

Executive Summary:

Within the NANODETECT Project, an advanced biosensor technology was used to develop off-line monitoring systems (sensors) and design the appropriate on-line models which combine the expertise of sensitive and specific molecular biological processes with the potency of microfluidic systems for application in liquid process streams. The developed biosensors interact with information technology tools and thus contribute to improved quality control systems within the European dairy industry as a case study.

The main challenge in monitoring for contaminants in the food chain is the lack of fast and user friendly off- or on-line monitoring systems respectively. Milk was chosen as reference process stream as it is subject to different levels of various contaminations of which the following quality and safety items were chosen as models: for mycotoxins aflatoxin M1; for drug residues aminoglycosides and finally for fraud high value goat milk blended with cheap cow milk. The developed biosensors are based on different types of immunoassays, depending on the envisaged application. Single modules were developed for the detection and quantification of specific contaminants which can be combined according to users' demands.

An off-line system was constructed and upgraded with a tailored software and automated decision support system, applicable for large industries as well as bench top devices for small companies with flexible production units. The developed technology enables analyses without expensive and time-consuming sample preparation and provides the opportunity of on-site analyses. The NANODETECT system is operable without use and disposal of harmful solvents and requires no safety measures. The data processing automated by the decision support system provides the calculation of measurements and evaluation of results. Furthermore, an integrated database enables various reporting options and ensures the traceability, which is necessary for each industrial sector of food processing. The modular design of the NANODETECT system forms the base for further applications. The modules are exchangeable by the development of parallel configuration, thus the system is adaptable to different fluids.

In the NANODETECT project, an appropriate number of industrial and academic food technologists, sensor technologists and quality managers cooperated in order to develop an innovative technology.

Due to unexpected problems with the auto-fluorescence of important prototype parts, the prototype finalisation was delayed for several months. Therefore, the beneficiaries applied for a project extension which was granted by the EC. Thanks to the additional 6 months, the project work plan and associate deliverables and milestones could be finalised as initially planned.

Project Context and Objectives:

The NANODETECT project contributed to the advancement of scientific know-how for solving problems within quality control in the European food industry by providing solutions for the early discovery of various types of contaminants in liquid food products. In the project, an advanced biosensor technology was applied for the development of a biosensor which combines the expertise of sensitive and specific molecular biological processes with the potency of microfluidic systems for application in liquid process streams. The developed biosensors interact with information technology tools and thus contribute to improved quality control systems within the European dairy industry as a case example.

The main problem in monitoring for contaminants in the food chain is the lack of fast and user friendly off- and on-line monitoring systems. In NANODETECT, milk was chosen as an example process stream because it is subject to different levels of various contaminations of which the following quality and safety items were chosen as models: Mycotoxins (Aflatoxin M1), Drug residues (aminoglycosides) and Fraud (high value goat milk blended with cheaper cow milk).

The developed biosensors work on the basis of microfluidic chips on which different types of contaminant specific immunoassays are carried out. Single modules were developed for the detection and quantification of specific contaminants which can be combined for parallel detection according to the users' requirements.

An off-line prototype with an optical detection unit for the fluorescence signal was constructed and optimised successfully with regard to the needs of SMEs who emphasise flexible production units.

The developed NANODETECT software has been developed for automated data processing and evaluation of results. A NANODETECT Decision Support System comprises evaluation of results as safe or not safe and gives a distinct colour signal when a sample is contaminated above the MRL. Furthermore, an integrated database enables various reporting options for the SMEs quality management units, which is required for each industrial sector of food processing. The developed technology enables analyses without expensive and time-consuming sample preparation and provides the opportunity of on-site analyses. The NANODETECT system is operable without use and disposal of harmful solvents and requires no safety measures.

The partners have concluded and documented a projection of an online NANODETECT prototype following the agreement with the EC, so that this development stage can be implemented immediately upon further technical progress.

The NANODETECT results were presented to a number of dairy SMEs as well as to large companies from the food sector during a final dissemination event. The project's impact is expressed by the high interest of the industry in fast and user friendly contaminant detection.

In the NANODETECT project, an appropriate number of industrial and academic food technologists, sensor technologists and quality managers cooperated in order to develop an innovative technology featuring the following advantages:

- Parallel detection of different contaminants
- User-friendly interface
- Lower costs
- High conversion rates
- On-site detection
- Easy to use, clean and to reactivate
- Exchangeable construction for multiple usage

Project Results:

The work within the NANODETECT Project initially started with defining the requirements and technical framework for the NANODETECT prototype. MCB in cooperation with TTZ defined the following requirements for the instrumental system: As coating material SU-8, a negative polymer based on epoxy, was chosen. This photoresist polymer was identified as suitable material for the microstructures due to its good biocompatibility enabling for the development of an immunoassay system onto its surface. A further advantage of SU-8 is its transparency that allows for optical detection and has good properties to fabricate complex structures for microfluidic devices by a standard photolithography process. Procedures for testing substrate materials were established by MCB and OPTOTEK. To facilitate the detection of the resulting optical or fluorescence signal, the microfluidic device should preferably be fabricated using transparent materials. Accordingly, MCB used materials such as glass and PMMA (Polymethylmethacrylate) for testing. Therefore a thin film SU-8 with a thickness around 5µm has been coated directly on glass and PMMA. The resulting samples were transparent and therefore suitable for the on-going experiments concerning fluorescence measurements. Potential difficulties arose from adhesion between SU-8 and PMMA. The adhesion of the photoresist to the substrate is of importance for fabrication of micro-channels. PMMA as a substrate is particularly suitable for the microfluidic device due to its transparency and low costs. However, the adhesion of SU-8 with PMMA was a critical point. A direct sealing of the two materials is difficult without surface modification. A possible solution: A suitable surface treatment must be found to markedly increase the adhesion between SU-8 and PMMA.

TTZ defined the optimal microfluidic structure in cooperation with MCB. Afterwards MCB produced different microfluidic structures accordingly which were tested via a flow through of raw milk by TTZ. The conclusion from the kick-off meeting was that a squarish microfluidic system would be the easiest way to couple it to the optical detection unit. However, for the biological system a rectangular system would be of advantage, as it possesses an increased surface for antibody binding and for this reason it would also provide a larger possibility to enrich potential contaminants in milk.

In close cooperation with TTZ and MCB, OPTOTEK defined two basic measurement setups. Transmission measurement presented a simpler solution, requiring however the use of transmissive microchannel chips. PMMA was therefore suggested as the alternative to a more frequently used silicon or Pyrex® wafer, presenting a much cheaper solution. Second setup was measurement in reflection with some additional benefits: the Microchannel chip did not have to transmit light and due to the reflective construction there were no additional problems related to the laser output. No additional optical filters were necessary.

As the fluorescent dye Phycoerythrin was chosen for labelling within the first micro-channel constructions the laser with a wavelength of 532nm was selected for illumination. Available emission and dichroic filters □ spectral characteristics have been studied, enabling sufficient transmission of fluorescent signal and reflection of the laser's illumination.

Preliminary experimental results were performed towards testing the laser, the emitter and dichroic filters as well as observation of strong fluorescent signals obtained from a single 500 micrometer micro-channel made of PMMA. This enabled for measurements in both, transmission and reflection. The micro-channel

was filled with fluorescent dye, resulting in a strong optical signal that was successfully measured with a spectrometer. This enabled for determination of emitted light spectra. As the final optical signal will be significantly lowered in case of actual microfluidic samples, further experimental evaluations were performed with the use of photodiodes and photomultipliers.

On the meeting in Ljubljana OPTOTEK, TTZ and MCB decided that the prototype will be constructed in a way which allows for the characterization of a single microfluidic chip. The photodiode and the photomultiplier were considered for further integration into the micro-optical system, whereas a single photo-detector will already be sufficient for the final prototype.

Finally OPTOTEK, MCB, and TTZ decided that the use of cheap micro-channel chips made of PMMA will be tested. This should enable for measurements in transmission as well as in reflection. On a Project meeting in Ljubljana it has been decided that the prototype will be constructed to perform the measurement of a single test sample at a time, exhibiting a cartridge for placement. This will allow the development of a cost effective prototype using a single laser for illumination and a single photodetector (photodiode or photomultiplier) for light detection. In direct contact with MCB, NORAYBIO analysed the most relevant aspects that will condition the development of the bio-informatics software.

