SPIDIA Newsletter 11/2012

SPIDIA Homepage (www.SPIDIA.eu)

News about the SPIDIA project, including up-to-date lists of events where to meet us, downloads of SPIDIA posters and presentations, links to other organizations and related initiatives are posted regularly on our homepage. There you can also find more information about the background of the project and about the SPIDIA partners. If you have questions or ideas, you can also get into contact with us using the „Contact Us" form. Feel free to visit us at www.SPIDIA.eu

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About Us

What is SPIDIA

SPIDIA (Standardisation and improvement of generic pre-analytical tools and procedures for *in vitro* diagnostics) is a four-year large-scale integrating research project that will work on the missing standardisation and improvement of pre-analytical procedures for *in vitro* diagnostics. SPIDIA's research and standardisation activities cover all steps from creation of evidence-based guidelines to creation of tools for the pre-analytical phase to testing and optimisation of these tools through the development of novel assays for sample quality biomarkers. The consortium is built by seven public research organisations, eight research companies and an official European standards organisation. SPIDIA's budget is € 13,000,000 with an EC contribution of € 9,000,000.

Why was SPIDIA initiated

In vitro diagnostics have enabled a significant progress in medicine. Further progress is expected by new technologies analysing cellular biomolecule profiles as nucleic acids, proteins, and metabolites. Studies have demonstrated that the profiles of these molecules can change drastically during transport and storage, thus making a reliable diagnostic or pharmaceutical research unreliable or even impossible. Therefore further progress is limited due to the lack of guidelines in sample collection, handling, stabilisation and storage of clinical samples and due to still missing new and improved sample technologies. The project SPIDIA aims to close this gap by providing guidelines, quality assurance schemes and innovative pre-analytical tools. These may also be of high importance for biobanking and biomedical research.

SPIDIA's approach

SPIDIA is organised around three activities. Each consists of multiple work packages. The first activity leads to pan-European quality assurance schemes and guidelines for the pre-analytical phase of *in vitro* diagnostics. Such documents will be based on evidence gathered during ring trials to be performed in order to elucidate problematic steps in pre-analytical procedures. These procedures will have a specific focus on DNA, RNA, protein, and metabolite targets isolated from tissue, tumour, whole blood, serum, and plasma samples. In addition, sample quality assurance biomarker(s) will be discovered to serve as indicators for artificial, post-collection changes of clinical and biological samples. Our second activity is dedicated to the discovery, development and integration of breakthrough technologies that strengthen weak steps and links in the pre-analytical phase of *in vitro* diagnostics. The results are intended to allow the association of classical and molecular diagnostics. This work includes the discovery of novel stabilisation technologies for tissues, blood, and non-invasive samples, such as swab samples to the integration of multiple pre-analytical steps into an automated workflow. Finally, our third activity focuses on management, ethics and spreading of excellence. This activity aims to perform training to disseminate information about discoveries and guidelines to the clinical, scientific and biobanking communities. It will also ensure ethical sensitivity and compliance.

SPIDIA's Project Progress

Comprehensive Characterisation of a Novel Tissue Stabilisation Technology Demonstrates Remarkable Improvements for the Quality of Tissue-Based Studies and Biomarker Development

Molecular characterisation of human disease requires analysis of multiple parameters ranging from histopathology to a broad spectrum of molecular biomarkers. The morphological characterisation is based on the analysis of formaldehyde-fixed and paraffin-embedded (FFPE) tissues but it is known that formalin fixation impairs molecular analyses which typically require frozen tissue samples. In the context of personalized medicine, upcoming molecular diagnostics and biomarker development, there is an increasing need for combined morphological and molecular analyses from the same tissue sample, especially when collection of freshly frozen material is impossible for medical, ethical or logistic reasons. SPIDIA´s industrial partners developed in a high-throughput approach a new technology (PAXgene Tissue System) for high-quality preservation of biomolecules and morphology in paraffin-embedded clinical tissue samples. Comprehensive characterisation of this technology by SPIDIA partners revealed excellent preservation of morphology, antigenicity, nucleic acids and phosphoproteins. Importantly, the quality of tissue-based molecular studies with PAXgene-fixed and paraffin-embedded (PFPE) tissue samples was not only significantly better than that obtained with formalin-fixed samples, but even similar to that from snap-frozen tissue, which represents the gold standard for molecular analyses. Results of these comprehensive studies were published in well-known, peer-reviewed journals:

