SPIDIA Newsletter 06/2012

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

Evaluation of a Novel Tissue Stabilisation Technology Allowing Morphological and Molecular Analysis from the Same Clinical Tissue Specimen

SPIDIA´s industrial partners developed a new tissue stabilisation technology for simultaneous high quality preservation of biomolecules and morphology in clinical tissue samples. To evaluate the quality of morphology and suitability of the novel SPIDIA tissue preservation technology (PAXgene Tissue System) for routine diagnostics, malignant and non-malignant human tissue specimens were collected by SPIDIA partners and divided into equal parts for formalin-fixation and paraffin-embedding (FFPE) or PAXgene-fixation and paraffin-embedding (PFPE). Routine hematoxylin-eosin, different special stainings and immunohistochemistry were performed. Morphological aspects of PFPE tissue were described by pathologists of SPIDIA partners and also scored relative to FFPE tissue using a virtual microscopy platform. It could be shown that in general morphology was well-preserved in PFPE samples. Results of these studies were published (Kap et al. PLoS One. 2011;6(11):e27704. Epub 2011 Nov 16. Histological Assessment of PAXgene Tissue Fixation and Stabilization Reagents). As next step the reliability and reproducibility of the histopathological diagnosis in PFPE samples will be evaluated in different diagnostic scenarios within morphology ring trials, involving renowned pathologists from throughout Europe.

After the first successful analysis of proteins extracted from PFPE tissue samples (Ergin et al., J Proteome Res. 2010 Oct 1;9(10):5188-96. Proteomic analysis of PAXgene-fixed tissues), SPIDIA partners performed a large-scale comparative study to analyse the preservation of the phosphoproteome. 16 different non-malignant and 4 different malignant tissue entities were investigated with 11 phosphorylation-specific antibodies. Recovered phosphoproteins showed 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. These findings clearly demonstrate that the PAXgene Tissue System preserves not only the proteome but most importantly also post-translational modifications like the phosphorylation of proteins, enabling extensive biomarker studies on clinical tissue samples.

Standardizing the Pre-Analytical Workflow for Tissue Samples

As described above, SPIDIA's industrial partners developed a new tissue fixation and stabilisation technology, which preserves tissue morphology and stabilises RNA, DNA and proteins (PAXgene Tissue). Meanwhile the technology was tested with more than 5000 tissue samples. In addition, a sample container concept was developed, that allowed the standardization of the workflow of tissue collection/stabilisation and transport. A standardized workflow for tissue fixation and stabilisation using the container device was established and dedicated extraction procedures for the isolation of RNA, miRNA, DNA and proteins from stabilised tissue material were developed.

One important aspect for the standardization of tissue sample collection and storage is the option to build tissue archives for retrospective studies of human disease progressions. Tissue archives from formalin-fixed, paraffin-embedded (FFPE) tissue material represent a huge value for biobanks and pharma companies as a source for well characterized human sample material for the

molecular evaluation of diseases. The most important disadvantage of the current archived material is the fixation prior to the paraffin embedding process, as formalin fixation leads to crosslinks between nucleic acids and other biomolecules, which hamper downstream analyses and limit the use of such material for molecular studies. In order to evaluate the performance of PAXgene Tissue fixed, paraffin embedded (PFPE) tissue material for long term achieving purposes, a study was initiated in the beginning of the SPIDIA project. Rat tissue from liver, kidney, spleen, lung, and intestine was either fixed with neutral buffered formalin or with the PAXgene Tissue System before processing and paraffin embedding. PFPE and FFPE samples were then stored at 22°C, 4°C, -20°C and -80°C for three years and morphology and RNA quality was investigated. After three years of storage, morphology was still preserved at all temperatures with both fixation methods. However, only from PFPE material RNA could be isolated and used for demanding downstream applications like amplification of up to 1 kb gene fragments in RT-PCR from all tissue samples, regardless of the storage temperature. In contrast, regardless of the storage conditions, RNA from FFPE tissue could not be used to amplify the large RNA fragments. We concluded that tissue samples fixed and stabilised with the PAXgene Tissue reagents can be stored as PFPE blocks of tissue even at ambient temperature for archiving purposes.