The SME Beneficiaries MGLANG and RINY defined the matrix of study: full fat cows' milk for cheese making and for curd cheese. Furthermore, RINY and MGLANG provided their HACCP concepts for further evaluation. Additionally RINY provided the Spanish legislation in relation with the project's aims for the preparation of an overview on legislation issues in order to assure that the prototype fits in.

Finally the biological system for each contaminant was defined as follows: The detection system is based on modified immunoassays (sandwich or competition) using monoclonal antibodies (mAbs) and one type of fluorescent label (the fluorescent protein phycoerythrin (PE) with an excitation wavelength of 490 nm and an emission peak at 575 nm). In case that the big protein PE (240 kDa) disturbs the assays, Alexa 532 (mw 721 Da; Abs/Em maxima of 532/554 nm) could be an option. Within the sandwich assay format (*Listeria* and bovine caseins), antibodies have to be coated onto the sensor's surface. In case of mycotoxins and antibiotics, protein conjugates have to be prepared for coating of the sensor surface.

Listeria monocytogenes

The biological system for the enrichment and detection of *Listeria monocytogenes* in raw milk is built up like a sandwich immunoassay. A capture antibody and a fluorescent labelled detecting antibody, both specific for *Listeria monocytogenes* are used. The detecting antibody is labelled with a fluorescent dye either Phycoerythrin or in case of detection difficulties with Alexa 532. The labelling procedure will be done in coherence of protocols provided by RIKILT.

Two methods are conceivable to detect the *Listeria* bacteria. First one is to label the detecting antibody with Phycoerythrin or Alexa 532 respectively. This method is the final selection according to the NANODETECT application requirements. The second one, in order to get first quantitative results, would be to elute the bacteria off the microfluidic system and detect them subsequently by real time PCR.

Mycotoxins

The mycotoxins have a significantly lower molecular weight and cannot be detected in the same way as *Listeria* as it is impossible that a second antibody binds to the antigen at the same time. The detection has to work on the basis of a competitive assay instead: the antigen (mycotoxin), or a BSA conjugate, is coated onto the surface of the reaction chamber. Monoclonal aflatoxin M1 antibodies labelled with phycoerythrin will be bound to the coated antigen in the absence of free Aflatoxin M1 and generate a strong fluorescence signal. If Aflatoxin M1 contaminated milk passes through the reactor, the labelled antibodies will competitively bind to the free antigen in the sample and thus lower the signal on the surface.

Drug residues

Drug residues will be detected according to the same methodology of Aflatoxin M1. The difference is the use of two types of antibodies, capture antibodies and reporter antibodies labelled with the fluorescent marker

Fraud

Fraud, for instance bovine proteins in goat milk, will be detected according to the same methodology as previously described for the pathogen *Listeria monocytogenes*.

Anti-bovine casein: RIKILT worked with hybridoma cell lines which produce two monoclonal antibodies (mAbs) raised against bovine kappa-casein. The development of these mAbs and the application in an optical biosensor for the detection of cows' milk in for instance higher priced milk of ewes and goats was selected.

Fluorescent labels

The different mAbs were labelled with PE (or Alexa 532) according to a standard operation procedure. The procedure was first tested by RIKILT with the selected anti-casein mAbs.

In order to assure proper dissemination and exploitation RIKILT established a list of stakeholders of the European dairy industry. For this purpose a list of nearly 400 stakeholders was compiled based on data available from internet, through the European Dairy Association as well as their National members.

As alternative the list of approved (section 9) establishments of the EU (http://ec.europa.eu/food/food/biosafety/establishments/list_en.htm) was used. This source contained an excessive number of addresses, thereby often without stating the corresponding postal address. It also did not contain an electronic address. This possible approach was put on hold

A personal approach of National Dairy Associations was then proposed and agreed upon. TTZ approached the Polish, French and German Associations. RINY approached the Spanish Association, MGLANG a regional German registered co-operative and RIKILT approached the UK, Dutch and Belgian Association respectively with the request to circulate the questionnaire among their members.

A questionnaire towards contamination and resulting desired analytical possibilities had been established by RIKILT in corporation with TTZ and NORAYBIO. The questionnaire existed of three different parts. Afterwards the questionnaire was tested by RINY and MGLAN. The time necessary to complete the questionnaire was considered too time consuming as it exceeds 2 hours. Thus a simplified questionnaire was condensed from the first one. The questionnaire and accompanying letter were established in English language and translated into German, Polish, French, Spanish, and Italian language afterwards. Evaluation of the questionnaire indicated that food safety to be the major issue, compromising both microbiologically and chemically safety. Quality characteristics show somewhat more variability. Chemical characteristics related to health like vitamins, minerals etc. are judged as less or even not essential. Evaluation towards preferences for model matrices revealed pasteurized milk as well as to some extend yoghurt is of great interest.

Furthermore dairy Associations from Belgium (BCZ-CBL), France (CNIEL), Germany (Milchindustrie-Verband EV, Genossenschaftsverband e.V) Netherlands (NZO) Poland (Krajowy Związek Spółdzielni Mleczarskich) and Spain (Federacion Nacional de Industrias Lacteas) were approached for support. The different federations represented in total more than 700 companies. The federations circulated the questionnaires among their members. In addition the Dutch federation invited the project members to discuss the project in a meeting with representatives of their members. The results of this meeting were added the analysis.

Development of the biological detection systems

After setting up the technical framework, the underlying detection principles for pathogens, mycotoxins, drug residues, and for fraud were established. All detection principles are based on biological assays. The basic step for all assays was the production of reliable antibodies. Thereby the production of antibodies was performed in three steps. First one was to culture the cells. This procedure took up to 4 weeks depending on the antibody. Afterwards the cell culture supernatant was clarified and concentrated. Finally the antibodies were purified. For instance BIOCULT produced and purified three different test samples of AFM1 antibodies. The preferred test sample for AFM1 was produced and needed to be re-purified. Furthermore BIOCULT produced and purified monoclonal antibodies against bovine k-casein and KLH-dihydrostreptomycin. Also antibodies against sulphonamide and gentamicin, both being antibiotics were produced.

Development of the pathogen detection unit system

For the detection of pathogens, using *Listeria monocytogenes* as reference, a sandwich ELISA was chosen. The general principle with reference to the project is shown in the figure below.

Figure 1: Overview on the sandwich ELISA principle used to detect pathogens

To ensure a most efficient enrichment of pathogens in raw milk MCB produced several different microfluidic structures that were tested in □first flow through□ experiments towards their suitability. Within these experiments three different types of structures were compared: i) column shaped structures ii) meander shaped channel structures and iii) channels with columns in between. Column shaped structures showed best performance, as for instance fatty components, among others present in milk, did not clog the structure. BIO CULT assessed the market for commercially available antibody-producing cell lines for anti-*Listeria monocytogenes* antibodies and TTZ checked several commercially available antibodies towards their suitability for the envisaged purpose. The theoretical design for detecting *Listeria monocytogenes* was transferred to the 96-well microtiter plate. The selection of suitable antibodies was comprehensive. Most commercial antibodies showed various cross reactions or had a poor affinity to their antigen. An extensive literature review was performed in order to identify high sensitive *L. monocytogenes* specific antibodies. This review revealed that there is only one antibody clone out of thirty: the mab 2b3 from Stephen Hearty et al. at Dublin City University in Ireland. Other antibodies were described to cause cross reactions with certain other bacteria. The use of mab2b3 antibodies minimized the cross reactions, but was finally not sensitive enough for the NANODETCT device.

Figure 2: Determination of the detection limit in a catching ELISA of *L. monocytogenes* 20600 with mab 2b3 and Vibrand® DyeCycle™.

The complexity of microbiological detection e.g. *L. monocytogenes* in continuous-flow systems, especially the indication of presence and no detection of past encounter, hindered a sensitive detection.

Development of the mycotoxin detection unit (TTZ)

The theoretical design for detecting aflatoxin M1 was also transferred to the 96-well microtiter plate (MTP) format. First experiments were performed in an ELISA format; later on all improvements were performed as fluorescence immunoassay (FIA). An overview on the FIA developed for detection of mycotoxins is shown in the figure below.

