MicroActive
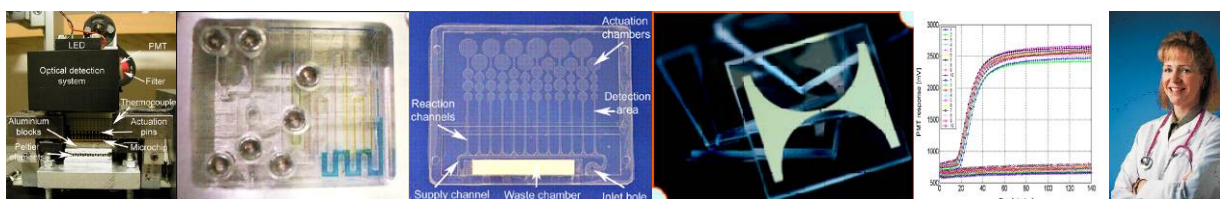## Automatic Detection of Disease Related Molecular Cell Activity

D7.18a) Final Activity report

WP 7 Project management

January 2009

Responsible: SINTEF, Norway

Date / Revision   2009-01-15 / Revision 2

Dissemination level[1]:   PU – Public

Contract No:	IST-NMT-CT-2005-017319
Acronym:	MicroActive
Title:	Automatic Detection of Disease Related Molecular Cell Activity
Instrument:	Specific Targeted Research Projects (STREP)
Research Area:	IST-NMP-2 Bio-sensors for Diagnosis and Healthcare
Coordinator:	SINTEF, Norway
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MicroActive
Automatic Detection of Disease Related Molecular Cell Activity

MicroActive is a specific targeted research project running under EU's 6th IST Framework Programme. The project started in December 2005 and will run until December 2008.

The MicroActive project develops an instrument for molecular diagnostics intended for use in the doctors' office. The instrument will first be used for patient screening for a group of viruses causing cervical cancer. Microfluidics and biotechnology form the basis for the development.

 SINTEF (Norway) coordinates the project. IMM (Germany), IMTEK (Germany), BioFluidix (Germany), NorChip (Norway) and Coombe Womens' hospital (Ireland) are project partners.

www.sintef.no/microactive

Contents

1 Publishable execution

1.1 Background

The MicroActive vision has been to develop an integrated system based on microtechnology and biotechnology for automated diagnosis of a wide range of diseases. Diagnostics can be moved from the central laboratories to the doctor's office. Health care can be made available for a larger group of citizens worldwide with a tool for quick and low-cost diagnostics. In the project the developed system has been tested on one type of diagnosis, namely diagnosis of cervical cancer. The analysis detects specific mRNAs, the transcription of active genes of different Human Papilloma Virus (HPV) types, which are strongly linked with cervical cancer.

Several countries have a screening program for early detection of cervical cancer. One standard procedure is that the doctor uses a brush for collecting cells from the cervix and then he or she transfers the cells and other components, such as mucus, to a buffer solution in a container. The container is sent to a laboratory for analysis with respect to abnormal cells. A normal procedure is that slides with cell smears are visually examined by pathologists for diagnosis. Following a positive diagnosis, some countries advice molecular methods to be used to support or falsify the first diagnosis. DNA or mRNA analysis requires elaborate laboratory routines with more than 20 manual operations and highly skilled laboratory personnel.

The MicroActive project has developed two instruments and two microfluidic chips that can be joined together into one system for a full analysis of a patient sample. The analysis is miniaturized and the analysis procedure for diagnostics of HPV is more automatized than those currently used in the laboratories. The procedure starts with metering up 3 ml of the buffer with patient cells and mucus from the standard container into a syringe. The syringe with the sample is then placed into the instrument with the sample preparation microfluidic chip. The extraction of mRNA, with a sufficient high quality for later NASBA amplification, is performed automatically in the chip and the output is about 50 microliter of elute. The only manual step of the current procedure is to mix this elute with reagents and then transferring it to the amplification/detection chip in the second instrument. The liquid is there automatically pulled into the chip, and split into separate amplification volumes. The mRNA for a specific HPV is amplified if the patient sample was positive for that active HPV virus, and then a fluorescent signal is monitored real time in the reaction chambers. From the signal a diagnosis is decided. The 20 manual steps have been reduced to 3 manual steps, and work is ongoing to avoid also these steps.

At the end of the project, the partners have to a large extent achieved the goals. Functional instruments and functional microfluidic chips have been used for final clinical tests. Using clinical cervical smear specimens from the established biobank, which were positive for HPV 16 and HPV 33, the functionality of the developed nucleic acid extraction with following on-chip amplification has been proven.

The scientific and commercial background for the project was a development already started and performed by the company NorChip AS and two of the research partners in a more restricted context. More partners from Europe were assembled in order to create a consortium with the needed expertise for the development of a total platform for automatic diagnostics, based on mRNA analysis

1.2   Project Objective

The objective of the project has been to develop a platform for automatic analysis of patient samples. The platform is based on biotechnology and microfluidics. The aim is a platform into which a patient sample is inserted and that has as output a positive or negative diagnostics for several disease markers simultaneously. The manufacturing methods of the platform are suitable for volume production and the system has a simple analysis protocol that is acceptable for a general practitioner doctor. The biological functioning of the platform is tested on a range of viruses that are related to cervical cancer and the functioning and sensitivity of the chips is compared to the "gold standard" methods that are in use today at the hospital laboratories.

Project objectives have been to:

- Develop an integrated system based on micro-technology and biotechnology for automated diagnosis of a wide range of diseases. The system will analyze biological samples and be specifically designed for use in primary health care.

- Validate the sensitivity of the system using cytological samples from women at risk of developing cervical cancer (the second most common female cancer) as test cases. Results from the new, automated system will be compared with gold standard hospital lab tests for HPV.

- Prepare for industrial production of the system.

1.3   Partners in Consortium

Stiftelsen SINTEF, research institute, Oslo, Norway
www.sintef.no

Main tasks have been design of amplification and detection chip, design of optical instrument and fluorescence detection system and technical coordination of the project. In addition SINTEF is the Project Coordinator in the MicroActive project holding the contract with the Commission.

NorChip AS, SME company, Klokkarstua, Norway
www.norchip.no

Main tasks have been the miniaturization of NASBA bio-chemistry on the amplification and detection chip and to qualify both chip systems for biological use. NorChip is the exploitation partner for the POCNAD^{TM} technology platform and holds the patents.

Institut für Mikrotechnik Mainz GmbH, research institute, Mainz, Germany
www.imm.de

Main tasks have been design of sample preparation chip and design of the instrument operating it. IMM has also performed validation of the bio-chemistry on the chip including some clinical validations.

Albert-Ludwigs Universität, Freiburg, Germany
www.imtek.de

Main tasks have been surface modification and pattering of the amplification and detection chip.

The Coombe Lying-in Hospital, Dublin, Ireland
www.coombe.ie

Main tasks have been to perform clinical tests on both chip systems, establish a bio-bank of clinical samples and compare the technology platform with "Gold standards". Coombe has also been responsible for the dissemination activities in the project.

BioFluidix GmbH, SME company, Freiburg, Germany
www.biofluidix.de

Main tasks have been surface modification and pattering of the amplification and detection chip. BioFluidix is also the exploitation partner for the spotting technology that has been improved in the project.

1.4 Work performed

The MicroActive project has during the three year project period developed and manufactured a table-top instrument for molecular diagnostics with corresponding disposable microfluidic chips. Microfluidics and biotechnology have formed the basis for the development. Proof-of-principle clinical patient diagnostics have been performed for a group of viruses, which causes cervical cancer, using the new instrument and new microfluidic chips. On-chip biological procedures have been compared to "gold standard" laboratory procedures.
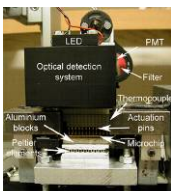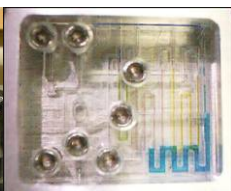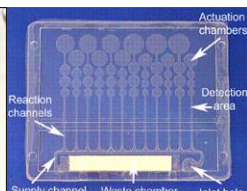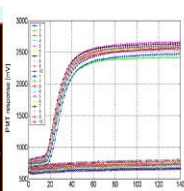
1.4.1 Methodologies & approaches employed

In order to develop a functioning automatic system for diagnostics from patient samples, experts in several scientific disciplines have been cooperating. The project has been divided into three phases; in the first phase several solutions for each of the sub-functions of the system were explored, in the second phase the functioning solutions were integrated, and in the last phase the system was tested using clinical specimens from a BioBank that was established in the project. In parallel with the technical solutions, the diagnostic based on macroscopic mRNA analysis was compared to cytology, histology and DNA based molecular diagnostics. In the project we developed solutions for

- Instrument technology; control of pumps and heating and on-chip process control, solutions using low-cost, standard components for production
- Optics for fluorescent detection, a new solution for multiple reaction chambers
- Polymer microsystem manufacturing, solutions for good biological and optical functioning and volume production
- Microfluidics for complicated reaction protocols, and functional sequences of mixing, splitting, metering etc.
- Bio-functionalization of chip surfaces for biological activity
- Efficient spotting of biological material for storage on-chip
- Spotting of fluidic features, with high precision and industrial volume capability
- Optimizing reagent concentrations for miniaturized biological reactions
- Building up a new BioBank with clinical specimen from women with positive cytology and healthy women
- Controlled comparison between on-chip results and "gold standard" macroscopic instrument results

During the project three new instruments have been built and more than 600 microfluidic chips have been manufactured and most of them used for technological development and biological testing.

Each partner has had responsibility for one area of research, although all development has been a collaborative effort between the technical and the medical partners of the project. This can be seen in the following table, indicating the partners involved in each field of development.

WP1	WP2	WP3	WP4	WP5	WP6

2008-12-05

Wp1 Automatic diagnostics system	Wp2 Sample preparation chip	Wp3 Amplification and detection chip	Wp4 Surface modification and patterning	Wp5 NASBA reactions	Wp6 Meeting the needs of end-users
Tasks: Instruments Clinical tests	Tasks: Microfluidics Chip design Valves Filters Manufacturing	Tasks: Digital microfluidics Chip design Valves Manufacturing	Tasks: Spotting Biosurfaces Dried reagents	Tasks: Miniaturization of the "hands-off" amplification reaction	Tasks: BioBank "Gold standards" Dissemination
Partners: SINTEF IMM NorChip Coombe	Partners: IMM NorChip Coombe	Partners: SINTEF NorChip	Partners: IMTEK BioFluidix SINTEF NorChip	Partners: NorChip SINTEF BioFluidix	Partners: Coombe All

1.4.2 Achievements to the current state-of-the-art

The project has achieved state-of-the-art scientific results in all of the disciplines involved. This has been proven by accepted publications on microfluidics, on-chip sample preparation, and miniaturized nucleic acid amplification and on clinical comparison of HPV detection technologies in international journals and at international conferences. Four publications are under preparation at the closing of the project: A paper on the method for biocompatible lamination of polymer microchips, a paper on automatic sample preparation on-chip, one on parallel actuation of nanoliter sized plugs and spotting in micro-channels and one paper on the total analysis system.

The collaborative effort of the project has resulted in a proof-of-principle of on-chip correct patient diagnostics of three HPV viruses based on NASBA mRNA amplification. Since the start of the project many groups have been working on similar systems for automatic analysis of several disease markers simultaneously. Most recently, a group reported on purification and NASBA amplification of a single bacteria tmRNA on-chip[1]. The analysis was quick, due to a very high concentration of mRNA in each organism (much higher in our clinical samples). The MicroActive development differs from this work in the proven sensitivity of the purification and amplification, by the commercial manufacturing methods for the microfluidic chips and by analysis of multiple markers simultaneously. In 2006 one conference paper on a detection chip (the amplifications were performed off-chip in small volumes) enteric bacterial pathogens[2] was published, however no further results from this group or papers referring to it have been published. This year a chip for magnetic bead

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[1] Dimov I.K., L. P. Lee et al., " Integrated microfluidic tmRNA purification and real-time NASBA device for molecular diagnostics", Lab on a chip, 8, 2071-2078, 2008

[2] Weigl BH, Gerdes J, Tarr P, et al, "Fully integrated multiplexed lab-on-a-card assay for enteric pathogens, 2 Microfluidics, BioMEMs, and Medical Microsystems IV Book Series: PROCEEDINGS OF THE SOCIETY OF PHOTO-OPTICAL INSTRUMENTATION ENGINEERS (SPIE) Vol 6112, p 11202-11202, 2006

purification and RT-PCR analysis of virus in serum and bacteria in blood was published[3]. All these papers use PDMS chips, a material well known for good biological compatibility and simple manufacturing, whereas a main concern of out work has been to develop a commercial diagnostic system. Also we have developed a full system, in comparison to the chips only.

We thus believe that the work performed within MicroActive is at the forefront in the field of development for a diagnostic system for low-concentration biomarkers, to be used in doctors' office.

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[3] Kang-Yi Lien; Wang-Ying Lin; Yu-Fang Lee; Chih-Hao Wang; Huan-Yao Lei; Gwo-Bin Lee, "Microfluidic Systems Integrated With a Sample Pretreatment Device for Fast Nucleic-Acid Amplification", Jour. Of Microelectromechanical Syst., Vol 17, 2, p 288-301, 2008

# 1.5 Results achieved during the project – fulfilment of objectives

The MicroActive project has during the three year project period developed and manufactured a table-top instrument for molecular diagnostics. Microfluidics and biotechnology have formed the basis for the development. Proof-of-principle clinical patient diagnostics have been performed for a group of viruses, which causes cervical cancer, using the new instrument and new microfluidic chips. Both instruments and chips are developed for production and for future use in a commercial system.

The MicroActive project has developed two instruments and two microfluidic chips that can be joined together into one system for a full analysis of a patient sample. The analysis is miniaturized and the analysis procedure for diagnostics of HPV is more automatized than those currently used in the laboratories. The procedure starts with metering up 3 ml of the buffer with patient cells and mucus from the standard container into a syringe. The syringe with the sample is then placed into the instrument with the sample preparation microfluidic chip. The extraction of mRNA, with a sufficient high quality for later NASBA amplification, is performed automatically in the chip and the output is about 50 microliter of elute. The only manual step of the current procedure is to mix this elute with reagents and then transferring it to the amplification/detection chip in the second instrument. The liquid is there automatically pulled into the chip, and split into separate amplification volumes. The mRNA for a specific HPV is amplified if the patient sample was positive for that active HPV virus, and then a fluorescent signal is monitored real time in the reaction chambers. From the signal a diagnosis is decided. The 20 manual steps have been reduced to 3 manual steps, and work is ongoing to avoid also these steps.

At the end of the project, the partners have to a large extent achieved the goals. Functional instruments and functional microfluidic chips have been used for final clinical tests. Using clinical cervical smear specimens from the established BioBank, which were positive for HPV 16 and HPV 33, the functionality of the developed nucleic acid extraction with following on-chip amplification has been proven.

The main achievements in the MicroActive project have been:
- Proof-of-principle diagnostics of HPV 16 and HPV 33: Positive clinical samples from the BioBank were inserted into the sample preparation chip / instrument, the eluate was mixed with reagents and transferred to the amplification/detection chip /instrument. The diagnostics of the chip system was positive for extracts that tested positive on the macroscopic NASBA diagnostics.
- Two functioning instruments that
  - Control microfluidic flows of patient samples and four types of buffers, mixing, splitting and metering
  - Control the in-chip temperature for nucleic acid extraction and for mRNA amplification
  - Detects the fluorescent signal from the amplification in real time
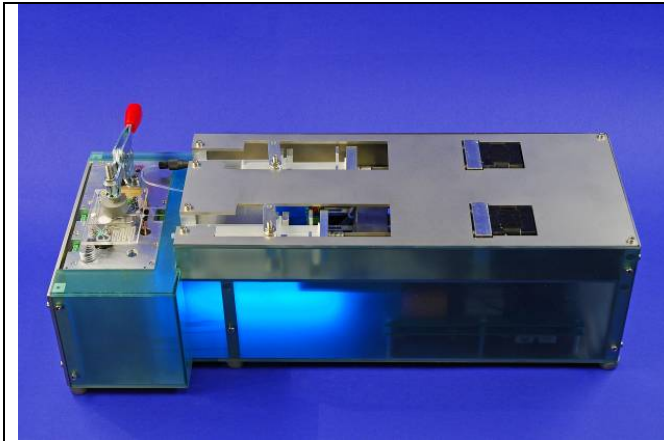
The Voyager instrument that controls flow and mixing of patient sample and four buffers, as well as the temperature in the sample preparation chip	The amplification/detection instrument that controls nucleic acid eluate splitting and metering as well as amplification and detection in eight parallel chambers
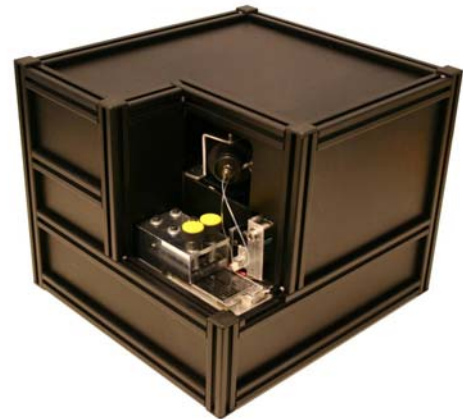
- The two instruments are designed to be integrated. When the instruments are integrated, the eluate droplet output of the sample preparation chip is transferred to the amplification/detection chip via a protected path. The transfer mechanism has been tested
- Two fluidically functioning disposable microfluidic chips, for use in the instruments
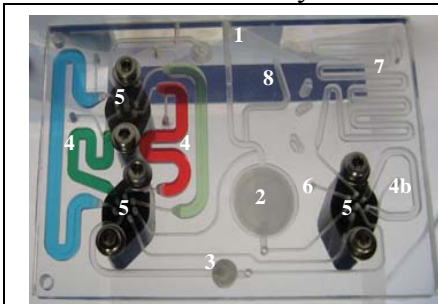
The sample preparation chip with on-board stored buffers, valves and filters	The amplification/detection chip with parallel reaction chambers
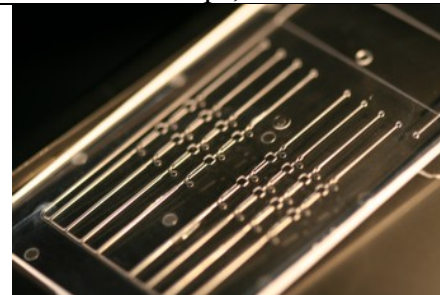
- The two microfluidic chips are biologically functioning; chip materials, chip surface roughness, biological surface coatings, Teflon spotting and dried reagents have been tested for biological compatibility and selected
- Manufacturing methods for the disposable polymer chips with coatings and reagent storage have been developed. The developed manufacturing methods result in chips that enables storage of reagents on chip, allows for fluidic control and that is manufacturable in higher volumes.
    - Amplification/detection chip manufactured by injection moulding
    - Sample preparation chips designed for manufacturing with injection moulding
    - Turning valves have been developed to deal with the complicated flow pattern of several buffers and the patient sample
    - A method for integrating functional biological filters have been developed
    - A manufacturing method for spotting of a new Teflon based solution precisely in channels have been developed

11

o   A method for spotting of different reagents (viscous) of precise volume and at a precise position has been developed

- Experiments with HPV positive cell line dilutions in the sample preparation chip shows that down to 5 cells can be successfully amplified and detected after nucleic acid extraction on-chip
- Experiments with HPV positive cell line dilutions in the amplification/detection chip shows that down to 1.25 cells can be successfully detected in each reaction chamber
- Experiments in the amplification/detection chip shows positive amplifications of cell lines and clinical samples using dried reagents
- Comparisons of output eluates of sample preparation chip with gold standard (Qiagen M48 BioRobot) extraction for following macro scale PreTect HPV Proofer amplification
- More than 90 sample preparation chips fluidically/biologically tested
- More than 380 NASBA chips fluidically/biologically tested
- Instruments and chips are in use outside the research laboratories

The objective of the project was to develop an instrument for use in the doctor's office. The instrument is still at a stage where a few manual operations are left, and the instruments will first be used for further research. Ongoing work is focusing on solving the last challenges of full automatization.

1.6   Impact on industry and research

1.6.1 Societal objectives: Quality of life

The low-cost, fully automatic diagnosis system developed in the project will:

- Widen the availability of advanced medical diagnostics for all citizens.

- Reduce the time from patient testing to diagnosis, avoiding anxiety and enabling earlier treatment.

- Precision of diagnosis - A more precise diagnosis will give a more precise treatment.

- Environmental pollution - All reagents and human samples are left inside the chip, use of reagents is minimized.

- Food safety - If chip is used for detection of bacteria in food.

1.6.2 Economic impact – public sector

Using the automatic diagnosis system as an alternative to today's diagnostic testing will imply cost savings for the public health authorities.

The most obvious cost savings relate to the tests themselves. Today, molecular testing typically costs 40-110 Euro per target per sample. Our automatic system will be a low cost instrument with no manual protocol, and have the potential to give a price of around 5-10 Euro per target per sample. In addition to the savings on direct costs per sample, there will be savings on the associated *administrative* costs, both in doctors' offices and at central laboratories. Today, staff must register data and send samples/results back and forth. With the

new automated system, this will no longer be necessary, and laboratory personnel will be free to spend time on other operations.

Less obvious - but more significant - savings will arise from reduced treatment costs. Lower-cost tests can be carried our more frequently, allowing earlier detection of disease. This will allow simpler, earlier treatment and even prevention. This is much cheaper than long-term care. Where molecular diagnostics replaces traditional methods, the accuracy of the tests is increased. The most suitable treatment can then be started immediately - instead of experimenting with potentially costly treatments that will fail.

The scale of cost savings depends on the diseases to which the technique is applied. The mRNA approach is applicable to a wide range of diseases, and the full impact of the project will become apparent as its results are applied to a wider range of diseases. Within the project itself, the technique will be tested on the specific case of cervical cancer. This is the second most common female cancer, so cost savings on that condition alone are highly significant. Currently used techniques result in a fairly high number of false positive results - with associated costs (not to mention patient anxiety). Some technical details about the applicability of the mRNA approach to this particular condition are supplied below.

Applicability of mRNA approach to cervical cancer (test case to be used in the project): HPV is a prerequisite for cervical cancer and the new NASBA based instrument detects the presence of mRNA coding for E6/E7 proteins of the human papillomavirus (HPV). These viral proteins down-regulate cell cycle regulatory proteins like P53 and Rb that are important tumour suppressors. Cells lacking these enzymes are highly cancer prone. Cytological tests are dominant for cervix scanning today. The number of false positive results for first time cytological screening is between 50 -75%. This will be decreased to 1% using the NASBA amplification chip.
HPV DNA tests will miss 5-10% of cervical cancer samples due to integration of HPV DNA followed by deletion of parts of the genome. HPV DNA tests will also identify 20-30% of normal women as positive, but more than 90% will be false positive answers, i.e. there is no production of E6/E7 oncoproteins present. The NASBA amplification procedure is therefore superior to both cytological methods and DNA based methods, due to test selectivity.

1.6.3 Economic Impact - Private sector / competitiveness

The market for automatic instruments is currently dominated by the USA. Cepheid has a commercial product, while many others such as Motorola and Xtrana are performing research on prototypes. European development is somewhat fragmented, and mostly university-based. The commercialization stage of the instrument of this project is on the level of the US companies prototypes.

The project will develop a system that can be mass produced, and so firmly establish European players in this market. The instrument will open up production industry opportunities in several areas:

- Optical instruments
- Mechanical/electronic instruments

- Polymer chips

- Biological reagents

The different parts of the automatic instrument developed in this project (polymer chips and actuation instrumentation) may also be used separately in the development of other similar products. According to FlowMap 2004[4], the medical diagnostics market size in 2001 was 23.8 billion Euros and the point-of-care market is the main growing segment. The type of system developed in this project is also applicable for biotechnology research (functional genomics) and for food quality monitoring. These markets are also expected to grow.

1.7   Further work

The instruments developed in the project will be used intensively also after the project, in order to optimize the biochemical processes on chip. Also the chip designs and the chip manufacturing will be used in the ongoing work. The spotting of future chips will be performed with the new BioFluidix spotter.

Collaboration between the partners will continue, but in a form that is more suitable for a concept that approaches the commercial phase.

A major publication involving the total system with biology and technology combined is planned to be published in spring 2009.

1.8   Project information

Coordinator: SINTEF
                   Strindvegen 4,
                   N-7465 Trondheim, Norway
                   www.sintef.no

Project logo:

Contact persons:

Mrs. Liv Furuberg	Mr. Dag Ausen
Tel.: + 47 2206 7546	Tel.: + 47 2206 7546
Fax: + 47 2206 7350	Fax: + 47 2206 7350
E-mail: liv.furuberg@sintef.no	E-mail: dag.ausen@sintef.no

Web-site: www.sintef.no/microactive

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[4] FlowMap - Microfluidics Roadmap for the Life Sciences, Editors: J. Ducrée and R. Zengerle, Books on Demand GmbH, Norderstedt, Germany, ISBN: 3-8334-0744-1, 2004

2 Dissemination and use

2.1 Overview of dissemination activities in the project

2.1.1 Scientific publications

All partners involved in the consortium have an excellent track record for publication in scientific peer reviewed journals. As the project has progressed a number of peer reviewed publications have been accepted for publication (Table 1). Technical articles, book chapters and two PhD theses have been published on microchip systems, lab on a chip devices and a comparison of current state of the art HPV detection systems, specifically NASBA with "gold standard" HPV testing approaches. The latter has generated considerable interest among the medical community and has been accepted for presentation at four international medical meetings. Table 1 and Table 2 list all the scientific publications generated by the consortium to date.

Together these articles have broad readership covering both the medical and scientific community, and will reach our target populations of scientists, engineers, biologists, physicists and of course clinicians.

At the project closure, three major articles based on the results of the MicroActive project will be published. One of these articles will contain the combined results of the sample preparation platform and the NASBA platform with clinical tests. The complete story of the micro total analysis system will be submitted to a journal of high impact factor. The two articles describing each of the two platforms separately will also be submitted to high ranked journals. Suggested journals for the three articles are; Nature Methods, Science, Lancet, EMBO J, PNAS and Cell.

