Executive Summary:

The AUTOCAST project has developed a new panel of mRNA biomarkers for early detection of cervical cancer, a novel low cost automated real time PCR/probe technology, in a microarray biochip format with a corresponding automated detection instrumentation for use as a rapid "point of care" diagnostic device in both a clinical and laboratory setting.

Cervical cancer and its associated virus, Human Papilloma Virus (HPV) is the model system which was used in the current project to exploit the potential of the novel real time PCR technology, namely the Mediator Probe Assay (MPA) to detect panels of multiple biomarkers, both viral and host cell markers, within a single test.

Within the project, AUTOCAST has developed the following:

(1) A cervical mRNA expression biomarker set for sensitive and specific detection of CIN2+ lesions in cervical smear specimens which has been validated in a large cohort of colposcopy referred patients with histologically confirmed disease status. The complete clinical validation of the selected biomarkers panel was performed on standard real time PCR instrumentation and yielded excellent results. For the detection of CIN2+ cases the biomarker panel specificity (0.93) and sensitivity (0.89) were better than standard screening methods including high risk HPV DNA tests (specificity 0.85, sensitivity 0.88) and/or cytology alone with ASCUS+ cutoff (specificity 0.83, sensitivity 0.70) or with LSIL+ cutoff (specificity 0.97, sensitivity 0.54) respectively.

(2) A novel real time PCR detection technology for use in solid phase microarray formats called the mediator probe assay (MPA) has been developed and its " proof of principle" was demonstrated for both liquid and solid phase chemistries. The Mediator Probe Assay (MPA) developed within project combines the enormous multiplex capability of microarrays together with the sensitivity of real-time PCR. Additionally the assay enables the use of a universal microarray whilst any gene could be detected by changing the primers and probes of the PCR.

(3) A unique PCR cartridge was designed which includes an optical and fluidic chip, with 8 channels, each channel suitable to multiplex up to 20 gene targets. The material for each chip was selected fulfilling production, thermal, optical, chemical, biomolecule immobilization and PCR biocompatibility requirements. Chips have been produced by injection molding and several bonding and coating options have been evaluated.

A low-cost total internal reflection fluorescence and epifluorescent PCR cycler/scanner has been produced which has automatic data handling capabilities.

Partial integration of these various technologies was tested on clinical specimens using the liquid phase, MPA and PCR scanner technology and targeting the application of the developed biomarker panel.

Project Context and Objectives:

Globally cervical cancer incidence rates have increased from 378,000 (256,000-489,000) cases per year in 1980 to 454,000 (318,000-620,000) cases per year in 2010, a 0.6% annual rate of increase. Cervical cancer death rates have been decreasing but the disease still killed 200,000 (139,000-276,000) women in 2010, of whom 46,000 (33,000-64,000) were aged 15-49 years in developing countries. It is usually preceded by a long phase of pre-invasive disease called cervical intraepithelial neoplasia [CIN]. This pre-invasive phase is characterised microscopically as a range of events progressing from cellular atypia to various grades of dysplasia, including cervical intraepithelial neoplasia (CIN) before progression to invasive carcinoma. This precursor phase is generally asymptomatic, and can occur over a long period of 10– 20 years.

Human papillomavirus (HPV) is the single most important aetiological agent in the pathogenesis of cervical cancer and pre-cancer. About 15-20 types are associated with cervical cancer, of which HPV16, 18, 31, and 45 accounts for 80% of cervical cancers. HPV vaccination has now become part of cervical cancer screening and prevention strategies world wide. There are two prophylactic vaccines currently available which protect against two specific high risk HPV types 16 and 18. With the introduction of HPV vaccination, the landscape of cervical pre-cancer will change over time. While the incidence of abnormal smears and high grade disease will decrease over time, it is anticipated that in the future, the incidence of some type of low grade abnormalities may increase as a consequence. Furthermore, in vaccinated populations, the lower prevalence of disease will directly impact on the performance characteristics (i.e. positive predictive and negative predictive values) of current diagnostic tests.

There are no specific clinical features or symptoms which indicate the presence of CIN. Initial diagnosis is usually made by cytological analysis of a Pap smear specimen. Cytological analysis of PAP smears is the current method employed by cervical screening programmes for the initial diagnosis of abnormality. The result of the PAP smear is what refers the women for additional screening in colposcopy. However it is well recognised that there are limitations to the existing PAP smear screening procedures. These include a significant incidence of false positive and false negative results. Efforts to improve the specificity and sensitivity focus on triage of cytology with HPV testing, which certainly can improve the sensitivity but issues with specificity still remain. As eluded to above in HPV vaccination populations, the rate of abnormal cytology will decrease, this will mean it will be more challenging for cytotechnologists to review cervical cytology samples. It’s likely therefore that HPV testing will become the primary screening event in vaccinated populations. There remains a lot of debate over the best method for triaging women who test HPV positive into high and low risk groups. In this context biomarkers will be an important consideration.

Currently histology is the gold standard procedure in terms of disease diagnosis. However histological diagnosis of CINs is complicated by a variety of cellular changes associated with inflammation, pregnancy and/or atrophy. These changes may mimic precancerous cervical

lesions, thereby making traditional cervical histology approaches, subjective and prone to variability. This is reflected in poor inter-observer agreement between pathologists. In particular, the differential diagnosis between immature squamous metaplasia and CIN1/2, or between low-grade [CIN1] and high-grade [CIN2/3] lesions, tend to be difficult. The limitations associated with histological diagnosis, impact greatly on clinical trials in the area of cervical cancer pre-cancer biomarker validation, HPV vaccination, and novel therapies, as histology is usually the end point in terms of disease. Accurate grading of CIN lesions is paramount for clinical management of patients because CIN 1 and CIN 2-3 lesions are treated differently. Furthermore, as histological diagnosis of cervical biopsy ultimately determines the decision to treat, combined with the low positive predictive value of the smear test which initially refers women into colposcopy, often results in unnecessary treatments. The estimate of this burden has been assessed in several studies, where up to 20% of low grade lesions were upgraded and 26% of high-grade lesions were downgraded after histological review. The issues relating to accurate diagnosis of CIN can result in over- or under-treatment of the lesion. Moreover, in early CIN lesions, the progression rates to high-grade lesions are low (CIN1 and 2 to CIN3 is 9-22% and to invasive cancer is 1-5%). This effectively means that many women unlikely to progress are being over-treated and followed up unnecessarily, causing a huge burden on healthcare systems. Collectively, this emphasises the need for specific biomarkers to aid objective CIN lesion grading, to identify true high-grade cervical disease, and to increase the specificity and PPV of disease detection.

Molecular methods (especially nucleic acids based assays) are powerful diagnostic tools for rapid detection of specific gene sequences in clinical samples. However, molecular diagnostic tests in general, such as the multiplex polymerase chain reaction (PCR), DNA sequencing and microarrays, are still labour intensive, time consuming and complex investigations. To overcome these limitations and to enable simpler use of these methods for medical diagnostics, the whole process of molecular tests requires simplification and revision with miniaturization and automation presenting a great potential for the integration of molecular assays in an easy-to-use and flexible point of care diagnostic system. In this project, we have developed a novel low cost automated real time PCR/probe technology, in a microarray biochip format with corresponding automated detection instrumentation for use as a rapid “ point of care” diagnostic device in both a clinical and laboratory setting.

Cervical cancer and its associated virus, Human Papillomavirus (HPV) is the model system which has been used to develop this novel automated cancer screening technology. The uniqueness of the current project is the potential of the novel real time PCR technology to detect panels of multiple biomarkers, both viral and host cell markers, within a single test. This will impact enormously on current cervical screening programs particularly in the context of HPV vaccination. Incorporation of these biomarker panels into cervical screening programs has the potential to stratify patients based on their HPV subtype, defining different treatment cohorts, resulting in improved patient management and reduced costs to healthcare providers.

