[2] Yu-Cheng Wu,[2] [1]*Applied Gene Technologies Inc., 6190 Cornerstone ct. East, Suite 101, San Diego, CA 92121, and* [2]*AsiaGen Corporation, Fl.4, No3, Nan-Ke 3rd. Rd., Tainan Science-Based Industrial Park, Hsin-Shi 744, Tainan County, Taiwan, R. O. C.*

Tessera Array Technology (TAT) is a simple, sensitive and cost effective method for the rapid identification of infectious agents in clinical samples by nucleic acid hybridization. In the TAT assay procedure, nucleic acids in the sample are labeled with signature compounds. The signature compounds are made of nucleic acid binding ligands, binding enhancers and detection moieties. The compounds are photoactivable to form covalent bonds between nucleic acids and ligands. The labeled sample is hybridized with immobilized probes. The label associated with the signature compound detects the hybrid. We describe an application of TAT assay system to detect *Mycobacterium tuberculosis* that is a better alternative to the commercial tests, which are complex and not cost effective. *Mycobacterium tuberculosis* (Mtb) is one of the leading infectious diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually. Detection of this organism takes several weeks or longer due to slow growth of Mtb. It is important to diagnose Mtb as quickly as possible, since *M. tuberculosis* is highly contagious. The specificity and sensitivity of the TAT assay allows the detection of Mtb

while differentiating it from *M. chelonae, M. sacrofulaceum, M. fortuitum, M. avium*, and *M. intracellulare* in clinical samples. Data for the specificity of different molecular probes will be presented. In summary, TAT provides a rapid, specific and cost-effective detection of *Mycobacterium tuberculosis.*

20

Design and Development of an Ashkenazi Jewish Mutation Panel Using MALDI-TOF Mass Spectrometer. H. Khoja,[1] J. Nagashima,[1] G. Giannoukos,[1] J. Leushner,[1] C. Oddoux,[2] and H. Ostrer,[2] M. McGinniss,[1] [1]*Sequenom Inc., 3595 John Hopkins Court, San Diego, CA 92121;* [2]*Human Genetics Program, NYU School of Medicine, 550 First Avenue, New York, NY 10016*

High carrier frequency genetic diseases such as Tay-Sachs, Cystic Fibrosis, Canavan disease, Gaucher disease, Niemann Pick, Familial hyperinsulinism, Mucolipidosis type IV, Familial Dysautonomia, Fanconi Anemia, and Bloom syndrome are well documented in the Ashkenazi Jewish population which comprises some 90% of American Jews. We have developed a modular, highly accurate, and high-throughput genotype screening assay panel for all these diseases using Sequenom's MassARRAY™ system that utilizes matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) for automated analysis and genotyping. Genotyping in the MassARRAY™ system is carried out by primer extension reactions in which a short gene-specific primer is annealed to the template (in most cases a PCR product) adjacent to the mutation. The primer is then extended by several bases across the mutation. The extension products are then deposited on the matrix spot of a spectroCHIP™ MS chip for high throughput analysis using a MALDI-TOF-MS. Since MS analyses directly measure the mass of the extension products, it is far more accurate, informative and amenable to high throughput automated genotyping and analysis than the indirect gel, hybridization, or fluorescent-based assays. Our MALDI-TOF-MS based panel is designed to test for 26 mutations of these 10 genetic diseases in a 5ul multiplex PCR reaction, followed by Sequenom's proprietary extension reaction optimized for 384 well format. The modular design of our panel allows the user to exclude one or more of the disease specific assays from the panel without adversely affecting the format or throughput. The user is also provided the freedom of easily repeating only the failed assays instead of repeating the entire panel. Our data, to date, on 96 wild-type controls, and 15 positive controls from Coriell Cell Repositories, and other sources, consistently indicate 100% genotyping accuracy, and greater than 96% assay success on first pass.

21

Evaluation of the APTIMA Combo 2 Assay for Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae.* Beatrisa Boyadzhyan, Tatyana Yashina, Jo Ann H.Yatabe, and Meeta Patnaik, *Specialty Laboratories, Inc., Santa Monica, CA.*