Figure 3: Schematic model of AFM1 FIA

The anti-AFM1 antibody of clone 5A11 provided by BIO CULT was considered as satisfying. After determining the appropriate antibody and labelling procedure with Phycoerythrin, the final antibody concentration was established. Several experiments were performed to analyse matrix effects and influences of temperature modelling. The sensitivity of the direct inhibitory fluorescence immunoassay (FIA) was the crucial parameter. To meet the envisaged sensitivity in range of the defined aflatoxin M1 level of 0.05 µg/ kg [European Commission, 2010], the developed FIA needed to be optimised. First, the common procedure of FIA was repeated. The incubation conditions were set as described in the prior section for pathogen detection.

Figure 4: Dependence of B/B₀ [%] on concentration of AFM1 in PBS [µg/l] in logarithmic scale: Direct FIA with single sample volume and incubation for 1 h at RT carried out in microtiter plate. Measurement was performed in microplate reader with $\lambda_{exc.}$ = 530 nm and $\lambda_{em.}$ = 590 nm. The error bars were based on the relative standard deviations (RSD).

The outcome of the curve fitting was a LOD of 0.009 µg/l AFM1 in PBS and an IC₅₀ value of 0.97 µg/l AFM1 in PBS. The linear working range varied from 0.17 to 3.17 µg/l AFM1 in PBS. The curve was adapted by reciprocal modelling. The result showed the critical limit of AFM1 in the developed assay did not reach the required sensitivity. To increase sensitivity of the inhibitory fluorescence immunoassay (FIA) in the 96-well format, the sample volume was enlarged. Due to the fact that the volumetric capacity of the FluoTrack[®] MTP-wells is limited, an increase of volume was reached by modification of incubation time and sample application. Finally, 100 µl of AFM1 contaminated sample blended with R-Phycoerythrin labelled anti- AFM1 antibodies was transferred in each well and incubated for 1 h at RT while shaking. After 20 min the incubation was interrupted, the sample was replaced by another 100 µl as described above and incubation was continued. This procedure was repeated after 40 min incubation time. Simultaneously, the assay was performed without volume increase to enable for comparison.

Figure 5: Dependence of B/B₀ [%] on concentration of AFM1 in PBS [µg/l] in logarithmic scale: Direct Fluorescence Immunoassay in microtiter plate: (?) single sample volume and incubation for 1 h at RT; (?) threefold sample volume, each with 20 min incubation time at RT. Measurement was performed in microplate reader with $\lambda_{exc.}$ = 530 nm and $\lambda_{em.}$ = 590 nm. The error bars were based on RSD.

An IC₅₀ value of 2.06 µg/l AFM1 in PBS and a linear working range from 0.20 to 7.90 µg/l AFM1 in PBS was calculated for a single sample volume. However, an IC₅₀ value of 0.13 µg/l AFM1 in PBS and a linear working range from 0.007 to 2.92 µg/l AFM1 in PBS resulted from the volume increase. The curve fitting for the single sample evaluation used the Harris model. The MMF model was appropriate for the enhanced sample volume. The linear working range was increased to work within the envisaged limits. After the assay performance in PBS buffer the FIA was performed using TT milk. Quality characteristics of TT milk comply with those of raw milk. Furthermore, TT milk was permitted to be marketed in opposition to raw milk [European commission 2004]. Compared to fresh drinking milk, neither TT milk nor raw milk was homogenized. The promising results of direct fluorescence immunoassays with the increased sample volume may enable for the conclusion that the envisaged sensitivity will be achieved by use of TT milk. Therefore, TT milk was used as matrix.

Figure 6: Dependence of B/B₀ [%] on concentration of AFM1 in TT milk [µg/l] in logarithmic scale: Direct FIA with threefold sample volume and incubation for 1 h at RT carried out in microtiter plate. Measurement was performed in microplate reader with $\lambda_{exc.}$ = 530 nm and $\lambda_{em.}$ = 590 nm. The error bars were based on the relative standard deviations (RSD).

For assay performance with TT milk by means of threefold sample volume the outcome of the curve fitting was a LOD of 0.003, an IC₅₀ value of 2.30 and a linear working range of 0.05 to 8.25 µg/l AFM1 in TT milk.

The relative standard deviations were slightly increased due to the inhomogeneous properties of TT milk, but the deviations remained in acceptable limits. Although, sensitivity was generally sufficient, the results of the FIA with TT milk were less sensitive than those retrieved using whole milk. Hence, optimal assay

conditions were ascertained and the required sensitivity increased. After optimization of the MTP format, the most critical parameters (antibody concentration and coating conditions) were adapted to the SU-8 surface on the wafers to the microfluidic system. The introductive test series using the microfluidic devices were undertaken to find proper flow velocities through the channels and to functionalize the SU-8 surface since the highest signal obtained was too weak. Several reagents and solutions were tested to activate the epoxy based surface. The solution of cerium(IV) ammonium nitrate (CAN) and acetic acid showed the best results for increasing the fluorescence intensity, which was examined via fluorescence microscopy, measured via laser detection and evaluated by OPTOTEK.

After successful performance and evaluation of the FIA with increase sample volume in microtiter plates the method was adopted to the microfluidic device. Within previous performed assays the chips were loaded with samples containing free AFM1 and R-PE labelled anti-aflatoxin antibodies. Afterwards incubation was carried out for 60 minutes at room temperature. For increased sample volume, the incubation was accomplished with a flow rate of 10 $\mu\text{l}/\text{min}$ (0.05 rpm) for 60 minutes. The final FIA was performed in the microfluidic device as follows. The required number of chips was placed in a petri dish and inlets were marked by coloured dots.

Figure 7: Chip (IMSAS University of Bremen, Bremen) with SU-8 microchannel (diameter: 1 mm)

The SU-8 surface needed to be functionalized. Therefore cerium(IV) ammonium nitrate solution (10% CAN, 4% Acetic acid) was prepared and transferred into the microfluidic device. Incubation was carried out for 60 minutes at room temperature in the absence of light. CAN solution was removed by dint of a pipette. Following the chips were washed with deionized water and dried carefully by air flow. Coating solution containing 2 $\mu\text{g}/\text{ml}$ AFM1-BSA was applied and incubated overnight at 4°C, when chips were surrounded by water soaked tissues and covered with aluminium foil to avoid dehydration. For the blanks blocking buffer was used. Following the coating procedure AFM1-BSA conjugate was removed and blocking buffer (2% BSA in PBS) was added. Blocking was conducted for 2 hours at room temperature. During the blocking step AFM1 standards in range of 100 ppb to 0.01 ppb and 20 $\mu\text{g}/\text{ml}$ anti-AFM1 antibody solution were prepared. All samples containing labelled antibodies had to be stored in a dark place prior to use. Blocking buffer was removed and the chips were placed in a Teflon® block for washing procedure. The Teflon® block was covered with aluminium foil and plastic slides. Ismaprenen tubes connected the peristaltic pump with the microfluidic device. Each chip was washed with TBST buffer. Subsequently, the standards were mixed with antibody solution. For blank and standard of zero value anti-AFM1 antibody solution was diluted to concentrations of 10 $\mu\text{g}/\text{ml}$. The chips were loaded with appropriate samples by dint of extra tubes. Incubation was carried out for 60 minutes.

Figure 8: Experimental setup of a 4 channel microfluidic device containing 4 chips: tube ends were dived into the sample or washing buffer, whereas the other end was connected with the Teflon-block. Via peristaltic pump, the fluid was pumped through the channels. The outflow was collected in a waste vessel.

Following chips were washed as previously described. The Teflon® block was opened and the chips were taken out. Remaining washing buffer was removed by a slight air flow. The fluorescence was examined and documented using a fluorescence microscope. Finally, chips and fluorescence intensity were analysed by OPTOTEK by use of their optical detector. The following results were observed with the fluorescence microscope.