- Viertler et al. J Mol Diagn. 2012 Sep;14(5):458-66. Epub 2012 Jun 28. A New Technology for Stabilization of Biomolecules in Tissues for Combined Histological and Molecular Analyses. http://www.ncbi.nlm.nih.gov/pubmed/22749745
- Kap et al. PLoS One. 2011;6(11):e27704. Epub 2011 Nov 16. Histological assessment of PAXgene tissue fixation and stabilization reagents. http://www.ncbi.nlm.nih.gov/pubmed/22110732
- Ergin et al. J Proteome Res. 2010 Oct 1;9(10):5188-96. Proteomic analysis of PAXgene-fixed tissues. http://www.ncbi.nlm.nih.gov/pubmed/20812734
- Groelz et al. Exp Mol Pathol. 2012 Jul 17 [Epub ahead of print]. Non-formalin fixative versus formalin-fixed tissue: A comparison of histology and RNA quality. http://www.ncbi.nlm.nih.gov/pubmed/22814231

Additionally, the reliability and reproducibility of the histopathological diagnosis in different PFPE tumor samples will be evaluated within ongoing morphology ring trials, involving renowned pathologists from throughout Europe.

Furthermore, SPIDIA partners performed a large-scale comparative study to analyse the preservation of the phosphoproteome and submitted a publication demonstrating that recovered phosphoproteins show very similar properties when compared to cryo-preserved samples by western blotting and reverse phase protein array (RPPA) and were superior to proteins from FFPE samples. Conclusively, the PAXgene Tissue System preserves post-translational modifications like the phosphorylation of proteins, enabling extensive biomarker studies on clinical tissue samples.

Finally, tissue microarrays (TMAs) were constructed by SPIDIA partners to evaluate the impact of standard formalin fixation and paraffin embedding and fixation with PAXgene Tissue, the impact of time in fixation and storage conditions on antigenicity. Several immunohistochemistry stainings with routine antibodies were performed at different SPIDIA labs. So far no negative impact of e.g. prolonged fixation time with PAXgene Tissue on antigenicity was observed which facilitates applicability of the new tissue fixation technology in a routine clinical setting. Some antibodies routine protocols used for FFPE samples need to be optimized for PFPE samples e.g. different retrieval procedures are needed.

Standardizing the Pre-Analytical Workflow for Tissue Samples

After the new PAXgene Tissue System (please see also previous article) for preservation of tissue morphology and stabilisation of RNA, DNA and proteins in tissue material was developed, a first tissue sampling container device, a so called "two chamber, one closure" concept was generated, a workflow for tissue collection and processing was established (see SPIDIA-Newsletter 03 from 10/2011, page 3) and very intensively tested within SPIDIA project. This sample container concept allowed the standardisation of the workflow of tissue collection/stabilisation and transport. Dedicated extraction procedures for the isolation of RNA, miRNA, DNA and proteins from stabilised tissue material were developed and evaluated within the SPIDIA working group. One important aspect of the standardisation approach was to restrict the container to the use with standard histocassettes, which define the size of the tissue at least in one dimension to 4 mm, so that penetration of the tissue fixative is assured in a certain time. It turned out during the evaluation of the container, that in some cases, the tissue size limits its use because it is not always possible to restrict the thickness of the tissue to these 4 mm.

Therefore, a second container concept was developed, which allows a greater flexibility for the tissue collection. Within this new concept, the sample container contains only the tissue fixative and not the tissue stabiliser (see SPIDIA-Newsletter 03 from 10/2011, page 2). The size of the container allows also preservation of larger pieces of tissue and it is pre-filled with the fixative. Tissue pieces up to 20 mm in each dimension, or up to 4 histocassettes each containing up to 6 biopsies would fit into the new container concept. As the container has only one chamber, the tissue stabiliser is distributed in bottles and the user must exchange fixative and stabiliser after a defined incubation time in this new workflow concept. As the use of larger tissue pieces bears the risk of incomplete fixation, a detailed workflow evaluation was started by QIAGEN in order to specify each processing step. Analyses comprised the evaluation of the tissue morphology as well as the quality of the nucleic acids, which were isolated from the fixed and stabilised tissue material. A detailed workflow protocol was established in that way, which showed equal performance to the workflow that was established with the "two chamber, one closure" container.

