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ANALYTICAL AND BIOMEDICAL APPLICATIONS

LJUBLJANA, OCTOBER 20TH, 2022

ABOUT US

This is the story of an innovative science platform for sharing knowledge. We are creating connecting conferences, pipetting education, webinars, workshops and more in the field of life science & analytical QC/development.

Our ambitious and multidisciplinary team from the areas of biotechnology, microbiology, chemistry, chemical technology, biochemistry, and technical science represent the foundation of our company's success.

The KEMOMIND science conference is one of the region's most interactive educational life-science events. Our idea is to support young researchers in their research careers actively. The 5th Kemomind science conference was separated into two parts:

1. Analytical: “Separation is what brings us together“
2. Biomedical: “Our DNA is Our Identity: from Genome Editing, Biobanking and Cell Biology"

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FOREWORD

Creating an impact in science

Organization Board of Kemomind

The KEMOMIND science conference is becoming one of the region's most interactive educational events. In collaboration with our partners, we had a unique opportunity to bring experts from Slovenia, Croatia, and abroad who shared their experiences with you.

> *We organized two parallel sessions correlated with the biomedical and analytical applications for the first time. We brought over 8 scientific topics and 21 presenters in one place.*

Our idea is to increase the level of integration in science in South Europe and to support young researchers in their research careers. The official language of the conference was English and Slovenian.

We want to thank all speakers for their time and for sharing their research results with the participants. Your impact is much appreciated, as it elevates the professionalism of the conference to a higher level. Lastly, we thank our partners Illumina, Hamilton Robotics, Phenomenex, IDT, NipponGenetics, Euformatics, Sartorius, Integra, CELLINK for supporting the Kemomind science conference.

ABSTRACTS

SEPARATION IS WHAT BRINGS US TOGETHER

Practical Approaches for Identifying Equivalent and Orthogonal Columns

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The selection of a suitable column for HPLC and UHPLC method development can be challenging. There is an immense number of columns to choose from, with almost 400 containing the ever-popular C18 phase alone and an ever-expanding offering of non-C18 stationary phases being introduced and marketed for everything from improved retention for polar analytes to improved peak shape for basic analytes. Columns that offer very similar selectivity can provide an alternative, or back up column, for a method. Columns having different selectivities may be desired when alternative, or orthogonal, separations are necessary, or when different elution orders are beneficial for complex separations. In this lecture we will review several different strategies for classifying and identifying similar and very different columns.

- Identifying columns with similar selectivity to your current column of choice
- Identifying columns with different or orthogonal selectivity to your current column of choice
- Understanding the resources that are available for selectivity data for HPLC and UHPLC columns

Simultaneous determination of chemical parameters for wine and cider quality control using HPLC-UV/RI system based on reverse-phase separation capabilities

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Ethanol, organic acids and sugars (mainly fructose and glucose) are components of wine and cider (apple wine), which significantly affect organoleptic properties, microbiological and chemical stability. Determining the content of these chemical parameters is ubiquitous in the process of fermentation as well as in final wine where quality controls have to be established. Fast, accurate, precise and easy to handle analytical methods for above mentioned organic compounds in the field of research are needed as well. Namely, when micro fermentations are performed for large scale screening for purposes such as yeast profiling, only small amounts of each sample could be taken from fermentation experiments, and as many as possible chemical parameters have to accurately and simultaneously determined.

In order to meet such analytical requirements, high-performance liquid chromatography coupled with UV and RI detector (HPLC-UV-RI) is needed. The simultaneous determination of organic acids and ethanol has so far been performed with high-resolution liquid chromatography (HPLC) coupled with a UV detector (for the determination of organic acids) and an RI detector (for the determination of ethanol), using a multimodal stationary phase which enables the determination of individual organic acids and ethanol at the same time (separation is performed on the basis of ion exchange and size exclusion chromatography).

Namely, HPLC-UV system incorporating stable and durable C18 reversed-phase (RP) columns enable highly reproducible results without sample pre-treatment. In addition, multimodal HPLC columns have been employed for simultaneous determination of organic acids, whereas ethanol and sugars can be also determined. On the other hand, simultaneous determination of individual sugars can be easily achieved using hydrophilic interaction liquid chromatography (HILIC) without sample pre-treatment.

Our research is focused on the development of an HPLC-UV-RI methods for the simultaneous, accurate and fast determination of major organic compounds based on reversed-phase separations. We have tested different reversed-phase columns in order to obtain optimal separation selectivity for above mentioned compounds in juices and alcoholic beverages.

Development of New LC-MS Method for Quantification of Sterols in Cell Models

Cene Skubic

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Cholesterol synthesis is a "housekeeping" metabolic pathway, and abnormalities in the late part of synthesis usually lead to an accumulation of sterol intermediates that cause severe malformations in humans. There are 19 predicted sterol intermediates in cholesterol synthesis, of which at least 13 have been experimentally confirmed. Apart from being the necessary precursor molecules, sterol intermediates are increasingly recognized as molecules with specific and important physiological functions. However, due to a lack of accessible methodology, knowledge about their roles in physiological and pathophysiological conditions remains scarce. Several sterols can act as potential RORC ligands, FF-MAS and T- MAS acts in promoting sperm maturation and 8,9-unsaturated sterols promote oligodendrocyte formation.
The obstacle in the analytics of non-polar sterols is that some have the same mass and MRM, so sufficient chromatographic separation is required. Our aim was thus to develop a method that would allow the separation and quantification of sterols with similar properties. We succeeded in developing a simple and robust LC-MS /MS for the quantitative analysis of 13 sterols from the late part of cholesterol synthesis using available standards.

We validated the method on various biological samples, such as the human HepG2 liver cell models with CRISPR-Cas9 knockouts of different genes from cholesterol synthesis, human blood serum, mouse liver tissue, and primary mouse hepatocytes.
To investigate the role of different cholesterol intermediates, we used the CRISPR-Cas9 system on HepG2 cells. We generated cell lines where in each a different gene from the late part of cholesterol synthesis was knocked out (KO), ie, CYP51, DHCR24, and SC5D KO. The KO–cell lines accumulate upstream sterol intermediates and do not have sterol intermediates located downstream of the deleted enzyme. Transcriptome changes in the KO cell lines were evaluated using Affymetrix microarrays. Analysis of differentially expressed genes (DEG), pathways enriched in KEGG, and TF enrichment was performed using TransFac 2.0 and ChEA3 databases. Cell proliferation assays and cell cycle analyses were also performed.
We were able to detect most of the targeted sterols in biological samples. As expected, the highest concentrations of sterols were present in cell/liver models with deletion of cholesterol synthesis genes, where sterols accumulate in large amounts via the deleted enzymes. In some samples, particularly human serum, the cholesterol concentration is several orders of magnitude higher than that of the other sterols and may interfere with the detection of lathosterol due to a large chromatographic peak, requiring quantification of both sterols with an adapted protocol.

IIn our cell models comparison of DEG among all HepG2 genotypes showed that 107 (or 8.9% of the total) are common to the three genotypes, but more than 65% of the total DEGs are altered in only one of the KO cell lines. This suggests a specific role of individual sterols, with different KOs causing changes in different signalling pathways, as also shown by KEGG pathway analysis. Accumulation of lanosterol and 24,25-dihydrolanosterol but not other sterols resulted in higher proliferation, cell cycle changes, upregulation of metabolic pathways, and TFs associated with cancer progression like NFKB, ESR1 and genes from WNT signalling like LEF1.

These results clearly indicate that individual sterols or groups of sterols play a specific role in the signalling pathways. We hypothesise that dihydrolanosterol activates signalling pathways that promote cell proliferation, block apoptosis, and affect cell cycle state, with transcription factors NFKB1 and ESR1 likely playing key roles, but the exact mechanisms remain to be unravelled.

