Microfluidic chip-based diagnostics of cervical cancer

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Morten Borch, Per Stenstad (related projects)
“MicroActive”
Automatic Detection of Disease Related Molecular Cell Activity

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Recent developments in the area of “point of care” diagnostics may allow the transfer of molecular diagnostics from central laboratories to the doctor’s office and to the homes of the patients.

Currently, molecular diagnostic methods often require a number of steps, such as laborious sample preparation, creation of master mixes, and the performance of several assays consecutively, each with their own complications. Such is the complexity of current molecular assays that they cannot be adapted to simple, portable formats such as the currently used pregnancy test or glucose meter.

The vision of the European funded project MicroActive has been to develop an integrated system based on microtechnology and biotechnology for automated diagnosis of a wide range of diseases. In the project, the developed system was tested successfully for the “proof-of-principle” detection of biomarkers indicative of cervical cancer. Specifically, the system was designed for the detection of transcription of oncogenic HPV E6/E7. The analysis detects mRNA (messenger ribonucleic acid), which is indicative of a biologically significant HPV (Human PapillomaVirus) infection. On-chip biological procedures have been compared to “gold standard” laboratory procedures.

MicroActive 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 minimised and the analytical procedures for diagnostics of HPV in more automated than those currently used in the laboratory. The procedure starts with spotting up 3 ml of the buffer with patient cervical smear containing cells and mucus from a standard test 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 (Nucleic Acid Sequence Based Amplification), is performed automatically in the chip and the output is about 50 microliter of aq. This liquid is mixed with reagents and then transferred to the amplification detector chip in the second instrument where the liquid is 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 measured in real time in the reaction chambers. From the signal a HPV E6/E7 mRNA positive result is determined.

Functional instruments and functional microfluidic chips have been used for tests on clinical specimens, in total more than 100 biological analyses on clinical samples were performed. Using clinical cervical cytology smear specimens from an established biobank, the functionality of the developed nucleic acid extraction with following on-chip amplification was demonstrated. Both instruments and chips are developed for production and for future use in a commercial system.

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 unwanted nucleic acid contamination.
The main objective for The MicroActive project was to develop an instrument for molecular diagnostics intended for use in the doctor’s office.

- Human Papilloma Virus
- Cervical cancer
- mRNA
- Two microchips
- Instrument
- Liquid based cytology specimens in (cervical epithelial cells)
- Diagnosis on 5 HPV viruses out
The technology platform is generic – HPV mRNAs were the markers chosen for proof-of-principle

- Platform for detection of groups of disease markers (that gives similar symptoms) such as our set of 5 mRNAs transcribing active genes of HPV types

- Screening
- Diagnostics
- Monitoring of treatment

Microfluidics: several virus can be detected simultaneously with high sensitivity
Human Papilloma Virus

HPV 16, 18, 31, 33, 45, 39, 51, 52, 56, 58, 59, 66, 68, and 73
High to medium risk of cancer

Cervical cancer is the second most common cause of mortality due to cancer among women worldwide

- Persistent infections – cancer
- Cytology based screening of population
- If cytology ambiguous – HPV (DNA or mRNA test)
- Vaccination of young girls (HPV 16 and HPV 18)
Cancer screening?

- Checking for disease when there are no symptoms
- Early stage, better chance of curing the disease
- **Pap smear, cytology (cervix)**

- Mammogram (breast)
- Colonoscopy (colon)
- Prostate-Specific-Antigen blood level (prostate)
- Genetic tests

- New technology:
  - Expensive with population testing
  - Avoid false positives!
BioBank with 518 patients - cervical smear liquid based cytology specimens

Used for

- Macroscopic comparisons
- PreTect HPV Proofer vs Digene Hybrid Capture 2
- Sensitivity 71.4 % vs 75.8 %
- Specificity 100 % vs 43.7 %
- (Journal of Virology 2009)

- On-chip
- Nucleic acid extraction experiments
- NASBA amplification experiments

Gold standard: 58 histological data
Microfluidic chips and instruments have been made, clinical specimens have been correctly diagnosed.

The two instruments can be put together.

Detection instrument.
The sensitivity challenge of miniaturization is met

- Is it possible to miniaturize the macroscopic mRNA HPV detection?

- Dilution series experiments showed:
  - Sample prep chip: extracts from down to 5 cell line cells (CaSki) is amplifiable
  - NASBA chip: down to 1.25 cell line cells (Caski) per droplet amplifiable

- Yes
We compared chip results with commercial macroscopic results, using clinical specimens.
Functions that have been integrated in the chips

Cell filter for cell concentration of “mucus” rich patient specimen
Cell lysis
Silica stationary phase nucleic acid capture (Boom)
Washes
Elution of nucleic acids
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Mixing with different dried reagents
NASBA isothermal amplification of mRNA
Hybridization to fluorescent beacons
Fluorescent detection

Specificity – primers for amplification + hybridization
Sensitivity – optical properties of chip / volume / new fluorescent detecting system
A sample preparation chip was manufactured

- Purification of nucleic acids
- Start material (3 ml): liquid based cytology
- Output (40 μl): mRNA suitable for NASBA amplification

Functions
- Cell filter
- Lysis buffer, wash buffers, elution buffer stored on-chip
- Nucleic acid capture filters

Macro: Qiagen M48

MicroActive chip (IMM)
The mRNA extracted on-chip could later be amplified using NASBA

- 22 patient specimens split into many samples. 49 mRNA extracts performed on-chip
- Later amplification in PreTect HPV Proofer (macroscale)
- Compared with Qiagen M48 BioRobot extracts in following macroscale amplification

<table>
<thead>
<tr>
<th>Number of measurements</th>
<th>49</th>
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<tbody>
<tr>
<td>Number of correct results on-chip compared with Qiagen M48 Biorobot</td>
<td>31</td>
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<tr>
<td>% correct results</td>
<td>63%</td>
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A NASBA amplification and fluorescent detection chip has been manufactured

Input: 20 µl of purified nucleic acids

Split fluid volume into droplets of 500 nl

Dried reagents stored on chip

3 droplet stop positions controlled by hydrophobic patches in channels
  Metering
  Dissolution of reagents and detection

SINTEF injection molded chip
NASBA of HPV-16 mRNA in 500 nl plugs in microchips

- Optimization of drying agents
- Wall roughness
- Wall coating

Macro: PreTect HPV Proofer
On-chip extracted mRNA was amplified in NASBA chip and detected

6 patient specimens, positive for HPV 16 and 33, split into aliquots, 22 NASBA chip experiments
For specimens extracted on chip, 19/21 (90.5%) of the filled channels amplified for HPV
Conclusion

- First time mRNA analysis of clinical specimen using microchip sample preparation and microchip amplification
- Proof-of-principle: on-chip NASBA based diagnostics is possible!

- A molecular marker based cancer diagnostics
  - That has higher specificity (and higher PPV) than DNA based methods (and cytology)
  - That is possible to miniaturize