SPIDIA Newsletter 03/2013

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

Contents

About Us

What is SPIDIA

SPIDIA (Standardisation and improvement of generic pre-analytical tools and procedures for *in vitro* diagnostics) is a 4.5-year large-scale integrating research project that is working 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.

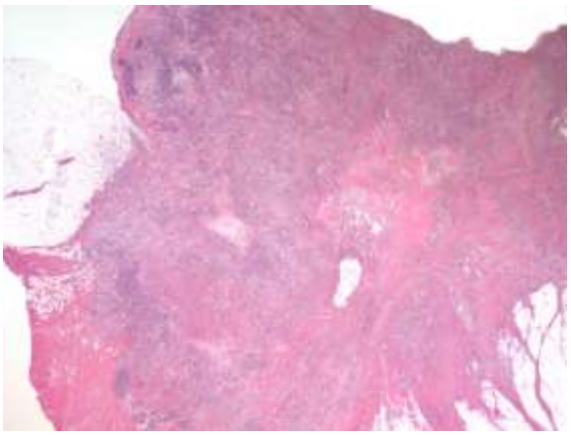
SPIDIA's Project Progress

Investigation, Improvement and Standardisation of Critical Aspects for Tissue Based Diagnostics

Characterisation of human disease requires analysis of multiple parameters ranging from histopathology to a broad spectrum of molecular biomarkers. Routine morphological evaluation 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. SPIDIA partners investigated the impact of pre-analytical variables in molecular analysis of fixed and paraffin-embedded tissues including different fixatives, fixation time, RNA extraction methods and storage of tissues on several widely used downstream methods including complementary DNA synthesis, quantitative reverse transcription polymerase chain reaction or microarray hybridisation. FFPE samples revealed e.g. major gene to gene variations as compared to the cryopreserved reference samples and routine methods for quality control were reliable for cryopreserved tissues but did not accurately predict the behaviour of mRNAs from FFPE samples. A manuscript summarizing these results for quality control from paraffin-embedded tissues is currently in review. These comprehensive investigations of the pre-analytical workflow should help to identify the most critical steps impacting on quality of analyses and, therefore, should become the target for improved quality control using new assays tested in SPIDIA labs.
Furthermore, SPIDIA partners developed and characterized in detail a new technology (PAXgene Tissue) for simultaneous preservation of biomolecules and morphology in paraffin-embedded tissue samples and demonstrated high quality preservation of morphology, antigenicity, nucleic acids and phosphoproteins:

- Viertler et al. J Mol Diagn. 2012; A new technology for stabilization of biomolecules in tissues for combined histological and molecular analyses http://www.ncbi.nlm.nih.gov/pubmed/22749745
- Groelz et al. Exp Mol Pathol. 2012; Non-formalin fixative versus formalin-fixed tissue: A comparison of histology and RNA quality http://www.ncbi.nlm.nih.gov/pubmed/22814231
- Kap et al. PLoS One. 2011; Histological assessment of PAXgene tissue fixation and stabilization reagents http://www.ncbi.nlm.nih.gov/pubmed/22110732
- Ergin et al. J Proteome Res. 2010; Proteomic analysis of PAXgene-fixed tissues http://www.ncbi.nlm.nih.gov/pubmed/20812734

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. In a large-scale comparative study to analyse the preservation of the phosphoproteome SPIDIA partners demonstrated that recovered phosphoproteins show very similar properties when compared to cryopreserved samples by western blotting and reverse phase protein array (RPPA) and were superior to proteins from FFPE samples. Results of this study have very recently been accepted for publication (Gündisch et al. PLoS One. 2013, in press; The PAXgene® Tissue System preserves phosphoproteins in human tissue specimens and enables comprehensive protein biomarker research).



Additionally, the reliability and reproducibility of the histopathological diagnosis in different PFPE cancer tissues are currently evaluated within several morphology ring trials in comparison with mirrored FFPE samples. So far, more than 60 renowned pathologists from Europe and the US participated in the SPIDIA morphology ring trials and data analysis is ongoing.