The technology described in the presented approach is based on a microfluidic disposable cartridge enabling real time polymerase chain reaction (PCR) amplification and detection. The disposable cartridge is processed by a small integrated low cost thermo-cycler and reader system which can perform the diagnostic test automatically. The diagnostic test relies on a panel of biomarkers, which are detected within the cartridge to allow for multi parameter analysis. The detection is based on the most sensitive TIRF method (TIRF = total internal reflection fluorescence) and has been modified to facilitate the the more common epifluorescent technique. By this, the sensitivity and specificity of detection can be improved and there is a potential to generate quantitative results.

The novel real time PCR approach, which incorporates a novel type of mediator probe assay, is applicable to a large variety of diagnostic markers and PCR based tests. This approach enables the use of a single generic microarray for multiple purposes. Thus the high volume fabrication of the disposable cartridge hosting the microarray can be largely facilitated and commercial uptake can be fostered. The developed technology has the potential to eliminate the bottlenecks of current immobilized real-time chemistries such as immobilised molecular beacons. The developed system and assay technology can not only be used in the medical field for cancer research and diagnostics, but will also be applicable for food safety, environmental monitoring or homeland security if a suitable biomarker panel is adopted. The applicability and performance of the developed prototype system in the AUTOCAST project has been demonstrated for detection of human papillomavirus viruses (HPV) and mRNA biomarkers in cervical cancer.

With the recent introduction of the Gardasil HPV vaccine (Sanoffi-Pasteur) in Europe, the combined use of cervical screening and a HPV prophylactic vaccination programme heralds a new era in cervical cancer prevention. The diagnostic device developed within the current project will advance and complement international cervical cancer vaccination and screening programs, through its ability to stratify patients based on their HPV and biomarkers status, in a point of care setting. As part of the project a clinical study has been performed on clinical specimens to validate the full system i.e. the new mediator probe assay for HPV detection in combination with the microfluidic cartridge technology. The novel system has been benchmarked against the gold standards currently used in the clinical laboratories.

Any new device to be used in primary screening protocols should include an aim to increase sensitivity and specificity and reduce inappropriate high referral rates from a targeted screening population. The currently available tools used in cervical screening (PAP smears, hybrid capture HPV testing etc.) in general have low positive predictive value and variable sensitivity (5). This has hindered widespread extension of HPV screening programmes.

Nevertheless, the combination of cytology and HPV testing significantly improve the accuracy of primary cervical screening. Therefore, there is a need for a sensitive and specific, high-throughput diagnostic device which will facilitate HPV and biomarker population

screening. The attractiveness of a microchip device with multiple reporter channels facilitating sub-type HPV analysis and other viral (load) and human biomarkers in a unified platform represents a major step forward in cervical cancer screening and diagnostics. Such a platform using a microarray device has the potential for development in other diagnostic systems (including other cancers), biosafety platforms and food technology.

Microarrays are in principle ideally suited as miniaturized assay platforms for such a multi parameter diagnostic device. What is required is the coupling of a multiplex real-time amplification step with detection on an array platform, heretofore not possible. This proposal attempts to develop a real-time PCR based array technology utilising novel mediator probe chemistry. The microchip will use HPV sub-type analysis and other human biomarkers as a proof of concept for this technology approach.

Main objectives

The aim of the AUTOCAST project was to develop and to validate a point of care (POC) diagnostic system for cancer screening, combining the methods of real time PCR and DNA microarrays. The complete system will comprise:

- A combined thermal cycler and optical detection system to perform PCR cycling and microarray detection simultaneously

- A microfluidic cartridge containing light guiding components to enable attenuated total reflection (ATR) and the immobilized microarray within a reaction chamber, which is amenable for PCR cycling and optical readout by TIRF

- A novel type of assay protocol (the mediator probe assay) to enable screening of any nucleic acid by a single universal/ generic microarray

- A new combinational approach for cervical cancer screening including both HPV and human gene biomarker testing

- A clinical feasibility and validation study to benchmark the performance of the new test to existing diagnostic platforms with respect to HPV diagnostics

Project Results:

Summary:

According to Annex I objectives the following were achieved:

- A combined thermal cycler and optical detection system to perform PCR cycling and microarray detection simultaneously.

An automated PCR microarray analyser has been developed within workpackage 4 of the project Autocast. The system includes an optical readout based on total internal reflection fluorescence (TIRF) and epifluorescence and a temperature control unit for PCR cycling. The whole system is controlled by electronics and software for a fully automated operation. The software has several updated version according to the project requirements and also to include several new features. Instruments were used at GenoID, Budapest and Trinity College Dublin, respectively. The third one remained in Freiburg to be used by IMTEK and Fraunhofer IPM. D4.4 deliverables and the associated tasks are completed. The Autocast real-time PCR microarray analyser BCR 603 was tested and characterised. The systems redesigned later (prototype) for the clinical validation was also characterized. The analyser' s specifications have been met according to the specification of deliverable 4.1, and it is used the characterisation methods in deliverable 4.2. D6.1, D6.2 and D6.3 deliverables and the associated tasks are completed. The D6.4 was joined with D6.2, because the delayed chip production, the reagent and performance validation of the demonstrator design was no longer feasible, so only the prototype system was validated.

- A microfluidic cartridge containing light guiding components to enable attenuated total reflection (ATR) and the immobilized microarray within a reaction chamber, which is amenable for PCR cycling and optical readout by TIRF.

For cartridge demonstrator the adequate plastic material has been determined that will withstand the operation conditions. The AutoCast Cartridge consists of the optical and the fluidic chip. The optical and fluidic chip had been delayed due to injection moulding problems, but later successfully completed. For cartridge prototype several bonding techniques were used, laser welding and adhesive bonding procedures were evaluated. The compatibility with mass production of the laser welding process makes it a very promising candidate as a final bonding technique; however it was incompatible with oxygen plasma treatment, which in turn was necessary for functional probe immobilisation of real-time PCR probes. Several contingency measures were introduced with limited success (selective coating, different non oxygen plasma dependent immobilisation methods, thermal bonding). Adhesive bonding was partly incompatible with the PCR process, and also it cannot support the production of larger numbers of chips, which are necessary for the clinical evaluation. Thermal bonding was also incompatible with oxygen plasma treatment. With delay, however, the D5.2, D5.3, D5.4, D5.5 and D5.6 deliverables and the associated tasks are completed.

- A novel type of assay protocol (the mediator probe assay) to enable screening of any nucleic acid by a single universal/ generic microarray.

The whole assay integration of the singleplex Mediator Probe Assay is presented. Five different concepts were evaluated to detect a liquid phase target gene spatially resolved on a solid support. All concepts are based on the Mediator Probe cleavage accompanied by the release of the Mediator. The different approaches comprise different enzyme classes or significantly modified oligonucleotides like multiple fluorophores or inversed internal linkage. The presented detection techniques were developed and evaluated in regard of manufacturing aspects. Regarding feasibility the concept consists of an immobilized hair-pin shaped oligonucleotide with fluorophore and quencher moieties (Universal Reporter). A prerequisite for intensive testing and optimization is the immobilization of the oligonucleotides using a thermostable coating of the chip surface (co-operation partner Austrian Institute of Technology), the sealing of the microfluidic and optical part of the cartridge by laser welding (co-operation partner Jenoptik Polymer Systems) was not fully achieved during the timeframe of the project. The low reproducibility of the results hampered our efforts to fully deliver D3.3. D3.1 is done partly and discontinued (this was a risk aversion contingency route), D3.2 deliverable and the associated tasks are completed.

- A new combinational approach for cervical cancer screening including both HPV and human gene biomarker testing.

