Postersession

Main Postersession:
Monday, 3rd April 2017 6:00pm - 10:00pm

Location:
Foyer - lower level

All posters will be displayed in all three poster sessions (Monday evening and two lunch poster sessions).
Poster setup is on Monday afternoon and all posters are available until Wednesday afternoon.

Poster numbers: PO 01 – PO 78

NGS PO 01 – PO 16

PO 01: Ligation-free small RNA sequencing libraries from clinical samples using SMART® technology

Sara Gonzalez-Hilarion[1], Nathalie Bolduc[2], Simon Lee[2], Rachel Fisch[2], Kahraman Tanriverdi[3], Evangelia Malahias[3], Ekaterina Mikhalev[3], Jane Freedman[3], Andrew Farmer[2]
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Small non-coding RNAs regulate gene expression via diverse mechanisms and facilitate fundamental cellular processes such as transcript splicing and protein translation. Moreover, small RNAs may act at sites different from their original production via incorporation and transport in bodily fluids. Obtaining an accurate portrait of small RNA expression levels from small sample inputs carries potential both for the fulfillment of basic research objectives and the development of novel therapeutics and clinical diagnostic solutions. Towards this end, we have developed a novel, ligation-free approach for the preparation of small RNA sequencing libraries that leverages 3' RNA polyadenylation followed by cDNA synthesis and template switching. This approach minimizes sample representation bias and is sensitive enough to accommodate inputs of as little as 1 ng of total RNA. Here we present data demonstrating the accuracy, sensitivity, and reproducibility afforded by the SMARTer® smRNA-Seq Kit for Illumina®. Using a library of synthetic miRNAs, we show that the SMARTer method generates a more accurate representation than the typical adapter ligation method. Furthermore, we successfully created high-quality libraries using 400 pg to 1 ng of RNA extracted from plasma and serum samples, demonstrating the suitability of the SMARTer approach for applications such as biomarker discovery.

PO 02: Complete "Sample-to-Result" Highly Automated NGS and qPCR Workflow for Clinical Diagnostics

Elian Rakhmanaliev, Tatiana Ivanova, Yin Kum Ng, Alex Yeo, Harry Suhardi, Caroline Villy, Mei Qi Yee, Kevin Tan, Amanda Fan, Andy Soh, Pramila Ariyaratne, Si Kee Tan, Jocelyn Wong, Jiang Xia, Grace Tan, Jasmin Ang, John Ong, Charlie Lee, Gerd Michel, Wen Huang
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Sanger sequencing and real-time PCR methods have been the standard molecular methods in clinical diagnostics for decades. Deep sequencing or Next Generation Sequencing (NGS) technology revolutionized the field of genomics, transcriptomics and metagenomics and is now rapidly becoming a routine method in different areas of clinical diagnostics such virology, oncology, drug-resistance monitoring, non-invasive prenatal tests, microbiology, precision medicine, etc.

Vela Diagnostics developed complete ("Sample-to-Result") highly automated multi-purpose *Sentosa* workflow, which consists of: 1) a robotic liquid handling system for nucleic acid extraction, PCR set-up and/or NGS library preparation (*Sentosa* SX101); 2) instruments for real-time PCR or template preparation and deep sequencing (Ion Torrent's PGM system); 3) kits for nucleic acid extraction, target specific real-time PCR-based tests, NGS library preparation assays and reagents for deep sequencing; 4) assay specific applications, and 5) data analysis and reporting software. Different diagnostic applications employ the same robotic platform for qPCR set-up and preparation of NGS libraries. In less than 5 years Vela Diagnostics developed 7 NGS-based viral and oncology assays and more than 20 qPCR-based viral, microbial and oncology CE-IVD tests, which can be run on the same system. In addition, several extraction kits were developed to isolate nucleic acids from various types of clinical samples, including FFPE, whole blood, plasma/serum, swabs, sputum, stool and urine.

Sentosa qPCR and NGS workflow appears as a robust and efficient *in vitro* diagnostics (IVD) tool for the detection and/or quantitation of a wide range of bacterial and viral pathogens as well as mutations in human genes. Such comprehensive combined NGS and qPCR workflow that can help fill some existing gaps in the quality of molecular diagnostics will take the necessary steps towards improving patient outcomes.

PO 03: Gene Expression Variance As A Proxy For Chronological Age

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Aging, previously thought to be a random process of entropic decay in living organisms, is now known to be genetically regulated. Since the discovery that inhibiting insulin/IGF-1 signaling in C. elegans extends lifespan, much work has focused on finding additional genetic modulators of lifespan. Here we present "drift" as a genetic indicator of chronological age which depends upon the harmonic correlated

expression of gene sets. Drift is defined as the variance of the relative expression values of a group of genes with respect to themselves at an earlier time. As genes need to work in concert with other genes, we hypothesized that increasing differences in the ratio of gene products within biological pathways would be detrimental to an organism's health and contribute to aging. We therefore sought to develop a genetic biomarker for chronological age which would be useful for interrogating an organism's expected chronological age without having to wait for the organism to perish. In addition, we aimed to use drift to uncover specific areas of biology which are critical to lifespan extension.

Gene expression values of C. elegans cohorts were measured via RNA-seq (Illumina). Mianserin, a small-molecule antidepressant, was known from previous studies to increase mean C. elegans lifespan and was used to generate long-lived N2 worms in the study. Two cohorts of worms were generated. Worms were fed either solvent or 50 uM mianserin on day one of adulthood, and RNA was harvested for sequencing on days 1, 3, 5, and 10 of adulthood. Genomes were aligned to the reference genome from WormBase. Data analysis was performed in Python and R.

Drift analysis was performed for 10879 C. elegans genes at a transcriptome-wide level. While 19196 genes were sequenced, some genes had to be removed from the analysis due to undetectable expression values, which interfered with the normalization procedure for calculating drift. For the 10879 analyzed genes, we found that their drift values increased log-linearly with chronological age, and was consistently reduced across the entire worm's lifespan when exposed to mianserin. This supports our previous findings that mianserin specifically increases the young period of a worm's life.

We also examined how well genes grouped together by Gene Ontology (GO) terms drifted in order to identify biological areas which may be significant modulators of C. elegans lifespan. Since we found that the entire transcriptome exhibited drift behavior, we compared GO term gene sets to size-matched gene sets randomly sampled from the population of all genes. This allowed us to more accurately estimate how well a GO term grouped genes of similar biological function together. We identified bacterial response genes, protein kinase activity regulators, and immune response genes to be most highly correlated with drift. In conclusion, we have created a molecular proxy correlating with chronological age which is sensitive to changes in worm lifespan.

PO 04: Efficient Isolation on Vero.DogSLAMtag Cell Line and Whole Genome Characterization by Next Generation Sequencing of Dolphin Morbillivirus

Simone Peletto[1], Claudio Caruso[1], Cerutti Francesco[1], Modesto Paola[1], Cristina Biolatti[1], Alessandra Pautasso[1], Carla Grattarola[1], Federica Giorda[1], Sandro Mazzariol[2], Walter Mignone[1], Loretta Masoero[1], Cristina Casalone[1], Pier Luigi Acutis[1]

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Dolphin morbillivirus (DMV) is regarded as an emerging threat being the cause of recent epidemics worldwide. In 1990-1992, a DMV outbreak in the Mediterranean Sea involved thousands of striped dolphins with high mortality. Another major epidemic event was the DMV outbreak in 2006–2008 affecting three cetacean species along the Mediterranean coast. The DMV genome from the 1990-1992 epidemics is the only cetacean Morbillivirus (MV) genome that has been completely sequenced. We report the first retrospective isolation of DMV from a striped dolphin stranded along the Italian coast in 2008. This viral isolate, representative of the 2006-2008 Mediterranean epidemic (hereafter DMV_IZSPLV_2008), efficiently growth on Vero.DogSLAMtag cells and was submitted to whole genome sequencing by NGS technology.

The striped dolphin was an adult female stranded along the Ligurian coast in October 2008. In 2015, the brain of the striped dolphin was processed for virus isolation using Vero.DogSLAMtag cell line. Libraries were submitted to NGS on an Illumina MiSeq platform. Reads passing the quality filter were aligned to the DMV reference genome (NC_005283) using the BWA software. Read mapping was visualized with Tablet to obtain a consensus genome. MEGA7 was used for maximum likelihood (ML) phylogeny.

Successful viral isolation on Vero.DogSLAMtag cell line was demonstrated by CPE observed after 5 days. The final genome length obtained from the read assembly was 15,667 nucleotides covering 99.78% of the DMV reference genome. Comparison of the DMV_IZSPLV_2008 and the 1990-1992 DMV strain sequences revealed 157 nucleotide mutations and 47 amino acid changes at different sites. The sequence similarity was 98.7% at the full genome level. Additionally, the DMV_IZSPLV_2008 isolate was aligned with two partial sequences identified in Spain during the 2006-2008 outbreak in a striped dolphin and a pilot whale; in this case, sequence similarities were 99.8% and 99.9%, respectively.

In MV diagnostics, viral isolation is still considered the "gold standard", even though efficient propagation of live virus is difficult to achieve. Our results seem to confirm an increased sensitivity of Vero.DogSLAMtag cells for isolating cetacean MV. The successful on-culture replication of the virus allowed performing NGS directly from the viral RNA, without prior PCR amplification. We therefore provide the scientific community a second DMV genome representative of another major outbreak, the 2006-2008 epidemics in the Mediterranean Sea. Analysis of nucleotide sequence similarity confirmed that the two viruses are highly correlated. Phylogenetic analysis suggests that the DMV strain circulating during the 2006-2008 epidemics emerged from the 1990-1992 DMV strain. Interestingly, genome comparison revealed that the neglected L gene, although encompassing ~40% of the DMV genome, indeed includes 74% (35 out of 47) of the diversity (amino acid changes) that differentiate the strains of the two epidemics.

PO 05: Developing a potential biomarker set of miRNAs in packed RBCs to detect autologous blood doping

Anna Haberberger[1], Veronika Mussack[1], Benedikt Kirchner[1], Irmgard Riedmaier[1,2], Christian Wichmann[3], Raymund Buhmann[3], Georg Wittmann[3], Reinhard Henschler[3,4], Michael W. Pfaffl[1]

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Autologous blood doping provides an athlete with an increased red blood cell (RBC) mass after transfusion of his own packed RBCs, which results in enhanced performance as compared to a non-doped athlete. Nevertheless, it is also associated with serious health threats like stroke or pulmonary embolism. Although this kind of doping is prohibited by the WADA (World Anti-Doping Agency) since 1986 there is still no reliable test to detect this kind of doping. The hypothesis was that the miRNA signature of packed RBCs differ from fresh whole blood due to blood processing, buffer, storage temperature, and storage time. In this study, concentration was directed only on the increased miRNAs of stored RBC units on the aspect that only increased miRNA levels could be measured after transfusion.

To verify this hypothesis, three venous blood samples were taken from 12 healthy and athletic subjects before blood donation and from their blood bags at several time points during blood storage. Here, all 12 subjects were investigated using small RNA Sequencing, and analyzed using the DESeq2 algorithm.

The resulting data shows a significant increase in more than 120 miRNAs in the packed RBCs compared to fresh whole blood, which is due to preservative solutions and processing. A list of the Top 20 increased miRNAs without natural variation and with the greatest p-values was drawn up. This list distinguishes between samples freshly collected from subjects and their packed RBCs after processing.

The next step will be the discovery of changes during storage until week 6 to adjust the preliminary miRNA signature according to these storage changes und to test this potential miRNA signature in an autologous blood doping study.

PO 06: Different Isolation Strategies of Exosomal Extraction Kits Have A Major Influence On Small RNA Capturing And Expression Profiles in Clinical Settings

Benedikt Kirchner[1,2], Dominik Buschmann[1,3], Stefan Kotschote[4], Michael Bonin[4], Marlene Reithmair[3], Gustav Schelling[5], Michael W. Pfaffl[1]

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Extracellular vesicles in general and exosomes in particular have become an important object of research especially in biomarker development, due to the special role they and their cargo play in cell-to-cell communications. Since isolation via differential ultracentrifugation is time-consuming and labour intensive, commercially available extraction kits take advantage of various biochemical and physical properties to isolate exosomes from biofluids or cell-culture supernatant. Although all isolation strategies (e.g. membrane affinity, precipitation, size exclusion chromatography) offer their own advantages and disadvantages a thorough evaluation on their impact on downstream applications like NGS is still missing, especially in the context of clinically relevant settings or samples like liquid biopsies.

Extracellular vesicles were isolated from 1ml serum of healthy individuals and critically ill patients (n=10 each) using 4 different commercially available isolation kit alongside differential ultra-centrifugation (n=8). Total RNA yield and integrity were evaluated using capillary gel electrophoresis and holistic small RNA expression profiles were generated by NGS. Extracellular vesicle isolation kit-specific influences were assessed by comparing library size, sequence length distribution, unsupervised clustering and differential expression analysis between sample matrices as well as isolation strategies.

Total RNA yield differed greatly (p-value = 0.002) between isolation strategies with precipitation (4505 ± 3329 pg/µl) greatly outperforming size-exclusion chromatography (157 ± 197 pg/µl). Sampling from critically ill patients reduced RNA yield for all methods by a factor of 1.5 to 3.8 (p-value = 0.002). Even more striking differences were revealed by small RNA NGS. Although all isolation strategies were able to distinguish between samples from healthy and critically ill individuals to a certain degree, mapped miRNA expression profiles varied significantly.

A major impact on small RNA expression profiles could be shown for all EV isolation kits and strategies, respectively. Our findings highlight the importance of further optimization and standardization of exosomal isolation methods in differing sample matrices and special attention needs to be paid to obtain reproducible and comparable biomarker signatures from liquid biopsies.

PO 07: NEXTERA XT DNA Library Preparation on PIPETMAX

Laura B. Simdon[1], Seth A. Hanson[1], Brandon Converse[2], Brendan Keogh[2], Scott Monsma[2], Tristan J. Berto[1]

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Reliable and reproducible library preparation is critical for successful next generation sequencing. In this project we automated the liquid handling steps of the Illumina Nextera XT DNA Library Preparation Kit workflow and then constructed replicate libraries using either a benchtop liquid handler or manual pipetting performed by an experienced technician. Five automated scripts were developed, corresponding to the Tagmentation, Amplification, Library Cleanup, Library Normalization, and Library Pooling portions of the workflow. The PIPETMAX was equipped with two motorized, multichannel air displacement pipette heads that enable precise single or multichannel pipetting from 1 µL to 200 µL. The compact benchtop instrument permits up to nine bed elements to be employed during an automated script, including tips, microplates, tube racks, and accessories such as an on-bed orbital shaker. A custom rack was constructed to hold SLAS-footprint labware in the portrait orientation (as opposed to the landscape orientation on a standard PIPETMAX tray) and was used in the Amplification plate setup script to enable multichannel pipetting of Illumina TruSeq primers into the proper matrix. As part of the Library Cleanup and Library Normalization procedures, the Agencourt® AMPure® XP PCR purification system was automated using an on-bed magnetic bead separator rack. The PIPETMAX instrument can carry out all steps of the AMPure XP PCR cleanup procedure without manual intervention, including raising and lowering the magnets, resuspending paramagnetic beads, and transferring all liquids such as sample, wash solution, 80% ethanol and resuspension buffer. The automated AMPure XP script also allows the end user to modify several variables such as number of samples to process, sample volume, bead volume, number of wash steps, and incubation times. The wizard-style TRILUTION micro software interface provides real-time updates on the run status and produces run reports that help reduce errors associated with manual data entry. To assess the library quality for NGS libraries constructed using the automated procedure vs. manual liquid handling, twelve Nextera XT DNA libraries were constructed using PIPETMAX and an additional twelve were prepared using manual pipetting. Each library was prepared from 1 ng of *E. coli* genomic DNA. Library size was assessed using an Agilent BioAnalyzer. All 24 libraries were pooled and sequenced in one lane of an Illumina MiSeq instrument. Each library was downsampled to 312,500 reads, which yielded >8x coverage for all 24 libraries. Both library preparation methods generated high quality data with >95% mapped reads and optimal quality scores. The percentage of mapped reads was almost identical for libraries prepared with automated liquid handling or manual liquid handling. The libraries prepared with PIPETMAX exhibited smaller standard deviation and variance, consistent with the reproducibility of liquid handling on this system.

PO 08: SiMSen-Seq: Simple, Multiplexed, PCR-based Barcoding of DNA for Sensitive Mutation Detection using Sequencing

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The detection of extremely rare variant alleles is becoming increasingly relevant in many areas of clinical and basic research, such as oncology, prenatal diagnostics and transplantation medicine. Introducing molecular barcodes during NGS library preparation to uniquely tag target DNA allows the subsequent removal of polymerase errors incurred during library generation and sequencing. The SiMSen-seq protocol allows flexible, easy generation of barcoded libraries with minimal DNA input using two rounds of PCR and enables the detection of rare variants below 0.1% allele frequency. While the generation of libraries using barcoded PCR primers is an elegant, simple and fast solution, the detection of rare variants from complex matrices such as human plasma or serum necessitates highly optimized PCR assays and conditions. Here, we present major improvements to the original SiMSen-seq protocol. Through assay optimization and enhanced workflows the dynamic range and sensitivity to detect rare mutations are increased, while losses of target molecules are minimized at the same time. We outline suggestions for assay development to generate highly sensitive and specific assays to maximize library quality and recovery of target molecules. We also developed a software for de-barcoding and error correction from raw sequencing data, which delivers detailed information on molecule counts and variant detection. Thus, the optimized SiMSen-seq workflow facilitates highly accurate variant detection in challenging sample types such as liquid biopsies.

PO 09: TempO-Seq: High-Throughput Surrogate and Whole Transcriptome Targeted Gene Expression Analysis

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TempO-Seq® is a novel highly multiplexed targeted RNA expression profiling assay that makes extremely efficient use of existing NGS platforms for readout. Its unique attributes enable many high-throughput applications where RNA-Seq or other methods are too limited in performance or throughput, or are cost-prohibitive.

Sample input does not require RNA extraction/isolation, and may be lysates from cells or tissue, including FFPE, or purified RNA. The assay is robust to degradation or fixation, generating equivalent results even with these challenging samples. TempO-Seq achieves exceptionally high performance (reproducible differential expression, single-base selectivity, and single-cell sensitivity) by utilizing annealing and ligation steps without the limitations of reverse transcription or pre-amp using a simple add-only assay protocol. TempO-Seq generates a reduced complexity barcoded and pooled NGS library, is robust to sequencing errors, and minimizes necessary bioinformatics, supporting very high throughputs with 384+ samples multiplexed per flowcell lane. The capture-free assay is simple to perform manually in 96-well format with 1 hr hands-on using common laboratory equipment including any PCR/qPCR instrument, and may be automated.

