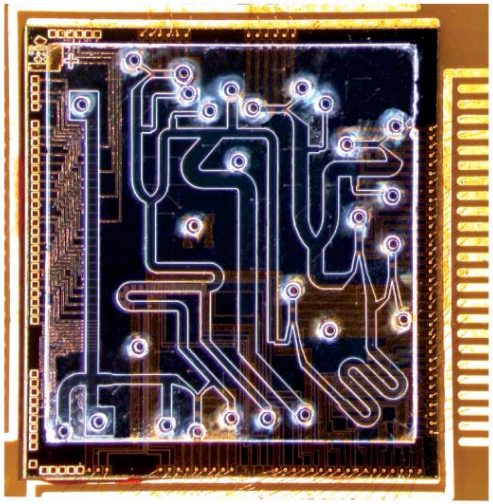
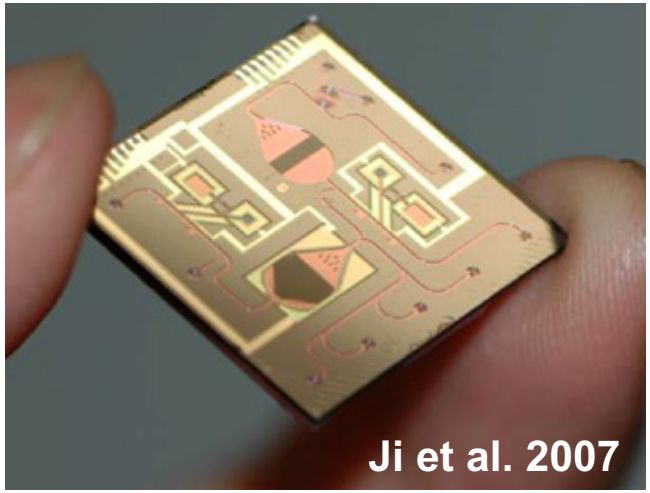
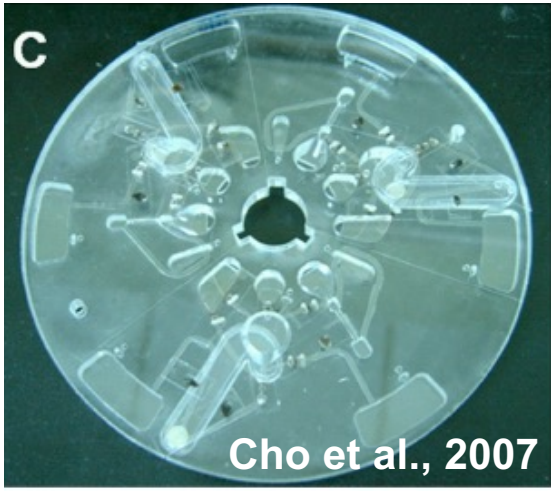


DOI: 10.1039/B505994A





Biomolecule analysis
Sample preparation
Integrated devices

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UNIVERSITY OF TWENTE.

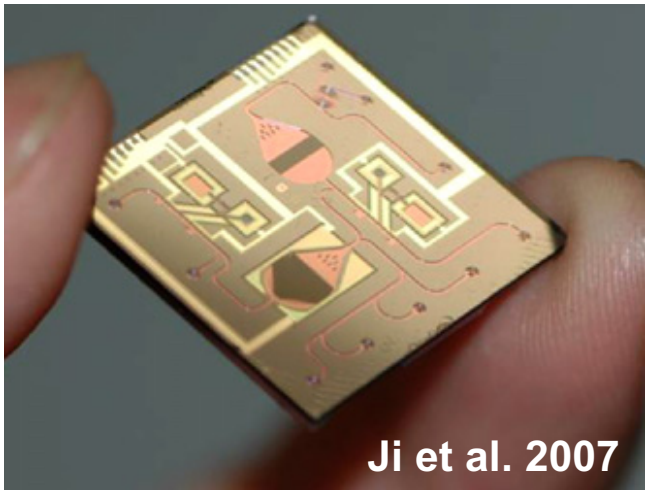
Content and scope of the course

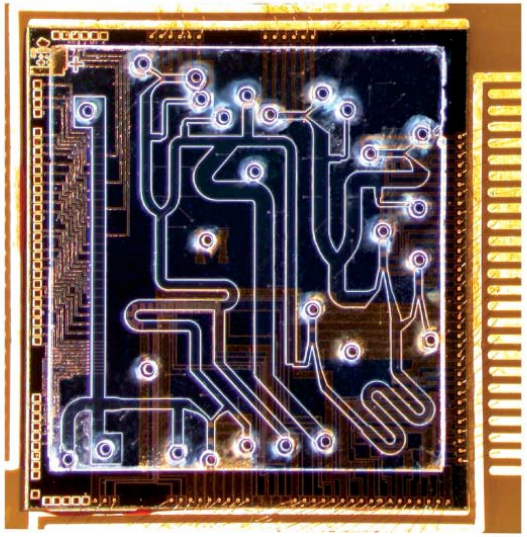
Outline

- Motivation
- Sample preparation steps
- Examples of integrated devices

Scope of the course

- Importance of sample preparation in an analytical process
- Which steps? How to perform them?
- Examples of integrated platforms for biomolecule analysis





