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ABSTRACTS

Technology developments and applications

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064: Keeping pace with the innovation: An easily adaptable technique for rapidly generating humanized animal models in the era of human genome

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The phenomenal increase in the amount of information about human genome is having a huge impact on biomedical research. Transgenic mice have become invaluable resource and it serves as the most powerful tool for analysing gene function and regulation in vivo. Through research utilizing the transgenic mouse or non-human primate as a model for human disease, investigators can translate basic biological phenomena into a human health perspective. A rapid procedure for making transgenic mice is also needed for screening thousands of human genes whose functions are yet unknown.

Pronuclear DNA microinjection in the oocyte is the most frequently used technique for generating transgenic animals. This and other techniques for making transgenic animals are fairly complicated, requiring trained personnel, costly infrastructure and a large number of zygotes harvested from animals that are then killed. Due to technical difficulties in obtaining substantial number of embryos from non-human primates and limitations in repeated surgical intervention in them, it is practically difficult to make much needed models of transgenic primates necessary to study several diseases specific to primates. All these drawbacks generate a need for developing a handy and cost effective technique for producing transgenic animals preferably with minimum or no loss of animal lives. We have developed a reproducible non-terminal technique for inserting genes in testicular spermatogonial cells through in vivo electroporation of the testis. The optimal result was obtained by injecting linearized DNA into the testis of male mice, followed by electroporation using 8^2 40 V electric pulses in alternating direction with a time constant of 0.05 s and an inter pulse interval of $\sim$1 s. The transgenesis was confirmed by PCR, Southern blot analysis and immunohistochemical studies of the progeny. More than 90% of males electroporated with any one of the four different constructs successfully sired transgenic pups. Such electroporated males provide a valuable resource for continuous production of transgenic founders. This method requires neither assisted reproductive techniques nor sophisticated laboratory setup and highly trained personnel. This ethically superior (deathless) and easily adaptable time saving procedure opens avenues for developing several transgenic mice in short span of time and broadens the scope of making transgenic sub-human primates. (This study was funded by Department of Biotechnology, India.)

065: Human whole genome sequencing by high-throughput, short read oligonucleotide ligation

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The advent of new ultra-high throughput sequencing methods allows contemplation of resequencing thousands of human genomes to extensively survey common and rare forms of genetic variation that could have implications in health and disease. We sequenced the genome of an individual of African origin with the Applied Biosystems SOLiDTM system. The method allows sequencing of single or paired ends 25–50 bp reads of 108–109 templates on a single array containing beads with clonally amplified templates. We collected over a billion reads in seven runs, amounting to a total of 32 Gbp of sequence, and obtained an average 12X haploid sequence coverage and 130X clone coverage. After aligning those reads to the hg18 reference assembly, over 95% of the genome is covered and 99.45% of the genome is spanned by a paired end clone. A novel error correction technique improves the accuracy of the aligned reads to >99.95%. With such accuracy, our results suggest that heterozygote identification can be efficiently achieved at 10–15X coverage levels, by comparison with known HapMap genotypes. We detected over 2 million SNPs, of which 81.4% are in dbSNP. We categorize the genes using the Panther ontology, and annotate the damaging potential of non-synonymous SNPs using PolyPhen. We discovered that transcription factors, ligases, growth factors, receptors, and RNA helicases are the molecular functions most under-represented for

damaging mutations, and GPCR genes involved in Olfaction, and genes for Immunity and defense are the biological functions most over-represented for damaging mutations. Olfaction and Immunity have previously been observed to be under recent positive selection in human populations; thus we recapitulate evidence of natural selection in a single human sample. 90,000 small insertions and deletions (<12 bp) were identified with the use of paired end reads. Indels are significantly underrepresented in translated exons. The analysis of the distance and orientation of the paired end reads allowed the identification of thousands of putative insertions and deletions ranging from 50 bp to several Kb. We predict 50 inversions (25% previously reported in other samples) and 4 gene fusions resulting from deletions (two previously reported). Depth of coverage analysis allows the inference of copy-number variants. Our results provide guidance for future studies to discover sequence and structural variants in human populations and cancer with short-read next generation sequencing.

066: Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing

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The Polymerase Chain Reaction (PCR) has become the cornerstone of molecular diagnosis, with almost every genetic test aiming to identify DNA sequence variation incorporating PCR. A commonly encountered problem is that variant DNA sequences exist in the presence of a large majority of wild type alleles such as when DNA is obtained from heterogeneous cancer biopsies or when fetal alleles are sought in maternal blood. As a result, downstream assays are severely limited in their ability to identify subtle genetic changes that can have profound impact in clinical decision-making and outcome. We describe Co-amplification at Lower Denaturation temperature (COLD-PCR), a novel form of PCR that amplifies minority alleles selectively from mixtures of wild-type and mutation-containing sequences irrespective of the mutation type or position on the sequence (Technical Report, Nature Medicine, May 2008 issue). In COLD-PCR, an intermediate annealing temperature is used during PCR-cycling to allow cross-hybridization of mutant and wild type alleles; hetero-duplexes, which melt at lower temperatures than homo-duplexes, are then selectively denatured and amplified at Critical Denaturation Temperature (Tc), while homo-duplexes remain double-stranded and do not amplify efficiently. To validate COLD-PCR, we used serial dilutions of DNA from tumor-derived cell lines and genomic DNA from a series of colon and lung cancer surgical specimens, and plasma-circulating DNA collected under IRB approval were utilized for validation on clinical specimens. By replacing regular PCR with COLD-PCR prior to application of a range of assays (Sanger sequencing; Pyrosequencing; MALDI-TOF; dHPLC; RFLP; and Taqman) we improved mutation detection sensitivity up to 100-fold and identified several additional TP53/KRAS/EGFR mutations in heterogeneous cancer samples. About 4 of 43 surgical samples and 3 of 10 plasma samples tested contained clinically important mutations that were not detected by any of the methods tested when preceded by regular-PCR, but they were detectable following COLD-PCR. Replacement of regular PCR with COLD-PCR provides a universal boost to all mutation detection technologies and enables them to be used with the required confidence in routine screening of cancer specimens for somatic mutations, including low-level mutation screening of surgical/FFPE tumor samples or bodily fluids. COLD-PCR is equally applicable to fields other than cancer, such as pre-natal diagnosis, in infectious diseases and in epigenetics.