Impact of the Preanalytical Workflow on Biomarker Expression …

…in Tissues

Precise quantitation of **protein biomarkers** in tissues has great potential for the development of personalized molecular targeted therapies. However, little is known about the impact of pre-analytical factors on protein stability. One aim of the SPIDIA consortium is to develop standard operating procedures for asservation of tissue specimen. In this study we are focussing on potential changes of proteins and phosphoproteins with regard to delayed fixation. A first manuscript with results from human samples has already been submitted for publication. Additional murine and rat liver samples were collected under different experimental ischemic conditions and either cryopreserved, formalin-fixed or fixed with the PAXgene Tissue System. The phosphoproteome of biological triplicates is currently being analyzed using quantitative mass spectrometry (LC-MS/MS) and reverse phase protein array (RPPA) technology and a manuscript is in preparation.

Affymetrix chip analysis was performed to analyse RNA stability in human tissue samples during the pre-analytical phase. Deregulated and stable genes were identified and a validation study by qPCR is ongoing. Potential **RNA profile quality biomarker** candidates will be cross-validated by different partners in order to increase reliability of new biomarkers which indicate the quality of clinical tissue samples. Special emphasis will be on the identification of stable reference genes and comparison with common housekeeping genes to improve future biomarker validation studies.

By analysing low molecular weight molecules new results indicate that remarkable metabolome disturbances exist during the preanalytical workflow, e.g. during cold ischemia times. According to these findings models are being defined to predict from the NMR metabolomic fingerprint the ischemia time in tissue samples with unknown preanalytical history.

…in Blood

To monitor pre-analytical variation of RNA in blood samples, a panel of **RNA quality biomarkers** has been successfully identified and validated in two separate biomarker development processes (including sample processing, microarray, biomarker candidate selection, qPCR assay design, biomarker assay prevalidation and precision measurement, extended validation). After an extended validation study with blood samples from 60 donors and a pilot study, four of these biomarkers are selected as RNA quality control biomarkers in the second SPIDIA RNA ring trial. A manuscript describing these RNA quality biomarkers is in preparation.

Workflow Standardisation for the Processing of Human Blood Samples

In order to standardize the complete pre-analytical workflow for human whole blood samples from sample collection up to the analysis of intracellular RNA from white blood cells via quantitative RT-PCR technique, QIAGEN developed a fully automated protocol for isolation of total RNA including miRNA from stabilised blood samples (PAXgene Blood RNA Tubes) and subsequent reaction setup for RT-PCR reactions. Protocols were developed on the basis of the existing QIASymphony SP robotic sample preparation platform as well as the QIAsymphony AS (Assays Setup) module, which takes the isolated RNA and generates a complete mastermix for quantitative RT-PCR reactions. The quality of the isolated RNA, including RT-PCR, was evaluated by AROS Applied Biotechnology A/S. QIAGEN developed four different RT-PCR assays, two duplex qRT-PCR assays, targeting the c-fos and the IL-1B gene transcript with 18 S rRNA as internal control gene, and two monoplex assays targeting the p53 and IL8 gene transcripts as manual RT-PCR assays first and then transferred them onto the QIAsymphony AS module. After it was verified, that the two new protocols work separately, QIAGEN performed a study with ten human blood donors to compare the complete manual workflow with the fully automated one. Blood was collected into PAXgene Blood RNA tubes and the CE-marked PAXgene Blood RNA kit was used for manual RNA extraction. Duplicate samples were processed per donor and method and RNA quality and RT-PCR performance were analysed. Both workflows showed equivalent results for RNA quality and RT-PCR results, indicating that also the combination of the two newly developed protocols worked according to the set specifications. As a next step, SPIDIA partners will test the new automated pre-analytical workflow with their assays and sample material.

Impact of DNA Quality on Long Range Multiplex PCR Performance for Biomarker Analysis for DNA Extracted from Blood

In collaboration with its SPIDIA partners, ImmunID analysed the impact of preanalytical steps on the quality and integrity of genomic DNA extracted from blood and on biomarker analysis by analysing a patient's immunity and immune repertoire diversity by its ImmunTraCkeR® Technology. Immune repertoire diversity analysis is performed using a long-range multi-N-Plex® PCR on genomic DNA extracted from peripheral blood mononuclear cells (PBMC).

One of the goals was to define preanalytical guidelines for the best development and clinical use of biomarkers. Work of SPIDIA clearly showed that preanalytical steps and conditions (i.e. temperature, storage time after blood collection, extraction methods, etc.) can impact on the quality of genomic DNA. Indeed, reduced DNA integrity (increased DNA fragmentation) during the preanalytical process impairs high molecular weight DNA amplification and biomarker performance (Fig 1).