In addition as the final phase of the project concludes, there are obvious topics which will be published in scientific peer reviewed journals in the coming 6-12 months. These include:

- RNA extraction capabilities of the sample preparation chip
- Sample preparation chip - technical description
- NASBA chip - technical description
- Spotting, drying and re-activation of enzymes/primers in fluid channels.
- Parallel mRNA amplification and detection.
- Comparison between the detection signal from NASBA chip and signal from macroscopic NASBA system
- Numerical simulations on fluid movement
- Experiments on repeatability and controllability of fluid flow in chips
- Numerical simulations on mixing in a droplet
- Experiments on droplet flow patterns (using PIV setup)

Table 1 List of Scientific publications (Appendix A)

No	Authors and Title	Journal	Citation
1.	Keegan H, Mc Inerney J, Pilkington L, Gronn P, Silva I, Karlsen F, Bolger N, Logan C, Furuberg L, O'Leary JJ, Martin CM. Comparison of HPV detection technologies; Hybrid capture 2, PreTect™ HPV-Proofer and analysis of HPV DNA viral load in HPV16, HPV18 and HPV33 E6/E7 mRNA positive specimens..	Journal of Virological Methods 2008	Accepted for publication in October 2008
2	Gulliksen, A. and Karlsen, F. Microchips for the diagnosis of cervical cancer	Microchip-based Assay Systems: Methods and Application, Chapter 6, Humana Press Inc. [5]	Humana Press Inc., Totowa, NJ, 1, 65 – 86 (2007)
3.	Gulliksen, A. Microchips for isothermal amplification of RNA. Development of Microsystems for analysis of bacteria, virii and cells	PhD thesis: University of Oslo, Dept of Molecular Biosciences, Oslo, Norway	2007
4.	Solli, L. A. Development of a lab-on-a-chip platform technology - experimental and numerical investigations on microfluidic aspects.	PhD thesis, Norwegian University of Science and Technology (NTNU), Trondheim, Norway	2007
5.	Furuberg L, Mielnik M, Gulliksen A, Solli L, Johansen IR, Voitel J, Baier T, Riegger L, Karlsen F RNA amplification chip with parallel micro-channels and droplet positioning using capillary valves [6]	Microsystem Technologies (Springer Berlin / Heidelberg)	No. 4-5, Vol.14, p. 673-681 (April 2008)

Table 2List of Scientific presentations

Completed activities (date)	Type / Reference	Type of audience	Location	Partner responsible / involved
2006-04-25	Direct and indirect (biomarker) detection of oncogene expression in future screening of invasive cervical cancer	Medical and scientific	France Europe	Coombe
2007-04-02/04	Invited talk at the SPIE European Symposium on Microtechnologies for the New Millennium	Scientific	Gran Canaria, Spain	SINTEF
2007-04-02/04	Conference proceedings for SPIE European Symposium on Microtechnologies for the New Millennium Smart Sensors, Actuators and MEMS III, Volume 6589-1B, 2007 The MicroActive project: Automatic detection of disease-related molecular cell activity	Scientific	Gran Canaria, Spain	SINTEF
2007-10-08/11	Sample metering and parallel liquid plug actuation for multiple biochemical assays. 2 pages paper in proceeding at The 11th International Conference on Miniaturized	Scientific	Paris, France	SINTEF

[5] http://www.springer.com/humana+press/biotechnology/book/978-1-58829-588-0 (ISBN: 978-1-58829-588-0)

[6] http://springerlink.com/content/n25840t11w158314/fulltext.pdf

2008-12-05

	Systems for Chemistry and Life Sciences, µTAS 2007			
2007-10-08/11	Storage and reactivation of enzymes in a disposable, self-contained lab-on-a-chip system, 2 pages paper in proceeding at The 11th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2007	Scientific	Paris, France	NorChip
2007-10-04/06	Platform Presentation at Eurogin, New Strategies in Cervical Cancer Prevention, Monte Carlo, Monaco 4-6th Oct, 2007. "Comparison of HPV Detection technologies; Hybrid Capture II (Digene), Pretect HPV Proofer (NorChip) and Real time PCR for Detection of HPV in cervical cytology specimens."	Scientific and medical	International	Coombe
2007-11-03/09	Poster Presentation at 24th International Papillomavirus Conference and Clinical Workshop in Beijing, China, "Evaluation of HPV DNA and mRNA Detection Technologies for Detecting HPV in Cervical Cytology Specimens."	Scientific and medical	International	Coombe
2008-01-07/09	Platform Presentation at The Pathological Society of Great Britain and Ireland, Winter Meeting, Oxford UK. "Evaluation of HPV DNA and mRNA Detection Technologies for Detecting HPV in Cervical Cytology Specimens".	Scientific and medical	International	Coombe
September 2008	Platform Presentation at The British Society for Clinical Cytology, Dublin, September 2008. Comparison of PreTect™ HPV-Proofer and Hybrid Capture 2 for HPV detection with viral load analysis of HPV16, HPV18 and HPV33 E6/E7mRNA positive specimens.	Scientific and Medical	International	Coombe
October 2008	Paper in the proceedings of the 12th International Conference on Minaturised Systems for Chemistry and Life Sciences, µTas 2008: "Automated chip-based extraction of HPV mRNA from cervical samples."	Scientific	World	IMM
2008-10-20/22	Development of automatic chip-based detection of mRNA. Discovery to Diagnostics Conference 2008, San Diego California	Scientific	World	NorChip
2008-10-20/22	Point-of-care nucleic acid diagnostics POCNAD™ or Lab-on-a-chip™ technology. Discovery to Diagnostics Conference October 20-22nd 2008, San Diego California	Scientific	World	NorChip

2008-12-05

2.2   Research community

Specifically in relation to the research community at large, there have been a broad range of dissemination activities which have been specifically targeted to the research community. These activities range from educational workshops and lectures (Table 4), to exhibitions at conferences and trade fairs as will be outlined in section 2.5 below, and presentations at national and international meetings (Table 2). This approach to dissemination by the consortium has ensured that a broad audience base has been addressed including medical, scientific and commercial audiences.

Within the first 6 months of the project commencement a MicroActive project website (www.sintef.no/microactive) was established and has been maintained throughout the project (Figure 1). The website contains information about the project itself, the individual partners who are involved with specific links to their relevant websites and publications and dissemination activities which have arisen from the project. The website targets both the research community and the wider community.

Figure 1Project web site (www.sintef.no/microactive) established in June 2006 and maintained through the lifetime of the project.

The MicroActive clinical partners (The Coombe Women and Infant's University Hospital) have established The Irish Cervical Screening Research consortium under the name CERVIVA. These partners have integrated the MicroActive project into one of they key project areas within CERVIVA and through all the public relations and outreach have referred to MicroActive project and its goals. These include at the official launch of CERVIVA the Irish Cervical Screening Research consortium, the CERVIVA website www.cerviva.ie which has links to the MicroActive project website (http://www.cerviva.ie/Project8.html). Finally, CERVIVA have participated in the Women's World Show and women's mini marathon 2008

18

in the RDS, Dublin[7] to increase awareness among women on cervical screening, HPV and HPV vaccination. Through this forum CERVIVA distributed information leaflets on Cervical Cancer, HPV and project underway within CERVIVA to 40,000 women.

Table 3 Description of major dissemination activities carried out towards the research community

Activity	Description
Scientific Presentations	All partners have been actively involved in presenting the MicroActive project at International Scientific and Medical meetings. Specific details are outlined in Table 4.
Workshops	SINTEF and IMM have been actively involved in scientific and educational workshops. The specific details are outlined in section 2.4, Table 8.
Trade Fairs	BioFluidix, IMTEK, NorChip and IMM have been active in exhibiting components of the MicroActive project at International Trade fairs. The specific details are outline in Table 5.

Table 4 Dissemination activities targeted to the research community

Completed activities (date)	Type / Reference	Type of audience	Countries addressed	Size of audience	Partner responsible / involved
2006-02-02	Consultation workshop "Personal Health Systems: the path from FP6 to FP7"	Scientific	Europe	Small	SINTEF
2006-02-02	Concertation workshop of IST projects on intelligent eHealth Systems for personalised medical care	Scientific	Europe	Small	SINTEF
2006	Contribution to eHealth booklet [8]	Scientific and Medical	Europe	Large	SINTEF
2006	eHealthNews.eu – project information [9]	Scientific	Europe	Large	SINTEF
2006-12-15	The Coombe Women's Hospital Research Symposium December 2008.	Medical and Scientific	Ireland	Small	Coombe
2007-04-02/04	Invited plenary talk + proceedings paper at the SPIE European Symposium on Microtechnologies for the New Millennium	Scientific	Gran Canaria, Spain	Large	SINTEF
2007-05-14/15	Invited plenary talk at R3 Nordic Symposium 2007	Scientific	Oslo Norway	Medium	SINTEF
2007-08-28/31	Oral presentation at the Nordic Research Course on Nanofabrication of Polymer materials	Scientific	Tallinn, Estonia	Small	SINTEF
2007-10-05	Presentation at Eurogin Conference, 2007	Scientific and Medical	Monte Carlo, Monaco	Large	Coombe

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[7] 30th May to 1st June, 2008

[8] http://ec.europa.eu/information_society/activities/health/docs/projects/fp6book/microactive.pdf

[9] http://www.ehealthnews.eu/content/view/881/66/

2007-10-08/11	Poster at The 11th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2007	Scientific	Paris, France	Large	SINTEF
2007-10-08/11	Poster at The 11th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2007	Scientific	Paris, France	Large	NorChip
November 2007	Article "GIT Labor-Fachzeitschrift"	Scientific	Germany	Large	IMM
2007-11-03/09	Poster Presentation at 24th International Papillomavirus Conference and Clinical Workshop in Beijing, China	Scientific and Medical	Beijing, China	Large	Coombe
2008-01-07/09	Platform Presentation at The Pathological Society of Great Britain and Ireland, Winter Meeting, Oxford UK. "Evaluation of HPV DNA and mRNA Detection Technologies for Detecting HPV in Cervical Cytology Specimens".	Scientific and medical	UK	Medium	Coombe
May 2008	The 6th international cancer conference, St. James's Hospital Dublin, May 2008	Medical and Scientific	International	Medium	Coombe
2008	Article in Microsystem Technologies	Scientific	European	Large	SINTEF
2008-09-08	Presentation at the British Society for Clinical Cytology Annual Meeting	Scientific	Dublin	Medium	Coombe
2008-10-07/11	Poster at The 12th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2008, San Diego	Scientific	USA / world	Large	IMM
2008-10-20/22	Oral presentation at Discovery 2 diagnostics Conference October 20-22nd 2008, San Diego California	Scientific	USA/ world	Large	NorChip
2008-10-20/22	Poster Presentation at Discovery to Diagnostics Conference October 20-22nd 2008, San Diego California	Scientific	World	Large	NorChip
October 2008	Biomedical Devices Institute [BDI], Dublin City University, October 2008.	Scientific	Ireland	Small	Coombe
October 2008	The Gynaecology Club of Great Britain & Ireland, Annual Meeting October 2008.	Medical	Ireland/UK	Small	Coombe
2008-11-3	Oral presentation at "Tag der Technologie 2008"	Scientific/ General	Germany	Medium	IMM
2008-11-4/7	Poster presentation at "Wissenschaftswoche", University of Mainz	Scientific/ General	Germany	Medium	IMM
2008-11-11/12	Oral presentation at "Young scientists workshop" at IMM	Scientific	Germany	Medium	IMM
2008-12-9/10	Oral presentation at "Diagnostics and Bioanalytical Devices", Frankfurt	Scientific	Europe	Medium	IMM

2.3   The doctors' community

An integral part of the MicroActive project was to obtain feedback and liaise with clinicians and end users of the system to obtain information in relation to their routine practices and their needs/opinions on system design and implementation. This was achieved at many levels through informal discussions, through distribution of a questionnaire and through presentations at medical conferences and meetings. Obtaining feedback relied heavily on dissemination of the project concepts and objectives. This was achieved through distribution of a project information flyer together with the doctor's questionnaire and at medical conferences and meetings (Table 6).

In addition the clinical partners within the consortium (Coombe) have integrated the MicroActive project into a large Irish consortium called CERVIVA (ICRSC-The Irish Cervical Screening research consortium)[10], as described above, which is a multi-investigator collaboration encompassing researchers at seven Irish universities, eight hospitals and ten commercial diagnostic/ biotechnology companies, one of them being NorChip.

Education also plays a key role in dissemination to clinical audiences. The future of diagnostics in medicine will rely heavily on education and outreach to the next generation of medical doctors and as such current medical students. At Trinity College, Dublin the consortium's clinical partners are striving to achieve this within the educational programme being delivered to medical students and the next generation of laboratory scientists. These partners now include the MicroActive project, where relevant, on the teaching curriculum as an example of the future of point of care diagnostics.

The medical community at large has also been targeted through presentations arising from the project at national and International conferences. The specific details in relation to these are listed in Table 6.

Table 5 Description of major dissemination activities carried out towards the doctor's community

Activity	Description
Questionnaire	Questionnaires and project information leaflets were distributed to doctors throughout Europe to obtain feedback on requirements for such a POC device.
Education	Microactive project has been included as teaching material for medical students and laboratory scientists as an example of future point of care diagnostics (Table 7).
Presentation at Medical conferences.	The results of the project have been presented at 8 Medical conference (Table 7)

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[10] www.cerviva.ie

Table 6 Dissemination activities towards the doctor's community

Completed activities (date)	Type / Reference	Type of audience	Countries addressed	Size of audience	Partner responsible / involved
2006-01-23	Press release by ICSRC on DMMC[11] webpage	Medical Scientific and general public	Ireland		Coombe
2006-03-15	Press Release by ICSRC[12] and Health Research Board, Ireland on cervical cancer research.	Medical Scientific and general public	Ireland		Coombe
2006-04-25	Direct and indirect (biomarker) detection of oncogene expression in future screening of invasive cervical cancer	Medical and scientific	France Europe	400	Coombe
2006-05-24/26	Cancer Biology to Cancer Medicine	Students	Ireland	50	Coombe
2006-07-03/06	Molecular Biology in Cytopathology	Medical and Scientific	UK	200	Coombe
2006-10-21	Project promotion at the Irish Gynecology Oncology Society Annual meeting-Distribution of questionnaires and information leaflets	Medical	Ireland	200	Coombe
2006-11-01	Launch of ICSRC, Burlington Hotel, Dublin	Public, medical and scientific	Ireland	Large	Coombe
2006-12-15	Project presentation, Coombe Women's Hospital Research Symposium, Dublin	Medical and Scientific	Ireland	100	Coombe
2007-10-04/06	Eurogin, New Strategies in Cervical Cancer Prevention, Monte Carlo, Monaco	Medical and Scientific	Monaco	500	Coombe
2007-11-03/09	24th International Papillomavirus Conference and Clinical Workshop in Beijing	Medical and Scientific	China	700	Coombe
2008-01-07/09	Pathological Society of Great Britain and Ireland, Winter Meeting, Oxford.	Medical and Scientific	UK	300	Coombe
May 2008	The 6th international cancer conference, St. James's Hospital Dublin, May 2008	Medical and Scientific	International	Medium	Coombe
2008-13-14/06	Basic and Advanced Colposcopy (BSCCP accredited).	Medical	Ireland	Small	Coombe
2008-09-08	Presentation at the British Society for Clinical Cytology Annual	Medical and Scientific	Ireland	Medium	Coombe
October 2008	The Gynaecology Club of Great Britain & Ireland, October 2008	Medical	Ireland	Small	Coombe

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[11] DMMC - Dublin Molecular Medicine Centre

[12] ICSRC - Irish Cervical Screening Research Consortium

2.4 Microtechnology courses

Dr. Liv Furuberg, teaches a Graduate course on "Microsystems design" at the University of Oslo. Parts of the MicroActive work have been included into lectures concerning microfluidics. In addition IMM, IMTEK and BioFluidix are all involved in teaching European courses on Microfluidics and Microarrays through FSRM Switzerland, and the MicroActive project results have been included as part of the teaching material.

Prof. Frank Karlsen (NorChip) is responsible for teaching of a graduate course in BioMEMS at Vestfold University College. The results of the µActive project are frequently used as examples within the course to illustrate the features of microchip functionality.

These courses are all targeted towards students, engineers, scientists and researchers working in the field of microtechnology. Table 7 lists all dissemination activities in relation to this specific area.

Table 7 Dissemination towards students, engineers, scientists and researchers

Completed activities (date)	Type / Reference	Type of audience	Countries addressed	Size of audience	Partner responsible / involved
2006-02-17	Project presentation	Young female scientists from the University of Oslo (Goal: Inspiration for future career in physics)	Norway	Small	SINTEF
2007 Summer Semester	Lecture on Microfluidics	Students	Germany	20	IMTEK
2007-11-11	FSRM course Microfluidics & Microarrays	Engineers	EU	10	IMTEK/Bio Fluidix
2008 autumn	UiO course material	Students	Norway	10	SINTEF
2008 autumn	Graduate course in BioMEMS	Students	Norway	Small	NorChip
2008-11-03/04	FSRM Course on Modelling and Simulation of Micro Fluidic Systems	Young researchers	Europe	Small	IMM
2008-11-13	FSRM course Microfluidics & Microarrays	Engineers	EU	10	IMTEK/ BioFluidix

2.5 Beyond the research community

Within the first 6 months of the project commencement a Microactive Project website (www.sintef.no/microactive) was established and has been maintained throughout the project (Figure 1 above). The webpage contains information about the project itself, the individual partners who are involved with specific links to their relevant websites and publications and dissemination activities which have arisen from the project. Web-statistics from the site has only been available since July 2008, counting around 150 different visits in the Q3-2008. To broaden the target audience a number of partners have included a link on their own institutions/ consortia web pages to the MicroActive project webpage.

A MicroActive flyer focussing on the sample preparation chip is distributed on fairs by IMM in order to attract future partners and customers for research and development projects and inform the audience about progress and current status of chip based analytical devices.

In addition the selected members of the consortium, BioFluidix, IMTEK and IMM have targeted commercial and industrial audiences through exhibitions at trade fairs (Table 9). Components of the MicroActive Project, including the sample preparation device have been included on trade stands at these exhibitions.

The concepts and ideas behind the MicroActive project have been disseminated on a broader level to a general audience through 2 radio interviews and a television broadcast in Norway, which described the concepts behind the project and showed some images of how such a point of care diagnostic for cancer screening will look. In addition, to coincide with the timely awarding of the Nobel Prize for Medicine 2008 to Professor Harald zur Hausen, for his discovery of human papilloma viruses causing cervical cancer, IMM have issued a press release in Germany on the MicroActive project.

In addition, NorChip as commercial partners in the project has made a promotional DVD which can be used to promote the MicroActive Lab-on-a-chip platform to potential users, investors and partners. The DVD has been produced and financed completely by NorChip beyond the MicroActive project. NorChip is positive to that the consortium partners promote the technology presented in the DVD, but the partners have to declare the use of the promotional DVD in each case to make sure that the publications do not conflict with NorChips business plan. During the presentation of the DVD it must be informed that NorChip has the commercial rights for the technology platform.

Table 8 Description of major dissemination activities carried out towards the public

Activity	Description
Website	www.sintef.no/microactive
Exhibitions at Trade fairs	Components of Microactive project have been exhibited on stands at International Trade fairs (Table 9).
Press releases	Tracing pre-cancer, IMM press release in front of MEDICA 2009 [13]
Promotional DVD	NorChip

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[13] http://www.imm-mainz.de/upload/dateien/PR%202008-10-30e.pdf

Table 9 Exhibitions and trade fairs

Completed activities (date)	Type / Reference	Type of audience	Countries addressed	Size of audience	Partner responsible
2007-01-21	Stand at Labautomation Palm Springs exhibition & conference	Scientific, Diagnostic, Technical	USA	Large	BioFluidix/ IMTEK
2007-10-9	Stand at BioTechnica Hannover exhibition	Scientific, Diagnostic, Biotech	Germany	13.000	BioFluidix/ IMTEK
2007-09-26	Stand at EuroBio Lille exhibition & conference	Scientific, Diagnostic, Biotech	France	approx. 5.000	BioFluidix
2007-10-09/11	Display of the sample preparation chip on IMMs stand at the BioTechnica 2007 trade fair	Technical	Germany	Large	IMM
2008-01-24/27	Labautomation Plams Springs	Scientific, industry	US / world	> 5.000	BioFluidix / IMTEK
April 2008	Analytica	Scientific, industry	Germany / world	Medium	BioFluidix
2008-04-21/25	Hannover Fair	Industry	Germany / world	> 100.000	BioFluidix / IMTEK
2008-10-7/11	BioTechnica, Hannover	Scientific/ Industry	Germany / world	Large	IMM
2008-11-3/7	Technologie-Tage Uni Mainz	Scientific/ General	Germany	Medium	IMM
2008-11-11	Pharmaforum, 11.Nov. 08, Saarbrücken	Scientific/ Industry	Germany / world	Medium	IMM
2008-11-19/22	Medica, Düsseldorf	Scientific/ Industry	Germany / world	Large	IMM

Figure 2 The sample prep instrument was demonstrated at the IMM stand at BioTechnica 7-9 October 2008 (http://www.biotechnica.de)

25

Table 10 Dissemination activities carried out so far in the project

Completed activities (date)	Type / Reference	Type of audience	Countries addressed	Size of audience	Partner responsible
2006-01-23	Press release by ICSRC on DMMC[14] webpage	Public	Ireland	Large	Coombe
2006-01-30	Project description on homepage	Public	All	Large	BioFluidix
2006 November	Radio interview (Kanal24) of Dr. Michal Mielnik, SINTEF [15]	Public	Norway	Large	SINTEF
2006-10-02	Television broadcast (feature in health magazine "PULS" at NRK, the Norwegian Broadcasting Corporation)	Public	Norway	Large	NorChip
2007-12-14	Christmas Mailing	Scientific, Diagnostic, Technical	Worldwide	200	BioFluidix
2008-10-17	Insight-article "Helse i hver sensor", Teknisk Ukeblad 35-2008	Technical	Norway	284.000	SINTEF
2008-10-29	Press release by IMM on MicroActive	General	Germany/ Europe	Large	IMM

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[14] DMMC - Dublin Molecular Medicine Centre, www.dmmc.ie

[15] 15 minutes interview broadcast in the Norwegian national radio station „Kanal 24" in a popular-scientific program called „Superstreng" (eng: super string). The interview was on the topic of micro- and nanotechnology in general, MicroActive results were given as an example on Lab-on-a-chip applications.

3 Publishable results

3.1 TopSpot® E-Vision

Personal microarrayer with integrated control

Description

The well proven TopSpot® E microarrayer now comes with an integrated optical quality control for online monitoring of the printing process and for quality assessment of the printed microarrays. Therefore the slide holder has been redesigned to enable optical assess to the microarray from below. Two independent optical cameras are integrated to document and to control the printing process at any stage.

Market applications

- DNA / RNA microarrays

- Protein / antigen microarrays

- Fabrication of bio-chips

Stage of development

Product, see Appendix A

Contact details

BioFluidix GmbH, Germany

Web: www.biofluidix.com
Phone: +49 761 203 7285
Email: info@biofluidix.com

TopSpot® E - Vision

Personal microarrayer with integrated quality control

The well proven TopSpot® E microarrayer now comes with an integrated optical quality control for online monitoring of the printing process and for quality assessment of the printed microarrays. Therefore the slide holder has been redesigned to enable optical assess to the microarray from below. Two independent optical cameras are integrated to document and to control the printing process at any stage.

SpotCheck™ feature

The new SpotCheck™ feature delivers live camera images from the actual printing position. Shape and position of the spots can be detected immediately after dispensing before the liquids evaporate. For optimization of the printing buffers and evaporation conditions the spots can be observed until completely vanished. Pictures can be stored from every print to document the production process and to ensure 100 % quality.

DropCheck™ feature

With the integrated DropCheck™ function stroboscopic images of the droplets can be taken in flight. These pictures provide important information on the quality of the print head's surface coating and the printing parameters. The choice of proper printing parameters for unknown solutions is largely facilitated by this feature!

New Software Release

The new software TopSpot® E 2.0 supports all the new optical features of TopSpot® E - Vision. But also customers using the standard TopSpot® E will appreciate the improved handling and display provided by the new release.

Applications

DNA / RNA microarrays

Protein / antigen microarrays

Fabrication of bio-chips

TopSpot® - Specifications	
Dosage volume:	0.5 nl - 1 nl (single shot volume, volume depends on liquid properties), CV < 5%
Array:	24 or 96 spots at a pitch of 500 µm
Substrates:	All standard slides
Positioning accuracy:	better 50 µm
SpotCheck™:	resolution ~ 900 dpi
DropCheck™:	resolution ~ 2400 dpi
Tested Media:	Aqueous solutions, DMSO, PBS, SSC, glycerol …
Order No.	TS 10010 A
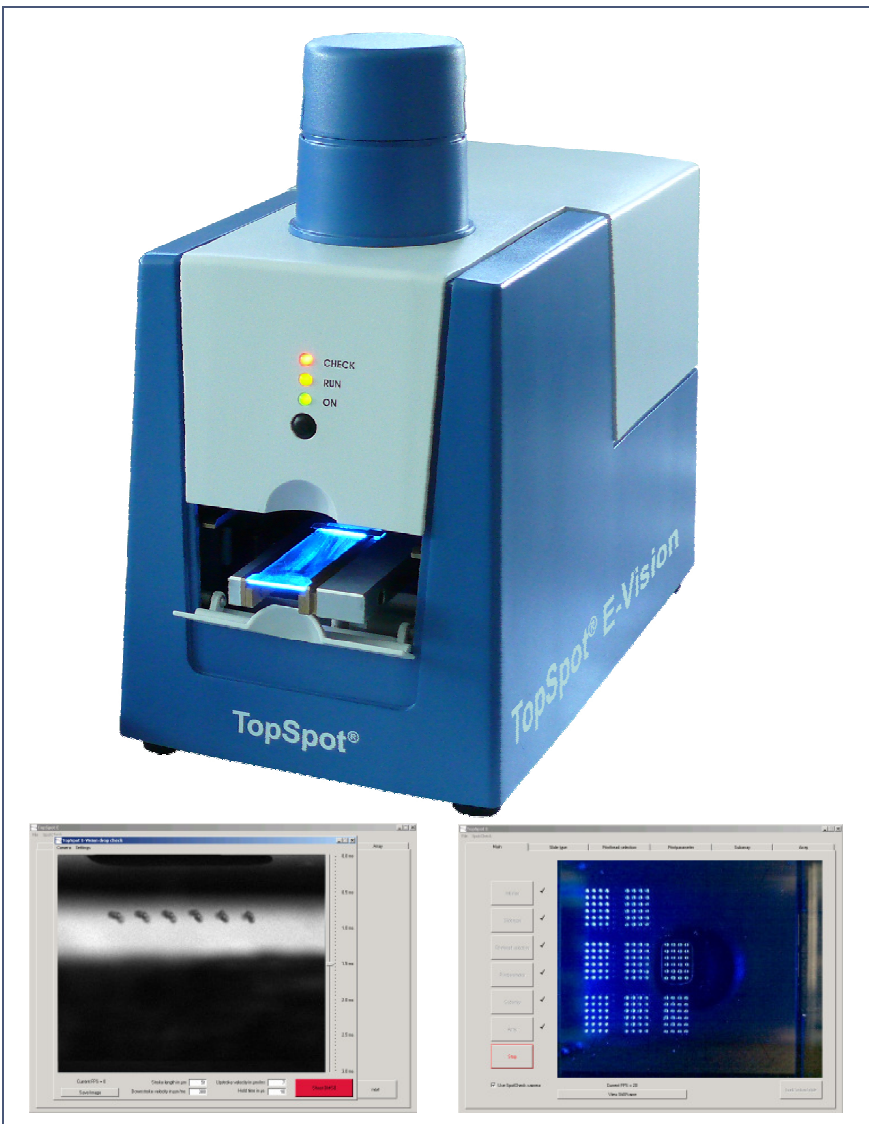
Contact	BioFluidix GmbH		2008 / 01
	Georges-Koehler-Allee 106	phone: + 49 761 203 7285	web: http://www.biofluidix.com
	79110 Freiburg; Germany	fax: + 49 761 203 7284	e-mail: info@biofluidix.com

3.2   BioSpot® systems

Spotting of lateral flow assays and diagnostic substrates

Description

The BioSpot® systems are precise nano pipetting and spotting workstations for small scale laboratory and medium scale production of diagnostic substrates. They allow for the contact-free dosage of liquid droplets in the range from a few nanoliters up to some microliter. Key element of the system is the PipeJetTM dispensing technology enabling media independent, non-contact pipetting and dispensing with highest precision. The PipeJetTM modules can be configured to work as dispensers fed from a bottle or tube. Alternatively they can be connected to the syringe pump to enable aspiration and dispensing. Re-configuring the system is done within a few minutes! Extending the system up to 8 individual dispensers is possible.