With minor modifications in the isolation procedure, our LC/MS method can be applied to a wide variety of biological samples, allowing unraveling the enigmatic role of sterols in health and disease. The transcriptome data from KO cell models together with downstream analyses allows us to further explore the unknown functions of sterols and find out in which biological processes they are involved.

Analyzing Complex Plant Material – Cannabis

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Cannabis is a plant that produces cannabinoids, terpenes, and many other secondary metabolites. The most wildly used and researched cannabinoids are cannabidiol (CBD) and Δ-9-tetrahydrocannabinol (THC). CBD is nowadays one of the most popular substances in pharmaceutical, food supplements, and cosmetic productions. CBD is an active pharmaceutical ingredient (API) in medicines with marketing authorization, a cosmetic ingredient, recognized by the European Commission in the CosIng database, and is pending permission in food supplement products in the European Union if the producers succeed to prove safety of their products. THC is the most notorious cannabinoid with more direct pharmacological action. Because of its psychoactivity, cannabis was historically scheduled as an illicit drug and was underresearched. On the other hand, THC is an indispensable API.

With increasing scientific information in the area, cannabinoid use is becoming better regulated. Due to the unavailability of cannabinoid medicines with marketing authorization in some markets, magistral medicines are available in Slovenian pharmacies. They are prescribed by a doctor for any indication and in any form at his judgment and responsibility. Prescribed formulations are individually prepared by the pharmacist for each patient without covering their expense by the health insurance system. Therefore, a lot of people are using homemade cannabis preparations, which contain various and sometimes unknown amounts of THC. Around 100 adult poisonings are treated at the University clinical center every year due to intoxication with cannabis products. This sheds the light on the great importance of the analytics the industry constantly implements in quality assurance. Many security risks are also connected to the production process, not to forget the fact cannabis is simultaneously an illicit drug.

Cannabinoids are present on the surface area of cannabis epidermal tissues, especially in female flowers. They are biosynthesized in the form of acids. To turn them into pharmacologically active substances, we need to carry out the decarboxylation process. During the research on CBD production, we found that the best decarboxylation conditions are heating the cannabis plant to 140 °C for 20 minutes. Cannabinoids can be used in the form of complex cannabis extracts or purified to CBD, THC, and other isolates (cannabigerol, cannabichromene, cannabinol, etc.).

We carried out solubility studies of CBD in different extraction solvents and pharmaceutical vehicles. CBD is well soluble in alcohols and some organic solvents, but not soluble in water. It can be dissolved in edible seed oils up to 40 % w/w. The most common way to obtain cannabis extracts from biomass is extraction with ethanol or supercritical CO2. We found that CBD is light-sensitive but more stable than THC, which quickly degrades under air exposure. The presence of seed oils can influence its stability.

Currently, in the EU, harmonized monographs to be published in the European pharmacopoeia for flowers and extracts are in the pipeline: Cannabis flowers, Cannabis extractum siccum, and Cannabis extractum spissum. Until their release, monographs for cannabis flower and cannabis extract are in use from the Deutsche Arzneimittel Codex (DAB). Those monographs include the HPLC methods for the determination of the cannabinoid content. We developed a more efficient HPLC method, which also proved to be superior to gas chromatography. Gas chromatography namely demands derivatization steps in order to distinguish between acidic and decarboxylated cannabinoids.

Special attention needs to be paid to the fact that cannabis is of natural origin, which means each organism is unique. The growth and production of secondary metabolites, including cannabinoids, are highly dependent on the growing conditions. Cannabis is vulnerable to contaminants such as microorganisms, which furthermore produce toxic metabolites, such as alfa- or ochratoxins. Cannabis is also known for its heavy metal accumulation, which is drawn from the soil. As with any other crops, regular monitoring of water quality and prevention of pests with hygienic measurements are better than curation. Use of pesticides demands costly analyses after harvest with a high risk of expendable crops. This is the reason why following Good agricultural and collection practice (GACP) is crucial for the cultivation of plants intended for pharmaceutical, food supplement, and cosmetic production.

To sum up, cannabis is indispensable medicinal material. It seems to be opening a new era in the establishment of a pharmaceutical quality systems to produce medicines of natural origin. Cannabis is a pioneer in understanding GMP guidelines for indoor cultivation. Big challenges occur due to its natural origin, complexity, regulatory status, and also the late start of its intense scientific research. Recent analytical advances are one of the reasons that cannabis science and the economy are developing rapidly.

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The Science and Technology of Water Purification

John Jerger

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Purified water is the most commonly used solvent in laboratories. Consistent water quality is critical for reproducibility and reliability of results between experiments and laboratories.

Topics discussed in this presentation:

- The importance of purified water for laboratories
- Contaminants in potable water
- Qualities of purified water and their applications
- Water purification technologies
- Best practice for working with purified and ultra-purified water

AVIDITY SCIENCE

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Pipetting techniques and pipetting errors origins, pipettes calibration

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Pipetting is widely used practice in most laboratories and air displacement pipettes are most common types of pipettes, whether they are mechanical or electronic, single- or multi- channel. There is an air column between the liquid and the piston, which is very sensitive to environmental conditions, such as temperature, air pressure and humidity. Consequently, those conditions can have major impact on pipetting results and must be taken into consideration.

Liquids also have different properties and require different pipetting techniques. Forward pipetting is most known and used, but it is suitable mostly for water and water solutions containing buffers. For most other liquids (organic solvents, viscous and foaming liquids, serum, blood, master mix, cell cultures) reverse pipetting is recommended.

In pipetting, there are two commonly used terms: accuracy and precision. Accuracy means how close the measured value is to the true value and is expressed as 'inaccuracy' or the 'systemic error'. Precision means how close replicate measurements are to each other and is it is expressed as 'imprecision' or 'random error'. Both values are stated in calibration certificate. Only properly maintained and calibrated pipette can give accurate and precise pipetting results.

Calibration is a set of operations for determining the relationship between the values shown by an instrument or measuring system, or the values shown by a tangible measure or reference material, and associated values, realized with standards, under certain conditions.

In July 2022 a new edition of the calibration standard was published, ISO 8655-2022, implementing some changes into calibration procedure in environmental requests. ISO 8655 is a general standard for the performance of calibrations. Calibration laboratory Kemomed has been accredited in 2005, offering maintenance, accredited calibration and non-accredited gravimetric tests for volumetric devices from all manufacturers.

In addition to the correct pipetting technique (forward or reverse pipetting), many other factors must be considered to achieve the highest accuracy and precision: pre-rinsing, right aspiration and dispensing angle, right immersion depth, use of right tips, pipetting speed etc. Pipette softness and ergonomic are also important. Electronic pipettes make pipetting easier and are more precise and accurate even when the pipettor has low experience.

Pipetting can be tiring job and can result in injuries and absence from work. Laboratory staff have 2-times more symptoms of WURLD (work related upper limb disorder) than other workers and 44 % of laboratory staff suffers from pipetting induced injuries. This can be reduced with use of soft and ergonomic pipettes, proper workspace preparation, proper posture and with pause and stretching.

