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Agricultural Biomarkers for Array-Technology

Working Group 1 nucleic acid micro-arrays

Working Group 2 protein micro-arrays

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Lawickse Allee 9
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Meeting Room: Kleine Veerzaal

ABSTRACTS

High-throughput genotyping for red cell and platelet blood group antigens by DNA micro arrays

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Background: In the Netherlands, all donors are serologically ABO and Rhesus (Rh)D typed. To provide antigen-negative red cells for certain groups of patients, the majority of donors is serologically typed as well for RhCcEe and K and a subset of donors for clinically relevant systems (e.g. Fy, Jk, MNSs). Red cells with very rare phenotypes are currently only available via the Blood Bank of the Council of Europe (frozen and in limited amount). Complete phenotyping of all blood donors, for all blood groups is too laborious and simply not feasible because of the lack of sufficient and high-quality typing reagents. In theory, high-throughput molecular genotyping facilitates the typing of a donor for all clinically relevant red cell and platelet antigens in a single test and provides a completely typed red cell and platelet inventory.

Study design and methods: For most blood group systems the molecular basis is known and found to be a single nucleotide polymorphism (SNP). In the last years, we developed a method facilitating high-throughput blood group genotyping by glass-based DNA micro arrays. In this assay, first a multiplex PCR is performed, which is designed in such a way that simultaneous amplification and fluorescent labeling is obtained for 30 gene fragments encoding red blood cell and human platelet antigens. Genotyping of these gene fragments is performed by allele-specific hybridisation of the dye-labeled fragments to probes spotted on defined positions on glass slides. The genotype (aa, ab or bb) can be deduced by comparing signal intensities of probes specific for the a- or b-allele, respectively. To test the setup of the genotyping method by microarray, a pilot study with human platelet antigen (HPA) typed donors was performed.

Results: Two blinded panels encompassing 94 donors were genotyped for HPA-1 through -5 and -15; no discrepancies were found compared to their serologic (HPA-1, -2, -3, -4 and -5) and genotyping (HPA-15, TaqMan, Applied Biosystems) results.

Conclusion: This study shows that the micro-array-based genotyping provides a reliable and fast genotyping platform. However, the current procedure is costly (hands-on time and reagents) and complete automation will be difficult.

The use of high-throughput donor genotyping as a daily routine in the blood bank not only will improve the direct availability of typed red cells and platelets, but also holds a promise of a future change in transfusion policy because it facilitates an increase in the match between donor and patient beyond ABO and D to prevent alloimmunisation. Introduction of so-called preventive matching will reduce the occurrence of severe transfusion reactions (for example delayed haemolytic transfusion reactions by Jka antibodies) and will prevent reduced survival time of transfused red cells in alloimmunized patients.

Rapid and simple detection of amplified mRNA/DNA using antibody microarrays and colloidal carbon nanoparticles

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Introduction:

Biosensor analysis set-ups usually comprise components such as a sampling device, an interface with interactive layer, a detector to record signals and a processing unit to analyse data. The signal is often based on optical or electrochemical principles and in many cases this requires a rather sophisticated module which often make the apparatus and/or the running costs quite expensive. We aim to develop a micro-array system that is rapid, simple to perform and cheap. Therefore, the format of a previously developed one-step nucleic acid lateral flow immunoassay (NALFIA) was translated into a micro-array system. Several micro-organisms were chosen as targets to assess the applicability of the method.

Methods:

The previously described NALFIA approach employs differently tagged primers, one labelled with biotin and the other one with a tag such as digoxigenin or fluorescein. Microarrays were prepared by spotting (TopSpot/E apparatus, 1 nL per spot; diameter \pm 200 μ m) of different amounts of antibodies onto several types of commercially obtained glass slides. Neutravidin was physically adsorbed onto colloidal carbon nanoparticles. After the sandwich assay, the arrays were analysed by conventional flatbed scanning and image analysis (TotalLab, nonlinear dynamics) and the pixel grey volumes were used for quantifying the binding yield of amplicon conjugated colloidal carbon.