Finally, results of these comprehensive SPIDIA studies will serve as a baseline data set for standardisation and improvements of the pre-analytical phase of biological samples and have been accepted as a New Work Item for a Technical Specification by the pre-analytical CEN/TC 140, the European Committee for 'Diagnostics' in October 2012.

Biomarker Expression in Human Tissues: Impact of the Preanalytical Workflow

Protein biomarkers need to be precisely quantitated in human tissue samples as they have great potential for the development of personalized molecular targeted therapies. However, little is known about the impact of pre-analytical factors on protein stability. On November 16th 2012, the SPIDIA consortium published results for a study aiming to characterize fluctuations of protein and phosphoprotein levels in human tissue samples during the preanalytical phase (Gündisch S, et al., J Proteome Res. 2012, http://www.ncbi.nlm.nih.gov/pubmed/23134551).

The conclusions of the study are: "Our data revealed that the degree of sensitivity of proteins and phosphoproteins to delayed preservation varied between patients and tissue types. General trends toward up- or down-regulation of most proteins were not evident due to pronounced interpatient variability but signal intensities of only a few proteins were altered from baseline in postresection samples. Our approach which combines systematic tissue collections and quantitative protein analysis will help to identify protein changes due to tissue handling. As increasingly more disease related protein biomarkers are being identified, it becomes crucial to demonstrate that these markers are not affected by pre-analytical variations in tissue processing."

With regard to **mRNA biomarkers** deregulated and stable genes were identified by Affymetrix chip analysis and successfully validated by RT-qPCR. Deregulated and stable mRNAs are currently being cross-validated by different partners in different labs in order to increase reliability of the findings.

By analysing **low molecular weight molecules** we found that remarkable metabolome disturbances exist during the preanalytical workflow, e.g. during cold ischemia times. A manuscript has been submitted introducing models to predict from the NMR metabolomic profile the ischemia time in tissue samples with unknown preanalytical history.

RNA Biomarker Expression in Blood: Impact of the Preanalytical Workflow

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. The data analysis of the confirmatory study of the second set of biomarkers is being finished and a manuscript is being written.

Workflow Standardisation for the Processing of Human Blood Samples

Pre-analytical systems typically integrate collection/transport, storage and extraction of the target biomolecules for the downstream analytical assay, but stop at this point typically. At the start of SPIDIA, there was no system available that integrates the pre-analytical steps and the processing of the analytical assay. There were automated systems available for the extraction of RNA from stabilized blood samples collected into PAXgene Blood RNA tubes, but all systems were limited to the stage where the pure RNA is isolated. There were many manual interactions necessary to link sample processing and assay setup. Besides the workload, these steps were prone to errors e.g. sample mix-up or incorrect setup of RT-PCR or PCR master mixes. Generally preferable for all handling steps is the automated approach, as it always reduces the risk of human error.

Based on the existing platform QIAsymphony SP (Sample Preparation; Fig. 1.) a new workflow for the automated isolation of RNA from PAXgene Blood RNA Tubes was developed (see SPIDIA Newsletter 11/2012 page 5). This new developed protocol was able to isolate quantitatively all species of RNA (including miRNAs) with high purity and integrity. The robustness against sample compounds that normally influence the RNA extraction was tested with critical samples, collected at the University of Florence. These samples were either enriched with lipids (lipemic samples) or proteins (bilirubin) which are known to interfere with RNA isolation procedures or consisted of samples with a high portion of prelysed red blood cells (hemolytic samples). None of the critical samples let to a failure of the RNA extraction procedure or to a negative interference in downstream analyses like qRT-PCR, indicating the good robustness of the extraction protocol.