Most importantly, the assay provides precise data with $\log_2 R^2 > 0.99$, average CVs < 15%, and essentially no background, which permits even small differences down to 1.2-fold ($p_{adj}<0.05$) to be accurately measured without interference from false positives and enables comparison between disease states, intercellular heterogeneity, or compound dose-response effects. Probe performance is independent of context within different pools, allowing panels to be customized without painstaking re-optimization while retaining the ability to compare results. Pre-validated assays can be supplemented with custom-designed isoforms, cSNPs, splice variants, fusion genes or other mutations.

We demonstrate TempO-Seq performance and cross-platform comparisons in a number of applications, including:

i) kits measuring ~2700 genes representing a human surrogate transcriptome assay (with which >50 samples can be run on a single MiSeq flowcell)

ii) the human whole transcriptome assay against 38,247 RefSeq IDs

iii) single-cell analysis

iv) degraded RNA and FFPE samples

v) non-human model species

PO 10: SIRV SUITE: RNA-Seq Quality Metrics and Comparison Platform

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Despite significant advances in transcriptome sequencing the majority of samples is processed without external standards. This hinders flawless comparisons between experiments and prevents whole RNA sequencing (RNA-Seq) to reach the next level and to develop into standardized analytical tools for clinical applications. Lexogen, a specialized transcriptomics company, addresses this problem by providing Spike-In RNA Variants (SIRVs) which condense the complexity of transcriptomes in a nutshell. Tiny amounts of SIRVs processed as part of the RNA samples allow for the evaluation and monitoring of RNA sequencing with respect to transcript isoform detection and quantification. However, because standards require standardized data processing Lexogen presents the SIRV SUITE, a new software package for SIRV data evaluation. It is easy to operate and can be integrated in every RNA sequencing pipeline. The SIRV SUITE computes the main quality metrics of gene expression measurements like coefficient of deviation, precision, accuracy and differential accuracy. The results are presented in overview figures, summarized in quality control forms, and saved in a data base. The linked metadata and “RNA-Seq fingerprint” SIRV quality metrics allow to systematically calculate the concordance of RNA sequencing experiments. Data can be compared to references on the bases of meaningful autonomous control subsets. The SIRV SUITE brings together spike-in derived NGS data, annotations and data evaluation in an easily navigable way.

PO 11: Suppression Before Amplification Of Highly Abundant mRNA To Profile Gene Expression From Microdissected Mammary Epithelial Cells

Claudia Bevilacqua[1], Nicolas Crapart[1,2], Cathy Hue-Beauvais[1], Barbara Brandao[1], Sophie Lemoine[3], Fanny Coulplier[3], Lucile Broseus[4], Patrice Martin[1]
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Laser capture microdissection (LCM) is a powerful tool to isolate homogenous cell populations from a heterogeneous tissue to analyze their expression profile. Due to the small amount of material collected in such a way, an amplification step has to be carried out before library preparation, to obtain a specific gene expression signature of microdissected cells using RNA-Seq. The main challenge of any amplification protocol is to preserve the relative transcript levels, enabling transcriptional levels and events to be faithfully represented. Today, several kits are available and each one is based on different amplification strategies. The quality (RIN value) and quantity of RNA to start from play an important role in the choice of the amplification kit to use. Moreover, RNA-Seq approaches on mammary epithelial cells (MEC) isolated by laser capture microdissection (LCM) showed that, at the peak of lactation, *ca.* 3/4 of the transcripts correspond to the 6 genes encoding the 6 main milk proteins: 4 caseins, a-lactalbumin and b-lactoglobulin (Canovas *et al.*, 2014). The objectives of this study was to evaluate the possibility to deplete the abundant transcripts prior amplification of cDNA for a RNA-Seq analysis to access very low expressed genes. For that we used Ovation Human FFPE RNA-Seq Library Systems (Nugen) based on the Inda-C technology. The RNAseq results were compared to that obtained with another kit based on the SMART technology (SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing, Clontech). The objective was to evaluate the impact of depletion on sensitivity and reproducibility starting from microdissected cells with a RIN value ranging between 6.5 and 7. Results show that the abundance of transcripts encoding the 6 major milk proteins in Nugen libraries depleted for both ribosomal RNA and milk protein transcripts was lowered to 3-4% of reads aligned on exons, compared to 75-80% in the SMART-seq library. In contrast, SMART-seq kit allows to obtain transcriptomic profiles starting from really small amount of RNA (1 ng) whereas 25 ng were necessary with the Ovation kit (Nugen).

PO 12: Implementation of a new automated Sample Quality Control tool in a Whole Exome Sequencing workflow

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Objectives: The German Cancer Research Center (DKFZ) is one of the largest biomedical research institutions in Germany. The High Throughput Sequencing Unit of the DKFZ Genomics and Proteomics Core Facility provides sequencing services for multiple applications.

This project demonstrates the use of an automated electrophoresis system as a quality control (QC) tool in a whole exome sequencing workflow. Mandatory for the experimental success of whole exome sequencing is the quality of the incoming genomic DNA (gDNA) material and the DNA samples at various stages of the library preparation workflow.

Methodology: Exome libraries were prepared according to the Agilent Low Input Sure-Select^{XT} Human All Exon v5 protocol from FFPE tumor tissue samples. The libraries were equimolar pooled. Each pool was sequenced on two lanes using the Illumina HiSeq 4000 System with 100 bp paired end sequencing. To ensure success quality control was verified with an Agilent 4200 TapeStation system of the received gDNA samples and during the library preparation.

Results: Intermediate QC steps were taken throughout the protocol to monitor library preparation for sequencing, such as evaluation of DNA after fragmentation, analysis of adapter-ligated and amplified DNA, and lastly, qualification of the final library. The initial QC of incoming gDNA was determined based on the DNA integrity number (DIN). All samples had a low DNA integrity, what is usual for DNA extracted from FPPE material. Due to the low quality of the DNA material, a modified fragmentation protocol was used. Modification of the fragmentation enabled to obtain meaningful sequencing results.

Conclusion: Quality control is an important part of NGS workflows, library preparation protocols recommend quantification and qualification of the DNA samples at various stages. The increasing sample throughput creates a need for automation especially in a core facility where many precious samples are proceeded with time pressure. The implementation of the automated electrophoresis system in the whole exome sequencing workflow enabled to increase the efficiency of the workflow and ensure good sequencing results.

PO 13: A SMARTer Approach to Profiling the Human T-Cell Receptor Repertoire Using the ICELL8 Single-Cell System

Sara Gonzalez-Hilarion[1], Sarah Taylor[2], Thomas Schaal[3], Jude Dunne[3], Maithreyan Srinivasan[3], Andrew Farmer[2]

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Profiling T-cell receptor (TCR) diversity is critical for understanding the adaptive immune system and can provide valuable insights in studies involving immuno-oncology, immune deficiency, autoimmunity, and vaccine response. While low-throughput approaches have yielded important insights concerning TCR repertoire dynamics, the development of next-generation sequencing (NGS) technologies has dramatically expanded the prospects for this research area. Using SMART® technology, we previously developed an NGS library-preparation method that employs a 5' RACE-like approach to capture full-length variable regions of TCR-alpha and/or TCR-beta subunits. In contrast with TCR profiling methods that use genomic DNA as starting material, our SMARTer® approach allows for analysis of expressed TCR sequences that are likely to encode functional receptors and utilizes single primer pairs for each subunit, thereby avoiding amplification biases associated with multiplex PCR. This approach was originally developed for RNA or bulk-cell inputs, requiring a minimum of 10 ng of RNA or 50 cells. In this poster, we present a modified library preparation protocol adapted for allowing high-throughput single-cell TCR-alpha/beta profiling using the WaferGen ICELL8 Single-Cell System. This approach, in combination with phenotyping, enables researchers to identify specific pairings of TCR alpha and TCR beta chains that comprise functional receptors in individual cells, and provides a starting point for classifying these individual T-cells based on function, maturity, and other complex parameters such as the timing of cytokine secretion.

PO 14: When the promises and perils of advanced molecular diagnostics unexpectedly go hand in hand: Genomic Analysis of Uterine Lavage Detects Early Endometrial Cancers and Reveals a Prevalent Cancer-Driver Mutation Landscape in Women without Cancer

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Introduction. Endometrial cancer is the most common gynecologic malignancy and incidence and associated mortality are increasing. Currently, there is no effective screening methodology or protocol for endometrial cancer. The Cancer Genome Atlas (TCGA) revealed many of the molecular defects that define this cancer. Based on these cancer genome results, and in a prospective study, we hypothesized that the use of ultra-deep, targeted gene sequencing could detect somatic mutations in uterine lavage fluid obtained from women undergoing hysteroscopy as a means of molecular screening and diagnosis.

Methods. Uterine lavage and paired blood samples were collected and analyzed from 102 consecutive patients undergoing hysteroscopy and curettage for diagnostic evaluation. Lavage fluid was separated into cellular and acellular fractions and cellular and cell-free DNA were isolated, respectively. Two targeted next-generation (NGS) gene panels, 56 genes and 12 genes, were used and orthogonal mutation validation was performed using dPCR and Sanger sequencing.

Results. 7 patients were diagnosed with endometrial cancer based on classic histopathology following their hysteroscopy procedure. 6 had stage IA cancer. All 7 patients had cancer driver gene mutations in lavage-isolated DNA and these mutations, above a specific allele frequency, were present in the matched tumors. Of the remaining 95 patients with benign/non-cancer pathology, 43 had no significant cancer mutations. Intriguingly, 51 patients had relatively high allele frequency, cancer-associated mutations. One of these negative histopathology cases returned 6 months later with stage IA endometrial cancer. Increasing age and post-menopausal status were significantly associated with the presence of driver/potential driver mutations.

Conclusions. Our study demonstrates that an NGS-based approach of genomic analysis of lavage fluid has the sensitivity to identify even early stage, microscopic foci of endometrial cancer. Moreover, this genomic approach identified one cancer patient, who originally had a negative histopathology diagnosis of tissue curettage, nearly 1 year prior to her returning with a positive histopathologic cancer diagnosis. Unexpectedly, we also reveal a prevalent landscape of cancer driver mutations in women without histopathologic evidence of endometrial cancer. Beyond identifying an opportunity to gain new insights into why some women with cancer driver mutations remain healthy and cancer-free while others develop endometrial cancer, these studies provide cautionary evidence of a Pandora's box in NGS-based cancer diagnostics.

PO 15: Influence of Genome Update and Methodology on Biomarker Discovery – RNA-Seq study in chicken

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In October 2016, a new assembly of the chicken genome, Galgal5, was released with 9000 new annotated genes (coding and non-coding). At that time, RNAseq data from a chicken study was analyzed with the former version Galgal4 to determine the mode of action of antibiotic in the intestine of birds, and evaluate potential biomarker(s). Therefore, it was necessary to reanalyze and compare the outputs with both genome versions for discovery of potential new target genes. In addition, two alignment applications, STAR Aligner and TopHat, were assessed in our methodology. Briefly, RNA was extracted from the ileum of chickens fed for 35-days either basal feed (control) or

supplemented with avilamycin (antibiotic). Five biological samples per group (with two technical replicates per sample) were used for RNAseq (Illumina HiSeq, stranded paired-end data, 30 M reads, 150 bp). Quality of the sequenced reads was evaluated in FastQC, and Trimmomatic was used to remove adapters and filter the reads. TopHat and STAR aligner were evaluated for alignments of the reads to the genome. To quantify the genes, Cufflinks was used. The final outputs were visualized and compared in Partek® Genomics Suite to obtain differentially expressed genes (DEG) (antibiotics vs. control using a 1way-ANOVA model, p-value with FDR < 0.05, fold change > 2 or < -2). The same pipelines were used to compare the two genome versions (reads mapped with either Galgal4 or Galgal5). The hierarchical clustering in Partek® was the same for the four final outputs (done with 2 aligners and 2 genomes). However, two biological replicates in the control had to be excluded for determining appropriately DEG between control and antibiotic groups. The total number of DEG per pipeline was as follows, Galgal4 in STAR = 139, in TopHat = 139, and Galgal5 in STAR = 142, in TopHat = 201. Different algorithms for the two aligners, and reannotations in the genome Galgal5 could explain the discrepancy in the results with TopHat. When using STAR aligner, 55 DEG were found in common between Galgal4 and Galgal5 (out of 139-142 DEG). Similarly, only 47 DEG were commonly found between genome versions with TopHat (out of 139-201 DEG). When comparing STAR aligner and TopHat with one version of the genome, 111 DEG were commonly found with Galgal4, and 128 with Galgal5. The resulting number of DEG in common across the four pipelines was 42. This shows that both aligners resulted in very similar outputs regarding DEG, but reads mapped with either Galgal4 or Galgal5 significantly affect the final results. A closer look revealed that many genes annotated in Galgal4 disappeared from the entry system in ENSEMBL database. Corrections done in the new assembly of the genome and the reannotation system greatly account for that. In conclusion, further downstream analysis to discover potential biomarker(s) should be performed with the results of Galgal5.

PO 16: Avoiding DNA adsorption to tube walls in NGS sample preparation

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Polypropylene (PP) is the ideal plastic material for (q)PCR tubes as PP is chemically inert, resistant to solvents and is well suited to injection moulding, allowing for (q)PCR tubes to be produced with very thin walls for fast heat transfer and therefore optimum (q)PCR results. Despite the very hydrophobic nature of PP, DNA has been shown to bind to PP tubes, especially in high ionic conditions. Different PP polymers can be used for producing PCR consumables, each of which have different characteristics. A particularly important characteristic of PP polymers is the surface charge, which influences binding of DNA in varying amounts.

Binding of DNA to PP is typically not an issue for (q)PCR as during amplification, any DNA stuck to the walls of the tube is released during the denaturation step, maintaining accessibility of the DNA for the reaction. This is, however, becoming more of an issue due to the progressing miniaturisation of reaction volumes and the development of new technologies such as Next Generation Sequencing (NGS). These techniques often use very small amounts of DNA and therefore require ultra-low DNA binding consumables to ensure specificity and consistency of the reaction.

4titude® Ltd. have developed both FrameStar® PCR plates and storage microplates that utilise a PP polymer with low binding characteristics which make them well suited for use in NGS and other sensitive techniques. No coatings are used to achieve the low binding characteristics of the products ensuring samples are safe from assay inhibition and degradation by leachables. Other options are available to reduce the DNA binding to tubes, including the use of polyallomer or siliconised tubes; however, these are not as readily available as PP and often demand a much higher price. Additionally, non-ionic detergents such as Triton-X and Tween 20 can be added to samples at a low concentration to prevent DNA binding to PP but not impact on qPCR reactions.

On comparison with low binding products from other vendors, serial incubation of a 1.1kb linear DNA fragment showed no significant loss of DNA in 4titude low binding plates, while some loss was seen from low starting concentrations in plates made of alternative materials or competitor low binding branded plates. Additionally, incubation temperatures of 4°C, 37°C and 65°C had no significant impact on DNA binding. 4titude low binding plates offer ideal properties for highly sensitive assays where low DNA input is vital and therefore any loss would have a significant impact on the success of the assay. Low binding plastics offer advantages not only for sensitive techniques such as NGS but also for the storage of samples and use in the forensic market.

GMO PO 17 – PO 21

PO 17: An Information System to Support Genetically Modified Organisms Analysis Strategies in Turkey

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According to the laws in Turkey, food crop products (cereal, oil seed, fruits and vegetables, etc.) have to be analyzed as genetically modification (GMO) by law. There are many laboratories that need to have a specific strategy about GMO analysis. In this project, our aim is to create Basic Information System for GMO Analysis to specifying GMO analysis strategy with using DNA based methods. With the basic information system of GMO analysis, laboratory and all parties who requests for analysis can easily create their own GMO analysis strategy. Basic information system for GMO analysis could be useful for laboratories and also provides optimization for risk assessment.

Molecular biology techniques used for fast, accurate, highly repeatable GMO content detection, identification and quantitation with multiple results are fundamentally divided into two groups; DNA-based analytical techniques and protein-based analytical techniques. The usage possibilities for traceability and control of DNA, RNA and protein isolation methods, PCR and ELISA techniques, DNA sequencing techniques, and finally microarray technique have been expanding increasingly. In terms of molecular biology, food, feed and seed samples with multiple components, not only plant or animal originated materials but also large and difficult materials. It's very important title of quick DNA, RNA or protein isolation from every sample, with high quality and quantity. Nowadays for GMO analysis; intensively used real time polymerase chain reaction technology (Real Time PCR, RT-PCR) have been accepted at national and international level and essential to provide quantitative results to be obtained. At the national level, there are many laboratories that need to have a specific strategy about GMO analysis. In this project, it's aimed to create Basic Information System for GMO Analysis to specifying GMO analysis strategy. For this purpose,

Approved GMO types in Turkey, GMO types confirmed by European Union GMO types and genes that can be analyzed in Turkey Statuses, analysis contents and locations of related laboratories At least one procedure for GMO analysis that has an international acceptance would be specified and placed on Information System of GMO Analysis. Under favor of Basic Information System of GMO Analysis, laboratory and all parts who requests for analysis can easily create their own GMO analysis technology. Usage of Fundamental Information System of National GMO Analysis would makes procedure unity between laboratories and also provides optimization for result assessment.

Acknowledgments: We are thankful to Hamideh Hammamchi, Gizem UYGUN, M. Türkan ŞAHİN who provided expertise that greatly assisted in the research.

PO 18: Identification and quantification of genetically modified organisms (GMO) from high throughput sequencing data

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We have explored the feasibility of detecting and quantifying genetically modified organisms (GMO) by high throughput sequencing (HTS) analysis. The rapid drop in sequencing costs can make GMO analysis by HTS affordable and cost-effective.

A GMO can be defined as an organism where the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination (EU DIRECTIVE 2001/18/EC). Thus, not all GMOs contain gene(s) from another species (trans-gene). The GMO soybean line DP-305423 is genetically modified to alter its lipid metabolism via RNAi, and the inserted sequences are solely soybean derived. This GMO can therefore be defined as a cis-gene. We sequenced a 10% (mass/mass) sample of DP-305423 in a non-GMO soybean background on an Illumina HiSeq.