Market applications

- Fabrication of lateral flow assays

- Spotting into microfluidic chips

- Pipetting of microwell plates

Stage of development

Product, see Appendix A

Contact details

BioFluidix GmbH, Germany

BioFluidix GmbH, Germany

Web: www.biofluidix.com
Phone: +49 761 203 7285
Email: info@biofluidix.com

2008-12-05

BioFluidiX

BioSpot® Systems

Spotting of lateral flow assays and diagnostic substrates

The BioSpot® systems are precise nano pipetting and spotting workstations for small scale laboratory and medium scale production of diagnostic substrates. They allow for the contact-free dosage of liquid droplets in the range from a few nanoliters up to some microliter. Key element of the system is the PipeJet™ dispensing technology enabling media independent, non-contact pipetting and dispensing with highest precision. The PipeJet™ modules can be configured to work as dispensers fed from a bottle or tube. Alternatively they can be connected to the syringe pump to enable aspiration and dispensing. Re-configuring the system is done within a few minutes! Extending the system up to 8 individual dispensers is possible.

The x-y-z motion control of the BioSpot® system features an active area of 160 x 130 mm (BioSpot® 160) or 600 x 260 mm (BioSpot® 600). The tray is equipped by default with a card holder for lateral flow-assay production. Customization of the working area is possible to hold substrates like microtiter plates, glass slides, tissue samples etc. Next to the experimental area there is some space accessible by the y-stage only which can be used according to the application requirements for additional substrates, cleaning baths or tubes and reservoirs to aspirate from.

The easy to use software provided with the BioSpot® enables arbitrary pipetting and dispensing sequences. Drawing of lines and printing of dots, arrays and even low resolution graphics is facilitated by the new bitmap import feature. Control buttons can be used to execute and abort the programmed batch sequences directly from the work space. All BioSpot® systems are open for customization of any kind: A camera for online optical quality control or stroboscopic imaging can be integrated as well as other dispensing devices, whether they are from BioFluidix or third party vendors.

BioSpot® - Specifications	
Dosage range:	5 nl - 100 nl single droplet volume 5 nl - 10 µl multiple droplets > 1 µl syringe based pipetting
Dispenser:	Up to 8 individual PipeJet P9, P4.5, or custom made dispensers (4.5 or 9 mm pitch)
Aspiration:	Single channel syringe pump or peristaltic pump
Probe supplying:	Tube or PipeJet-Tip™
Mech. repeatability:	+/- 50 µm
Tray size: (holds membranes, slides or well plates)	BioSpot® 160: 165 x 130 (mm) BioSpot® 600: 600 x 280 (mm)
Working area :	BioSpot® 160: x 160, y 130, z 40 (mm) BioSpot® 600: x 600, y 260, z 40 (mm)
Tested media:	Aqueous solutions, solvents, DMSO, beads, cells, PEG, THF, Acetonitryl, Ethanol, Oils, ...
Electrical connections	90 - 250 V @ 50 / 60 Hz, USB 2.0

pipeJet

Applications	Materials
Fabrication of lateral flow assays	All liquid contaminated materials:
Spotting into microfluidic chips	• are low cost disposables • can be sterilized
Pipetting of microwell plates	• are USP class VI compliant

Contact	BioFluidix GmbH		2008 / 01
	Georges-Koehler-Allee 106	phone: + 49 761 203 7282	web: http://www.biofluidix.com
	79110 Freiburg; Germany	fax: + 49 761 203 7284	e-mail: info@biofluidix.com

Appendix A – Project papers and posters

1. *Comparison of HPV detection technologies: Hybrid capture 2, PreTech^{TM} HPV Proofer and analysis of HPV viral load in HPV16, HPV18 and HPV33 E6/E7 mRNA positive specimens*, H.Keegan et.al., **paper in Journal of Virological Methods** (2008)

2. *RNA amplification chip with parallel micro-channels and droplet positioning using capillary valves*, L.Furuberg et.al., **paper in Microsystem Technologies** (2008)

3. *Development of an Automatic Chip-based Detection of mRNA*, L.Solli et.al., poster at **Discovery to Diagnostics Conference 2008**

4. *Evaluation of HPV DNA and mRNA Detection Technologies for Detecting HPV in Cervical Cytology Specimens*, H.Keegan et.al., Synoposes of papers, **Journal of Pathology Vol 214 (S1), p2A.** (Jan 2008)

5. *The MicroActive project: Automatic detection of disease-related molecular cell activity*, L. Furuberg et.al., **SPIE European Symposium on Microtechnologies for the New Millennium**

6. *Storage and reactivation of enzymes in a disposable, self-contained lab-on-a-chip*, A.Gulliksen et.al., **The 11th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2007**

7. *Sample metering and parallel liquid plug actuation for multiple biochemical assays*, M.Mielnik et.al., **The 11th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2007**

8. *Automated chip-based extraction of HPV mRNA from cervical samples*, T.Baier et.al., **The 11th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2008**

9. *Project information*, **ICT for Health – Resource book of eHealth Projects** (FP6)

Comparison of HPV detection technologies: Hybrid capture 2, PreTect™ HPV-Proofer and analysis of HPV DNA viral load in HPV16, HPV18 and HPV33 E6/E7 mRNA positive specimens

Helen Keegan [a,*], Jamie Mc Inerney [a,b], Loretto Pilkington [a], Petter Grønn [c],
Ivan Silva [c], Frank Karlsen [c], Noel Bolger [a], Catriona Logan [a,d], Liv Furuberg [e],
John O'Leary [a,b], Cara Martin [a,b]

[a] Department of Pathology, Coombe Women and Infants University Hospital, Dublin 8, Ireland
[b] Department of Histopathology, University of Dublin, Trinity College, Dublin 2, Ireland
[c] Norchip AS, 3490 Klokkartsua, Norway
[d] Microbiology Department, Our Lady's Childrens Hospital, Crumlin, Dublin 12, Ireland
[e] SINTEF ICT, MiNaLab Facility, Gaustadalleen 23C, 0373 Oslo, Norway

A B S T R A C T

Human papillomavirus (HPV) testing using molecular methods in liquid based cytology (LBC) specimens may be useful as an adjunct to cervical screening by cytology. We compared the positivity rate of the commercially available HPV DNA method hybrid capture 2 (hc2) and the commercially available E6/E7 mRNA method PreTect™ HPV-Proofer in cytological specimens ($n = 299$).

LBC specimens collected ($n = 299$) represented the following cervical cytological disease categories: Normal ($n = 60$), borderline nuclear abnormalities (BNA) ($n = 34$), CIN1 ($n = 121$), CIN2 ($n = 60$), CIN3 ($n = 24$). Overall, 69% (205/299) of the cases were positive by hc2 and 38% (112/299) of the cases were positive by PreTect™ HPV-Proofer. Concordance rates between the two tests were highest in the high-grade cytology cases (CIN2: 67% and CIN3: 83%) and the normal cytology cases (88%) and lowest in the BNA and CIN1 categories (56% and 52%). HPV DNA viral load analyses were carried out on HPV16 ($n = 55$), HPV18 ($n = 9$) and HPV33 ($n = 13$) samples that were positive by PreTect™ HPV-Proofer.

The sensitivity and specificity of PreTect™ HPV-Proofer and the hc2 DNA test for the detection of high-grade cytology (i.e. CIN2+) were 71.4% and 75.8% vs 100% and 43.7%, respectively.

The relatively low detection rate observed by PreTect™ HPV-Proofer in the whole range of cytological positive cases, combined with a relatively higher specificity and PPV, suggests that PreTect™ HPV-Proofer may be more useful than hc2 for triage and in predicting high-grade disease.

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1. Introduction

Cervical cancer is the second most common cause of mortality due to cancer in women worldwide. Cervical cytology screening for cervical intraepithelial neoplasia (CIN) has reduced the incidence of mortality worldwide and most dramatically in countries where cervical screening programmes have been implemented (van der Aa et al., 2008; Gunnell et al., 2007). However, cervical cytology has limited sensitivity and despite the introduction of liquid based cytology (LBC), the sensitivity of cytological testing for cervical disease relies on multiple screening events and referral to colposcopy, in particular in cases of low-grade or borderline cytology (Wright et al., 2006).

Infection with oncogenic human papillomavirus (HPV) is the main causative agent in the development of cervical cancer. HPV can be detected in 99.7% of invasive cervical carcinomas and high-risk HPV genotypes 16, 18, 31, 33 and 45 are the most commonly identified types in cervical tumours worldwide (Muñoz, 2000; Clifford et al., 2006). Therefore, it has been proposed that the performance of cervical screening programmes could be enhanced through the incorporation of molecular HPV testing (Cuschieri and Cubie, 2005). Most commercially available HPV tests are DNA based and involve the detection of a region of the L1 major capsid-forming gene. The hybrid capture test (hc2), Digene Ltd., UK, is a hybridisation based assay which has the ability to detect HPV DNA from 13 high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and

* Corresponding author. Tel.: +353 14085674.
  E-mail address: keeganh28@gmail.com (H. Keegan).

68. hc2 is currently the only HPV test that has USA Food and Drugs Administration (FDA) approval for in vitro diagnostic use and has been approved for use as an adjunct to cervical screening in the US in women aged 30 years and over (Wright et al., 2004). However, as the overall prevalence of HPV DNA in cervical smear specimens is very high, particularly in young women (Coupé et al., 2008; Insinga et al., 2007; Keegan et al., 2007), and as most infections are transient, the potential use of pan-HPV DNA testing for early detection of cervical cancer is limited and would only marginally reduce the follow-up colposcopy and histology. This highlights the need to investigate additional prognostic markers such as HPV E6/E7 mRNA and HPV viral load for use in cervical screening programmes.

Unlike the hc2 test, which detects HPV DNA, the PreTect™ HPV-Proofer test, developed by NorChip AS (Klokkarstua, Norway), has the ability to detect HPV E6/E7 mRNA transcripts from the five most commonly found oncogenic HPV types: 16, 18, 31, 33 and 45. Expression of E6 and E7 mRNA from high-risk HPV types and the production of functional oncoproteins has been found to be necessary for conversion to malignancy, through the modulation of a number of host tumour suppressor and host regulatory products, such as p53 and retinoblastoma (pRB) (Münger and Howley, 2002). The PreTect™ NASBA assay has also been adapted to a diagnostic biochip format for the detection of HPV16 mRNA (Gulliksen et al., 2005). A further generation of this biochip is under development by the MicroActive Consortium (www.sintef.no/microactive). As the oncogenic potential of an HPV infection depends on the production of E6/E7 viral oncoproteins, the detection of HPV E6/E7 mRNA transcripts may be a more specific test in the determination of underlying pre-cancer disease and the risk potential of an HPV infection.

HPV viral load determination has been suggested as a biomarker for use in the area of cervical screening with adjunctive HPV testing, however its potential application has not yet been determined. Numerous studies have been performed to determine the significance of HPV viral load as a diagnostic or prognostic indicator. It has been postulated that the high viral load resulting from active viral replication may support viral persistence (Ylitalo et al., 2000). Viral load has also been used to determine the likelihood of lesional regression and viral clearance in abnormal cytology (van Duin et al., 2002). The significance of HPV viral load has been particularly associated with HPV16 viral load determination. Some studies have demonstrated that the grade of cervical disease is associated with HPV16 viral load (Lo et al., 2005) and HPV16 viral load has been shown to have the potential to predict the risk of cervical cancer before the development of CIN (Ylitalo et al., 2000; Josefsson et al., 2000). However, other studies have shown that in cervical carcinoma cases, patients with a higher viral load had a more favourable prognosis (Biedermann et al., 2004). This may be related to the process of viral integration into the human genome, which commonly is associated with viral episome loss (Pett et al., 2004). Thus, a correlation between viral load and prognosis may be found up to CIN 1 and 2; however, for the further progression towards CIN3 and cervi-

cal cancer, viral load does not seem to be significant as a prognostic indicator.

In this study, the use of hc2 and PreTect™ HPV-Proofer for the detection of HPV in cervical LBC specimens and in a subset of specimens with histological diagnosis were compared. HPV16, HPV33 or HPV18 DNA viral load in patients with a positive HPV E6/E7 mRNA result was also examined.

2. Materials and methods

2.1. Clinical specimens

Clinical specimens were obtained through the Department of Cytology at the Coombe Women's Hospital, Dublin, Ireland, during 2006. All samples were anonymised and ethical approval for the study was obtained from the Research Ethics Committee at the Coombe Women's Hospital. Cytological diagnosis was performed by specialised cytotechnologists and cytopathologists and diagnosis was consistent with BSCC (British Society for Clinical Cytology) guidelines for classification of abnormal smears (Evans et al., 1986). In total, 299 specimens were examined. The cytological diagnosis for these samples ranged from normal ($n = 60$) and BNA (borderline nuclear abnormalities) ($n = 34$) to CIN grades 1 ($n = 121$), 2 ($n = 60$) and 3 ($n = 24$). In Bethesda terminology, BNA corresponds to atypical squamous cells of undetermined significance (ASCUS), CIN1 corresponds to Low-Grade Squamous Intraepithelial Lesion (LSIL) while CIN2 and CIN3 correspond to High-Grade Squamous Intraepithelial Lesion (HSIL). Histological results following cervical biopsy were available for patients who underwent colposcopy following cytology ($n = 58$). Following preparation of a smear for cytological testing, total nucleic acids or DNA alone were extracted as described below.

2.2. HPV DNA testing by hybrid capture 2

DNA was extracted from an aliquot of 4 mL PreservCyt sample using the Sample Conversion Kit (Digene Ltd., UK) for HPV testing by hybrid capture (hc2, Digene Ltd., UK). The HPV DNA status of the specimens was assessed using the hc2 HPV kit for high-risk HPV detection of types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, according to manufacturer's guidelines.

2.3. HPV E6/E7 mRNA testing by PreTect™ HPV-Proofer

An aliquot of 5 mL PreservCyt was processed for total nucleic acid extraction using the Qiagen M48 BioRobot™ extraction method (Qiagen Ltd., UK). Cell lysis was performed prior to BioRobot™ extraction. Briefly, cells were centrifuged for 12 min @ $1\,130 \times g$, and washed in 1 mL 100% ethanol. Qiagen lysis buffer (Buffer RLT: 400 μL) was added to the cell pellet and the sample was vortexed for 1 min. Samples were transferred to the M48 BioRobot for nucleic acid extraction using the "Custom NorChip" programme in the Qiasoft™ software and the MagAttract™ RNA Cell Mini M48 kit (Qiagen Ltd., UK). Sample volume was set to 400 μL and elution volume was set to 50 μL. Extracted nucleic acids were stored at −80 °C. Samples were assessed for HPV E6/E7 mRNA from types 16, 18, 31, 33 and 45 and for an internal housekeeping control gene U1A, using the PreTect™ HPV-Proofer kit (NorChip AS, Norway), according to manufacturer's guidelines.

2.4. HPV16, HPV33 and HPV18 TaqMan® quantitative PCR

PCR primers and TaqMan® MGBNFQ (minor groove binders non-fluorescent quenchers) probes to the E6 region of HPV16, HPV33 and HPV18 genome were designed using Primer Express

Table 1
Sequences of HPV TaqMan® primers and probes.

Primer/Probe	Sequence (5′–3′)	Length (bp)	Dye
HPV16 E6F	gaacaacattagaacagcaatacaacaa	28	–
HPV16 E6R	tggcttttgacagttaatacacctaatt	28	–
HPV16 TP	ccgttgtgtgatttgt	16	6-FAM MGBNFQ
HPV33 E6F	tgatttgtgccaagcattgg	20	
HPV33 E6R	ttttgcattccacgcactg	19	
HPV33 TP	agacaactatacacaacattgaacta	26	6-FAM MGBNFQ
HPV18 E6F	gaggccagtgccattcgt	18	–
HPV18 E6R	tgtttctctgcgtcgttgga	20	–
HPV18 TP	caaccgagcacgacag	16	6-FAM MGBNFQ

Software Version 2.0 (Applied Biosystems, Foster City, CA, USA) (Table 1), and their specificities were confirmed using BlastN (www.ncbi.nlm.nih.gov/blast). All TaqMan® PCR probes were labeled with fluorescein molecule FAM at the 5′ end (Table 1).

The β-actin housekeeping gene was used as performance and integrity control in a TaqMan® PCR using the TaqMan® β-actin Control Reagents (Cat. No. AB 401846) from Applied Biosystems (Foster City, CA, USA). For HPV16 and HPV18, individual TaqMan® PCR reactions were performed in triplicate in a total volume of 25 μL using 1X Universal PCR Master Mix (Cat. No. AB4304437), 300 nM primers, 200 nM of TaqMan® probe and 1 μL of nucleic acid template, and for β-actin using 200 nM primers and 100 nM of TaqMan® probe. For HPV33, TaqMan® PCR was performed in triplicate in a total volume of 20 μL using 1X Universal PCR Master Mix, 300 nM primers, 200 nM of TaqMan® probe and 1 μL of nucleic acid template. TaqMan® PCRs were performed on an Applied Biosystems 7500 thermal cycler using the recommended Universal PCR Master Mix cycling conditions which were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 1 min. Controls for TaqMan® PCRs included: no template control (water added as template), human control DNA, HPV16, HPV33 and HPV18 E6 plasmid clones. HPV copy number normalization was carried out using β-actin as a housekeeping gene. HPV quantitative TaqMan® PCR was performed by generating standard curves for HPV16, HPV33 and HPV18 E6 genes. This was achieved using serial dilutions of clones of E6 generated from HPV16, HPV33 and HPV18 over a linear dynamic range of 1×10^6 to 1×10^0 copies of HPV per reaction. Total nucleic acid extracted from cytology specimens as described above was amplified and quantified off standard curves.

2.5. Data analysis

HPV detection rates, concordance rates, and sensitivity, specificity and positive predictive values for detecting cytological abnormalities, CIN2+ were calculated for both HPV tests hc2 and PreTect™ HPV-Proofer. As histological data was not available for all cases and the primary objective was to compare HPV tests for the detection of cytological abnormalities we used cytology as the gold-standard test for the majority of these calculations except where otherwise stated. Sensitivity and specificity were calculated using the following formula whereby:

	Test +	Test −	Sum
Gold standard +	a	c	g
Gold standard −	b	d	h
Sum	e	f	n

Population size: n, sensitivity: a/g, PPV: a/e, specificity: d/h.

3. Results

3.1. Comparison of HPV detection by hybrid capture 2 (hc2) and PreTect™ HPV-Proofer

Cytological diagnoses of specimens recruited to the study are outlined in Table 2. Cytological diagnosis was performed by specialised cytotechnologists and cytopathologists and diagnosis was

Table 2
Cytological diagnosis of cervical specimens (n = 299).

Cytology	Normal	BNA[a]	CIN[b] 1	CIN2	CIN3
No. of samples (n = 299)	60 (20%)	34 (11%)	121 (41%)	60 (20%)	24 (8%)

[a] Borderline nuclear abnormality.
[b] Cervical intraepithelial neoplasia.

Table 3
Comparison of HPV detection technologies for the detection of HPV in cytology specimens (n = 299).

Cytology	HC2		PreTect™ HPV-Proofer		Concordance
	+	−	+	−	
Normal (n = 60)	5/60	55/60	2/60	58/60	88%
BNA[a] (n = 34)	16/34	18/34	5/34	29/34	19%
CIN1[b] (n = 121)	100/121	21/121	45/121	76/121	40%
CIN2 (n = 60)	60/60	–	40/60	20/60	67%
CIN3 (n = 24)	24/24	–	20/24	4/24	83%

[a] Borderline nuclear abnormality.
[b] Cervical intraepithelial neoplasia.

consistent with BSCC (British Society for Clinical Cytology) guidelines for classification of abnormal smears (Evans et al., 1986) which uses the following categories to grade cytological specimens Normal, CIN grades 1–3 and Borderline Nuclear Abnormalities (BNA). The HPV status of the clinical specimens was determined by hc2 and by PreTect™ HPV-Proofer.

The overall prevalence of HPV in the study sample population (n = 299) was 68.6% by hc2 and 37.5% by PreTect™ HPV-Proofer. HPV DNA was detected in a higher percentage of specimens from each cytological category than HPV mRNA (Table 3). HPV DNA was detected in 8.3% of normal, 47.1% of BNA, 83% of CIN1, and 100% of CIN2 and CIN3 specimens, while HPV mRNA was detected in 3.3% of normal, 14.7% of BNA, 37% of CIN1, 67% of CIN2 and 83% of CIN3 specimens. There was good concordance between the methods for the detection of HPV in cases with high-grade cytology CIN3 (83%) and normal cytology (88%) (Table 3; Fig. 1). Concordance rates in the CIN2 category were 67% while the BNA/CIN1 category was lower at 19% and 40%, respectively.

3.2. Clinical sensitivity and specificity of hc2 and PreTect™ HPV-Proofer

In this cohort of specimens, which represents a broad spectrum of cervical pre-cancer disease categories, the positive predictive value and specificity of the hc2 DNA test was 97.6% and 91.7%, respectively, compared with 98.2% and 96.7% of the PreTect™ HPV-Proofer E6/E7 mRNA test for the detection of overall abnormal cytology. The sensitivity of hc2 and PreTect™ HPV-Proofer were 83.7% and 46%, respectively. If we specifically focus on high-grade disease (cytological CIN2+), the sensitivity of PreTect™
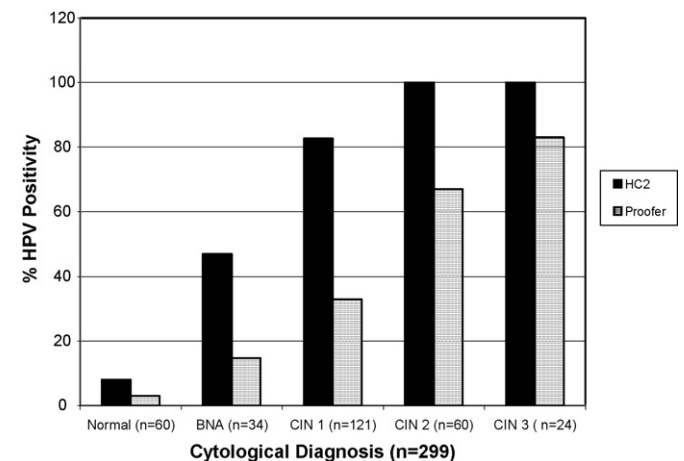

Fig. 1. Comparison of HPV detection technologies: hybrid capture (hc2) for the detection of HPV DNA and PreTect™ HPV-Proofer for the detection of HPV E6/E7 mRNA in cervical cytology specimens.

Table 4
HPV genotype prevalence by PreTect™ HPV-Proofer (n = 112).

Cytology	PreTect™ HPV-Proofer genotype (no. of positive samples)										Total
	16	18	31	33	45	16&33	18&33	16&18	31&33	33&45	
Normal n = 60	2										2
BNA n = 34	2		1	1		1					5
CIN1 n = 121	24	3	1	7	6	2	1	1	1		46
CIN2 n = 60	26	5	3	4	1					1	40
CIN3 n = 24	13	2		2		3					20

HPV-Proofer increases to 71.4% with a specificity of 75.8%, while the sensitivity and specificity of the hc2 DNA test are 100% and 43.7% respectively. These figures were calculated using cytology as the gold-standard method. The primary objective of this study was to compare HPV detection technologies in cytology specimens and to correlate this with cytological diagnoses. The majority of specimens examined in this study were normal and low-grade disease (n = 215). Therefore, in the majority of cases, tissue biopsies following colposcopy were not available. However, histological data on a small subset of the study group (n = 58) were available for analysis and comparison.

Using the follow-up histology result (CIN2+) as the gold standard for these specimens, clinical sensitivity, specificity, and positive predictive value (PPV) could be calculated. Given that histology data was only available for women with a cytologically high-grade lesion the authors recognise that this type of calculation will have a strong verification bias towards cytology. With this in mind, the sensitivity and PPV of hc2 were 100%, and 87.9%, respectively, and of PreTect™ HPV-Proofer, 74.5%, and 92.7% respectively.

3.3. HPV genotype distribution in cervical cytology specimens

In total, 112 of the 299 cases (37%) tested were positive for HPV E6/E7 mRNA using the PreTect™ HPV-Proofer assay. HPV16 was the most prevalent single type infection, representing 60% of the positive cases, followed by HPV33 (12.5%), HPV18 (9%), HPV45 (6.3%) and HPV31 (4.5%) (Table 4). In addition, co-infection with more than one HPV type was observed in 10/112 (9%) of the positive cases. This co-infection was predominantly with HPV16 and 33 (6 of 10 co-infected cases). These multiple infections with two or more types of HPV were found across each of the cytology disease categories; BNA (1/34), CIN1 (5/121), CIN2 (1/60) and CIN3 (3/24) (Table 4).
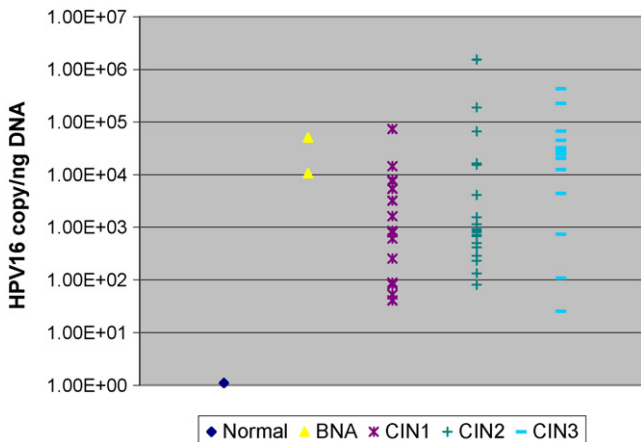

Fig. 2. Distribution of HPV16 viral load in cervical cytology specimens.

3.4. HPV16, HPV33 and HPV18 viral load quantitation by TaqMan® PCR

HPV16 DNA viral load was determined in 55 cytology specimens positive for HPV16 E6/E7 mRNA by PreTect™ HPV-Proofer. The range of HPV16 copy numbers across all of the specimens varied from 0 to 1.5×10^6 copies/ng. We did not detect a statistically significant difference in HPV16 viral load across the cytology categories (P = 0.03) (Fig. 2). However, it should be noted that the number of HPV16 positive cases in each group was low. The viral load of HPV33 was determined in 13 specimens positive for HPV33 E6/E7 mRNA by PreTect™ HPV-Proofer. The range of HPV33 copy numbers across all of the specimens varied from 6.49×10^2 to 1.6×10^5 copies/ng (Table 5) and there was no correlation between HPV33 viral load and cytology. The viral load of HPV18 was determined in nine specimens positive for HPV18 E6/E7 mRNA by PreTect™ HPV-Proofer. The range of HPV18 copy numbers across all of the specimens varied from 1×10^2 to 1.8×10^4 copies/ng (Table 5) and no correlation was seen between HPV18 viral load and cytology.

4. Discussion

Adjunctive molecular testing of cytology specimens for HPV has mainly involved the use of DNA based assays, in particular the hc2 test. However, as HPV DNA is highly prevalent and the life-time risk for HPV is estimated to be up to 80%, more specific biological markers for early detection of cervical cancer are needed. It is now widely known that the high-risk HPV oncoproteins E6 and E7 are necessary for conversion to and maintenance of malignancy. With this in mind, the PreTect™ HPV-Proofer assay has been designed to detect type-specific E6/E7 mRNA transcripts of the five most commonly found high-risk types in cervical carcinomas (Molden et al., 2007). Our study compares the detection of high-risk HPV by hc2 and PreTect™ HPV-Proofer in liquid based cervical cytology specimens ranging in their cytological diagnoses from normal to CIN3. Follow-up histology results were available for 58 samples with cytological CIN2 or CIN3. HPV DNA viral load analyses were performed on a subset of HPV16, HPV33 and HPV18 E6/E7 mRNA positive specimens with various cervical cytological diagnoses.