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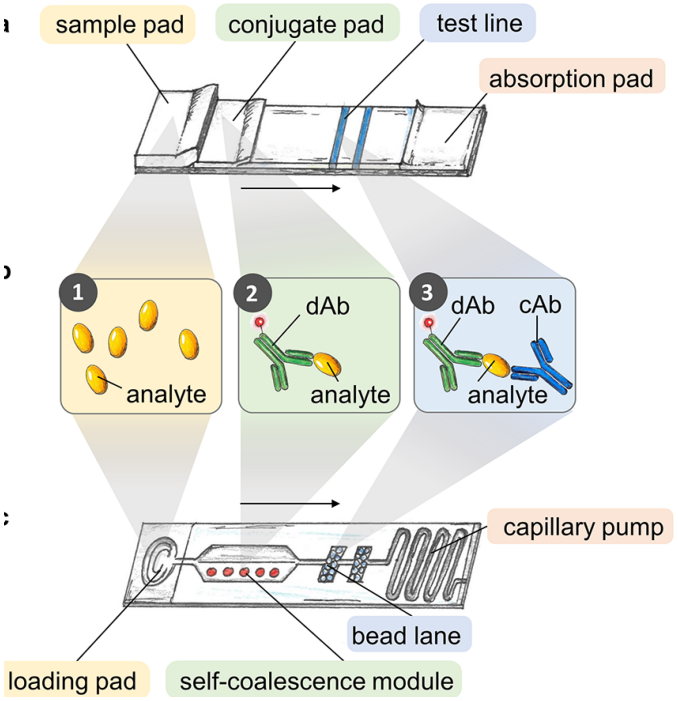


Hemmig et al., Anal Chem, 2020

Analysis purposes – Many application fields

1. **Medical/biological analysis**
   - **Pharmaceutical** industry: discovery of early biomarkers (peptides, metabolites, protein/DNA modifications)
   - **Diagnostic** purposes: early disease detection (DNA mutations, higher level of biomarkers (peptides/proteins)), point-of-care devices
   - **Research**: *e.g*, fundamental understanding of disease onset and progression, proteome database.

2. **Forensics**: DNA

3. **Homeland safety**: bioterrorism, detection of biological threats (pathogens – intact, protein-based; after lysis, DNA-based)

4. **Food**: Checking product origin

5. And **many others**…

Analysis strategy

http://www.pfizer.ie/personalized_med.cfm

Sample preparation

- Deal with the complexity of the initial sample/matrix
- Allow the detection of (low-abundant) biomarker(s) of interest

Sample Preparation

⇨ "**Accessibility**" of the sample: many steps from the "crude-real-world" sample to what is analyzed on the chip.

- **sample extraction and purification** (matrix removal)

⇨ Sample **processing**: **biochemical reactions** (digestion, PCR amplification (DNA/RNA only)…)

⇨ Sample **concentration/amplification**: sensitivity of the detection method *vs*. real sample concentration (in the sub-µM towards < pM range)

1 cell (~ sphere of 10 µm Ø) = 1 pL

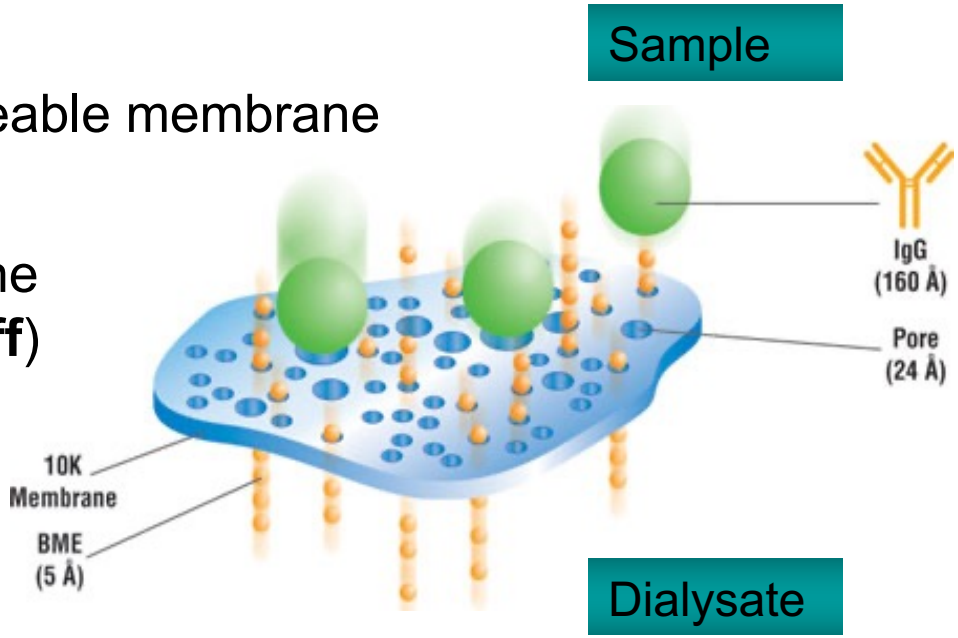
- Proteins/species of interest: few copies/cell
- No amplification step
- [high abundant species] / [low abundant species] = 10^6 !!!