Introduction: *Chlamydia trachomatis and Neisseria gonorrhoeae* are two of the most prevalent sexually transmitted pathogens worldwide. Recently, nucleic acid amplification (NAA) tests have become widely used for their greater sensitivity and accuracy and have improved the ability to detect *C.trachomatis* and *N.gonorrhoeae* infections. **Objectives:** The purpose of this study was to evaluate the performance of APTIMA Combo 2 Assay for *C.trachomatis* and *N.gonorrhoeae* detection in urethral and endocervical swabs and urine specimens from both sexes and compare that with currently available assays. **Materials and Methods:** The GEN-PROBE APTIMA Combo 2 Assay is a second generation of NAA test that utilizes Transcription-Mediated Amplification (TMA) technology for the *in vitro* simultaneous qualitative detection and differentiation of ribosomal RNA (rRNA) from *C. trachomatis* and/or *N.gonorrhoeae*. An Automated Liquid-Handling System (Tecan Genesis RSP 150) was used as automated pipettor. The performance of Combo 2 Assay was evaluated with a total of 327 urine and 212 swab specimens for *C. trachomatis* and *N.gonorrhoeae* received for analysis by different methods in our laboratory, including Cell Culture, Direct Fluorescent Antibody (DFA) and LCR Amplification Assays. **Results:** The comparison of Combo 2 and LCR showed that out of 320 urine specimens tested for *C.trachomatis* 29 (9.1%) were positive for both assays; however, Combo 2 detected 6 (1.9%) more positives. Out of 327 urine specimens tested for *N.gonorrhoeae*, 6 (1.8%) were found positive by both methods and Combo 2 detected 2 (0.6%) more positives. A good correlation was found between TMA and LCR methods tested on swab specimens, 4/63 (6.4%) and 2/62 (3.2%) were detected positive by both methods for *C. trachomatis* and *N.gonorrhoeae*, respectively. The comparison of Combo 2 with Cell Culture for *C. trachomatis* was done on 64 swab specimens. Six (9.4%) were positive by both methods, and additional 7(10.9%) specimens were detected positive by Combo 2 assay. Out of 68 swab specimens tested for *N.gonorrhoeae* by TMA and Cell Culture, 1 (1.5%) was positive by both methods, and 3(4.4%) more positives were detected by TMA only. Combo 2 assay was compared also with DFA method for *C. trachomatis* on 81 swab specimens, where 3 (3.7%) positives where detected by Combo 2 which were missed by DFA. Intra- and inter-assay study of TMA method demonstrated good reproducibility for both pathogens. **Conclusion:** Thus, we found out that APTIMA Combo 2 Assay for simultaneous detection of *C.trachomatis* and *N.gonorrhoeae* is more sensitive then cell culture and DFA methods for swab specimens, has similar sensitivity as LCR for swab, however it is more sensitive then LCR for urine specimen. In addition, it is robust, easy to perform, and overall more sensitive then other commercially available methods for the detection of *C.trachomatis* and *N.gonorrhoeae*.

22

The Use of Novel HP^2 Capture Probes to Increase Specificity of DNA Hybridization. Susan Bortolin, Roman L. Zastawny, and Richard A. Janeczko, *Tm Bioscience Corporation, 439 University Avenue, Toronto, ON, M5G 1Y8 Canada.*

Precise control of DNA hybridization is the key step in developing any successful nucleic acid-based test. Here we demonstrate the utility of HP^2 capture probes in discriminating closely related sequences. HP^2 probes are oligonucleotide probes composed of a highly stable intramolecular duplex stem-loop structure adjacent to a specific target capture region. This capture region is capable of folding back onto itself forming a second less stable duplex. The less stable duplex functions as the thermodynamic energy barrier that must be overcome for hybrid formation to occur. This structure may also be imposed on conventional linear probes. To demonstrate the discriminatory power of HP^2 in multiplexed systems where the likelihood of false positives is high, a model system was designed whereby a 24mer capture sequence was hybridized to its perfectly matched target and to 13 different mismatched targets designed to have at least 66.7% homology. Hybrid formation was monitored using indirect chemiluminescence. If an arbitrary cut-off signal equal to 10% that generated by a perfectly matched hybrid is set as the maximum allowable limit required for discrimination, then HP^2 effectively discriminated 10 of the 13 mismatched targets whereas the HP control probe lacking the second duplex was unable to discriminate any of the 13 mismatches. The same sequences were then hybridized to a linear looped capture probe and compared to its linear control probe. The linear probe effectively discriminated 3 of the 13 mismatched targets while the looped structure discriminated all 13 mismatched targets. The results clearly demonstrate the exquisite power of HP^2 and linear looped probes in increasing the specificity of hybridization. As the complexity of DNA detection increases, controlling mismatched hybridization events inherent to all highly multiplexed systems is critical for accurate data interpretation. HP^2 and linear looped probes will prove especially useful in addressing this issue.

23

Accelerated DNA Hybridization and Mutation Analysis in Low-Conductivity Environments. C. Gurtner, E. Tu, N. Jamshidi, T. Reyes, R. Haigis, S. Duffy, C.F. Edman, T. Onofrey, R.B. Wallace, and M.J. Heller, *Nanogen Inc, San Diego, CA.*