The overall prevalence of HPV DNA (68.6%) as detected by hc2 was greater than that of E6/E7 mRNA (37.5%) as detected by PreTect™ HPV-Proofer, and this was the case for all cytological categories including CIN2 and CIN3. This is to be expected, as not all HPV DNA infected cells will have high-level expression of E6/E7 mRNA. In addition the limitations of the PreTect™ HPV-Proofer test with respect to the smaller number of HPV genotypes detected (5 high-risk types) compared with hc2 which detects 13 high-risk types, may contribute to the higher detection rate observed for hc2. Similar data presented by Andersson et al. (2006) showed that 69% of women with any grade of cytological abnormality, detected in a population-based primary screening setting, were HPV DNA positive using the Quantovir HPV detection system (Quantovir AB, Sweden) while 40% were HPV E6/E7 mRNA positive by PreTect™ HPV-Proofer (Andersson et al., 2006). More importantly, in the

present study, HPV prevalence in women with BNA was 47% and 15% for HPV DNA and E6/E7 mRNA, respectively and in women with CIN1, 83% and 37%, respectively. This preliminary data would suggest that secondary screening using PreTect™ HPV-Proofer may have a higher triage effect and may be more cost effective, reducing the number of positive cases that are referred to colposcopy. However, a larger trial with appropriate patient follow-up is required to investigate this. Also, a high rate of concordance between HPV DNA and mRNA detection was observed in specimens with normal cytology and CIN3 cytological categories. In the normal cytological category, the 88% concordance rate predominantly reflected HPV negative results and the rate of detection of HPV DNA (8.3%) was higher than that of E6/E7 mRNA (3.3%).

For samples in the BNA and CIN1 categories, a much lower rate of concordance was observed between the HPV DNA test and the mRNA test (18% and 40%, respectively) (Table 2, Fig. 2). This is to be expected given that less than 20% of BNA and CIN1 cases progress to pre-cancer and that the two technologies detect different targets (DNA vs E6/E7 mRNA), with hc2 detecting a larger number of HPV genotypes (13 high-risk HPV types) than the PreTect™ HPV-Proofer assay (5 high-risk HPV types). There is evidence to suggest that detection of a HPV mRNA result as opposed to a DNA result within these categories may be indicative of an infection of higher biological significance and a positive HPV E6/E7 mRNA result may be of greater prognostic value (Molden et al., 2005). This study, which looked specifically at the BNA/CIN1 categories using PreTect™ HPV-Proofer, found that a positive HPV mRNA result in women with low-grade abnormalities, increased the relative risk of a histological CIN2+ diagnosis by 69.8-fold (Molden et al., 2005). Another study which used both DNA and mRNA detection techniques on normal cytology specimens has demonstrated that HPV mRNA detection is less sensitive but more specific than HPV DNA for the detection of disease during a 2 years follow-up (Cuschieri et al., 2004). In the same study, women who were positive for both HPV DNA and mRNA at baseline were more likely to have a persistent HPV infection. In the American ASCUS LSIL study (ALTS), it was concluded that the detection of HPV DNA by hc2 in women with LSIL was of limited value in their clinical management (Schiffman and Solomon, 2003). Combining the data from these published studies would suggest that detection of mRNA as opposed to HPV DNA in women with BNA/CIN1 cytology might reduce the number of women who are referred to follow-up without compromising the identification of women most likely to progress to CIN2+ or invasive cancer. In our study, a higher rate of concordance between the hc2 and the PreTect™ HPV-Proofer test was observed in the CIN3 (83%) categories (Fig. 1, Table 3), however this study lacks patient follow-up and has limited information in relation to histological outcomes. Based on these preliminary findings a larger trial examining the impact of HPV DNA and mRNA testing across all disease categories with appropriate patient follow is urgently required.

The overall aim of this study was to examine the utility of HPV DNA and mRNA detection technologies for detecting HPV in cytological specimens. For this reason, calculations in relation to specificity, sensitivity and PPV are based on cytology as gold-standard method for detecting cytological abnormalities. Overall, the sensitivity of the DNA based test for the detection of abnormal cytology was 83.7% while the sensitivity of the PreTect™ HPV-Proofer assay was much lower at 46% indicating that fewer false negatives were detected by the hc2 assay. Indeed the high negative predictive value of the hc2 assay has made it very suitable for use in large scale clinical studies such as the ALTS trial (Schiffman et al., 2003). The positive predictive value and specificity of Pre-Tect HPV-Proofer (98.2% and 96.7%) were greater than those of hc2 (97.6% and 91.7%) for the detection of abnormal cytology. Focussing specifically on the detection of high-grade disease, i.e. CIN2+ in

cytology, the sensitivity of the mRNA test (71.4%) was lower than that of the DNA test (100%), however the specificity of the mRNA test (75.8%) was much greater than that of the DNA test (43.7%). A recent study by Varnai et al. (2008) reported similar rates of sensitivity (87%) and specificity (56%) of the PreTect™ HPV-Proofer test for the detection of prevalent cytological disease (HSIL). In CIN2+ cases with histological follow-up ($n=58$), the sensitivity of the DNA test (100%) was greater than that of the mRNA test (74.5%) for the detection of CIN2+, however the PPV of the mRNA test was higher than that of the DNA test (92.7% vs 87.9%). The higher PPV of a PreTect™ HPV-Proofer positive result may be explained biologically, as the production of neoplastic lesions requires a stable expression from the E6 and E7 genes and a subsequent high and stable production of the transforming viral oncoproteins. HPV DNA detection at a single time point by hc2 indicates however viral presence only and would not give any information about the presence of a transforming infection.

In our study population, HPV16 was the most predominant single type infection (59%), followed by HPV33 (12.5%), HPV18 (9%), HPV45 (6.2%) and HPV31 (4.5%. HPV16 was the most prevalent HPV type in all cytological categories, whereas the prevalence of the other HPV types detected by PreTect™ HPV-Proofer varied. The rate of co-infections was 8% (9/112), with HPV16 and 33 being the most commonly occurring co-infection. Other studies that have used the PreTect™ HPV-Proofer assay have detected multiple type infections in 2.8% of cases of various grade cytology (Andersson et al., 2006) and in 5.6% of women with normal cytology but a positive DNA result for either HPV16, 18, 31, 33 or 45 (Cuschieri et al., 2004). By comparison, it is estimated that 20-30% of women infected with HPV DNA will have multiple type infections regardless of cytology (Moscicki et al., 2006). In a recent Irish study, which genotyped HPV positive samples from general practitioners in the Dublin area by sequencing of the L1 gene, the most commonly detected HPV types found that are included in the PreTect™ HPV-Proofer assay were HPV16 (20%) followed by HPV18 (12%) and HPV33 (8%) (Keegan H et al., 2007), which corresponds with the findings of our study. The higher prevalence of HPV in our study population reflects the greater number of high-grade cytology specimens examined.

In addition to HPV type, there is evidence to suggest that HPV viral load can be used to determine the grade of CIN, increasing with disease severity (Healey et al., 2001; Sun et al., 2002; Dalstein et al., 2003). The ALTS study, conducted on 2198 women with ASCUS, showed that high viral load can be used to identify women with ASCUS who had an underlying CIN which was confirmed on histological follow-up (Sherman et al., 2003). In our study, no correlation between HPV viral load and grade of abnormal cytology was observed for HPV16, HPV18 or HPV33 infected patients; however, it should be noted that viral load was only assessed in cases expressing E6/E7 mRNA. Moreover, there is further evidence to suggest that there is no correlation between HPV viral load and expression of HPV16 or HPV18/45 E6/E7 mRNA in cases with abnormal cytology (Andersson et al., 2006). This may be explained by viral integration into the host genome and the observed loss of viral replication upon integration (Doorbar, 2006). It is therefore plausible that in patients with high-grade disease, the HPV DNA viral load is relatively low compared to mRNA expression. In addition, increased stable expression of E6/E7 is commonly a result of deregulated transcription of integrated HPV DNA copies, in turn being independent of the number of integrated copies.

One concern in evaluating the use of HPV viral load as a biomarker for cervical screening is the method of cervical scraping that is used. In our study, the range of viral load was much less for HPV18 and HPV33 positive specimens than for HPV16. It has been shown previously, that HPV18 resides in the lower layers of the epidermis and is to a higher degree associated with glandu-

lar lesions and adenocarcinomas than squamous cell carcinomas (Castellsagué et al., 2006). Thus, sampling methods may explain the lower overall HPV18 viral load. Alternatively, the natural history of HPV infections may be type specific and is yet not well understood.

In conclusion, no correlation between HPV viral load and grade of abnormal cytology was observed for HPV16, HPV18 or HPV33 E6/E7 mRNA positive patients. Further studies are needed in order to reveal whether testing for viral load may have a potential value for use in cervical cancer screening. Moreover, our results suggest that the detection of E6/E7 mRNA by PreTect HPV-Proofer may indicate underlying high-grade cervical abnormalities and pre-cancer, supported by the higher specificity and PPV compared with the hc2 DNA test for the detection of abnormal cytology, in particular for the detection of CIN2+.

References

Andersson, S., Hansson, B., Norman, I., Gaberi, V., Mints, M., Hjerpe, A., Karlsen, F., Johansson, B., 2006. Expression of E6/E7 mRNA from 'high risk' human papillomavirus in relation to CIN grade, viral load and p16INK4a. Int. J. Oncol. 29, 705–711.

Biedermann, K., Dandachi, N., Trattner, M., Vogl, G., Doppelmayr, H., Moré, E., Staudach, A., Dietze, O., Hauser-Kronberger, C., 2004. Comparison of real-time PCR signal-amplified in situ hybridization and conventional PCR for detection and quantification of human papillomavirus in archival cervical cancer tissue. J. Clin. Microbiol. 42, 3758–3765.

Castellsagué, X., Díaz, M., de Sanjosé, S., Muñoz, N., Herrero, R., Franceschi, S., Peeling, R.W., Ashley, R., Smith, J.S., Snijders, P.J., Meijer, C.J., Bosch, F.X., International Agency for Research on Cancer Multicenter Cervical Cancer Study Group, 2006. Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. J. Natl. Cancer Inst. 98, 303–315.

Clifford, G., Franceschi, S., Diaz, M., Muñoz, N., Villa, L.L., 2006. Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. Vaccine 24 (Suppl. 3), S26–S34.

Coupé, V.M., Berkhof, J., Bulkmans, N.W., Snijders, P.J., Meijer, C.J., 2008. Age- dependent prevalence of 14 high-risk HPV types in the Netherlands: implications for prophylactic vaccination and screening. Br. J. Cancer. 98, 646–651.

Cuschieri, K.S., Cubie, H.A., 2005. The role of human papillomavirus testing in cervical screening. J. Clin. Virol. 32 (Suppl. 1), S34–S42.

Cuschieri, K.S., Whitley, M.J., Cubie, H.A., 2004. Human papillomavirus type specific DNA and RNA persistence—implications for cervical disease progression and monitoring. J. Med. Virol. 73, 65–70.

Dalstein, V., Riethmuller, D., Prétet, J.L., Le Bail Carval, K., Sautière, J.L., Carbillet, J.P., Kantelip, B., Schaal, J.P., Mougin, C., 2003. Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. Int. J. Cancer. 106, 396–403.

Doorbar, J., 2006. Molecular biology of human papillomavirus infection and cervical cancer. Clin. Sci. (Lond.) 110, 525–541.

Evans, D.M., Hudson, E.A., Brown, C.L., Boddington, M.M., Hughes, H.E., Mackenzie, E.F., Marshall, T., 1986. Terminology in gynaecological cytopathology: report of

the Working Party of the British Society for Clinical Cytology. J. Clin. Pathol. 39, 933–934.

Gulliksen, A., Solli, L.A., Drese, K.S., Sörensen, O., Karlsen, F., Rogne, H., Hovig, E., Sirevåg, R., 2005. Parallel nanoliter detection of cancer markers using polymer microchips. Lab. Chip. 5, 416–420.

Gunnell, A.S., Ylitalo, N., Sandin, S., Sparén, P., Adami, H.O., Ripatti, S., 2007. A longitudinal Swedish study on screening for squamous cell carcinoma and adenocarcinoma: evidence of effectiveness and overtreatment. Cancer Epidemiol. Biomarkers Prev. 16, 2641–2648.

Healey, S.M., Aronson, K.J., Mao, Y., Schlecht, N.F., Mery, L.S., Ferenczy, A., Franco, E.L., 2001. Oncogenic human papillomavirus infection and cervical lesions in aboriginal women of Nunavut, Canada. Sex. Transm. Dis. 28, 694–700.

Insinga, R.P., Dasbach, E.J., Elbasha, E.H., Liaw, K.L., Barr, E., 2007. Progression and regression of incident cervical HPV 6 11 16 and 18 infections in young women. Infect. Agent. Cancer 2, 15.

Josefsson, A.M., Magnusson, P.K., Ylitalo, N., Sørensen, P., Qwarforth-Tubbin, P., Andersen, P.K., Melbye, M., Adami, H.O., Gyllensten, U.B., 2000. Viral load of human papillomavirus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. Lancet 355, 2189–2193.

Keegan, H., Ryan, F., Malkin, A., Griffin, M., Lambkin, H., 2007. Human papillomavirus prevalence and genotypes in an opportunistically screened Irish female population. Br. J. Biomed. Sci. 61, 18–22.

Lo, K.W., Yeung, S.W., Cheung, T.H., Siu, N.S., Kahn, T., Wong, Y.F., 2005. Quantitative analysis of human papillomavirus type 16 in cervical neoplasm: a study in Chinese population. J. Clin. Virol. 34, 76–80.

Molden, T., Kraus, I., Skomedal, H., Nordstrøm, T., Karlsen, F., 2007. PreTect HPV-Proofer: real-time detection and typing of E6/E7 mRNA from carcinogenic human papillomaviruses. J. Virol. Methods. 142, 204–212.

Molden, T., Nygård, J.F., Kraus, I., Karlsen, F., Nygård, M., Skare, G.B., Skomedal, H., Thoresen, S.O., Hagmar, B., 2005. Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV-Proofer and consensus PCR: A 2-year follow-up of women with ASCUS or LSIL Pap smear. Int. J. Cancer 114, 973–976.

Moscicki, A.B., Schiffman, M., Kjaer, S., Villa, L.L., 2006. Chapter 5: Updating the natural history of HPV and anogenital cancer. Vaccine. 24 (Suppl. 3), S42–S51.

Münger, K., Howley, P.M., 2002. Human papillomavirus immortalization and transformation functions. Virus Res. 89, 213–228.

Muñoz, N., 2000. Human papillomavirus and cancer: the epidemiological evidence 2000. J. Clin. Virol. 19, 1–5.

Pett, M.R., Alazawi, W.O., Roberts, I., Dowen, S., Smith, D.I., Stanley, M.A., Coleman, N., 2004. Acquisition of high-level chromosomal instability is associated with integration of human papillomavirus type 16 in cervical keratinocytes. Cancer Res. 15, 1359–1368.

Schiffman, M., Solomon, D., 2003. Findings to date from the ASCUS-LSIL Triage Study (ALTS). Arch. Pathol. Lab. Med. 127, 946–949.

Sherman, M.E., Wang, S.S., Wheeler, C.M., Rich, L., Gravitt, P.E., Tarone, R., Schiffman, M., 2003. Determinants of human papillomavirus load among women with histological cervical intraepithelial neoplasia 3: dominant impact of surrounding low-grade lesions. Cancer Epidemiol. Biomarkers Prev. 10, 1038–1044.

Sun, C.A., Liu, J.F., Wu, D.M., Nieh, S., Yu, C.P., Chu, T.Y., 2002. Viral load of high-risk human papillomavirus in cervical squamous intraepithelial lesions. Int. J. Gynaecol. Obstet. 76, 41–47.

van der Aa, M.A., Pukkala, E., Coebergh, J.W., Anttila, A., Siesling, S., 2008. Mass screening programmes and trends in cervical cancer in Finland and the Netherlands. Int. J. Cancer 122, 1854–1858.

van Duin, M., Snijders, P.J., Schrijnemakers, H.F., Voorhorst, F.J., Rozendaal, L., Nobbenhuis, M.A., van den Brule, A.J., Verheijen, R.H., Helmerhorst, T.J., Meijer, C.J., 2002. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. Int. J. Cancer 98, 590–595.

Varnai, A.D., Bollmann, M., Bankfalvi, A., Speich, N., Schmitt, C., Griefingholt, H., Kovács, K., Klozoris, C., Bollmann, R., 2008. Predictive testing of early cervical pre-cancer by detecting human papillomavirus E6/E7 mRNA in cervical cytologies up to high-grade squamous intraepithelial lesions: diagnostic and prognostic implications. Oncol Rep. 19, 457–465.

Wright Jr., T.C., Massad, L.S., Dunton, C.J., Spitzer, M., Wilkinson, E.J., Solomon, D, 2006. American Society for Colposcopy and Cervical Pathology-Sponsored Consensus Conference 2006. 2006 consensus guidelines for the management of women with cervical intraepithelial neoplasia or adenocarcinoma in situ. Am. J. Obstet. Gynecol. 197, 340–345.

Wright Jr., T.C., Schiffman, M., Solomon, D., Cox, J.T., Garcia, F., Goldie, S., Hatch, K., Noller, K.L., Roach, N., Runowicz, C., Saslow, D., 2004. Interim guidance for the use of human papillomavirus DNA testing as an adjunct to cervical cytology for screening. Obstet. Gynecol. 103, 304–309.

Ylitalo, N., Sørensen, P., Josefsson, A.M., Magnusson, P.K., Andersen, P.K., Pontén, J., Adami, H.O., Gyllensten, U.B., Melbye, M., 2000. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study. Lancet 355, 2194–2198.

TECHNICAL PAPER

RNA amplification chip with parallel microchannels and droplet positioning using capillary valves

**Liv Furuberg · Michal Mielnik · Anja Gulliksen ·
Lars Solli · Ib-Rune Johansen · Jörg Voitel ·
Tobias Baier · Lutz Riegger · Frank Karlsen**

Abstract We present results from the MicroActive project which develops an instrument for molecular diagnostics. The instrument is first tested for patient screening for a group of viruses causing cervical cancer. Two disposable polymer chips with reagents stored on-chip are developed and will be inserted into the instrument for each patient sample analysis. The first chip will perform nucleic acid extraction from patient epithelial cervical cells, while mRNA amplification and fluorescent detection takes place in the second chip. This paper reports results on the amplification chip. Purified sample is inserted into the chip and split into ten smaller droplets for simultaneous amplification and detection of ten viruses. The droplets move in parallel channels, each with two chamber extensions containing dried reagents. Experimental results on parallel droplet movement using one external pump combined with hydrophobic restrictions show that the parallel droplet positions can be controlled. There are four valves with increasing burst pressures between 800 and 4,500 Pa in each parallel channel, positioning the droplets in metering zones and reaction chambers. The re-hydration times for the dried reagents in micro chambers have been monitored. After sample insertion, uniform concentration of the reagents in the droplet was reached after respectively 60 s and 10 min. These times are acceptable for successful amplification. Finally we show positive amplification of HPV type 16 viruses in a micro chamber.

L. Furuberg (✉) · M. Mielnik · I.-R. Johansen · J. Voitel
SINTEF Microsystems and Nanotechnology, Forskningsveien 1,
0314 Oslo, Norway
e-mail: liv.furuberg@sintef.no

A. Gulliksen · L. Solli · F. Karlsen
NorChip AS, Industriveien 8, 3490 Klokkarstua, Norway

L. Furuberg · A. Gulliksen
University of Oslo, 0316 Oslo, Norway

T. Baier
Institut für Mikrotechnik Mainz GmbH, Carl-Zeiss-Straße 18-20,
55129 Mainz, Germany

L. Riegger
IMTEK, Georges-Koehler-Allee 103, 79110 Freiburg, Germany

1 Introduction

The aim of the MicroActive project (MicroActive 2006) is to develop an instrument for molecular diagnostics intended for use in the doctors' office. The instrument will first be used for patient screening for a group of viruses causing cervical cancer, but the technology platform will have a wide applicability. Microfluidics and biotechnology form the basis for the development. Fully automated diagnosis systems based on microfluidics will widen the availability of advanced diagnostics for all citizens due to both higher availability and lower cost (Yager et al. 2006). Also, reducing the time from patient testing to diagnosis avoids anxiety and enables earlier treatment.

Human papillomavirus (HPV) is a group of sexually transmitted viruses that is related to the development of cervical cancer (Jenkins 2001; Walboomers et al. 1999). Cervical cancer is the second most common cancer type among women worldwide. Oncogenic proteins related to the five HPV-types 16, 18, 31, 33, and 45 are related to more than 97% of all cases of HPV-induced cervical cancer in Europe and these HPV viruses are the target of the NorChip PreTect HPV-Proofer©. The task of MicroActive is to miniaturize and automate the protocol of the PreTect

HPV-Proofer[©], namely mRNA amplification and detection. Compared to commonly used approaches (e.g. DNA PCR amplification and immunoassay methods), mRNA detection largely avoids false positive results and has a high sensitivity (Lie et al. 2005; Kraus et al. 2004; Norchip 2007). It can currently be used to detect pre-cancer, cancer, STD and a range of respiratory diseases, to mention a few. New bio-markers are continuously being developed.

Within the MicroActive project the partners are working on:

1. Development of one disposable microfluidic chip for sample preparation including reservoirs containing all liquid reagents necessary to perform sample preparation consisting of cell concentration, lysis and nucleic acid purification.

2. Development of a second disposable microfluidic chip for multiple target amplification and fluorescent detection with dried spotted reagents stored in microchannels.

3. Development of manufacturing methods for spotting and drying of reagents, surface coating and patterning, and polymer chip lamination that prevents inhibition of the bio-molecular processes.

4. Performing multi target detection from a single sample. This is possible due to simultaneous amplification and detection in separate parallel detection channels. Each parallel channel contains dried reagents for amplification of a disease (here: HPV type) specific marker.

5. Performing isothermal amplification of mRNA by nucleic acid sequence-based amplification (NASBA) (Compton 1991).

6. Performing tests on clinical samples, using test-chips for separate functions.

7. Providing the repeatable and stable fluid control required by a commercial system through use of simple pumps in combination with surface modification.

8. Developing an instrument without manual protocols.

9. Testing the instrument on clinical specimens and compare to gold standards.

10. Addressing factors such as reliability, usability and cost of the total instrument—factors which are crucial to acceptance by health care professionals.

In this paper we will focus on reporting experimental results on separate tests of the fluidic and biological functions on the second chip, the NASBA amplification and fluorescent detection chip, related to the points 2–7 above. The advantage of this NASBA amplification chip compared to previous microfluidic solutions for nucleic acid amplification is firstly that 11 mRNA markers can be detected simultaneously. Many symptoms may be caused

by a range of viruses that now can be tested in one run. By splitting the sample droplet into e.g. 11 smaller droplets, each droplet can be mixed with dried primers identifying one marker and the 11 amplifications are performed in parallel channels before the positive/negative signal is read out separately for each channel. Cepheid (2007) performs PCR simultaneous amplification of four genes in one chamber, but this test is restricted to four genes only, due to detection of fluorescence at four separable wavelengths. The alternative to NASBA amplification of mRNA is reverse-transcriptase PCR (RT-PCR) which has not been an active field within microfluidics (Lien et al. 2007). NASBA has another advantage over RT-PCR, being a constant temperature reaction; it requires a simpler temperature control.

Earlier, positive NASBA reactions with wet, premixed reagents have been demonstrated on chip (Gulliksen et al. 2004, 2005). The new aspect of the present work is to dry the reagents in the micro chambers and to perform the amplifications in metered, separate nanoliter sized droplets. Droplet based analysis in a lab-on-a-chip was described by Burns (1998). We have reported splitting of the sample containing nucleic acids into 11 metered droplets in another paper (Mielnik et al. 2007). Metered sample droplets will be pushed into different channels where they mix with different dried regents for multiple analyses. Work on multiple analyses using different types of stored reagents have been performed by Weigl et al. (2006), for air drying and reactivation of PCR mixes in conventional macro scale volumes using 96-well plates. PCR in microchips have been performed by numerous groups, focusing e.g. on temperature cycling speed, miniaturization and chamber architecture and post-amplification detection (Zhang et al. 2006). For self-contained systems with pre-stored reagents, both liquid reagents and dried reagents have been introduced as plausible solutions for long-term storage of reagents for immunoassays on-chip (Linder et al. 2005).

Here we report experiments on parallel droplet movement control using one external pump only, combined with valves based on hydrophobic restrictions (Oh and Ahn 2006). We show seven droplets moving in seven parallel channels, each stopping controllable in three reaction chambers along the channel. Due to heating requirements for the reactions and localized heaters, it is important that all droplets are in the same reaction chamber at the same time. We also monitored the re-hydration times for the two necessary dried reagents using fluorescent markers and a confocal microscope. After sample droplet insertion, concentration of the reagents in the liquid droplet was not changing significantly in time after respectively 60 s and 10 min. These times are acceptable for successful amplification. Finally we have shown positive amplification of

HPV type 16 using dried enzymes stored in micro chambers. The re-activation of dried reagents is very sensitive to the environment, spotting and drying procedure, and positive amplifications are considered to be a breakthrough for the project.

2 NASBA amplification chip

In the NASBA amplification chip, purified RNA is mixed with dried reagents, heated and amplified. The amplified RNAs are detected by fluorescence. The input to the NASBA amplification chip is purified nucleic acids in elution buffer. The aim is that the sample preparation eventually will be performed in the sample preparation chip. However, the NASBA amplification chip can also be combined with laboratory routines for nucleic acid extraction. In the amplification experiments with droplets and dried enzymes reported in the end of this paper, HPV 16 oligos were amplified.

The input droplet of the NASBA amplification chip will be split into smaller reaction droplets. Each volume will be mixed with different reagents, so that a different mRNA strand is amplified in each reaction volume. In this way, the sample can be simultaneously analyzed for several HPV viruses, each giving either high risk of cervical cancer. In the amplification processes, a fluorescent beacon is attached to the amplicons, and the fluorescent signal of each reaction chamber is monitored, giving a positive or a negative HPV status for each HPV type.

Biochemical experiments have initially been performed in order to decide upon the design of the amplification chip. In particular, it was found that the reagents necessary for each of the NASBA amplifications must be dried in two separate chambers. It was also found that the chamber storing the second reagent mix also could be used for fluorescent detection, because the reagents dissolved so quickly that the optical signal was not disturbed. Thus, two reaction chambers are needed for each parallel HPV analysis. In order to provide a sufficiently strong fluorescent signal, the sample volume for each amplification was chosen to be 500 nl.

A prototype amplification chip has been designed and manufactured. The design (see Fig. 1) is based on numerous tests on sub-functions. Here we report on the sub-function tests performed on different test chips. Experiments on parallel motion control of the droplets, the re-hydration of dried reagents, and the RNA amplification, using dried enzymes are reported. A sketch showing the functions of the chip is seen in Fig. 1. Sample with extracted nucleic acids in buffer will be transferred from the sample preparation chip to the amplification chip. The output volume of the sample preparation chip is
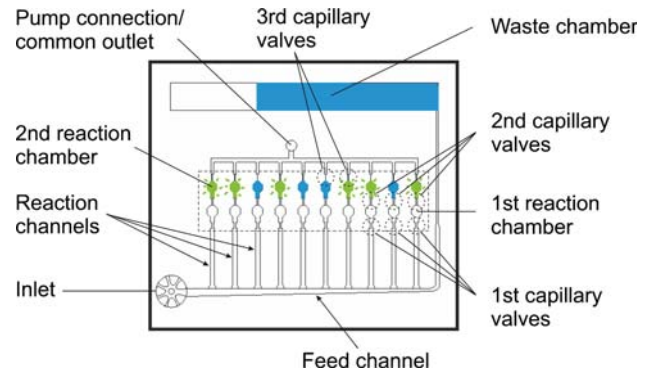

Fig. 1 Sketch of the NASBA amplification chip. The *star*-shaped inlet is in the *lower left* corner. The horizontal feed channel leads to the ten parallel reaction channels and to the large waste chamber on the *top* of the chip. The hydrophobic valves are spotted at the three restrictions in each parallel channel. The lower/first restrictions are wider than the upper/last ones. Dried master mix will be spotted in the first chamber and dried enzymes will be spotted in the second chamber. A peltier element is placed under the area of the *dashed line*, and the optical module will be scanned across the upper chambers. In the figure, the parallel reaction droplets have reached the second reaction chamber

approximately 20 µl, the current design of the amplification chip allows for input volumes between 14 and 35 µl. The sample enters the amplification chip via a star-shaped inlet and is pulled into a feed channel by capillary action. As the liquid moves through the feed channel, the ten vertical reaction channels are filled with sample up to the first capillary valve. The excess sample enters the rightmost waste channel and is pulled into the large, upper waste chamber. The waste chamber contains a filter which absorbs the surplus sample, so that the feed channel and the waste channel are drained. At this stage, ten equally sized sample droplets are snapped off and reside in the ten parallel reaction channels. One pump is connected to the system via one common outlet for all 10 parallel channels. The parallel droplets are pulled from the metering sections of the channels into the first reaction chamber, overcoming the resistance caused by the 1st capillary valves. When all droplets have crossed their respective capillary valves, the pumping stops. No sample droplets enter the second chamber yet, because the second set of capillary valves is designed to be stronger (having higher burst pressure) than the first. At this point, all sample droplets reside inside their 1st reaction chambers, and are allowed to mix with the dried reagents. After a prescribed time, the pumping is recommenced. When the pressure difference reaches a level corresponding to the strength of the second set of capillary valves, the droplets sequentially overcome the restrictions and travel to the second reaction chamber. When all droplets reside in the second chamber, the pumping is stopped and the chip is heated to 41°C. The sample droplets are left in this reaction chamber to mix

with dried enzymes, and the NASBA reaction is initiated. During the amplification process the reaction chambers are scanned sequentially by the optical module, and the fluorescence signal is recorded.