Results:

A comparison was made between the final binding yields obtained with UltraStick slides (amino-propyl-tri-ethoxy silane surface), XanTec slides (preactivated hydrogel coating) and Whatman FAST slides (coated with nitrocellulose). Physical adsorption of capture antibodies onto FAST slides gave the best results. Spots were scanned and the corresponding spot grey volume clearly increased as a function of antibody spotting concentration. Serial dilutions of PCR material were incubated on the arrays in the presence of various volumes of 0.2% (w/v) colloidal carbon nanoparticles - neutravidin conjugate in a total volume of 70 μ L and incubated at room temperature for 7.5 min to several hours. Initial results indicated that approximately 0.5 to 2 μ L of PCR material, 1 μ L carbon conjugate and incubation for 15 min to 1 hour is sufficient to obtain significant results by flatbed scanning and image analysis. Several application examples will be shown such as for *E.coli* and *Listeria (monocytogenes)*.

Discussion:

As compared to conventional quantification on DNA micro-arrays, for example based on fluorescence, the present approach is sufficiently rapid and does not require expensive apparatus. In addition to a PCR set-up, the necessary equipment consists of a micro-arrayer and a flatbed scanner. The disposable part consists of slides and reagents. In addition to PCR an adapted protocol is being studied to use the colloidal nanoparticle based micro-array in combination with isothermal NASBA (i.e. RNA) amplification, which would further simplify the total procedure.

Microarrays: Which Technology for Which Application?

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Microarrays have a short yet hectic history. Initially designed for the study of regulatory processes in human medicine they are now also increasingly used in diagnostics. Their use covers all areas of medical and biological research and development. The wide use mirrors the fact that many of the initial problems of this new technology are now solved. Among these are for example the slide surfaces, on which the probes are attached, the specific technology of attachment to the solid surface, the length of the probes, methods of labelling of the target sequences, the conditions of hybridisation to the probes and finally the analysis methods of the hybridisation patterns. Many different solutions partly using different technological approaches were developed to be ready for marketing. I will give a short overview of some of these diverse realizations of microarrays with a focus on their relevance to the field of agronomical diagnostics.

Quantitative multiplex detection of plant pathogens using PRI-lock probes and universal, ultra-high-throughput real-time PCR on OpenArrays™

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Current technologies for multiplex, quantitative analyses frequently suffer from compromises between the level of multiplexing, throughput and accuracy of quantification. In general, for the detection of nucleic acids, microarrays provide very high level of multiplexing, but less accurate quantification and usually low throughput. At present, real-time, quantitative PCR provides the most reliable means of target quantification, and it is suitable for the analysis of a relatively high number of samples. The achievable level of multiplexing, however, is low.

Nano-scale technology, provides high-density and low-volume microchambers, which could accommodate very high number of reactions, performed under standard conditions. Many of these systems are still at the experimental phase, and are not capable of monitoring the fluorescent signals in real time for each microwell, which is required for quantification.

Recently, a conceptually new, ultra-high-throughput platform has become available for real-time PCR, capable of accommodating more than 3000 reactions per array. The OpenArray™-s have 48 subarrays, allowing parallel testing of up to 48 samples, and each subarray contains 64 microscopic through-holes of 33 nL volume. The primers are pre-loaded into the holes, while the sample along with the reagents are auto-loaded due to surface tension, provided by the hydrophilic coating of the holes and the hydrophobic surface of the array.

Plant Research International recently has developed PRI-Lock probes for multiplex detection which provide flexibility, and bridge the gap between target-specific recognition and high-throughput amplification using universal but unique primer pairs and a generic TaqMan probe. PRI-lock probes are long oligonucleotides, similar in structure to padlock probes. They contain artificially selected primer sites and a TaqMan probe region, flanked by target complementary regions.

In this study, we have characterized the quantitation power of circularizable ligation probes, and report the development of a high-throughput, quantitative multiplex diagnostic assay based on the described principle.

Development and evaluation of *gyrB* DNA microarray for detection of and identification of pathogenic bacteria

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To determine the potential of the microarray-based method for the detection and identification of bacterial pathogens a pilot microarray was developed and evaluated. An adapted protocol for the sequence-specific end labelling of oligonucleotides was used in combination with a set of gyrase subunit B (*gyrB*) specific probes. The microarray was validated using a representative set of targeted strains as well as several related negative controls. Environmental applicability was tested with a set of environmental samples, both spiked and non-spiked with targeted strains.

We were able to show this diagnostic microarray to be capable of parallel detection of a broad range of pathogenic bacteria from complex environmental samples with high levels of both specificity and sensitivity. Specificity of the detection was further enhanced by the introduction of the novel competitive oligonucleotides concept. Sensitivity level was determined to be between 0.1 and 1%.