Workflow Standardization for the Processing of Human Blood Samples

As human whole blood is an important sample material for diagnostic purposes, the SPIDIA working group aimed to standardize the pre-analytic workflow for human whole blood samples from collection to the analytical steps for RNA based assays. Therefore, QIAGEN developed a fully automated protocol for isolation of total RNA including miRNA from stabilised blood samples (PAXgene Blood RNA Tubes) based on the existing QIASymphony® SP robotic sample preparation platform and magnetic particle technology. A first successful evaluation of the protocol including the evaluation of RNA yield (including miRNAs), purity and RT-PCR performance in various assays was performed at AROS Applied Biotechnology A/S.

QIAGEN started to further integrate the analytical workflow using the QIAsymphony AS robotic system that takes the isolated RNA and generates a complete mastermix for quantitative RT-PCR reactions. Four different assay setup protocols were programmed by QIAGEN. 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 a next step these assays will be tested in completely automated workflows including the RNA preparation followed by a direct PCR setup in comparison to manual procedures.

In addition, DiaGenic has made successful evaluations of the PAXgene blood RNA isolation protocol for the QIAcube instrument, in terms of RNA yield, purity, integrity and performance (prediction values and test results) on the gene expression signature for Alzheimer's disease developed in DiaGenic. The QIAcube workflow slightly shifted prediction values when compared to the manual workflow but did not change the test results. Next, both, the QIAcube workflow and the new QIAsymphony automated workflow will be tested on a new gene expression signature developed by DiaGenic using clinical samples collected at UNIFI.

The Second SPIDIA Blood Ring Trial Programme has Started

The aim of the SPIDIA blood ring trials is to generate evidence for developing quality guidelines for the pre-analytical phase of blood samples. The first SPIDIA ring trial was completed in March 2011. The second SPIDIA blood ring trial has started in October 2011 with the enrolment of participants for the three different quality programs: DNA from whole blood and plasma samples as well as RNA from blood samples (called "DNA", "DNAplas" and "RNA"). The design of this second ring trial took the results obtained by a pilot study (Günther et al., Clin Chim Acta. 2012 Apr 11;413(7-8):779-86. Implementation of a proficiency testing for the assessment of the preanalytical phase of blood samples used for RNA based analysis) and obtained by the first ring trial into consideration. Additional measures were taken to further improve the quality of the sample material used for the ring trials. Numerous invitations for participation in the ring trial programmes were sent out to potential European laboratories including all participants of the first ring trial of which the majority agreed to participate also in the second ring trial (see Table 1).

SPIDIA PROGRAMME 2nd ring trial	No OF APPLICATIONS	1ST RING TRAIL APPLICATION PERFORMING THE 2nd ONE
DNA	126	87
DNAplas	61	39
RNA	121	80
total	308	206

Table 1. Details of SPIDIA 2nd ring trial participants representing 223 participating laboratories.

In total 223 laboratories were enrolled, 94 of them performed more than one programme. Almost 50% of the applicants belong to university laboratories followed by research institutes and regional hospitals as shown in Figure 1.

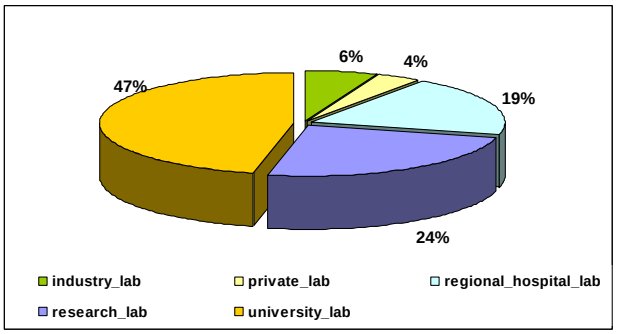


Figure 1. Type of institutions to which the laboratories belong.

