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On the cover this month: James Watson and Francis Crick with their model of DNA. Many individuals would argue that the "molecular" era began in 1953 when Watson and Crick published their famous paper in Nature. However, it was another 30 years before the words "molecular" and "diagnostics" appeared together in the literature. Molecular diagnostics, which has been empowered by continual advancements in technology, now provides most laboratory tests in infectious disease and genetics, and an increasing number in oncology. It has been 6 years since molecular diagnostics was first highlighted in a Special Issue of *Clinical Chemistry*. Because molecular diagnostics remains "a revolution in progress," we have devoted a second Special Issue to this rapidly changing field. Begin your journey with the thoughtful preamble written by Chiu, Lo, and Wittwer.

Extreme PCR: Efficient and Specific DNA Amplification in 15–60 Seconds
By Jared S. Farrar and Carl T. Wittwer

PCR analysis originally required 2-4 hours. In the 1990s the authors of this study developed "rapid-cycle PCR" that was completed in 10-30 minutes. In this paper, they introduce "extreme PCR" that is completed in 15-60 seconds, enabled by increasing primer and polymerase concentrations ten- to twentyfold. Efficient, specific, sensitive, and high-yield PCR products were obtained from human genomic DNA in less than 1 minute, opening up the potential for DNA diagnostics in only a few minutes.

Translating Sanger-Based Routine DNA Diagnostics into Generic Massive Parallel Ion Semiconductor Sequencing
By Adinda Diekstra, et al.

The authors of this study demonstrate the development and validation of a generic automated ion semiconductor sequencing workflow that can be used in a clinical setting and can substitute for Sanger sequencing. Standard amplicon-based enrichment remains identical to PCR for Sanger sequencing. A novel post-enrichment pooling strategy was developed, reducing sequencing costs up to 60-70%. This workflow might be applicable in any laboratory that is interested in using a benchtop next-generation sequencing machine, without having to invest in different enrichment designs. In combination with appropriate data analysis, this approach exhibited an average sensitivity of 99.61% and specificity of 99.98%, which is comparable to Sanger sequencing.

DNA Ligase-Based Strategy for Quantifying Heterogeneous DNA Methylation without Sequencing
By Eugene J.H. Wee, et al.

DNA methylation is a potential source of disease biomarkers. This work describes a novel approach named EpiQ to measure heterogeneous DNA methylation, that is, the methylation patterns on individual DNA strands or epialleles. Currently only single molecule approaches, such as next generation sequencing, can provide detailed epiallele information. EpiQ is a simpler alternative single molecule-like approach using DNA ligases and enhanced thermo-instability of short mismatched DNA probes for the relative quantification of epialleles. Having comparable analytical performance to next generation sequencing, this method can potentially facilitate the use of epialleles as biomarkers. EpiQ can also easily be adapted for miniaturized chip-based platforms.

Noninvasive Prenatal Testing for Wilson Disease by Use of Circulating Single-Molecule Amplification and Resequencing Technology (cSMART)
By Weigang Lv, et al.

Reliable and accurate technologies for noninvasive prenatal testing of monogenic diseases are urgently needed. The authors of this study developed and validated a novel method called circulating Small Molecule Amplification and Re-sequencing Technology, abbreviated as cSMART, for quantification of mutant alleles in plasma. As proof of concept, they applied cSMART to 4 pregnancies at risk for Wilson Disease and showed that the fetal genotypes assigned by cSMART were concordant with the fetal genotypes diagnosed by traditional invasive testing of amniocytes. cSMART has potential as a universal method for noninvasive prenatal testing of monogenic diseases since the method only requires knowledge of the parental mutations.

Screening Newborn Blood Spots for 22q11.2 Deletion Syndrome Using Multiplex Droplet Digital PCR
By Dalyir Pretto, et al.

This study successfully demonstrates the application of droplet digital PCR for the diagnosis of 22q11 deletion syndrome in large population studies including newborn screening. Droplet digital PCR allows the detection of copy number variation of marker genes COMT and PIK4CA within the deleted region of chromosome 22q11 from bloodspot DNA. This approach provides a fast and cost-effective diagnosis of 22q11 deletion syndrome, and potentially of other microdeletion syndromes, that is promising for early detection through newborn screening. The early identification can positively impact the clinical outcome of a patient by presenting opportunities for timely medical interventions.

Circulating Long Noncoding RNA TapSAKI Is a Predictor of Mortality in Critically Ill Patients with Acute Kidney Injury
By Johan M. Lorenzen, et al.