A new expression biomarker set for cervical cancer screening has been established. The established biomarker geneset fulfils the criteria to complete the workpackage D2.3, but we had postponed the due date of the D2.3 to investigate some other alternative possibilities, and establish contingency genesets for further use. The D2.3 delivered significantly improved results. For the detection of CIN2+ cases the biomarker panel specificity [0.93 95%CI 0.90-0.96] and sensitivity [0.89 95%CI 0.86-0.91] were better than the values of HR-HPV test [specificity 0.85 95%CI 0.81-0.88, sensitivity 0.88 95%CI 0.85-0.90] or cytology alone with ASCUS+ cutoff [specificity 0.83 95%CI 0.77-0.88, sensitivity 0.70 95%CI 0.66-0.73] or with LSIL+ cutoff [specificity 0.97 95%CI 0.93-0.99, sensitivity 0.54 95%CI 0.51-0.55]. Bayesian point estimates of positive (PPV) and negative (NPV) predictive values for a hypothetical, natural history based, screening population with 1% of CIN2+ prevalence are [PPV: 0.12, 0.05, 0.04, 0.17; NPV: 0.999, 0.998, 0.996, 0.995], respectively. Favouring high screening specificities to keep screening costs low for special settings an optimal cutoff value for the biomarker panel test was selected. This was achieved at a compromise of sensitivity for CIN2+ [0.50 95%CI 0.48-0.50], however the point estimate of PPV improved significantly to 0.55 and the corresponding NPV is still high (0.994). Nevertheless this cutoff still provides high sensitivity for the detection of cervical carcinoma [0.83 95%CI 0.70-0.86].

D2.1, D2.2, D2.3 deliverables and the associated tasks are completed.

- A clinical feasibility and validation study to benchmark the performance of the new test to existing diagnostic platforms with respect to HPV diagnostics.

The overall output of this deliverable is to provide a Biobank which consists of a comprehensive collection of well characterised cervical cytology smear samples collected from a defined population of women with cervical abnormalities. The AUTOCAST Real-time PCR Microarray Analyser, novel mediator probe assay chemistry and 3 prototype chip formats have been successfully demonstrated for detection of HPV16 and mRNA biomarkers in cervical smear specimens. Also we have successfully demonstrated a multiplex HPV PCR detection, with simultaneous amplification of 14 high-risk HPV genotypes in a single channel. To access its value a case-control study using Hungarian and Irish cervical screening populations was set up. Cases comprised 750 women who developed CIN2+ or worse confirmed by histology, and were stratified by age group (under 29 and over) and disease grade (CIN2, CIN3, and carcinoma). Controls (n=250) were women who identified disease free by cytology and were the same age on average as cases, the controls were also stratified by age. Biomarker reverse transcriptase PCR measurements using routine exfoliated cytological samples, high-risk HPV testing, cytology and/or histology were performed and used to evaluate different protocols of testing including combinations of the tests with different test cut-offs. The Bayesian estimates of critical parameters of test performance were calculated and compared for the different stratified groups. D7.1, D7.2 and D7.3 deliverable and the associated tasks has been delivered and reported on schedule

Detailed S/T results

Here we present a short summary of the results we have generated for each of the specific tasks across the 7 workpackages. Overall we present the development of a new diagnostic cervical cancer in vitro diagnostic device capable of HPV and mRNA biomarker detection in clinical samples. .

WP1 Selection of biomarker panel and device requirements

Preanalytical constrains

Within this task we defined the optimal sampling medium for processed and use with the AUTOCAST system. The sampling type and medium we have chosen is liquid-based cytology smears specimen collected preferably into ThinPrep PreservCyt, solution. This will complement another project (SPE_SAFE) we have running alongside AUTOCAST, funded through a Hungarian national grant which is developing a combined sampling and extraction device for RNA and DNA within the same timeframe as the AUTOCAST project. This device is designed to provide long ambient sample stability. Common exploitation is proposed for both foregrounds.

We have also defined the required sensitivities we require both for signal intensities of fluorescent detection and PCR analytical sensitivities, in both cases the projection is that the prototype AUTOCAST device should be capable of achieving similar sensitivities to conventional liquid phase real-time PCR reactions. The reaction volume which we will use in the system is 5 ul per detection channel, comprising of 4 ul reaction mastermix and 1 ul nucleic acid sample volume. In order to achieve good analytical sensitivities for the whole

sample volume the SPE_SAFE is expected to deliver a highly concentrated nucleic acid solution. The current AUTOCAST chip design has 8 channels with 20 immobilisation areas in each channel for the detection probes. The current instrument is capable of reading at 2 wavelengths so the maximum number of the reactions is 20 reaction in one channel, altogether 160 reactions per chip. While the complete multiplex real-time PCR reaction was not demonstrated within the timeframe of the project, the initial proof of concept studies have demonstrated the feasibility of this high level multiplexity approach. . The results have demonstrated mediator probe surface detection, multiplex HPV amplification and sensitive detection of liquid phase biomarker expression. Nevertheless for the proposed HPV multiplex detection (for 20 genotypes), typing and viral load determination (quantitative detection), the established liquid phase mediator probe reaction is capable of working at a multiplicity of 2. The 8 channels are divided between positive control, negative control and duplicated target detection for both biomarker and HPV detection tasks. The plan was for the other wavelength to be used for spotting control. A universal thermal cycling profile has been developed for the mediator probe assay. The overall aim is that the cycling time is kept below one hour to deliver a viable point-of-care instrument, this is achievable with this universal setup, however the current multiplex HPV protocol takes a bit longer. The detection dynamic range is large enough to cover several magnitudes and does not limit the overall detection performance. The software is already available for control of detection and thermal cycling, and will deliver background and negative control corrected evaluated amplification results.

Evaluation of required technologies

For benchmarking, development of reference materials and reactions was carried out: Genoid supplied BF and IMTEK with HPV reference materials, for the biomarker reactions Genoid established a TaqMan based reaction format for two-site evaluation of the biomarker genes (TCD and Genoid). IMTEK made reference reactions and material available for Genoid and TCD regarding the mediator probe chemistry including probe designs for both mediator and molecular beacon reactions. Regarding material selection and technological decisions, evaluation of the possible immobilisation chemistries in accordance with the optical, thermal, chemical, biochemical features of the cartridge material was carried out. Physical design requirements were set as described above. To ensure a low cost device, the technologies are carefully designed, low cost, high productivity injection molding, laser welding, two-step (chemistry and probes are spotted in two consecutive step in the same technology step), top-spot immobilisation was the planned approach.

Selection of biomarker panel

Selection of genes for the biomarker panel was carried out. The established classification biomarker panel of 20 genes were initially validated on an independent set of samples from TCD biobank (136 samples) and at TCD using independent instrument and personnel and later was validated at Genoid on larger population. All of the cases comprised 750 women who developed CIN2+ or worse confirmed by histology, and were stratified by age group (under 29 and over) and disease grade (CIN2, CIN3, and carcinoma). Controls (n=250) were women who identified disease free by cytology and were the same age on average as cases, the controls were also stratified by age. For the detection of CIN2+ cases the biomarker

panel specificity [0.93 95%CI 0.90-0.96] and sensitivity [0.89 95%CI 0.86-0.91] were better than the values of HR-HPV test [specificity 0.85 95%CI 0.81-0.88, sensitivity 0.88 95%CI 0.85-0.90] or cytology alone with ASCUS+ cutoff [specificity 0.83 95%CI 0.77-0.88, sensitivity 0.70 95%CI 0.66-0.73] or with LSIL+ cutoff [specificity 0.97 95%CI 0.93-0.99, sensitivity 0.54 95%CI 0.51-0.55].

WP3 Assay chemistry

A novel assay chemistry which allows simultaneous real-time amplification and detection of multiple targets in paralled was developed. This new technology is called the Mediator Probe Assay (MPA). This mediator probe mediated detection enables the fabrication of universal oligonucleotide arrays for simultaneous real-time PCR detection.