The subdivision into countries of the participants is illustrated in Figure 2-4.

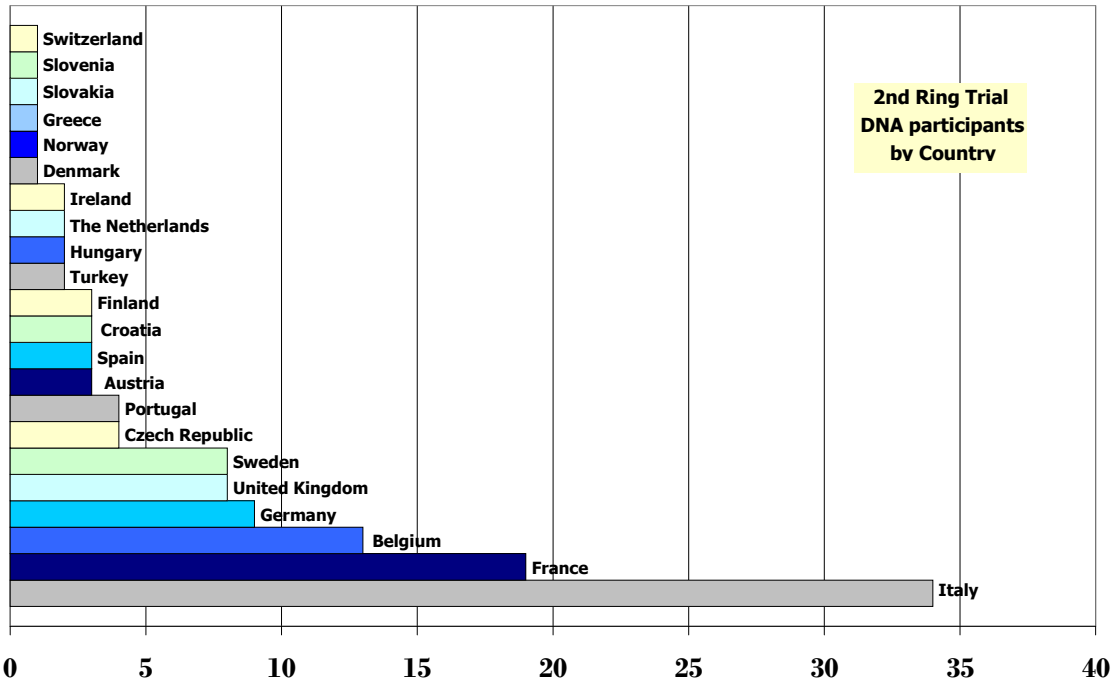


Figure 2.Total participants to the DNA 2nd ring trial subdivided by country.