A GMO is identifiable by the junctions between the artificially inserted sequence and the flanking host genome. Each read was mapped to a database consisting of the inserts and flanking sequences of 43 GMOs plus the soybean genome (RefSeq accession GCF_000004515.4). Only unambiguous hits with MAPQ ≥ 20 were considered.

We used expected frequencies to calculate the statistical significance of reads bridging a junction. The following equations were used to calculate the expected frequencies:

Eq. 1: $F_1 = 1 + (0.25)^{n \times 0.99} \times (l - n)$ *Eq. 2:* $F_2 = (0.25)^{m \times 0.99}$ and *Eq.* 3: $F = F_1 \times F_2$

where *n* and *m* is the length of the part of the read that can be mapped to one side of the junction and *l* is the length of the sample genome (here = 1.115 Gbp for soybean; *eq. 1*). To place the first part of the read, n, is equivalent the expected frequency of finding a random n-mer anywhere in the soybean genome plus 1, since we know that we have sequenced soybean and that the sequence is present in soybean (*eq 1*). When the first part is placed, is placing the second part, m, equivalent to place a m-mer at a specific location in the soybean genome (*eq. 2*). Total expected frequency of a random read bridging the junction is the multiplied frequencies (*eq. 3*). We decided to apply a frequency threshold of ≤0.01 to identify significant reads and thus identify a GMO.

Coverage ratios can be used to estimate the GMO concentration. Coverage over the entire insert can be used for a trans-gene where the inserted sequence is absent in the host genome. This is not possible for a cis-gene where the inserted sequence is present elsewhere in the host genome. The junctions, however, are unique and the coverage over the junctions relative to the coverage across the host genome can therefore be used to estimate the GMO content.

A total of 165,831,876 paired-end raw reads generated and mapped to the GMO/soybean database with BBMap (version 36.59) after QC. Twenty-one reads were found to bridge the 8 junctions in DP-305423 with a median expected frequency, F, of 1.8×10^{-39}. The GMO content was estimated to 10.8%, which is close to the certified assigned concentration of 10%.

PO 19: Optimized Analysis Procedure For Monitoring Transgenic Rapeseed Along Transportation Routes In Bavaria

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At present, no genetically modified (GM) rapeseed line (event) is authorized for commercial cultivation in the European Union, whereas some herbicide tolerant events are approved for their use as food and feed. Spillage of transgenic rapeseed during transportation to processing plants would be considered as an unauthorized release. The occurrence of transgenic rapeseed due to seed losses during transportation was already observed in several countries where cultivation or even import of GM rapeseed is banned. Therefore, the presence of transgenic rapeseed lines along transportation routes in Bavaria is being investigated within a project since 2014. For this purpose, rapeseed plants are sampled at sites where the probability of seed spillage is comparatively high. In 2016, the sampling locations comprised an inland harbor in Bamberg, the access roads of the only central oil mill of Bavaria situated in Straubing as well as the vicinity of decentral oil mills. In the initial analysis procedure, DNA isolation from rapeseed leaves was performed using an automated method, which was previously aligned to DNA extraction for leaves. Afterwards, an element-specific multiplex qPCR assay was applied to limit the number of potential events which could be identified in subsequent event-specific singleplex qPCR assays. Since this approach requires large amounts of DNA and high expenditure of time, DNA extraction method from leaf samples was optimized and five official event-specific singleplex qPCR assays were combined to a multiplex qPCR setup. The pentaplex assay should allow the simultaneous detection of transgenic rapeseed lines approved in the European Union for their use as food and feed: GT73, MON88302, MS8, RF3, MS8xRF3 and T45. Hydrolysis probes were labeled with different dyes (FAM, HEX, TEX615, Cy5, TAMRA). Fluorophores were chosen by consideration of their spectral properties. Overlapping of the emission spectra should be kept as low as possible to avoid crosstalk between the detection channels. Primer and probe concentrations were varied to determine optimized conditions for the detection of the five targets. The multiplex qPCR assay was validated by examination of the limit of detection, the specificity, the robustness and the efficiency of the method. The limit of detection was five copies per reaction for each target. The individual detection systems fulfilled the acceptance criteria for amplification efficiency and coefficient of determination. The multiplex qPCR assay showed high specificity to the targeted sequences and was robust to small deliberate deviations from the experimental conditions. The appliance of the method for the analysis of the sampled rapeseed plants turned out to be time-effective and led to savings in DNA input. The occurrence of transgenic rapeseed along transportation routes in Bavaria was not observed in 2016 as none of the 297 sampled plants was tested positive for GM rapeseed.

PO 20: Development Of Specific qPCR Assays For The Detection Of Genetically Modified Alfalfa J101, J163 and KK179

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Alfalfa (*Medicago sativa*), as one of the world's most important forage crops, was genetically modified (gm) in order to gain tolerance against the herbicide glyphosate (Round Up; events J101 and J163) or to alter the lignin composition of the plant (event KK-179). In 2015, about 13 % of the total alfalfa cultivation in the United States (US) was gm.

A recent study has shown the spreading of gm alfalfa on feral plant sites in the US, which might lead to a contamination of conventional alfalfa products, which are also exported to the European Union (EU). Biotech crops need to be authorized for import or cultivation in the EU and according to the non-tolerance policy, non-authorized gm crops may not be placed on the EU market. Biotech alfalfa is not deregulated in the EU and is therefore in the focus of official surveillance authorities.

Detection and identification of gm crops is currently performed using real-time PCR (qPCR) assays, targeting the junction sequences between the plant genome and the inserted DNA fragment (event-specific detection). Up to now, no qPCR assays for the detection of gm alfalfa and no reference material are available. Therefore, we designed plasmids as reference material and developed qPCR assays for the detection and identification of three alfalfa events based on patent sequence information.

The plasmids for events J101 and J163 contain the 5' and 3' junction sequence, a Col E1 origin and a *kanamycin resistance* gene. The KK179 plasmid contains the whole inserted DNA fragment and the 5' and 3' junction sequences, as well as a Col E1 origin and

an *ampicillin resistance* gene. Primers and hydrolysis probes were designed targeting the junction sequences of the respective alfalfa events. The assays were in-house validated using a 7900HT instrument and the Promega GoTaq Probe qPCR Master Mix. Probes were labelled with a FAM reporter dye and two quenchers (ZEN: internal quencher; IBFQ: 3' quencher). The following assay specifications were determined: optimal oligo concentration, limit of detection, robustness, efficiency and specificity.

Robustness tests indicate very stable assay performance after changing the master mix and after varying performance parameters (master mix volume ±1 µL, annealing temperature ±1 °C). No unspecific amplification signal was observed after analysis of DNA from different plant species and from other biotech crops. Analyses revealed a limit of detection of 5 copies/PCR for all assays.

A ring trial still needs to be set up in order to show the transferability of the assays and to verify the optimized PCR conditions. As soon as certified reference material for the three alfalfa events is available, the developed detection systems should be applied to verify their suitability with actual sample material.

PO 21: GMO Analysis of Soy Lecithin and Products Containing Soy Lecithin

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Lecithin is a natural lipid that can be found in all living cells and it is primarily obtained from plants that contain oils, primarily soybean. It is used for applications in food mostly as emulsifier, feed and pharmaceuticals. The raw lecithin is separated from the soybean oil, and further can be purified in several steps.

In practice, lecithin counts as a difficult matrix for DNA isolation. Using of the CTAB based DNA isolation protocol for lecithin in usual way, as it is applied for other samples routinely, will result in poor quality and concentration of DNA which would be inapplicable for real time PCR reaction and further genetic modifications (GM) analyses. The quality of DNA depends on the processing level to which raw lecithin was subjected.

Adjusting the isolation procedure to this type of samples brought positive results, and it was examined and confirmed by the several dozens of samples. The most successful results were obtained when the whole amount of the supernatant in first steps of isolation was used, while merely changes in sample weighs did not give the desired results. The DNA concentration isolated in such way was >10 ng/µl, and the purity was in acceptable range. The satisfying DNA isolate was confirmed with lectin (soy taxon specific gene) amplification curves detected in the real time PCR reaction.

Difficulties emerge in analyses of samples containing small amounts of lecithin, such as products supplemented with soy lecithin, as well as products that contain only a several percent of lecithin such as chocolates, cookies, wafers etc. Isolation of DNA from cookies containing soy lecithin as emulsifier, resulted in a good quality and quantity of DNA (>30 ng/µl), but lectins were not amplified in real time PCR reaction.

This problem has been overcome by product partitioning in the easily separable units which contain lecithin. Further, the isolation procedure was adjusted in the same manner as it was done for lecithin. From these separate product units, the sufficient quantity and quality of soy DNA was obtained, which was confirmed by amplification of lectins in real time PCR reaction.

Investigation and confirmation of above statements were performed on 42 samples of different products during 2016. All examined products contain less than 5% lecithin, as the only soy-derived component in them. In 40 cases (95%) it was possible to complete the GM analyses.

In conclusion, the expertise of analysts and a good knowledge of the principles and techniques of GM testing methods, enable adaptation of DNA isolation procedure to specificities of sample. Also, in the analyses of GM, in addition to the basic analytical principles prescribed by methodology, it is important to be familiar with nature of sample and its composition.

Optimisation & Standardisation PO 22 – PO 29

PO 22: Biocev GeneCore: More Than Just Gene Expression Profiling

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We present services of BIOCEV Gene Core - the best equipped core facility and service provider in the field of gene expression in Central Europe. We have broad experience in quality control (QC e.g. Fragment Analyser) in a single cell analysis (automated cell picking ALS Cellcelector), high-throughput and digital PCR (Fluidigm Biomark, BioRad QX200 Droplet Digital PCR System) and NGS library preparation.

We emphasise quality control, which is often neglected. Effective QC is based on the use of molecular tools to control contamination (RNA/DNA spikes), genomic background (ValidPrime) and quality of RNA (ΔAmp, RIN). We also take part in development of these methods to facilitate analysis of gene expression starting from bulk samples, down to the level of individual cells (direct lysis).

In addition to conventional qPCR analysis, we focus on single cells expression profiling and multi-analyte approach. Analysis of DNA/RNA/protein in parallel in one sample even on the single cell level provides comprehensive tool to map gene expression and characterizes types of cells and to determine the degree of differentiation and to study the pathological condition.

Currently we offer assistance with library preparations and experimental design of RNA-Seq experiments, which are key preconditions for a successful project.

PO 23: Automated, high-throughput quantitative PCR for accurate and economical copy number determination

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Copy number variation (CNV) has become a popular area of genetic research with an increasing number of studies highlighting the importance of CNV in relation to human health. One example in the field of pharmacogenomics is the cytochrome P450 2D6 (CYP2D6) gene. Deletions and duplications of CYP2D6 can impact the metabolism of many clinically relevant drugs, therefore making it a popular target for continued research. The IntelliQube® real-time qPCR instrument in conjunction with dual labeled BHQ® probe-based assays provides an accurate, high-throughputsolution for assessing CYPD26 CNV. Utilizing Array Tape® technology, the IntelliQube integrates liquid dispensing, thermal cycling, and detection with qPCR analysis in miniaturized reaction volumes. In this study, we assess the performance of custom BHQ qPCR assays run on the IntelliQube for determination of multiple copy number targets within the CYP2D6 gene. Accuracy and reproducibility of the platform was assessed using purified gDNA samples from cell lines obtained from the NIGMS

Human Genetic Cell Repository at the Coriell Institute for Medical Research. Copy number results were compared to previously published literature. The results demonstrate that the IntelliQube, when used in conjunction with BHQ probes, provides a powerful solution for accurate, automated, high-throughput CNV research and analysis.

PO 24: Comparison of Manual vs. Automated qPCR Reaction Plate Setup

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Quantitative real time polymerase chain reaction (qPCR) is a powerful technique for detecting differences in the copy number of nucleic acids in biological samples. qPCR techniques may be paired with reverse transcription and are used for applications such as transcript expression, detection of viruses, copy number variation (CNV), food safety testing and more. Each step in the extremely sensitive qPCR procedure can potentially contribute to experimental error. Accurate and precise pipetting can minimize sources of variability. In this work we compared qPCR results for reaction plates set up using manual pipetting or automated using Gilson PIPETMAX® qPCR Assistant v1.3. PIPETMAX is a robotic pipetting workstation with motorized multichannel PIPETMAN® technology accommodating volumes from 1 to 200 µL. The system supports many types of labware including as microfuge tubes, reservoirs, strip tubes, 96 well plates and 384 well plates. The software interface, TRILUTION® micro v2.3, runs on a tablet or PC and includes wizard-style assistants that automate common laboratory procedures. PIPETMAX qPCR Assistant automates plate preparation for qPCR, including preparation of master mixes, standard curve and sample dilutions, and reaction setup. The qPCR Assistant interface guides the user through experimental parameters including selection of controls, dilution factors, and number of replicates, and primer/template combinations. qPCR Assistant generates run documentation that can be incorporated into laboratory notebooks, including master mix recipes, a detailed pipetting guide, list of labware used in the experiment, and graphical representations of reaction plate configuration. Additionally, the software generates thermocycler run files that are compatible with all major real- time PCR instruments; this permits direct import of the sample information into the thermocycler and eliminates the need for data entry at that step, resulting in significant time savings for the user while assuring traceability of the data. qPCR reactions were prepared in 384 well plates (manually or using qPCR Assistant) to assess transcript levels in a universal cDNA as well as human cDNA samples from seven tissue types. Primer pairs were chosen from a panel of pretested assays for human reference genes. Transcript levels, as assessed by Cq value, were comparable for reactions set up using manual or automated pipetting. Variance was lower for reactions prepared on PIPETMAX. PIPETMAX qPCR Assistant fits into the qPCR workflow and provide standardized and reliable methods to help labs meet the MIQE publication requirements. This easy to use automation solution delivers reproducible automated pipetting, while reducing recordkeeping errors and user- to-user variability.

PO 25: Opening Pandora's box – unmasking the need for a continuous sample processing control for qPCR based fecal marker analysis

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PCR based techniques for the rapid detection of trace level DNA in environmental samples have gained considerable popularity. The method of qPCR itself has become a widely used routine technology and the challenges now centre on numerous limitations of the pre- and post-assay workflows. The aim of this study was the implementation of a detailed process control for the bacterial cell filtration, DNA-extraction and qPCR-performance characteristics of water samples from a habitat with a very diverse and changing matrix composition to provide a robust basis for confirmed interpretation of qPCR data. The aquatic habitat selected was Lake Neusiedl, located in the Eastern part of Austria, an important recreation area for the large urban area of Vienna. High and fluctuating levels of salt, humic acid, and suspended solids (TSS) characterize the water matrix of Lake Neusiedl. Therefore, this habitat represents a perfect model to study the basic performance characteristics of qPCR-based diagnostics on water samples with changing matrix conditions. The recovery efficiency through the processes of extraction and purification has only rarely been monitored for whole sample sets and when recovery efficiencies have been recorded they ranged from 1-100%. In our study we therefore added a filtration/extraction process control to each sample by spiking with a defined cell standard (DeTaCS). In addition, a strict evaluation of PCR-inhibition by using an internal amplification control and different dilutions for each sample was performed. DNA was extracted using a phenol-chloroform and CTAB based method. Results clearly demonstrated that inhibition of the PCR reaction played only a minor role in the samples investigated. In contrast, the presence of high amounts of (inorganic) TSS led to a complete loss of DNA during the process of DNA-extraction (i.e. recovery rates ≤1%). To test the hypothesis that the adsorption of DNA to surface-reactive matrix components during DNA-extraction was the causing mechanism, spiking experiments with different amounts of sodium pyrophosphate and salmon sperm were conducted in the laboratory. Addition of these adsorption site competitors helped to recover DNA concentrations to near control levels, however, with different effects on qPCR performance. Higher amounts of sodium pyrophosphate resulted in an inhibition of the PCR reaction. The results of the presented study impressively show the importance of using a stringent and continuous process control on a "sample-per-sample" basis. Using this approach we were able to unmask the challenges of diverse matrices of samples from a single habitat and could adapt the DNA-extraction protocol in order to get comparable qPCR results. We postulate that the herein observed challenges are rather the rule than the exception and thus propose the continuous use of a stringent filtration/extraction process control as basis for a robust quality management of qPCR-based environmental diagnostics.

PO 26: Influence of PCR consumables on the accuracy of real-time PCR experiments

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Classical PCR and qPCR plates are one-component plates made out of polypropylene (PP). PP is the best plastic material for PCR tubes as it is chemically inert and allows for the production of ultra-thin tube walls which is important for fast temperature transfer. While PP has become the standard material for PCR consumables some of its properties question its suitability for applications like qPCR and NGS:

The material characteristics of PP exhibit a Vicat Softening Temperature (VST) of 90°C and a coefficient of thermal expansion of 180×10^{-6} K^{-1} which are potential weaknesses for its usage at typical (q)PCR temperatures.

When used for (q)PCR, not only do the plates soften during the denaturation step, but measurements also show that the plates expand by up to 2mm in the diagonal plane (from room temperature to 95°C) and they shrink again as the temperature decreases. Therefore, the plate will undergo expansion and contraction in every cycle, placing significant tension on the plate seals. As a result, contact between the seal

and plate will be particularly weakened in the corner positions and outer rows leading to evaporation from the plate in these areas, while centre wells will only be affected minimally. This differential evaporation effect is especially eminent when adhesive seals are used (as opposed to heat seals).

Evaporation has a significant effect on the reaction conditions resulting in noticeable effects, especially for qPCR. Identical samples can exhibit significant differences in their Ct values, depending on their position on the plate. This often remains unnoticed as triplicates are typically placed in neighbouring wells which are affected by similar levels of evaporation.

A solution to the problem of evaporation related qPCR inaccuracies is the usage of two-component plates. These plates consist of tubes made out of PP but a frame made out of polycarbonate (PC). PC does not show significant temperature-dependant expansion and contraction as the VST of this material is 147°C and the coefficient of thermal expansion is $70x10^{-6}$ K^{-1}.

PO 27: Cell-free DNA assessment for clinical purposes: optimization of blood collection and DNA extraction methodologies

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Background: Assessment and characterization of circulating, cell-free (cf)DNA is a promising tool in various clinical settings, including prenatal diagnosis, detection of metastasizing tumors, and detection of rejection after organ transplantation. The reliability of this tool depends on the specificity by which the low-frequency genotype (either fetal-, tumor- or donor-organ-specific) can be quantified within the total circulating DNA pool of the host. Reliable assessment of cfDNA content first requires optimization of the protocol.