Figure 9: Fluorescence-microscopic image of fluorescence immunoassay in microfluidic device with columns. Medium: PBS, maximum signal (0 ppb AFM1), incubation: 1 h, RT, without flow

Figure 10: Fluorescence-microscopic image of fluorescence immunoassay in microfluidic device with columns. Medium: PBS, maximum signal (0 ppb AFM1), incubation: 1 h, RT, with continuous flow

The hemicycles, visible on the right and left side of the image, represented inlet and outlet in direction of flow. The bright spots in the middle of the microfluidic channel were generated by the high auto-fluorescence of the columns. The fluorescence signal was increased by the continuous flow as proved in this task. Accordingly, further fluorescence immunoassays in microfluidic device were performed with the continuous flow.

The chip design was modified retroactively, because the images and measurements of fluorescence signals identified a significant inhomogeneity. The improved design of the microfluidic devices was characterized by the absence of columns. Consequently, the homogeneity of fluorescence signals was increased.

Figure 11: Fluorescence-microscopic images of fluorescence immunoassays in microfluidic device with columns (first image) as well as without columns (second image). The medium was PBS buffer; incubation of sample with 0 ppb AFM1 in PBS was carried out with continuous flow.

The hemicycles, visible on the right and left hand side of the images, represented inlet and outlet in direction of flow. The bright spots in the middle of the microfluidic channel with columns were generated by the high autofluorescence of the columns.

The fluorescence intensity of microfluidic devices with columns was a little brighter than the fluorescence intensity of those without columns. In contrast, the homogeneity of the observed fluorescence signals was more obvious in case of microfluidic device without columns. The microfluidic devices with columns possessed round structures of high fluorescence around the columns; otherwise, there were definite non-fluorescent areas between. Fluorescence microscopic examinations gave a significant review about the intensity and distribution of fluorescence within the microfluidic channel, but no quantitative determination. Therefore, microfluidic devices were evaluated by OPTOTEK with laser excitation.

Within one performance of the fluorescence immunoassay in microfluidic devices only four of these chips could be used simultaneously because of the limitation of the Teflon holder. Four concentrations of free AFM1 had to be chosen, hence, fluorescence signals were not normalized. At first the FIA was performed in microfluidic devices with columns.

Figure 12: Dependence of fluorescence signal [arbitrary unit] on concentration of AFM1 in milk [$\mu\text{g/l}$] in logarithmic scale: Direct fluorescence immunoassay in microfluidic device with columns: (?) incubation with continuous flow. Measurement was performed by laser excitation. The error bars were based on SD.

The scale of figures was adjusted to the properties of measurement (broken y-axis). The blank value (which is not displayed in figure) pointed out the high auto-fluorescence of the microfluidic devices with columns. The displayed data demonstrated sensitivity in range of the critical limit, but the increased standard deviation ascertained the expected inhomogeneity of the fluorescence signal. After assay

performance in the microfluidic device with columns, those without columns were used. The assay conditions were set as before.

Figure 13: Dependence of fluorescence signal [arbitrary unit] on concentration of AFM1 in milk [$\mu\text{g/l}$] in logarithmic scale: Direct fluorescence immunoassay in microfluidic device without columns: (?) incubation with continuous flow. Measurement was performed by laser excitation. The error bars were based on SD.

For better examination of displayed data the scale of figures was adjusted to the properties of measurement (broken y-axis). Microfluidic devices without columns had less auto-fluorescence than those with columns. In spite of decreasing fluorescence intensity, assay sensitivity was taken for granted.

In contrast to chips with columns, the homogeneity of the fluorescence signal was increased by use of microfluidic devices without columns. The average standard deviations of microfluidic devices without columns were about 9% less than those with columns.

Therefore, the design of microfluidic devices without columns was appropriate for the developed fluorescence immunoassays and was implemented for further application.

Development of the drug residue detection unit

The following three monoclonal antibodies (Mabs)-producing hybridoma cell lines were selected and transferred to BIOCULT, who carried out the large scale production of the antibodies ($> 10 \text{ mg}$):, anti-gentamicin and two anti-sulfonamide derivatives. For the drug detection unit Luminex100 flow cytometer in combination with the Multi Analyte Profiling (xMAP) technology was used in lab scale. This system was closest to the theoretical design of the NANODETECT system. The xMAP technology used small carboxylated polystyrene microspheres ($5.6 \text{ }\mu\text{m}$ beads), which were internally dyed with a red and an infrared fluorophore. By varying the ratio of the two fluorophores, up to 100 different color-coded bead sets could be distinguished, and each bead set could be coupled to a different biological probe. In combination with flow cytometry, it was possible to simultaneously measure up to 100 different biomolecular interactions in a single well. The carboxylated bead surface allowed simple chemical coupling of capture reagents such as antibodies or drug-protein conjugates. The binding of PE-labelled antibodies to the beads was measured by the fluorescence intensities. Protein-drug conjugates were prepared for coupling to the Luminex beads, and later on to the NANODETECT sensor surface, and for the detection, a second antibody (goat anti-mouse (GAM)) labelled with PE was used. Gentamicin was detected at the MRL using ten times diluted milk samples (figure).

Figure 14: Calibration curves of gentamicin in the Luminex100 flow cytometer in the inhibition assay format using gentamicin-BSA coated magnetic beads and PE for the detection. Calibration curves were prepared in buffer and in 10 times diluted milk samples.

Following the successful assay performance in lab scale the detection of drug residues was transferred to the microfluidic devices.

Development of the fraud detection unit

The fraud assay was developed in the Luminex® format as well. Inhibition and sandwich immunoassay were developed for the detection of cows' milk in goats' milk. The assays were based on two monoclonal antibodies tailor developed against bovine κ -casein. Applying 100x diluted goat's milk, the measurement ranged for the inhibition and sandwich immunoassay formats between 0.1 and 1 % cows' milk and both formats were used to transfer the application to the NANODETECT device. Initial experiments were also performed with paramagnetic nanoparticles. At first, SU-8 slides were tested, therefore SU-8 coated PMMA and glass slides were rinsed in ethanol for 5 minutes and air dried. A serial dilution of κ -casein, from 200 to 1.56 $\mu\text{g/ml}$, was prepared. The slides were marked on the back side with 2-3 mm circles.

The slides were incubated for one hour at RT. During this step the samples dried in on the slides. The slides were rinsed with water (MQ) and then transferred to a petridish. For blocking, PBS/0.2 % BSA was added and further incubated for another hour at RT. Afterwards the slide was washed with MQ and dried using Whatman paper. Antibody/R-PE conjugate was added to PBS. From this solution 10 μl were added to each previous spot (1 to 8). The slides were again incubated for 1 hour at RT. Then they were washed with water and stored in the dark until measurement with the PerkinElmer Microarray scanner.

Figure 15: Scanner results with κ -casein-coated Glass and PMMA slides

The binding of antibody/R-PE conjugate to the κ -casein spots on the chip, was obvious. However, background signals were present around the initial spots. This might be due to the fact that the slides were dried using a Whatman® paper which maybe damages the SU-8 layer somehow. Another cause might be that the blocking was not sufficient or inefficient. The experimental setup was as described in the previous experiment with a few modifications. The slides with 5 μl κ -casein spots were incubated in a closed petridish, so samples could not dry up. Following 2 μl of antibody/R-PE conjugate was added to 600 μl of PBS. Slides were washed with PBS-Tween. Afterwards slides were air dried with pressured air after each step in the protocol. Due to the fragility of the glass slides PMMA slides were mainly used. After these experiments, fraud was successfully detected with the sandwich immunoassay in diluted milk. The PMMA-SU-8 slide was washed with ethanol and air dried. 60 μl of antibody was added to 940 μl of PBS and 10 μl of this solution was spotted on the SU-8 surface by pipette. The slide was placed in a closed dark chamber and incubated for 1 hour at RT. After incubation, the slide was placed in 1 % PBSAT and blocked for 1 hour. The PBSAT was discarded and the slide was washed in PBST for 5 minutes. The slide was washed with water and air dried. To each spot 20 μl of diluted milk samples (mixtures of goats' and cows' milk, same as in experiment 5) were added. Milk samples were diluted 10, 100 and 500 times. The slide was placed in a closed dark chamber incubated for 1 hour at RT. After the incubation the slide was washed with water, placed in PBST and washed for 30 minutes. The slide was washed with water and the air dried. Secondary antibody/Alexa 532 (10 μl in 1 ml of PBS) was added on top of the slide covering all the spots. The slide was washed with water and with PBST for 15 minutes. The slide was washed with water, air dried and scanned.