⇨ needed **sensitivity** in the **low fmol–amol** range

SAMPLE PREPARATION STEPS

Sample Dialysis for Matrix Elimination

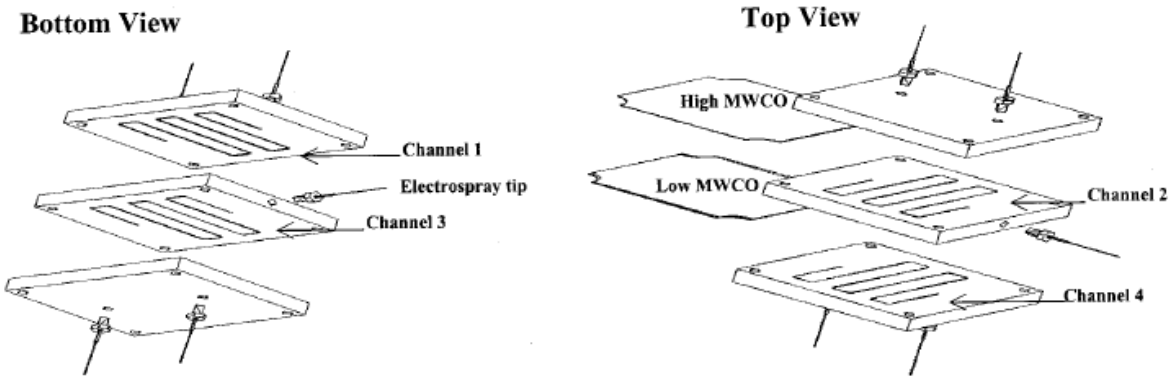
- Sample **clean-up** with semi-permeable membrane
- Diffusion of small molecules into the dialysate (MW < membrane **cut-off**)

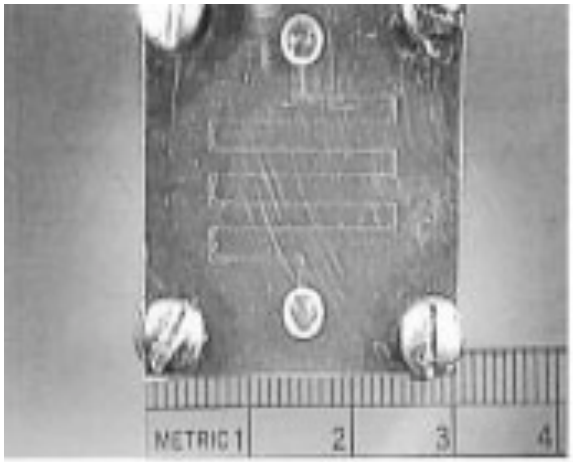


- **Membrane**:
  - often cellulose-based,
  - **MW cut-off** 100 to 300 000 Da (~ molecule size)
- Speed determining parameters:
  - MW and concentration of compounds (pH!),
  - Thickness of membrane, surface area,
  - Temperature
- **Continuous cleaning**:
- Suited for *in vivo* & in-line application

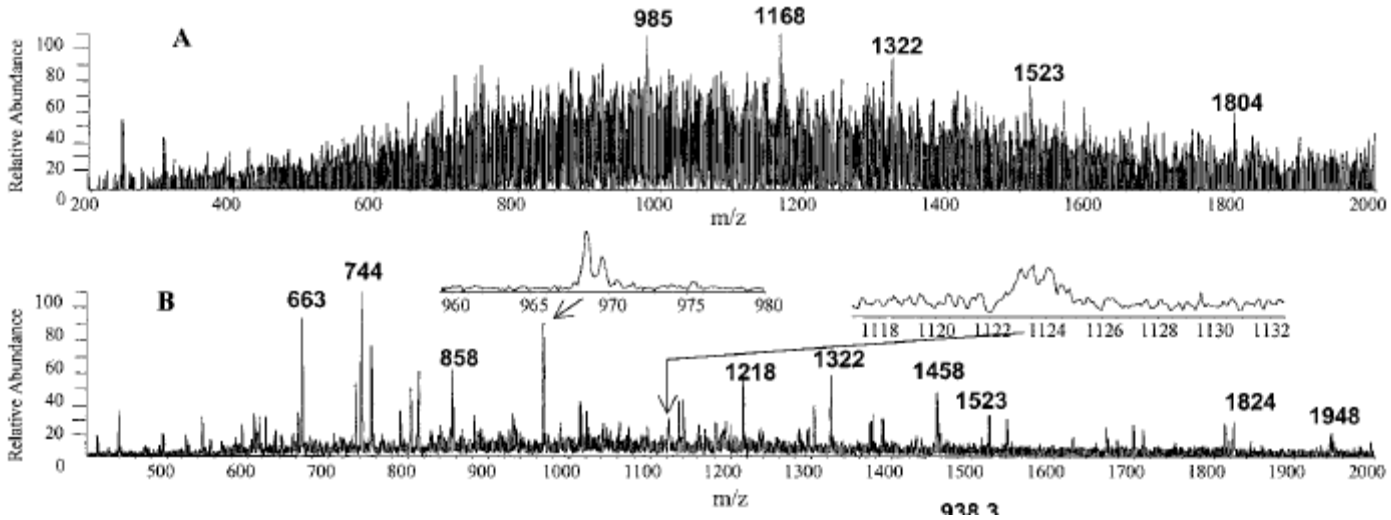
Dual Sample Dialysis before MS Analysis

- **MS analysis** hindered by **salt** (low MW) and **matrix** (high MW) contamination
- PC-based system with 2 **cellulose membranes** (low and high MW cut-offs)
  - ⇨ **dual dialysis** before on-line MS analysis





E. Coli cell lysate: 1 mg/mL total protein concentration



Low MWCO = 8,000
High MWCO = 50,000

20-fold increase in the signal-to-noise ratio!!!