Methods: We collected blood of healthy volunteers in different anti-coagulant tubes (Heparin, Citrate, regular EDTA, EDTA-Streck, PAXgene), and incubated for several time periods (0h, 24h, 48h) before cfDNA extraction using the genomic (g)DNA blood mini kit or circulating nucleic acid (CNA) kit (both from Qiagen) from the plasma. Total DNA yields were determined by a Qubit fluorometer, and HCK gene copies were quantified by digital droplet PCR.

Results: The use of the CNA kit resulted in an average 2.1-fold higher cfDNA yield (~5.5 ng per mL of plasma) compared to the gDNA kit. The type of blood tubes used and prolonged blood incubation may cause release of gDNA from the patient's nucleated blood cells, thereby possibly affecting specificity in detecting low frequency targets. With Streck and PAXgene tubes, DNA yields remained constant between 0 and 48 hours blood incubation (1.2 ± 0.4 fold). The use of Heparin tubes resulted in the highest increase (24h: 16.4 ± 19.0 fold; 48h: 46.2 ± 70.8 fold), whereas EDTA tubes resulted only in a slight fold increase after incubation (24h: 1.7 ± 0.1 fold; 48h: 3.4 ± 0.5 fold).

Conclusion: The specialized CNA kit gives enhanced cfDNA yields and is preferred for downstream processing of blood plasma. Results for Streck and PAXgene confirm that these preservant-containing tubes prevent gDNA release from white blood cells. Regular EDTA tubes may offer a cost-effective alternative for cfDNA analysis of blood plasma, processed within 24 h, which already has been archived in laboratories.

PO 28: Compatibility of Magnetic Nanoparticles with Polymerase Chain Reaction

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Nanoscale materials have seen a rapid increase in interest for the area of biotechnology and biomedicine. An important class of nanomaterials is nanoparticles based on magnetic materials, which can be manipulated by an external alternating magnetic field. Owing to their exceptional properties they serve promising biomedical application such as a contrast agent for magnetic resonance imaging (MRI), drug/gene carriers for targeted drug/gene, therapeutic agents for hyperthermia, or tissue repair. The modelling and the mimicking of biochemical processes that combine nanoparticles is a new and interesting area of research. [1,2,3]

The aim of this work is to design and implement microscopic biochemical reactors, in the form of cell-sized capsules that can perform biochemical reactions such as the reproduction and amplification of nucleic acids *in vitro* through the polymerase chain reaction (PCR) and be controllable remotely in a contactless way. Magnetic iron oxide nanoparticles (MNPs) which act as the local source of heating by exposing to an RF alternating magnetic field are combined with the PCR mixture (DNA template, enzyme, primers, dNTPs, etc.) within core-shell capsules. In this specific contribution, we present an investigation of the compatibility of the MNPs with the PCR reaction components and identify the relevant conditions for effective PCR even in the presence of high concentrations of MNPs.

MNPs were fabricated successfully[4] with various types of functional groups including sodium citrate dehydrate ($Na_3C_6H_5O_7.2H_2O$), dextran 70 (from Leuconostoc spp., approx. Mw 70 kDa), 3-aminopropyl-triethoxysilane (APTES) and characterized in terms of concentration, size distribution, surface morphology, and radiofrequency heating ability. The response to the RF alternating magnetic field is proportional to the size and concentration of the nanoparticles. Results have shown that dextran coated MNPs were stable in the PCR reaction mixture even in high concentration while the remaining types formed aggregates. Future experiments are focused on dextran coated MNPs in sufficiently high concentrations to achieve the required PCR temperature by exposure to an RF alternating magnetic field.

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PO 29: Conception And Optimization Of A Detection Method Of Filovirus Species By Combining The Use Of Inosines And HRM Analysis.

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Our institute develops dual research to respond both to the armed forces and to the civilian uses. Different research programs are thus conducted in the field of Nuclear Biological Chemical (NBC) risks. Our unit is a core facility that offers various molecular biology tools, with the associated technical expertise. We also develop innovative research programs, linked to the NBC fields. The aim of this study was to develop an original method to quickly detect and identify viral species, in a small biological sample. More precisely, we focused on the detection of a Filovirus species, in a case of a suspect viral haemorrhagic fever. We have tried to design a pan Filovirus PCR primer pair which can detect all selected Filovirus species, by using "universal base", inosine or 5-nitroindole and the High Resolution Melting curves analysis. The different primers designed were then tested on a synthetic sequence.

The first step was to find the best zones on 5 Ebola genus species and 4 Marburg genus species to design these pan Filovirus primers. The

Filoviridae family presents a 19 kb genome, in the form of a linear single stranded RNA, that codes for seven genes. If the functional homology is strong, the nucleotide homology is quite low. Only 2 homology zones were found to be compatible with the design of universal primers. We have designed two pairs of primers (for an average size of 20 nucleotides), where 5 to 8 bases were replaced by a "universal base" : inosine or 5-nitroindole.

The second step was to test and validate these primers pairs. It has soon appeared that the primers containing 5-nitroindole did not allow any amplification. Several steps of optimization and adaptation were necessary to obtain a valid and reliable amplification for primers containing inosines. We began to test these optimizations on one species : ZEBOV, in order to get reliable amplification. Once an optimized condition was defined, we applied it to all the species. It appeared that the primer pairs containing less inosine bases gave better amplification than the other. This condition allowed to amplify all species, even if the Tm profiles were not perfectly reproducible for all of them.

In order to improve the reproducibility and the sensibility, another strategy was chosen : testing a semi nested PCR. It consists in a first PCR of 35 cycles to preamplify all matrices, followed by a second PCR on these diluted products. With this new strategy, we obtained an increased sensibility and a better reproducibility.

In conclusion, the use of inosine in PCR primers allows to amplify all selected Filovirus species. Further investigations are necessary to carry out this detection strategy.

Advanced Molecular Diagnostics PO 30 – PO 43

PO 30: Evaluation of SNPs based on DNA extracted from urine as part of the development and use of a kit of non-invasive biomarkers to monitor respiratory health of young children

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Respiratory health of children is among the priorities of environmental health programs. Both genetic and environmental factors are affecting the children's likelihood to develop respiratory diseases. Monitoring exposure, effect and susceptibility in children's cohorts, using biomarkers is of critical importance for health care management purposes, public health decision making, and primary prevention activities.

The most commonly used source of biomarkers is serum but it requires blood sampling, which for ethical and practical reasons is not feasible in most epidemiological studies involving young children. Therefore, using non-invasive biomarkers like nasal lavage fluid (NALF) or urine is a necessary alternative for monitoring their respiratory health. NALF reflects upper airways alterations found in nasal epithelium while changes occurring in the deep lung are only accessible through the urine as a source of protein biomarkers. Some studies have associated genetic polymorphisms to the development of hypersensitivity response or sickness. Furthermore, few prospective studies have investigated how epigenetics affected by environmental exposure influences the health response. Future biomonitoring studies should integrate all proteinaceous, genetic and epigenetic aspects to optimize the use of health biomarkers and consequently to reduce the potential risk of respiratory disorders for substrata of the population.

Our study involves an existing biobank of 850 non-invasive samples (NALF & urine) coming from 5-years old children linked with a questionnaire including data of environmental and lifestyle factors and respiratory health data. Currently, specific genetic modifications correlated with the development of asthma are being measured in the children's biobank. Following the DNA extraction from urine, real-time PCR, is being used for the detection of DNA polymorphisms (e.g SNP CC16 A38G). Statistical analysis will be performed to correlate frequencies of the SNPs with risks of allergic sensitization of children. In a second stage, protein biomarkers (e.g. CC16) related to respiratory health will be measured with the MRM-technology. Additionally, epigenetic modifications potentially related to adaptive response to environmental insults or to respiratory health will be explored. These will include gene methylation and the expression of miRNAs. Exosome isolation from NALF and urine will be explored in order to "concentrate" potentially unquantifiable or undetectable biomarkers.

The final goal of this project is to design a kit of non-invasive protein, genetic and epigenetic biomarkers for allowing a better survey and diagnosis of the respiratory health of children at an early stage. This information is necessary to support a pro-active public health policy with the aim of improving respiratory health of children, and hence of older people with emerging health disorders which are suspected to have their origin in young children.

PO 31: Modaplex, a novel technology for the multiplex analyses of CpG and miRNA signatures in a single qPCR run

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Background: As the hallmark of cancer, DNA methylation is known to be widely involved in the processing of cancer. microRNAs are a group of small RNAs which can moderate the expression of mRNAs, and thus involved in cancer related pathways. Although, there are currently several techniques available to analyse DNA methylation and miRNAs, most of them are based on singleplex measurements. The development of biomarkers using multiple DNA methylation and miRNA signatures calls for a reliable multiplex method that can analyse several DNA methylation sites and miRNAs in a single platform. Here, we present the Modaplex technique, a novel and sensitive multiplex technology, which allows for a simple and rapid interrogation of the methylation status of four CpG sites and the relative quantification of four miRNAs simultaneously in a single PCR run.

Methods: For relative quantification 1) a CpG multiplex assay including one control for highly methylated status and one bisulfite conversion control was designed to amplify methylated and non-methylated genomic DNA, and 2) an assay for amplifying miRNA as well as two reference miRNAs and one miRNA degradation control was developed. The universal run protocol allows both assays to be run simultaneously using the QIAGEN Modaplex Technology.

Results: The Modaplex Methylation Assay* was designed for relative quantification of the amount of methylated vs. non-methylated DNA for four relevant CpG sites. Assay capability to detect the methylation status was determined using bisulfite converted methylated and non-methylated DNA from human blood. The Modaplex miRNA Assay* is also a relative quantification assay, which determines the expression of four miRNA markers. In order to test the capability of the miRNA assay, miRNAs from cell lines and human plasma/blood were used. First performance characteristic studies involving amplification efficiency, and limit of detection of both assays were carried out on human sample material.

Conclusion: The discovery of an altered epigenetic status such as DNA methylation and miRNA expression during carcinogenesis might provide new markers for diagnosis. Development of a single platform that can simultaneously analyse multiple methylation sites and miRNA expressions with a fast turnaround time has the potential to facilitate diagnostical and therapeutical clinical research.

*Modaplex Methylation and miRNA Assays are for Research Use Only. Not for clinical diagnostic use.

PO 32: Molecular Diagnosis of Toxoplasmosis in Serologically Positive Pregnant Women

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Recent acquired *T.gondii* infection is a serious problem in pregnant women which present a high risk of *Toxoplasma* transmission to the fetus with serious damages. Serological diagnosis relies on the detection of anti-*Toxoplasma* immunoglobulin; however, serology may be unreliable especially when serological testing for IgG/IgM antibodies fail to differentiate between a recent and a past infection. In this case, we rely on PCR technique.

The purpose of this study was to compare serology assay and PCR using B1 gene for the early detection of *T. gondii* in pregnant women. Sera samples were collected from 85 pregnant women and measured by Elisa for specific IgG and IgM antibodies. DNA was extracted from 17 PBL samples and then amplified by PCR. A total of 41 out of 85 women was seropositive : 24 (58.53%) were IgG+/IgM- and 17 (41.46%) were IgG+/IgM+. PCR assay contribute to confirm toxoplasmic infection and detected *Toxoplasma* DNA in 4 women presenting IgG+/IgM+. All samples from control patients were negative. This study demonstrates the usefulness of the PCR in the diagnosis of toxoplasmosis pregnant women. This method may be suitable for routine screening of *T.gondii* infection in the clinical laboratory in association with serological tests.

PO 33: qRT-PCR Methods to evaluate the seasonal variations of relative expression of testicular LhcgR mRNA in adult male Libyan Jird (*Meriones libycus*)

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The real time quantitative PCR was used to investigate the seasonal variations of testicular LhcgR mRNA expression in desert rodent (*Meriones libycus*) during the breeding and non breeding seasons. We used the relative SYBR Green compared to endogenous gene beta actin expression method. This technique was used for the first time to evaluate gene expression level in this species. The relative *Lhcgr* mRNA expression level was higher during non breeding season ($2.10 \times 10^{-3} \pm 0.35 \times 10^{-3}$) than during the breeding season ($0.92 \times 10^{-3} \pm 0.15 \times 10^{-3}$), ($p<0.05$). Q.RT-PCR gave us the sensitive rate of gene expression to study the seasonal variations of LhcgR expression in Libyan Jird.

PO 34: Real Time PCR to Evaluate the Effect of the N-Acylsulfonamide Bis-oxazolidin-2-ones on *Toxoplasma gondii's* Infected Swiss Mice

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Toxoplasma gondii (T.gondii) is a significant cause of congenital disease and an important AIDS opportunistic pathogen. Current available drugs do not give satisfactory results and often have only a static and several adverse side effects. The need to develop and evaluate new drugs is critical. The purpose of this study is to investigate the *in vivo* effect of two new chiral N-acylsulfonamide bis-oxazolidin-2-ones (BOSP and BOSV) on *T.gondii*'s infected Swiss mice. The anti-T.gondii RH strain activities of the new molecules were investigated in mice after molecules treatment at different concentrations by using a real-time PCR-based assay for the detection and quantification of the parasite. Oligonucleotide primers and a fluorescence-labeled TaqMan probe were used to amplify the *T. gondii* B1 gene. *Toxoplasma*-specific DNA was demonstrated in all samples from infected mice. The parasite quantity showed that BOSP molecule has better effect ($0,78.10^3 \pm 0,27$) than BOSV one ($1,83.10^3 \pm 0,19$) but did not completely inhibit the parasite proliferation. The molecules inhibited the parasite in a dose dependent manner. The intensity of amplification products increased ($3,85.10^3 \pm 0,31$) when treatment started late after infection. These findings suggest continuous parasite replication despite the treatment. In conclusion, our results showed a promising treatment effect of the tested molecules. Otherwise, the present study revealed that concentration and duration of tested molecules treatment are major factors that influence the course of *Toxoplasma* infection in infected mice.

PO 35: A Quantitative Real-Time PCR For Pharmacokinetic Analysis Of The Immunotherapeutic TLR9 Agonist Lefitolimod

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Background: Lefitolimod (MGN1703) is a synthetic DNA-based TLR9 agonist that serves as an immune surveillance reactivator (ISR) in immune-oncology and infectious diseases. It is currently tested in several clinical trials, of which a pivotal phase III trial for first line maintenance treatment of patients with metastatic colorectal carcinoma is the most advanced. To analyze the pharmacokinetic (PK) profiles in different species during preclinical and clinical development processes a hydrolysis probe qPCR was developed and successfully employed.

Methods: A hydrolysis probe qPCR was developed and validated for lefitolimod, a dumbbell-shaped covalently-closed molecule with two identical single-stranded loops linked by a palindromic double-stranded stem and an overall size of only 116 nucleotides. To avoid variation in efficacy of sample preparation due to the short and covalently-closed DNA molecule, serum or blood samples of different species were used directly for qPCR without any purification or extraction steps. The small size of lefitolimod but also the identical single-stranded loops connected by a double-stranded stem with a palindromic sequence posed difficult challenges for qPCR. In addition possible negative influences from serum or blood components had to be avoided.

Results: Despite the above described challenges a reliable assay could be developed and validated. PK measurements of lefitolimod in animal toxicity studies and a phase 1 clinical trial with healthy volunteers (MGN173-C4) were performed. The quantitative determination of lefitolimod was possible with high intra-assay precision and accuracy. The inter-assay precision and accuracy was established by analyzing 6 QC sets together with a complete set of calibration standards at 3 different days. The results show that the determination of lefitolimod is possible with high inter-day accuracy and precision as well. Linearity is given between $1x10^{-10}$ g/µl and $1x10^{-14}$ g/µl (100 ng/ml – 0,01 ng/ml). In human serum samples from the MGN1703-C04 trial standard PK parameters could be determined: The mean C_{max} was 189 ± 101 ng/mL (range: 53.4 to 449 ng/mL) and the mean AUC_{0-t} was 5001 ± 2518 ng•hr/mL (range: 1264 to 10984 ng•hr/mL). Although there was a moderately wide range in the C_{max} and AUC_{0-t} values, the T_{max} and $t_{1/2}$ were similar for all subjects. T_{max} ranged from 8 to 16 hours,

with a median of 14 hours. The values for $t_{1/2}$ ranged from 9.36 to 17.9 hours, with a median of 12.7 hours.

Conclusions: The data show that the hydrolysis probe qPCR method is able to analyze PK parameters of the short, dumbbell-shaped and covalently-closed DNA-based TLR9 agonist lefitolimod from serum or blood samples of different species during preclinical and clinical development.