Several approaches using molecular beacon chemistry in solid and liquid phase were also evaluated as a contingency to reduce the risk, should the MPA concept fail. These were later abandoned once the MPA proof of concept was demonstrated.

Small volume real-time liquid phase reaction

Low volume real-time liquid phase reaction were tested for molecular beacon chemistries (using red wavelength excitation/emission), which was comparable to normal volume reaction. The optimal polymerase and buffer pair was selected to achieve effective mediator probe cleavage.Liquid-phase mediator probe cleavage was demonstrated..

Solid phase adaptation of molecular beacon assay

Solid phase molecular beacon chemistry was a contingency measure and was published to be successfully implemented in other PCR reactions, so this was discontinued as a task.

Development of mediator probe chemistry

Successful implementation of mediator probe technology was achieved after investigation of several assay principles. The mediator hybridizes at an immobilized hair-pin oligonucleotide exhibiting a specific mediator complementary site. The polymerase extends the mediator whereby the 5-terminus of hair-pin oligonucleotide is digested and the quencher molecule is cleaved off. The polymerase elongates the primer until the polymerase activity is inhibited by an abasic site (optional) or by steric hindrance due to the hair-pin or internal linker. The polymerase dissociates from the oligonucleotide, the elongated Mediator is available for the next hybridization event. Assay performance is correlated to immobilization efficiency and reproducibility of the Universal Reporter molecule. Several concentrations of the Universal Reporter were tested. The concentration of the Mediator Probe was optimized to 200 nM. Increasing the concentrations neither improved the sensitivity nor increases the fluorescence signal in the assay. The data show a clear correlation between the fluorescence signal plot and the initial DNA copy number. Target DNA down to 10 copies per reaction can be

detected. In approx. 95% of the controls no signal increase was observed showing the specificity of the demonstrated Mediator Probe Assay. The cleavage of the Universal Reporter is indispensable for a successful accomplishment of the Mediator Probe Assay, especially on solid phase. This implies several requirements for the substrate material, coating and immobilization chemistry:

- thermostability

- selective orientation and immobilization via the linking group

- non-interfering fluorescence interaction

- overall feasibility for the assay implementation

Proof-of-concept studies for the Mediator Probe Assay utilizing immobilized Universal Reporter molecules within a functionalised microwell (global coating) were reported in Delieverable D3.2. To transfer the Mediator Probe Assay technology to a polymer cartridge with spatially resolved features a simplified setup is presented. An array comprising a limited set of different oligonucleotides is printed on a polymer slide. Furthermore, the polymer slide is bonded with a structured substrate to a microfluidic cartridge. After bonding (Biofluidix) the cartridge was washed (according to a protocol provided by AIT). After heat-activation of the polymerase and cooling down to the annealing temperature, the Mediator hybridizes to its complementary sequence site within the Universal Reporter sequence and is processed by the polymerase. The 5 terminus of the Universal Reporter is degraded resulting in an increased fluorescence level. Due to a well-considered array layout, influences like coating, printing buffer, and applied oligonucleotide concentration on thermostability, selective orientation, quenching effects and assay feasibility can be identified and quantified. The intensities of the immobilized oligonucleotides differ significantly. As the control oligonucleotide is modified with a fluorophore the highest signal intensity is detected. The duplexing capability was demonstrated. Two different Mediator molecules trigger signal generation at a specific Universal Reporter. These findings allow the conclusion that principally higher multiplexing degrees are feasible. However, experiments combining target amplification and solid phase detection were not achieved within the timeframe of the project..

<u>WP 4.0 Real-time PCR microarray analyser</u>

The TIRF based real-time thermocycler array reader (real-time PCR microarray analyser) has been developed and tested.. The instrument is capable of reading microarrayed fluorescent signals in real time. In parallel to the assay development, the instrument has been optimized for the diagnostic microarray readout including on chip PCR and real time detection. The partners required instruments for their work at an early stage within the project. Therefore, two stages of development were planned. In a later project stage, the final version of the instrument (second generation) was built up, taking into consideration the experience with the first generation. The second generation fulfils the criteria for clinical validation.

Definition of requirements

The system specifications were defined as a first step of instruments development. Aspects of fluidics and production techniques were taken into account. The requirements include the data of optical detection such as number of dots (20 per channel and 8 channels), area to be read out (8x10 mm), required limit of detection (comparable to state-of-art scanners), wavelength ranges. The temperature requirements for PCR cycling have been defined to make possible amplifications below one hour time limit to support point-of-care applications. The cartridge handling includes a locking mechanism using drawer design and the user interface is a computer program, which makes possible to manipulate all important experimental parameters.

Definition of test procedures for optical and thermal assessment

The definition of test procedures has been defined regarding spot sizes surface, the dynamic range and the optical cross talk. Thermal properties include the accuracy and stability of temperature control. All these parameters were measured during analytical evaluation of the prototypes devices.

Demonstrator instrument

On the basis of the specifications, instruments were realised. Simple and automated way to couple the light in after inserting the cartridge into the instrument has been successfully implemented, special optical coupling structures have been designed, tested and realised in the polymer technology. The coupling efficiency and its variations have been characterised and optimised. Homogeneous illumination of the readout area has been achieved, which is necessary for a comparable detection of fluorescence intensity. A low cost, the sensitive detection has also been realised by very simple means in order to keep fabrication costs of the system extremely low. An optimised high aperture imaging optics including spectral filtering has been developed on the basis of commercially available components. High-speed temperature cycling has also been implemented. The instruments have been developed, CE marked and analytically validated. Due to the delay in the delivery of the final Autocast chip, complete overall validation of the system has not been completed to date.

Prototype instrument

In a later project stage, the final version of the instrument (second generation) was built up, taking into consideration the experience with the first generation. In parallel to the instruments development, the cartridge was developed. Therefore, the first instrument operates with standard glass slides, the second generation works with the final cartridge. The second generation was used for clinical validation and proof of concept studies These instruments are operated by clinical lab personnel, requiring a high degree of user friendliness and robustness. Training and maintenance task are necessary to guarantee a

successful validation process. Instruments were used at GenoID, Budapest and Trinity College Dublin, respectively. The third one remained in Freiburg to be used by IMTEK and Fraunhofer IPM. The Autocast real-time PCR microarray analyser BCR 603 was developed, tested and modified. The systems redesigned for the clinical validation was characterized. The analyser's specifications have been met according to the specification of deliverable 4.1, and it is used the characterisation methods in deliverable 4.2. D6.1, D6.2 and D6.3 deliverables and the associated tasks are completed. The D6.4 was joined with D6.2, because the delayed chip production, the reagent and performance validation of the demonstrator design was no longer feasible, so only the prototype system was validated.

WP5 Microfluidic cartridge design

Design and fabrication of the disposable microfluidic cartridge including the microarray for multi parameter analysis has been achieved. Establishing a fabrication technology and supply of cartridges to the other work packages throughout the project were delivered and optimization of the performance in terms of thermal, fluidic and optical properties were carried out.

Selection and validation of materials

Adequate plastic material has been determined which withstands the later operation conditions. The material has been qualified in functional tests regarding mechanical and optical characteristics (low autofluorescence and birefringence), resistance to chemicals and temperature resistance. Furthermore, the material has to meet the relevant criteria for an economic as well as a steady injection moulding process, e.g. in relation to the shape design (micro fluidic cartridge, micro array immobilization) and sealing. The material has also tested regarding PCR and immobilisation conditions.

Cartridge design

The cartridge consists of an optical chip and a fluidic chip. Both of them are injection moulded. The optical chip incorporates an optical quality readout area and waveguide structures. The fluidic chip has eight detection channels and to sacrificial channels inlets and outlets. The sacrificial channels are applied to prevent clogging of detection channels due to gluing the components, which method is used before the laser welding is available. Before the PCR, channels must be sealed or closed, this has been established.