DEVELOPMENT AND APPLICATION OF HITChip - DIVERSITY MICROARRAY FOR THE HUMAN INTESTINAL MICROBIOTA

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The human gastrointestinal tract is inhabited by numerous microorganisms comprising a complex and dynamic ecosystem termed microbiota. Since the microbiota composition differs between individuals and gastrointestinal location, as well as changes in time, it is crucial to have a high throughput method which would enable identification and quantification of this enormous biodiversity. Here we present a newly developed Human Intestinal Tract Chip (HITChip) – a diversity microarray that covers more than 1,000 human intestinal bacteria reported in culture-dependent and culture-independent studies. The HITChip is produced by Agilent Technologies using an *in situ* synthesis method and consists of more than 5,500 oligonucleotide probes with a narrow range of melting temperatures. Each organism is represented on the HITChip with 6 probes that are targeting 2 hyper-variable regions of the 16S ribosomal RNA. Those probes are linked with a database containing information about phylogenetic position of the organism they target, which is of major importance when uncultured organisms are identified. The HITChip is suitable for two color hybridization, and thus in a single experiment two samples can be analyzed. Besides identification of the intestinal bacteria, the HITChip allows quantification of the groups of bacteria on the different phylogenetic level namely phylum, order, family and genus. The HITChip can give information about the relative increase/decrease of single species at different time points. In combination with group specific PCR it is possible to determine the composition of less abundant groups of the intestinal microbiota, as it was performed for the *Lactobacillus* and *Bifidobacterium* communities of 2 individuals. Analysis of the 16S ribosomal RNA gene amplicons obtained on DNA and cDNA isolated from a faecal sample showed slight differences between diversity and activity of intestinal microbiota of one person. About hundred bacteria were recognized as present or active. Monitoring the temporal variation of the microbiota in three healthy individuals during a period of 6 years showed that microbiota composition is relatively stable, which is in line with previous fingerprinting based observations. However, some variation in the composition could be observed and this was overseen when other, less precise techniques are applied. Overall, our study demonstrated that the HITChip can detect and quantify members of the gastrointestinal tract microbiota at the species level at high throughput level, which makes the HITChip suitable for implementation in intervention studies.

Microarray technology to detect antibiotic resistance genes

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For the detection of antibiotic resistance genes in microorganism an antibiotic resistance (AR) thematic microarray was developed. This AR-microarray consists of more than 250 probes, 50 or 60 nucleotides long, representing over 400 AR genes involved in resistance to the following classes of antibiotics: Aminoglycoside, Extended Spectrum β -Lactamase (ESBL), Chloramphenicol, Macrolide Lincosamides Streptogramin (MLS), Sulfonamide, Tetracycline, Trimethoprim, and Vancomycin.

The specificity of a large set of the oligonucleotides was tested using a variety of reference strains with known pheno- and genotypes.

In total nearly 300 *Salmonella* and Lactic Acid Bacteria (LAB) strains were screened for the presence of AR-genes. Various multiple drug resistant (MDR) strains were characterized, but also AR-genes were identified with the microarray that have never before been described in certain bacterial species. The results also showed that the microarray data were not always in complete concordance with the phenotypic profiles of the strains. Nevertheless the developed thematic microarray showed to be very suitable for the screening of various different microbial strains for the presence of AR-genes.

At the moment the applicability of this AR-microarray for environmental samples is being tested.

Luminex immunoassays for detection of plant pathogenic organisms

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Immunoassays were developed based on the Luminex xMAP® technology, for multiplex detection plant pathogenic organisms. Plant samples were incubated with antibody coated fluorescent beads and with secondary antibodies, conjugated with a reporter dye (Alexa Fluor 532). Samples were analyzed with the Luminex 100ST analyzer, in which one laser identifies individual beads and a second laser the reporter dye. These assays can be completed within 1 hour, are very robust, user-friendly, use low labor input and are suitable for high throughput screening.

For some bacteria in potato- and flower bulb extracts, the detection limit was 100-1000 cfu ml⁻¹ in a Luminex enrichment assay which was 10-100 times better than of an enrichment-ELISA. Without enrichment, the sensitivity of the Luminex assay and ELISA were largely similar and ranged between 10⁶ and 10⁷ cells ml⁻¹. For potato viruses the sensitivity of the Luminex assay is approx. 10 times better when compared to standard ELISA.