We describe a convenient and rapid research method for mutation analysis of amplified DNA based on electrophoretic transport and concentration of DNA in a non-hybridizing low-conductance environment. In this method, PCR amplified material is chemically or thermally denatured and directly applied to a disposable 96-well or 384-well plate that contains a streptavidin-loaded hydrogel layer. The hydrogel layer at the bottom of the plate serves multiple purposes as concentration, separation and hybridization medium. The well plate itself is located in a simple electrophoresis system that is filled with a non-hybridizing low-conductance buffer such as L-histidine. By application of a voltage across the electrophoresis system DNA samples are quantitatively transported to the hydrogel and the amplified sequences of interest are retained at the surface of the gel by a biotin/streptavidin interaction. Unbiotinylated complementary strands and other components of the amplification reaction are physically removed from the sequence of interest by simple electrophoretic action or dilution. For analysis, fluorescence labeled reporter probes are electrophoretically transported to the sequence of interest and hybridized by introduction of salt by one of several methods. A higher voltage is then applied to create stringent conditions that remove mismatched reporter probes to allow for single nucleotide point mutation (SNP) discrimination. Fluorescence signals are detected in a standard fluorescence plate reader. In the research setting, we have successfully applied this method to the analysis of a series of SNPs including EH-1, Factor V, Factor II, IL-4, and hemachromatosis. A blind research study performed on 60 Factor V patient samples was in 100% accordance with results obtained from a competitive technology. This electric-field-driven assay can be easily integrated with standard pipetting and imaging tools and requires less than half an hour post-PCR assay time.

24

Clinical Sample Preparation Using Pressure Cycling Technology Mark M. Manak, Chunqin Li, C. C. Tai, Nathan P. Lawrence, Richard T. Schumacher, and Feng Tao, *Boston Biomedica, Inc., 217 Perry Parkway, Gaithersburg, MD 20877.*

A novel sample preparation system has been developed based on cycled pressure (Pressure Cycling Technology, or PCT), using an instrument, Barocycler™, for alternately generating high and low pressure (≥ 1 atm) environments and disposable devices, PULSE™ tubes, for extracting biomolecules. The PULSE™ tubes may serve as storage tubes at –70 °C for both raw samples prior to processing or processed samples with nucleic acids and proteins available for downstream applications. The PCT system can achieve high-level release of DNA, RNA and proteins from a variety of biological samples

including 'hard-to-lyse' specimens, such as mycobacteria, yeast, and tissue from animals and plants. In many cases, the homogenization and release of biomolecules is completed with 5 cycles of pressure within 5-10 minutes. For clinical applications, this system can be used to release nucleic acids from pathogens or from tumor tissue for analysis. In some cases, crude lysate can be used directly for amplification and detection without a requirement for additional purification steps. Our data indicate that PCT can release reverse transcriptase (RT) from HIV virus in its cell culture without addition of any chemicals. The RT activities recovered in PCT treated samples are equivalent to or higher than those from common cell lysis procedures. In general, PCT sample preparation may be used to replace current mortar and pestle or sonication procedures for extraction of biomolecules. The PCT sample preparation method is rapid and efficient, simple-to-use, self-contained, compatible with existing purification methods and assays, and adaptable to high volume processing.

25

Rapid, Automatable Genotyping Using Chemically Labile Nucleotides and MALDI-TOF Mass Spectrometry. Martin Zillmann, Jia Wolfe, Tomohiko Kawate, Jeff Olson, David Sarracino, Valerie Stone, and Lyne Breault, *Variagenics, Inc., 60 Hamshire Street, Cambridge, MA 02139-1548, www.variagenics.com.*

A simple, 4-step procedure will be described by which: (1) novel base analogs are directly incorporated during amplification of sample, (2) amplicons are chemically cleaved at sites of analog incorporation to produce analyzable fragments, (3) cations are removed by ion-pair chromatography, and (4) samples are analyzed by MALDI-TOF mass spectrometry. The novel, base analogs (7-deaza, 7-nitro-dATP, 5-hydroxy-dCTP, 7-deaza, 7-nitro-dGTP, and 5-amino-dUTP) have been synthesized and are efficiently utilized by several thermostable DNA polymerases, including Pfu DNA polymerase. In the presence of analog and the complement of natural triphosphates, analog is incorporated throughout the amplicon, replacing its natural counterpart. After amplification, amplicon is fragmented by successive treatment with an oxidizing agent and organic base. The resulting fragments are prepared for analysis by MALDI-TOF mass spectrometry using a commercially available, 384-well format solid-phase extraction plate and ion-pairing agents. Samples are dissolved in MALDI matrix and spotted onto a hydrophobic anchor target. Automated analysis can generally be accomplished in less than 15 seconds per sample. The entire protocol for 384 samples can readily be performed in an 8 hour workday, either manually or robotically. Several strategies are available for assay design, increasing the flexibility of the technique and allowing the generation and analysis of multiplexed samples. Assays can be designed to either produce diagnostic fragments with only mass difference, or fragments with both length and mass difference. In addition, other fragments produced by the cleavage reaction can be used to confirm that the desired region is being genotyped. The quality of mass spectra can be improved dramatically by decreasing the length of the amplicon or the size and dispersion of the diagnostic fragments.