PO 36: Expression and Localization of the Thrombospondin Family Members During Corpus Luteum Formation and Function in the Bovine Ovary

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The objective of this study was to characterize the expression patterns and localization of the thrombospondin family members (THBS1, THBS2) and their receptors (CD36 and CD47) in bovine corpus luteum (CL). First, the corpus luteum (CL) was assigned to the following stages: days 1-2, 3-4, 5-7, 8-12, 13-16 and >18 of the estrous cycle and of pregnancy (month 1-2, 3-4, 6-7 and > 8). Second, the corpora lutea were collected by transvaginal ovariectomy before and 0.5, 2, 4, 12, 24, 48 and 64 h after inducing luteolysis by injecting a prostaglandin F2alpha (PGF) analog. The mRNA expression of examined factors was measured by RT-qPCR, steroid hormone concentration by EIA, and localization by immunohistochemistry. The mRNA expression of THBS1, THBS2, and CD47 in the CL during the estrous cycle was high, but decreased significantly during pregnancy. After induced luteolysis, thrombospondins increased significantly to reach the maximum level at 12 h for THBS1, 24 h for THBS2, and 48 h for CD36. The temporal expression and localization pattern of the thrombospondins and their specific receptors in the bovine corpora lutea during the different physiological phases of the estrous cycle and induced luteolysis appear to be compatible with their inhibitory role in the control of ovarian angiogenesis

PO 37: Gene Expression Signatures Of Bovine *In Vitro* Embryos Produced At The Air-liquid Interphase On Oviduct Epithelial Cells

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Ongoing development in the field of *in vitro* culture (IVC) methods for embryos is driven by increasing knowledge of the effects of IVC conditions on offspring. Besides morphological alterations, several studies have shown aberrant gene and protein expression by comparing *in vivo* and *in vitro* developed embryos. A novel embryo culture system where embryos are co-cultured without IVC medium on differentiated bovine oviductal epithelial cells (BOEC) is hypothesised to resemble more closely the *in vivo* situation than conventional IVC. In the present study, we evaluated the gene expression of development-related genes to analyse the effect of embryo co-culture with BOEC. Embryos developed under serum-containing *in vitro* conditions (IVC-S), in a commercial serum-free medium (IVC-SF), or in a co-culture system with BOEC (IVC-BOEC) until the 8-cell (n= 3 pools of five embryos per group) and the blastocyst stage (n= 6 per group), respectively. The gene expression of pools of 8-cell embryos, and single blastocysts was assessed using a 48.48 Dynamic Array™ on a Biomark HD instrument. Target genes were selected based on published transcriptomics and RT-qPCR data of developing bovine embryos. Clustering of the embryos by developmental stage was confirmed by principle component analysis, where there was no indication of a culture condition-induced effect. At each developmental stage, nine differentially expressed genes were detected by comparing IVC-S, IVC-SF, and IVC-BOEC embryos. At the 8-cell stage, the expression of CDH1 and NOS2 (4.8- and 1.8-fold upregulated in IVC-BOEC vs IVC-S, respectively) indicates an interaction of embryos with BOEC. The expression of APEX1 (2.1-fold upregulated in IVC-SF vs IVC-S) demonstrates the adverse effect of culturing embryos under serum conditions. This is in concordance with the expression of BAX (2.5- and 3.2-fold upregulated in IVC-BOEC vs IVC-S and IVC-SF, respectively), PLAGL1 (3.4- and 2.4-fold upregulated in IVC-BOEC vs IVC-S and IVC-SF, respectively), and SMPD2 (3.1-and 2.8-fold downregulated in IVC-SF vs IVC-S and IVC-SF, respectively). Blastocysts also displayed support of the BOEC, given the expression of CCL26 (2.6-fold upregulated in IVC-BOEC vs IVC-S), CDH1 (2.1-fold upregulated in IVC-BOEC vs IVC-SF), and NID2 (4.4- and 6.5-fold downregulated in IVC-BOEC vs IVC-S and IVC-SF, respectively). Similar to the 8-cell embryos, the serum conditions altered gene expression of genes involved in metabolism. In the blastocysts, this is reflected by differential gene expression of GLUT-5 (2.3- and 2.7-fold downregulated in IVC-SF vs IVC-S and IVC-SF, respectively), SREBP1 (1.9- and 2.3-fold upregulated in IVC-BOEC vs IVC-S and IVC-SF, respectively), and CYP51A1 (3.2-fold downregulated in IVC-BOEC vs IVC-S). Collectively, the findings show distinct gene expression patterns in response to the environment of the early developing embryo, underlining the plasticity of the embryo during this critical developmental phase.

PO 38: Deregulated Genes in Hematopoietic Stem Cells Isolated from Spleen and Peripheral Blood of Patients with Myelofibrosis

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An important issue in myeloproliferative diseases research is to test the hypothesis that the neo-angiogenesis, which is observed in spleen and bone marrow, involves endothelial cells derived from the myeloid, neoplastic clone (Rosti V. *et al.* Blood 2013). In this study we analyzed the gene expression profile in CD34+ hematopoietic cells from the spleen and peripheral blood of patients with myelofibrosis (MF) and healthy individuals (HI). The analyzed transcripts were selected from literature among those that characterize the neoangiogenic gene signature and those involved in deregulated expression pathways in MF.

By using quantitative reverse transcription-PCR, we measured the expression levels of 141 targets and 5 reference genes, in CD34+ cells isolated by immunomagnetic separation from the spleen and peripheral blood of 4 patients and 3 HI. The same genes were also analyzed in circulating endothelial progenitor cells (EPCs), which were isolated from peripheral blood of 4 MF patients and 4 HI and subsequently expanded in vitro. The lists of differentially expressed genes were analyzed through several tools (i.e. STRING, ENRICHR and GSEA).

The most significant upregulated genes in CD34+ splenic cells from patients, included transcripts (*GATA1, HBB, TAL1, GATA2, PTGS1*) belonging to the molecular signature of CD34+ cells isolated from the bone marrow (BM) of patients with chronic myeloid leukemia (Diaz-Blanco E. *et al.*, Leukemia 2007). The genes *GATA1, TAL1, ITGB3, GATA2* and *PF4* were also found overexpressed in a study designed to characterize the genes essential to the development of megakaryocytes (Tenedini E. *et al.,* Blood 2004). These transcripts are part of an expression pattern characteristic for immature stem cells as well as megakaryocyte-erythrocyte progenitor cells. Another group of transcripts overexpressed in patients spleens, which includes some of the above mentioned genes (*CD34, ANGPT1, PF4, GATA2, PTGS1*), has been observed in a comparison between circulating CD34+ cells from patients with MF, and CD34+ cells isolated from the bone marrow of HI (Guglielmelli P. *et al.,* Stem Cells 2007). Moreover, in partial disagreement with the initial hypothesis, a group of genes

implicated in vascular development (*FLT1, THBS1, FN1, CTGF, IL8, EPHA2, PROK2, LOXL2*) were found significantly underexpressed in CD34+ cells isolated from the spleen of patients compared to HI. For these reasons, we analyze the gene expression pattern in EPCs. These cells, although not belonging to the mutated clone, express hematopoietic specific transcripts linked to immunity and inflammation. We observed that the disease modifies their phenotype by increasing the expression of some genes, including *PTGS1*.

Our results point toward a model in which, in the spleen of patients with MF, an altered hematopoietic stem cell differentiation could induce an inflammation-mediated angiogenesis through the overexpression of *PTGS1* in cells of the splenic tissue.

PO 39: A Microbead-based Assay for the Real-Time Detection of Multiple HPV Genotypes

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Background Human papillomaviruses (HPV) infect skin cells and mucous membranes and usually cause a local cell proliferation after a successful infection. An infection with low-risk type HPV leads in most cases to benign proliferations such as epithelial cysts or skin warts. High-risk types cause lesions that may lead to the progression of malignant tumors (e.g., cervical cancer). Others have shown that HPV-DNA can be found in about 90% of all cervical carcinomas. In current diagnostics, viral DNA is detected in patient samples using the polymerase chain reaction (PCR) or DNA-hybridization assays.

Objective: A multiplex quantitative PCR system, which can used determine the type and approximate viral load level of HPV infections might be a valuable tool in diagnostics and fundamental research. We aimed to develop a multiplex quantitative PCR assay based on microbead encoded targets for the detection and quantification of HPV. The possible benefits are an increased sample throughput while reducing costs and effort.

Methods A primer/probe system for the detection of HPV was established for the microbead-based quantitative PCR of the VideoScan platform. Negative and positive controls were integrated into the assay to improve the reliability. Furthermore, the absolute quantification of HPV copies in patient samples was accomplished with the QX200 Droplet Digital™ PCR (ddPCR) system from Bio-Rad®. All data were analyzed using the *chipPCR* [1] and *MBmca* [2] packages for the R statistical computing language as described elsewhere [3,4].

Results and Conclusion For the HPV detection, a short conserved region (65 bp) of the L1 gene was appropriate. An absolute quantification of virus copies was made ahead of the assay establisment through a ddPCR system. The quantification and differentiation of the HPV 16 and HPV 18 genotypes was possible on the basis of sequence differences between specific hydrolysis probes, both in the liquid phase and on the micorbead-surface. An internal amplification control (Hypoxanthin-Guanin-Phosphoribosyltransferase 1) and an external extraction control (β-globin) were integrated in the assay and could be used as reliable assay controls (100% recovery rate). With this assay it was feasible to detect five different targets in one reaction. Since we used two distinct microbead populations per target we achieved a quasi ten-plex assay.

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PO 40: VitiPatho a qPCR-Method for the prediction of thresholds for fungizide application.

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Plasmopara viticola is a strictly biotrophic oomycete that causes downy mildew, which is one of the most important grapevine diseases. Control of the disease is most often achieved by fungizide application, which may have severe environmental consequences. To optimize the use of fungizide the „State Institute for Viticulture and Oenology of Freiburg“ is offering predictive meteorological data via a system called „VitiMeteo“. In order to obtain insight into the dynamic of host responses to grapevine downy mildew (*Plasmopara viticola*), we in addition developed a monitoring system for air-borne fungal pathogens „VitiPatho“. Spores of *Plasmopora viticola* were sampled using a modified micobiological air samling system MBASS30. 100 Liter of sampled air were collected directly into a 200µl PCR tube containing lysis buffer. Samples were then stored on ice for further treatment in the lab. After lysis samples were diluted and specific target sequences were amplified by qPCR using a Taq-polymerase from myPOLS, kindly provided by Ramon Kranaster. The PCR-reaction was checked for specifity and linearity. Relative quantification was calculated on the basis of the obtained ct-Values during the growing season in 2016.

Results:

1. *Peronospora viticola* infection could be detected as early as end of May (5 to 6 leaves) on the vineyard „Wohnhalde“.
2. The onset of *Peronsopora viticola* infection on the vineyard of the „Lorettoberg „ was about two weeks later in comparison to the location „Wohnhalde“.
3. Measured DNA from collected air samples of *Peronospora viticola* increased during infection by a factor of 1000.
4. During the Peronospora infection, the protection of plants by different fungizide could be visualized by low signals of *Peronospora viticola* DNA compared to the infected plants at the same vineyard investigated.

PO 41: A Novel High-multiplex Homogeneous PCR Assay Format

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Multiplex PCR has become an increasingly popular method to provide more, clinically relevant answers from the same sample. In many clinical settings, achieving multiplex answers from the same sample provide benefits both in terms of cost, speed, added clinical value as well as preservation of limited samples. In cases such as sepsis, RSV-testing, gastrointestinal testing and many others, a broad spectrum of agents are relevant for testing to assess possible infections. However, PCR readout is commonly limited to the current maximum of 4-5 fluorophores on most instruments. We have developed MeltPlex - a homogenous assay method to allow read-out of more than 20 answers from a single PCR reaction.

Material/methods: MeltPlex utilizes a system of labelled probes allowing each to be read out by subsequent melting curve analysis by more than 5 probes per fluorophore. By utilizing meltcurve readout of modified probes – one for each target - rather than the only amplicons, the system adds an extra level of specificity to meltcurve analysis. Reaction and melting analysis is performed without the need to re-open PCR reaction tubes.

Results: We will present proof-of-principle of the method and demonstrate its relevance in routine clinical infectious agent testing.

Conclusions: MeltPlex comprise a robust, high-multiplex, homogeneous system to provide 20+ readouts per PCR reaction.

PO 42: Detection Of *EGFR* Mutations In Cell-Free DNA From Patients Affected By Lung Cancer: Prenalytical And Analytical Aspects

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Lung cancer is the leading cause of cancer death worldwide. EGFR mutations are driver oncogenic mutations against which EGFR tyrosine kinase inhibitors (TKIs) represent an effective treatment. Recently AIOM guidelines indicated cell-free DNA (cfDNA) as a surrogate for the determination of EGFR status in patients without available tumor sample.

Our aim is to evaluate different approaches for the identification of EGFR mutations in cfDNA from lung cancer patients in view of a clinical application. In particular we focused on mutations associated with sensitivity to EGFR TKIs, such as deletions in exon 19 and the mutation p.L858R in exon 21, and on the mutation p.T790M in exon 20, responsible for resistance to treatment.

We considered the preanalytical phase by comparing two methods, manual and automated, for cfDNA extraction: the automatic procedure showed a better yield than the manual.

We compared the analytical performances of two methods based on real-time quantitative PCR (qPCR) with those of a digital PCR (dPCR) approach. From the results obtained on reference standard samples at known percentages of mutated alleles dPCR appeared as the most sensitive method, being capable of detecting L858R mutation and a deletion in exon 19 in a sample containing 0.1% mutated DNA.

Preliminary data on patients affected by lung cancer showed concordant results between the two qPCR methods. Some discrepancies in the mutational status of EGFR were found between tissue and cfDNA.

Upon optimization and standardization of methods, non-invasive analysis of cfDNA might replace biopsy for a precision medicine approach in lung cancer.

PO 43: Allelic Frequency of a 24-bp Duplication in Exon 10 of the CHIT1 Gene in the General Iranian Population by HRM Analysis

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Background: The human chitinase chitotriosidase enzyme, which is encoded by the CHIT1 gene, is produced by macrophages and may be important in immune responses to chitin-containing organisms, such as fungi. Plasma chitotriosidase activity is used to diagnose and monitor some forms of lysosomal storage disorders, such as Gaucher disease. This metabolic disorder is an autosomal recessive lysosomal storage disease. Clinical manifestations of the disease include hematological and skeletal complications including bone pain (BP) and bone crisis, medullary expansion, osteopenia, osteolysis, osteonecrosis, and pathological fractures. However, homozygous duplication of a 24-bp region in exon 10 of the CHIT1 gene eliminates enzyme activity and may complicate disease monitoring. The high prevalence of this mutation highlights the need to determine its frequency in different populations and screen patients for this mutation in order to verify whether chitotriosidase activity is a reliable marker of lysosomal storage disease. This study investigated the allele frequency of the 24-bp duplication in the general Iranian population. *Methods:* To identify the 24-bp duplication in exon 10 of the CHIT1 gene (H allele), genotyping of DNA extracted from peripheral blood leukocytes of 577 healthy Iranians was performed using PCR amplification and High Resolution Melting (HRM) PCR techniques. *Results:* In this study, heterozygous and homozygous duplications were detected in 183 (31.7%) and 35 (6.1%) subjects, respectively. In addition, the allelic frequency was 21.9% (95% confidence interval). *Conclusion:* Our study indicates that genotype analysis by HRM-PCR is a fast, reliable, and highly accurate screening approach for identifying the 24-bp duplication in CHIT1 exon 10. Due to the wide range of duplication frequencies among different ethnic groups, new biomarkers are necessary for assessing genetic characteristics of lysosomal storage disorders in different populations.

qPCR data Analysis PO 44 – PO 46

PO 44: Development of an R Package for qPCR Data Management with the Real-time PCR Data Markup Language

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Objective Real-time quantitative PCR (qPCR) is one of the most widely applied methods in molecular biology, diagnostics, forensics and genetical testing. The guideline for Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) [1] was established to facilitate the comparison of experimental results obtained from qPCR. The MIQE guidelines suggests the Real-time PCR Data Markup Language (RDML) as the standard qPCR interchange data metadata and values between applications [2]. RDML is a vendor independent and freely available file format. It is based on the eXtensible Markup Language (XML).

Aim and methods The cross-platform statistical computing language R is the de facto standard in applied statistical bioinformatics, provides comprehensive tool-sets for reproducible research and is suitable for standalone desktops or servers. Although there are R packages for qPCR and melting curve analysis available, it was not previously possible to seamlessly process RDML files [3].

Results We developed the cross-platform open source RDML package for the statistical computing language R. RDML is compliment to RDML ≥ v.~1.2 and provides functionality to (i) import RDML and native data from various PCR machines vendors; (ii) extract sample information (e.g., targets, concentration); (iii) transform data to various formats of the R environment; (iv) generate human readable experiment summaries; and (v) to create RDML files from user data. In addition, RDML offers a graphical user interface, designated rdmlEdit, to edit RDML files and perform simple manipulation of the PCR curves like background subtraction and Cq calculation. rdmlEdit can be used as tool from a browser or integrated development environments such as RStudio. RDML has import functionality for additional file formats such as comma separated values. Since the software is based on R, all available packages can be combined with function from the RDML package.

Conclusion The RDML package is a tool that can be used from the command-line to design advanced analysis pipelines. Moreover, the implemented graphical user interface enables the users to perform rapid management of their qPCR data. The RDML package is available at https://cran.r-project.org/package=RDML

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PO 45: A comparison of nonlinear fitting methods to raw qPCR amplification curves: performance, caveats and implications for *Cq* and *E* estimation.

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In the last decade, several algorithms have been developed for the estimation of quantification cycles (*Cq*) and amplification efficiencies (*E*) that employ whole-curve fitting of the qPCR amplification trajectory (compiled in [1]). In the majority of cases, this is conducted by *ad-hoc* fitting of four- or five-parameter sigmoidal models (e.g. logistic, log-logistic, Boltzmann, Richards) that can or cannot account for curve asymmetry around the “point of inflection” [2]. However, the scientific literature provides a plethora of nonlinear models that have not found their way into qPCR analysis or are not yet implemented in routine software, such as six-parameter (incorporating linear baseline shift) and seven-parameter (incorporating quadratic baseline shift) models, linear-exponential (Spiess, unpublished) and bilinear-exponential [3] hybrid models, as well as recursive growth models [4]. Non-parametric alternatives also exist, such as exact or smoothing splines [5]. In the application of these models, it is essential to use Information Criteria (AIC, BIC) to estimate the goodness-of-fit, as the increasing number of parameters inherently results in improved (over-)fitting [6]. In addition to standard nonlinear fitting, weighted fitting can dramatically improve fit quality, because the increasing dispersion of data points in relation to their magnitude (heteroscedasticity) can be compensated by giving less fitting weight to curve regions displaying high variance, e.g. baseline and plateau phase. This weighting compensation can also be included in the fitting process by iteratively estimating the optimal weighting parameters (generalized nonlinear models). All the above methods employ nonlinear least-squares, which minimizes the sum-square of *vertical* residuals. Along these lines, we also investigate the rarely employed but highly important method of orthogonal nonlinear least squares [7] which exhibits optimal fitting in the steep slope region of the qPCR curves by minimizing *orthogonal* residuals. For all these methods, we show their performance, advantages and drawbacks on high-numbered technical replicate datasets, especially with respect to the elimination of system-intrinsic *Cq* value periodicities previously identified by us [8].

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PO 46: The Role of Amplification Efficiency and its Uncertainty in Assessing Relative Expression by the $\Delta\Delta C_q$ Method: A Nonlinear Least-Squares Approach that Overcomes Deficiencies in a Widely Used SAS Program

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Relative expression ratios are commonly estimated in real-time qPCR studies by comparing the quantification cycle for the target gene with that for a reference gene in the treatment samples, normalized to the same quantities determined for a control sample. For the "standard curve" design, where data are obtained for all four of these at several dilutions, nonlinear least squares can be used to assess the amplification efficiencies (AE) and the adjusted $\Delta\Delta C_q$ and its uncertainty, with automatic inclusion of the effect of uncertainty in the AEs. A simple algorithm is illustrated for the KaleidaGraph program and used to illustrate flaws in an SAS program that has been used for this purpose in the last decade.