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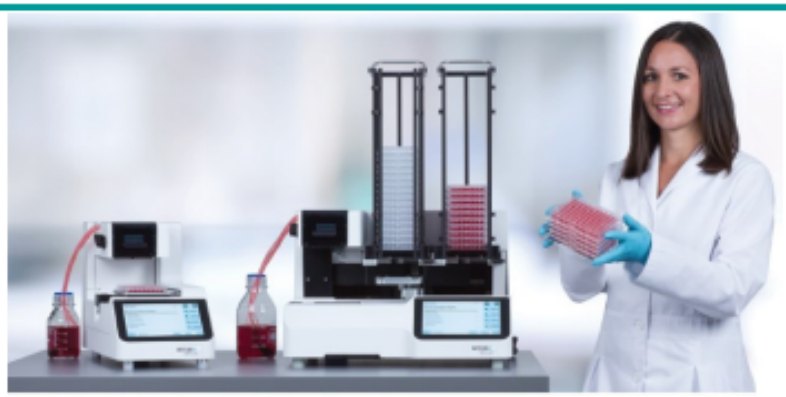

INTEGRA BIOSCIENCES

ABSTRACTS

OUR DNA IS OUR IDENTITY: FROM GENOME EDITING, BIOBANKING AND CELL BIOLOGY

Targeted Next Generation Sequencing for the Diagnosis of Familial Erythrocytosis

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Erythrocytosis is a blood disease defined by an increased number of red blood cells, haemoglobin (Hb) and haematocrit (Hct). Erythrocytosis has a diverse background and is rarely the result of an acquired or inherited single gene variant (monogenic disorder). Polycythemia vera (PV) is an acquired erythrocytosis due to a somatic variant in the gene JAK2. Congenital or Familial Erythrocytosis (ECYT) is the result of a germline variant in one of the nine genes involved in the oxygen sensing pathway in the kidneys (VHL, EGLN1, EPAS1, EPO), in erythropoietin signal transduction in pre-erythrocytes (EPOR) or in the regulation of haemoglobin-oxygen affinity in mature erythrocytes (HBB, HBA1, HBA2, BPGM). Germline variants are often found in patients diagnosed at a young age or in patients with a family history of erythrocytosis (1). However, in more than 60 % of cases, the genetic background of disease development remains undetected, suggesting that other genes and mechanisms must be involved in disease development (2). In the last decade, next generation sequencing (NGS) has been widely used for the diagnosis of this rare disease, as it allows parallel sequencing of multiple genomic regions.

Our research aims to improve the diagnostic yield in patients with idiopathic (unexplained) erythrocytosis in Slovenia. First, we updated the current national diagnostic algorithm to identify patients with Familial Erythrocytosis among those with idiopathic erythrocytosis. Second, we developed a dedicated NGS panel targeting genes involved in erythropoiesis and iron metabolism. Third, we applied the newly developed targeted NGS to erythrocytosis patients selected in the 8-year retrospective study.

The national diagnostic algorithm for Polycythemia vera was expanded into a three-stage diagnostic algorithm for erythrocytosis in collaboration with the Department of Haematology at the University Medical Centre Ljubljana, Slovenia. In the first stage, true erythrocytosis is confirmed by enrolling patients with elevated Hb (> 185 g/L in men, > 165 g/L in women) and/or elevated Hct (> 0.52 in men, > 0.48 in women) at two examinations more than two months apart. In the second stage, Polycythemia vera is ruled out and secondary causes of acquired erythrocytosis are carefully reviewed. In the third stage, patients who remain idiopathic are referred to targeted NGS for possible evaluation of their genetic background (3). The described diagnostic algorithm has also been established within the self-developed ViDis platform, which enables the visualisation and exchange of medical algorithms (4).

Detailed revision of the mechanisms involved in normal erythropoiesis and the pathophysiology of erythrocytosis, highlighted the role of 24 erythrocytosis- and 15 hereditary haemochromatosis-associated genes (Figure 1). Targeted NGS of 39 genes included the exon regions for all genes, while for some genes (EPO, VHL) the first intron, promoter and enhancer regions were also examined. Libraries were prepared using the Nextera DNA library preparation kit (Illumina). Enrichment was performed by probe hybridisation with a custom gene panel (Integrated DNA Technologies), followed by sequencing (MiniSeq, Illumina) (5). Sanger sequencing of all clinically relevant regions was developed to validate the variants identified by NGS.

The list of genes and regions is regularly updated based on recent publications. The flexibility of gene panel exome sequencing with pre-designed probes provides the opportunity to promptly update new variants in known and newly identified candidate genes associated with erythrocytosis.

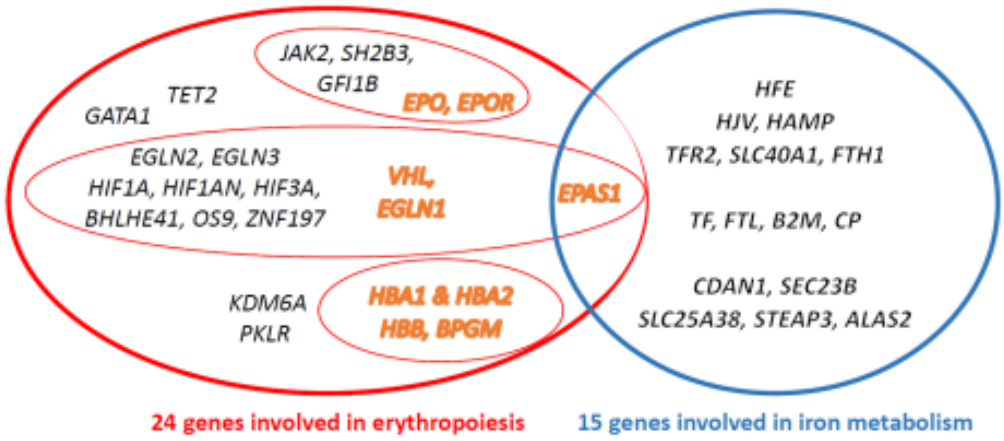

Figure 1. Genes included in the targeted NGS for erythrocytosis.

An updated three-stage national diagnostic algorithm was used to identify patients with suspected for Familial Erythrocytosis among those referred to the outpatient haematology clinic at the University Medical Centre over an eight-year period between March 2011 and April 2019. A retrospective analysis revealed that of 655 patients who met the criteria for true erythrocytosis, 116 patients met the criteria for non-clonal (JAK2 negative) erythrocytosis. A detailed review of the patients' medical records revealed that 34 patients had acquired secondary erythrocytosis due to a chronic condition such as obstructive sleep apnoea, lung, heart, or kidney disease. In 15 patients, including five families, the erythrocytosis remained idiopathic despite all relevant investigations and they were referred further for genetic investigation using targeted NGS. Other patients were still incompletely characterised at the time of inclusion in the study and are currently being further investigated (3).

Targeted NGS was later performed in 25 patients and two controls. Several variants were identified that are known or suspected to be associated with erythrocytosis or haemochromatosis. However, only one patient was identified with the pathogenic heterozygous variant in the EPAS1 gene (c.1609G > A, p.Gly537Arg) with proven cause of Familial Erythrocytosis type 4 (ECYT4, OMIM ID 611783), previously reported in several unrelated individuals with erythrocytosis. All other variants identified were novel variants of unknown significance (VUS) in the coding region of the EGLN1, EPAS1, JAK2 and SH2B3 genes, which may promote the development of erythrocytosis (6). The low-frequency intron variant in the JAK2 gene with an autosomal dominant inheritance pattern was identified in two affected family members who may result in a splicing alteration (5). In addition, a high frequency of two heterozygous variants in the HFE gene associated with hereditary haemochromatosis type 1 (HFE1; OMIM ID 235200) was found in 10 of 25 patients. In addition, a pathogenic variant in the SEC23B gene associated with congenital dyserythropoietic anaemia type 2 (CDAII; OMIM ID 224100) was identified in two unrelated patients. All identified pathogenic or VUS variants were further confirmed by Sanger sequencing.