QIAGEN further integrated the QIAsymphony AS (Assays Setup; Fig. 1) module, into the workflow, which takes the isolated RNA and generates a complete mastermix for quantitative RT-PCR reactions. After finalisation of both protocols, QIAGEN tested the completely automated workflow, including RNA extraction and RT-PCR setup on the respective QIAsymphony modules, using ten different donors, against the complete manual workflow for RNA extraction and RT-PCR setup with equivalent results. Finally, SPIDIA partners also compared manual and automated workflows with their downstream assays.

***Figure 1**: QIASymphony SP AS : Sample preparation and assay setup module*

DIAGENIC analyzed blood from 14 donors, collected into PAXgene Blood RNA tubes and compared the automated sample preparation on the QIAsymphony SP module with the manual extraction protocol and compared also the manual and automated RT-PCR setup on the QIAsymphony AS module, using 4 different assays from the Alzheimer-disease panel to compare automated and manual workflows.
The Biotechnology Institute of the Czech Academy of Sciences received the residual RNA from the comparative study between QIAsymphony and manual extraction from QIAGEN and applied different assays from the **RNA quality biomarker** program, developed within SPIDIA on the Fluidigm BIOMARK as well as the BIO-RAD CFX384 platform.
The analyses from SPIDIA-partners also showed equivalence of manual and automated RNA extraction as well as manual and automated RT-PCR reaction setup. With these final workflow comparisons, the work within the work package was finalized.
A fully automated workflow for RNA extraction from stabilized blood samples and subsequent RT-PCR reaction setup was successfully developed within SPIDIA.

Second SPIDIA Blood Ring Trial

The aim of the SPIDIA blood ring trials is to generate evidence based quality guidelines for the pre-analytical phase of blood samples. After having the first SPIDIA blood ring trial completed in 2011, a second SPIDIA blood ring trial was started. The second ring trial covered again the three different quality programs: DNA from whole blood and plasma samples as well as RNA from blood samples (called "DNA", "DNAplas" and "RNA").

Improvements have been introduced for the second ring trial mainly related to the time and temperature of sample storage, the blood collection and the shipping conditions. In particular, we adopted the dedicated shipping boxes to keep low temperature (ranging from 2 to 8 °C) for 48h (Fig.2A) instead of the 24h as in the first SPIDIA ring trial (Fig.2B).

A B

Figure 2: *Shipping box of the second ring trail (**A**: left image; 2-8 °C for 48h) versus shipping box of first ring trail (**B**: right image; 2-8 °C 24 h)*

The recruitment of the participant laboratories to the second SPIDIA ring trial officially closed on November 18, 2011. We obtained 315 applications corresponding to 223 laboratories of different European countries (Table 1). 67% of these laboratories participated also in the first SPIDIA ring trial. The number of total applications in the first SPIDIA ring trial was 324, corresponding to 220 laboratories.

Second SPIDIA ring trail	Applications
DNA	127
DNAplas	66
RNA	122
Total	315

Table 1: *Number of applications of the second SPIDIA ring trial (The DNAplas program includes also the DNA program, therefore the final DNA applications were 127 plus 66 = 193).*

The return rate was over 90% for all quality programs and comparable to those of the first SPIDIA ring trial. The return rate is summarized in Table 2.

DNA		**DNAplas**		**RNA**	
Sent to participant	*Returned from participant*	*Sent to participant*	*Returned from participant*	*Sent to participant*	*Returned from participant*
127	119	61*	56	119*	109
Return rate = 94%		Return rate = 92%		Return rate = 92%	

Table 2: *Return rate of the samples. *Because of some renunciations, this number represents the number of effective applications*