Figure 16: Scanner results (using 2 heat maps) of the sandwich immunoassay format using diluted goats' milk samples (10, 100 and 500x) with different concentration of cows' milk (0 (spot 1), 1 (spot 2), 2 (spot 3), 5 (spot 4), 10 (spot 5), 25 (spot 6), 50 (spot 7) and 100 % (spot 8))

The inhibition and the sandwich immunoassays for the fraud unit worked on the SU-8 slides and were ready to be transferred to the NANODETECT device.

In the meantime, experiments were performed to include paramagnetic nanobeads into the project and into a device with curved microchannels. The results were promising, but the procedure was only applicable with an additional commitment of resources.

Therefore, the previous developed fraud assay was performed in microfluidic devices. The microfluidic devices were placed in the Teflon holder and washed with ethanol in the serial setting for 10 minutes. The chips were then washed with water for 10 minutes in serial setting and after that another 10 minutes with PBS. 100 μ l of κ -casein antibody was added to 1900 μ l of PBS and 1.7 ml was introduced into the serial fluidic device setting. Then the inlet and outlet were connected to make the serial system circular and the solution was pumped around overnight at room temperature. The serial setting was made parallel again and the device was washed and blocked using PBST-BSA for 60 minutes. The buffer was switched to PBST and the slides were washed for 10 minutes. With the connections in parallel, the different samples were introduced in each slide. The tubing was then made circular and the samples were pumped in circular flow for 2 hours. The samples were then drained from the slides and the system was washed in parallel with PBST still in parallel setting for 15 minutes. The system was made serial again and washed with PBS for 5 minutes. Secondary antibody/Alexa® 532 conjugate (20 μ l) was added to 1980 μ l of PBS. This was introduced in the system (1.7 ml) and pumped in circular flow for 1 hour. The non-bound label was drained from the slides and the system was washed in parallel setting with PBST in serial setting for 15 minutes. The system was washed with PBS for 10 minutes. The system was washed with double distilled water for 10 minutes. The slides were taken out of the device and the remaining double distilled water was removed from the slides with pressured air. The slides were ready for measurement and send to OPTOTEK. Laser excitation spot of 2mm in diameter was used to ensure no side walls of the channels were interfering with the measurement. Every microchannel was measured five times, repositioning it manually after every measurement (black solid squares). Red empty circles represent average of these measurements.

Figure 17: Dose-response curve of the fraud unit as obtained at OPTOTEK.

The individual cow/goat milk concentrations points were well defined and comfortably detected. \square White spot \square , however, could cause problems and preventing it improved the results even further. Fraud of 2 % or more cow milk in goat milk was detected confidently, thus the measurements were successful. Later, the protocol of the FIA to detect cow milk in goat milk (also for drug and AFM1) was adjusted to the requirements of the NANODETECT prototype. This will be described in subsequent tasks.

Construction of the NANODETECT system

During the last year of the project, the prototype has been modified in order to facilitate the measurement of the extremely low fluorescence signal emitted by the bio-molecules. The NANODETECT prototype has been modified according to the experience made by using the first version of the prototype. This modification includes the implementation of new peripheral devices such as a stepper motor (linear stage), bubble traps and reformation of the fluid connection. Furthermore, the embedded software has

been adjusted according to the final design including two important new phases in the prototype operation. The final prototype has been successfully integrated and also optimized. Several experiments have been performed to identify the presence or absence of specific substances in raw milk.

Final Design of the Microfluidic Devices

As previously described several materials were examined as possible materials for the fabrication of the microfluidic devices. Properties such as biocompatibility, uniformity and optical properties including their auto fluorescence in required wavelength were precisely investigated. After this intensive research, PMMA GS was selected as the substrate material for the fabrication of the microfluidic chips, because it showed the lowest background fluorescence and also, because it showed best surface homogeneity compared to other polymer materials examined in this project.

The material for the microstructures was mainly selected according to specific feature of enabling the immobilization of biomolecules onto its surface. According to experimental results, the photopolymer SU-8 fulfilled required conditions and offered also enormous benefits for the fabrication of the microstructures. Besides the biocompatibility of SU-8, it can be used as adhesive layer and in addition to that, this photopolymer exhibited high chemical resistance that maintains its characteristics, even when it is subject to high temperatures and when it has direct contact with strong chemical substances. The significant disadvantage of SU-8 is its auto fluorescence at the emission wavelength. Hence, additional materials were tested in order to find another material with similar chemical properties. However the photoresist SU-8 was the best suitable material to enable the immobilization of biomolecules on its surface. Moreover, this material showed excellent chemical properties to fabricate structures with a high aspect ratio, which is mandatory for the final design of the fluidic system.

The fabrication process was based mainly on a standard photolithography process; in which on the top wafer were defined the microfluidic channel structures using SU-8 photopolymer and the bottom substrate was used to define the inlet and outlet of each microfluidic device. SU-8 2005 was used as adhesive laser, whereas SU-8 3050 was used to generate the microstructures.

Once, top and bottom wafer were processed individually, bonding process took place integrating in this manner, the microfluidic device as represented in the following figure.

Figure 18: Fabrication process using SU-8 and PMMA.

In the course of the project, MCB has fabricated approximately 300 microfluidic chips with different diameter as scanning window. Microfluidic devices with 3mm of diameter were selected to be used in the final prototype. This chip geometry was selected, because the flow profile of a fluid passing this channel-shape showed the best homogeny, thus ensuring an uniform distribution of the biomolecules along the whole channel. It was also experimental demonstrated qualitatively by using a standard light microscope and qualitatively by using the optical detector developed by OPTOTEK and implemented in the prototype. Chips with 3mm of diameter were the best suited microfluidic systems for the enrichment of biomolecules. Figure 18 shows fabricated PMMA microfluidic devices with dimensions of 14 x 8 x 4 mm (length, width, height). Fabricated systems were sent to the project partner TTZ and RIKILT for testing.

Figure19: Fabricated PMMA microfluidic devices with dimensions of 14 x 8 x 4 mm (length, width, height)

Optical Detection System

In process of realization of the detection system there some challenges to overcome primarily connected with extremely low fluorescence signal of measured samples. A very important part of that process was closely following the development of the assays, measuring samples provided by the project partners on every stage of progress and gradually adjusting the detection system according to freshly obtained data.

The most straight forward method to improve measuring of low fluorescence signal, provided that the sensitivity is not a problem, was to lower the background to absolute minimum. □Background□ in this example is a group name for multiple fluorescence signals with many different sources that spoil successful sample detection or at least diminish detector dynamic span. The easiest and most obvious precaution was to prevent surrounding light from entering the detector by installing suitable sealings on each problematic junction of the detector. In the final prototype the light shielding is twofold. Apart from the light sealing of all the components, detector and other light emitting electronics, inside the casing also the casing itself is light sealed.

Another unwanted fluorescence source from within the detector was loose excitation laser rays that excite auto-fluorescence of inner detector walls. Solution to this problem was a carefully designed optical system that guides laser light accurately to the sample excitation spot, disposes excess light and guides fluorescence light of the measured sample to the PMT detector. Good results were achieved in that area by installing dedicated filter set, designing optical system, appropriately shaping laser polarization and implementing laser source fluctuation compensation system.

The part of the background that was most difficult to get rid of was undoubtedly the fluorescence of the optical components and microfluidic substrates itself. Especially in the case of finding suitable material for microfluidic chips it was a long way with close collaboration with all project partners.

Measurements and results

The autofluorescence values of PMMA+SU8 and PMMA+UC6772+SU8 type microchannels were somewhat unexpected. Fluorescence was the same in both microchannel types despite that the same type wafers□ autofluorescence differed a lot. For the comparison also the middle area of very old microchannels with obsolete design were measured.

There was no apparent bleaching. All PMMA wafers were 2mm thick. Background signal was not subtracted. The measurements were done with excitation laser ray of 3mm in diameter. Therefore, because also highly fluorescent edges were excited, 2mm microchannels showed higher signal. Edges of two microchannels were also measured and the signal was very high as expected. Microchannels with the lowest autofluorescence were PMMA+UC6772+SU8 type. When optical and biological advantages were summarized, PMMA+SU8 type chips were chosen as final design.