Congenital erythrocytosis type 4 was diagnosed in a young patient with erythrocytosis without a known familial background, who suffered from several thromboembolic complications prior to NGS diagnosis. In addition to viscosity, the patient also had other risk factors for thromboembolic complications that may promote thromboembolic events, including obesity, smoking and hormonal influences. Addressing all of these factors could prevent further complications. The examination of the patient's child and parents did not confirm heredity (7).

In summary, in close cooperation with the University Medical Centre Ljubljana, we have successfully introduced a diagnostic algorithm for Familial Erythrocytosis in Slovenia and implemented it in clinical practice. The three-stage algorithm is a useful tool for the daily clinical evaluation of erythrocytosis. Secondary causes of erythrocytosis are currently being systematically reviewed and patients in whom disease remains idiopathic are referred for genetic testing. Targeted NGS was a necessary advance in existing diagnostics and we were able to confirm the genetic cause in one patient. However, the discovery of new variants with as yet unknown significance in Slovenian patients encourages the development of functional approaches to determine the association of the variants with the disease. For further investigation of disease etiology in patients with unexplained erythrocytosis, a broader approach is recommended, such as an expanded gene panel, WES or WGS.

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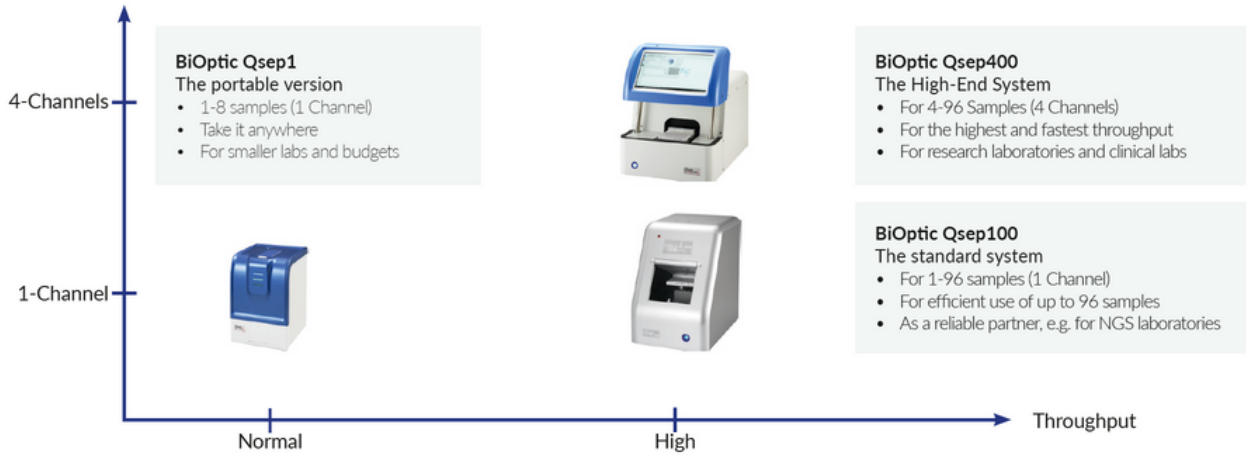
3. Choose the appropriate method and run. In just a few minutes, users can get reliable results in diverse formats (PDF, Word, Excel), including peak and gel charts.

The three Qsep Fragment Analyzer models differ in the number of samples to be analyzed per run. Handling, technology, and the type and quality of the applications that can be carried out are identical for all models.



Applications Of Ngs At National Laboratory Of Health, Environment And Food In Slovenia

Aleksander Mahnic, Ph.D.

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National Laboratory of Health, Environment and Food (NLZOH) is the central and largest Slovenian public health laboratory that handles environmental protection, diagnostic and public health microbiological activities, chemical and microbiological analyses of different types of samples, and performs research activities. This talk will cover chosen research and public health-oriented applications of Next Generation Sequencing at NLZOH.

From the start of SARS-CoV-2 pandemic, NLZOH coordinates Slovenian project of SARS-CoV-2 variant monitoring with whole genome sequencing (WGS) of the entire viral genome. Collected genome data aided at guiding social and public health measures throughout the epidemic. The variant spread in Slovenia largely overlapped with that observed in neighboring countries, except for the unusually successful spread of variant B.1.258.17 in Slovenia in early pandemic. In depth genome analysis and infectivity testing on Vero cells did not confirm any advantage of this variant compared to variants predominating at the same time in neighboring countries.

At NLZOH we are at early stages of establishing a comprehensive collection of bacterial strains with antimicrobial resistances (AMRs). The accessibility of strains isolated from clinical samples as well as different environmental sites and food will enable a monitoring system, which will elucidate the transfer of AMRs and help us identify their reservoirs. Early results showed low overlap between carbapenem resistant Pseudomonas aeruginosa genotypes isolated from hospitalized patients and wastewater treatment plants.

Finally, at the Department for microbiological research, we are focusing our research on the analysis of complex microbial populations, especially gut microbiota through the development of simple gut models as well as semi-large population studies. The research was led from Clostridioides difficile associated gut alterations to studies of microbiota in healthy Slovenian population in relations to different disease-specific modulations. In the talk we will present our main findings from the study including 121 hospitalized patients with different gastrointestinal-related health complications.

Critical steps along the NGS workflow dry lab

Christophe Roos, Ph.D.

Euformatics

Validating an NGS test in a laboratory requires sufficient knowledge of the process and the right tools to ensure that validation can be carried out efficiently and with a high reproducibility rate. For most laboratories, it is also imperative to adhere to relevant ISO standards as they may also be looking to acquire official laboratory accreditation. In this case, one crucial aspect is the quality management documentation of the different processes that can affect the outcome for a patient. With sample quality control all the data required is readily and continuously at hand, both on a per-sample basis and a higher level, and triggers for automatic warning can be set.

For running a validation, the DNA amount loaded onto the sequencing chip is critical, since the sequencer manufacturer has routines for assessing the DNA amount based on observations on cluster size and signal intensity as well as other parameters obtained after the run. As a result, the DNA amount will also affect the coverage on target and can therefore be monitored on the level of baits or amplicons and down to single base pairs using sample quality control.

The read depth (DP) is another one of the most important parameters for obtaining sufficient measurement data to perform a proper variant calling. The required read depth will depend on multiple factors such as level of detection (LOD, the lowest variant allele frequency that must be detected), the complexity of the genomic ROI, and the number of observations required to consider a called variant to be true.

By combining the automated functionality of sample quality control to routinely assess and measure the key parameters with its ability to both store the data for reporting purposes and chart it for comparisons, a laboratory can run an iterative process to arrive at the stage where their tests are running correctly and at a level where they can be sure that the data is trustworthy to be used in a diagnostics procedure.

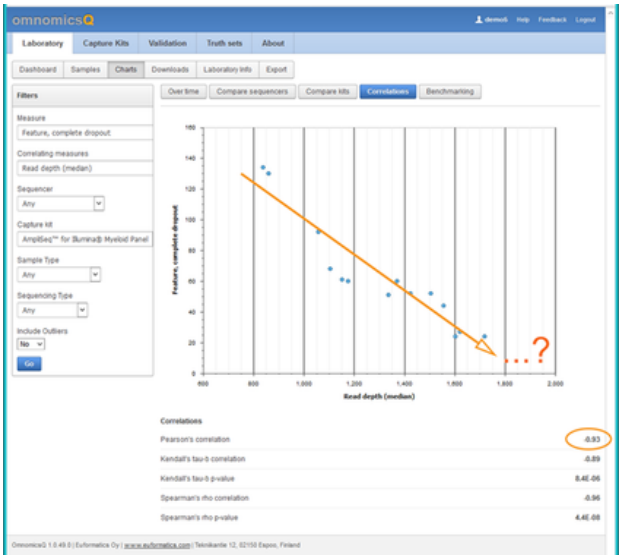

Figure 1: correlation between different tested read depths and coverage on target in sample quality control.