Exosomes & non-coding RNAs PO 47 – PO 53

PO 47: Urine microRNAs as potential diagnostic and prognostic biomarkers of urinary bladder cancer

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Bladder cancer is the most common cancer of the urinary tract. More than 90% of bladder cancers are urothelial carcinoma, which are divided into non-muscle-invasive and muscle-invasive forms. Non-muscle-invasive tumors frequently recur (50-70%) and can also progress to invasion form (10-15%). These patients are monitored by cystoscopy and may have multiple resections over many years. Improved monitoring method is needed, ideally via urine analysis, which could reduce the morbidity and costs associated with long follow up. Currently there is no molecular biomarkers which could diagnose or accurately predict disease progression. We aimed to develop a clinically applicable, specific and sensitive panel of urine microRNAs enabling detect bladder cancer and predict risk of progression to muscle-invasive form.

Within the exploratory phase of study we have analyzed expression profiles of 1733 miRNAs in urine supernatant of 16 bladder cancer patients (6 muscle invasive, 5 high-grade muscle non-invasive, 5 low-grade muscle non-invasive), 17 controls, 10 RCC patients and 4 urinary tract infections (UTI) using Affymetrix miRNA microarrays. Diagnostic and prognostic potential of selected microRNAs was further validated on independent samples in training phase (50 bladder cancer patients, 15 controls) and validation phase (100 bladder cancer patients, 55 controls, 45 renal cancer patients) using specific TaqMan assays and qRT-PCR method.

Global expression profiling identified set of 76 miRNAs able distinguish bladder cancer patients from healthy controls ($P < 0,01$), thereof 64 highly up-regulated and 12 down-regulated. Moreover 23 miRNAs were able distinguish invasive and non-invasive forms of UCUB ($P < 0,01$) and 18 miRNAs high-grade and low-grad non-invasive ($p < 0,01$). Set of 12 miRNAs with highest expression level and statistical significance was validated in training phase of study. Based on the results the panel of three miRNAs (miR-31, miR-93, miR-191) was profiled. In validation phase we confirmed diagnostic potential and ability of this urine miRNA-based panel to diagnose patients with bladder

cancer with high sensitivity and specificity (AUC = 0,8794, sensitivity = 82%, specificity = 80%).

Our data have shown that urinary microRNAs could serve as sensitive and specific biomarkers of urinary bladder cancer and could be useful tool to increase sensitivity of standard cytological examination and reduce costs associated with long-term follow-up of bladder cancer patients. This work was supported by Ministry of Health of the Czech Republic, grant nr. 15-31071A, 15-34678A and CEITEC 2020 (LQ1601). All rights reserved.

PO 48: Is Quantitative microRNA Expression Profiling Using Fluidigm Microfluidic Dynamic Arrays Reliable? Taqman microRNA Assay Evaluation.

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High-throughput profiling methods are being used to save biological material, time and hopefully also money. During our experiment we used two high-throughput instruments to measure microRNA expression: 3D-Gene (Toray) for a discovery phase of rectal cancer biomarkers search and BioMark, a high-throughput qPCR instrument from Fluidigm, for the validation phase. During the initial search for a reliable microRNA system, we excluded Exiqon platform due to its exclusive use of SYBR green chemistry. The SYBR green is not compatible with microfluidic instruments. There were several articles supporting use of Taqman microRNA system together with BioMark, therefore we decided to take this direction. Here we describe how we evaluated probes from Life Technologies to be reliable for BioMark. Twenty patients were sampled for rectal tumour tissue and adjacent healthy rectal tissue. Using 3D-Gene microRNA array, 2000 microRNA were screened for differential expression. Seventy one candidate microRNAs were selected for next, validation, phase of experiment. None or very late amplification (Cq > 45) was detected using 23 Taqman microRNA probes. There might be two reasons for the lack of amplification. One is that the probe does not work, which was tested by temperature gradients. The other reason can be limited correlation between microarray system and qPCR that is reported in the literature. The correlation can differ from 0.48 to 0.94. Other 13 assays provided very late amplification (Cq > 36). All these mentioned probes were excluded from further validation. The second half of Taqman microRNA assays were evaluated. The assay precision, efficiency, limit of quantification were measured. Because only limited amount of biological material can be used for high-throughput experiment, pre-amplification is required. This additional step was also evaluated. Finally, we ended up with several variables for each assay that had to be compared. Principal component analysis help us to identify 21 reliable microRNA assays. In summary, the assay evaluation revealed that 70% of obtained Taqman microRNA assays could have not been used for BioMark validation phase. However, 21 approved assays (30%) enabled us to get consistent and reliable results that were obtained from subsequent experiments.

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PO 49: Analysis of microRNA isoforms (isomiRs) in Galaxy and their differences in expression levels

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In general, the analysis of small RNA-Sequencing (RNA-Seq) data is more challenging compared to standard RNA-Seq. Moreover, an additional problem when working with livestock species, such as the pig, is that the annotation of small non-coding RNAs (ncRNAs), e.g., microRNAs (miRNAs), is far from being complete. In humans, a great variety of ncRNAs including miRNAs are known, which can be used as orthologue information for the annotation of sequences derived from other mammalian species. This helps to increase the percentage of annotated miRNA sequences and their various isomiRs. IsomiRs are miRNA variants that differ at the 3' and/or 5' end, which results from imprecise and alternative cleavage during the pre-miRNA processing and post-transcriptional modifications represented by added or deleted nucleotides. These different isomiRs can exhibit different stability, sub-cellular localization, and can have a different target spectrum.

This study introduces a data analysis pipeline to filter, annotate, and detect miRNAs and their different isomiRs. The workflow is mainly based on standard Galaxy tools and a number of in-house scripts. Well-annotated related species information was used to improve the annotation of each sequence found in small RNA-Seq results. The pipeline consists of basic analysis steps to check for quality, filtering, and clipping the adapter sequence. Afterwards all sequences were collapsed to unique sequences and the corresponding read counts. These reads were mapped with BLASTn-short to align them to all transcripts of our sequenced species, including ncRNAs and related well-annotated species. The collection of BLAST databases contained sequences from mirBase (precursor and mature miRNAs), sequences from NCBI and Ensembl, including ncRNAs and protein-coding transcripts, as well as tRNAs and piRNA cluster sequences. All BLAST results were filtered and joined by removing duplicated hits, which resulted in ~45000 mapped sequences out of ~68000 unique filtered sequences. The annotated sequences were further analyzed for isomiR differences. To do that, 3' end and 5' end modifications were detected and analyzed by type. Finally, expression levels were compared within isomiR groups and towards the corresponding mature miRNA. Using this annotation pipeline, 1581 unique sequences could be assigned to 266 different miRNAs. The highest expression levels were found for the canonical miRNA form (mature miRNA) (~70%) within the miRNA groups including the mature miRNA (172 group members), followed by isomiRs with a 3’ end modifications with (~23%). In groups with isomiRs without the presents of the canonical miRNA (94 group members), the 3’ end modified isomiRs had the highest expression levels with 74%, and 21% at 5’ end followed by 5% on both sides.

PO 50: Development of miRNA Profiling Capacity Using Reverse Transcription–Quantitative PCR (RT-qPCR) by Prestizia, A miRNA Platform For Biomarkers Signature Discovery

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Context and objectives: The discovery of microRNAs (miRNA) in body fluids paves the way for the characterization of novel non-invasive biomarkers. The relative abundance of those miRNA can be quantified thanks to several platforms and technologies. However the final validation of their expression uses the RT-qPCR method. The biomarker discovery nowadays is based on high-throughput pipelines, and for miRNA, a commercial solution is available with the Life Technologies TaqMan Array Human MicroRNA cards A and B. Although the two cards provide 760 miRNA, more than 2,000 are now described in the literature and specific databases. Our aim was to increase the capacity of miRNA profiling by designing new custom cards. In parallel we developed a standardized workflow to analyze simultaneously the data provided by both the commercial and custom cards.

Methodology: The design of the new custom cards was achieved by using the miRBase information. To validate the newly designed cards, we used the same set of standardized positive control (human tissues RNA and human serum pool) and negative control. This allowed us to determine and assess the detection capacity, reproducibility, sensitivity, differential expressions and specificity. Finally we tested matched serum and whole blood samples from different donors.

Results: The detection capacity was first tested on a large number of new miRNAs (4 custom cards with a total of 1,440 miRNAs): 516 miRNAs were detected and were subsequently used to design a second version of two custom cards “C and D”. Analytical performances of

these two custom cards were compared to the commercially available A and B cards.

The aim of miRNA expression profiling studies is to quantify differences between sample groups; we showed that we were able to quantify differentially expressed miRNAs between two sample types.

Conclusion: Finally, we have successfully validated 2 new cards doubling the high-throughput profiling capacity by RT-qPCR from 760 to 1,300 miRNAs.

PO 51: Two-tailed RT-qPCR: a Novel Method for Highly Accurate MiRNA Quantification

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MicroRNAs are a class of small non-coding RNAs that serve as important regulators of gene expression at the posttranscriptional level. MiRNAs are stable in body fluids and pose great potential to serve as biomarkers. Here, we present a highly specific, sensitive and cost-effective system to quantify miRNA expression based on two-step RT-qPCR with SYBR-green detection chemistry called Two-tailed RT-qPCR. It takes advantage of novel, target-specific primers for reverse transcription composed of two hemiprobes complementary to two different parts of the targeted miRNA, connected by a hairpin structure. The introduction of a second probe ensures high sensitivity and enables discrimination of highly homologous miRNAs irrespectively of the position of the mismatched nucleotide. Two-tailed RT-qPCR has a dynamic range of 8 logs and a sensitivity sufficient to detect down to a hundred of target miRNA molecules. It is capable to capture the full isomiR repertoire, leading to accurate representation of the complete miRNA content in a sample. The reverse transcription step can be multiplexed and the miRNA profiles measured with Two-tailed RT-qPCR show excellent correlation with the industry standard TaqMan miRNA assays (R^2 = 0.985). Moreover, Two-tailed RT-qPCR allows for rapid testing with a total analysis time of less than 2.5 hours.

PO 52: Tetraspanin CD9 Modifies the RNA Cargo of Prostate Extracellular Vesicles

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Introduction: Extracellular vesicles (EVs) have gained interest as a promising avenue for cancer biomarkers in recent years. EVs are small spherical shaped vesicles, which are secreted from their tissue of origin and contain high amounts of noncoding RNAs. The process for RNA loading into EVs is still not fully understood, but there are likely to be multiple mechanisms potentially based on cell type and disease. Potential components that may play a role in cargo sequestering are tetraspanins. They have been shown to be involved in key cancer-related cellular processes, with studies showing that expression of the tetraspanin CD9 correlate with prostate tumour type, stage and patient outcome. A decrease in CD9 expression is indicative of poor prognosis and metastasis. Further, CD9 is abundant on EVs that represent a source of biomarkers readily accessible by non-invasive means. This project seeks to determine how CD9 expression in prostate cancer influences the EV cargo to identify novel prognostic biomarkers.

Methods: CD9 expression was knocked down in RWPE1 (normal prostate cells) and increased in PC3 (bone metastasis from prostate cancer). EVs were collected after 48h by ultraconcentration from supplement-free cell culture media. NanoSight NS300 was used to evaluate size and concentration by nanoparticle tracking analysis. Total RNA was extracted with Trizol and evaluated using total RNA Agilent 2100 bioanalyzer chips, RNA was converted to labeled cDNA and hybridized to Affymetrix Human Transcriptome arrays, gene level differential expression ($p<0.05$) and alternative splicing analyses were performed (event score ≥ 0.7).

Results: Increasing the expression of CD9 in PC3 cells resulted in increased incorporation of CD9 in EVs and the differential incorporation of 2532 transcripts. Whereas decreasing CD9 expression in RWPE1 cells and the resultant EVs altered the incorporation of 486 transcripts into the EVs. A highly incorporated transcript family, particularly in the CD9 knockdown cells, is the small nucleolar RNAs, whose function is to chemically modify RNA. The alternative splicing analysis suggests that increased CD9 expression in prostate cancer cells and EVs results in incorporation of 10 alternatively spliced transcripts in EVs, whereas decreased CD9 expression in normal prostate cells generates EVs with 7 unique splicing events. What is not known, as this stage is if these transcripts are full length or are fragments.

Conclusion: These transcripts therefore represent novel potential EV biomarkers due to being differentially incorporated by expression of the metastasis suppressor CD9. From this it may be possible to identify a biomarker signature that will improve outcomes for prostate cancer patients.

PO 53: EXÖBead: an efficient method of isolating exosomes from small sample volumes without ultracentrifugation

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Exosomes are small vesicles (30-150 nm) secreted from different cell types and found in various biofluids, such as blood, urine, saliva and CSF. Exosomes contribute to cell-cell communication, antigen presentation or tumor progression by carrying cellular proteins, RNA/DNA, glycans and lipids. Differential ultracentrifugation is still regarded the 'Gold Standard' for isolation exosomes. However, ultracentrifugation is a laborious and time-consuming method that requires specialized equipment and operational expertise. Several alternative methods such as polyethylene glycol (PEG) or antibody-conjugated beads were developed to isolate exosomes without ultracentrifugation. These methods reveal different abilities of exosome RNA or protein isolation. A major issue for PEG isolation is the purity of exosome due to PEG remains. Isolation based on antibody-conjugated beads, however may damage exosomes by using acidic or alkaline reagent to break antigen-antibody interactions.
To solve these issues, we create a non-antibody coated magnetic bead, EXÖBead, that is able to isolate exosomes in a very elegant and efficient way without ultracentrifugation. We incubated EXÖBead with minimal volumes of pre-cleared cell culture medium or serum and analyzed the pulled-down fraction by flow cytometry (FACS), western blot, bioanalyzer and nanoparticle tracking analysis (NTA). Our result showed that exosome protein marker CD63 can be detected by FACS in exosome-EXÖBead complexes from 100 µl to 1 ml human serum and 200 µl to 1 ml RAW 264.7 cells culture medium. Additionally, the expression of exosomal proteins Alix and Rab5 was substantiated by western blot using the exosome-EXÖBead complex from 200 µl mouse serum and 200 µl to 1 ml B16F10 cells culture medium. For RNA analysis, we used a commercial extraction kit to extract RNA from exosome-EXÖBead complex. The pattern of exosomal RNA and its cDNA from EXÖBead isolation is similar with differential ultracentrifugation (120,000 g pellet). Furthermore, we designed a specific elution buffer for EXÖBead without using any acidic or alkaline reagent. To test the ability of EXÖBead elution buffer to release exosomes from beads, we performed NTA analysis to measure exosome size and its distribution. NTA data showed the major peaks of eluted exosomes to be at 65 nm, 105 nm and 155 nm. The size of exosomes eluted from EXÖBead is similar to exosomes isolated by ultracentrifugation. In conclusion, EXÖBead provide an easy, fast and reproducible isolation method for researcher working with small amounts of starting material.

Digital PCR PO 54 – PO 58

PO 54: Droplet Digital PCR: analysis approach for the detection and absolute quantification of lactic acid bacteria

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This study demonstrates the application of the droplet digital PCR (ddPCR) method, namely the absolute cell quantification of lactic acid bacteria in multi species cultures. Lactic acid bacteria comprise a wide range of species that produce lactic acid as the major metabolic end product of carbohydrate fermentation. Multi species lactic acid bacterial starter cultures are traditionally used for the production of a variety of cheeses and fresh fermented milk products. These starter cultures are indispensable for complex fermentation processes that not only require the acidification of the milk but also to produce the desired flavor and to extend the shelf life of the product. Standard microbiological analyses based on cultivation on selective agar media can identify lactic acid bacteria but they are laborious and time-consuming. Additionally, they clearly have some limitations in discriminating species showing similar physiological characteristics or to quantify particular species in complex cultures that can comprise different lactic acid bacteria species. Therefore, a method was supposed to be established that can quantify the species of interest in a mix of species independently from the background cell concentration in order to replace the time-consuming and laborious culturing techniques. For this purpose the droplet digital PCR method was applied using the instrument QX200 (Bio-Rad). A comparison study between plate count analyses of lactic acid bacteria multi species cultures and ddPCR revealed that the absolute cell counts were by trend higher using ddPCR. Therefore, the absolute quantification of cells is expressed as absolute genomic units (AGU). It could be shown that the inter-run variability of ddPCR was low with 2-8 %. In addition, a fiveplex ddPCR assay was set up in order to quantify simultaneously five different species in a multi species starter culture. Using this fiveplex PCR, the absolute number of AGU's of different species common in several product samples can be quantified within a single ddPCR reaction.

PO 55: Clinical samples collected in Streck Cell-Free DNA blood collection tubes are suitable for liquid biopsy testing using BEAMing digital PCR

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Mutation profiling of circulating cell-free DNA (cfDNA) extracted from plasma samples has advanced to a powerful diagnostic tool in oncology. Making liquid biopsy tests broadly and easily available requires the transport of whole blood to a central testing laboratory. In standard K_2EDTA tubes, white blood cells start to lyse shortly after blood collection, thereby releasing genomic DNA into the plasma and diluting the already small amounts of circulating tumor DNA (ctDNA). Streck Cell-Free DNA blood collection tubes (cfDNA BCTs) were specifically designed to maintain blood cell integrity and cfDNA stability for several days. Thus, cfDNA BCTs present a promising alternative to standard K_2EDTA tubes and have been integrated in many liquid biopsy workflows.

However, qualification data for the use of Streck cfDNA BCTs in oncology is limited and mainly based on blood collected from healthy individuals as well as data extrapolated from the prenatal testing field. The data generated from these samples may not represent the unique dynamics of clinical oncology specimens and therefore a study of true clinical oncology samples is required to support the use of cfDNA BCTs in routine practice.

In this study, we evaluated the cfDNA integrity of matched blood samples collected in cfDNA BCTs vs standard K_2EDTA tubes from colorectal, pancreatic and non-small cell lung cancer patients (N = 53). Blood drawn into cfDNA BCTs was either processed immediately or 3 days after phlebotomy. DNA quantification was followed by BEAMing digital PCR (OncoBEAM™) on KRAS, NRAS and EGFR mutations and compared to matching specimens collected in K_2EDTA tubes.

Our results suggest that cfDNA yield as well as the genomic DNA background is not affected by prolonged storage of clinical samples in cfDNA BCTs for up to 3 days. In all sample sets containing mutant ctDNA, the detected mutational load was comparable between cfDNA BCTs and K_2EDTA tubes.

In conclusion, this study represents a comprehensive clinical evaluation of cfDNA BCTs vs EDTA tubes for ctDNA profiling. In conjunction with the findings of our previously presented cfDNA BCT shipping condition studies, our data supports the compatibility of clinical oncology specimens collected in cfDNA BCTs with the BEAMing technology.