26

Eight Color System for Detection of Highly Multiplexed PCR Reactions. Gregory Kintz, Lee Christel, Linda Thompkins, Martin Jones, and Bill McMillian, *Cepheid Inc, Sunnyvale, CA*

A new prototype system for the detection of highly multiplexed real time fluorescence PCR reactions has been demonstrated. The key innovation in this design is an optical system capable of detecting eight different fluorescent dyes. These dyes, when coupled by conventional means to specific nucleic acid hybridization probes, will allow the detection of up to 8 discrete target DNA sequences. This capability will be useful in diagnostic applications such as SNP detection in cancer and antibiotic resistance mutations in bacteria. The commercially available SmartCycler™ rapid thermal cycling system has the ability to detect four different targets in a single real time fluorescence PCR reaction. The new system is based on several proven and robust technologies used in the SmartCycler™. The reaction tube and heating and cooling system are identical to the ones used in the SmartCycler™. The optical system was redesigned using a collection of eight LEDs with wavelengths spanning the spectral region from 380 nm to 700 nm was used as excitation sources for the target dyes. Dielectric coated filters produced a narrow excitation wavelength centered on the absorption peak of the dyes. Detectors filtered with dielectric coatings are used to detect the fluorescence signal. Dyes were selected from commercially available sources based on their spectral separation from the UV to the near IR and their compatibility with chemical coupling methods. The dyes tested in the current system include Pacific Blue, Coumarin, FAM, Alexa 532, Alexa 546, Texas Red, Alexa 633 and Alexa 660. The detection levels are typically about 1 nM for the listed dyes. The deconvolution of the raw signal data is required to determine the actual signal levels from the target dyes. The cross talk from adjacent dye channels is characterized as a function of the relative brightness on the target dyes. Typical cross talk values are less than 10% from adjacent channels. The new system demonstrates the ability to accurately measure more targets in a single highly multiplexed reaction.

27

The Combination of Rolling Circle Amplification and Differentially Labeled Microspheres Enables the Development of Ultrasensitive Multiplexed Immunoassays. M.C. Mullenix, X. Feng, R. Sivakamasundari, R. M. Krishna, and S.P. Piccoli, *Molecular Staging Inc.*

Microspheres, also known as latex beads, polystyrene beads and microbeads are a proven solid phase immunoassay format used in both research and clinical diagnostics. They serve as a solid phase onto which antibodies, antigens or DNA probes are immobilized. Though technically a solid phase, microsphere based systems offer near fluid phase binding kinetics and improved sensitivity over traditional microtiter plate format assays. Fluorescent microspheres are prepared by incorporating fluorescent dyes during the manufacturing process. The process can be controlled to provide microspheres with very specific fluorescence intensities. When two fluorescent dyes are mixed during the manufacturing process a large number of specific ratios of fluorescence intensities can be produced. The various fluorescence intensities and intensity ratios can easily be differentiated using a flow cytometer. Separate immunoassays can be developed on different types of fluorescent microspheres and then combined to provide multiplex assays. Several commercial assays are available in this format. In Rolling Circle Amplification (RCA) based immunoassays, a concatamer of single stranded DNA is produced which remains covalently attached to the antigen-antibody complex. The covalent attachment makes RCA an ideal detection system for use in flow cytometry based fluorescent microsphere assays. To establish the compatibility of RCA with multiplexed fluorescent microsphere format assays, commercial microsphere based test kits were purchased and modified to incorporate various RCA based detection systems. The RCA amplification had no effect on the ability of the flow cytometer to classify the microspheres on the basis of internal fluorescence characteristics. RCA consistently increased the sensitivity of numerous cytokine immunoassays by 100 fold, frequently detecting 0.1 pg/mL. To demonstrate multiplexing in the RCA amplified microsphere assay four cytokines were specifically detected simultaneously using a mixture of separately optimized microsphere assays. Our results demonstrate that incorporation of Rolling Circle Amplification in flow cytometer based microsphere immunoassays greatly improves assay sensitivity and maintains specificity in multiplex assays.

28

Functional Surface Coatings with Low Nonspecific Binding Properties. S. W. Metzger and M. J. Lochhead, *Accelr8 Technology Corporation*