EU-eBIOSENSE, electrical bio-sensor arrays for the analyses of mycotoxins and toxigenic fungi

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Detection tools for *Fusarium* spp. and their mycotoxins are constantly evolving. In the EU consortium eBIOSENSE, electrical chip technology, molecular biology and advanced immunology will be integrated for fast, cheap and robust detection of mycotoxins as well as the corresponding fungi. The platform is based on conductivity of the electrochemical mediator amino-phenol, which is released from electrically inactive para-amino-phenol-phosphate by alkaline phosphatase. Conductivity is measured by interdigitating electrodes, which facilitate redox-cycling and micro-diffusion, leading to a 100-fold signal amplification.

Two different platforms are being developed: a protein-based chip for the detection of ochratoxin A, fumonisin and DON and a DNA based chip for the detection of fumonisin producing species, e.g. *F. verticillioides* and *F. proliferatum*.

To detect mycotoxins we raised highly specific monoclonal antibodies in Llama, which have the unique feature that they consist of only heavy chain moieties. Characterization of the antibodies is currently in progress.

DNA based chips have been designed on the basis of sequence data obtained from the polyketide synthase gene (*fum1*) of *F. verticillioides*, *F. proliferatum*, *F. nygamai* and *F. globosum*, all *Fusarium* species known to produce fumonisin. Through the selection of proper regions, capture- and detection-oligonucleotides have been designed that allow the discrimination between *F. verticillioides* that occurs mainly on maize and *F. proliferatum* that is much more polyphagous.

Currently the detection of *Fusarium* DNA using the electric chip is being optimized to improve sensitivity and robustness. This electric chip approach might in time lead to extensive automation of early warning systems for *Fusarium* spp. in cereals and other commodities.

This work is partly financed by the EU project LSHB-CT-2004-512009 and the Dutch ministry of Agriculture, Nature and Food Safety. Program LNV 397-1.

Analysis of the microbial community dynamic associated to Take-All decline using 16S taxonomic microarrays

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The decline of take-all disease (*Gaeumannomyces graminis* var. *tritici*) may take place during wheat monocropping. So far, the analysis of microbial populations associated with take-all decline has focused mainly on culturable antagonistic fluorescent *Pseudomonas* spp. producing the antifungal compound 2,4-diacetylphloroglucinol, and relatively little has been done to take into account other bacterial populations. The objective of this study was to assess the changes in the rhizobacterial community linked with take-all decline of wheat, based on an approach combining taxonomic 16S rRNA-based microarray, *rrs* cloning-sequencing and quantitative PCR. The 16S rRNA-based microarray contains about 600 probes targeting bacteria (including several new specific probes for *Pseudomonas* groups, particularly including biocontrol strains) at various taxonomic levels. Rhizosphere samples were collected from plots grown with wheat for one year (treatment I; low level of take-all disease), five years (treatment V; high level of disease) or ten years (treatment X; low level of disease, suppressiveness reached). First, principal component analysis of microarray results for the total bacterial community discriminated mainly between treatments V and X. Probes targeting the *Pseudomonas* genus gave higher signals in treatment V, in agreement with quantitative PCR data. Second, microarray results obtained after PCR of *Pseudomonas* populations discriminated mainly between treatments I and X. Overall, treatment X appears to be associated with certain types of *Pseudomonas* (which comprise biocontrol strains), as well as particular non-*Pseudomonas* taxa. These tools are promising for analysis of bacterial diversity associated with disease-suppressive soils.

Converting a complex profile into a simple diagnostic test

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Introduction

Breast cancer patients with similar pathological staging can have markedly different rates of disease-free and overall survival. A key challenge in breast cancer management is to accurately determine a patient's risk of developing distant metastasis at the time of primary diagnosis. This information can then be used to tailor metastasis-preventing treatment for high-risk patients.

We have developed and extensively validated a 70-gene breast cancer prognosis profile using oligonucleotide microarrays containing ~25,000 genes^{1,2}.

Aim

To convert a robust gene expression profile, discovered on complex microarrays, to a high-throughput, extensively validated clinical diagnostic tool.

Methods

A custom made microarray with 8 sub-arrays was developed ("MammaPrint® 8-pack"), in association with Agilent Technologies, USA. Each sub-array, printed on a 1"x3" slide, contains 1,900 microarray probes and allows 8 simultaneous hybridizations. 1,611 biological oligonucleotides, including normalisation genes and the 70 gene profile in triplicate are present in each sub-array, along with carefully selected control features, used to monitor hybridisation quality. To confirm the prognostic ability of the 70 gene expression profile in a MammaPrint sub-array format, we analyzed 168 patient samples used in previous studies^{1,2}. Samples were hybridized against a uniform breast cancer reference in duplicate, incorporating a dye-swap into the hybridisation protocol. Outcome prediction of the 168 sample cohort was carried out by determining the correlation of each patients unique 70-gene profile to the average expression profile of 44 good prognosis samples. Paired risk profiles determined using both 25k complex and 1.9k Mammaprint microarrays were analysed to quantify the inter-platform reproducibility of the test.