PO 56: Digital PCR quantification of the 7S DNA per mitochondrial genome proportion in non-invasive sources of human cell types

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The mitochondrial displacement (D-) loop is a triple-stranded region found in the major non-coding region of many mitochondrial genomes. It is formed by stable incorporation of a third DNA strand known as 7S DNA [1]. This extra strand – approximately 650 nucleotides in length – starts around the origin of heavy strand replication (O_H) and terminates just after the termination-associated sequence. The few reported proportions of 7S DNA-containing molecules ranging from 10% in cultured human cells to 90% of *Xenopus* oocytes (reviewed in [1]) have exclusively been performed by semi-quantitative methods before the era of quantitative PCR (qPCR) or digital PCR (dPCR).

Here we determined the proportion of 7S DNA per mitochondrial genome in a number of human cell types sampled non-invasively from healthy individuals. Copy number concentrations of target nucleic acids were determined by digital PCR – the gold standard for their precise quantification – using the chip-based platform QuantStudio™ 3D Digital PCR System in combination with the QuantStudio™ 3D AnalysisSuite™ Cloud Software (Thermo Fisher Scientific) [2].

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PO 57: Poisson Plus Quantification for the QuantStudio™ 3D Digital PCR System

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Digital PCR, a next generation nucleic acid quantification technique works by spreading the target material across a large number of partitions. The average number of molecules per partition is estimated using Poisson statistics, and this partition average is converted into concentration by dividing by partition volume. In this standard approach, identical partition sizing is assumed. At higher concentrations, violations of this assumption result in the underestimation of target quantity, when using Poisson modeling. An extension to the Poisson model, the Poisson-Plus Model, is presented here; which, if statistics of the volume variation are well characterized, accommodates for this underestimation. Parameters of the model were measured on the chip array based QuantStudio 3D Digital PCR System. The volume variation was measured using the ROX fluorescence signal level as a proxy for effective load volume per through-hole. Monte Carlo simulations demonstrate the efficacy of the proposed correction. Empirical measurement of model parameters characterizing the effective load volume on QuantStudio 3D Digital PCR chips is presented. The model was then used to analyze digital PCR experiments from QuantStudio 3D Digital PCR System and showed improved accuracy in quantification results. At the higher end of the concentration spectrum, the modeling must take effective volume variation into account to produce the most accurate estimate. The extent of the difference from the standard to the new modeling is proportional to the extent of volume variation in the effective load of your reactions.

PO 58: Detection of TMPRSS2:ERG Fusion Transcript Using TaqMan Assays and the QuantStudio 3D Digital PCR System

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Current biomedical research aims at personalized cancer therapeutics, for example in prostate cancer. Due to the high prevalence of the TMPRSS2:ERG fusion which occurs in more than 50% of cases, it seems to be a suitable biomarker for monitoring prostate cancer which can be detected in less invasive sample material such as blood and urine. Use of digital PCR is currently being demonstrated to be a highly sensitive method for reproducible and robust measurements without the use of standard curves. Especially the QuantStudio 3D Digital PCR System from Applied Biosystems has a high point-of-care potential due to the closed, compact and easy to handle system. Aim of this project was to establish a system for the detection of TMPRSS2:ERG fusion on this Digital PCR platform, which can be deployed as a future-oriented method. A TaqMan Fusion and a Gene Expression Assay were used to detect mutant and wildtype alleles, and their performance was optimized by changing the temperature-time profile. RNA isolated from fusion positive VCaP and fusion negative LNCaP cell lines were used as reference material. In wet-lab experiments it was possible to detect TMPRSS2:ERG transcripts with a detection limit of 0.05% fusion portion in a correspondingly high wildtype background. Spike-in experiments allowed the confirmation of an easy workflow. This is a first step forward to apply the assay to prostate cancer samples for monitoring disease and to further validate the significance of the TMPRSS2:ERG fusion as a suitable biomarker.

For Research Use Only. Not for use in diagnostic procedures.

MicroGenomics PO 59 – PO 67

PO 59: Toward elucidation of the biological function of S100 calcium binding protein A9 in monocytes using single cell sorting and qPCR methodologies

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Background: We previously found that relatively high tissue expression of S100 calcium binding protein A9 (S100A9) early after clinical kidney transplantation is associated with favorable outcome at the long run. S100A9 is abundantly expressed by circulating monocytes and tissue macrophages. To clarify the biological function of S100A9, we now aim to both functionally and phenotypically characterize S100A9 high expressing and -low expressing monocyte subsets.

Method: We addressed our aim by two approaches: I) application of SmartFlare™ (Merck Millipore) RNA detection probes bound to nanoparticles. These should be taken up within 16 hours by live monocytes, and fluoresce once they bind intracellular S100A9 mRNA targets; II) performing intracellular staining and sorting for S100A9 protein by flow cytometry (FACS), and subsequent qPCR profiling of the cell fractions. For both purposes, $CD14^+$ human monocytes were employed, which had been enriched from Ficoll-isolated mononuclear cells of buffy coats.

Results: Monocytes incubated with S100A9-specific SmartFlares were gated on high, intermediate, and low signals by flow cytometry. The three different fractions did not differ in intracellular S100A9 protein, and also not in S100A9 mRNA expression levels after FACS sorting. Therefore, cell fractions were not furtherinvestigated in functional tests. Intracellular protein staining normally disrupts the RNA and poses a challenge in the reliable molecular profiling of cellular mRNA content. We found that a specialized buffer containing 5% RNAse inhibitor during cell sorting kept mRNA degradation to a minimum (Cq increase < 3).Most importantly, $S100A9^{high}$ fractions expressed significantly higher (~ two-fold) S100A9 mRNA levels than $S100A9^{low}$ fractions.

Conclusion: The SmartFlare technology does not generate monocyte fractions that differently express S100A9. Intracellular S100A9 protein staining of monocytes enables sorting of high- and low-expressing cell subsets. This will enable further phenotypical characterization of the subsets.

PO 60: Determining the Limit of Detection of Rare Targets Using Digital PCR

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Detection and quantification of mutant alleles in tumor tissue allow for disease monitoring, the evaluation of drug efficacy and guide decisions on future treatment plans. Testing for the presence of mutations in circulating free DNA (cfDNA) is one of the less invasive research methods available at this time. Digital PCR presents a research tool for mutation detection in cfDNA at a sensitivity level of 1% and below. Challenges associated with digital PCR experiments for rare allele detection include understanding the limit of detection of the assay and platform. This work compares false positive assessment strategies using the signal levels of the no-amplification cluster. Once the false positive call rate is established, this work outlines a method to determine the limit of detection of the assay and platform, at a given level of confidence. Given the number of partitions, the interrogated volume and the false call rate, the tradeoffs between sample load and sensitivity are also discussed. The mathematics outlined to calculate the theoretical limit of detection is applied on a set of assays from Thermo Fisher Scientific covering the KRAS codon mutations commonly found in tumor tissues. Experimental results showing a detection

of at least 0.1% mutation rate are presented as examples. Test samples were created using both mutant plasmid and mutant genomic DNA mixed with wild-type genomic DNA at a predefined percentage. For Research Use Only.

PO 61: Single Cell Gene Expression Profiling in Breast Cancer Cells with the Her2/neu Gene Knockout by CRISPR-Cas9

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The Her2/neu gene is amplified and overexpressed in 15%–30% of breast cancers. The overexpression of this oncogene is strongly correlated with decreased survival, increased cancer relapse, and poor prognosis. Although therapy has been successfully developed to target this oncogene, a better understanding of this oncogene will provide further insight for breast cancer biology and future drug development. In recent years, the CRISPR-Cas9 system has emerged as an efficient method for genetic engineering, enabling targeted gene knockout with minimal off-target effects. In this study, we employed a plasmid-based CRISPR-Cas9 system to knock out the Her2/neu gene in breast cancer cells. Puromycin selected, GFP-positive (two indicators of the incorporation of the CRISPR-Cas9 system), Her2-negative individual cells were index-sorted using a BD FACSMelody™ system into individual wells of a 96-well BD™ Precise plate with sample barcoding and molecular indexing. A whole transcriptome amplification (WTA) assay was performed to obtain a gene expression profile for each of the Her2/neu knockout cells. Her2 gene knockout was performed on two cell lines: T47D, a ductal carcinoma-derived breast cancer cell line with no Her2 amplification that shows low to intermediate Her2/neu expression; and SKBR3, an adenocarcinoma-derived breast cancer cell line known for Her2/neu gene amplification and overexpression. For comparison, WTA assays were also carried out on parental cells without gene editing. Comparison of the gene expression profiles reveals downstream changes in gene expression correlated to the Her2 knockout. The profiling of gene expression in Her2/neu CRISPR-Cas9 knockout cells on a single cell level promises to provide insight into the mechanisms in these aggressive cancers that could help future drug discovery.

PO 62: T cell differentiation dynamics profiled by massively parallel single cell RNA capture and highly multiplexed PCR

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The immune system consists of a complex network of cell populations that can go through different spatial and temporal transitions. Cell surface markers and flow cytometry have aided the characterization of immune subsets. However, restrictions in the number of available antibodies and multicolor cytometry channels greatly limit access to phenotypic information that illuminate function and cell identity. Single cell whole transcriptome analysis offers a higher resolution approach to uncover novel regulators and markers obscured by bulk sample analysis. Yet several limitations exist for such approach for T cells. T cells, especially in an un-activated state, have low numbers of transcripts for surface markers and transcription factors used to define lineage and function, and thus deeper sequencing is required to accurately detect genes of interest. Moreover, a great proportion of sequencing reads consist of ribosomal and housekeeping genes, making meaningful clustering based on cell identity difficult. Here we demonstrate using a highly multiplexed PCR approach to characterize T cell lineage and function with greater sensitivity for T cell specific genes and higher cost effectiveness. The BD™ Resolve targeted T cell assay allows the surveying of more than 300 T cell genes in hundreds to more than ten thousand of individual cells per experiment. In this study we were able to distinguish between T cells that were differentiated into different CD4 effector subsets using cluster analysis. Within each condition, we were able to identify cells in different activation and differentiation states. With much fewer sequencing reads than what is needed for the whole transcriptome approach, we detected low abundance T cell markers that are hard to uncover in whole transcriptome sequencing. The BD Resolve single cell targeted approach is a high throughput method to study the functional states of T cells in a high dimensional and cost effective way.

PO 63: High Throughput Single-Cell Gene Expression Analysis Reveal Cell Type Specific Differences in Response to Different Human Peripheral Blood Preservation Protocols

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Gene expression analysis of peripheral blood has been widely used to identify biomarkers for detecting, monitoring, and predicting diseases. However, it has been known that gene expression changes in blood can be induced by different blood handling and processing methods, thus skewing experimental results. Past studies examining effects of blood handling and preservation have mostly been conducted with bulk RNA using microarrays, and it is not commonly known whether certain cell populations in blood are more prone to gene expression changes. Here, we used BD Resolve, a tool for massively parallel single-cell RNA sequencing library preparation, and shallow sequencing, to compare whole transcriptome profiles of ~10,000 freshly isolated peripheral blood mononuclear cells (PBMCs) each from three blood donors, as well as blood cells isolated and/or stored in the following conditions: freshly isolated, cryopreserved, or liquid life AQIX® storage. With such high resolution single cell analysis, we found donor to donor differences, not only in population sizes of immune subsets, but also in gene expression differences in each of the subset. Additionally, we found that certain immune subset, such as monocytes, were more susceptible to expression changes depending on preservation techniques. Together these results will help develop proper methods for handling and preserving blood samples for future large scale high throughput single cell gene expression studies.

PO 64: Preamplification using dUTP and Cod UNG allows for reliable detection and quantification of limited samples sizes in the presence of contaminating DNA amplicons

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Detection and quantification of rare DNA and RNA molecules in limited sample sizes, including liquid biopsies, fine-needle aspirates and single cells, usually requires preamplification. This makes downstream analyses especially sensitive to PCR generated contamination. Here, we study the feasibility to perform preamplification with dUTP in the presence of Cod uracil-DNA N-glycosylase (Cod UNG), allowing elimination of contaminating DNA amplicons. Cod UNG can be completely and irreversibly heat inactivated, making it suitable for preamplification applications. Using optimized qPCR assays, we show that using dUTP instead of dTTP in the reaction mix yields similar dynamic range, reproducibility, sensitivity and amplification efficiency. Furthermore, we demonstrate that Cod UNG degrades essentially all contaminating uracil-containing amplicons regardless of initial concentration in all samples for most assays tested without inhibiting downstream reactions. Finally, we validate that dUTP and Cod UNG can easily be included in the workflow for targeted single-cell gene expression profiling. In conclusion, a standardized introduction of dUTP and Cod UNG in the experimental workflow poses an attractive solution to largely avoid generation of erroneous data when analyzing limited sample sizes containing few target molecules, in clinical routine assessments as well in research studies.

PO 65: Achieving Unparalleled Sensitivity and Reproducibility in Single-Cell Transcriptomics

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Differential expression (DE) analysis utilized for single-cell comparisons has become one of the key methods for studying transcriptome variability, especially when homogeneous cell populations are elusive, such as in cancer research, developmental biology, neurobiology, and immunology. SMART (Switching Mechanism at the 5' end of the RNA Template) technology has emerged as the most sensitive solution for processing the small amounts of mRNA present in a single cell. Here we discuss the use of the highly performant SMART-Seq v4 chemistry in the new SMART-Seq v4 3' DE Kit to enable differential expression analysis in a more efficient and cost-effective manner. SMART-Seq v4 chemistry incorporates LNA technology in order to produce high-quality, reproducible sequencing data with superior identification of genes, including those with low expression. By combining these features with cellular indexes, pooling, and 3' end-capture sequencing, we enable researchers to obtain high-quality gene expression data from single cells or ultra-low input amounts of RNA without having to sequence the entire transcriptome.

PO 66: Resolving Molecular Networks and Dynamics Involved in CD8+ T-cells Function and Differentiation in Acute and Chronic Infections on a Single-Cell Level

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CD8 T-cells are major players in the adaptive immune defence against intracellular pathogens (including viruses and intracellular bacteria) as well as in tumor surveillance. In the context of acute viral infections like influenza and yellow fever, naive antigen-specific CD8 T-cells differentiate into highly functional effector and memory CD8 T-cells. In result, the generated effector cells swiftly eliminate the viral infections, while the memory cells provide effective protection in case of secondary infection caused by the same virus. In contrast, some viruses like HIV, HBV and HCV in humans as well as LCMV clone 13 in mice are able to establish chronic infections associated with the development of CD8+ T-cells with diminished functional activity, a state often referred as T-cell “exhaustion”. The latter represents a unique state of CD8 T-cell differentiation which is likely to be effective but hyporesponsive, providing the host with the ability to control the viral load without causing severe bystander pathology. Otherwise the excess cytotoxic T-cell activity due to persisting antigen presence can bring the host to even more life-threatening condition compared to chronic viral presence. Some of the general CD8 T-cell hallmarks in different chronic infections compared to acute infection are unique transcriptional program, immune signaling, migration as well as metabolism. Despite the amount of accumulated knowledge about CD8 T-cell phenotype and function in chronic infection, we are still far away from establishing successful strategies for therapeutic reactivation of the immune system in order to eradicate established chronic infections. In our opinion, this might be a reflection of the usually applied population-based assessment of CD8 T-cell gene expression profiles, which is prone to generating biased and even false results due to giving only average values. The main question we want to address is which molecular networks and mechanism are involved CD8 T-cell function and differentiation in acute and chronic infections on a single cell level. To address that question we decided to generate gene expression profiles of CD8 T-cells from different acute and chronic infection settings and time points using single-cell mRNA sequencing approach. So far we are optimizing the method and we have started to obtain single-cell gene expression profiles from different settings. Besides the deep single-cell information on molecular networks and mechanism, we hope that the single-cell approach we apply will be informative concerning cellular dynamics and subpopulation taking place in different acute and chronic infections. Finally, the ultimate outcome will be to exploit that knowledge for generating new therapeutic strategies for successful intervention in immune response against problematic viral infections.

PO 67: Combining Microgenomics Methods To Study How Gut Microbiota Influences Gene Expression In The Hypothalamo-Pituitary-Adrenal Axis In Rats.

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Objective: The gastro-intestinal tract hosts a complex microbial community, the gut microbiota, which is nowadays regarded as a full organ taking part to the host physiology. The gut microbiota is in particular increasingly recognized as a factor that can shape brain and behaviour. We and others showed that the gut microbiota regulates the hypothalamo-pituitary-adrenal (HPA) axis reactivity, as reflected by a greater corticosterone systemic concentration in germfree rodents than in conventional ones, following an acute stress. The objective of the present study was to develop a microgenomics workflow combining the use of laser capture microdissection (LCM), pre-amplification and reverse transcription real time PCR (RT-qPCR) to analyse which HPA axis genes are regulated by the gut microbiota.

Methodology: We subjected germfree and conventional rats to an acute stress (strongly illuminated open-field for 6 min), killed them by decapitation and collected and froze the brain, the pituitary gland and the adrenal glands. Non stressed rats served as controls. Different combinations of analytical methods were used in those 3 tissues, according to the distribution and density of the cells of interest. Brain and adrenal gland sections were stained with cresyl violet to localize the key areas, namely the hypothalamus paraventricular nucleus (PVN) in the brain, and the cortical zona fasciculata in the adrenal glands. Then, distinct cell harvesting techniques were applied: micro-punching for the PVN, and LCM of cell clusters for the adrenal gland. As the corticotropic cells are scattered in the anterior pituitary gland, an immunohistochemistry method coupled with LCM was developed to identify and capture corticotropic cells as specifically as possible. Around 50 corticotropic single cells were isolated and processed with the TaqMan® PreAmp Cells-to-C_T™ kit. The expression level of a panel of 48 genes, selected according to their role in the HPA axis reactivity, was analysed using TaqMan® Array Micro Fluidic Cards; qPCR was carried out in all sorted samples. A target-specific pre-amplification was applied in the adrenal gland cell clusters and single cells.

Results and conclusion: Bayesian Structural Equation Modelling indicated that gene expression in the 3 HPA axis tissues was overall influenced by the gut microbiota status (germ-free or conventional) and the stress status (acute stress or no stress) of the rats. In conclusion, this microgenomics workflow has allowed to address the issue of gene expression analysis in a heterogeneous set of tissues involved in a specific neuroendocrine pathway.