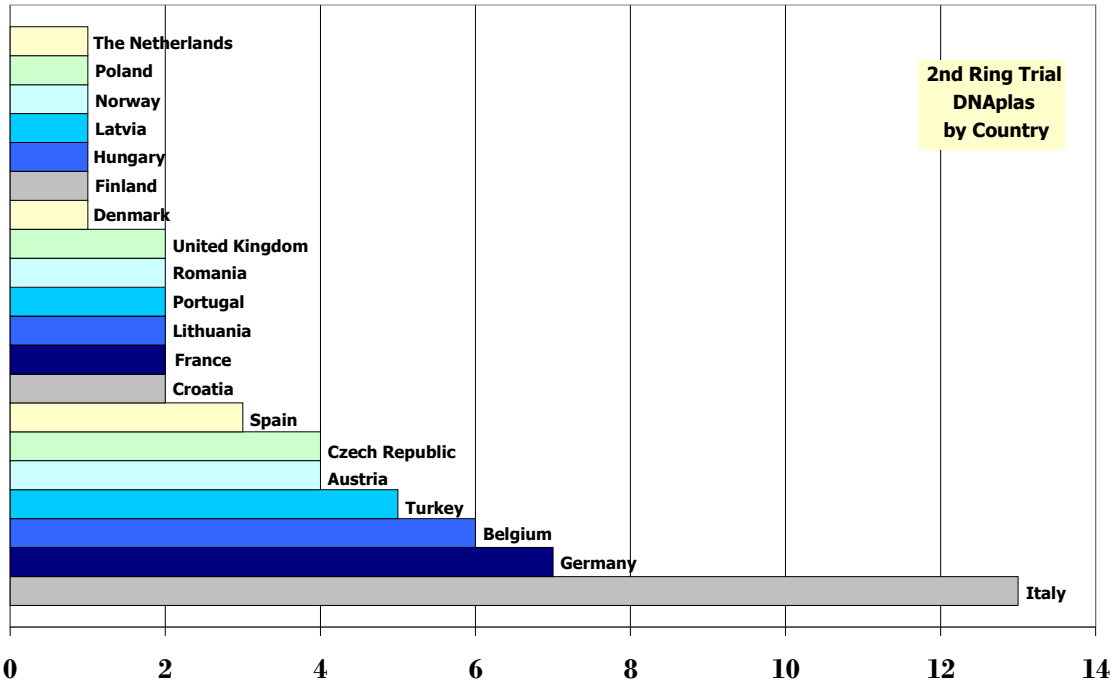


Figure 3. Total participants to the DNAplas 2nd ring trial subdivided by country.

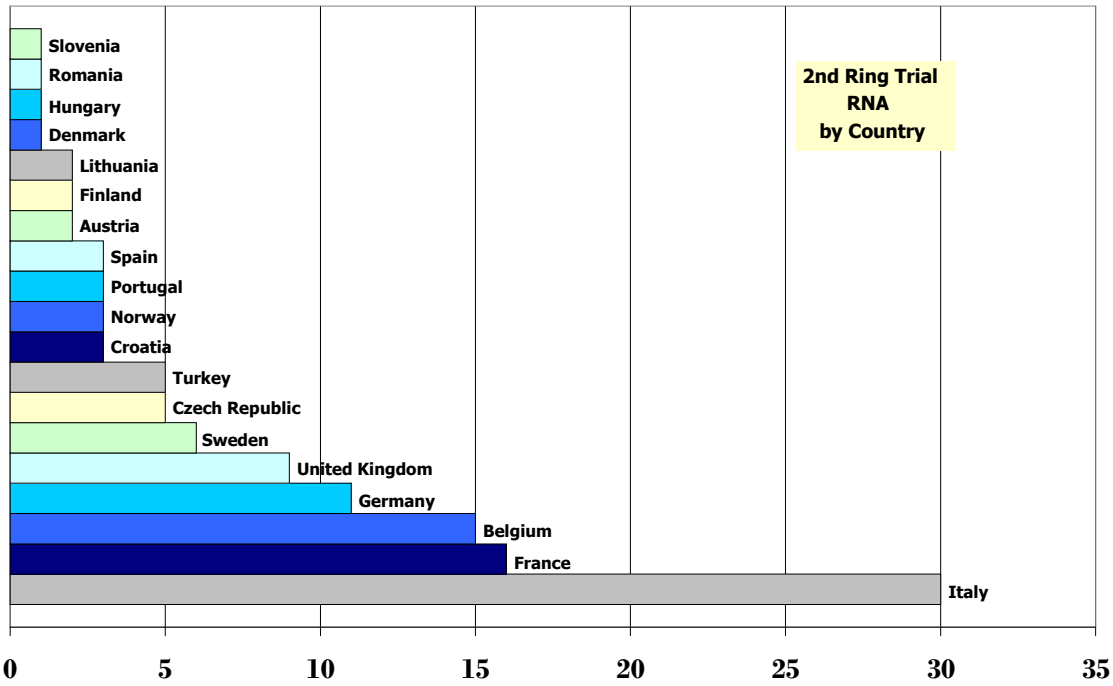


Figure 4.Total participants to the RNA 2nd ring trial subdivided by country.