References:

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- (2) van de Vijver MJ, He YD, van't Veer LJ et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002; 347(25):1999-2009

Early results of CGH on *Agrobacterium* tiling arrays: the lessons from a short oligo design.

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Bacterial species are genomically coherent clusters of strains, which are thought to be adapted to the same or very similar ecological niches. Our project aims at finding "species specific" genes involved in the ecological specialisations of closely related but readily distinguishable species by comparative genome studies. The chosen tool for such a genomic study are microarrays covering the entire genome of a reference strain against which would be hybridized DNA from strains belonging i) to the same species in order to determine the species core genome, then ii) to more distantly related species in order to determine the genus core genome as well as the accessory but species specific genome.

The study was done with *Agrobacterium spp.*, which consists of species displaying a large range of genome diversity, and by using pangenomic microarrays constructed with 29-mer oligo-probes - in a complete tiling design - against the completely sequenced strain C58.

However, as the possibility for an absence of hybridization increases with genome divergence, a simulation (based on AFLP and MLSA results) was done to estimate whether the 29-mer oligo-probes are sensitive enough to provide significant hybridization signals when hybridized with DNA from more distantly related strains.

The preliminary results of the CGH analysis we'll present focus on technical aspects such as : 1) the hybridization sensitivity of 29-mer probes to mismatches of target DNA and its impact for CGH studies of distantly related strains (and thus a criticism of the chosen microarray design); 2) a study of the variation of hybridization intensities according to probe sequence features even in absence of mismatches (a problem we found as critically hampering quantitative studies with short oligo microarrays as the 16S microarrays).

Detecting viruses using microarrays

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Microarray methods seem well suited to screening plant and other material for virus infection. Unlike other pathogens (and pests) viruses do not contain conserved sequences that can be amplified using PCR for detection and identification. In the case of fungal and bacterial pathogens (ITS or 16S) and invertebrate pests (COX) conserved regions of sequence can be amplified using PCR; following sequencing a database of sequence can be interrogated to identify the species. In the case of viruses since no similar 'universal' sequences are available screening is done by performing tests in parallel – something microarray techniques were initially developed to achieve.

The talk will be in two parts, the first will describe the end of the European Union funded microarray project DiagChip (www.diagchip.co.uk), in which an array was developed for the detection of the quarantine pathogens of potato. The project ended with a 'ringtest' of the protocols and arrays developed in seven different diagnostic laboratories around Europe. The results will be presented and discussed. The second part of the talk will introduce a new microarray project 'Defra biosecurity chip' which aims to develop a standardised microarray approach to detecting all the viruses of interest to the Department for the Environment, Food and Rural Affairs (Defra). The consortium made up of seven partners will deliver a multiple target assay covering 600+ known viruses of vertebrates, invertebrates and plants, including zoonotic viruses, which will be made available to government agencies as a useable tool.

Protein chips for screening of protease-protease inhibitor interactions.

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In this study, we analyzed *in vitro* protease inhibitory activity of a set of plant (from potato and wild Solanum species) Kunitz-type protease inhibitors, which were obtained as recombinant proteins expressed in *E. coli*. A collection of kunitz-type (2 class A, 5 class B, and five class C) protease inhibitors was screened for the inhibition of serine proteases (trypsin, chymotrypsin, elastase), aspartic protease (cathepsin D) and cysteine proteases (papain, cathepsin B). Inhibition assays in eppendorf tubes were evaluated by incubating the inhibitors with trypsin and chymotrypsin, and measuring the absorbance after the cleavage of benzoyl-Arginyl-naphthylamide (BANA) and benzyl-Phenylalanyl-substrates.

The interaction with the proteases was tested by spotting the set of protease inhibitors on epoxy slides. Protein-protein interactions were studied using trypsin, chymotrypsin and cathepsin B in solution: proteases were labelled with Cyanine3-NHS in bicarbonate buffer pH 9 at 37°C for 1 h, stopped with TRIS-glycine pH 7.5, separated by elution on a P-100 sepharose, then stored at -20°C in glycerol 20%.