Solid phase molecular diagnostic techniques require exquisite control of biomolecular interactions with surfaces. Specificity, signal to noise ratios and detection limits of these analytical systems, including nucleic acid and protein-based diagnostics, are limited by surface non-specific binding. Inhibition of non-specific binding is thus a critical performance feature in the design of improved synthetic materials that contact and operate in biological fluids. While non-specific binding to surfaces is most often undesired, *specific* biomolecule, particle or cell binding at surfaces often *is* desired. The goal is to bind only one type of molecule, particle, or cell, and to do so in a manner that preserves its recognition activity and native structure. We have developed OptiChem™, a ligand-conjugated surface coating that can be applied to virtually all materials commonly used in bio-analytical devices including plastics, glass and metals. OptiChem™ coated substrate has significantly lower non-specific binding than conventional BSA blocked surfaces. Furthermore, non-specific surface binding is virtually eliminated under high loads of up to 10 μg/ml of total soluble protein. We have used the well-known streptavidin-biotin interaction as a model receptor-ligand pair to demonstrate the specific binding capabilities of the OptiChem™ coating. The surface attains a highly reproducible streptavidin capture agent surface density through simple bulk dilution methods and the biotin functionalized OptiChem™ selectively binds streptavidin with up to 100 fold better efficiency than traditional adsorptive chemistries. Reduction of non-specific binding and control of biotinylated receptor density translates into increased signal to noise ratios, thus improving upon conventional surface chemistries resulting in faster assay turnaround and lower consumption of valuable or rare samples. The OptiChem™ coating eliminates the need for lengthy adsorption times, blocking steps, or incubations at elevated temperatures increasing assay throughput and assay flexibility. The capacity for coating various substrates, ready scale-up of convenient fabrication and low preparation costs make the surface chemistry ideal for many types of solid phase molecular diagnostic tools.

29

Apparatus for Sample Preparation of RNA from Biological Fluids for RT-PCR Amplification and Detection. Jesus Ching,[1] Peter Nguyen,[1] Kendra Paul,[1] Ron Chang,[1] Dokhi Nargessi,[2] Matt Pourfarzaneh,[2] and William McMillan,[1] [1]*Cepheid, 1190 Borregas Ave., Sunnyvale, CA 94089;* [2]*Cortex Biochem, 1933 Davis Street, San Leandro, CA 94577.*

An apparatus for preparing RNA from biological fluids for RT-PCR amplification and detection is presented. The major components of the apparatus consisted of an injection molded container (50 μL) for retaining beads and machined acrylic chambers for containing buffer and wash solutions. A

heating block with a controller was coupled to the container holding the beads. The range of input sample volume for the device was 50 μL to 1 mL. When assembled, the apparatus was a manually operated fluidic cartridge for sample preparation of RNA for analysis. A solid phase material from Cortex Biochem was designed specifically for binding RNA. The sample containing RNA was mixed with binding buffer and flowed through the beads inside the cartridge. Next, the beads with the bound RNA was rinsed with a wash buffer. Compared to other nucleic acid isolation strategies, no alcohol or organic solvents were used in this protocol. After washing, the bound RNA was eluted with buffer. Heat was evaluated as a means to denature the RNA for elution. The isolated RNA was subjected to reverse transcription and real-time PCR using the Smart Cycler®. Model systems for evaluating RNA recovery on the benchtop system were viral RNA (i.e., MS2) in white blood cell lysate and colon total RNA in buffer. The amount of recovered RNA was calculated from a standard curve of known RNA standards after RT-PCR. The percent recovery of MS2 from white blood cell lysate was 31%. The percent recovery of colon total RNA from buffer was approximately 90-100%. The elution of RNA with heat gave a smaller elution volume than without heat. The described apparatus isolated RNA in 6-8 minutes from various fluids without organic solvents, alcohol, or centrifugation. The RNA isolation apparatus demonstrates potential for application in molecular diagnostics.

30

TPMT (Thiopurine Methyltransferase) Genotype-Phenotype Correlation in IBD Patients. Ellen Taylor, Mariko Matsutani, Richard Bogardt, E. Robert Wassman, *Prometheus Laboratories, Inc., San Diego, CA.*

Relevance: Thiopurine methyltransferase (TPMT) metabolizes 6-mercaptopurine (6-MP) and its pro-drug, azathioprine (AZA). TPMT genotyping and monitoring of the phenotype through enzymatic and metabolite assays are a valuable application of clinical pharmacogenomics both to avoid toxicity and achieve optimal therapeutic response. **Objective:** Our objective was to identify specific factors unique to a clinical setting leading to genotype/phenotype variability in the pharmacogenomic evaluation of the TPMT status of patients. **Methodology:** WBC DNA is specifically amplified by PCR for TPMT alleles, restriction digested with Bsl-1, Mwo-1, and Acc-1, and separated by electrophoresis. RBC enzyme activity is assayed for incorporation of exogenous methyl groups into thiopurines by HPLC with fluorescence detection. **Results:** The distribution of genotypes is consistent with prior population studies, and between cases genotyped (N=3566) only, or genotyped and phenotyped (N=219).

Table 1. Distribution of genotypes/phenotypes.