Regarding the DNA and DNAplas program the samples were sent to the participants during January 2012. Participants were asked to extract DNA within 3 days after arrival according to their current standard procedures, storing blood and plasma at 4 °C. After DNA purification and spectrophotometric measurement, participants had to send the DNA sample to the SPIDIA reference laboratory until February 20, 2012.
Concerning the SPIDIA RNA program, blood samples were sent in March and April 2012 to the participants and RNA samples were received by the SPIDIA reference laboratory within May 2012. Depending on the request on the application form, each participant laboratory received two blood samples either in EDTA tubes or in PAXgene Blood RNA tubes. We asked to extract and store RNA at pre-specified times and temperatures:
a) immediately after arrival (Tube C) and b) 24h after arrival (Tube D). All laboratories that received the blood in PAXgene Blood RNA tubes had to store Tube D at room temperature (RT) for additional 24h after the arrival. The laboratories that received the blood in EDTA tubes had to store the Tube D at RT or at +4°C. Tube D was used only to investigate the effect of the pre-analytical factors (temperature and time) on the quality of extracted RNA and it will not be included in the laboratory-specific report.

The analysis and interpretation of the results of the second SPIDIA ring trail is ongoing.

Where to meet us

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

Upcoming events

- **qPCR and NGS 2013 Event - Next Generation Thinking in Molecular Diagnostics**
  March 18-22, 2013
  Munich, Germany
  Poster presentation:
  - V. Korenková: Evaluation of the new plasma stabilisation technology for circulating cell-free DNA

  http://www.qpcr-ngs-2013.net/

- **ESGI Satellite Meeting: Outcome of BBMRI, ESGI, and SPIDIA relevant to biological sample processing for next generation sequencing**
  March 19-20, 2013
  Berlin, Germany
  Oral presentation:
  - K. Zatloukal: SPIDIA: Standardization of preanalytical requirements for sequencing

- **SPIDIA International Public Workshop "Standardisation and Improvements of Generic Pre-Analytical Tools and Procedures for in vitro Diagnostics"**
  March 20, 2013
  Hilden, Germany
  Oral presentation:
  - U. Oelmüller: SPIDIA Project Overview - EU SPIDIA Project Update – Standardization and Improvement of Generic Pre-Analytical Tools and Procedures for In-Vitro Diagnostics
  - H. Moore: Biospecimen Research at the U.S. National Cancer Institute
  - F. Rousseau: Molecular Biomarker Validation Topic (title to be announced)
  - R. Chadwick: Privacy and Identification: The Challenges Ahead
  - M. Pazzagli: SPIDIA Pan-European Blood DNA Ring Trial
  - F. Malentacchi: SPIDIA Pan-European Blood / Plasma Cell Free Circulating DNA Ring Trial
  - S. Gelmini: SPIDIA Pan-European Blood RNA Ring Trial
  - P. Verderio: SPIDIA Pan-European Ring Trials – Statistical Data Analysis
  - M. Pazzagli: SPIDIA-Prot: Effects of the pre-analytical phase on protein stability
  - R. Wyrich: New Blood Stabilization and Processing Technology
  - K.-F. Becker: SPIDIA´s Results on Molecular Changes in Tissue Samples during the Pre-analytical Phase
  - C. Viertler: Impact of Pre-Analytical Variables and Preservation Techniques on Tissue-Based Studies
  - D. Grölz: A New Tissue Stabilization Technology for Preservation of Biomarkers and Morphology
  - K.-F. Becker: Preservation of Proteins and Phosphoproteins in Formalin-Free Tissue Specimens for Diagnosis and Research
  - C. Viertler: The SPIDIA Tissue Morphology Ring Trials
  - P. Turano: Metabolomics in SPIDIA
  - L. Krieger: Dissemination and Exploitation of Results of R&D-Projects into Standardisation
  - P. Riegman: QA and QC in Biobanking and Biobanking Networks
  - U. Oelmüller: Closing Words