Xiang et al., 1999

Diffusion-based Sample Purification

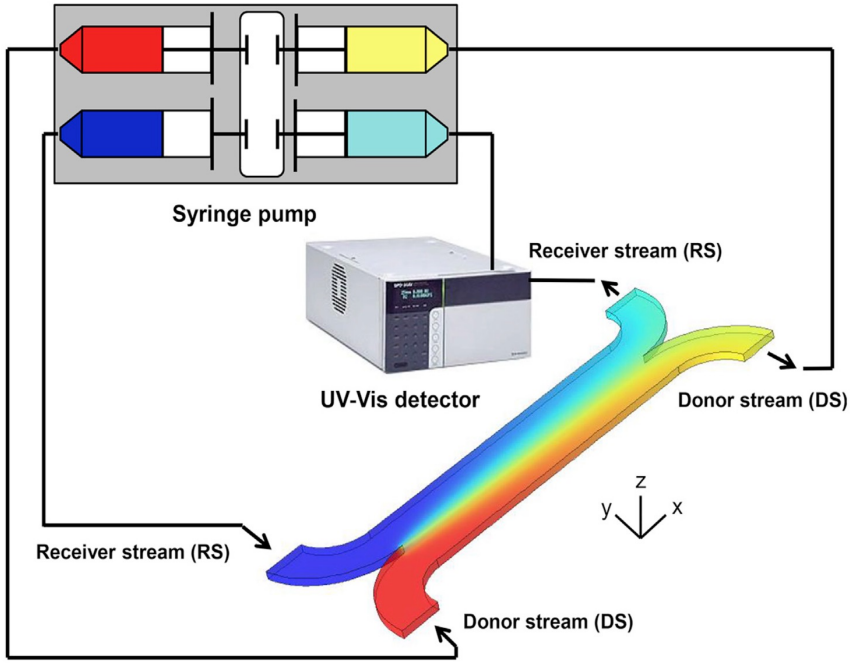
Einstein-Smoluchowski equation (derived from Fick's law of diffusion)
d_{diff} = average distance travelled by diffusion

$$d_{diff} = \sqrt{2\,D\,t}$$

With D = diffusion coefficient
$\Rightarrow d_{diff}$ ~ molecule size

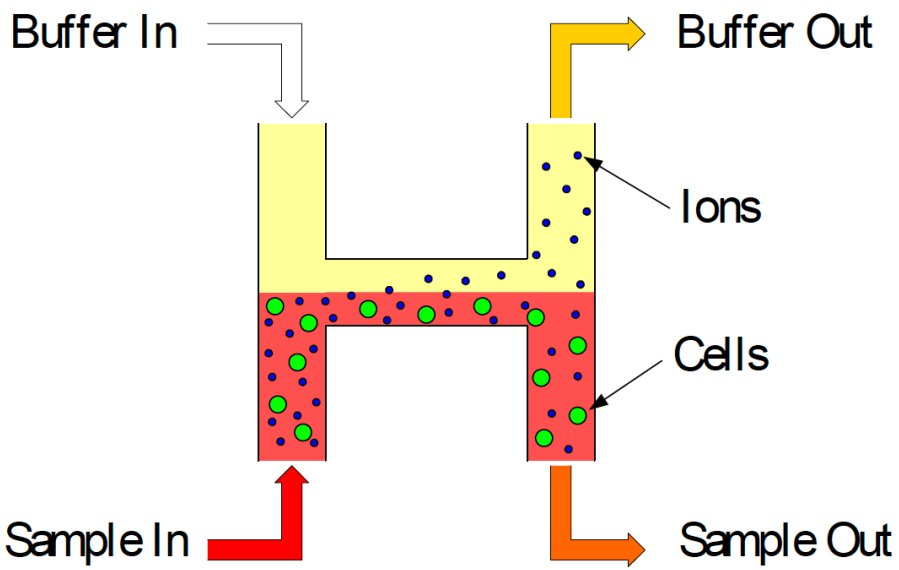
Small molecules; D = 10^{-9} m^2/s
t = 1 s $\Rightarrow$ d_{diff} = 45 µm
2 s $\Rightarrow$ 63 µm
5 s $\Rightarrow$ 100 µm

Large molecules (proteins); D = 10^{-11} m^2/s
t = 1 s $\Rightarrow$ d_{diff} = 4.5 µm
2 s $\Rightarrow$ 6.3 µm
5 s $\Rightarrow$ 10.0 µm