Figure 20: Comparison of various materials fluorescence

To optimise the optical filters, spectral transmissivity of the dichroic and emission bandpass filters has been measured to get insight into possible incompatibilities with the detector guiding optics design and assess dichroic filter transmissivity properties as dependence of angle of incidence. This study also served as fundament when deciding about technology of assembly of final NANODETECT detector.

Every filter's transmissivity (except laser clean-up filter) was measured with three angles of incident light: normal and 5 degrees offset in both directions. As expected, dichroic filter was most problematic because its properties vary the most. In extreme case it started to leak (its transmission curve spreaded over laser source λ 532nm). All filters were relative non-problematic for assembly and their characteristics were relatively position independent except the dichroic, which had to be aligned very carefully.

Figure 21: Spectral transmittance of used filters compared to excitation and emission specter of R-Phycoerythrin. Laser wavelength is 532nm. λ Central position λ with bandpass filter means 0 degrees incidence angle but with dichroic filter it means 45 degrees incidence angle.

The dependence of fluorescence signal to laser attenuation had to be identified and its use to improve the final prototype optical detector accuracy was assessed.

Highly fluorescent R-Phycoerythrin wafer was used as measured sample. The fluorescence signal was measured with many different laser attenuations. The result showed that dependence is perfectly linear over all laser power range. Therefore, it was demonstrated that improving the detector accuracy by recording laser beam intensity fluctuations and compensating in post processing was possible.

Figure 22: Fluorescence signal to laser attenuation dependence. Abscise is logarithmic.

Fluorescence detector - validation of background signal

The background signal measured by the fluorescence detector had to be validated. A low background was essential for proper detector operation. Because low background was easily disturbed by improper slide positioning, it was important to measure necessary positioning accuracy. It also has to be shown that background was uniform inside the error boundaries.

Figure 23: 2D fluorescent signal strength scan in the plane perpendicular to incident laser beam in empty Teflon holder (left graph) and empty PMMA slide (right graph).

The obtained results show that the background did not change if the Teflon® holder (with or without slide) was displaced in perpendicular directions, which lies in plane perpendicular to the excitation laser beam, from its central position to 0,25mm in each direction. As expected, outside that boundary the background suddenly increased.

Fluorescence detection device

The setup consisted of green (532nm) 30mW laser and appropriate filter set for PE fluorescence measurement. To lower the laser excitation power, in some measurements additional ND filters were added. The signal was measured with Photo Multiplier Tube (PMT). The current on PMT was evaluated roughly every 0.6 second and every measurement lasted for about 8 seconds.

Figure 24: Actual fluorescence detection system (upper photo) with laboratory implementation of motorized linear stage (lower part of upper photo). Scheme of the detector design is in the lower drawing.

Apart from low background, for accurate detection it was also important to use laser source with stable optical output. Because such laser sources are normally big and expensive, it was decided to use additional optical element (photodiode) to record fluctuations and compensate them later in data post processing.

Figure 25: Optical element responsible to collect data about laser optical power fluctuations in various stages of development.

The position of the PMMA slide is crucial for successful detector operation. Allowed error margin of only few tenths of a millimetre made manual manipulation of slides for end user very difficult and complicated. It was decided that this process should be automated. OPTOTEK chose Standa® motorized linear stage and designed adapter to fit MCB's Teflon holder. Fine positioning system was implemented in the adapter allowing quick and accurate alignment of linear stage in two perpendicular directions once the detector was in the NANODETECT prototype box.

Figure 26: Standa® motorized linear stage (left). Standa® motorized linear stage with integrated adapter and Teflon holder (right).

Nanodetect fluorescence detector - measurement and post processing

In order to differentiate very low fluorescent signals, the whole experimental setup, measuring equipment, measuring process and post processing of data had to comply with some special demands. The basic idea behind those processes is getting quality, stable PMT output values that sufficiently represent fluorescent signal from the measured sample with minimum exposition to excitation laser light.

The PMT is driven and read using the Data Acquisition Unit by sending/reading the commands/values over RS232 port. The PMT output is a value from 0 to 4095 (in arbitrary units), representing the measured fluorescent signal. However, the output value is not uniform and can be manipulated using two PMT parameters, Integration Time and Dead Time (units are 1/100 of millisecond). When the reading sequence is started, PMT collects the fluorescent signal for amount of time specified in Integration Time, waits for another amount of time specified in Dead Time and then it returns the single value. During measuring of fluorescent sample the reading sequence is repeated with constant Integration Time and Dead Time parameters for 10-15 seconds. All PMT values are recorded and post processed. Single value and its uncertainty (error), representing the final result of the measured sample is obtained using statistics on these values (average and standard deviation).

For the sake of simplicity, the values of Integration Time and Dead Time parameters are changed in a way that their sum is always 0,5s. It is important to understand that connection between Integration Time and output PMT value is linear.

To minimize the PMT output fluctuations Integration Time and Dead Time parameters were used. It shows that the fluctuations of the output PMT value are getting smaller when approaching the upper limit. To get accurate results (i.e. small fluctuations), the samples should be measured in that regime and the Integration Time and Dead Time parameters should be set properly. Because the measured samples can have very different fluorescence, setting of Integration Time and Dead Time parameters have to be done for every new measured sample individually. That's why every measurement has two stages. In the first stage, sample fluorescence is roughly evaluated using some default Integration Time and Dead Time parameters values – low enough not to damage the PMT and high enough to get some measurable signal. Based on known default Integration Time parameter and rough evaluation of PMT output value, the new Integration Time parameter value is computed and used in second stage where the real measurement takes place. The new Integration Time parameter value is written in output file's header. Later it is used in post processing to correct PMT values to some universal constant Integration Time parameter value that is the same for all sets of data and insures direct comparability of all measured values.

In parallel with fluorescence measurement, also excitation laser intensity measurement is measured with the photodiode that is driven through the second RS232 port. For every PMT output value, also value between 0 and 1023 is stored, representing the excitation laser intensity in arbitrary units.

Also excitation laser light intensity readings were used to minimize PMT output fluctuations. So the other very important part of ensuring low errors of measurement results is stabilizing the excitation laser. To make it indirectly (optically) it is very complicated and expensive, therefore it was decided to implement it in post processing based on laser intensity measurement that is done in parallel with fluorescence measurement. Again, the basic assumption is the linear dependence between laser excitation light intensity and PMT output value. In post processing, the PMT output values are corrected to a fixed excitation laser light intensity which is the same for all measurements and for all the samples. Hence, this ensures the numerical compensation for laser fluctuation during the measurement (lower final value error) and the direct comparability of all PMT output values from different measured samples. Altogether, both types of corrections are independent from each other and can be applied in arbitrary order. When applied, averaging and standard deviation computation should be carried out to get the representative and directly comparable values of measured samples. It had to be considered that after applying those corrections also – universal constant Integration Time parameter value – and – universal constant excitation laser light intensity – are part of the result. Thus, the result is relative to those two parameters.

The NANODETECT Prototype

By using the first design of the NANODETECT prototype, the initial results were not promising. Consequently, a step-by-step evaluation has been performed to determine the necessary improvements. Based on the experience made by using the initial design, the prototype has been optimized. After performing required optimization, the basic operation of the prototype was also modified and

implemented in the embedded software. The prototype operation and representation of the state machine of embedded software are also presented in this section.

Based on the experience made with the first prototype, a difficulty has been observed concerning the formation of air bubbles within the microfluidic chip. Some methods have been selected and implemented in the prototype to reduce the formation of these undesired air bubbles along the microfluidic channel. Bubble traps provided by Trace Analytics were implemented into the prototype. They were selected as their material composition, for instance PEEK and PTFE, are inert and also chemical resistant. These bubble traps were also chosen because they ensure no dead volume, thus avoiding the accumulation of rest of milk or PBST inside of the bubble trap. An adequate membrane of the bubble trap had to be found, which is compatible with the pressure provided by the micro pumps. After testing different types of membranes, an appropriate one was found. Bubble traps work effectively. Air bubbles could not be formed during pumping operation. However, when microfluidic devices are replaced by new ones for performing next measurements, unavoidable air enters into the fluidic connector forming air bubbles into the microfluidic channel. The risk of air bubbles can be minimized by filling the chips with fluid prior to application.