067: Characterization of whole blood gene expression profiles in sickle cell disease with enhanced transcript detection using globin mRNA reduction

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Room temperature whole blood mRNA stabilization procedures, such as the PAX gene system, are critical for the application of transcriptional analysis to population based clinical studies. Global transcriptome analysis of whole blood RNA using microarrays has been proven to be challenging due to the high abundance of globin transcripts that constitute 70% of whole blood mRNA in the blood. This is a particular problem in patients with sickle cell disease, secondary to the high abundance of globin-expressing nucleated red blood cells and reticulocytes in the circulation. In order to more accurately measure the steady state whole blood transcriptome in sickle cell patients we evaluated the efficacy of reducing globin transcripts in paxgene stabilized RNA samples for genome-wide transcriptome analyses using oligonucleotide arrays. We demonstrate here by both microarrays and Q-PCR that the globin mRNA depletion method resulted in 55- to 65-fold reduction in globin transcripts in whole blood collected from healthy volunteers and sickle cell disease patients. This led to a dramatic improvement in microarray data quality with increased detection rate of expressed genes and improved overlap with the expression signatures of isolated peripheral blood mononuclear (PBMC) preparations. The differentially modulated genes from the globin depleted samples had a higher correlation coefficient to the 116 genes identified to be significantly altered in our previous study on sickle cell disease using PBMC preparations. Additionally, the analysis of differences between the whole blood transcriptome and PBMC transcriptome reveals important erythrocyte genes that participate in sickle cell pathogenesis and compensation. The combination of globin mRNA reduction after whole-blood RNA stabilization represents a robust clinical research methodology for the discovery of biomarkers for hematologic diseases and in multicenter clinical trials investigating a wide range of nonhematologic disorders where fractionation of cell types is impracticable.

068: Evaluation of factors affecting the success of multiplex PCR

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A set of 200 genomic PCR primers was tested in multiplex experiments. All primers were working flawlessly in single-plex experiments, as determined by gel electrophoresis. In multiplex environment about 5% of 2-plex experiments and 25% of 4-plex experiments failed to produce at least one of expected PCR products. By using statistical model we determined the factors that have correlation with the degradation of the quality of multiplex PCR. The most important factor was the number of possible alternate genomic binding sites of primers, as determined by nearest-neighbour thermodynamic model. The same factor is also correlated with the degradation of the quality of single-plex PCR, but the effect is much stronger in multiplex conditions. We also found, that the hybridization affinity between primers of different PCR, calculated by nearest-neighbour thermodynamic model, is correlated with the degradation of the quality of multiplex PCR, although the effect is much lower than the effect of genomic hybridization. These results can be used in

the design process of primers for multiplex PCR. Lower quality primers can be eliminated already in design phase. Additional testing for unwanted hybridization between different PCR primer pairs can be used as criterion in designing multiplex groups.

069: A new assay in functional genomics: Monitoring cell differentiation in live cell chips by electric cell-substrate impedance sensing

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There is a great demand for cell-based in vitro screening systems for drug target validation and generation of resources for cell therapies. Our assay is focused on the formation of contacts between cells and between cells and the extracellular matrix, which is important for cell differentiation processes relevant in cancer therapy and tissue repair. Cellular interactions are measured with a method called electric cell-substrate impedance sensing (ECIS). By applying alternating current and measuring the voltage across an electrode, the impedance can be calculated and broken down into a series resistance and capacitance. Insulating membranes of spreading cells block and constrain the current flow, resulting in measured variations in the impedance. We showed the applicability of this system with different cell types and chip designs (Maercker et al. in Eng Life Sci 8: 1–8, 2008). Mesenchymal stem cells (MSCs), isolated from bone marrow, were transferred to a specialized multi-well live cell chip with integrated electrodes which allowed us to measure cellular interactions by ECIS. The attachment of MSCs was tested after in vitro induction of differentiation into bone or fat tissue. Within 24 h, we were now able to distinguish different differentiation stages, which also were visualized by light microscopy in parallel. Specific impedance profiles already were visible within the first two hours after starting growth in differentiation medium. In a "wound healing assay" in the live cell chips, adipogenic cells showed a reduced migration activity compared to non-differentiated MSCs. With HaCaT keratinocytes, we tested cell attachment on the level of gene expression. After induction (TetOn system) of the expression of nidogen, a protein critical for extracellular matrix formation, cells showed stronger adhesion than without nidogen expression, which was documented by characteristic impedance spectra. Compatible RNAi silencing experiments are under way. With our measurements we are able to discriminate between cell types, cell densities, and differentiation status. It even is possible to describe the function of single adhesion molecules. Therefore, this noninvasive cell-based assay most probably not only will help us to improve biological resources for advanced cell therapies. Simulation of the homing process of stem cells in an in vitro assay also is a promising setup for large-scale gain-of-function or loss-of-function screenings in functional genomics.