Biophysical Journal *116*, 595–609, February 19, 2019

H-filter

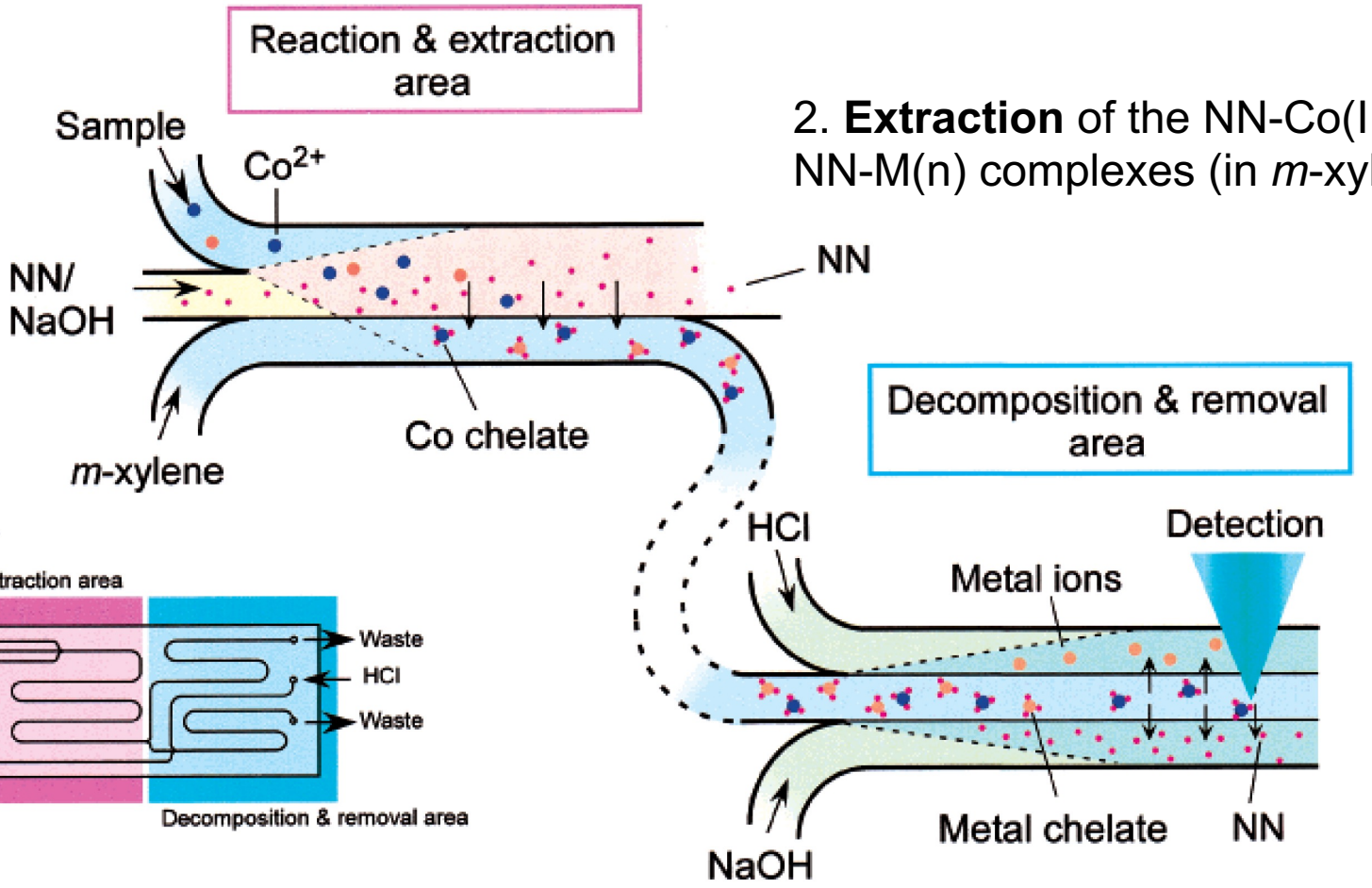


Proc. of SPIE Vol. 6003 60030N-1

Continuous-Flow Chemical Processing

Goal: Determination of the amount in Co(II) in environmental samples.

1. **Complexation** of Co(II) and M(n) with NN



2. **Extraction** of the NN-Co(II)/ NN-M(n) complexes (in *m*-xylene)

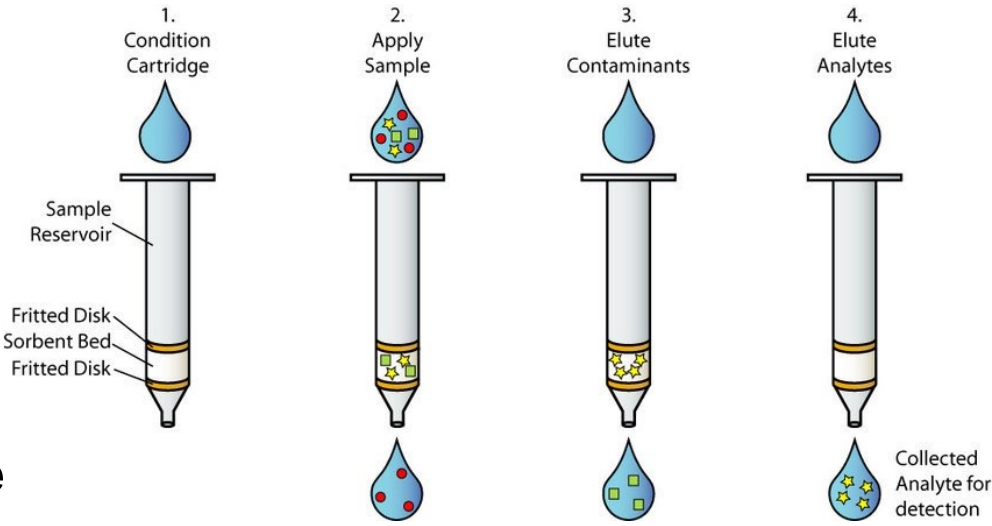
3. **Decomposition** of the NN-M(n) complexes in acidic or alkaline solutions

Tokeshi et al., 2002

Solid Phase Extraction (SPE)

- **Goals**
  - Specific retention of analytes
  - Removal of contamination/impurities
  - Elution in a small volume ⇨ concentration of the analytes

⇨ **Need for a stationary phase**



www.specartridge.com

Stationary phase

- Functionalized solid support
- High **affinity** for the analytes to be purified/concentrated
- **Large surface area** to provide enough interaction sites with the analytes

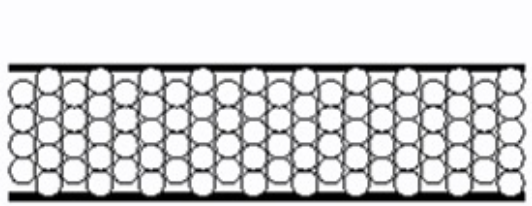
⇨ **Efficient and specific retention** of targeted analytes