Normal activity	*1/*1	(>23.6 EU)	89.0%
Intermediate activity	*1/*2	(6.7-23.6EU)	1.4%
	*1/*3A		6.4%
	*1/*3C		1.4%
Low activity	*3A/*3A	(<6.7 EU)	1.8%

In 208 of the cases, we observed good correlation between the genotype and the phenotype. In 11 of the cases, we observed variability between the genotype and the phenotype. Four cases were homozygous or heterozygous "deficient" genotypes with higher than expected enzyme levels. The remainder were *1/*1 homozygotes with intermediate enzyme levels. **Discussion:** In a clinical setting, significant value is obtained by the combined use of genotype, phenotype, and metabolite levels in pharmacogenomic optimization of patient therapy. Our reference lab observed 95% correlation between the TPMT genotype and phenotype. Both physiological and pre-analytical factors contribute to the variability demonstrated in a clinical setting versus a research setting. The physiological factors include intrinsic and extrinsic activators and inhibitors as well as differential expression of heterozygous alleles. The preanalytical variables include prior transfusion and specimen stability. We believe the preanalytical factors deserve further investigation in order to determine whether they are a more significant cause of variability between TPMT genotype and phenotype in the clinical setting.

31

Genotyping Assay Transfer to Pyrosequencing: What We Learned Along the Way. Maria C. Gentile Davey, Steven C. Cassar, and David R. Grimm, *Abbott Laboratories, Abbott Park, IL*

Pharmacogenetics (PG) is the study of the role that genetic variations play in an individual's response to drug treatment. When genetic variations (or polymorphisms) are present in drug metabolism or transport genes, they can influence how a patient responds to a drug. To date, 32 assays have been developed in the Abbott PG Laboratory that detect polymorphisms in the genes of several families of drug metabolizing enzymes (DMEs) linked to variability of drug response. These assays were originally based on AS-PCR, PCR-RFLP, TaqMan or long PCR. We recently purchased a PSQ 96 instrument from Pyrosequencing and we have initiated the process of converting our assays to this new platform. The Pyrosequencing method is based on strand synthesis sequencing with quantitative detection of pyrophosphate generation. Data collected so far indicate that this methodology is vastly superior to our previous assay methods in areas such as reproducibility, flexibility, efficiency, cost and throughput. We report our progress related to this conversion and our assessment of this new technology.

32

Homogeneous MassARRAY™ Platform (MALDI-TOF Mass Spectrometry) for Automated High-Throughput Genotyping Analysis of *GPIIIa*, *ACE*, and *PAI-1*. B. Zhang,[1] E.C. Lau,[1] M. Patnaik,[1] M.J. McGinniss,[2] and T. Stewart,[2]
[1]*Specialty Laboratories, Inc. and* [2]*Sequenom, Inc.*

We have developed a minipanel of genetic tests for the evaluation of predisposition to arterial thrombosis. This minipanel comprises platelet glycoprotein (*GPIIIa*), angiotensin-converting enzyme (*ACE*), and plasminogen activator inhibitor-1 (*PAI-1*) genotyping assays. We used a proprietary SEQUENOM homogeneous MassARRAY™ (hME) format which is both highly sensitive and accurate to detect these gene mutations. The hME assay improves on the proprietary MassEXTEND™ assay by eliminating the need for sample transfers with all reactions occurring on a single plate. A common polymorphism, T1565C, in exon 2 of the platelet glycoprotein (*GPIIIa*) gene results in a proline for leucine substitution at amino acid residue 33. The *GPIIIa* gene encodes a polymorphic protein with platelet alloantigens Pl^{A1} and Pl^{A2} as the most common allelic isoforms. *GPIIIa* gene was amplified by PCR from genomic DNA, followed by the MassEXTEND™ reaction and detection by MALDI-TOF Mass Spectrometry (MS). MALDI-TOF MS separates and detects molecules by the intrinsic physical property of mass-to-charge ratio (m/z), eliminating the need for gel electrophoresis or fluorescent labeling. We determined the *GPIIIa* genotypes of 100 anonymous individuals by the hME method. Among these 100 individuals, there were 21 heterozygotes and 4 homozygotes carrying the *GPIIIa* T1565C mutation. Compared to PCR/*RFLP* method, both the accuracy and precision of genotyping by the hME method were 100%. In addition, we have determined the *ACE* insertion/deletion and the *PAI-1* 4G/5G genotypes of 100 anonymous individuals using the hME method. The hME assays were performed on 384 well chips, and the time required for processing 384 whole blood specimens to genotyping results was 2 days. The amount of DNA required for the hME assay was extremely small compared to other PCR assays. Reliable test results were obtained from 3.5 ng to 100 ng of genomic DNA by our hME method, whereas genotyping by PCR-*RFLP* required approximately 250 ng of genomic DNA. The robustness of the hME platform has enabled automated, high-throughput, and virtually error-free genotyping of *GPIIIa, ACE, and PAI-1*.