3 Test chip manufacturing

Test chips for both fluidic and biological experiments on sub functions of the amplification chip were manufactured in cyclic olefin copolymer (COC). The chips used for testing of the hydrophobic valves were milled and laser ablated, whereas test chips for re-hydration of dried reagents and for NASBA amplification experiments were hot embossed.

After manufacturing, the chips were cleaned in an ultrasonic bath, air dried, and O_2 plasma activated prior to coating with 0.5% polyethylene glycol (PEG) in methanol (Sigma Aldrich Norway AS, Norway). The PEG layer on the microchip surfaces after coating rendered the channel walls hydrophilic, with a contact angle to DI water measured to be approximately 30°.

The hydrophobic valves were created in bottlenecks of the microchannels by spotting 0,5% Teflon 1600 AF (DuPont) using the PipeJet spotting system (BioFluidix, Germany). The contact angle of DI water on Teflon surface was measured to be approximately 110°. Satisfactory coating of the valve structures was confirmed after spotting by microscope inspection. After coating and spotting, the chips were sealed with adhesive tape.

4 Sample droplets moving in parallel channels with hydrophobic valves

We have tested the feasibility of controlling the positioning of all sample droplets in the parallel channels by using one common pump only, combined with channel restrictions and hydrophobic patches separating the hydrophilic reaction chambers. The test chips contained seven parallel channels; the choice of seven channels was arbitrarily chosen for demonstrating the effect. The final prototype will as mentioned contain ten parallel channels. Each test channel had a width of 800 and a depth of 200 μm. All channels had four lateral restrictions with widths 380, 150, 75 and 33 μm, widths decreasing in the downstream direction (see Fig. 2). A total of five chips were tested.

The chips were mounted in an aluminum frame and connected to a second chip containing a branch-like channel structure for distribution of the pump under pressure to all parallel channels, see Fig. 3. A syringe pump (PHD2000, Harvard Apparatus) was used to apply suction to the common chip outlet, withdrawing air from the
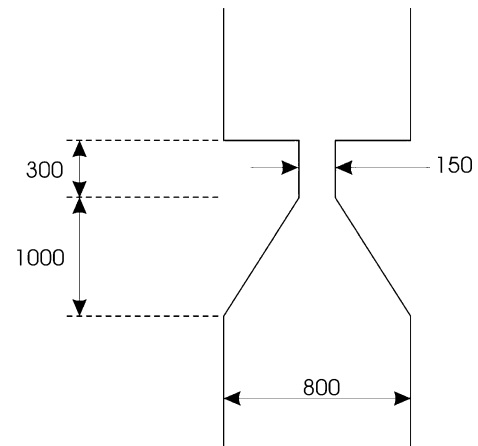

Fig. 2 Details of capillary valve geometry (here, valve width is 150 μm). All dimensions in μm. The capillary valves contain a tapered part and a narrow restriction. The tapered part is included to aid spotting of the fluorpolymer for hydrophobization
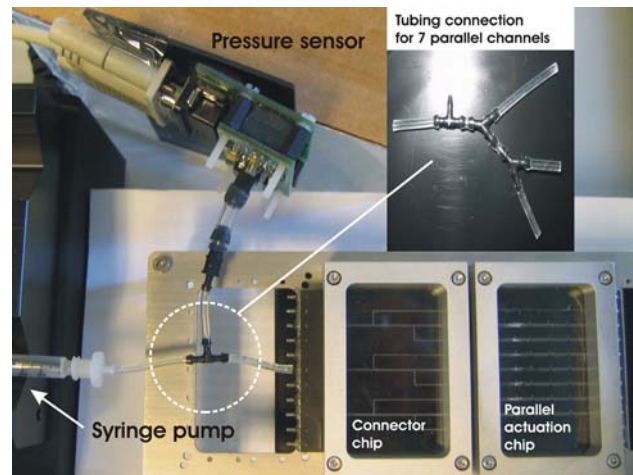

Fig. 3 Test setup for monitoring of parallel droplet movement. The syringe connected to the pump is seen to the *left*, the MEMSCAP pressure sensor *upper left*. Tubing connects the pump and pressure sensor to the aluminum frame with the fluidic connectors, the pressure distribution chip and the rightmost parallel droplet actuation chip. The presented image shows the setup for actuation of three channels in parallel. For actuation of seven channels simultaneously, the tubing connection between the syringe, the pressure sensor and the aluminum frame is exchanged with the connection shown in the *upper right* corner of the image

system at a rate of 10 μl/min. A pressure sensor (TP3100 001A 0P from MEMSCAP) was used for monitoring the pressure in the tube connection to the parallel channels. A close-up image of the parallel actuation chip with liquid droplets in front of the first restriction is shown in Fig. 4.

The pressure as a function of time at the common pump was recorded during operation and is shown in Fig. 5. The pressure plot shows the absolute pressure. During pumping, the pressure in the system decreases until the pressure
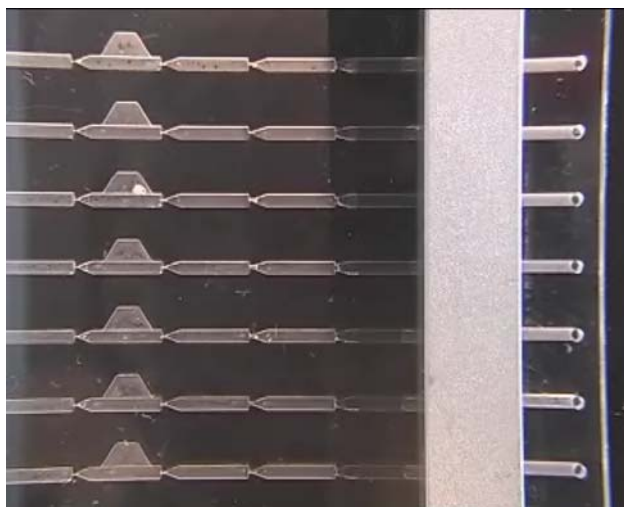
Fig. 4 The test chip for parallel droplet movement control. Seven parallel reaction and amplification test channels are seen, each with four restrictions with hydrophobic spots. Valve pressure strength increases from *right* to *left*. In the image, 500 nl droplets are positioned before the rightmost, weakest valve
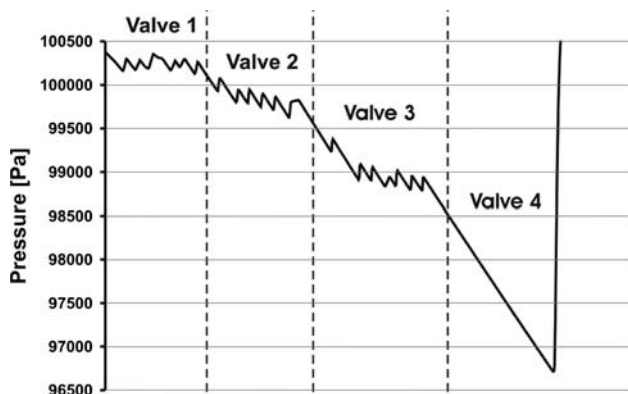

Fig. 5 Pressure characteristics (absolute pressure) of droplet movement through the parallel actuation chip containing seven parallel channels. A constant flow rate (suction) of 10 μl/min is applied at the common downstream end of the chip. At the fourth valve, the first droplet to break the valve effectively ventilates the system, and hence the pressure in the system returns to atmospheric conditions

difference across the front water/air meniscus becomes higher than the burst pressure of the first hydrophobic valve. When the first droplet breaks through its valve, it enters the first chamber and the pressure in the common downstream region is slightly relaxed due to the reduced volume between the liquid droplets and the syringe. The parallel liquid droplets sequentially pass their respective first valves; the order in which the droplets move is arbitrary, depending on slight variations in burst pressure of the seven valves due to manufacturing tolerances and surface roughness effects. No droplet passes to the second chamber
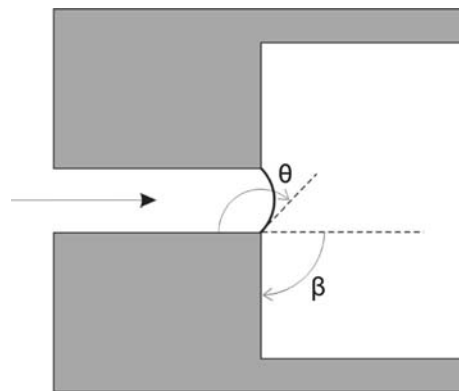

Fig. 6 Definition of wetting angle θ and diverging angle β

before all droplets reside in the first chamber, because of the higher strength (burst pressure) of the second set of valves. This is clearly visible in Fig. 5, where the pressure level of the first seven valves is higher than that of the second valves. Once all sample droplets reside in the first chamber, they proceed to cross their respective second valves as the pumping continues. Passing of the fourth valve results in one pressure rise only, because once a single sample droplet crosses this valve, the system is vented to atmospheric pressure and further pumping does not cause any more movement of any droplet.

The pressure necessary to overcome a hydrophobic restriction for a liquid with surface tension γ and wetting angle θ is given by the Young-Laplace equation, which in the case of a rectangular channel can be written as (Probstein 1994):

$$\Delta p = -2\gamma \cos(\theta)\left(\frac{1}{w} + \frac{1}{h}\right) \tag{1}$$

where w and h are the width and depth of the restriction, respectively. Here, identical wetting angle of all four walls is assumed. In the present case, the capillary valve consists of a hydrophobic restriction terminated by a suddenly diverging channel section (with diverging angle β), see Fig. 6.

In order to drive the liquid forward, the applied pressure difference across the meniscus must be sufficient not only to overcome the strength of the hydrophobic restriction, but also to force the meniscus to achieve wetting angle θ with the diverging wall. In such case, the pressure required to drive the liquid forward may be expressed as:

$$\Delta p = -2\gamma \left(\frac{\cos(\theta)_I}{w} + \frac{\cos(\theta)}{h}\right) \tag{2}$$

where $\theta_I = \min[(\theta+\beta), 180°]$ (see e.g. Cho et al. 2007). For a channel with hydrophobic walls, $\theta > 90°$ and in our case β is 90°. Then $\theta_I = 180°$ and thus the pressure required to overcome the valve becomes:

$$\Delta p = 2\gamma \left(\frac{1}{w} - \frac{\cos(\theta)}{h} \right) \tag{3}$$

the pressure necessary to overcome the capillary valves was estimated from Eq. (3) and compared to experimentally obtained pressure values. The average burst pressures of the four valves are plotted in Fig. 7. The data (open squares) represents average values from five chips with DI water as the working liquid. The error bars indicate the standard deviation of the differential pressure value. The solid line represents the theoretical values calculated from Eq. (3). As evidenced by the data in Fig. 7, good comparison between the experimental and theoretical values for the burst pressure is achieved.

In addition to the use of DI water as working liquid, one experiment (open circles in Fig. 7) using pre-mixed sample with NASBA reagents was conducted to verify the system functionality. The measured burst pressures are seen to be slightly below that found for DI water. This is attributed to the slightly lower wetting angle of the sample mixture, which was measured to be approximate 95°.

The parallel droplet experiments demonstrate that the burst pressures for the sequential valves do not overlap, and that the principle of parallel droplet positioning using one single pump and hydrophobic valves is feasible.

5 Re-hydration of dried master-mix and enzymes

The NASBA amplification chip will have the all necessary reagents stored on chip in a dry state. In each parallel channel the two chambers separated by hydrophobic valves will contain dried reagents. The first chamber will store the NASBA nucleotide ion-adjusted master mixture (master mix) that is specific for each HPV type mRNA
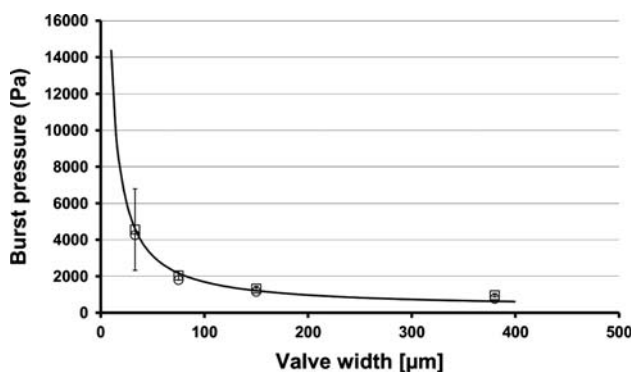

Fig. 7 Burst pressures of the capillary valves as function of valve width. Symbols: *open square* DI water; *open circle* reagents; *solid line* represents the analytical values for water. Contact angle of DI water on Teflon was measured to be approximate 110°. The pressure data for each valve represents an average of 35 measurements (5 chips with 7 parallel channels each)

amplification, and the second chamber containing the dried enzymes. One crucial question is whether the dried reagents will dissolve into the sample droplet in an acceptable time, or if further mixing by e.g. moving the droplet back and forth over the dried reagents is needed. Here we report on experiments monitoring the dissolution of the master mix and the enzymes.

The spotting of reagents and enzymes into the 500 nl hot embossed reaction chambers was performed using the spotter Nanoject II from Drummond Scientific Company. A volume corresponding to the required amount of reagents for a 500 nl sample size was spotted sequentially into the chambers, 30 nl at a time. The spotted liquid was allowed to dry for approximate 30 s between each deposited droplet. In this manner, a well-defined lump of reagents was deposited on the bottom surface of the reaction chambers. After spotting, the chips with dried reagents were stored at room temperature for at least three days prior to the re-hydration experiments.

In the actual master mix, molecular beacons which contain both a fluorophore and a quencher are present in the solution. The quenchers suppress the fluorescent signal of the fluorophore as long as the targeted mRNA is not present; upon detection, the fluorophore and the quencher are separated within the molecular beacon, releasing the fluorescent signal (in presence of external excitation). For the re-hydration experiments, molecular beacons (excitation/emission 490 nm/520 nm) without quenchers were added to the solution prior to spotting and drying. In this manner, the master mix was fluorescent without the need of actual amplification, and its re-hydration from solid state and diffusion into the sample could be monitored. In contrast to the master mix, the enzymes do not contain any fluorescent components. In order to permit fluorescent detection of the re-hydration and diffusion process of the dried enzymes, the antibody IgG (Southern Biotech, Birmingham, AL) fluorescently labelled with FITC (excitation/emission 495/515 nm) was added to the enzyme suspension prior to spotting and drying in the reaction chambers. The fluorescent IgG tracer (molecular weight 150 kDa) thereby modeled the largest enzyme AMV-RT (molecular weight 160 kDa).

The processes of re-hydration of the dried master mix and enzymes in the liquid samples were investigated experimentally by confocal laser scanning microscopy (CLSM). A Leica DM RXA epifluorescent microscope equipped with Leica TCS 4D confocal unit was used for the measurements. The imaging of the reaction chambers was performed via an HC PL Fluotar objective with 5-fold magnification and $NA = 0.15$. The low magnification was necessary in order to image the entire reaction chamber within the field of view of the microscope. As a consequence, the depth-wise resolution of the measurements was

Fig. 8 The upper sequence of CLSM images shows the temporal ▶
evolution of fluorophore concentration, initially dried with the master
mix at the bottom of the chamber. The focal plane is positioned
150 μm above the bottom of the chamber. Time between images is
12 s. The image at $t = 0$ is taken without the aqueous solution present
in the chamber, with the focal plane at the bottom wall of the
chamber; hence the high fluorescence intensity in the image. The
lower image sequence shows the concentration time evolution of
fluorescent IgG at the same height. The fluorescent IgG was initially
dried at the bottom of the chamber with the NASBA enzymes. Time
between images is here 60 s

limited, with optical slice thickness ∼ 100 μm. An Omni-chrome Series 43 ArKr laser was used for sample illumination. The fluorescence filters were set for FITC detection, with excitation peak at 488 nm and emission at >510 nm.

The reaction chamber containing dried master mix was flooded (Goldschmidtboeing 2006) by injecting 1.5 μl of the relevant solution containing 15% DMSO and 375 mM Sorbitol in water into the microchip via the chip supply channel. Sequences of images at a plane 150 μm above the bottom wall of the chamber were acquired in 12 s time intervals (total chamber depth was 200 μm). The re-hydration process of the dried master mix is shown in Fig. 8. As can be seen by inspection of the images, the fluorescence intensity is essentially unchanged beyond 60 s, although some weak local fluorescence intensity variations can still be discerned in the image sequence beyond that time. Thus, the re-hydration and diffusion of the fluorescent species is sufficiently completed after 60 s. After emptying the reaction chamber, no fluorescent reactant residues were detected at the chamber bottom, thus confirming the successful re-hydration process.

For the dried enzymes, the time before an equilibrium concentration of fluorescent IgG at 150 μm height was reached, was approximately 10 min.

In order to investigate whether the dissolution time of the dried reagents is dominated by the re-hydration or by the diffusion process, we compare these results with the theoretical estimate for the diffusion time of similarly sized molecules from the bottom of the chamber to the measurement plane, i.e. a distance of 150 μm. The average distance of diffusion L of a particle with a diffusion coefficient D is proportional to the square root of time t:

$$L = \sqrt{2Dt}. \tag{4}$$

The Stokes–Einstein diffusion coefficient for a spherical particle suspended in an aqueous solution is given by:

$$D = \frac{kT}{3\mu\pi d_p} \tag{5}$$

where k is the Boltzmann constant, T is the absolute temperature, μ is the dynamic viscosity and d_p is the particle diameter. We have estimated the diameter of the
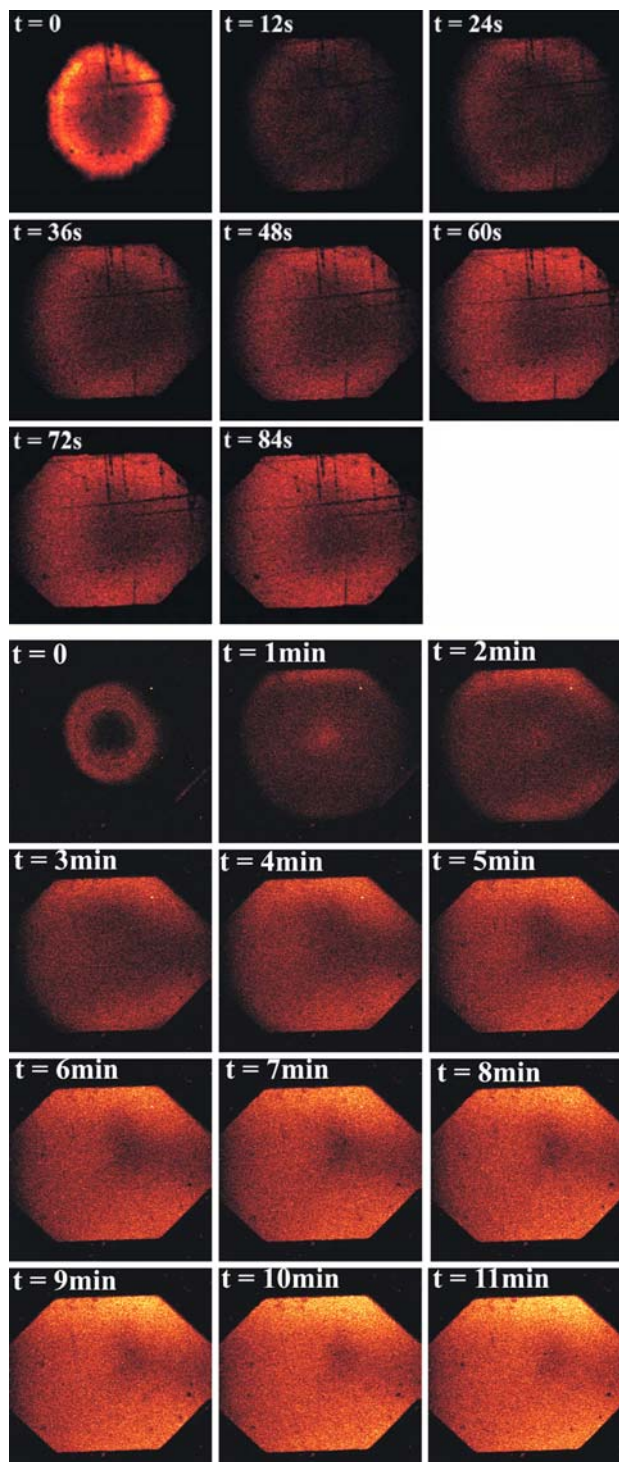
fluorescent molecular beacons in the master mix to be $d_{MB} \approx 2.8$ nm, while the diameter of the fluorescently labelled IgG is estimated to be $d_{MB} \approx 7$ nm. Using these sizes, we estimate an average diffusion time of 70 s for the fluorescent beacons to reach the 150 μm plane. The larger IgG proteins will have an average diffusion time of approximately 3 min.

Although the estimates given above are crude, they indicate that the diffusion time for the molecular beacons roughly corresponds to the experimentally observed time needed to obtain a homogeneous suspension of master mix in DMSO/sorbitol/water solution. This, in turn, implies that the dissolution process is diffusion-limited. Re-hydration itself is rapid, occurring nearly instantaneously as compared to the time scale of diffusion.

For the enzymes, we observe that the time required to achieve a homogeneous concentration of fluorescent IgG is significantly larger than the estimated diffusion time. This implies that the re-hydration of the enzymes is slow and dominates the process of dissolution. Nevertheless, complete dissolution was observed.

Dissolution of the enzymes will be the final step on the NASBA chip prior to detection of the fluorescent signal. Microscope inspection of the reaction chamber after re-hydration revealed that no residue of the dried material is left on the chamber wall. Therefore, the bottom of the reaction chamber does not disturb the optical detection of the amplification process, and it may be performed in the same chamber as the dissolution of the enzymes. The amplification is time-consuming, requiring approximately 90 min before unambiguous decision about presence/absence of the targeted species can be reached.

Based on these experiments we conclude that no active mixing mechanisms are necessary neither for the dissolution of the master mix, nor the enzymes. In both cases, the mixing time is most probably sufficiently fast for the functionality of the NASBA chip.

6 NASBA amplification in nanoliter chambers with dried enzymes

The critical test of the NASBA amplification chip is whether it is possible to obtain and detect successful NASBA amplification of oligos or HPV mRNA using separate sample volumes of 500 nl and dried reagents. Tests have been performed on re-hydration and re-activation of dried enzymes. The enzymes needed for the NASBA reaction are AMV-RT, RNaseH and T7 RNA polymerase (PreTect HPV-Proofer kit, NorChip). COC hot embossed 500 nl reaction chambers with feeding and ventilation channels were coated with PEG. In contrast, COC milled chips with the same geometries had no positive amplifications; this may be due to insufficient PEG coating of rough surfaces and thereby the adsorption of enzymes on the walls, which inhibits the reaction.

The enzyme solution was spotted in the micro-chambers, on top of the PEG. The microchips were spotted with enzymes using the Nanoject II from Drummond Scientific
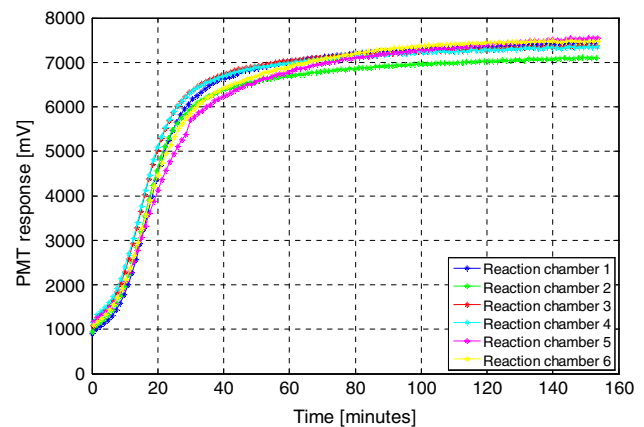

Fig. 9 Amplification curves of the six 500 nl reaction chambers run simultaneously on one microchip employing a sample of positive control for HPV type 16 (0.1 μM)

Company, dried in room temperature for up to 1 day and then sealed with adhesive tape.

The amplification reaction was performed by first manually mixing the NASBA reagents, except the enzymes, and a positive control sample of human papillomavirus (HPV) type 16. The mixture was incubated at 65°C for 3 min followed by 3 min at 41°C. The sample droplets were pulled into the microchip by an under-pressure and positioned in the reaction chambers with dried reagents with the help of hydrophobic spots applied in the narrow channels leading in and out of the chamber. A custom-made instrument (Gulliksen et al. 2005) recorded the fluorescent signal of the amplification reaction at 41°C, scanning 6 reaction chambers on one chip (Gulliksen et al. 2007). Figure 9 shows positive amplification curves of the 500 nl reaction chambers.

The present work demonstrates on-chip storage of dried enzymes which are reactivated upon re-hydration of sample. These results are promising with regard to the development of disposable self-contained microchips for NASBA.

7 Conclusions

We have presented experimental results on test chips, examining sub-functions of a prototype microchip for simultaneous detection of multiple mRNA targets. It was shown that successive passive valves based on hydrophobic channel restrictions could have increasing burst pressures well separated and above pinning pressures. This enabled the simultaneous droplet positioning in parallel channels to be controlled by one external pump only. The two types of dried reagents (master-mix and enzymes) necessary for NASBA amplification were spotted, dried and re-hydrated in micro-chambers. Re-hydration with fluorescent markers

showed that the dried reagents were sufficiently re-hydrated on time scales acceptable for on-chip NASBA reactions. The most important result in the project so far is that spotted and dried enzymes in a nanoliter-sized reaction chamber can be re-hydrated and re-activated and may result in positive NASBA amplifications. To our knowledge, this is the first time the NASBA enzymes have been successfully dried and re-activated on-chip.

The next step in the project is to use the prototype NASBA amplification chip with all functions included, in order to verify that the fluidic and biochemical sub functions work when they are combined.