The second DNA and DNAplas ring trials were started on January 23 with the shipment of the samples to the participating laboratories. The participants extracted DNA according to their current standard procedures but within timeframes specified by SPIDIA. The isolated DNA samples were returned to SPIDIA laboratories by mid of February. The SPIDIA partners have started the analysis of DNA quality and quantity.

The second RNA ring trial started in March. Due to the complexity of the ring trial setup, it was not possible to ship the samples to all laboratories at the same day, therefore the laboratories were divided into two groups. For the first group the samples were sent on March 26 and for the second group on April 2. As for the first ring trial, the laboratories received blood either without any stabilisation (just with EDTA as an anticoagulant) or in a dedicated tube, that stabilises the intracellular RNA (PAXgene Blood RNA tube), in order to evaluate the effects of the stabilisation technology on the RNA quantity and quality. Participants sent the isolated RNA back to the SPIDIA lab in Florence until May 2. SPIDIA partners are currently analyzing the RNA quality by various downstream tests.

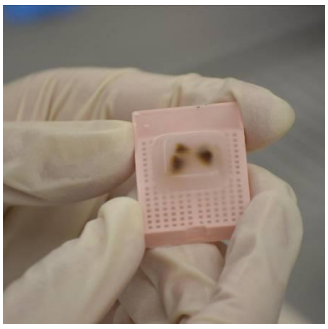
The results of the ring trials will be documented in individual reports for the participants.

Identification and Validation of Quality Biomarkers Monitoring the Pre-Analytical Workflow for Biological Samples.

RNA and Proteins in Tissue

The quality of human tissue specimens can have a significant impact on analytical data sets for biomarker research. To fully understand the impact of warm ischemia time and a delay of the tissue preservation on proteins and phosphoproteins, ischemic human and animal

tissue samples have been investigated. Multiple approaches, both targeted (reverse phase protein array) and non-targeted (tandem mass spectrometry), were used to achieve a comprehensive insight into the proteome and phosphoproteme of ischemic tissues. The first data suggest that identification and validation of standardized quality markers that indicate inadequate tissue handling is very complex. High inter-patient variability was the major hurdle for proper statistical analysis and identification of quality protein biomarkers. A first manuscript comprising the results of ischemic human tissue samples has been submitted and a second manuscript dealing with the results of ischemic animal tissue samples is in preparation. Furthermore, the evaluation of additional ischemic tissue samples and of the impact of other pre-analytical factors like the preservation method is ongoing.



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 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.

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RNA in Blood

Quality control of blood sample handling is an important step in clinical diagnostics. However, no reliable biomarkers have been discovered to date that indicate changes occurring during pre-analytical procedures in blood. An important objective for SPIDIA is to identify RNA quality biomarkers for monitoring pre-analytical variations in blood samples. We have successfully identified and validated a set of biomarkers for monitoring pre-analytical variation of RNA in blood samples. These biomarkers are currently undergoing an extended evaluation and validation in a large cohort of blood samples (60 individual donors). Another aim of the project is to apply these RNA quality biomarkers as quality control parameters in the second Blood RNA ring trial (see also page 4).

In addition to the existing quality biomarkers, new biomarker assays of the candidates selected from a second microarray study were designed and validated with qPCR. New promising candidates are selected for the further precision study.

Metabolomics in Tissue and Serum Samples

Low molecular weight molecules were identified that change the most during the pre-analytical phase with respect to freshly collected tissue samples. The results will be used to put together metabolites into chemically coherent subsets, to assist interpretation of the undesired side-reactions occurring during tissue management within a clinical/biobank environment and provide inputs for the definition of reliable, validated and harmonized SOPs.

In a clinical setting, the metabolic signature of metastatic colorectal cancer has been identified in the NMR spectra of sera. This signature is strong enough to predict overall survival. A number of metabolites have been identified that are responsible for the fingerprint of metastatic colorectal cancer, offering insights into the biochemistry of the disease. More information can be found in Bertini I, Cacciatore S, Jensen BV, Schou JV, Johansen JS, Kruhøffer M, Luchinat C, Nielsen DL, Turano P. Metabolomic NMR fingerprinting to identify and predict survival of patients with metastatic colorectal cancer. Cancer Res. 2012; 72: 356-364.