The sample quality control comprehensive NGS quality management system delivers the insights lab management and quality managers need to achieve top-tier confidence in sample data quality and lab performance by automating, centralising and contextualising quality control data management and analysis.

- Validate and verify assays with the assay validation module
- Apply widely to RNA and DNA (WGS to panels) with any kit, sequencer or platform.
- Track sample quality using traffic light notification for every sample according to stored user-defined SOPs.
- Report, chart and benchmark lab performance progress and changes.
- Compare performance to database of peers.
- Track performance of lab sequencers and kits.
- Implement CAP/AMP Standards and Guidelines, EuroGentest Guidelines and ISO 15189/17025 standards.

The basics: Genom Editing with CRISPER/CAS

Abdelilah Mekhloufi, Ph.D.

IDT

CRISPR-based homology-directed repair (HDR) is an invaluable tool to facilitate precise, specific mutations in a genomic region of interest. While many methods have been reported for improving HDR efficiency, achieving precise changes via HDR remains a challenge particularly for large knock-ins. HDR repair outcomes are most efficient with single-stranded DNA (ssDNA) templates when small insertions, deletions, or SNP changes are desired edits. For these applications, synthetic oligonucleotides (ssODN) are commercially available with modifications for enhanced efficacy in HDR. Here we present expansive data sets to achieve HDR with custom-synthesized Alt-R HDR Donor Oligos, which are chemically modified IDT Ultramers™ for improved HDR. These experiments have resulted in the development of the Alt-R HDR Design Tool, a novel bioinformatics tool that has been trained on empirical data for the design of HDR donor templates in an easy-to-use, open-access web format. The Alt-R HDR Design Tool supports designs to generate insertions, deletions, and point mutations in genomic DNA and enables Cas9 guide selection with a paired DNA template containing silent mutations. Larger insertions can be generated via HDR using enzymatically generated ssDNA or double-stranded DNA (dsDNA) donor templates. Here, we present work demonstrating that improved efficiency in HDR rates for large insertions is obtained when dsDNA donor templates include novel end-modifications. These modifications improve the frequency of HDR and reduce homology-independent (blunt) insertion events that can occur at both on- and off-target CRISPR edits. Finally, we demonstrate improved HDR rates when using Alt-R HDR Enhancer V2, a small molecule that increases the rate of HDR in varied cell types including iPSCs and primary human T-cells. Together the use of Alt-R modified repair templates and the Alt-R HDR Enhancer V2 improved HDR rates up to 5- to 10-fold across knock-in experiments.

The sample quality control comprehensive NGS quality management system delivers the insights lab management and quality managers need to achieve top-tier confidence in sample data quality and lab performance by automating, centralising and contextualising quality control data management and analysis.

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Alex Kruis, Ph.D.

AciesBio d.o.o.

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Enhanced gene editing by Coiled-coil Recruitment of an Exonuclease to CRISPR/Cas

D. Lainšček[a, b] **V. Forstnerič** [a], **V. Mikolič** [c, d], **Š. Malenšek** [a, d], **P. Pečan** [a, d], **M. Benčina** [a, b], **M. Sever** [c, d], **H. Podgornik** [c, e], **R. Jerala** [a, b].

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The CRISPR/Cas system is a highly potent tool which has revolutionized genome engineering and regulation of gene transcription in various cells and organisms. This gene-editing tool consists of a guide RNA (gRNA) and Cas9 endonuclease. Cas9 catalyzes the formation of double-strand DNA breaks, which are then repaired by different cell mechanisms. Error-prone Non-homologous end joining occurs, resulting in random indel (insertion-deletion) mutations, which can lead to functional gene inactivation by either frameshift or deletions. To achieve greater indel mutations that can lead to bigger gene disruption, CRISPR system can be coexpressed in cells with DNA exonucleases, which cause increased recessions of DNA following DNA breaks. We show that joint action of the CRISPR system with different exonucleases significantly increases the percentage of indel mutations at various targeted genes. Of the different exonucleases tested, the E.coli-derived exonuclease III (EXOIII) exhibited the best performance in terms of indel formation. To further improve the rate of indel mutations, Cas9 and EXOIII were brought into the proximity via coiled-coil forming heterodimeric peptides (CCExo). This resulted in increased indel formation compared to the classical CRISPR/Cas system as well as more efficient than cointroduction of non-interacting and genetically fused Cas9-EXOIII.

We discovered that not only the percentages of indel were increased also the size of gene deletions were significantly enhanced compared to conventional CRISPR/Cas system. Genome editing rate was augmented when using heterodimeric coiled-coil forming peptides with higher binding affinity. By high through put method, called CIRCLE-seq we have proven that increase in genome modification does not bare any dangers as no additional undesired DNA cleavage was observed.

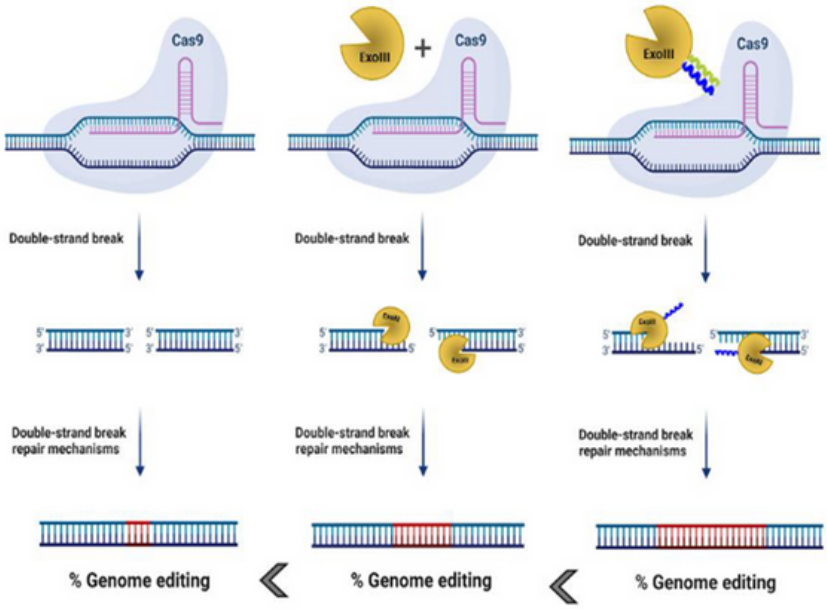

Figure 1. Schematic presentation of the principle of enhanced gene inactivation via the CCExo system.

The robustness of CCExo was determined for different genes in various cell lines, as well in human primary cells and in somatic adult cells. Finally we performed a case study for the use of the CCExo system as a potential anti-cancer therapeutic tool. The Philadelphia chromosome, which occurs in leukemic cancer cells, is the result of characteristic the reciprocal genome translocation t(9:22) and is responsible for higher proliferation of tumorous cells. Using the CCExo system, we achieved a higher degree of indel mutations at the translocation site, which resulted in greater killing of cancer cells, thus providing a useful potential anti-cancer therapeutic tool. This was also confirmed in vivo by using xenograft animal cancer model.

We successfully confirmed that enhanced genome editing tool, CCExo, holds a tremendous potential not only in treating CML cancer but potentially also in other diseases with genetic etiology.

Efficacy evaluation of steric block oligonucleotides for splice correction

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Oligonucleotide therapeutics are nucleic acid polymers with the potential to treat or manage a wide range of diseases such as Duchenne muscular dystrophy, familial amyloid neuropathies, acute hepatic porphyria, macular degeneration, cancer. Majority of oligonucleotide therapeutics focus on gene silencing, splice modulation and gene activation. Oligonucleotides mainly interact with target molecules via complementary Watson-Crick base pairing and selectively target any gene with minimal or at least predictable off target effects. Oligonucleotides as therapeutic drugs come in various forms, from antisense oligonucleotides and small interfering RNAs to anti-miRNA oligonucleotides.