33

Rolling Circle Amplification Technology Allows High Sensitivity Detection of Nucleic Acid Targets in Cells and Tissues. V. Wheeler, J. Montano Tu, H. Ferguson, Jr., and S. P. Piccoli, *Molecular Staging, Inc., New Haven, CT.*

Rolling Circle Amplification Technology (RCAT™) is an isothermal amplification method that has demonstrated an increased sensitivity for the detection of proteins in cells and tissues. Nucleic acid targets have been analyzed by RCAT™ using bi-specific oligonucleotide probes. Bi-specific probes consist of two regions; one region hybridizes to a target-specific sequence and the other region functions as a platform to hybridize to the rolling circle and initiate synthesis of the amplified product. These probes have been used to detect mRNA for EGFR, and kappa and lambda chains in formaldehyde fixed cells and tissues. Serial dilutions of bi-specific probes for EGFR RNA detection in breast sections resulted in a 25-fold increase in sensitivity. Bi-specific probes have also been used to detect integrated viral sequences at low copy number. In addition to the increased sensitivity over conventional *in situ* hybridization provided by RCAT™, there was no increase in non-specific background. The ability to multiplex is an additional attraction of RCAT™, providing opportunities to detect multiple RNA targets or RNA and DNA targets simultaneously. Detection of the RCA product has been accomplished via fluorescence or colorimetric methods, thus providing sensitivity as well as versatility.

34

Lack of Evidence for Jaagsjekte Sheep Retrovirus in Human Bronchioloalveolar Carcinoma by PCR. K.M. Hiatt and W.E. Highsmith

Human bronchioloalveolar carcinoma (BAC) is a lung cancer that appears to be increasing in incidence and has no confirmed carcinogenic association. Histologically, it appears identical to an ovine lung cancer known as sheep pulmonary adenomatosis (SPA) which is known to be caused by jaagsiekte sheep retrovirus (JSRV). To date, no studies have shown this virus, or any other, to be associated with human BAC. However, a recent report demonstrated that 30% of human BACs and none of the benign lung lesions studied express a protein that cross-reacts with an antibody directed to the JSRV capsid protein[1]. Additionally, a JSRV receptor has been localized to chromosome 3p21.3[2]. If confirmed, this would represent a major breakthrough in our understanding of the causes of non-tobacco related lung cancers. We evaluated 24 human lung cancers for the presence of JSRV DNA. The samples were formalin fixed, paraffin embedded tissue from 18 BACs, 4 adenocarcinomas with focal BAC pattern, one moderately to poorly differentiated squamous cell

carcinoma with focal BAC pattern and one poorly differentiated adenosquamous carcinoma with atypical bronchioloalveolar hyperplasia. We anticipated that approximately 30% of these tumors would have integrated JSRV DNA if the JSRV capsid protein cross-reactivity, shown previously, reflected the presence of JSRV. None of the tissue demonstrated evidence of integrated JSRV *gag* DNA by PCR using primers reported by Palmarini et al.[3] Amplification of DNA from paraffin embedded lamb muscle tissue using these primers, which amplify both JSRV and endogenous ovine retroviral sequences, gave a positive signal at a one to one million dilution in human DNA. This study suggests that, though BAC is histologically identical to SPA, it is not caused by the Jaagsiekte sheep retrovirus. This study does not exclude an etiology involving a retrovirus that is antigenicly similar to JSRV, but with a unique *gag* gene sequence.

1. De Las Heras et al. Eur Respir J 2000;16:330–2.
2. Rai et al. J Virol 2000:74:4698–704.
3. Palmarini et al. J Gen Virol 1996;77:2991–8.

35

SNP-based DNA Fingerprinting by ASO-RDB Flow Through Hybridization. J. W. O. Tam, Y. K. Woo, J. K. F. Chow, and S. S. Xie, *Department of Biochemistry, The University of Hong Kong, Hong Kong , China.*

DNA fingerprinting by RFLP first introduced in 1986 for human identification was subsequently applied to other organisms. In human it was widely accepted as the best forensic tool for identification of suspects in criminal cases, paternity disputes and often used as the distinct human ID code. Recently the relatively time consuming RFLP method is mostly replaced by the high throughput automation process. Using PCR amplification of analyzing the number of short tandem repeat (STR) from 13, and 16 loci in the human genome, single cell identification is possible. However, it is still relatively expensive because it requires the use of sophisticated equipment. Single nucleotide polymorphisms (SNP) should have as high, if not more, discriminating power as VNTR or STR systems for forensic identification. With the use of ASO-arrays, the number of SNP to provide adequate discriminating power is easily attainable. We use our membrane-based semi-array ASO-RDB Flow-Through hybridization format to achieve such goal (method details will be included in poster or see US patent #5741647). In principle we could use the SNP of sufficient number anywhere in the genome for discriminating purpose. However, this may compromises the accuracy of paternity and kinship analyses because of the variability of mutation rate in different part of the genome. Hence we chose polymorphism sites in the coding region where the mutation rate is low to ensure the inherence nature for kinship identification. In this report we present our preliminary data of 35 SNPs from 9 highly polymorphic chromosome loci. In construction of the polymorphic frequency database, on each sites we have sequenced DNA samples from 50-150 unrelated individuals. The kinship analyses of 20 families were performed in parallel with the STR Profiler Plus human identity kit and the results were 100% in agreement. Although more data will be needed for validation, this SNP-based Flow Through format may prove to be a good alternative for human identification. (This investigation was supported by ITF grant of the Hong Kong Industry Department No. AF/204/99)