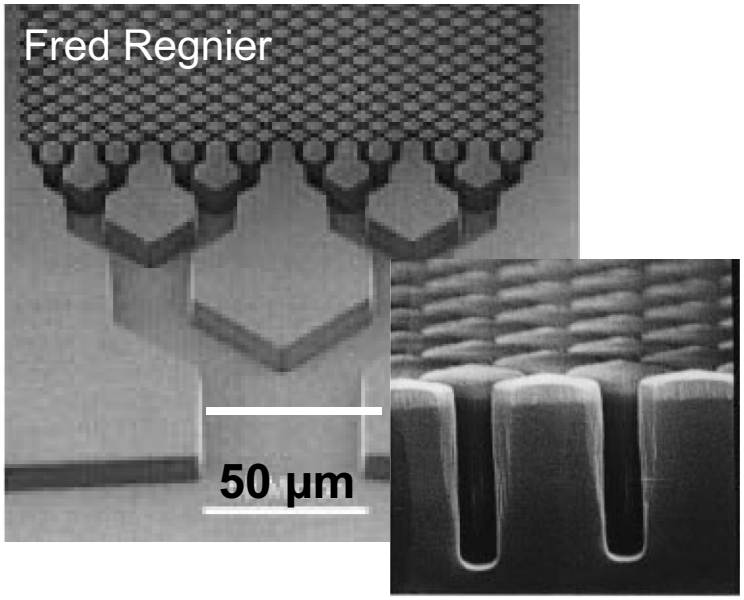
Solid Phase Extraction (SPE)

Use of a **functionalized solid support** for analyte trapping and concentration: combination of **affinity**/selective trapping and **purification/concentration**

1.Packing of porous and functionalized **particles** in a microchannel section

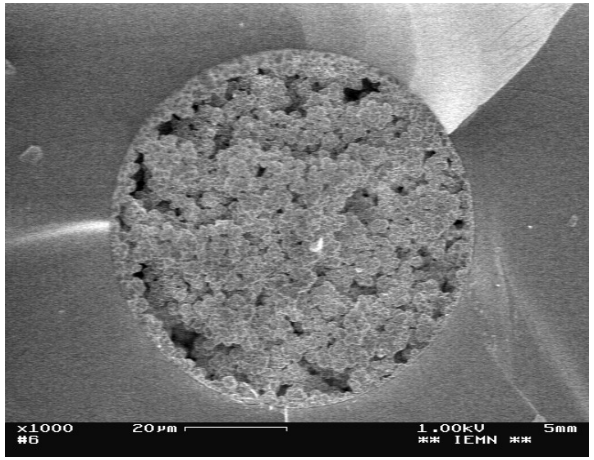
☹ particle density control + frits





2. **Structuration using microtechnology**: etching of pillars.

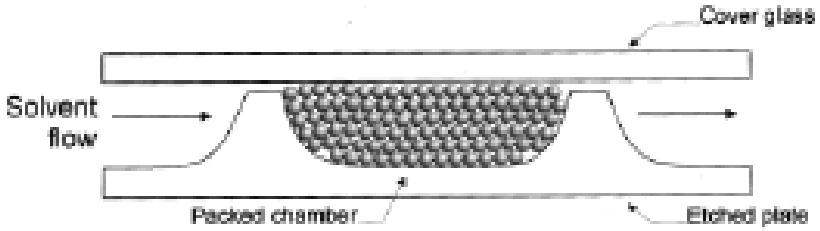
☹ Too low surface area



3. **Monolithic macroporous phase** prepared *in situ* using a radical polymerization process.