Construction and tooling and development of the moulding process

JOPS constructed tools for moulding both the optical and fluidic chips. Reproducible moulding of optical chip has been achieved. Production process optimization contained the following key aspects:

- improvement of the tool operation

- adaption of machine and systems engineering for realizing the process requirements

- defining a stable working point and process window for injection moulding and injection compression process

The following research and work has been performed. Integration of suitable heating and cooling systems with a minimum installation size and high efficiency for implementing the variotherm processes in the tools; taking account of the optical component requirements by maintaining the stiffness of the optical contour inserts. Evaluation and installation of necessary draft angles in the area of the fluidic channels and coating of the tool inserts for reducing the component grip and guaranteeing a damage free ejection. Researches for improving the polishing as well as the surface quality of the optical coupling and reflective surfaces. Evaluation and adjusting the prestressing of the frame plates for ensuring the filling and compression operation. Testing and optimizing the overall function of the tools. Purchasing, installation and testing applicable compression software for machines and hardware adaption. Selection, purchasing, installation and testing applicable heating and cooling technology for realizing the required variotherm process. Evaluation the influence of the process parameters on the component quality. The following parameters and machine settings had an impact on the process result: injection rate, injection and holding pressure, change-over-point to holding pressure, melt temperature, mould temperature or mould temperature profile, cooling time, clamping force and clamping force profile, stamping stroke, stamping start and stamping speed, development and qualifying process according QS 9000 and ISO 13485.

Optimized & qualified moulding process

To assure that a forming tool or mould together with the appropriate machine and the production process has the ability to manufacture tool- and mould-bounded parts that meets the requirements of the AUTOCAST system, several steps were involved. Basic components of the qualifying process: assignment and release of manufacturing drawings, realization of the tool or mould, risk assessment, qualification (Installation Qualification, Operation Qualification), initial sample testing, short-term process analysis (Ppk-Analysis), and production handover were performed. A statement regarding the achieved quality and stability of the process is possible with a Short-term process analysis (Ppk-Analysis). The chosen processes were evaluated and analyzed accordingly. The single measurement values of the mechanical dimensions as well as of the birefringence resulted in very good to satisfactory PpK-values. Based on this, the capability of the chosen and analyzed processes was demonstrated.

Chip pre-treatment and immobilization chemistry

Providing a suitable coating is highly challenging as polymer surfaces behave differently compared to glass surfaces. Further, coatings which feature high immobilization efficiency must not necessarily be suitable for the solid-phase mediator probe assay, especially when looking at specificity or thermal stability. Thus, coatings which require more complex (i.e., multi-step) application procedures which can complicate chip fabrication have been

investigated. Finally, this led to the identification of potential coating candidates (termed advanced coatings).

Spotting and validation of the microarray

The printing of the assay-specific reagents on the immobilization coating must fulfil two

requirements. First, the printing concept itself must comply with the boundary conditions of the Autocast cartridge design and allow for rapid chip production. Second, independent of the applied coating, the reagent droplets must be printed accurately into the respective channels. Both requirements are met as the current printing concept and instrumentation (TopSpot D) allows for the printing of up to 250 chips per day while ensuring that the reagent spots stay well inside the channels even if assuming worst case scenarios. The array quality (i.e. fluorescence intensity and S/N-ration) has been determined by conventional microarray hybridization experiments.

Development of the sealing process

During the project, chips were mostly joined by adhesive bonding (please refer to deliverable D5.2) and by this process, strongly bonded chips could be produced with high yields. However, this process did only offer limited throughput thus limiting the number of chips that could be produced for the clinical trials. It was thus decided to develop a joining process based on laser welding which offers excellent scalability due to the short required process time per chip. Finally, a new method was developed which featured the best yields and lead to spotted and sufficiently welded cartridges. In this approach, the optical chip was first covered by a laser cut PSA (pressure sensitive adhesive) foil. Then, the complete coating process chain (i.e., plasma treatment and multiple wet chemical processing steps, please refer to deliverable D5.5) could be applied to these chips. It was demonstrated that this method works in principle and valid joining processes can be established this way, with occasional errors and interference effects to be eliminated.

<u>WP6 System integration</u>

During the prototyping of the device (HPV biochip) it was essential to monitor the fulfilment of the requirements of the prototype and the final device. Analytical validation of the prototypes included: thermal, optical and fluidic validation, software validation and performance validation / cross validation.

Definition of test procedures and thermal and optical characterisation

Measurement of temperature with sensors was carried out. The specified temperatures should be reached in the reagents inside the channels of the chip. As during the PCR cycling no temperature measurement inside the channel is possible, the sensor for the temperature control is fixed on the surface of the peltier element. With PT100 (and other sensor types) the

temperature inside the chip can be reached as specified. However the measured temperatures inside the chip are not reproducible in the specified range. Further improvement of the heat transfer between the instrument and the chip should improve the reproducibility. The ramp rates inside the chip have a peak rate of maximum 4°C/second and an average of maximum 2°C/second. To enhance the ramp rates several improvements were tested: the thickness of the chips is reduced, the temperature transfer was optimized and an enquiry for peltier elements with higher power was carried out. Despite the inaccuracy of temperatures it can be shown that a reached temperature is stable and has only very little fluctuations. If cycling is carried out, the temperature variation from one cycle to the other is very low, under 0.5 °C, which is satisfactory for the planned applications. For the determination of the limit of detection (LOD), the signals are the grey scale values of the image taken by the CCD camera. The result is a mean grey value of 68 whereas the lowest concentration has a value of 124.

The calculated LOD is at a concentration of 0.03 ?g/ml. PCR is executed and results are shown. The lowest detectable concentration using E. coli plasmid was under 100 copies/?l. To increase the dynamic range, all measurements use several images taken with different integration times, with this technique, a dynamic range of more than three orders of magnitude is achieved.

Analytical validation of cartridges and reagents

A hydrolysis probe PCR is executed automatically on the instrument, using E. coli plasmid (pTYB1) as template. The concentrations of the template were 1000 copies/?l, 100 copies/?l and 10 copies/?l in the reaction. Several experiments were executed for reducing the holding time for the PCR steps in the BCR 603. The fastest PCR run was done in about one hour.

Software validation

The function of the software is not only to communicate with the user, but control and execution of the whole process. After starting the software, it is checked if the components are connected and working. The user interface is disabled until this test is done. Error messages will pop-up if one component is not working. Every time a component should be used and could not be detected the software stops at this status and shows an error message. Other tests are done like checking the user inputs for the right syntax. In chips temperature measurement, ramp rate determinations,

overshooting measurements were used to validate the software. The gained information was used to improve functionally and performance f the software. During the project the software gradually included the following features: extension of the dynamic range by using different integration times, automatic feature recognition and calculation of PCR curves and analytical values. The software and the interfaced platform were validated by functional tests by comparison with gold standard molecular diagnostic procedures.

WP7 Clinical validation

In order to design a HPV Point of Care Diagnostic device that is generally accepted among the medical community, it will be necessary to convince the medical and scientific community that the new system is as or more reliable than their current conventional diagnostic methods.

Extension and Maintenance of Cervical Specimen BioResource of clinical specimens (TCD)

Bioresource of cervical smear specimens has been extended and the repertoire of tests performed increased to include HPV genotype analysis. A comprehensive collection of liquid based cervical cytology smear samples and nucleic acid specimens has been collected and material from up to 2,000 patients is available within the CERVIVA biobank at TCD. The total nucleic acid has been banked at -80ºC until required for validation of the AutoCast biomarker panel. To date our CERVIVA biobank consists of specimens (ThinPrep and nucleic acid specimens) from over 2,000 women attending for first visits at the colposcopy clinic at the Coombe Women and Infants University Hospital, Dublin, Ireland. The cytological diagnosis and other testing modalities (Hybrid Capture 2, Roche Linear Array HPV Genotyping Test and others) have been made at the baseline. The majoroity of women have had a colposcopic procedure performed after taking the initial smear specimen. These procedures included biopsy (Punch or LOOP), or LLETZ treatment (Large Loop Excision of the Transformation zone), and histological data from these patients is established. The overall output of this BioBank is a well characterised comprehensive collection of cervical cytology smear samples and the corresponding nucleic acid extract, with defined cytology and HPV DNA and mRNA status. This BioBank of specimens is available for all primary testing, validation, and proof of principle experiments at each stage of the AutoCast chip and chemistry development.