Where to meet us

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

Upcoming Events

- **Biobank Technology Workshop:**
  June 27, 2012
  Hilden, Germany
  Oral presentation:
  - 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
  Poster presentations:
  - D. Grölz: PAXgene® Tissue fixation technology for simultaneous preservation of morphology and biomolecules

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

- **ESMRMB 2012:**
  29th Annual Scientific Meeting:
  October 4-6 2012
  Lisbon, Portugal
  Oral presentations:
  - C. Luchinat: MR metabonomics and personalized medicine

  http://www.esmrmb.org/

Past Events

- **Reverse Phase Protein Array Global Workshop**
  October 10-11, 2011
  Houston, Texas, USA
  Poster presentation:
  - K.-F. Becker: Impact of Delayed Fixation on Protein Profiles in Clinical Tissue Specimen
  - K.-F. Becker: A Novel Fixative Allows Morphological and Molecular Analysis from the Same Clinical Tissue Specimen

- **Symposium of the Society for Histochemistry 2011**
  October 12-15, 2011
  Munich, Germany
  Oral presentation:
  - S. Gündisch: Evaluation of PAXgene-fixed, Paraffin-embedded Tissues for Morphological and Molecular Analysis

  http://www.helmholtz-muenchen.de/histochemistry2011/home/index.html

- **ESBB Inaugural Conference:**
  November 16-19, 2011
  Marseille, France
  Oral presentation:
  - U. Oelmüller: EU Project SPIDIA - Standardization and Improvement of Generic Preanalytical Tools and Procedures

  http://www.esbb.org/nov2011/

- **QIAGEN Biobanking Expert Meeting:**
  November 24, 2011
  Hilden, Germany
  Oral presentation:
  - U. Oelmüller: SPIDIA Presentation in Opening Session

- **Munich Biomarker Conference:**
  November 29, 2011
  Munich, Germany
  Poster presentation:
  - S. Gündisch: Impact of pre-analytical factors on protein and phospho-protein profiles in tissue samples

  http://events.bio-m.org/munich_biomarker_conference

- **BRN symposium:**
  February 22-23, 2012
  Bethesda, Maryland, USA
  Oral presentation:
  - U. Oelmüller: EU SPIDIA Project Update – Standardization and Improvement of Generic Preanalytical Tools and Procedures for In-Vitro Diagnostics

  Poster presentations:
  - C. Viertler: The impact of tissue pre-analytics and a new stabilisation technology on the quality of tissue-based molecular studies
  - M. Kap: Evidence Based Biobanking; From Patient to Storage
  - S. Gündisch: Delay to preservation does not induce a systematic phosphoprotein response during tissue processing

  http://www.brnsymposium.com/

- **Symposium on Biosample Quality:**
  May 9, 2012
  London, United Kingdom
  Oral presentation:
  - D. Grölz: EU SPIDIA Project - Pre-analytical handling of biosamples; optimising biobank sample quality for protein and nucleic acid studies

  http://www.ncri.org.uk/ccb/

- **Sektion Molekulare Diagnostik der DGKL:**
  May 10-11, 2012
  Tutzing, Germany
  Oral presentation:
  - U. Oelmüller: Bedeutung des SPIDIA Projekts für Biobanken

- **Deutsche Pathologentagung 2012:**
  May 31 - June 03, 2012
  Berlin, Germany
  Poster presentations:
  - S. Gündisch: Delay to preservation does not induce a systematic phosphoprotein response during tissue processing

  http://www.pathologieberlin2012.de/

- **ISPD Meeting:**
  June 03-06, 2012
  Miami, Florida, USA
  Poster presentation:
  - M. Horlitz: Automated Large-Volume Extraction of Circulating, Cell-free DNA Using the QIAsymphony SP Instrument

  http://www.ispdhome.org/conference/2012/

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