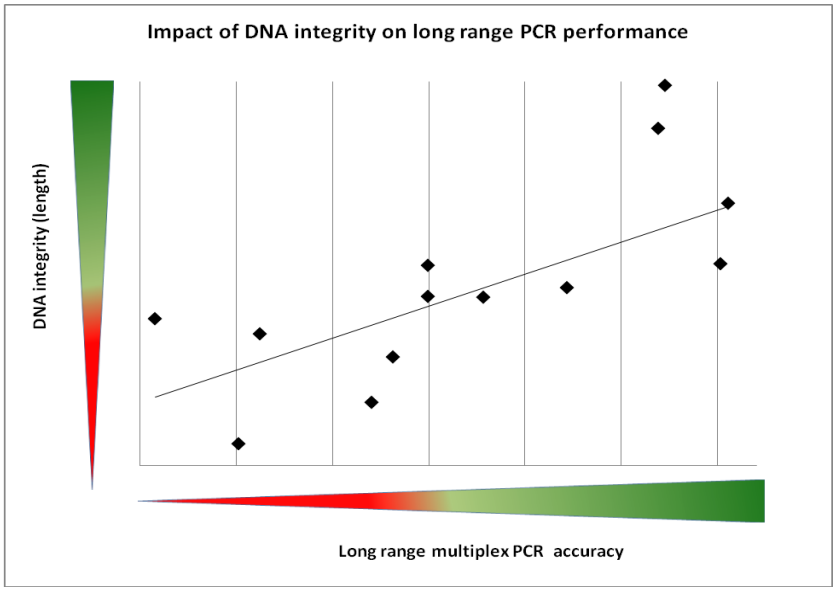

Figure 1: Impact of DNA integrity on long range PCR performance

The preanalytical process is a key but challenging step to which special attention should be paid in order to ensure a good performance of long range PCR and of biomarkers analysis to ensure the best clinical relevance.

SPIDIA OPEN WORKSHOP

Standardisation of Sample Pre-Analytics for Molecular Diagnostics and Biomarker Development
In a public workshop the scientific background, key activities and main results of SPIDIA were presented. This was accompanied by talks and discussions with international experts in the field of biospecimen research, molecular diagnostics and biomarker development. Scientific topics included:

- Critical pre-analytical variables of biological samples
- Novel tissue fixation technologies
- RNA analysis in blood and tissue samples
- Protein and phospho-protein profiles
- Metabolomics
- Development and validation of molecular biomarkers

The workshop was held at the Medical University of Graz, on Wednesday, October 10th 2012.

The programme was as follows:

- Irmgard Lippe: Opening words
- Uwe Oelmüller: EU SPIDIA Project Update – Standardisation and Improvement of Generic Pre-analytical Tools and Procedures for In-Vitro Diagnostics
- Helen Moore: Biospecimen Research at the U.S. National Cancer Institute
- François Rousseau: The Role of Interdisciplinary Networks in Molecular Diagnostics Biomarker Validation
- Mario Pazzagli: The Pre-Analytical Phase for Molecular Methods in Blood Samples
- Hui Zhang: RNA Quality Biomarkers to Monitor Pre-Analytical Variation in Blood Samples
- Kurt Zatloukal: Pre-Analytical Parameters Impacting on Molecular Analyses of Tissues

- Karl-Friedrich Becker: Impact of Pre-Analytical Factors on Protein and Phospho-Protein Profiles in Tissue Samples
- Christian Viertler: Improvements of Tissue Pre-Analytics for High Quality Tissue-Based Molecular Studies
- Peter Riegman: Adapting Routine Tissue Freezing Protocols for a Better Collection for Medical Research Purposes
- Giorgio Stanta: Molecular Analysis for Clinical Research and Diagnostics in Archive Tissues
- Berthold Huppertz: Biobank Graz: Automation and Effective Sampling and Storage to Maximize Sample Quality
- Paola Turano: Don't Alter the Individual Metabolome
- Beate Kamlage: Quality Control Needs of Biobank Samples for Reproducible Metabolomics
- Uwe Oelmüller: Closing words

SPIDIA training course held in CERM

The SPIDIA training course "Practical Introduction to Metabolomics" took place in Sesto Fiorentino (Florence, Italy) from 18 to 20 July 2012. It was organized and hosted by CERM (Magnetic Resonance Center), a center for research, knowledge transfer, and higher education of the University of Florence. In the institute nuclear magnetic resonance (NMR) is applied to answer fundamental questions in the field of Life Sciences. Five years ago the institute established a metabolomic laboratory, with a dedicated NMR instrument and a small group of young and enthusiastic researchers. The course was held in the frame of the educational activities of the SPIDIA project, and was particularly suited to students and young researcher interested in a general introduction to the field of metabolomics.