- **3rd International Workshop on Protein Analysis of Tissues**
  April, 11-12, 2013
  Munich, Germany
  Oral presentation:
    - D. Grölz: Formalin-Free Tissue Fixation: Preservation of Biomarkers and Morphology for Biomarker Discovery and Biobanking

  http://www.helmholtz-muenchen.de/proteinanalytik2013

- **97. Jahrestagung der Deutschen Gesellschaft für Pathologie e.V.**
  May, 23-26, 2013
  Heidelberg, Germany
  Presentation:
    - K. Grundner-Culemann: Delayed tissue preservation does not induce a systematic phosphoprotein response

  http://congress.cpb.de/index.php?id=804

- **Euromedlab Pre-Congress Satellite Meeting "THE QUALITY OF MOLECULAR METHODS IN THE AGE OF PERSONALIZED MEDICINE"**
  May 18, 2013
  Florence, Italy
  Oral presentation:
    - U. Oelmüller: Standardization of the Preanalytical Phase for Molecular Methods

  http://www.milan2013.satellitemeetings.org/index/firenze

- **ASCO Annual'13 Meeting**
  May 31- June 4, 2013
  Chicago, IL, USA
  Poster presentation:
    - A. Nocon: Automated large-volume extraction of circulating, cell-free DNA (ccfDNA) to improve the sensitivity of tumor biomarker detection

  http://chicago2013.asco.org/

Past events

- **SPIDIA Open Workshop "Standardisation of Sample Pre-Analytics for Molecular Diagnostics and Biomarker Development" during biannual SPIDIA Meeting:**
  October 10, 2012
  Graz, Austria
  Oral presentations:
  - I. Lippe: Opening words
  - U. Oelmüller: EU SPIDIA Project Update – Standardisation and Improvement of Generic Pre-analytical Tools and Procedures for In-Vitro Diagnostics
  - H. Moore: Biospecimen Research at the U.S. National Cancer Institute
  - F. Rousseau: The Role of Interdisciplinary Networks in Molecular Diagnostics Biomarker Validation
  - M. Pazzagli: The Pre-Analytical Phase for Molecular Methods in Blood Samples
  - H. Zhang: RNA Quality Biomarkers to Monitor Pre-Analytical Variation in Blood Samples
  - K. Zatloukal: Pre-Analytical Parameters Impacting on Molecular Analyses of Tissues
  - K.-F. Becker: Impact of Pre-Analytical Factors on Protein and Phospho-Protein Profiles in Tissue Samples
  - C. Viertler: Improvements of Tissue Pre-Analytics for High Quality Tissue-Based Molecular Studies
  - P. Riegman: Adapting Routine Tissue Freezing Protocols for a Better Collection for Medical Research Purposes
  - G. Stanta: Molecular Analysis for Clinical Research and Diagnostics in Archive Tissues
  - B. Huppertz: Biobank Graz: Automation and Effective Sampling and Storage to Maximize Sample Quality
  - P. Turano: Don't Alter the Individual Metabolome
  - B. Kamlage: Quality Control Needs of Biobank Samples for Reproducible Metabolomics
  - U. Oelmüller: Closing words

- **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

- **China Biobank Development Strategy Workshop**
  November 8-11, 2012
  Shanghai, China
  Oral presentations:
  - K. Zatloukal: Biobanking and the future of medicine
  - K. Zatloukal: Standardization and interoperability in biobanking

- **Multiscale systems biology cancer Workshop**
  November 12-13, 2012
  Leiden, Netherlands
  Oral presentation:
  - K. Zatloukal: Workshop Kick-off – metabolic disorders in interaction with cancer: linking tissue to prognosis

- **Methodology of cancer clinical trials**
  December 12-13, 2012
  Brussels, Belgium
  Oral presentation:
  - K. Zatloukal: European perspective on biobanking

- **Kick-Off Meeting BBMRI-LPC**
  February 10-12, 2013
  Amsterdam, Netherlands
  K. Zatloukal

- **EU Science Conference: Global Challenges, Global Collaboration**
  March 4-8, 2013
  Brussels, Belgium
  K. Zatloukal

- **ERINHA Workshop**
  March 11-15, 2013
  Spiez, Switzerland
  K. Zatloukal

Figure 3: *SPIDIA Partners at the SPIDIA meeting in October 2012 in Graz, Austria*

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