Some of the protease inhibitors showed low affinity binding to trypsin or chymotrypsin, due possibly to:

- 1: an incorrect folding (a difference in MW size between recombinant proteins and presence of double bands was observed by SDS-PAGE) and
- 2: the binding to the slide affected the ability to interact with proteases.

Physical interaction with trypsin and chymotrypsin were detected in the protein chip assay for many PIs that did not show inhibition in *in vitro* assays: hence binding to protease and accessibility of substrates to the protease active site are two distinct features. These experiments show that inhibition assays in solution indicates more clearly protease inhibition for each PI, but on chip protein-protein interaction data may provide additional information between PIs (as well as to check protein secondary structure after expression in *E. coli*). In this case, the chips were useful to show that cathepsin B, a cysteine protease, was actively bound by PIs, a data subsequently confirmed by the *in vitro* inhibition assays. Further experiments are ongoing to produce high-density spots and test inhibitors captured by specific antibodies on the chip for homogeneous presentation of proteins.

Nucleic acid microarray data analyses in potato – virus interaction studies

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In recent years cDNA microarrays have been employed to monitor gene expression on a scale much larger than previously possible. Nevertheless, several of the approaches still need to be optimized. We improved some steps in gene expression quantification using cDNA microarrays, including total RNA isolation, genomic DNA degradation, cDNA synthesis and data analysis. Several computer programs and statistical packages were used in data analysis. ArrayPro, R, MEV and MapMan turned out to be the most useful ones for analyzing and visualizing large data sets obtained with TIGR 10000 clones potato cDNA microarrays in studies of gene expression in the response of susceptible and resistant potato (*Solanum tuberosum* L.) cultivars at different times after infection with PVY.

POSTERS

Poster 1

PADLOCK PROBE TECHNOLOGY: Vision of a Universal Multiplex Diagnostic System for Versatile Applications.

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Padlock probes (PLP) are long oligonucleotides, whose ends are complementary to adjacent target sequences. Upon hybridisation to the target, the two ends are brought into contact, allowing PLP circularisation by ligation. PLPs provide extremely specific target recognition, which is followed by universal amplification and microarray detection. Since target recognition is separated from downstream processing, PLPs enable the development of flexible and extendable diagnostic systems, targeting diverse organisms. To adapt padlock technology for diagnostic purposes, we optimised PLP design to ensure high specificity, eliminating ligation on non-target sequences under real-world assay conditions. We designed and tested 11 PLPs to target various plant pathogens at the genus, species and sub-species levels, and developed a prototype PLP-based plant health chip. Excellent specificity was demonstrated towards the target organisms. Assay background was determined for each hybridisation using a no-target reference sample, which provided reliable and sensitive identification of positive samples. A sensitivity of 5 pg genomic DNA and a dynamic range of detection of 100 were observed. The developed multiplex diagnostic system was validated using artificial mixtures of genomic DNAs of characterized isolates. The demonstrated system is adaptable to a wide variety of applications ranging from pest management to environmental microbiology.

Poster 2

Application of Immunomagnetic Separation Live/Dead (IMS L/D) method for direct detection of *Pectobacterium carotovorum* subsp. *atrosepticum* in plant tissue

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Pectobacterium carotovorum subsp. *atrosepticum* (Pca) is a pectolytic pathogen belonging to the *Enterobacteriaceae* family which causes economically important losses in potato crops in temperate climate regions. In this study we present a method of identification of Pca and differentiation of the live and dead bacterial cells. The technique is based on immunomagnetic separation (IMS) of target bacteria from plant tissue and soil samples using magnetic microbeads labeled with Pca specific antibodies and in the second step - on staining the bacteria cells with two fluorescent nucleic acid dyes: SYTO[®] 9 - live cells) and propidium iodide (PI)- dead cells. The method enables to detect Pca cells with the detection level of 10⁴ – 10⁵ cfu/ml in 10% homogenate of plant tissue and differentiation of the visualised bacteria into live and dead cells. The efficiency of the proposed method is comparable with commercial ELISA tests. The IMS L/D is a promising method which may be used for detection and differentiation of different plant pathogenic bacteria.

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Poster 3

Application of Multiplex PCR in the detection of bacteria from the genus *Pectobacterium* in potato plant tissue

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Pectobacterium carotovorum subsp. *atrosepticum* (*Pca*), *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Pectobacterium chrysanthemi* (*Pch*) cause important losses of potato crops all over the world.