Long noncoding RNAs are novel intracellular noncoding ribonucleotides regulating gene expression. By using a complex massive long noncoding RNA profiling approach, the authors identified a new long noncoding RNA, termed TapSAKI, as an important novel diagnostic and prognostic marker of acute kidney injury. Circulating TapSAKI levels were detected by quantitative real-time PCR. TapSAKI specifically identified patients with acute kidney injury; baseline levels of TapSAKI were correlated with parameters of disease severity and were increased in nonsurvivors compared to survivors. TapSAKI was identified as a strong independent prognostic factor for 28-day-survival by multivariate Cox proportional hazards regression analysis and Kaplan-Meier curve analysis.

Variability of the Reverse Transcription Step: Practical Implications
By Stephen A. Bustin, et al.

Most molecular diagnostic tests that use RNA biomarkers for the early detection of disease, prognosis, or response prediction report only small differences between normal and diseased tissue. This is despite several relatively old reports that refer to the variability of the reverse transcription step. The authors of this study have quantified the reproducibility of the reverse transcription step to focus on the practical implications of that variability. They have used a range of different reverse transcriptases, samples, and RNA at different concentrations and of differing quality, together with SYBR Green I and a probe-based chemistry. Their investigation demonstrates that the variability of the reverse transcription step is sufficiently large to call into question the validity of many of the published data that rely on measurements of cDNA.

Clinical Exome Performance for Reporting Secondary Genetic Findings
By Jason Y. Park, et al.

This retrospective study examined a 56-gene subset from a total of 57 whole exome sequencing data sets and determined the adequacy of sequencing coverage for variant interpretation. All exons from 56 genes were examined for adequacy of coverage. In addition, the nucleotide positions of 18,336 potentially significant Human Gene Mutation Database variants were also examined for adequacy of coverage. Data from clinical exome tests revealed 7 genes where inadequate coverage was seen for more than 50% of Human Gene Mutation Database variant locations. This study demonstrates the possibility of inadequate coverage when using clinical exome sequencing for the interpretation and reporting of gene subsets.

The Landscape of MicroRNA, Piwi-Interacting RNA, and Circular RNA in Human Saliva
By Jae Hoon Bahn, et al.

Salivary RNAs have been identified as biomarkers for several diseases. However, the entire spectrum of extracellular RNA from saliva has not been fully characterized. Using high-throughput RNA sequencing, the authors of this study conducted an in-depth bioinformatic analysis of noncoding RNAs in human cell-free saliva of healthy individuals. They observed highly reproducible profiles of microRNA and piwi-interacting RNA expression across individuals and an enrichment of piwi-interacting RNAs in cell free saliva. This work is the first to characterize and validate circular RNAs in extracellular body fluid. The study results provide a landscape of noncoding RNA species in human saliva, which should pave the way for further biomarker discoveries.

Refinement of Variant Selection for the LDL Cholesterol Genetic Risk Score in the Diagnosis of the Polygenic Form of Clinical Familial Hypercholesterolemia and Replication in Samples from 6 Countries
By Marta Futema, et al.

The authors of this study performed an extensive analysis to optimize the LDL-cholesterol genetic risk score design for diagnosis of polygenic hypercholesterolemia and to select the most cost-efficient method for clinical purposes. They present for the first time results of the LDL-cholesterol genetic risk score analysis in children diagnosed with Familial Hypercholesterolemia, as well as results of the first-to-date replication of these findings in adults. Results of this study indicate that individuals with clinical diagnosis of Familial Hypercholesterolemia but with no identified mutation have a more than 95% chance of having a polygenic cause of hypercholesterolemia.

Chromosomal Instability in Cell-Free DNA Is a Serum Biomarker for Prostate Cancer
By Ekkehard Schütz, et al.

This study examines chromosomal regional ploidy heterogeneity in prostate cancer. The rationale for the study is that serum-free DNA derived from apoptotic tumor DNA is a reliable surrogate for the tumor as a liquid biopsy. A preliminary study in breast and prostate cancer indicated that each hormonally associated neoplasm was associated with its own unique chromosomal “hot spots” of DNA sequence imbalance. Using massively parallel sequencing the authors were able to distinguish prostate cancer from controls and from nonmalignant prostate disease with an accuracy of 83% and 90%, respectively. The 20 prostate regions of regional DNA ploidy heterogeneity were associated with several genes of fundamental significance to the process of malignant transformation.

Validation of DNA Methylation to Predict Outcome in Acute Myeloid Leukemia by Use of xMELP
By Gerald B.W. Wertheim, et al.

DNA methylation has emerged as an important factor in predicting the behavior of many cancers; however, methylation patterns are not routinely assessed in the clinical laboratory. The authors of this study have developed a methylation assay (termed "xMELP") that can be performed in the clinical laboratory with the turnaround times necessary for initiation of therapy in acute myelogenous leukemia. They describe xMELP as well as a new classifier for predicting prognosis in acute myelogenous leukemia and demonstrate its analytical characteristics in a variety of assay validation experiments. Most importantly, they show that their classifier predicts survival in an independent cohort of acute myelogenous leukemia patients.