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Late Submissions PO 68 – PO 78

PO 68: TaqMan™ Assays for Detection of Fusion Transcripts

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Chromosomal aberrations such as translocations are frequently found in human cancers (1). Translocations or trans-splicing may result in a chimeric gene expressing a fusion transcript/protein that affects regulatory pathways and stimulates cancer growth (2,3). A well-known example is the BCR-ABL fusion which is the result of a translocation of gene ABL on chromosome 9 to the BCR cluster on chromosome 22 (4,5). The BCR-ABL fusion products are the cause for 90% of chronic myeloid leukemia (6,7). Traditional methods for identifying translocations include FISH and karyotyping, none of which can quantify the expression level of the fused genes. We have developed quantitative real-time PCR assays—Taqman™ Gene Expression assays to detect and measure fusion transcripts. We designed assays that span the breakpoint regions but avoided placement of primer/probe directly on the breakpoints. As proof of principle, several assay designs were tested against the plasmids containing the translocation variant and the tissue samples with the translocation event. We demonstrate that the fusion assays can detect many BCR-ABL fusion variants in leukemia samples. These data indicate that TaqMan™ Fusion Assays with an easy and ready-to-go workflow have great sensitivity. The standardized assay format and protocol with an optimized master mix allows researchers to generate reproducible data.

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PO 69: QuantSeq 3' mRNA Sequencing Bridges the Gap Between Microarrays and Conventional RNA-Seq Methods and Yields Good Correlation Between High and Low Quality (FFPE) Samples

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Array-based quantification of gene expression was long considered the gold standard but now is rapidly replaced by sequencing-based methods. With the discontinuation of microarray systems, attention is now on RNA-Seq as the method-of-choice for gene expression (GEX) profiling. QuantSeq is an oligodT primed NGS library preparation protocol generating only one fragment per transcript. Unlike microarrays it allows for the detection of non-annotated features and enables accurate gene expression quantification across many orders of magnitudes independent of the RNA quality (including FFPE samples).

Standard mRNA-Seq protocols aim to cover the whole transcript and result in a heavy 3' bias when used on degraded RNA. Comparison between high quality and low quality RNA samples is therefore hampered and often biased. QuantSeq 3' mRNA-Seq focuses on counting 3' ends and hence delivers reliable and reproducible results independent of the RNA quality. The high concordance of gene expression detected in cryo and FFPE RNA samples demonstrates that QuantSeq is the method of choice for gene expression quantification independent of the RNA input quality. It provides improved gene expression quantification over traditional methods along with characterization of novel RNA features.

PO 70: QuantSeq as an Efficient Tool for Gene Expression Profiling and Targeted Sequencing: High Multiplexing,Sensitivity, and Reproducibility with Lowest Costs and Efforts

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Concurrently with the rapid progress of Next Generation Sequencing (NGS) technologies, RNA-Seq has been established as the new standard for transcriptome analysis. Although the sequencing costs per base have been substantially reduced, sample preparation, sequencing, and data processing present major investments of both cost and time in high-throughput screenings. QuantSeq provides a technology to prepare highly strand-specific NGS libraries directed towards the 3' end of polyadenylated RNAs within 4.5 h and can be further tailored to a variety of sequence specific applications. It requires only low input ranging from 0.1–500 ng of total RNA and can be efficiently used for low-quality samples (including FFPE). Single and dual indices with up to 4 x 96 barcodes enable a high degree of multiplexing. Since only 1 fragment per transcript is generated, FPKM calculations are unnecessary, and a recommended read depth of 2-5 million short, single-end reads per sample is sufficient for expression profiling of eukaryotic transcriptomes. Data analysis is simplified since no junction detection is necessary, and transcript abundances are given by read counts. Furthermore, Lexogen's collaboration with Bluebee now provides QuantSeq users also with a cloud-based, non-expert access to data analysis up to differential gene expression.

PO 71: Application of allele-specific qPCR and NGS for detecting epigenetic adaptations at the *FKBP5* locus: Relevance for stress-related diseases

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The risk for developing stress-related diseases is shaped by the interaction of environmental and genetic risk factors. The underlying molecular mechanisms integrating environmental and genetic cues have not yet been fully elucidated. In this study, we aimed to test, whether an intronic 3.3 kb structural variant in the *FKBP5* locus has A) effects on *FKBP5* gene regulation and, if yes, B) the potential to

moderate the risk for developing a stress-related disease. We observed that the deletion allele of the structural variant was associated with a higher induction of *FKBP5* expression after activation of the glucocorticoid receptor (GR) in lymphoblastoid cell lines. Furthermore, we could show via allele-specific qPCR that the structural variant can moderate the effect of the previously described functional single nucleotide polymorphism rs1360780[1] on the GR-induction of *FKBP5*. In order to explore how these transcriptional effects on *FKBP5* induction are reflected on the DNA level, we analyzed changes of the chromatin structure (chromosome conformation capture) and DNA methylation (targeted bisulfite sequencing, Infinium Methylation 450K array). By applying a circularized chromosome conformation capture (4C) approach, we detected an interaction within the insertion allele to the FKBP5 transcription start site. Moreover, we found indications for the structural variant to alter the CTCF-related structural basis[2] of *FKBP5* through modulating its interaction frequencies. Within the regions of this structural basis and the transcription start site of *FKBP5*, we also observed differentially methylated sites depending on the allele status for the structural variant. Finally, we investigated whether the structural variant has relevance for stress-related diseases such as posttraumatic stress disorder (PTSD). We found that the deletion allele is associated with higher PTSD symptoms but only in patients who experienced child abuse. Taken together, our data indicates that this structural variant moderates the extent of *FKBP5* induction after GR activation and is accompanied by changes at multiple epigenetic level of the *FKBP5* locus which can lead to *FKBP5* disinhibition and therefore increased risk for developing a stress-related psychiatric disease.

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PO 72: Overexpression of MiR-590, MiR-19a, and MiR-19b in Human Breast Carcinoma Is Associated with Poor Differentiation of Tumors, Negative Expression of Hormonal Receptors, and Worse Survival in Patients Treated by Chemotherapy

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Background: Changes in expression of ABCD2, CYP2B6, PRC1, SLC19A1, SLCO1A2, and SOD3 in breast carcinomas have been shown to associate with worse prognosis or chemotherapy outcome of breast carcinoma patients. MicroRNAs are short non-coding RNAs frequently deregulated in carcinomas and can be easily detected as biomarkers in tumor cells or biofluids. Aim of this work is evaluate the expression profile and clinical significance of miRNAs predicted to be regulators of the above identified candidates of poor prognosis in breast carcinoma patients.

Methods: Together 15 miRNAs targeting mRNA of candidate genes (ABCD2: miR-590-3p and 5p, miR-340, miR-30d, miR-519b; CYP2B6: miR-590-3p and 5p, miR-21; PRC1: miR-194, miR-19a, miR-19b, miR143; SLC19A1: miR-299, miR-335; SLCO1A2: miR-382, miR-320a, miR-320c; SOD3: miR-491) were selected with help of databases for miRNA target prediction (miRanda, TargetScan, DIANA, and PicTar). MiRNA expression profile was assessed by qPCR in total RNA isolated from breast carcinoma tissues (N=95), correlated with mRNA levels of candidate genes, and associated with clinical data of patients.

Results: Levels of miR-30d (ABCD2), miR-590-3p, and 5p (both for CYP2B6) correlated negatively with their targets. MiR-19a and 19b correlated positively with PRC1 expression. Significantly higher levels of miR-590-3p, 5p, miR-19a, and 19b were found in high-grade tumors, in larger tumors (miR-19a, 19b) and in tumors without expression of estrogen and progesterone receptors. Patients treated with chemotherapy with higher levels of miR-590-5p, miR-19a, and 19b had shorter disease-free survival than patients with lower levels in tumors (*P*=0.037, 0.017, and 0.006, respectively).

Conclusion: MiR-590, miR-19a, and miR-19b are potential biomarkers of efficacy of adjuvant chemotherapy and their levels are further analyzed in blood of breast carcinoma patients. A functional analysis follows also to verify inhibitory effect of miR-590 on CYP2B6 levels in carcinoma tissues.

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PO 73: A new core facility for high throughput qPCR : qPCR-HD-GPC

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The Fluidigm® Biomark™ HD system is available as a high throughput quantitative PCR device on the qPCR-HD core facility of the Institute of Biology of the Ecole normale superieure.

When compared to traditional real time PCR technologies, the Biomark™ HD System and the microfluidic chips from Fluidigm® offer a considerable improvement in terms of capacity, allowing researchers to produce more data in less time (up to 9,216 simultaneous qPCR reactions or 37k by digital PCR). High throughput qPCR platform raises several questions in terms of sensitivity (minimal input), absolute quantification and data processing.

To address these questions, we designed experiments with scales of zebrafish total RNAs as samples, and Taqman® probes as assays. RNAs were reverse transcribed with various RT enzymes to compare their sensitivity. In order to precisely quantify cDNA molecules, amplicons were cloned in plasmids and used as standards to calculate PCR efficiency for each probe from calibration curves. This process was automated thanks to dedicated R scripts.

We propose a comprehensive solution to perform high throughput qPCR and data analysis. Our framework integrates previously designed methods for quantifications and comparison as well as some improvements

PO 74: Detection Of Solid Tumor-Specific Mutant Transcripts In Blood Derived Extracellular Vesicles.

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INTRODUCTION

Extracellular vesicles (EVs) are secreted by different cell types into several body fluids (e.g. urine, spinal fluid and blood). These EVs contain protein and nucleic acids of cells they originate from. The goal of the current study was to investigate whether EVs secreted by cancer carry specific tumor cell characteristics that may be used for treatment decision making.

METHODS

A workflow was established using conditioned medium of 5 breast cancer cell line models and subsequently applied on blood of 12 metastatic cancer patients. Blood was collected in EDTA, BCT and CellSave tubes and processed into plasma. Less than 1mL plasma or 5mL culture medium was used to isolate EV-RNA using the exoRNeasy Kit (Qiagen) followed by a DNAse I treatment and cDNA generation. The resulting EV-cDNA from cell lines was evaluated by qPCR with a 96-gene expression panel. In addition, cell line and patient blood-derived EV-cDNA was characterized with the QuantStudio 3D Digital PCR system (Thermofisher) for tumor-specific gene mutant and wildtype transcripts (target genes). All 12 patients included in this study had a known tumor-specific mutation in their primary tumor, of which 10 patients previously were shown to have the mutation also detectable in cell-free plasma DNA (cfDNA).

RESULTS

The analyses of 96 genes in our cell line models showed that the EV-RNA expression profiles in general adequately reflected the matched parental mRNA profiles, however, 38 genes were differentially expressed of which 8 upregulated in EV-RNA. Two cell lines with a *PIK3CA* mutation in their DNA revealed comparable variant allele frequency (VAF) for *PIK3CA* mutant transcripts between cell line mRNA and matched EV-RNA in BT20 (30% vs 32%) and T47D (89% vs 84%). In patient plasma collected in BCT and Cellsave tubes no target gene copies were found whereas in 11 EDTA derived EV-RNA samples target genes copies were detected. Additionally, EV-RNA mutant transcripts were demonstrated in two of the 12 patients, i.e. a lung cancer patient with an EGFR T790M mutation (VAF: 61%) and a colon cancer patient with a KRAS G13D mutation (VAF: 6%).

CONCLUSION & DISCUSSION

Our study shows that EV-RNA can be used to perform expression analysis of multiple genes. Moreover, cell line derived EV-RNA reflects the mRNA profile of their parental cell line suggesting that tumor EV-RNA in patients' blood can harbor tumor-specific characteristics. In line with this, we detected somatic mutations in EV-RNA derived from both cell lines and patient plasma samples. For plasma, however, it is important to collect patient blood in EDTA tubes, since preservatives in other tube types interfere with downstream EV-RNA analyses. In addition, the amount of tumor specific EVs in plasma and target gene copies might explain why the mutation detection sensitivity in patient EV-RNA (20%) is lower compared to cfDNA (85%). This may be improved by increasing the plasma input (>1mL) for EV-RNA extraction.

PO 75: Profiling of Circulating MicroRNA in Patients Presenting with ST-segment Elevation Myocardial Infarction

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Introduction and aims: Acute myocardial infarction (AMI) is a life-threatening complication of coronary artery disease. Insufficient blood supply and oxidative stress result in necrosis of cardiac tissue, pathological remodeling and left ventricular dysfunction. Early diagnosis of AMI is necessary to rapidly restore blood flow to the heart to limit the extent of myocardial necrosis, which largely impacts patient outcome [1, 2]. Therefore, new biomarkers that can early detect of AMI are urgently needed. Recent studies have revealed the existence of free and stable circulating, myocardial-derived microRNAs (miRNAs) in human peripheral blood. The circulating miRNAs have been shown to be a sensitive biomarkers in the diagnosis of AMI. The aim of the present study was to identify differentially expressed circulating miRNAs in ST-segment elevation myocardial infarction (STEMI) patients.

Materials and methods: Sixteen patients with STEMI were included. The blood was collected on the 1st day of myocardial infarction (admission) and after 4–6 days (discharge). Control group comprised 8 patients with stable coronary artery disease, without history of myocardial infarction. miRNA expression profile analysis was performed using Exiqon Serum/Plasma Focus microRNA PCR panel in plasma samples.

Results: The profiling study identified 45 species of plasma miRNAs ($p < 0.05$) that were differentially expressed on admission compared to control group. On discharge 15 species of plasma miRNAs ($p < 0.05$) were differentially expressed compared to control group. Seven miRNAs ($p < 0.05$) were shared between the two comparisons (1st day of myocardial infarction vs. control group and 4–6 days vs. control group). Bioinformatics analysis by DIANA-miRPath v3.0 demonstrated that 26 Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways were significantly enriched ($p < 0.05$, FDR corrected) among seven differentially expressed plasma miRNAs, including fatty acid biosynthesis and metabolism, ECM-receptor interaction, lysine degradation, p53 signaling pathway and TGF-beta signaling pathway.

Conclusions: Our results suggest that miRNA expression profiling provides better understanding of the changes that occur in the acute phase of MI in the myocardium and circulating miRNAs can be used as potential biomarkers for AMI.

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PO 76: HiFit : robust data analysis method for High throughput qPCR

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Real-time quantitative polymerase-chain-reaction (RT-qPCR) is frequently used as a standard technique for various applications such as research or clinical diagnostic. To date, several data analysis strategies have been proposed to extract meaningful information from single RT-qPCR curves. Most of them are in fact semi-automated because they were developed in the context of low-throughput RT-qPCR data where each reaction can be visually investigated and its analysis manually corrected. We observed that portability of those methods to high throughput was far to be straightforward mainly due to their lack of robustness for high throughput platforms. In fact several of them could simply not be made fully automated. To address this question we have developed an absolute high throughput qPCR data analysis approach based on a robust fitting of a four or six parameters sigmoid model. We take advantage of the throughput such that the search of the optimal parameters for each curve is achieved using information gathered from the fitting of large sets of curves obtained from the whole dataset. Our approach brings the level of robustness required to address high throughput qPCR data

PO 77: The Development of Genomic Reference Materials for Quantitative Diagnostics

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The adoption of downstream applications to perform genetic tests for the quantification of nucleic acids, rare event and copy number variation detection is accompanied by the need for genomic reference materials (GRMs) that expedite the comparison of results across different platforms and laboratories, and allow the standardisation of diagnostic testing.

The National Institute for Biological Standards and Control (NIBSC) is the only producer of World Health Organization (WHO) international standards for genomic diagnostics, and is the only European producer of human genomic DNA reference materials for *in vitro* diagnostic use. WHO international standards are intended for use as primary reference materials in the calibration of secondary reference materials, kits, and assays; CE-marked GRMs are intended as 'run-controls' in diagnostic assays. The NIBSC programme was initiated with the development of GRMs for qualitative genomic diagnostic assays, but in recognition of the increasing requirement for quantitative assays, particularly for cancer diagnostics where biomarker levels determine treatment and prognosis, the need for quantitative standards to both calibrate and determine assay sensitivity is critical.

Here we consider the role of QPCR, digital PCR (dPCR) and next-generation sequencing (NGS) in the development of nucleic acid -based GRMs for cancer diagnostics. The WHO international reference panel for *BCR-ABL* was established using QPCR and serves to standardise RNA-based quantification of the Philadelphia chromosome. The alignment of increasingly sensitive QPCR and dPCR methods is facilitated by the availability of the standards. More recently, the WHO international reference panel for *JAK2* V617F was developed by utilising dPCR. An international collaborative study allowed both the derivation of consensus values for each of the materials (% *JAK2* V617F) and a comparison between multiple methods. The proposed WHO international reference panel for *KRAS* codons 12 and 13 was recently produced with the application of dPCR to derive values (% *KRAS* mutations). The international collaborative study will particularly compare these data with NGS values. In recognition of the need to detect mutations at low levels in multiple genes and distinguish between likely pathogenic and non-pathogenic mutations, and polymorphisms by NGS, we next propose to develop a CE-marked cancer mutation-detection process control panel, consisting of tumour cell line genomic DNAs with hundreds of mutations at a range of levels.

PO 78: Impact of GSTM1 Polymorphism on the Susceptibility to Prostate Cancer in the Algerian Population

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The prostate cancer (PCa) is the most frequent malignant pathology in older men of more than 50 years. Several polymorphisms affecting genes involved in the process of detoxification of the xenobiotics could be associated to the PCa. Among these genes, the family of Glutathion S - transférases (GSTs) was particularly studied as prognostic biomarkers. Therefore, it is necessary to identify markers to better predict the occurrence and the progression of the Disease. The objective of this study is to determine the contribution of the polymorphism GSTM1 in the occurrence of the prostatic cancer, in an Algerian population.

In our study, association between genetic polymorphism of GSTM1 and PCa was analysed in a case-control sudy of 90 individuals. A total of 49 patients PCa and 41 healthy subjects as control were used. All patients provided informed consent for participation in the study. Genotypic analysis was carried out with samples of peripheral blood. Genomic DNA was extracted using a " Flexi Gene DNA Kits " and then amplified by polymerase chain Reaction (PCR) using specific primers to detect GSTM1 polymorphism.

The results demonstrate that 38.77 % of the CaP patients present the null genotype while 61.22 % present an active genotype. For the control group, 14.63 % are of null genotype and 85.36 % of active genotype. This study suggests that the GSTM1 null type polymorphism may be associated with a significant increase risk of PCa (OR=3.69, IC: 1.306 - 10.44) in the Algerian population.

Our data show a significant relation between PCa and GSTM1. This result may be helpful for detection of susceptible individuals.