36

Clinical Validation of a High-Throughput DNA Diagnostic Test for Three CFTR Mutations (I148T, 1898(+1)G→A, 2184delA) Using Mass Spectrometry. J.O. Wang, N.T. Kummer, M.J. McGinniss, G.A. Howes, and M. Patnaik, *Specialty Laboratories Inc. and Sequenom Inc.*

Specialty Laboratories has developed and validated a Phase II Cystic Fibrosis (CF) assay for three mutations in the standard mutation panel recommended by the American College of Medical Genetics (ACMG) using the SEQUENOM'S patented MassARRAY™ system. These mutations compliment the Phase I Cystic Fibrosis (CF) Genotyper® assay launched by Specialty Laboratories in 2000 for the 31 other mutation recommended by the ACMG. The SEQUENOM system relies on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for automated genotyping and analysis. The assay is designed to genotype the CF mutations: I148T, 1898(+1)G>A, and 2184delA. This technology uses PCR amplification, primer extension, and mass spectrometry to directly characterize wild type and mutant alleles without the need for labels or nucleic acid fragment separation steps. In addition, the SpectroTYPER™ software automatically analyzes the spectrum of primer extension products and assigns genotypes based on varying levels of probability from conservative (highest), moderate, aggressive and indeterminate (lowest). Tests are repeated for any indeterminate genotype assignments. Clinical validation studies were performed and evaluated prior to assay acceptance at Specialty. Validation parameters examined were: influence of varying DNA extraction methods and DNA concentrations, testing of defined CF mutant and wildtype control samples, intra and inter assay reproducibility, and interlaboratory results comparisons. The assay showed 100% correlation with defined oligo samples in 199 tests for the I148T genotype, 199 tests for the 1898(+1)G>A genotype and 194 tests for the 2184delA genotype. By using defined clinical samples as standards, the assay has 100% sensitivity and specificity. Based on data from defined clinical samples, the positive predictive value and negative predictive value were both 100%. In the clinical setting, eighty-three patient samples have been tested, resulting in one 1898+1GA heterozygous positive patient and the rest wild type. The Phase II panel assay is the first clinical implementation of SEQUENOM-based MALDI-TOF-MS assay at Specialty laboratories and the first for a US commercial laboratory.

37

Cytochrome P450 (CYP450) Genotyping on the Codelink™ Bioarray. Sherri Longenbach-Huber,[1] Stephanie Safgren,[2] Teresa Raich,[3] Jim George,[3] Matthew M. Ames,[2] Dennis J. O'Kane,[1] *Depts. of [1]Laboratory Medicine and Pathology and [2]Oncology Research, Mayo Clinic, Rochester, MN; and [3]Motorola Life Sciences, Northbrook, IL.*

Introduction. Functional gene deletions and inactivating polymorphisms in CYP450 are responsible for many serious adverse drug reactions. Numerous CYP450 allelic variants are known, making polymorphism identification time-consuming and difficult. Use of a CYP450 genotyping array that detects all the major allelic variants simultaneously would facilitate detection of individuals at risk of adverse drug reactions. **Methods**. The Codelink™ CYP450 bioarray used in this study (Motorola Life Sciences) incorporates probe sets for seven CYP450 genes and 75 SNPs. Isolated genomic DNA was amplified for CYP450 genes in 12 separate PCR reactions in CodeLink pre-dispensed PCR primer plates. The PCR reaction products were pooled, purified, and fragmented using DNase I. The fragmented amplicons were then genotyped by allele-specific single-base extension using biotinylated acyclo terminator nucleotides and stained using streptavidin-Alexa-Fluor conjugate. The bioarrays were scanned for fluorescence and genotype calls performed using CodeLink Analysis software. **Results.** A panel of 30 DNA samples, previously validated for CYP2C19*2A, CYP2C19*3 and CYP2D6*17 through DNA sequencing and/or RFLP analysis, were used to evaluate the accuracy of the CYP450 bioarray. Calls were obtained for all 30 samples for the 3 SNPs except for one or both polymorphisms in 3 samples for CYP2C19 and in 1 sample for CYP2D6. Several errors in dye-terminator DNA sequence results were corrected using the CYP450 bioarray. A single disagreement with the DNA sequence data was noted (99% call accuracy). A rare CYP2D6 *17 homozygous polymorphism was discovered in one sample during the analyses.

Conclusions. Genotyping accuracy using the Codelink™ CYP450 bioarray equals or exceeds the accuracy of DNA sequencing and RFLP analysis and the results are available for next-day reporting.