Circulating Tumor Cell Enumeration with a Combination of Epithelial Cell Adhesion Molecule– and Cell-Surface Vimentin–Based Methods for Monitoring Breast Cancer Therapeutic Response
By Arun Satelli, et al.

Circulating tumor cells are considered to be the seeds of metastasis. However there are no markers to detect epithelial mesenchymal transitioned circulating tumor cells. This study identified cell-surface vimentin as a marker for epithelial mesenchymal transitioned cancer cells and used an antibody against cell-surface vimentin for this study. These findings take into account circulating tumor cells of both epithelial and epithelial-mesenchymal phenotype while monitoring the therapeutic response in breast cancer patients, thus providing a highly sensitive and specific method for clinical management of patients.

Single-Tube, Highly Parallel Mutation Enrichment in Cancer Gene Panels by Use of Temperature-Tolerant COLD-PCR
By Elena Castellanos-Rizaldos, et al.

Multiplexed detection of low-level mutations presents a technical challenge, when targeted resequencing in cancer gene panels is employed. Analysis of mutations below approximately 2-5% abundance is problematic due to increased "noise" from sequencing errors. The authors have developed a multiplex temperature-tolerant-COLD-PCR approach that uses cancer gene panels developed for massively parallel sequencing. By introducing modified nucleotides, fast-COLD-PCR is also adapted to enrich for melting temperature-increasing as well as melting temperature-decreasing mutations in a single tube. Using custom-made and commercial gene panels the authors demonstrate that fast-temperature-tolerant-COLD-PCR enriches mutations on all examined targets simultaneously and has a limit of detection of approximately 0.01-0.3% mutation abundance.

Gene Expression Profiling of Circulating Tumor Cells in Breast Cancer
By Emanuela Fina, et al.

The enumeration of circulating tumor cells provides clinically relevant information, but the molecular characterization of these cells could give further insights into disease progression to help overcome problems related to temporal and spatial tumor heterogeneity. Studies have only recently started to molecularly characterize circulating tumor cells. The cDNA-mediated annealing, selection, extension, and ligation assay facilitates the interrogation of a large number of genes using tiny amounts of circulating tumor cell-isolated RNA. The approach described in this paper enables the generation of technically reliable gene expression profiles from isolated circulating tumor cells from which biologically useful information can be obtained. The method is reproducible and suitable for prospective studies to assess the clinical utility of circulating tumor cell molecular profiles, provided that at least 25 circulating tumor cells can be isolated.

Next-Generation Digital PCR Measurement of Hepatitis B Virus Copy Number in Formalin-Fixed Paraffin-Embedded Hepatocellular Carcinoma Tissue
By Jing-Tao Huang, et al.

The authors of this study used a droplet digital PCR system to measure the hepatitis B virus copy number in 131 formalin-fixed paraffin-embedded hepatocellular carcinoma samples. Hepatitis B virus copy numbers were successfully determined for all clinical formalin-fixed paraffin-embedded tissues, and they ranged from 1.1 to 175.5 copies per microliter. Their results demonstrated that the copy numbers of hepatitis B virus were correlated significantly with tumor-node-metastasis classification, with Barcelona-Clinic Liver Cancer classification, and with the serum cholinesterase. The findings of this study indicate that hepatitis B virus infection is a key factor that influences tumorigenesis in hepatocellular cancer by regulating tumor occurrence and development.

Quantitative Cell-Free Circulating *BRAF*^{V600E} Mutation Analysis by Use of Droplet Digital PCR in the Follow-Up of Patients with Melanoma Being Treated with BRAF Inhibitors
By Miguel F. Sanmamed, et al.

Most of cutaneous melanomas harbor the *BRAF*^{V600E} mutation, which can be released to plasma. The authors of this study studied plasma levels of cell-free *BRAF*^{V600E} in advanced melanoma patients during treatment with BRAF inhibitors. *BRAF*^{V600E} was detected by digital droplet PCR at a fractional abundance of 0.005% in wild type gene. Agreement between tumor tissue *BRAF*^{V600E} and basal cell-free *BRAF*^{V600E} in plasma was 84.3%. Baseline cell-free *BRAF*V600E correlated with tumor burden and plasma levels decreased significantly after the first month of therapy. At progression there was a significant increase in the concentration of mutant copies. Lower plasma levels of basal cell-free *BRAF*^{V600E} were associated with better overall survival and progression-free survival.