Antisense oligonucleotide drug acting as steric block mask specific sequences within target transcript and interfere with transcript RNA-RNA and/or RNA-protein interactions. The most widely used application of steric block antisense oligonucleotides is in the modulation of alternative splicing in order to selectively exclude or retain specific exons. In these cases oligonucleotide masks a splicing signal such that it becomes invisible to the spliceosome, leading to the alterations in splicing decisions.
The objective of our study was to investigate the activity of the synthetised steric block oligonucleotides for splice correction by treating model cell lines with oligonucleotides and evaluating their the efficacy. We have demonstrated that synthesized oligonucleotides increase full lenght trancript levels and decrease the levels of the truncated transcript. Our results also show that all synthesized oligonucleotides have comparable effect and result in 2-fold increase in full lenght protein expression.

Next-generation Chimeric Antigen Receptor (CAR) T Cells

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Engineered T cell-based therapies are advanced cancer immunotherapy approach with genetically modified T cells. The two most widely used immune receptors are a Chimeric Antigen Receptor (CAR) and tumor-reactive T-cell Receptor (TCR). Based on successful clinical trials in relapsed or refractory pediatric acute lymphoblastic leukemia (ALL) and adult high-grade B-cell lymphoma, CD19-targeting CAR T cells received FDA and EMA approval in 2017 and 2018 respectively. The success of CAR T cells is countered by challenges in efficacy in solid tumors and in hematologic malignancies that induce dysfunctional T cell states. To overcome some of the challenges, we have developed a genetic approach that combines activation-inducible production of an accessory molecule, along with constitutive CAR expression in a single lentiviral vector called Uni-Vect. By knocking out the endogenous TCR using the CRISPR/Cas9 method, we rendered CAR signaling an exclusive activator of the system. Here we present two distinct therapeutic applications of Uni-Vect. In a first therapeutic approach, we introduced inducible expression of IL-12 (iIL-12 CAR T) and demonstrated remarkably enhanced anti-tumor responses in established solid tumors in vivo. In a second therapeutic approach, we modulated CAR T cell-intrinsic properties by transient activation-inducible transcription factor expression (iTF-CAR T) and demonstrated improved therapeutically relevant T cell states and in vivo expansion. Overall, our work provides a foundation for clinically actionable next-generation cellular immunotherapies.

Declaration of interests: AS, ADP, CHJ and DJP are co-inventors on PCT International Patent Applications by The Trustees of the University of Pennsylvania, which incorporate discoveries and inventions described here.

Funding: Perelman School of Medicine at the University of Pennsylvania, Parker Institute for Cancer Immunotherapy, A.S. also received funding from Slovenian Research Agency (ARRS) and from the National institute of biology

Why and how: Regulatory T cells for immunotherapy of autoimmune diseases

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Autoimmune diseases affect people of all ages and often significantly impair their quality of life. Patients often require intensive, lifelong medical interventions that result in only partial remissions. In the search for safe and effective therapies, there is great motivation to develop cell-based immunotherapies. Ideally, such treatments would reduce dependence on immunosuppressive drugs, restore immune response, and potentially stimulate tissue repair. The answer to reduce dependence on immunosuppressive drugs and to restore immune response already lies in the repertoire CD4+FOXP3+ regulatory T cells (Treg), which are essential for maintaining immune homeostasis.

Due to their multiple suppressive mechanisms affecting a broad spectrum of immune cells, Treg are currently being explored to develop cell therapies for autoimmune diseases and solid organ transplant rejection. Clinical studies have shown that polyclonal Treg cells can control autoimmune responses after adoptive transfer. In preclinical models, antigen-specific Treg cells were superior to polyclonal ones, which has driven the development of antigen receptor-engineered Treg cells.

To understand the molecular mechanisms involved in the protective role of genetically engineered Treg cells and to evaluate the strengths and limitations of this approach, we used a preclinical mouse model of central nervous system (CNS) autoimmune disease - experimental autoimmune encephalomyelitis (EAE) induced by immunization with a peptide derived from myelin oligodendrocyte glycoprotein (MOG).

Treg cells engineered to express TCRs that recognize the MOG35-55 peptide, protected mice from developing EAE. After disease onset, they initially accumulated in draining lymph nodes and upregulated LAG-3, CTLA-4, and PD-1. The genetically engineered Treg cells also significantly reduced the acute autoreactive CD4+ T cell response in the treated mice, as evidenced by the decreased number of MOG - reactive proinflammatory CD4+ T cells in the blood at day 9 after immunization. They also persisted in target organs for more than 30 days after immunization and showed an effector/memory CD44highCD62Llow phenotype compared with endogenous Treg cells. To define the molecular characteristics of autoreactive Treg cells in the CNS of diseased mice, we performed single RNAseq and bulk transcriptome analyzes of autoreactive Tregs and conventional T cells (Tconv) at the peak of the EAE. Among other genes, Il10, Ctla4, and Areg were highly expressed in Treg cells in the CNS compared with their Tconv counterparts. Using genetic approaches we generated Treg cells in which genes were silenced (Il10, Ctla4, Areg). We demonstrated that Il10 and Ctla4 were nonredundantly required for protection against EAE, whereas Areg had no effect. Engineered Treg cells thus protect against autoimmunity in the CNS via multiple immune mechanisms. This highlights their immense potential for intercepting autoimmune responses and developing cell-based therapies.

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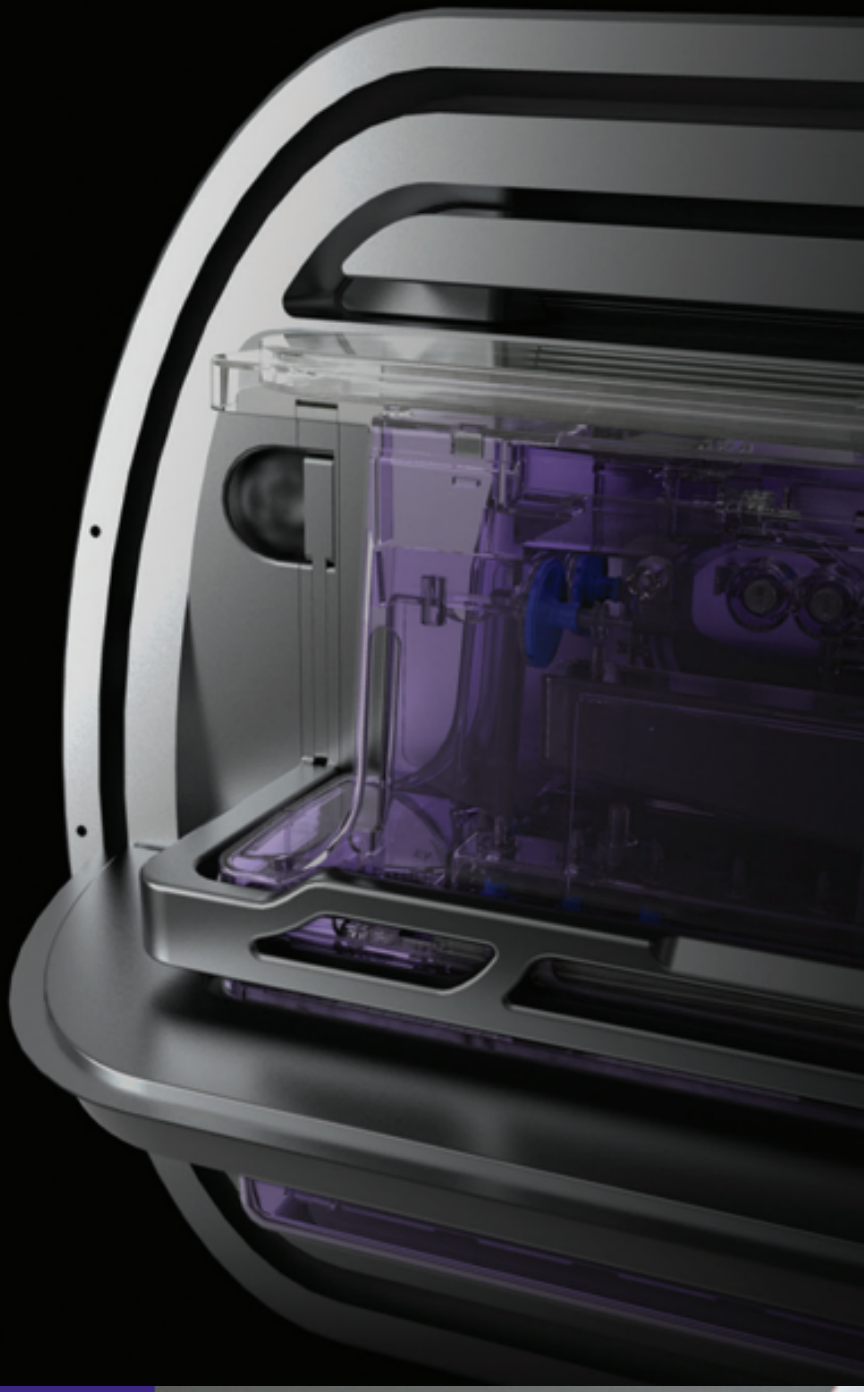