The first measurements showed that background fluorescence varied from one microfluidic device to another and this significant difference was not expected. In order to prove that the microfluidic devices have similar auto fluorescence intensities, an additional washing procedure has been introduced to the process. In addition to PBST, distilled water has also been pumped through the microfluidic chips to remove residues of unbounded particles, for instance fat and protein fractions derived from milk. After pumping water, microfluidic devices were measured again.

One difficulty observed in the first version of the prototype was the non-efficient transfer of volume sample into the microfluidic device caused by the dead volume in the fluidic connections. In order to determine the dead volume, initial volume and final volumes of each assay unit have been experimentally measured. In order to reduce the dead volume and thus improve the efficiency of the prototype, the fluidic connection had been modified. The fluidic path has been modified from a parallel connection to a serial connection. By means of a serial connection, the use of multiple connectors is not required, thus reducing considerably the dead volume in the system. In this way, all reagents flow to the microfluidic device using a serial continuous flow as depicted in the following figure. The dead volume was reduced considerably.

Figure 27: Schematic diagram of the modified fluidic connection. Liquid samples are pumped in series into the microfluidic device.

Schematic representation

The NANODETECT prototype consists basically of the optical sensor, pump system, a temperature sensor, microfluidic devices with the respective fluidic connections and a stepper motor (linear stage), which is able to move the microfluidic device in front of the optical sensor for measuring.

The prototype is also composed of an extern touch screen Panel □ PC as graphical user interface (GUI), which is able to control the detailed set-up such as access to the features and applications of the system.

The display could also provide users the visualizing of data resulting from the optical detector with an interactive interface.

Figure 28: Schematic representation of the actual NANODETECT prototype

All components of the NANODETECT prototype were housed in a robust and non-transparent ABS casing, which fulfils the most important requirement of light tightness. Original box was modified to facilitate testing of raw milk under realistic in-place conditions. The cover could be open easily by turning the wing screws for easy access by the operator to change microfluidic devices or to carry out potential necessary adjustments. In addition, the inside of the light box was painted flat black to avoid any reflections of light that could interfere with the operation of the detector.

The system power switch, USB, LAN network and RS232 connection were mounted externally for easy access for software update via RS232 or LAN and also for saving measurements via USB.

Figure 29: Photo of the optimized NANODETECT prototype

Figure 30: Inside views of the final prototype

Embedded software

Based on the concept of developing an embedded and wearable system for NANODETECT, MCB had developed a software to be implemented in the graphical user interface (GUI) based on a touch screen Panel PC. This Panel PC from taskit GmbH uses Linux as operating system. It serves among others for further development of an automated state machine. This state machine composed in eight steps, where either pumps or the optical system are running. The state machine can be paused and restarted, when the stop button is pressed during running and run on if pressed again.

Figure 31: Schematic representation of embedded software of the prototype

The DSS (Decision Support System)

NORAYBIO developed a data processing system including definition of input formats, data parsing and processing as well as a calibration process that enables the calculation of threshold values, in terms of PMTs, associated to the legal concentration limits. A testing system evaluates the samples against the calculated thresholds and a database saves all results. The DSS, developed by FERA, is fully integrated with NORAYBIO's module. It comprises 4 basic functions: data importing, calibration, comparison and reporting. It is built using Microsoft's .NET4 and ClickOnce software to make it as universally available as possible. It is available at <http://nanodetect.eu/dss>. It is easy to download and install. The following figure illustrates the landing page from where the software can be downloaded. It assumes the user already has Windows Installer 3.1 and Microsoft .NET Framework 4 Client Profile (x86 and x64), allowing the DSS to be launched immediately; but, if the user does not have this software installed, an Install button will do this for the user.

Figure 32: Landing page of DDSS

Once installed, the user moves to the homepage, illustrated in the next figure. The menu on this page gives the user the option to import data files generated by the NANODETECT device and to produce reports. The user can set and adjust limits as well as thresholds and examine data for individual farmers, over time etc.

Figure 33: Home page for the DSS and enlarged options menu for the home page

On the Import Data screen, the system is pointed to an appropriate directory and will list all the files it recognises as NANODETECT files in the central box. Data are imported in matched pairs, one data set for reference and one data set for measurement. The user highlights files and drags them into the appropriate slots on the right of the screen, allowing a measurement file and reference file for each of the three parameters which may have been sought (aflatoxin, drug or fraud). Identifying data such as farmer name, experiment number, date and description may be added by the user or are selected automatically from the files chosen.

Once the data are imported, they can be analysed against set thresholds within the database, giving an output. Calibrations based on reference data are performed in a similar manner. It is also possible from this output screen to examine the raw data and calculations performed on it to generate the result, using the tabs at the top of the screen.

Figure 34: Typical results output, showing no detection above threshold for fraud or drug contamination but an exceedance of legal limit for Aflatoxin M1.

Reporting options are comprehensive. The next figure shows the summary results over a time period, which can be set, for a particular farmer (in this case simply called □Laboratory Sample□). Though few results are shown, clearly if readings had been taken at regular intervals, daily or weekly for example, a long run of results could be illustrated showing the levels of contamination for each analyte over time.

Figure 35: Results summary over fixed time period for a given farmer

The system fulfils all the requirements defined in the beginning of the project. It takes output from the raw data files obtained from the optical detection unit and transforms it to give a qualitative answer to the user. Output levels are compared against a database of known or acceptable values for a given contaminant/matrix combination and a number of output options are then available. The DSS permits warnings/alerts to be issued if levels fall outside acceptable bounds. The DSS also acts as the on-line recording system, providing time line through flow evidence of monitoring, due diligence and background levels of noise and contamination.

Test and implementation of the NANODETECT biosensor

The objective of this work package was the demonstration of functionality of the prototype under practical conditions. This has been done by comprehensive trials with the working prototype carried out by the SMEs RINY and MGLANG in order to evaluate of the efficiency of the new method under practical conditions and compare its sensitivity and reliability with state of the art methods.

Both SMEs were trained in the prototype application by researchers from ttz and RIKILT. Technical problems which occurred during these trials were solved by MCB in cooperation with OPTOTEK

Implementation of the NANODETECT system

Even though the laboratory of MGLANG is not accredited, it is working according to the Good Laboratory Practice (GLP) and has implemented internal quality assurance procedures for their routine analysis. For the implementation of the NANODETECT system, MGLANG contributed with their requirements for the use of the NANODETECT system in their laboratory practise with a special focus on the reliability and reproducibility of the results in the frame of their internal of quality assurance programme.

Validation of the NANODETECT system for routine analysis

The NANODETECT prototype was already tested by the beneficiaries MCB, TTZ and RIKILT, but until now no experiments with raw milk under realistic conditions have been before the validation trials by RINY. Raw milk is not purchasable for costumers because of the regulation by law. Previous tests were performed by use of powdered milk, milk standards or pasteurized milk to ensure the constant composition of media. Task 5.2 required use of raw milk to adapt this task to the routine diagnostic of the end user.

In this work raw milk samples from different herds in Spain were tested. For executing the validation procedure, milk samples were contaminated with different concentrations of mycotoxins and blending substances and analysed in parallel with commercially available technologies and with the NANODETECT device. Before the validation, all samples were analyzed in RINYA for D° Acidity, pH, drug fast test (Drug Fast T.), fat (F), protein (P), dry matter (D.M.), somatic cellules (S.C.), bacteriology (BAC), drug slow test (drug slow t.) and cryoscopic point (C.P.) using standard methods.

For the validation study, the samples were contaminated with different concentrations of mycotoxins and blending substances and analyzed in parallel with commercially available technologies and the NANODETECT device. For the detection of drug LC-MS/MS method (SOP A1040) from RIKILT was used as validated method. Aflatoxin M1 was determined via cleaning by immunoaffinity chromatography and detection by high-performance-liquid chromatography (§ 64 LFGB L 01.00-76). Determination of fraud is not regulated; therefore RIKILT used the Luminex MAGPIX system.

Preparation of standard operation procedures

Standard Operation Procedures (SOPs) provide instructions for regular analytical processes in detail. They ensure the consistent performance of routine analyses and newly developed methods regarding good laboratory practise (GLP). Accordingly, SOPs retain the quality control and quality assurance of analytical processes, procedures and related results.