References

Burns MA, Johnson BN, Brahmasandra SN, Handique K, Webster JR, Krishnan M, Sammarco TS, Man PM, Jones D, Heldsinger D, Mastrangelo C, Burke DT (1998) An integrated nanoliter DNA analysis device. Science 282:484–487

Cepheid (2007) http://www.cepheid.com

Cho H, Kim HY, Kang JY, Kim TS (2007), How the capillary burst microvalve works. J Colloid Interface Sci 306:379–385

Compton J (1991) Nucleic acid sequence-based amplification. Nature 350:91–92

Gulliksen A, Mielnik M, Hovig E, Karlsen F, Furuberg L, Sirevåg R (2007) Storage and reactivation of enzymes in a disposable, self-contained Lab-on-a-chip system. In: Proccedings of MicroTAS 2007

Gulliksen A, Solli L, Karlsen F, Rogne H, Hovig E, Nordstrøm Tand Sirevåg R (2004) Real-time nucleic acid sequence-based amplification in nanoliter volumes. Anal Chem 76:9–12

Gulliksen A, Solli L, Drese KS, Sörensen O, Karlsen F, Rogne H, Hovig E, Sirevåg R (2005) Parallel nanoliter detection of cancer markers using polymer microchips. Lab Chip 5:416–419

Goldschmidtboeing F, Rabold M, Woias P (2006) Strategies for void-free liquid filling of micro cavities. J Micromech Microeng 16(7):1321–1330

Jenkins D (2001) Diagnosing human paillomaviruses: recent advances. Curr Opin Infect Dis 14:53–62

Kraus I, Molden T, Ernø LE, Skomedal H, Karlsen F, Hagmar B (2004) Human papillomavirus oncogenic expression in the dysplasticportio; an investigation of biopsies from 190 cervical cones. Br J Cancer 90:1407–1411

Lie A, Risberg B, Borge B, Sandstad B, Delabie J, Rimala R, Onsrud M, Thoresen S (2005) DNA versus RNA based methods for human papilloma virus detection in cervical neoplasia (2005). Gynecologic Oncology 97:908–915

Lien K, Lee W, Lei H, Lee G (2007) Integrated reverse transcription polymerase chain reaction systems for virus detection. Biosens Bioelectron 22(8):1739–1748

Linder V, Sia SK, Whitesides GM (2005) Reagent-loaded cartridges for valveless and automated fluid delivery in microfluidic devices. Anal Chem 77:64–71

MicroActive (2006) http://www.sintef.no/microactive

Mielnik M, Furuberg L, Voitel J (2007) Sample metering and parallel liquid plug actuation for multiple biochemical assays. In: Proceedings of microTAS 2007

Norchip (2007) http://www.norchip.com/

Oh KW, Ahn CHJ (2006) A review of Microvalves. Micromech Microeng 16:R13–R39

Probstein RF (1994) Physicochemical hydrodynamics. 2nd edn. Wiley, New York

Walboomers JMM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJF, Peto J, Meijer CJLM, Munoz N (1999) Human papilloma virus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189(1):12–19

Weigl BH, Gerdes J, Tarr P, Yager P, Dillman L, Peck R, Ramachandran S, Lemba M, Kokoris M, Nabavi M, Battrell F, Hoekstra D, Klein EJ, Denno DM (2006) Fully integrated multiplexed lab-on-a-card assay for enteric pathogens. Proc SPIE 6112:1–11

Yager P, Edwards T, Fu E, Helton K, Nelson K, Tam MR, Weigl BH (2006) Microfluidic diagnostic technologies for global public health. Nature 442:412–418

Zhang C, Xu J, Ma W, Zheng W (2006) PCR microfluidic devices for DNA amplification. Biotech Adv 24:243–284

Development of an Automatic Chip-based Detection of mRNA

L. Solli[1], A. Gulliksen[1], T. Baier[2], R. Gransee[2], T. Hansen-Hagge[2], M. Mielnik[3], I-R. Johansen[3], L. Furuberg[3], L. Rigger[4], P. Koltay[5], C. Martin[6], H. Keegan[6], J. O'Leary[6] and F. Karlsen[1]

[1]NorChip AS, Klokkarstua, Norway, [2]Institut für Mikrotechnik Mainz GmbH, Germany, [3]SINTEF ICT, Oslo, Norway, [4]Department of Microsystems Engineering (IMTEK), Albert-Ludwigs-Universität Freiburg, Germany, [5]BioFluidix GmbH, Freiburg, Germany, [6]Coombe Women & Infants University Hospital, Dublin, Ireland
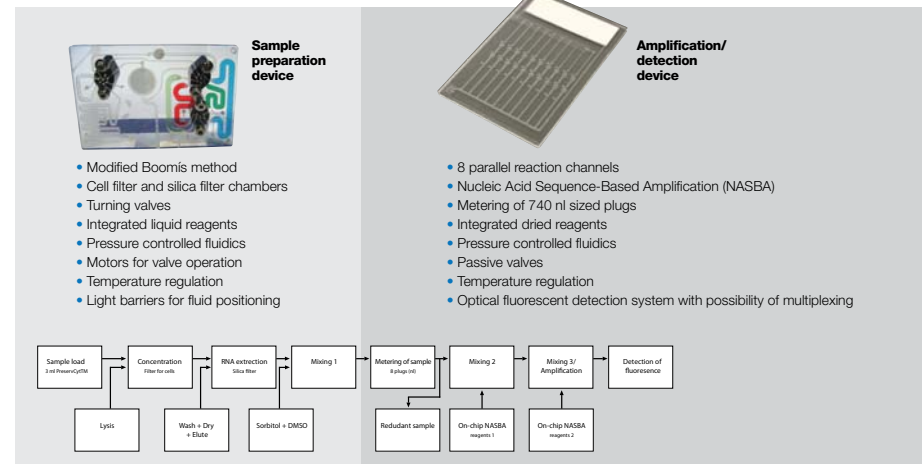
A direct link exists between the different types of disease and the presence or absence of single or multiple mRNA transcripts. Expression patterns of different mRNA transcripts simultaneously may be related to increased risk of cancer, the presence of pre-cancer, lack of immune defense, increased risk of heart failure, specific allergies or the presence of active infection.

This project is developing an automated point-of-care platform for on-chip sample preparation, nucleic acid extraction, amplification and fluorescent detection. All necessary reagents are stored on chip. After insertion of the chip into the device and loading of the sample onto the chip the procedure proceeds without further user interaction.

Two modular devices, one for sample pretreatment and one for amplification/detection are currently being tested individually before integration into a single setup. Molecular biomarkers for Human Papillomavirus (HPV) are used as a model system in the platform.

Description of work:

Sample preparation device
- Modified Boom's method
- Cell filter and silica filter chambers
- Turning valves
- Integrated liquid reagents
- Pressure controlled fluidics
- Motors for valve operation
- Temperature regulation
- Light barriers for fluid positioning

Amplification/detection device
- 8 parallel reaction channels
- Nucleic Acid Sequence-Based Amplification (NASBA)
- Metering of 740 nl sized plugs
- Integrated dried reagents
- Pressure controlled fluidics
- Passive valves
- Temperature regulation
- Optical fluorescent detection system with possibility of multiplexing

Results:

- The device has been successfully tested on various cell lines and clinical samples which express HPV mRNA.
- Device performance was validated by NASBA of the eluate using the PreTect® HPV-Proofer kit.

Clinical sample	HPV16	U1A
1	Positive	Positive
2	Positive	Positive
3	Positive	Negative
4 (Neg. control)	Negative	Negative

Cell line/ Cell count	CaSki	MS751	HeLa
50.000	HPV16 Positive	HPV45 Positive	HPV18 Positive
5.000	HPV16 Positive	HPV45 Positive	HPV18 Positive
500	HPV16 Positive	HPV45 Positive	HPV18 Positive
50	HPV16 Positive	HPV45 Positive	HPV18 Positive
5	HPV16 Positive	HPV45 Negative	HPV18 Positive

- The device has been successfully tested on dilution series of CaSki cells (table) and clinical samples positive for HPV16 (graph) with primers and probes integrated on chip.

| No. of extracted cells	No. of cells in the macro scale well	No. of cells in the reaction on	Spot volume (nl)	Results singleplex mix on chip	Results macro scale
50.000	5.000	125	125	8/8	1/1
50.000	5.000	125	100	6/6	1/1
5.000	500	12,5	125	4/4	1/1
5.000	500	12,5	100	7/7	1/1
500	50	1,25	125	8/8	1/1
500	50	1,25	100	7/7	1/1
50	5	0,125	125	4/6	1/1
5	0,5	0,0125	100	0/5	1/1

Conclusions and outlook:

A combined system is intended to serve as a point-of-care system for the detection of gene activity directly in a physician's office, thus avoiding time consuming analysis by specialized laboratories. The system is not limited to cervical samples and opens the way for a range of applications.

The system can be adapted to other fields of operation where analyses of complex biological samples in the field in a short time are desirable. This includes for example:

- **Food analysis**
- **Personalized medicine/ POC**
- **Forensics**
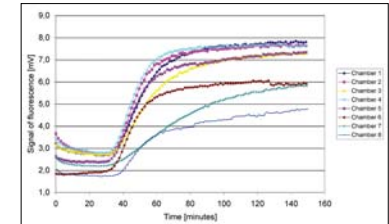
Acknowledgements:

Evaluation of HPV DNA and mRNA Detection Technologies for Detecting HPV in Cervical Cytology Specimens

{P} H Keegan[1], J McInerney[3], L Pilkington[1], P Gronn[2], I Silva[2], F Karlsen[2], N Bolger[1], J O'Leary[3], C Martin[3]

[1]*Coombe Women's Hospital, Dublin, Ireland,* [2]*Norchip AS, Klokkarstua, Norway,* [3]*University of Dublin, Trinity College, Dublin, Ireland*

HPV infection is the primary agent in the development of CIN and cervical cancer, with screening programmes moving towards the introduction of HPV testing as part of the screening process. In this study we evaluated two HPV detection technologies for detection of HPV in liquid based cytology specimens. These included HPV DNA by Hybrid Capture II (Digene, HCII), which detects 13 high-risk HPV types and E6/E7 mRNA expression by PreTect HPV Proofer (Norchip), which detects HPV 16, 18, 31, 33, and 45.

In summary, 205/299 cytology specimens representing the broad spectrum of the disease (Normal-CIN3) were positive for HPV DNA and 113/299 specimens were positive for E6/E7 mRNA. We report higher concordance rates between both technologies in CIN3 cases (83%) and Normal cases (88%) than in the BNA or CIN1-2 disease categories. The positive predictive value (PPV) and specificity of the HCII DNA test (41% and 43.7% respectively) were lower than that of the PreTect HPV Proofer mRNA test (53.6% and 75.6%) for detection of high-grade disease (CIN2+), indicating that PreTect HPV Proofer may be more useful than HCII for predicting high-grade disease.

This study forms part of the MicroActive Consortium funded under the EU 6th framework *eHealth* Initiative

Clinical Measurement of Prognostic Immune Signature in Follicular Lymphoma by RT-PCR based Gene Expression Profiling and Immunohistochemistry Demonstrates Favourable T-cell and Unfavourable Macrophage Infiltration

{P} RJ Byers[1], E Sakhinia[4], P Jacob[2], C Glennie[2], S McDermott[2], JA Hoyland[5], L Menasce[3], J Radford[1], T Illidge[1]

[1]*Division of Cancer Studies, University of Manchester, Manchester, United Kingdom,* [2]*Dept of Histopathology, Manchester Royal Infirmary, Manchester, United Kingdom,* [3]*Dept of Histopathology, Christie Hospital, Manchester, United Kingdom,* [4]*Molecular Diagnostic Centre, Manchester Royal Infirmary, Manchester, United Kingdom,* [5]*Division of Tissue Injury, University of Manchester, Manchester, United Kingdom*

Gene expression profiling studies have demonstrated immune response gene signatures predictive of outcome in follicular lymphoma (FL) and there is a need for validation of these signatures and for their translation to clinical use. However, measurement of these genes in routine practice remains difficult and to date there have been very few studies validating the hypothesis. In this project we used real-time PCR measurement of gene expression levels in globally amplified polyA cDNA to analyse of immune response signatures in FL. We used real-time PCR to measure expression levels (normalised to the mean of 4 housekeeping genes) of 36 candidate *Indicator* genes, selected from microarray studies, in polyA cDNAs prepared using polyA PCR from 58 archived human frozen lymph nodes, together with immunohistochemistry for CD3, CD4, CD7, CD8, CD10, CD20, CD21 and CD68 in parallel formalin fixed paraffin embedded tissue samples to measure immune response in FL. Immunohistochemical positivity was measured by a semi-automated image analysis method using spectral unmixing to identify areas of immunopositivity. Kaplan-Mier survival analysis was performed against the normalised real-time PCR expression levels of each of the genes and against the percentage immunohistochemical postivity for each of the antibodies except for CD68 survival analysis for which was performed for cases with either 15 or less or more than 15 CD68 positive cells per high power field (hpf). High levels of CCR1, a marker of monocyte actication, were associated with a shorter survival interval (p<0.02), whilst immunohistochemistry demonstrated association of high numbers of CD7 positive T-cells with longer survival interval (p<0.02) and of high numbers of CD68 positive macrophages with a shorter survival interval (p<0.032). The results confirm the role of the host immune response in outcome in FL and identify CCR1 as a prognostic indicator and marker of immune switch between macrophage and T-cell dominant response. The methods used are clinically applicable, whilst the clinical utility of polyA DNA and real-time PCR for measurement of gene signatures and the strength of this approach as a molecular block are confirmed.

The MicroActive project:
automatic detection of disease-related molecular cell activity

Liv Furuberg[a], Michal Mielnik[a], Ib-Rune Johansen[a], Jörg Voitel[a], Anja Gulliksen[b,c], Lars Solli[b,d], Frank Karlsen[b], Tobias Bayer[e], Friedhelm Schönfeld[e]; Klaus Drese[e], Helen Keegan[f,g], Cara Martin[f,g], John O'Leary[f,g], Lutz Riegger[g], Peter Koltay[h]

[a]SINTEF Microsystems and Nanotechnology, Forskningsveien 1, 0314 Oslo, Norway,
[b]NorChip AS, Industriveien 8, 3490 Klokkarstua, Norway
[c]University of Oslo, Dept of Molecular Biosciences, 0316 Oslo, Norway
[d]NTNU, Dept of Energy and Process Engineering, 7491 Trondheim, Norway
[e]Institut für Mikrotechnik Mainz GmbH, Carl-Zeiss-Straße 18-20, D - 55129 Mainz, Germany
[f]Coombe Women's Hospital, Dolphin's Barn Dublin 8, Ireland
[g]IMTEK, Georges-Koehler-Allee 103, 79110 Freiburg, Germany
[h]BioFluidix, Georges-Köhler-Allee 106, 79110 Freiburg, Germany

ABSTRACT

The aim of the MicroActive project is to develop an instrument for molecular diagnostics. The instrument will first be tested for patient screening for a group of viruses causing cervical cancer. Two disposable polymer chips with reagents stored on-chip will be inserted into the instrument for each patient sample. The first chip performs sample preparation of the epithelial cervical cells while mRNA amplification and fluorescent detection takes place in the second chip. More than 10 different virus markers will be analysed in one chip. We report results on sub-functions of the amplification chip. The sample is split into smaller droplets, and the droplets move in parallel channels containing different dried reagents for the different analyses. We report experimental results on parallel droplet movement control using one external pump only, combined with hydrophobic valves. Valve burst pressures are controlled by geometry. We show droplet control using valves with burst pressures between 800 and 4500 Pa. We also monitored the re-hydration times for two necessary dried reagents. After sample insertion, uniform concentration of the reagents in the droplet was reached after respectively 60 s and 10 min. These times are acceptable for successful amplification. Finally we have shown positive amplification of HPV type 16 using dried enzymes stored in micro chambers.

Keywords: Biomedical, sensor, molecular diagnostics, microfluidics, lab-on-a-chip, dried reagents, spotting, valve

*liv.furuberg@sintef.no; phone +47 93059326; fax +47 22 067321; www.sintef.no

1. INTRODUCTION

The aim of the MicroActive project [1] is to develop an instrument for molecular diagnostics intended for use in the doctors' office. The instrument will first be used for patient screening for a group of viruses causing cervical cancer, but the technology platform will have a wide applicability. Microfluidics and biotechnology form the basis for the development. Fully automated diagnosis systems based on microfluidics will widen the availability of advanced diagnostics for all citizens due to both higher availability and lower cost [2]. Also, reducing the time from patient testing to diagnosis avoids anxiety and enables earlier treatment.

Human Papillomavirus (HPV) is a group of sexually transmitted viruses that is related to the development of cervical cancer. Cervical cancer is the second most common cancer type among women worldwide. More than 80% of all adults will have at least one infection with HPV during their lifetime. However, nearly all these infections may be considered as the "cervical flu", and have nothing to do with cervical cancer risk. The development of cervical cancer is related to oncogenic proteins produced by transformed cells in the women's cervix. These cells are influenced by certain genetic sequences from a limited group of HPV-viruses and may develop into cervical cancer over a period of 10-25 years. Oncogenic proteins related to the five HPV-types 16, 18, 31, 33, and 45 are related to more than 97% of all cases of HPV-induced cervical cancer in Europe. The NorChip Pre Tect HPV proofer technology [3-6] detects the oncogenic expression, and is therefore an accurate and cost-effective test for cervical cancer prevention. The task of MicroActive is to miniaturize and automate the protocol of this macroscopic mRNA amplification and detection. Compared to commonly used approaches (e.g. DNA PCR amplification and immunoassay methods), mRNA detection largely avoids false positive results and has a high sensitivity. It can currently be used to detect pre-cancer, cancer, STD and a range of respiratory diseases, to mention a few. New bio-markers are continuously being developed.

Within the MicroActive project the partners will:

1) Develop one disposable microfluidic chip for sample preparation including reservoirs containing all liquid reagents necessary to perform sample preparation consisting of cell concentration, lysis and nucleic acid purification

2) Develop a second disposable microfluidic chip for multiple target amplification and fluorescent detection with dried spotted reagents stored in micro-channels.

3) Develop manufacturing methods for spotting and drying of reagents, surface coating and patterning, and polymer chip lamination that do not inhibit the bio-molecular processes.

4) Perform multi target detection from a single sample. This is possible due to simultaneous amplification and detection in separate parallel detection channels. Each parallel channel contains dried reagents for amplification of a disease (here: HPV type) specific marker.

5) Perform isothermal amplification of mRNA by nucleic acid sequence-based amplification (NASBA) [7].

6) At early stages of the project, perform tests on clinical samples, using test-chips for separate functions.

7) Develop an instrument without manual protocols.

8) Test the instrument on clinical specimens and compare to gold standards.

9) Address factors such as reliability, usability and cost of the total instrument – factors which are crucial to acceptance by health care professionals.

10) Provide the repeatable and stable fluid control required by a commercial system through use of simple pumps in combination with surface modification.

In this paper we will focus on reporting the first experimental results on the fluidic and biological functions on the second chip, the NASBA amplification and fluorescent detection chip. Earlier, positive NASBA reactions with wet, premixed reagents have been demonstrated on chip [8, 9]. The new aspect of this work is to dry the reagents in the micro chambers and to perform the amplifications in metered, separate nanoliter sized droplets. The NASBA amplification chip is thus based on sample metering and droplet based microfluidics as described by Burns et al [10]. In our case the separate metered sample droplets are pushed into different channels where they mix with different dried regents for

multiple analyses. Work on multiple analyses using different types of stored reagents have been performed by Weigl and co-workers [11] for air drying and reactivation of PCR mixes in conventional macro scale volumes using 96-well plates. For self-contained systems, both liquid reagents and dried reagents have been introduced as plausible solutions for long-term storage of reagents on-chip [12].

Here we report experiments on parallel droplet movement control using one external pump only, combined with hydrophobic valves. We show 7 droplets moving in parallel channels, each stopping controllable in three reaction chambers along the channel. Due to heating requirements for the reactions, it is important that all droplets are in the same reaction chamber at the same time. We also monitored the re-hydration times for two necessary dried reagents using fluorescent markers and a confocal microscope. After sample droplet insertion, uniform concentration of the reagents in the droplet was reached after respectively 60 s and 10 min. These times are acceptable for successful amplification. Finally we have shown positive amplification of HPV type 16 using dried enzymes stored in micro chambers. The re-activation of dried reagents is very sensitive to the environment, spotting and drying procedure, and positive amplifications are considered to be a breakthrough for the project.

Work related to the sample preparation chip is not reported here, but a biobank has been established that stores patient cervical cell samples. Biobank samples are currently used for macroscopic comparisons between the HPV mRNA diagnostics method which the project is miniaturizing, and laboratory gold standards for cervical cancer diagnostics. Patient samples have also been processed in microfluidic test chips performing cell capture and lysis. The chosen procedure for cell capture and lysis has resulted in mRNA of sufficient concentration and quality for amplification, further optimization is under way. The first chips for nucleic acid purification have also been tested, in this case the quality of the extracted mRNA was too low and a new approach is currently explored. A prototype including all fluidic functions of the sample preparation chip has been manufactured and tested with stored liquid reagents, pumps and heating. The chip functioned roughly as expected; improvements will be made in the next generation prototype.

2. NASBA AMPLIFICATION CHIP

In the NASBA amplification chip, the purified sample output of the sample preparation chip is split into separate, smaller reaction volumes. Each volume is mixed with different reagents, so that a different mRNA strand is amplified in each reaction volume. In this way, the sample can be simultaneously analyzed for several HPV viruses, each giving either high- or medium risk of cervical cancer. In the amplification processes, a fluorescent beacon is attached to the amplicons, and the fluorescent signal of each reaction chamber is monitored, giving a positive or a negative HPV status for each HPV type.

Biochemical experiments have initially been performed in order to decide upon the design of the amplification chip. In particular, it was found that the reagents necessary for the NASBA amplification must be dried in two separate chambers. It was also found that the chamber storing the second reagent mix also could be used for fluorescent detection, because the reagents dissolved so quickly that the optical signal was not disturbed. Thus, two reaction chambers are needed for each parallel HPV analysis. In order to provide a sufficiently strong fluorescent signal, the sample volume for each amplification was chosen to be 500 nl.

A prototype amplification chip has been designed and manufactured. The design is based on numerous tests on sub-functions, below we report on the parallel motion control of the droplets, the re-hydration of dried reagents, and the amplification, using dried enzymes. A sketch showing the functions of the chip is seen in figure 1. Sample with extracted nucleic acids in buffer will be transferred from the sample preparation chip to the amplification chip via a disposable connection. The output volume of the sample preparation chip is approximately 20μl, the current design of the amplification chip allows for input volumes between 14μl and 35 μl. The sample enters the amplification chip via a star-shaped inlet and is pulled into a feed channel by capillary action. As the liquid moves through the feed channel, the 10 vertical reaction channels are filled with sample up to the first capillary valve. The excess sample enters the rightmost waste channel and is pulled into the large, upper waste chamber. The waste chamber contains a filter which absorbs the surplus sample, so that the feed channel and the waste channel are drained. At this stage, 10 equally sized sample droplets are snapped off and reside in the 10 parallel reaction channels. One pump is connected to the system via one common outlet for all 10 parallel channels. The parallel droplets are pulled from the metering sections of the channels into the first reaction chamber, overcoming the resistance caused by the 1st capillary valves. When all droplets have crossed their respective capillary valves, the pumping stops. No sample droplets enter the second chamber yet, because

the second set of capillary valves is designed to be stronger (having higher burst pressure) than the first. At this point, all sample droplets reside inside their 1st reaction chambers, and are allowed to mix with the dried reagents. After a prescribed time, the pumping is recommenced. When the pressure difference reaches a level corresponding to the strength of the second set of capillary valves, the droplets sequentially overcome the restrictions and travel to the second reaction chamber. When all droplets reside in the second chamber, the pumping is stopped and the chip is heated to 41 °C. The sample droplets are left in this reaction chamber to mix with dried enzymes, and the NASBA reaction is initiated. During the amplification process the reaction chambers are scanned sequentially by the optical module, and the fluorescence signal is recorded.

Figure 1. Sketch of the NASBA amplification chip. The star-shaped inlet is in the lower left corner. The horizontal feed channel leads to the 10 parallel reaction channels and to the large waste chamber on the top of the chip. The hydrophobic valves are spotted at the three restrictions in each parallel channel. The lower/first restrictions are wider than the upper/last ones. Dried master mix will be spotted in the first chamber and dried enzymes will be spotted in the second chamber. A peltier element is placed under the area of the dashed line, and the optical module will be scanned across the upper chambers. In the figure, the parallel reaction droplets have reached the second reaction chamber.

3. TEST CHIP MANUFACTURING

Test chips both for fluidic and biological experiments on sub functions of the amplification chip have been manufactured. All chips were manufactured in cyclic olefin copolymer (COC). The chips used for testing of the hydrophobic valves were milled and laser ablated, whereas test chips for re-hydration of dried reagents and for NASBA amplification experiments were hot embossed.

After manufacturing, the chips were cleaned in an ultrasonic bath, air dried, and O2 plasma activated prior to coating with 0.5% polyethylene glycol (PEG) in methanol (Sigma Aldrich Norway AS, Norway). The PEG layer on the microchip surfaces after coating rendered the channel walls hydrophilic, with a contact angle to DI water measured to be approx. 30°

The hydrophobic valves were created in bottlenecks of the microchannels by spotting 0,5% Teflon 1600 AF (DuPont) using the PipeJet spotting system (BioFluidix [xx]). The contact angle of DI water on Teflon surface was measured to be

approximately 100º. Satisfactory coating of the valve structures was confirmed after spotting by microscope inspection. After coating and spotting, the chips were sealed with adhesive tape.

4. SAMPLE DROPLETS MOVING IN PARALLEL CHANNELS WITH HYDROPHOBIC VALVES

We have tested the feasibility of controlling the positioning of all sample droplets in the parallel channels by using one common pump combined with channel restrictions and hydrophobic valves [13]. The chips contained 7 parallel channels (800 μm × 200 μm, width × depth), each with four lateral restrictions of 380 μm, 150 μm, 75 μm and 33 μm (see figure 2). A total of 5 chips were tested.
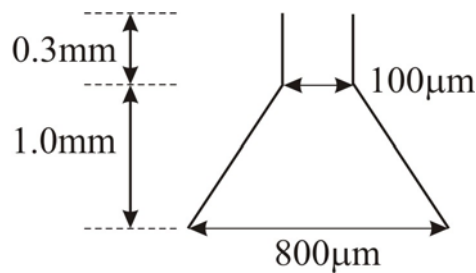
Figure 2. Details of capillary valve geometry (here, valve width is 100μm). The capillary valves contain a tapered part and a narrow restriction. The tapered part is included to aid spotting of the fluorpolymer for hydrophobization.