The first day, after an introductory lecture, held by Leonardo Tenori (FiorGen Foundation), Anna Artati (Helmholtz Zentrum München) showed the mostly used mass spectrometry strategies in metabolomics, with example of procedures for profiling and quantification of metabolites in bio-fluid. The second day was dedicated to the training activities in samples preparation and NMR spectra acquisition, with a four hours training in the morning. In the afternoon a lecture of Claudia Napoli (Bruker Italy srl) showed how to achieve full standardisation and automation in NMR metabolomic studies and a lecture of Rui Wang-Sattler (Helmholtz Zentrum München) illustrated interesting application of mass spectrometry based metabolomics for the identification of metabolites that are specifically linked to age, gender, smoking and type 2 diabetes. The third day started with a lecture about statistics and data analysis in metabolomics, held by Birk Schutz (Bruker Biospin). Finally, Claudia Napoli trained the participants in the use of Amix software (Bruker) for NMR spectral analysis.

The training activities were highly interactive, allowing room for questions and discussion among the participants, and to ensure that the participants had ample time to acquire the new concepts.

Where to meet us

SPIDIA Partners give regularly presentations about the project and their results on various congresses.

Past Events

- **Biobank Technology Workshop:**
  June 27, 2012
  Hilden, Germany
  Oral presentations:
  - U. Oelmüller: Welcome and opening session
  - D. Grölz: PAXgene Tissue: New fixation method for multimodale biomarker discovery
  - K. Zatloukal: Standardization and interoperability in global biobanking
  - P. Riegman: The importance of sample quality and standardization efforts in biobanking for medical research

  http://www.qiagen.com/events/biobankworkshop/

- **European Congress of Pathology:**
  September 08-12, 2012
  Prague, Czech Republic
  Oral presentation:
  - D. Grölz: PAXgene® Tissue fixation technology for simultaneous preservation of morphology and nucleic acids

  http://www.esp-congress.org/

- **Human Proteome Organization:**
  September 09-13, 2012
  Boston, MA, USA
  Poster presentation:
  - S. Sighart: Delay to preservation does not induce a systematic phosphoprotein response during tissue processing

  https://netforum.avectra.com/eweb/StartPage.aspx?Site=HUPO&WebCode=HomePage

- **Austrian Association of Molecular Life Sciences and Biotechnology:**
  September 17-19, 2012
  Graz, Austria
  Poster presentation:
  - C. Viertler: A New Tissue Stabilisation Technology for High-Quality Tissue-Based Molecular Studies

  http://www.oegmbt.at/index.htm

- **Austrian Proteomic Research Symposium:**
  September 24-26, 2012
  Graz, Austria
  Poster presentation:
  - C. Viertler: A new technology for simultaneous preservation of biomolecules and morphology in tissues facilitates biomarker development

  http://aprs2012.tugraz.at/

- **Live Webinar Event:**
  September 27, 2012
  Webinar Event:
  - L. Rainen and D. Grölz: Simultaneous, Formalin-Free Preservation of Tissue Biomarkers and Morphology for Biomarker Discovery and Biobanking

- **ESMRMB 2012:**
  October 04-06, 2012
  Lisbon, Portugal
  Oral presentation:
  - C. Luchinat: MR metabonomics and personalized medicine

  http://www.esmrmb.org/

- **SPIDIA Open Workshop "Standardisation of Sample Pre-Analytics for Molecular Diagnostics and Biomarker Development" during biannual SPIDIA Meeting:**
  October 10, 2012
  Medical University of Graz
  Graz, Austria
  (please see also page 6)

- **ASCO-NCI-EORTC Meeting "Markers in Cancer":**
  October 11-13, 2012
  Hollywood, FL, USA
  Poster presentation:
  - A. Nocon: Automated large-volume extraction of circulating, cell-free DNA to improve the sensitivity of tumor biomarker detection

  http://markersincancer.org/

- **AMP:**
  October 25-27, 2012
  Long Beach, CA, USA
  Poster presentation:
    - D. Grölz: Formalin-free Tissue Fixation for Multimodal Biomarker Analysis

http://www.amp.org/meetings/2012/index.cfm

Figure 2: SPIDIA Partners at the SPIDIA meeting in October 2012 in Graz, Austria

Colophon
SPIDIA, QIAGEN GmbH - QIAGEN Strasse 1 - 40724 Hilden - Germany