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Alternative In Vitro 3d Cell Models For Genotoxicity Testing

Martina Štampar a., **Bojana Žegura** a.

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Liver cell lines cultured in two-dimensional (2D) monolayers are the most often used experimental system for investigating the adverse effects of xenobiotics in vitro. However, most hepatic cells cultured in 2D lack relevant hepatic properties. They express low levels of phase I (activation) and phase II (detoxification) metabolic enzymes required for metabolic activation of indirect-acting genotoxic compounds, which makes extrapolation of results to in vivo conditions questionable. This is of particular importance in genotoxicity testing as unreliable results may lead to the requirement to be confirmed in vivo in animal studies. Therefore, it is very important and essential to develop improved in vitro cell-based systems that can more realistically mimic in vivo cell behaviour and provide more predictive results for in vivo conditions. Three-dimensional (3D) models have improved cell-cell and cell-matrix interactions and preserved complex in vivo cell phenotypes. Moreover, compared to 2D models, 3D hepatic models exhibit higher levels of liver-specific functions, including metabolic enzymes and enable prolonged exposures, due to their increased stability, maintaining high cell viability and morphology over a period of several weeks. Altogether, newly developed hepatocellular 3D cell models, represent a more advanced experimental model for genotoxicity studies as well as for safety testing of new chemicals and products due to their more complex structure and improved metabolic capacity. Nevertheless, the 3D cell models need be further characterised and validated in terms of cell division and response to genotoxic stress to better know their behaviour and properties. The 3D models have the potential to bridge the gap between in vitro and animal testing/models.

Let's Go 3d: The Future Of Prostate Cancer Models

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Tissues are three-dimensional (3D) entities as well as the tumor that arises within them. Therefore, to study the complexity of tumor model it is necessary to develop more realistic model than classical 2D monolayers used so far. Even though traditionally used for decades, because of vast benefits they provide, 2D cell cultures do not meet the demands in terms of being able to effectively mimic complex cellular signaling, angiogenesis, invasion and metastasis present in cancer. Moreover, 2D monolayer represents overly simplified extracellular matrix (EMC) which has been proven unsuccessful in identifying preventative anti-cancer treatments. The aim of our research is to establish a platform for development of multicellular 3D spheroid models of prostate cancer which can easily be produced and maintained in the laboratory setting. More precisely, by producing such a model we hope to elucidate the tumor-stromal cell interaction in prostate cancer, which is of great importance for prostate cancer research, however it is difficult to study in 2D environment. One of the relevant pathways in prostate cancer progression is the Hedgehog-GLI (HH-GLI) signaling pathway which is crucial for normal embryonic development, stem cell maintenance and tissue homeostasis in adult organisms, yet it seems hyperactivated in various tumors, including prostate cancer. The final goal is to better understand the differences in HH-GLI signaling pathway in co-culture of prostate cancer and stromal cells while comparing three-dimensional (spheroid) models with two-dimensional (adherent) setting used so far.

We have produced various 3D models (spheroids) of prostate cancer, using adenocarcinoma prostate cell line LNCaP and cancer-associated prostate fibroblasts WPMY-1 as a monoculture or co-culture by the means of hanging drop system. Cells are grown separately for 72 hours depending on the cell line, at 37 °C, 95% relative humidity in a 5% CO2 and air atmosphere. Afterwards, each cell line is counted at density of 1x106/ml, labeled with membrane-integrating fluorescent dyes, and mixed in diverse ratios before setting them into hanging drops for spheroid formation. These spheroids are subsequently trypsinized and analyzed on the MUSE Cell Analyzer to measure cell ratios and cell viability, in order to establish optimal experimental conditions (cell number per sphere, duration of cultivation). Furthermore, spheroids are subjected to the cryosectioning. Each spheroid is collected by pipetting and embedded in the CryoFix gel, followed by freezing in liquid nitrogen vapor. Frozen blocks are cut on the cryotome and preserved at -80°C until staining. Cryosections of 3D spheroids are then stained by immunofluorescent staining for HH-GLI pathway proteins important for the prostate cancer development and epithelial/mesenchymal markers, to determine the localization of proteins in the 3D model and the HH-GLI pathway activity in tumor vs. stromal cells. We have established a simple, cost effective and straightforward protocol for development of multicellular 3D spheroid models of prostate cancer which can easily be produced and maintained in the laboratory setting.

Cancer and stromal cells are forming spheroids of different shapes and sizes. Immunofluorescent staining for HH-GLI pathway proteins and epithelial/mesenchymal markers demonstrated correlation with previous expression analyses of the key genes in question. It is visible that stromal cells are changing their characteristics to the higher extent compared to the cancer cell lines. Results obtained so far are pointing out the importance of interaction between the cancer cells and cancer associated fibroblast in the prostate cancer progression.