The chips were mounted in an aluminum frame and connected to a second chip containing a branch-like channel structure for distribution of the pressure to all parallel channels, see figure 3. A syringe pump (PHD2000 from Harvard Apparatus) was used to apply suction to the common chip outlet, withdrawing air from the system at a rate of 10 μl/min. A pressure sensor (TP3100 001A 0P from MEMSCAP) was used for monitoring the pressure in the tube connection to the parallel channels. A close-up image of the parallel actuation chip with liquid droplets in front of the first restriction is shown in figure 4.
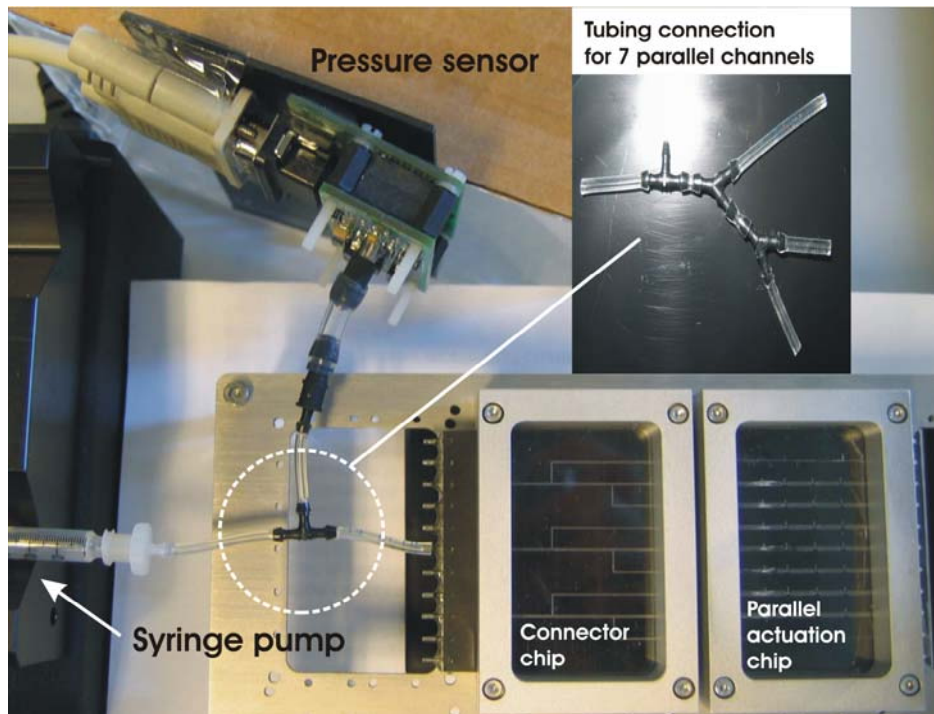
Figure 3. Test setup for monitoring of parallel droplet movement. The syringe connected to the pump is seen to the left, the MEMSCAP pressure sensor upper left. Tubing connects the pump and pressure sensor to the aluminum frame with the fluidic connectors, pressure distribution chip and the rightmost parallel droplet chip. The presented image shows the setup for actuation of three channels in parallel. For actuation of 7 channels simultaneously, the tubing connection between the syringe, the pressure sensor and the aluminum frame is exchanged with the connection shown in the upper right corner of the image.
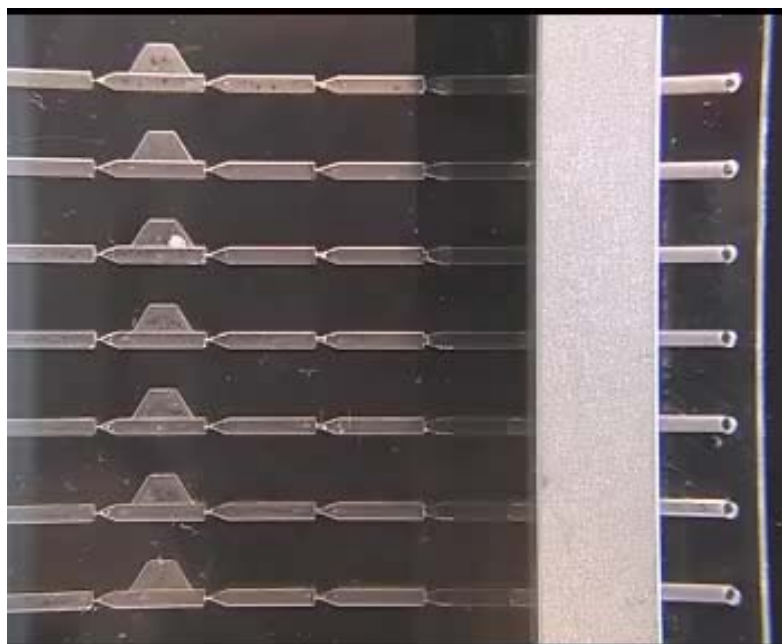

Figure 4. The seven parallel reaction and amplification test channels. Four restrictions with hydrophobic spots can be seen in each channel. Valve pressure strength increases from right to left. In the picture, 500 nl droplets are positioned before the rightmost, weakest valve.

The pressure as a function of time at the common pump connection for all channels was recorded during operation and is shown in figure 5. The pressure plot shows the absolute pressure. During pumping, the pressure in the system decreases until the pressure difference across the front water/air meniscus becomes higher than the burst pressure of the first hydrophobic valve. When the first droplet breaks through the valve, it enters the first chamber and the pressure in the common under pressure region is slightly relaxed due to the reduced volume between the liquid droplets and the syringe. The parallel liquid droplets sequentially pass their respective first valves; the order in which the droplets move is arbitrary, depending on slight variations in burst pressure of the seven valves due to manufacturing tolerances and pinning effects. No droplet passes to the second chamber before all droplets reside in the first chamber, because of the higher strength (burst pressure) of the second set of valves. This is clearly visible in figure 5, where the pressure level of the first seven valves is clearly higher than that of the 2nd valves. Once all sample droplets reside in the first chamber, they proceed to cross their respective 2nd valves as the pumping continues. Passing of the 4th valve results in one pressure rise only, because once a single sample droplet crosses this valve, the system is vented to atmospheric pressure and further pumping does not cause any more movement of any droplet.
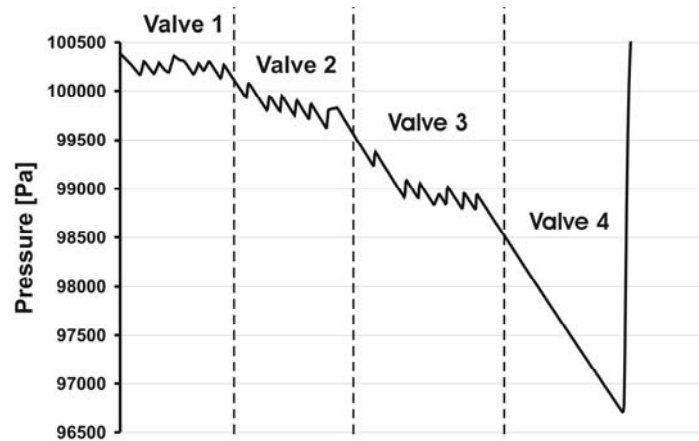

Figure 5. Common pulling pressure of parallel valves as a function of time. Parallel droplets are pulled at constant rate of 10 μl/min. Absolute pressure trace of seven parallel droplets moving through the parallel actuation chip. At the fourth valve, the first droplet to break the valve effectively ventilates the system, and hence the pressure in the system returns to atmospheric conditions.

The average burst pressures of the four valves are shown in figure 6. The data (open squares) represents average values from 5 chips with DI water as the working liquid. The error bars indicate the standard deviation of the differential pressure value. In addition to the use of DI water as working liquid, one experiment (open circles in figure 6) using pre-mixed sample with NASBA reagents was conducted to verify the system functionality. The measured burst pressures are seen to be slightly below that found for DI water. This is attributed to the slightly lower wetting angle of the sample mixture, which was measured to be approx. 95°.

The parallel droplet experiments demonstrate that the burst pressures for the sequential valves do not overlap, and that the principle of parallel droplet positioning using one single pump and hydrophobic valves is feasible. After these experiments were finished, biochemical experiments showed that two reaction chambers in sequence are sufficient. Therefore, in the design of the final device, the number of valves can be reduced from 4 to 3, thus giving the possibility to separate the levels of burst pressure of the valves even further.
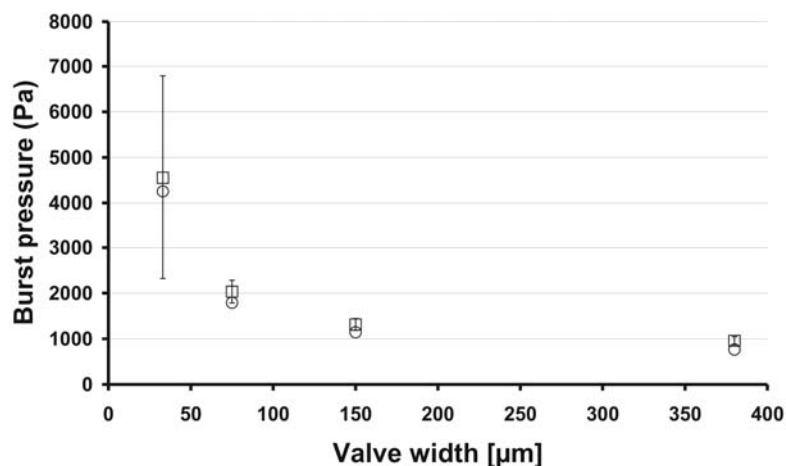
Figure 6. Burst pressures of the capillary valves as function of valve width. Symbols: □ DI water; ○ reagents; solid line represents the analytical values for water. Contact angle of DI water on Teflon was measured to be approx. 100º. The pressure data for each valve represents an average of 35 measurements (5 chips with 7 parallel channels each).

5. RE-HYDRATION OF DRIED MASTER-MIX AND ENZYMES

The NASBA amplification chip will have the all necessary reagents stored on chip in a dry state. In each parallel channel there will be two chambers with dried reagents, the first with the NASBA nucleotide ion-adjusted master mixture (master mix) that is specific for each HPV type mRNA amplification, and the second chamber containing the dried enzymes. One crucial question is whether the dried reagents will dissolve into the sample droplet in an acceptable time, or if further mixing by e.g. moving the droplet back and forth over the dried reagents is needed. Here we report on experiments monitoring the dissolution of the master mix and the enzymes.

The spotting of reagents and enzymes into the 500 nl hot embossed reaction chambers was performed using the spotter Nanoject II from Drummond Scientific Company. A volume corresponding to the required amount of reagents for a 500 nl sample size was spotted sequentially into the chambers, 30 nl at a time. The spotted liquid was allowed to dry for approx. 30 seconds between each deposited droplet. In this manner, a well-defined lump of reagents was deposited on the bottom surface of the reaction chambers. After spotting, the chips with dried reagents were stored at room temperature for at least three days prior to the re-hydration experiments.

In the actual master mix, molecular beacons which contain both a fluorophore and a quencher are present in the solution. The quenchers suppress the fluorescent signal of the fluorophore as long as the targeted mRNA is not present; upon detection, the fluorophore and the quencher are separated within the molecular beacon, releasing the fluorescent signal (in presence of external excitation). For the re-hydration experiments, molecular beacons (excitation/emission 490nm/520nm) without quenchers were added to the solution prior to spotting and drying. In this manner, the master mix was fluorescent without the need of actual amplification, and its re-hydration from solid state and diffusion into the sample could be monitored. In contrast to the master mix, the enzymes do not contain any fluorescent components. In order to permit fluorescent detection of the re-hydration and diffusion process of the dried enzymes, the antibody IgG (Southern Biotech, Birmingham, AL) fluorescently labelled with FITC (excitation/emission 495nm/515nm) was added to the enzyme suspension prior to spotting and drying in the reaction chambers. The fluorescent IgG tracer (molecular weight 150 kDa) thereby modeled the largest enzyme (molecular weight AMV-RT, 160 kDa).

The processes of re-hydration of the dried master mix and enzymes in the liquid samples were investigated experimentally by confocal laser scanning microscopy (CLSM). A Leica DM RXA epifluorescent microscope equipped with Leica TCS 4D confocal unit was used for the measurements. The imaging of the reaction chambers was performed via an HC PL Fluotar objective with 5-fold magnification and NA = 0.15. The low magnification was necessary in order to image the entire reaction chamber within the field of view of the microscope. As a consequence, the depth-wise

resolution of the measurements was limited, with optical slice thickness ~100µm. An Omnichrome Series 43 ArKr laser was used for sample illumination. The fluorescence filters were set for FITC detection, with excitation peak at 488nm and emission at >510nm.

The reaction chamber containing dried master mix was flooded [14] by injecting 1.5µl of the relevant solution containing 15% DMSO and 375 mM Sorbitol in water into the microchip via the chip supply channel. Sequences of images at a plane 150 µm above the bottom wall of the chamber were acquired in 12s time intervals (total chamber depth was 200µm). The re-hydration process of the dried master mix is shown in figure 7. As can be seen by inspection of the images, the re-hydration and diffusion of the fluorescent species is essentially completed after 60s. After that time, no significant changes in the fluorescent signal from the solution (neither with regard to intensity nor homogeneity) were observed. For the dried enzymes, the time before an equilibrium concentration of fluorescent IgG at 150 µm height was reached, was 10 minutes.
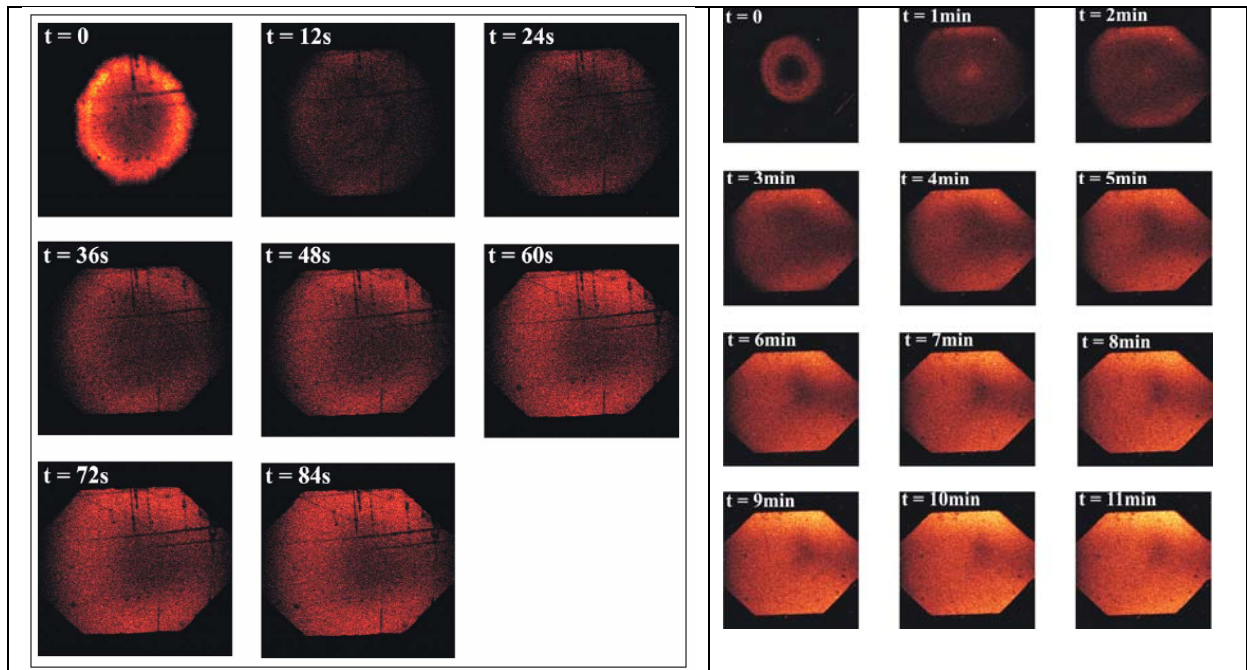

Figure 7. The left sequence of CLSM images shows the temporal evolution of fluorophore concentration, initially dried with the master mix at the bottom of the chamber. The focal plane is positioned 150 µm above the bottom of the chamber. Time between images is 12s. The image at t=0 is taken without the aqueous solution present in the chamber, with the focal plane at the bottom wall of the chamber; hence the high fluorescence intensity in the image. The image sequence on the right shows the concentration time evolution of fluorescent IgG at the same height. The fluorescent IgG was initially dried at the bottom of the chamber with the NASBA enzymes. Time between images is here 60 s.

In order to investigate whether the dissolution time of the dried reagents is dominated by the re-hydration or by the diffusion process, we compare these results with the theoretical estimate for the diffusion time of similarly sized molecules from the bottom of the chamber to the measurement plane, i.e. a distance of 150 µm. The average distance of diffusion L of a particle with a diffusion coefficient D is proportional to the square root of time t:

$$L = \sqrt{2Dt} \ .$$

The Stokes – Einstein diffusion coefficient for a spherical particle suspended in an aqueous solution is given by:

$$D = \frac{kT}{3\mu\pi d_p}$$

where k is the Boltzmann constant, T is the absolute temperature, μ is the dynamic viscosity and d_p is the particle diameter. We have estimated the diameter of the fluorescent molecular beacons in the master mix to be $d_{MB} \approx 2.8$ nm, while the diameter of the fluorescently labelled IgG is estimated to be $d_{MB} \approx 7$ nm. Using these sizes, we estimate an average diffusion time of 70 s for the fluorescent beacons to reach the 150 μm plane. The larger IgG proteins will have an average diffusion time of approximately 3 minutes.

Although the estimates given above are crude, they indicate that the diffusion time for the molecular beacons roughly corresponds to the experimentally observed time needed to obtain a homogeneous suspension of master mix in DMSO/sorbitol/water solution. This, in turn, implies that the dissolution process is diffusion-limited. Re-hydration itself is rapid, occurring nearly instantaneously as compared to the time scale of diffusion.

For the enzymes, we observe that the time required to achieve a homogeneous concentration of fluorescent IgG is significantly larger than the estimated diffusion time. This implies that the re-hydration of the enzymes is slow and dominates the process of dissolution. Nevertheless, complete dissolution is observed without the aid of external actuation.

Dissolution of the enzymes will be the final step on the NASBA chip prior to detection of the fluorescent signal. Microscope inspection of the reaction chamber after re-hydration revealed that no residue of the dried material is left on the chamber wall. Therefore, the bottom of the reaction chamber does not disturb the optical detection of the amplification process, and it may be performed in the same chamber as the dissolution of the enzymes. The amplification is time-consuming, requiring approximately 90 min before unambiguous decision about presence/absence of the targeted species can be reached.

Based on these experiments we conclude that no active mixing mechanisms are necessary neither for the dissolution of the master mix, nor the enzymes. In both cases, the mixing time is most probably sufficiently fast for the functionality of the NASBA chip.

6.   NASBA AMPLIFICATION IN NANOLITER CHAMBERS WITH DRIED ENZYMES

The critical test of the NASBA amplification chip is whether it is possible to obtain and detect successful NASBA amplification of oligos or HPV mRNA using separate sample volumes of 500 nl and dried reagents. Tests have been performed on re-hydration and re-activation of dried enzymes The enzymes needed for the NASBA reaction are AMV-RT, RNaseH and T7 RNA polymerase. The hot embossed 500 nl reaction chambers in COC were also coated with PEG. Milled reaction chambers had no positive amplifications; this may be due to insufficient PEG coating of rough surfaces and thereby the adsorption of enzymes on the walls, which inhibits the reaction.

The NASBA reagents were provided in the PreTect HPV-Proofer kit (NorChip AS, Norway). A stabilizing agent, 0.5% PEG 8000 (Sigma Aldrich Norway AS), was added to the enzyme solution prior to spotting and drying [15]. The microchips were spotted with enzymes using the Nanoject II from Drummond Scientific Company, dried in room temperature for up to 1 day and sealed with adhesive tape.

The amplification reaction was performed by first manually mixing the NASBA reagents, except the enzymes, and a positive control sample of human papillomavirus (HPV) type 16. The mixture was first incubated at 65˚C for 3 minutes followed by 3 minutes at 41˚C. The microchip containing the dried enzymes was tempered to 41˚C before 1 μl reaction mixture was applied to each of six reaction chamber in parallel on the microchip. The sample droplets were positioned in the reaction chambers by applying hydrophobic spots in the narrow channels leading in and out of the chamber. A custom-made instrument recorded the fluorescent signal of the amplification reaction at 41˚C [3], scanning 6 reaction chambers on one chip. Figure 8 shows amplification curves of the 500 nl reaction chambers for reactivation of spotted and dried enzymes.
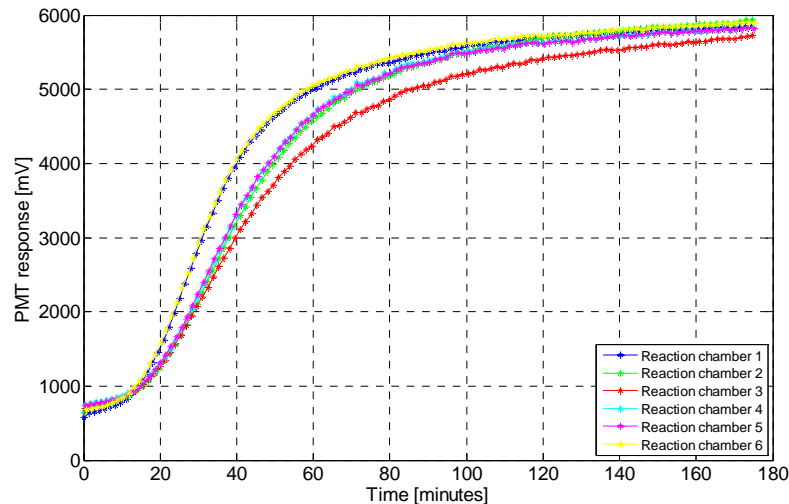
Figure 8. Amplification curves of the six 500 nl reaction chambers run simultaneously on one microchip employing a sample of positive control for HPV type 16 (0.1 μM). The dried enzymes including PEG 8000 were re-hydrated after 1 day at room temperature. No amplification was observed when PEG 8000 was excluded in the drying procedure.

The present work demonstrates on-chip storage of dried enzymes which are reactivated upon re-hydration of sample. These results are promising with regard to the development of disposable self-contained microchips for NASBA.

7. CONCLUSIONS

We have presented experimental results on sub-functions of a microchip for simultaneous detection of multiple mRNA targets, specifically HPV viruses that predispose for cervical cancer. It was shown that by manufacturing passive valves consisting of channel restrictions with hydrophobic spotting, the simultaneous movement of droplets in parallel channels could be controlled by one external pump, only. Tests were also performed on the re-hydration of the reagents needed for NASBA amplifications. Pre-tests had shown that master mix and enzymes had to be dried in two separate chambers. Spotting and drying of reagents in chambers, and later re-hydration with fluorescent markers, showed that the dried reagents were re-hydrated on time scales acceptable for on-chip NASBA reactions. The most important result in the project so far is that re-activation of dried enzymes in a nanoliter-sized reaction chamber is possible and may result in positive NASBA amplifications. To our knowledge, this is the first time the NASBA enzymes have been successfully dried and re-activated on-chip. The next step in the project is to use the prototype NASBA amplification chip with all functions included, in order to verify that the sub functions work when they are combined. Also, the re-activation of dried master mix will be developed.

ACKNOWLEDGEMENTS

REFERENCES

1. MicroActive FP6 STREP, contract IST-NMP-CT-2005-0173319 http://www.sintef.no/microactive

2. P. Yager, T. Edwards, E. Fu, K. Helton, K. Nelson, M. R. Tam and B. H. Weigl, "Microfluidic diagnostic technologies for global public health", *Nature* 442, 412-418 (2006)

3. A. K. Lie et al., " DNA versus RNA based methods for human papilloma virus detection in cervical neoplasia", *Gynecologic Oncology* 97, 908-915 (2005)

4. Walboomers et al. "Human papilloma virus is a necessary cause of invasive cervical cancer worldwide", *J. Pathol.* 189(1), 12-19 (1999)

5. I. Kraus, T. Molden, L. E. Ernø, H. Skomedal, F. Karlsen and B. Hagmar, "Human papillomavirus oncogenic expression in the dysplasticportio; an investigation of biopsies from 190 cervical cone*"*, *British Journal of Cancer* 90, 1407-1411 (2004)

6. http://www.norchip.com/

7. J. Compton, "Nucleic acid sequence-based amplification," *Nature* 350, 91 (1991).

8. A. Gulliksen, L. Solli, F. Karlsen, H. Rogne, E. Hovig, T. Nordstrøm and R. Sirevåg, "Real-time Nucleic Acid Sequence-Based Amplification in Nanoliter Volumes", *Anal. Chemistry* 76, 9-12 (2004)

9. A. Gulliksen, L. Solli, K. S. Drese, O. Sörensen, F. Karlsen, H. Rogne, E. Hovig and R. Sirevåg, "Parallel nanoliter detection of cancer markers using polymer microchips", *Lab Chip* 5, 416-419 (2005)

10. M. A. Burns, B. N. Johnson, S. N. Brahmasandra, K. Handique, J. R. Webster, M. Krishnan, T. S. Sammarco, P. M. Man, D. Jones, D. Heldsinger, C. Mastrangelo, and D. T. Burke, "An integrated nanoliter DNA analysis device", *Science*, 282, 484-487 (1998)

11. B. H. Weigl, J. Gerdes, P. Tarr, P. Yager, L. Dillman, R. Peck, S. Ramachandran, M. Lemba, M. Kokoris, M. Nabavi, F. Battrell, D. Hoekstra, E. J. Klein and D. M. Denno, "Fully integrated multiplexed lab-on-a-card assay for enteric pathogens,", *Proc. of SPIE* 6112, 1-11 (2006)

12. V. Linder, S. K. Sia and G. M. Whitesides, "Reagent-loaded cartridges for valveless and automated fluid delivery in microfluidic devices", *Anal Chem*, 77, 64 (2005)

13. K. W. Oh, C. H. J. Ahn, "A review of Microvalves", *Micromech. Microeng* 16, R13-R39 (2006)

14. F. Goldschmidtboeing, M. Rabold, and P Woias, "Strategies for void-free liquid filling of micro cavities", *Journal of Micromech. and Microeng.*, 16 (7), 1321 - 1330 (2006)

15. E. Garcia, J. R. Kirkham, A. V. Hatch, K. R. Hawkins and P. Yager, "Controlled microfluidic reconstitution of functional protein from an anhydrous storage depot", *Lab Chip* 4, 78 (2004)

STORAGE AND REACTIVATION OF ENZYMES IN A DISPOSABLE, SELF-CONTAINED LAB-ON-A-CHIP SYSTEM

A. Gulliksen[1, 2], M.M. Mielnik[3], E. Hovig[4], F. Karlsen[1], L. Furuberg[3] and R. Sirevåg[2]

[1] NorChip AS, NORWAY, [2] University of Oslo, NORWAY
[3] SINTEF, NORWAY, [4] The Norwegian Radiumhospital, NORWAY

ABSTRACT

For a point-of-care chip, it is important to have reactants stably stored on-chip. In this study, deposition and drying of a solution of three enzymes have been examined. The time for diffusion/rehydration of fluorescently labelled mouse IgG in the dried enzyme solution was ~10 minutes. Microchips with native cyclic olefin copolymer (COC) surfaces showed large adsorption of mouse IgG, while polyethylene glycol (PEG) coated surfaces showed adequate protein resistance. Successful amplification on chip in 500 nl reaction chambers was obtained for spotted and dried enzymes when mixed with 0.5% PEG 8000.

Keywords: NASBA, mRNA amplification, fluorescence detection, diagnostics

1. INTRODUCTION

In this work, which is part of a project aimed at the development of a self-contained disposable microchip, we have explored an approach for stabilization and storage of three labile enzymes (AMV-RT, RNase H and T7 RNA polymerase) in a single formulation on-chip for the isothermal amplification of mRNA and ssDNA by nucleic acid sequence-based amplification (NASBA) [1]. Similar work has been performed by Weigl and co-workers [2] for air drying and reactivation of PCR mixes in conventional macro scale volumes using 96-well plates. While PCR is only dependent on the activity of one enzyme for amplification, NASBA requires 3 functional enzymes. NASBA has previously been demonstrated on-chip with premixed reagents [3]. The work reported herein evaluates the reactivation of enzymes after drying on-chip. To our knowledge, this is the first time the NASBA enzymes have been successfully dried and reactivated in a microchip. For self-contained systems, both liquid and dried reagents have been proposed as plausible solutions for long-term on-chip storage of reagents [4, 5].