The following Standard Operation Procedures specify the implementation of the NANODETECT system. The detection procedure for each contaminant requires a specific description. Hence, the overall process is represented by three SOPs prepared by ttz and can be found in D21.

Comprehensive dissemination and exploitation measures were carried out by all beneficiaries during the whole course of the project. The dissemination activities are summarised in D26 while the exploitation of the project results is described in the PUDF.

Potential Impact:

The NANODETECT project has been launched to support the competitiveness of a knowledge-based European bio-economy, particularly addressing tailored biochemical processes and innovative nanodevices in smart control applications for increased levels of quality assurance, food safety and transparency of product properties.

Thus, the NANODETECT approach has been centered on the development of an innovative nanosensor for the analysis and control of quality parameters within in the food chain. The key driving context therefore lies with the urgent need for fast, robust and reliable methods to identify various types of contaminants especially in liquid foods. Due to the production processes, which require large storage capacities, extensive stapling, and the use and blending of various raw material batches, it is highly important to provide new tools to the industry that help to identify and to eliminate unsuitable material streams, which are either unfit for human consumption, high in undesirable residues or even subject to fraud, when cheaper raw materials are blended with high value products.

Given this background, the NANODETECT consortium has set its first investigatory focus on milk as an example process stream, because it is subject to various of the before-mentioned types of contamination. The quality and safety item models chosen and addressed by the project have included:

- Pathogenic microorganisms (*Listeria monocytogenes*),
- Mycotoxins (Aflatoxin M1),
- Drug residues (sulphonamides and aminoglycosides),
- Fraud (high value goat milk blended with cheaper cow milk).

The model systems investigated, the modules developed and the NANODETECT sensor prototype work on different type of immunoassays, followed by respective detection and quantification of specific contaminants. The available systems are linked to simple output signals as well as software-/IT-systems that allow the user to recognize, read and interpret results as well as to interact with the system and subsequent product steps based on the analytical results obtained during fast measurements. Given the flexible nature of the use of immunoassays, which are sensitive and specific molecular biological processes - in the case of NANODETECT applied with the potency of nanotechnology □ the systems are also suitable for other liquid process food streams or other analytical measurements where the sample can be easily and quickly diluted. Individual parts of the developed systems may even hold the capacity for transfer to other process controls, measurement environments or analytical systems which has been identified with various stakeholders in the frame of conferences and symposia during the run-time of the project. On the other hand, the NANODETECT project results have shown that next to the feasibility and functionality of the chosen approach and test systems, the individual adaptation to the industrial environment requires additional development on hard-ware components to achieve high levels of robustness in demanding online systems. This opens the opportunity for component suppliers (e.g. cooling systems, automation specialists, pumping systems and controls) to cooperate with the NANODETECT consortium in the future for new tailor-made systems for various industrial branches; e.g. in the medical/diagnostic or in the drinking water sector.

Hence, the NANODETECT results have great relevance to a number of industrial target groups and users, including:

- end-users involved in the milk sector, especially production, transportation, blending, production of milk-based liquid foods or solid foods, which require extensive quality checks of the incoming raw-material milk;
- end-users in other liquid food productions (e.g. beverages, natural extracts, liquid ingredients, chemical or cosmetic components);
- end-users in other agro-food applications (e.g. aquaculture);
- end-users producing or processing other foods and materials that are highly sensitive to pathogens, toxins, residual contaminants or fraud (foreign DNA-detection).

In fact, the project beneficiaries were approached by numerous companies from the food sector which are highly interested in quantitative mycotoxin detection in produce like nuts and grain to be realised as early warning systems for continuous raw material checks. Suchlike RTD projects will be started as soon as a dedicated call will have been identified.

The main benefit for this group is also of high economic and socio-economic impact as new analytical methods will allow them

- to optimize their logistics (time-saving release of raw-materials into the production, dispatch, sales),
- to avoid discard of large product volumes, which have been contaminated in stapling, storage or blending,
- to avoid product recalls and to increase consumer trust and safety regarding undesirable or even harmful product contamination,
- to reassure consumer trust and understanding of a safe and transparent food value chain, where fraud is especially strictly controlled and immediately identifiable.

Hence, the main targets to increase the competitiveness of agro-food SMEs, to support future avoidance of food discard which is estimated at several hundred million tons annually in Europe [based on a study by Federal Ministry of Food, Agriculture and Consumer Protection] and to safe-guard the current level of employment (estimated at more than 2.6 million people) has been reached and well prepared for the above-mentioned key users and implementable applications.

In addition, the NANODETECT system also addresses

- laboratory and analytical service providers centrally working for the food industry in the before-mentioned topics who are interested in reducing the time-span from measurement to result and in improving user-friendliness and work conditions based on versatile small-scale bio-sensors;
- component specialists for future developments;
- industrial interest groups, technology promoters and industrial consultancy services supporting above end-users who are willing to inform their clients about new state-of-the-art achievements with high implementation and strong efficiency potency.

The NANODETECT consortium itself consists of dairy SMEs, specialists in immunochemicals, biological detection systems, microoptical laser systems, evaluation software systems, academic, private and public research partners supporting the food industry, who are actively seeking contact and joint exploitation opportunities for

- pathogen detection units,
- mycotoxin detection units,
- drug residue detection units,
- fraud detection units,
- nanostructure reactors and activated surfaces,
- detection and steering software systems,
- data transfer and processing tools as well as decision support systems,
- complete NANODETECT sensor system.

This outcome has also been the consequence of a series of dedicated dissemination measures which included printed publications, online publications and active contributions to symposia, conferences and trade shows where vibrant networking with industrial stakeholders has enabled the NANODETECT consortium to strengthen their developments and their path of later exploitation. It is particularly noticeable that the NANODETECT project and consortium has achieved □ next to its scientific publications which are in the nature of a collaborative project □ a very good coverage in and high interest from online media and also broadly read magazines with biotechnology or food technology background. These media cover more frequently research on a very applied level which is believed to be closer to market; hence, high confidence in fruitful and fast exploitation and implementation has been placed in favor for the NANODETECT project.

Via its newsletter also a number of industrial associations has been reached and kept in the loop-hole regarding the new developments, in particular:

- European IAG: European Dairy Association (EDA)
- Netherlands: Nederlandse Zuivel Organisatie (NZO)
- Germany: Milchindustrie Verband e.V. (MIV), Berlin, Genossenschaftsverband e.V. (GENO)
- Belgium: Belgische Confederatie Zuivelindustrie (BCZ-CBL)
- UK: Dairy UK
- Ireland: Irish Dairy Industries Association (IDIA)
- Spain: FEDERACIÓN NACIONAL DE INDUSTRIAS LÁCTEAS (FeNIL)
- Poland: Krajowy Związek Spółdzielni Mleczarskich
- France: IDF France (International Dairy Federation) and □CNIEL□ (Centre National Interprofessionnel de l'Economie Laitière)

In summary, it can be concluded that the NANODETECT project has set a successful basis for the post-project exploitation and valorisation of its results. This can be seen extremely positive since the impact of a successful NANODETECT implementation has also socio-economic relevance beyond the before-mentioned support of industrial users, the dairy sector and agro-food branch in more general, and analytical practice in quality assurance for specific pathogen-, toxin-, residue-sensitive foods or products prone to be subject of fraud. As highlighted above, the industrial benefits of a safe and transparent food

value chain mirror back to a positive impact for European consumers who can enjoy major socio-economic benefits from a greener and more sustainable food production, including:

- a better handling of analytical chemicals and the management of residual contamination due to the superior modalities of bio-sensor technology, which is after concluded optimisation a clear, greener production advantage,
- less food wastage, which is a major driver for shortening of resources and problematic in terms of discard and discard management,
- the chance to regain trust in food production systems after a high number of recent food scandals since all major scares (pathogens, toxins, residual contaminants, fraud) can be addressed.

Finally, the NANODETECT project has □ whilst broadening own expertise by the joint and European-funded research efforts □ also dedicated and invested time in the educational training of pupils and students, who had the chance to experience the power and possibilities of bio-based knowledge and developments, as well as the scientific advancement and promotion of young scientists, who had the opportunity to finalise their academic degree and/or PhD in the course of the project.

List of Websites:

www.nanodetect.eu