The aim of this work was to develop efficient Multiplex PCR assays based on combinations of five pairs of primers specific for different species and subspecies of *Pectobacterium*: for *Pca*: ECA1f/ECA2r, Y₄₅/Y₄₆; for *Pcc*: Y₁/Y₂ and for *Pch*: ADE1/ADE2, SJ₁/SJ₂, which would allow for quick, fast and direct detection of mixed infection of potato plants and tubers caused by these pathogens.

The work showed the possibilities of simultaneous and specific detection of two different subspecies of *P. carotovorum*: *Pca*, *Pcc* and species *P. chrysanthemi* in multiplex PCR. Amplification conditions for different combinations of primers for *Pca* and *Pch* simultaneous detection were described. In addition conditions for simultaneous detection of three different *Pectobacterium* in one reaction mixture were also described.

Presented Multiplex PCR assays give possibilities to reduce costs and time of specific pectinolytic bacteria detection and identification directly in infected potato tissue.

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Poster 4

T. Malinowski, not received

Poster 5

Design of microarrays for detection of plant viruses – short oligonucleotide approach

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Microarray technology evolved into a series of different experimental methods and approaches. We compare here two basic approaches, namely the microarrays based on DNA amplicons (DNA microchip) with those using short oligonucleotides (oligonucleotide microchip) as capture probes.

In our project, we focus on the seven most widely distributed potato viruses: PVY and PVA potyviruses, PVX potexvirus, PVS and PVM carlaviruses, potato leafroll luteovirus and mop-top potamovirus.

In the first approach, viral RNA was isolated from infected indicator plants and was amplified by RT-PCR with specific primers. After PCR reamplification, purified amplicons (400 - 600 bp) were used as capture probes and spotted on the chip. In this way we successfully tested viruses PVS and PLRV.

The second, new, approach consisted in using short synthetic single stranded oligomers (40nt) instead of PCR products as capture probes. A microchip detecting potato viruses PVA, PVS, PVM, PVX, PVY and PLRV in both single and mixed infections was developed and tested. The chip was also designed to distinguish between the main strains of PVY and PVS. Results of initial tests with PVY^{NTN} and PVY^O strains using several different probes for one virus are presented. Possibilities and advantages of the new oligonucleotide based microarray approach in plant viral diagnostics are discussed.

Acknowledgements

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Poster 6

P. Poltronieri (see abstract oral presentation)

Poster 7

Nanogravimetric sensor for specific DNA detection

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The detection and quantification of specific DNA sequences is of great importance in numerous applications, such as in medical research and clinical diagnosis, environmental monitoring and food analysis. In the past few years, the immobilization of DNA strands on electrode surfaces of different types has been the subject of the development of DNA sensors or DNA chips. Some different methods for immobilizing DNA onto electrode surfaces have been used: controlled potential adsorption, adsorption of DNA by evaporation of solvent during drying of DNA solution, direct covalent binding, entrapment in a polymer matrix or indirect binding by the use of intermediate systems, through self-assembly of the mercapto-modified DNA. Among other sensing devices, piezoelectric quartz crystals are suitable for direct and label-free real-time monitoring of affinity interaction of biomolecules. The quartz crystal microbalance (QCM) is an ultra-sensitive weighing device that utilizes the mechanical resonance of piezoelectric single-crystalline quartz. QCM's are capable of measuring mass changes as small as a fraction of a monolayer or single layer of atoms. The decrease of the resonance frequency correlates to the mass accumulated on quartz crystal surface. QCM are used for the detection of specific antigens, for the hybridization between differently immobilized DNA probes and complementary DNA strands, for the characterization specific interactions between proteins and phages.

In recent years, the use of self-assembled monolayers (SAMs) in various fields of research is growing rapidly. Many biomedical fields apply SAMs as an interface layer between a metal surface and a solution. Au substrate is most often used for the formation of SAMs because it is reasonably inert and well attracts organic sulfides. The aim of this study was to apply QCM for the registration of binding of synthetic 5'-amino-alkyl ssDNA fragments to carboxyl-terminated SAM and for DNA hybridization.

Poster 8

An Oligonucleotide Array for Differentiation of *Fusarium* species

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We have developed an array for identification of the most important *Fusarium* species. The array consists of species- or chemotype specific capture oligonucleotides (COs) derived from the ITS, EF1 α or Tri12 genomic regions. Using this array it was possible to identify individual species or chemotypes of *Fusarium*, however, in a few cases, cross-hybridisation was observed.