Validation of HPV mediator Probe and molecular beacon technology for detection of HPV clinical specimens.

Specimens within the resource bank have already been used to evaluate the sensitivity and specificity of the current gold standard HPV detection methods and Genoid background HPV detection technologies. The existing GenoID molecular beacon real time HPV PCR test to the gold standard HPV detection technology hc2 and the full spectrum HPV test from GenoID, furthermore specific HPV genotyping were confirmed by comparison with the reference genotyping test HPV Linear Array. We have demonstrated the real time PCR based approaches to HPV detection for cervical screening are comparable in many aspects to the gold standard HPV test hc2, which uses in vitro nucleic acid hybridization assay and signal amplification and to the more standard PCR hybridisation assays such as the full spectrum HPV test (FS-HPV) (GenoID) and HPV linear array (Roche). The sensitivity for hc2, FSHPV and MBRT HPV was 98.3%, 97.4% and 93.9% respectively, while the positive predictive value for hc2, FSHPV and MBRT HPV was 94.1%, 94.1 and 97.3% respectively.

The MBRT HPV test appears to have a slightly higher PPV than the other tests. A bank of HPV positive samples (n=241) with full HPV genotype assessment has been performed. As expected HPV 16 was the most prevalent type in this population representing 66.4% of the HPV positive cases, followed by HPV31 (22.1%), and HPV33 (18.3%)/ The multiple infection rate among the population genotyped was 58% which is high compared with a routine screening population, but this is explained by the high prevalence of HPV in the selected population.

The evaluation of the liquid phase Mediator Probe assay in comparison with the current gold standard HPV detection technologies was also carried out. The performance of the liquid phase mediator probe assays for detection of HPV 16, 18, 31 and 33 in cell lines and clinical specimens was demonstrated. 1-10 cells per reaction or 210fg/l of CaSKI DNA, 25pg/l of SiHA cells sensitivities were demonstrated.. The limit of detection for amplification of HPV18 DNA was comparable, at 78.3 copies per 10 l reaction (95 % confidence interval (CI): 47.0 to 372.5), which was comparable to hydrolysis assay (TaqMan PCR).

A clinical evaluation of the HPV16 liquid phase mediator probe assay on 185 cervical smear specimens indicated there was 79.8% concordance between HPV16 MP assay result and Roche Linear Array result for detection of HPV16 positive samples. Similarly, when compared with the GenoID real time molecular beacon assay for HPV16/18 duplex assay the HPV 16 mediator probe assay showed concordance of 80.1%.

The HPV16 mediator probe assay showed a high positive predictive value of 94.9% but a sensitivity that was lower (41.1%) than the other commercially available HPV detection technologies for detection of histologically confirmed high grade disease. This is due to detection of a single genotype by the HPV16 mediator probe assay compared to the other tests which detect a greater number of genotypes. The sensitivity of the mediator probe assay can be improved by increasing the number of Mediator Probes to include the panel of 14 high risk HPV subtypes.

Proof of Principle validation of novel HPV Biochip (prototype system) for detection of HPV in clinical specimens.

Three AUTOCAST prototype chip formats were used for the " proof of concept" clinical validation. methods for HPV detection. Assessment of the concordance with standard HPV and biomarker detection approaches and determination of the reproducibility and robustness of mediator probe assay and the AUTOCAST real time PCR instrument were carried out. The specimens used for validation of the system were from a range of cytological disease categories broken down as follows; .negative 2.3% (2/77), Borderline nuclear abnormalities (BNA) 6.5% (5/77), CIN1 9.1% (7/77), CIN2 27.3% (21/77), and CIN3 54.5% (42/77). Histology results were available for 70 of these women. Overall, 2.8% of the women had negative histology, 11.4% of the women had CIN1 and 85.7% had CIN2+. Control material for the initial validation experiments included DNA and cDNA generated from a HPV positive

cell line CaSki (HPV 16 and 18 positive squamous cell carcinoma). All samples were screened for HPV initially using hc2 (Qiagen) or the Cobas 4800 HPV test, specific HPV genotyping was then performed using the Linear Array HPV test and the molecular beacon real time PCR assay from GenoID. On chip, 85.5% (47/55) were positive for HPV16. This compares with positivity rates of 96.4% (53/55) for Linear Array HPV Genotyping Test, 96.4% (53/55) for Hybrid capture 2 and 96.4% (53/55) for the GenoID molecular beacon real-time test for HPV16/18. The corresponding mediator probe assay for HPV16 performed in liquid phase on the AB7900 instrument, demonstrated

100% positivity for HPV16. The sensitivity and positive predictive value of the HPV16 mediator probe on chip for detection of CIN2+ as a true positive were 92.3% and 97.3% respectively. Overall, 24 HPV16 positive patient samples, selected based on their biomarker expression patterns on TaqMan® PCR were tested on prototype 2 chips (n=24) using the AUTOCAST Real Time PCR Microarray Analyser. Of the samples tested on chip, 83.3% (20/24) were positive for HPV16 and 87.5% (14/16) [that were positive for all 4 biomarkers by TaqMan® PCR on AB7900 instrument] were positive on chip using the Real Time PCR Microarray Analyser. The current capacity of the AUTOCAST system in terms of multiplexity was demonstrated by concurrent amplification of HPV16 DNA and cDNA for 4 mRNA biomarkers (Serpin, TP63, KLK8 and RTKN2) in respective channels of the second prototype chip. Analytically, the AUTOCAST system showed good reproducibility between runs and across each chip with limit of detection for the HPV 16 mediator probe assay on chip at 10 cells per reaction. This compares well to the capability of TaqMan PCR given that sensitivity of the mediator probe reaction on chip may currently be limited by epifluorescence detection.

Potential Impact:

Global cervical cancer incidence increased from 378,000 (256,000-489,000) cases per year in 1980 to 454,000 (318,000-620,000) cases per year in 2010, a 0.6% annual rate of increase. Cervical cancer death rates have been decreasing but the disease still killed 200,000 (139,000-276,000) women in 2010, of whom 46,000 (33,000-64,000) were aged 15-49 years in developing countries. It is usually preceded by a long phase of pre-invasive disease called cervical intraepithelial neoplasia [CIN]. This pre-invasive phase is characterised microscopically as a range of events progressing from cellular atypia to various grades of dysplasia, including cervical intraepithelial neoplasia (CIN) before progression to invasive carcinoma. This precursor phase is generally asymptomatic, and can occur over a long period of 10– 20 years.

Human papillomavirus (HPV) is the single most important aetiological agent in the pathogenesis of cervical cancer and pre-cancer. About 15– 20 types are associated with cervical cancer, of which HPV16, 18, 31, and 45 accounts for 80% of cervical cancers. HPV vaccination has now become part of cervical cancer screening and prevention strategies world wide. There are two prophylactic vaccines currently available which protect against two specific high risk HPV types 16 and 18. With the introduction of HPV vaccination, the landscape of cervical pre-cancer will change over time. While the incidence of abnormal smears and high grade disease will decrease over time, it is anticipated that in the future, the incidence of some type of low grade abnormalities may increase as a consequence. Furthermore, in vaccinated populations, the lower prevalence of disease will directly impact on the performance characteristics (i.e. positive predictive and negative predictive values) of current diagnostic tests.