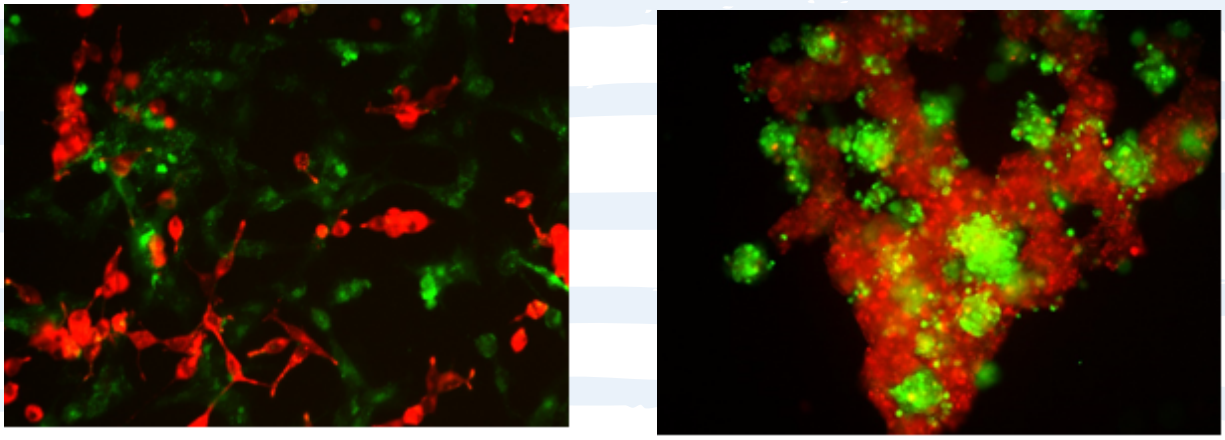
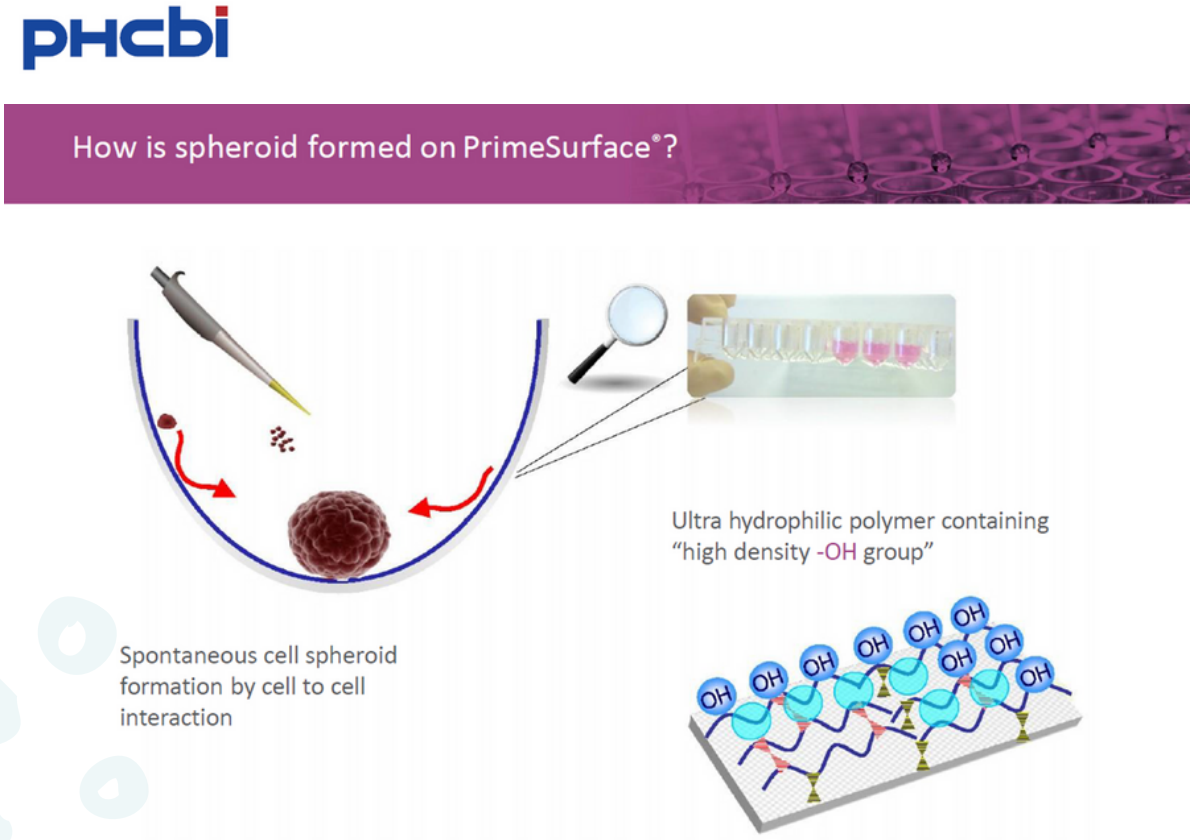


Figure 1. Comparison of a 2D (left) and 3D (right) co-culture of adenocarcinoma prostate cell line LNCaP (red) and cancer-associated prostate fibroblasts WPMY-1 (green) (unpublished results).



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Cross-border project for the efficient management of biobanks, C3B

prof. dr. Vladka Čurin Šerbec

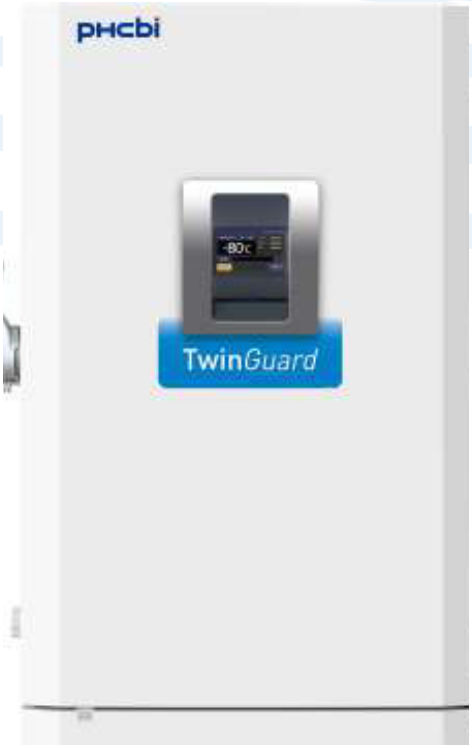
Zavod Republike Slovenije za transfuzijsko medicino
Blood Transfusion Centre of Slovenia

The common challenge of the Programme Area is the uncoordinated field of biobanking in the public health system, which makes it challenging to manage and exchange the biological samples needed for standardized research and clinical studies.

The project's primary goal is to upgrade the capacity of cross-border cooperation between institutions by encouraging public authorities and key stakeholders in the field of biobanking to establish standard solutions for process coordination and more efficient cross-border management of biobanks and biobank networks. The cross-border platform will be set up following the GDPR, national legislation/law, and EU acquis and ethical standards.

The target groups are legislators, public administration, regulators, companies, health systems, universities, and research institutes.

The Future of Biobanking



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SAFE® 2D tubes can be used everywhere, where the focus is on storing, transporting and reliably tracking samples safely in the long or short term and in a space-saving manner.

The long-term storage of human as well as animal and plant-based biomaterials probably comes closest to what is generally understood as biobanking. In contrast to epidemiological and clinical studies, this type of storage is typically used in research projects, in which the research objectives are still largely undetermined at the time of sampling.

This context often means that biobanks are often tied to institutes and centres for clinical chemistry and laboratory medicine of large hospitals and university hospitals. Biomaterial samples such as blood and urine, which were collected for routine diagnostic examinations, are transferred to long-term storage for research purposes.

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Fully Automatic Tube Laser Marker

HAMILTON

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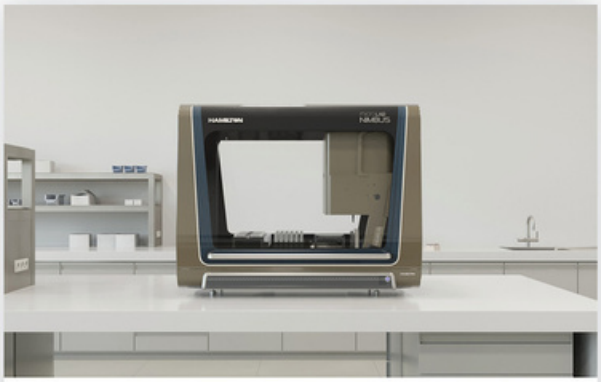

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NOTES

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Kemomed Sales Slovenia

Team Biomedicine

Team Kefo - Biomedicine Croatia

Team Analytica

IMPRESSIONS
from the conference


2. Validating [what]
Workflow: wet lab
before
DNA
Capture
Library


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