Poster 9

Towards a diagnostic microarray for European quarantine bacteria

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The European Mediterranean Plant Protection Organization (EPPO) list includes 26 quarantine bacteria comprising Xanthomonas, Erwinia, Pseudomonas, Clavibacter and Curtobacterium strains. The aim of this study is the fast detection and discrimination of these bacteria using the microarray technique. In a first approach 16S rDNA gene probes were designed. Hybridization of these probes with Cy3 labeled 16S rDNA PCR products from various strains revealed the discrimination between divergent genera (e.g. Pseudomonas, Xanthomonas) and even to the species level in some cases (e.g. *Pantoea stewartii*, *Ralstonia solanacearum*). For the differentiation of related species and among subspecies (e.g. *Xanthomonas translucens*, *X. oryzae*, *X. arboricola*) additional probes are needed. For this purpose degenerated primers of housekeeping genes (RNA polymerase beta subunit *rpoB*, cell division protein *ftsZ*) were designed. Differences in the DNA sequences may provide probes that allow a further differentiation of the tested strains.

Poster 10

Genes involved in carbon metabolism are differentially expressed after PVY^{NTN} and PVY^N infection in potato

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Viral infection of plants causes development of symptoms like stunting, leaf distortion, dark greening and chlorosis and has an impact on crop yield and therefore on crop industry. Physiological changes in those plants include alterations in the levels and / or partitioning of different metabolites, especially sugars. Accumulation of sucrose, starch and chloose is involved in development of chlorotic symptoms on inoculated leaves, since it has effects on pathways involved in pigmentation and hormone function.

Sensitive potato cultivars (*Solanum tuberosum* L.) Igor and Nadine infected with *Potato virus Y^{NTN}* (PVY^{NTN}) show chlorotic spots on inoculated leaves and necrotic ring-spots on tubers (PTNRD disease) while PVY^N infection does not cause development of symptoms. Early response (30min, 12h and 48h after inoculation) of genes involved in PTNRD symptoms development in inoculated leaves were investigated using potato cDNA microarrays (TIGR), where cDNAs from PVY^{NTN} and PVY^N inoculated leaves were hybridized on one microarray.

Preliminary microarray data analysis revealed differential expression of genes coding the enzymes of sucrose and starch synthesis, degradation and transport. Genes in both pathways appear to be up- or down-regulated in plants inoculated with PVY^{NTN} in comparison to the response in plants inoculated with PVY^N. Genes for fructokinase in sucrose degradation pathway are in both cultivars at all three time points up-regulated. In the starch pathway, 30 min after inoculation genes coding AGPase and 4-alpha-glucanotransferase are up-regulated in both cultivars. In the sucrose pathway Nadine has a

transient response. 30 min after inoculation genes coding hexokinase and sucrose-phosphate synthase are up-regulated, while after 12 h they are down-regulated. 48 h after inoculation those genes are again up-regulated.

According to our preliminary results, cultivars Igor and Nadine have different patterns in expression of genes involved in starch and sucrose metabolism. Up-regulation of fructokinase implicates accumulation of fructose-6P in leaves inoculated with PVY^{NTN} relative to the response in plants inoculated with PVY^N.

Poster 11

Detecting single nucleotide polymorphisms using DNA arrays for plant pathogen diagnosis

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The lack of rapid and reliable means for routine pathogen identification has been one of the main limitations in plant disease management and has pushed the development of culture-independent, molecular approaches. Currently, DNA array technology is the most suitable technique for high-throughput detection and identification, as well as quantification, of multiple pathogens in a single assay. Closely related pathogens that may have completely different host ranges or pathogenicity often differ in only a single to a few base pairs in genes that may be targeted for identification. Therefore, the ability to discriminate single nucleotide polymorphisms (SNPs) should be pursued in any diagnostic assay. In this study, we showed the utility of DNA array technology to detect SNPs while accounting for specific criteria such as the position of the mismatch, the sequence of the oligonucleotide, and the length and amount of labeled amplicons that are hybridized. When disregarding mismatches at the extreme ends of the oligonucleotides, cross hybridization to single mismatch oligonucleotides is rare when processing environmental samples that contain genetic material from unknown sources. In addition to plant pathology, this study is relevant for any field of research where DNA arrays are used to detect mutations or polymorphisms, ranging from human diagnostics to environmental microbiology and microbial ecology.