2. EXPERIMENTAL

For the experiments, COC chips were employed Figure 1 (a). The microchips were O_2 plasma activated prior to coating with 0.5% PEG in methanol (Sigma Aldrich Norway AS, Norway). The NASBA reagents were provided in the PreTect HPV-Proofer kit (NorChip AS, Norway). A stabilizing agent, PEG 8000 (Sigma Aldrich Norway AS), was added to the enzyme solution prior to spotting and drying. For the enzyme wall adsorption and the diffusion experiments, fluorescently labelled mouse IgG (Southern Biotech, Birmingham, AL) was used as a model molecule. The microchips were spotted with enzymes, dried in room temperature for up to 2 days before they were sealed with adhesive tape. The amplification reaction was performed by manually mixing the NASBA reagents, including the positive control sample of human papillomavirus (HPV) type 16. The mixture was first incubated at 65°C for 3 minutes followed by 3 minutes at 41°C. The microchip containing the dried enzymes was adjusted to 41°C before 1 µl reaction mixture was applied to each

reaction chamber on the microchip. A custom-made instrument recorded the fluorescent signal of the amplification reaction at 41˚C [3].
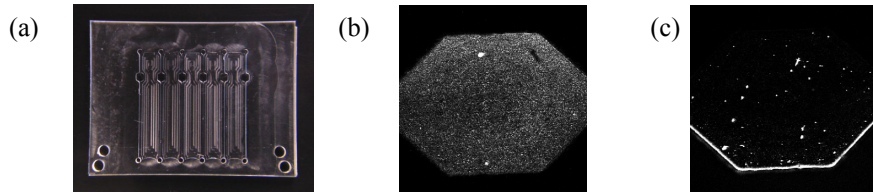

Figure 1. (a) COC microchip fabricated using hot embossing. The outer dimensions of the chip were 43 mm × 32 mm, while the dimensions of the reaction chambers were 1.6 mm × 1.6 mm × 0.2 mm, corresponding to a volume of 500 nl. (b), (c) Adsorption of fluorescently labelled mouse IgG on native COC and PEG coated COC surfaces. (b) The native COC surface was only plasma activated. No amplification could be observed for microchips with native COC surfaces. (c) The PEG coated COC surface showed adequate resistance to the fluorescently labelled IgG. PEG coated surfaces gave successful amplification reactions.

3. RESULTS AND DISCUSSION

Adsorption of enzymes to the microchip surface, as well as the process of rehydration of the dried enzymes in the liquid sample, were investigated experimentally using confocal laser scanning microscopy (CLSM). Both native COC and PEG coated surfaces were incubated with NASBA reaction mixture including fluorescent IgG at 41˚C for 2.5 hours followed by a washing step with water. The native COC surfaces showed large adsorption of IgG, while PEG coated surfaces showed adequate protein resistance, Figure 1 (b) and (c).

For the diffusion measurements, the enzyme solution was mixed with fluorescent IgG prior to spotting and drying. Rehydration and diffusion of the fluorescent IgG species were essentially completed after approximately 10 minutes, see Figure 2.
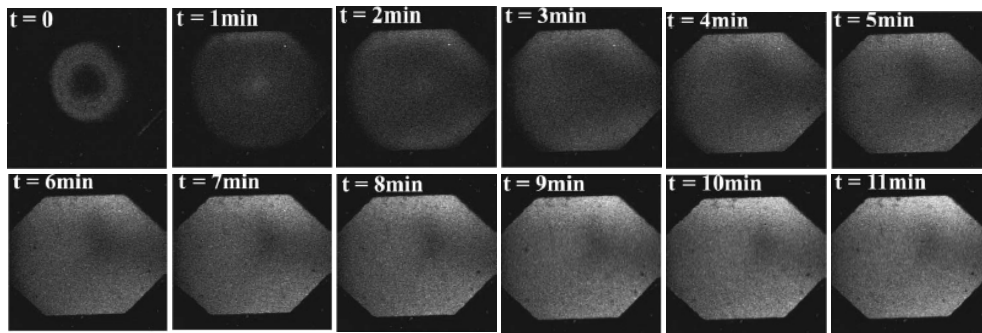

Figure 2. Rehydration of dried enzymes, temporal evolution. The time between frames was 1 minute. The dissolving liquid was a solution of all the NASBA reagents except the enzymes. The dried enzyme spot was inside a 500 nl chamber. All images were taken with the focal plane positioned 150 μm above the bottom wall of the reaction chamber.

The estimated time of diffusion time for a distance of 150 μm was ~3 minutes. The observed longer time required, may indicate that the process was dominated by rehydration and not by diffusion. The fluorescent IgG tracer (150 kDa) modelled the largest enzyme (AMV-RT, 160 kDa).

Figure 3 shows amplification curves of the 500 nl reaction chambers for reactivation of spotted and dried enzymes containing 0.5% PEG 8000. No amplification was observed when PEG 8000 was excluded in the drying procedure. The 10 minutes required for enzyme dissolution was shown to be acceptable.
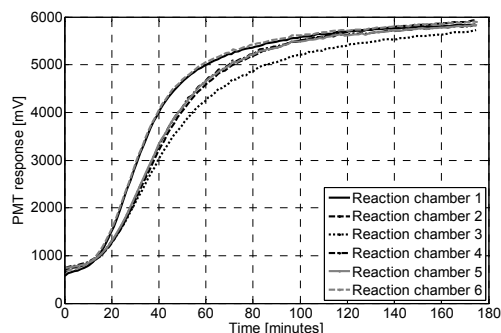

Figure 3. Amplification curves of the six 500 nl reaction chambers run simultaneously on one microchip employing a sample of positive control for HPV type 16 (0.1 µM). The dried enzymes including PEG 8000 were rehydrated after one day at room temperature.

4. CONCLUSIONS

The present work demonstrates on-chip storage of dried enzymes which are reactivated upon rehydration of sample. These results are promising with regard to the development of disposable, self-contained microchips for NASBA.

REFERENCES

[1] J. Compton, *Nucleic acid sequence-based amplification*, Nature, 350, 91 (1990)
[2] B. H. Weigl, J. Gerdes, P. Tarr, P. Yager, L. Dillman, R. Peck, S. Ramachandran, M. Lemba, M. Kokoris, M. Nabavi, F. Battrell, D. Hoekstra, E. J. Klein and D. M. Denno, *Fully integrated multiplexed lab-on-a-card assay for enteric pathogens*, Proc. of SPIE, 6112, 1 (2006)
[3] A. Gulliksen, L. Solli, K. S. Drese, O. Sörensen, F. Karlsen, H. Rogne, E. Hovig and R. Sirevåg, *Parallel nanoliter detection of cancer markers using polymer microchips*, Lab Chip, 5, 416 (2005)
[4] E. Garcia, J. R. Kirkham, A. V. Hatch, K. R. Hawkins and P. Yager, *Controlled microfluidic reconstitution of functional protein from an anhydrous storage depot*, Lab Chip, 4, 78 (2004)
[5] V. Linder, S. K. Sia and G. M. Whitesides, *Reagent-loaded cartridges for valveless and automated fluid delivery in microfluidic devices*, Anal Chem, 77, 64 (2005)

SAMPLE METERING AND PARALLEL LIQUID PLUG ACTUATION FOR MULTIPLE BIOCHEMICAL ASSAYS

M. M. Mielnik[1], J. Voitel[1], L. A. Solli[2] and L. Furuberg[1]
[1]SINTEF, Dept. of Microsystems and Nanotechnology, Oslo, NORWAY
[2]NorChip AS, Klokkarstua, NORWAY

ABSTRACT

We demonstrate a passive microfluidic system for simple and efficient metering, handling and control of parallel nanoliter samples on-chip. The system consist of two chips; one for sample loading, splitting and metering (based on capillary forces only), and another for sample plug movement in parallel reaction channels. The latter is based on a set of capillary valves and one single external pressure source only. The strength of the capillary valves determines the logic movement of the parallel sample droplets on-chip, permitting robust system control. In general, the presented system is applicable to a variety of multi-step reaction protocols with a large number of parallel channels.

Keywords: microfluidics, capillary valve, lab-on-a-chip, sample control

1. INTRODUCTION

Passive fluidic manipulation is a promising approach in Lab-on-a-Chip (LOC) systems due to the inherent mechanical simplicity of the resulting device, rendering it well suited for mass production with a minimum of (costly) assembly needs. Such manipulation has been previously explored by e.g. [1], [2], and [3].

Here, we present a passive system designed for on-chip amplification of mRNA and ssDNA by Nucleic Acid Sequence-Based Amplification (NASBA, see [4]), in our case requiring two separate isothermal steps and mixing with two different dried reagents stored on-chip. We demonstrate a passive system providing efficient multi-step control of liquid samples in several separate, parallel microchannels. Specifically, simultaneous fluidic control of seven parallel channel systems is demonstrated, permitting the analysis of a single sample for e.g. seven different markers.

3. EXPERIMENTAL

The experiments were performed using two types of test chips, one for metering (fig.1a) and another for control of sample plug movement in parallel channels (fig.1b). The chips were manufactured in Cyclic Olefin Copolymer (COC) by micromilling (features >100µm) and laser ablation (features of 100µm and below). Because native COC is hydrophobic, the chips were coated with 0.5% polyethylene glycol (PEG) in methanol (Sigma Aldrich). The contact angle of DI water on PEG surface was approx. 30º. The valve structures consisted of a tapered part and a narrow restriction channel, see fig. 2. Teflon fluorpolymer (AF1600, DuPont) was spotted using PipeJet spotting system (BioFluidix) onto the tapered part of the valve, filling the narrow valve structures by capillary action. The contact angle of DI water on Teflon surface was measured to be approx. 110º. After coating and spotting, the chips were sealed with adhesive tape.

Both DI water and pre-mixed NASBA reagents with sample were used as working liquids to verify the chip functionality.

The parallel sample plug movement chip (fig.1b.) was mounted in an aluminium frame with fluidic interconnects, and coupled to a syringe pump (PHD2000, Harvard Apparatus) which was used to apply suction to the common chip outlet, withdrawing air from the system at a rate of 10 μl/min. A pressure sensor (TP3100 001A 0P from MEMSCAP) was used for monitoring the pressure in the common tube connection to the parallel channels (see fig. 1b).
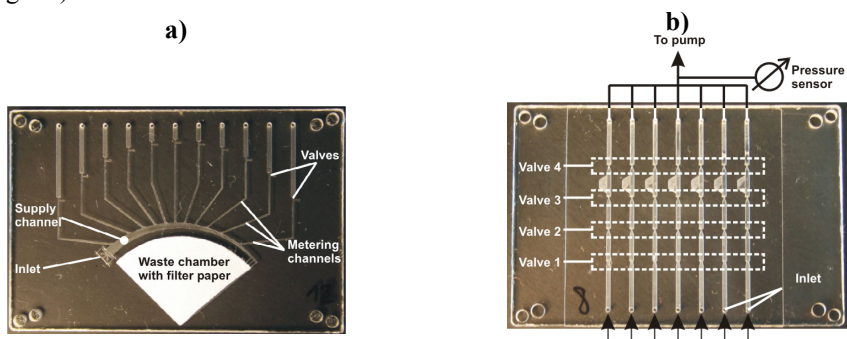

Figure 1: **a)** Sample metering chip. The sample is introduced at the inlet, filling up the supply channel (depth 200μm) and the eleven individual metering channels (75μm×300μm, volume 335nl) up to the hydrophobic capillary valves. Excess sample is drawn into the waste chamber, which contains an absorbing filter paper. **b)** Parallel reaction/actuation chip. The capillary valves increase in strength in the downstream direction, with valve widths of 380μm, 150μm, 75μm and 33μm. The depth of all channels is 200μm. The width of the parallel channels is 800μm.
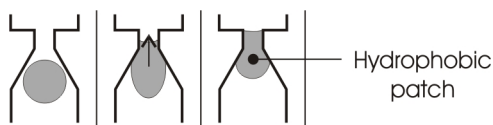

Figure 2: Capillary valve geometry. Hydrophobization was achieved by spotting a droplet of Teflon in the tapered part of the valve, filling the narrow restriction by capillary forces.

4. RESULTS AND DISCUSSION

The metering chip (fig.1a) was tested using different sample volumes at the inlet. It was found that the smallest sample volume permitting proper chip operation was 17μl. For this and larger volumes, the sample was drawn into the supply channel, filled all metering channels up to the position of the capillary valves, while excess sample was absorbed into the waste chamber. During the accompanying drainage of the supply channel, the liquid contained in the parallel metering channels was pinched off at the inlets, leaving a precisely metered sample plug of 335nl in each metering channel. The whole process required approximately 2 minutes from sample insertion till completed absorption of waste. Smaller sample volumes resulted in incomplete filling of metering channels, or incomplete drainage of the supply channel to waste. When the waste chamber was ventilated to the atmosphere, a smaller sample volume (15μl) could be applied at the chip inlet.

The parallel plug control chips (fig.1b) consisted of 7 parallel channels, each containing 4 hydrophobic valves of increasing strength. During operation, sample plugs introduced at the inlets all passed valve 1 before any plug passed valve 2, etc., such that all plugs resided at the same downstream position in the channels. In this manner, parallel, multi-step motion of the sample plugs was achieved.

A typical pressure trace of the movement of seven parallel plugs through the system is shown in fig.3a. For each valve, seven pressure minima can be observed, indicating the passing of a liquid plug through its respective valve. Figure 3b shows average burst pressure data collected from 5 chips. The solid line is the plot of the Young-Laplace equation representing the burst pressure required to overcome a capillary burst valve, see [5]. As can be seen from the figure, the valve performance is well predicted by the theoretical approximation.
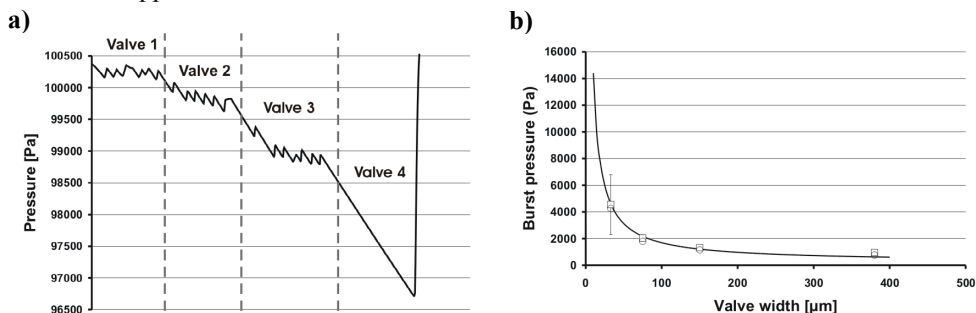
a)

b)

Figure 5: **a)** Pressure trace of seven parallel plugs moving through the parallel actuation chip. At the fourth valve, the first plug to break the valve effectively ventilates the system, and hence the pressure in the system returns to atmospheric conditions. **b)** Pressure characteristics of the capillary valves. Symbols: □ DI water; ○ reagents; solid line represents the analytical values for water.

5. CONCLUSIONS

A passive system capable of robust, parallel sample control on-chip using a single external pressure source has been demonstrated. The system relies on capillary valves of varying strength. The presented method is applicable to a variety of multi-step reaction protocols and can be extended to a large number of parallel channels.

REFERENCES

[1] K. Handique, D.T. Burke, C.H. Mastrangelo, M.A. Burns, *Nanoliter Liquid Metering in Microchannels using Hydrophobic Patterns,* Anal. Chem. 72 (17), pp. 4100-4109 (2000).

[2] C.H. Ahn, J-W. Choi, G. Beaucage, J.H. Nevin, J-B. Lee, A. Puntambekar, J.Y.Lee, *Disposable Smart Lab on a Chip for Point-of-Care Clinical Diagnostics,* Proc. IEEE 92 (1), pp. 154-173 (2004).

[3] S-H. Lee, C-S. Lee, B-G. Kim, Y-K. Kim, *Quantitatively controlled nanoliter liquid manipulation using hydrophobic valving and control of surface wettability,* J. Micromech. Microeng. 13, pp. 89-97 (2003).

[4] J. Compton, *Nucleic-acid sequence-based amplification*, Nature 350 (6313), pp. 91-92 (1991).

[5] H. Cho, H.Y. Kim, J.Y. Kang, T.S. Kim, *How the capillary burst microvalve works*, J. Colloid and Interface Science 306, pp. 379-385 (2007).

AUTOMATED CHIP-BASED EXTRACTION OF HPV mRNA FROM CERVICAL SAMPLES

Tobias Baier[1], Thomas Hansen-Hagge[1], Rainer Gransee[1], Anne Crombé[1], Sabine Schmahl[1], Klaus S. Drese[1], Petter Grønn[2], Lars Solli[2], Inger Marie Falang[2], Cara Martin[3], Helen Keegan[3], Liv Furuberg[4]

[1]*Institut für Mikrotechnik Mainz GmbH, Germany*
[2]*Norchip AS, Norway*
[3]*Coombe Womens Hospital Dublin, Ireland*
[4]SINTEF Microsystems and Nanotechnology, Norway

ABSTRACT

A Lab-On-Chip system with an operating device was developed for the automated extraction of mRNA from cell samples in methanol based solution (PreservCyt™). The target application is extraction of mRNA from cervical smears for detection of HPV-infections. The device accepts 3 ml of sample and performs the extraction in a disposable polymer chip of credit card size. All necessary reagents for lysis, washing and elution are stored on-chip and the extraction is performed in two filter stages for cell pre-concentration and nucleic acid capture. Tests with HeLa cell lines confirm the extraction of HPV-mRNA by the system.

KEYWORDS: Sample preparation, RNA extraction, HPV, Point-of-Care

INTRODUCTION

Cervical cancer is the second most predominant form of cancer among women in developed countries [1]. Nearly all cases of this cancer are directly linked to previous infection with one or more of cancer-inducing types of human papilloma virus (HPV) [2]. Detection of persistent HPV infections is thus desirable in order to initiate treatment before the disease can develop. Moreover, opposed to HPV-DNA detection, the detection of HPV E6/E7 mRNA expression indicates a transforming infection and higher oncogenicity [3]. Against this background, we present a chip-based automated platform for the extraction of nucleic acids, including HPV mRNA, from cervical samples (fig.1).

EXPERIMENTAL

The device accepts 2-5 ml of a suspension of fixated cervical smear cells in a methanol based solution (PreservCyt™). The sample is injected into a disposable chip (fig. 1b) by a syringe and cells are collected on a nylon filter as first step. These cells are then chemically lysed by flushing a high-molar caotropic salt solution through the filter chamber. The lysate passes a solid phase extraction (SPE) chamber containing a stack of silica filter membranes, where the mRNA is retained. Two washing steps remove cell debris and salt. After air-drying of the filter stack, the captured RNA is eluted by rinsing with water. In order to minimize user interference

during the sample preparation procedure all liquids necessary for the operation are stored on chip.

The fluid actuation is maintained by two syringe pumps (fig.1a). The sample is supplied in a disposable syringe. A second reusable syringe is used for pressure driven actuation of assay reagents as well as for air-drying before elution. The fluid control is performed by three turning valves. A heater elevates the temperature both during cell lysis and air-drying of the SPE chamber. The device is controlled by a customised LabView program running on a laptop. After insertion of the chip and the syringe containing the sample into the operating device the extractions proceeds without further user intervention.

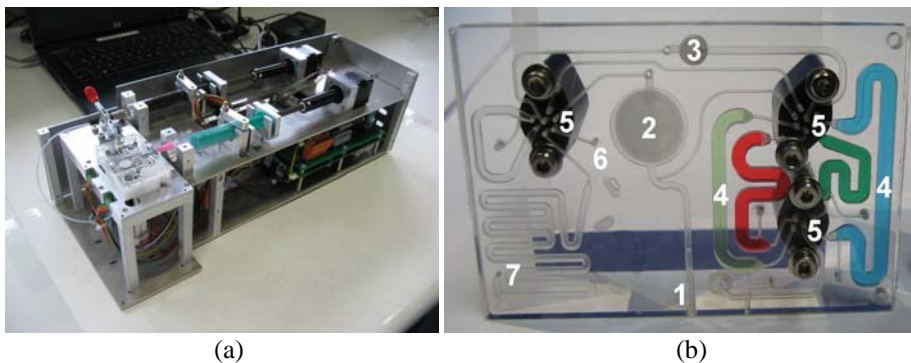(a)   (b)

Figure 1a: Operating device for the automated extraction of mRNA from cervical samples, containing two syringe pumps for fluid actuation, motors for valve operation, a heater and several light barriers for motor control and fluid positioning as well as the electronic control. The device is operated by a customized LabView program.
Figure 1b: Chip for sample preparation. The basic functional units contain (1) sample inlet, (2) cell filter, (3) SPE chamber, (4) reagent storage section, (5) turning valves, (6) waste outlet, (7) sample outlet. The chip material is cyclic olefin copolymer (COC).

RESULTS AND DISCUSSION

The device has been tested on HeLa cell lines which express HPV-18 mRNA. In order to validate the device performance the eluate from the instrument was amplified by Nucleic Acid Sequence Based Amplification (NASBA) using the PreTect® HPV-Proofer kit (NorChip AS, Klokkarstua, Norway). A sample read out from the on-line fluorescence detection during the NASBA is shown in figure 3, demonstrating a positive amplification result and thus a successful mRNA extraction. Presently, the device is investigated and optimized for its robustness and extraction efficiency as well as its performance with clinical samples.
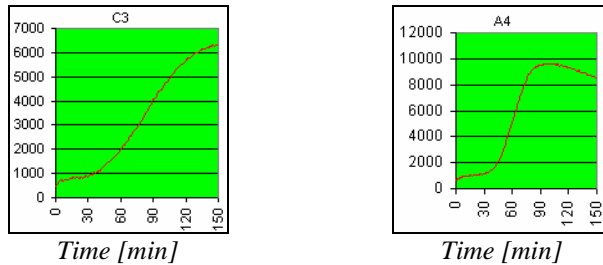
Time [min]   *Time [min]*

Figure 3. NASBA result showing the fluorescence intensity vs. time. Fluorescence intensity corresponds to RNA-yield.
C3: Sample positive for HPV18: 10^5 Hela-cells chemically lysed on the chip and eluted with 60µl water. Amplification using PreTect® HPV-Proofer kit (NorChip AS, Klokkarstua, Norway) and 5µl RNA-eluate.
A4: Positive control for HPV18. Amplification using PreTect® HPV-Proofer kit and 5µl RNA-sample.

CONCLUSIONS

The presented sample preparation device has been developed within the EU funded project MicroActive [4, 5]. This project aims at integrating the sample preparation device with a second automated instrument for on-chip parallel NASBA amplification and detection of several HPV types. The combined system may thus serve as a point of care system for the detection of HPV infection directly in a physician's office, avoiding the often delayed analysis by a specialized laboratory. However, the presented sample preparation device is not limited to cervical samples and opens the way for a range of similar sample treatment applications.

ACKNOWLEDGEMENTS

REFERENCES

1. D.M. Parkin, F. Bray, J. Ferlay, P. Pisani, "Estimating the world cancer burden: Globocan 2000", Int J Cancer, **94**, 2, 153-156 (2001).
2. J. M. M. Walboomers, et al., "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide.", J. Pathol., **189**, 12–19, (1999).
3. I. Kraus, et al., "Presence of E6 and E7 mRNA from human papillomavirus types 16, 18, 31, 33, and 45 in the majority of cervical carcinomas", J Clin Microbiol. **44**, 4, 1310-7, (2006).
4. www.sintef.no/microactive
5. L. Furuberg et al. "RNA amplification chip with parallel microchannels and droplet positioning using capillary valves", Microsystem Technologies, **14**, 4-5, 673-681, (2008).

MicroActive

Automatic Detection of Disease Related Molecular Cell Activity

MicroActive will develop an instrument for molecular diagnostics intended for use in the doctors' office. The instrument will in the first instance be used to screen patients for a group of viruses, known as human papilloma virus, which is implicated in cervical cancer. Microfluidics and biotechnology form the basis for the development..

Objectives of the project

Currently many common diseases require that samples are sent to remote labs for diagnosis. This is costly, time consuming, increases patient anxiety and delays the start of treatment. MicroActive will make it feasible to carry out automatic, accurate diagnosis at the local doctor's office.

MicroActive will achieve this by using bio-marker mRNA detection. Compared to commonly used approaches (e.g. PCR amplification and immunoassay methods), mRNA detection avoids false positive results and has a high sensitivity. This approach is currently used to detect cervical pre-cancer, cancer, STDs and a range of respiratory diseases, to mention a few. In addition, recent advances in the field of molecular biology and high throughput technologies are generating hundreds of potential bio-markers every day. MicroActive will:

- Develop an integrated system based on microtechnology and biotechnology for automated diagnosis of a wide range of diseases. The system will analyze biological samples and be specifically designed for use in primary health care.
- Validate the sensitivity of the system using cytological samples from women at risk of developing cervical cancer (the second most common female cancer) as test cases. Results from the new, automated system will be compared with gold standard hospital lab tests for human papilloma viruses (HPV).
- Prepare for industrial production of the system.

Project Description

Within the MicroActive project the partners will:

- Develop one chip for sample preparation with all the necessary liquid reagents integrated.
- Develop a second chip for amplification and detection of HPV with dried spotted reagents stored in micro-channels.
- Develop disposable chips so there will be no risk of contamination between samples.
- Develop manufacturing methods for spotting and drying of reagents, surface coating, patterning, and polymer chip lamination that will not inhibit the biomolecular processes.
- Perform multi target detection from a single sample. This is possible due to simultaneous amplification and detection in separate parallel detection channels.
- Test more than 5 different bio-markers of (in the first instance biomarkers of HPV infection) from one sample droplet.
- Performance of system will be tested. At early stages of the project, perform tests on clinical samples. This is already underway at, using test-chips for separate functions
- Develop an instrument without manual protocols.
- Test the instrument on clinical specimens and

'A low-cost, fully automated diagnosis system will widen the availability of advanced diagnostics for all citizens'

Scenario

Year 2009: Anne visits her doctor for her cervical smear test. Three years ago she had to wait for weeks while the pap-smear was analyzed at a central laboratory. This time the doctor selects a polymer chip for cervix screening from his fridge. A droplet of a solution containing Anne's epithelial cells is applied to the polymer chip and the chip is inserted into the MicroActive instrument on his desk. Two hours later the doctor tells her that her test is negative; no mRNA activity was found for the 5 markers of high cancer risk human papilloma virus types. This result has a lower probability for false positive results than those obtained from traditional tests.

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compare to gold standards
- Address factors such as reliability, usability and cost of the total instrument which are crucial to acceptance by health care professionals.
-

Expected Results & Impacts

A low-cost, fully automated diagnosis system will widen the availability of advanced diagnostics for all citizens. The end result will reduce the time from patient testing to diagnosis, lessen patient anxiety and facilitate earlier treatment.

Using an automatic diagnosis system as an alternative to today's diagnostic testing will imply cost savings for the public health authorities.

The result of the MicroActive project is an automated diagnostics instrument that will be unique because:

- It is based on sensitive detection of RNA bio-markers. This method offers high clinical sensitivity to cellular activity related to disease, and largely avoids false positive results.
- It provides a generic technology platform, consisting of a re-usable instrument and two disposable chips:
  - o The chips are disposable to avoid contamination between samples.
  - o Chip no. 1 will include reservoirs with all necessary reagents to perform sample preparation consisting of cell concentration, lysis and nucleic acid purification.
  - o Chip no. 2 will include all reagents to perform multiplex amplification and fluorescent detection of mRNA. The enzymes and primers will be stored in a dry state for long-term stability. The primers are disease specific.
- It provides new methods for spotting and drying of enzymes and primers for storage in the micro-channels.
- It provides the repeatable and stable fluid control required by a commercial system through use of simple pumps in combination with surface modification.

MicroActive

Automatic Detection of Disease Related Molecular Cell Activity

Project co-ordinator: SINTEF

Contact person: Liv Furuberg

Tel: +47 22067587

Fax: +47 22067321

Email: liv.furuberg@sintef.no

Website: www.sintef.no/microactive

Partners:

NorChip AS (Norway)

Institut für Mikrotechnik Mainz GMbH (Germany)

IMTEK, University of Freiburg (Germany)

BioFluidix GmbH (Germany)

The Coombe Lying-in Hospital (Ireland)

Timetable: from 12/06 – to 12/09

Total cost: 3.200.000 €

EC funding: 1.600.000 €

Instrument: STREP

Project Identifier: FP6-2005-IST-017319

Keywords: Biomedical sensors, molecular diagnostics, nanotechnologies microfluidics.