There are no specific clinical features or symptoms which indicate the presence of CIN. Initial diagnosis is usually made by cytological analysis of a Pap smear specimen. Cytological analysis of PAP smears is the current method employed by cervical screening programmes for the initial diagnosis of abnormality. The result of the PAP smear is what refers the women for additional screening in colposcopy. However it is well recognised that there are limitations to the existing PAP smear screening procedures. These include a significant incidence of false positive and false negative results. Efforts to improve the specificity and sensitivity focus on triage of cytology with HPV testing, which certainly can improve the sensitivity but issues with specificity still remain. As eluded to above in HPV vaccination populations, the rate of abnormal cytology will decrease, this will mean it will be more challenging for cytotechnologists to review cervical cytology samples. It’s likely therefore that HPV testing will become the primary screening event in vaccinated populations. There remains a lot of debate over the best method for triaging women who test HPV positive into high and low risk groups. In this context biomarkers will be an important consideration.

Currently histology is the gold standard procedure in terms of disease diagnosis. However histological diagnosis of CINs is complicated by a variety of cellular changes associated with inflammation, pregnancy and/or atrophy. These changes may mimic precancerous cervical lesions, thereby making traditional cervical histology approaches, subjective and prone to variability. This is reflected in poor inter-observer agreement between pathologists. In particular, the differential diagnosis between immature squamous metaplasia and CIN1/2, or

between low-grade [CIN1] and high-grade [CIN2/3] lesions, tend to be difficult. The limitations associated with histological diagnosis, impact greatly on clinical trials in the area of cervical cancer pre-cancer biomarker validation, HPV vaccination, and novel therapies, as histology is usually the end point in terms of disease. Accurate grading of CIN lesions is paramount for clinical management of patients because CIN 1 and CIN 2-3 lesions are treated differently. Furthermore, as histological diagnosis of cervical biopsy ultimately determines the decision to treat, combined with the low positive predictive value of the smear test which initially refers women into colposcopy, often results in unnecessary treatments. The estimate of this burden has been assessed in several studies, where up to 20% of low grade lesions were upgraded and 26% of high-grade lesions were downgraded after histological review. The issues relating to accurate diagnosis of CIN can result in over- or under-treatment of the lesion. Moreover, in early CIN lesions, the progression rates to high-grade lesions are low (CIN1 and 2 to CIN3 is 9-22% and to invasive cancer is 1-5%). This effectively means that many women unlikely to progress are being over-treated and followed up unnecessarily, causing a huge burden on healthcare systems. Collectively, this emphasises the need for specific biomarkers to aid objective CIN lesion grading, to identify true high-grade cervical disease, and to increase the specificity and PPV of disease detection.

Recent advances have opened new avenues for the biomarker based cervical cancer screening. The AUTOCAST project has been taken significant step toward these goals.

The project delivered significant results including: a novel cervical cancer screening technology, which has already attracted significant attention, a cheap and transportable point-of-care device (POC device), which participates in several ongoing future developments, a patented real-time PCR technology and several, critical, enabling know-how. The original goal to develop a POC with application of HPV and biomarker assay technologies is partially achieved, we have achieved the full integration of the instrumentation, and without solid phase, the integration of the biomarkers assay technologies and the new real-time PCR detection technology. HPV multiplex amplification is also achieved at 14 high-risk and 6 low-risk genotypes were amplified simultaneously. Several members plan to use the generated foreground for commercial or commercialisation targeting follow-up projects. Genoid, the SME in role of the exploitation of the project results, has already started the commercialisation process.

Successful implementation of mediator probe technology was achieved after investigation of several assay principles. The Mediator hybridizes at an immobilized hair-pin oligonucleotide exhibiting a specific Mediator complementary site. The polymerase extends the Mediator whereby the 5’ -terminus of hair-pin oligonucleotide is digested and the quencher molecule is cleaved off. The polymerase elongates the primer until the polymerase activity is inhibited by an abasic site (optional) or by steric hindrance due to the hair-pin or internal linker. The polymerase dissociates from the oligonucleotide, the elongated Mediator is available for the next hybridization event. Assay performance is correlated to immobilization efficiency and reproducibility of the Universal Reporter molecule. The assay principle has the potential to produce universal PCR coupled arrays for high parallel detection of analyses.

Several important know-how were generated during the project including injection moulding of microstructures, material selection, coating, printing and bonding.

TIRF based real-time PCR instrumentation have been developed as a new imaging and detection technique for monitoring and diagnosis which relies strongly on multidisciplinary interaction. We have achieved the full integration of the instrumentation (instrument and cartridge) and without the solid phase, the integration of the biomarkers assay technology and the new real-time PCR detection technology. Further developments in the solid phase technology are foreseeable after the end of the project. These technologies are enabling technologies and their impact would be exploited by combining all of these technologies in a future commercial product. Genoid is currently planning such developments, its IP portfolio including multiplex real-time HPV detection technologies and the currently generated foregrounds (instrument and cartridge) would be integrated in the planned product. Nevertheless the instrument and the mediator probe technology on their own would lead to commercial products, some of them already foreseeable and are developing in follow-up projects.

At the economical level, the cost-effectiveness of the combination of cytology and HPV testing is already considered. The final goal of the exploitation is a marketable product and the consortium was committed to understand the constrains of economical aspect of the final screening product, and this issue have been taken into account during the full duration of the project. Regarding cervical screening the developed biomarker technology is promising, in triage settings and even in primary screening it has the largest potential PPVs estimated using Bayesian estimates based on the clinical study conducted during the project. PPV can be directly translated to costs, because it strong influence on the colposcopic referral rate. Nevertheless the developed instrument and cartridge has low costs as well, which is warranted by design and the used technologies. Genoid considering a special version of the integrated technologies for development countries, where the colposcopic referral cost are unacceptable.

AUTOCAST adopted a multidisciplinary approach to integrate existing and newly derived biological data available on cervical cancer and pre-cancer biomarkers and up-to-date engineering approaches, to develop and validate a point-of-care device for cervical cancer detection. The approach have been taken facilitated identification of a cervical cancer/pre-cancer specific panel of biomarkers which using the proposed technology has potential for use in personalised medicine in molecular diagnostics and genetic profiling. This approach has the potential to greatly increase the capability of cervical cancer screening and validation. In addition it allowed for identification and validation of new biomarkers for cervical pre-cancer and cancer screening which will be of utmost importance for cervical screening programmes going forward, in which vaccination and primary HPV screening will be the foundations. While cervical cancer and pre-cancer are the model disease we will use in AUTOCAST, the approach can be applied to any disease type. The market requirements for this type of approach are on several levels; health, in vitro diagnostics market and on the biopharmaceutical industry.

Impact of AUTOCAST Project.

- TIRF based real-time PCR instrumentation have been developed as a new imaging and detection technique for monitoring and diagnosis which relies strongly on multidisciplinary interaction. We have achieved the full integration of the instrumentation (instrument and cartridge). and without the solid phase, the integration of the biomarker assay technology and the new real-time PCR detection technology. Further developments in the solid phase technology are foreseeable after the end of the project.

- The detection technology can be applied to other biological or disease conditions providing point-of-care detection systems of the underlying biological phenomena (reducing time and cost).

- Successful implementation of mediator probe technology was achieved. The assay principle has the potential to produce universal PCR coupled arrays for high parallel detection of analytes.

- We have developed and validated the biomarker and HPV based cervical cancer technology with outstanding results, which is a high-throughput bioassay and outperforms existing approaches.

- The proposed approach will reduce costs in two ways: it will provide reliable, quantitative, multi-content diagnostic screening approaches which will reduce overall costs by reducing the number of colposcopic events. The point-of-care approach is better suited for developing countries, where the lack of reliable logistic infrastructure could prevent other type of organised screening.

- Several important know-how were generated during the project including injection moulding of microstructures, material selection, coating, printing and bonding.