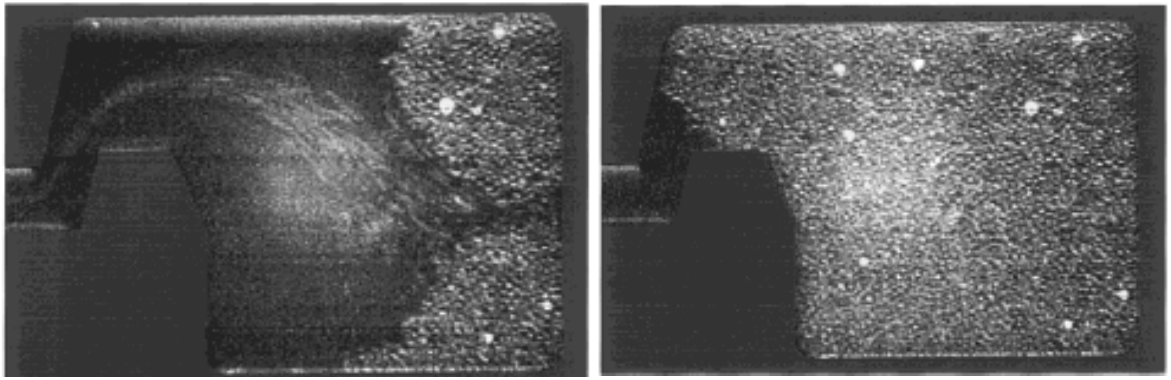
Bead-based Approach

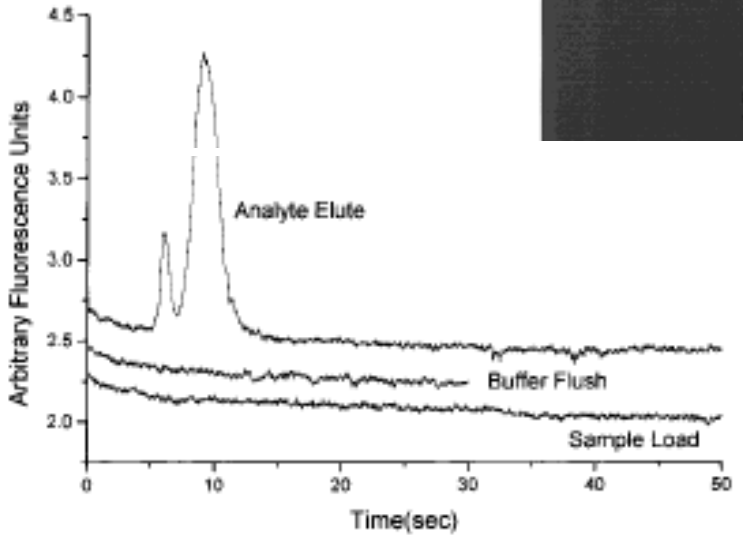
- Reverse-phase-based sample purification and concentration (**SPE device**)
- Hydrophobic beads (C18-coating) packed in a channel



Addition of high structures to trap the beads

Electrokinetic **packing** of the beads in the SPE chamber





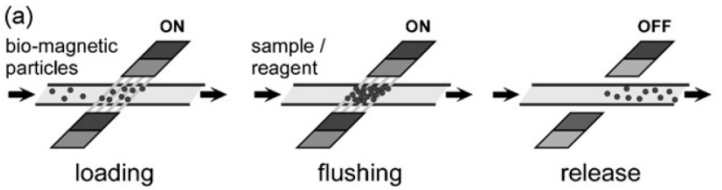
Concentration (80-500 times) of 2 fluorophores: Fluorescein and BODIPY

Anal. Chem. 2000, 72, 585–590

Multiplexed Bioassay using Magnetic Beads

Goal: Multiplexed analysis of samples based on affinity purification/trapping

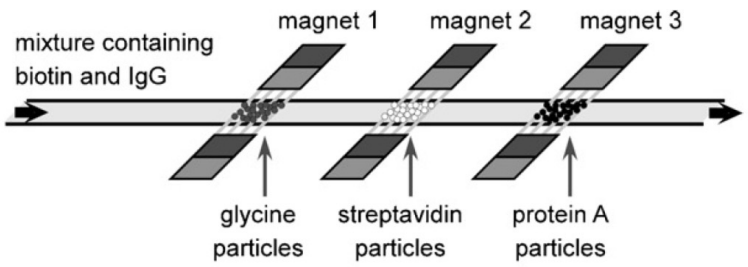
Principle:
Use of a plug of coated **magnetic beads** for **affinity trapping** of analytes

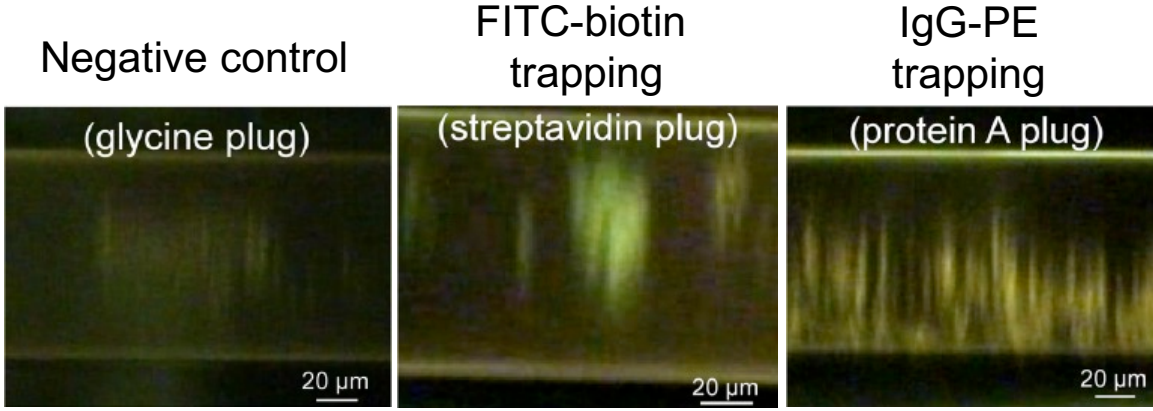


- **Magnetic force** ⇔ gradient of magnetic field
- Trapping ⇔ F_{mag} >> F_{drag} ⇨ flow-rate optimization

$$\mathbf{F}_{\mathrm{mag}} = \mu_0 \cdot \mathbf{M}_{\mathrm{s}} \cdot \mathrm{grad}\mathbf{H}$$