- Diagnostic information gained from cytology specimens will be of greater clinical utility and will result in earlier detection of disease.

- Histological categories potentially will be re-defined on the basis of new molecular biomarkers.

- Cytology samples are better suited for assessment of biological processes than the currently used biopsy derived histological specimens. The developed biomarker/HPV panel provides measurements in a quantitative manner (LBC/cytology specimens).

- We have developed and validated the biomarker and HPV based cervical cancer technology with outstanding results, which is a high-throughput bioassay and outperforms existing approaches.

- The developed cervical cancer technology has been proved that its use potentially a better strategy for both in primary screening and triage settings. These findings were benchmarked in a clinical study.

- The key problem of today cervical cancer screening strategies is the low PPV. In the benchmark study the PPV point estimates indicate better values than other approaches could achieve, reducing screening associated costs significantly with still high sensitivities. Lower cost can be translated to more comprehensive screening protocols, and overall lower cancer incidences.

Scientific impact at the European level

AUTOCAST is an interdisciplinary project which will address several aspects of the call objective including developing combined biomarker sets for point-of-care detection in cervical screening. The repertoire of skill by the team members across 4 countries, combined with the expertise from two SME' s would clearly not be not be covered by sourcing the competence in a single country alone. The consortium members are well embedded in their respective research and / or commercial environments. Consortium members such as GenoID and Biofluidics have a track record of application focused research led by commercial partners. TCD and IMTEK are very tightly linked into their commercial environments. The TCD partners established CERVIVA The Irish Cervical Screening Research Consortium and are closely aligned in their research objectives with CervicalCheck, The Irish Cervical Screening Programme.

On the commercial side, the diagnosis and treatment of cervical pre-cancer and cancer is rapidly advancing scientific field. With advent of HPV vaccination there are even more rapid changes affecting disease burden, clinical diagnosis and management. GenoID' s biomarker panel is a unique and leading edge technology, which provides the medical basis of this proposal, however we realise that new approaches are continuously emerging jeopardising the success of such technologies on the market. However the consortium are confident about its value, and there is no directly competing diagnostic approach which has been identified to date.

Regarding the technologies involved in this project an even more competitive environment is the mainstay. This innovative and powerful combination of technologies is likely to have profound impact on the future of protein biomarker screening and it is also unique in its kind.

Societal Impact

AUTOCAST will have a significant impact with regard to health. Our result is a state-of-art technology combining TIRF detection, the novel mediator probe chemistry, cartridge design and new biomarker panel. Integration of these technologies provides a quantum leap in speed and efficiency for cervical cancer screening. We are using cervical pre-cancer as the disease application; however, the scope of technical basis of the project goes far beyond cervical pre-cancer and cancer.

The output of the project has the potential to change cervical cancer and pre-cancer diagnosis and clinical studies. A number of large studies are underway internationally which are looking at assessing the long term effect of HPV vaccination with extended follow-ups. It is anticipated that in the long term vaccination will result in an increased demand for new type of technologies and methods for cervical screening and pre-cancer diagnostics.

The proposal in this project adopts a point-of -care based approach for detection of cervical pre-cancer biomarkers which will have enormous impact in two key areas:

- In diagnosis and grading of cervical pre-cancerous lesions, which will result in better disease stratification and identification of true high grade lesions. This will benefit clinical studies, improve clinical management of cervical disease and reduce cost associated with unnecessary treatments.

- As an enabling methodology combining several technologies has the potential to eliminate bottlenecks and speed up diagnosis, eventually increasing access to the healthcare system.

Impact on In Vitro Diagnostic Market

The progress in in-vitro diagnostics (IVD) has become a key economic driving force in the industrial countries and will create new mass markets not only in Europe but also in the newly industrialized countries like India or China. One of the biggest success stories in IVD was the introduction of HPV tests.

This is very competitive market there are new trends shaping and driving the global Human papillomavirus market and it is expected that emerging players significantly alter the market positioning of the current market leaders. It is estimated that the global HPV therapeutics market was valued at $2.8 billion in 2010, and is forecast to grow at a compound annual growth rate (CAGR) of 6.0% over the next seven years, to reach $4.2 billion by 2017. This moderate growth rate is primarily attributed to a weak pipeline landscape. There are only 25 HPV therapeutic molecules in the pipeline and most of them are in the early stages of clinical development, so they are going to make an impact on the market after 2017. Moreover there are only one first-in-class molecules in Phase III, and that too is a prophylactic vaccine. Overall there is moderate growth in the HPV therapeutics market. As a consequence of this moderate growth outlook in therapeutics, the role of primary and secondary prevention is emphasized and targeted diagnostics find opportunities in this market.

Analysts and industry experts agree that HPV testing is a hot spot for growth and will likely continue to be for the next few years. Accordingly, companies like Roche, Third Wave Technologies Inc. (Madison, WI), Gen-Probe Inc. (San Diego) and SensiGen (Ann Arbor, MI) are actively entering the market. Qiagen is the undisputed market leader in the US$230m market for HPV diagnostics, which is predicted to grow to US$620m by 2015. But players like Gen-Probe and Quest will contribute most to that 15% annual growth rate, and will challenge Qiagen' s leading position, says the reports.

However due to inherent issues with HPV testing in various testing scenarios, new diagnostic tools with potential to improve current screening and diagnostic protocols are emerging. Nevertheless primary cervical cancer prevention has strong negative effect on the secondary

prevention of cervical lesions, accordingly new methods and protocols are proposed to leverage this effect to increase positive predictive value of screening and maintain effective cervical cancer prevention measures. In this evolution of methods and protocols, as mentioned above HPV testing already gained a significant role, but cytology is still the mainstay for most screening programs. The new panel of cellular biomarkers could potentially replace tedious and subjective cytology (GenoID). The test has the potential to distinguish normal healthy from diseased with cervical intraepithelial neoclassic grade 2 or worse (CIN2+) women based on the gene expression status of exfoliated cervical cells. However to fully replace cytology in its entire role, grading information is mandatory, which necessitates multiple measurements to access phenotypic behaviour and possibly involves proteomic analysis, which reflects phenotypes in a more direct ways.

Detection technologies like the developed TRIF based PCR technology and the universal PCR array capable mediator probe technology could also bring sustainable change to the IVD and biopharmaceutical markets. By using these technologies it is now possible to drastically reduce the price per test and to address the upcoming new markets. This technological combination allows for the very accurate detection and a universal approach regarding manufacturing of many IVD relevant parameters. This allows cheap, simple and quick detection of the analyte via an immobilized universal recognition element. Furthermore, no specialized personnel are needed to perform the respective testing, as it was constrained by the point-of-care format.

The targeted markets for infectious disease testing was > 3 billion $ in 2010. Personalized Healthcare however is expected to save more than 14 billion $ within the next years. This will significantly increase projects and deals between pharma and diagnostics and even shift the value from therapy to diagnostics. Today, the typical “ blockbuster” drug is effective in only 40 to 60 % of patients prescribed them. With this statement the distinct need of new diagnostic tools for the emerging field of personalized diagnostics (or companion diagnostics) becomes very clear.

The WHO mentions very important infections like influenza, tuberculosis, hepatitis, Meningococcal disease, Severe Acute Respiratory Syndrome (SARS) and many more. Also, the WHO conducts programmes and projects dealing with cervical cancer and HPV infections. Obviously many of these infections are especially of great importance to some newly industrialized countries. Furthermore, these countries emergently need diagnostic tools for economic and fast point-of-care diagnostics. High-through put PCR is rapidly increasing in importance as the biopharmaceutical industry’s focus shifts from single detection to multiparameter diagnostics. Throughout the life science community, it is increasingly realised that multiparameter diagnostics is equivalent to the study of future therapeutics. This generates a growing demand for point-of-care multiparameter diagnostics.

List of Websites:

http://www.genoid.net/autocast/