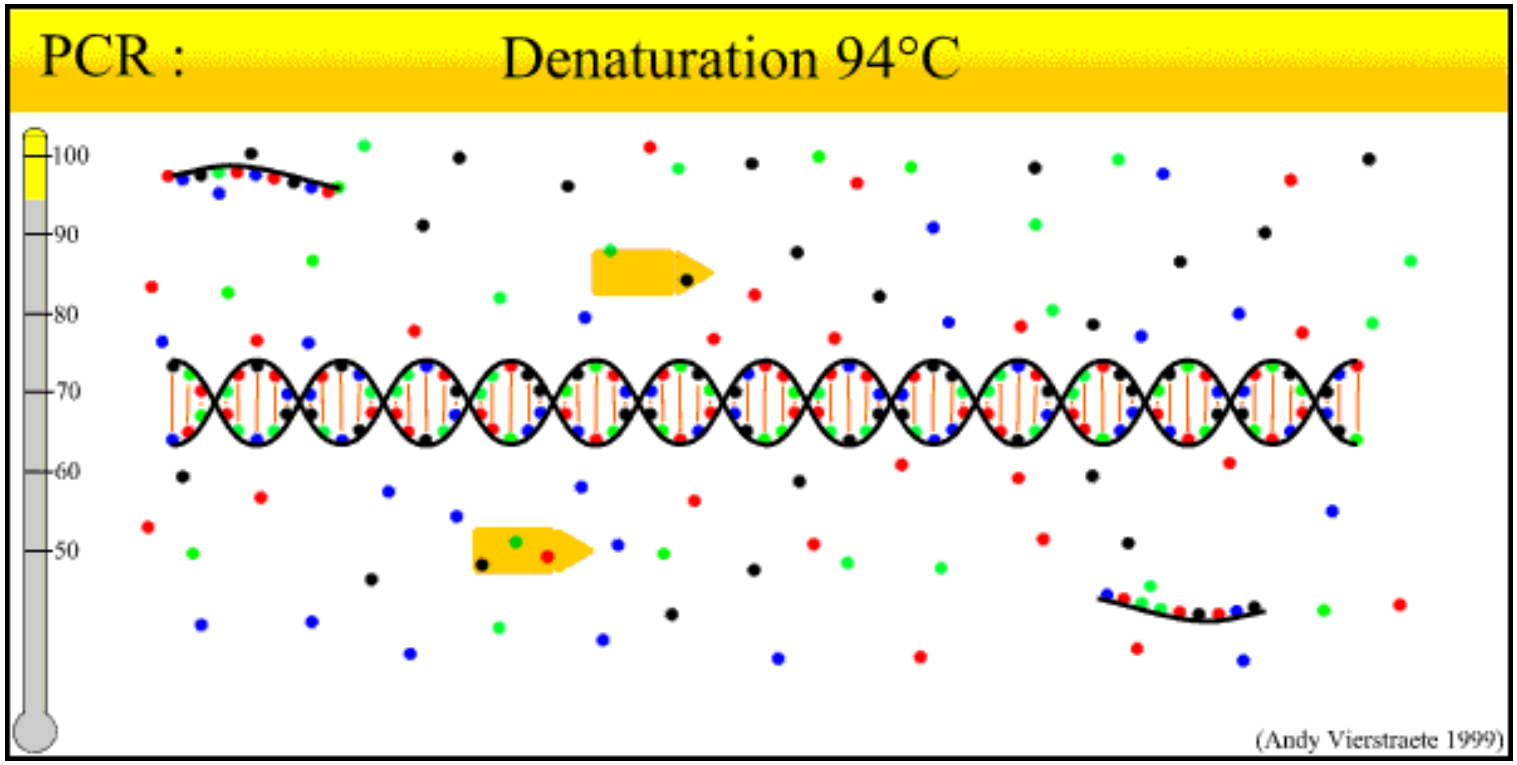
$$\mathbf{F}_{\mathrm{drag}} = 6\pi\eta r\Delta\mathbf{v}$$





Bronzeau et al., 2008

Polymerase Chain Reaction



animation: pcranimatie.gif

PCR= polymerase chain reaction, a method to "amplify"DNA and RNA

Polymerase Chain Reaction and Microfluidics

- Need to **control the temperature** and to create zones at 50, 70 and 90˚C

But: Microsystem ⇨ fast heat exchange

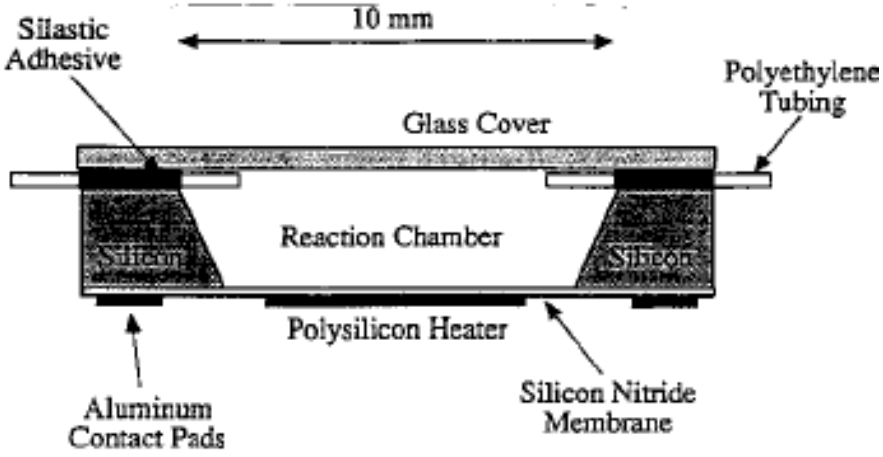
⇨ Fast and controlled temperature cycling (advantage of miniaturization):

  - Denaturation ≥ 90 °C, extension ~ 70°C, annealing ~ 50°C
  - Reproducibility of temp. cycle, temp. control
  - Placement and method of heating (direct contact, non-contact)
  - Cooling? (passive, active)

- **Device material**
  - No adsorption of the enzymes on the device walls!
  - Silicon with passivation layers (SiO_2 best, Si, Si_3N_4 most failures)
  - Glass, passivation (hydroxyethyl cellulose, polyethylene glycol, polyvinylpyrrolidone)

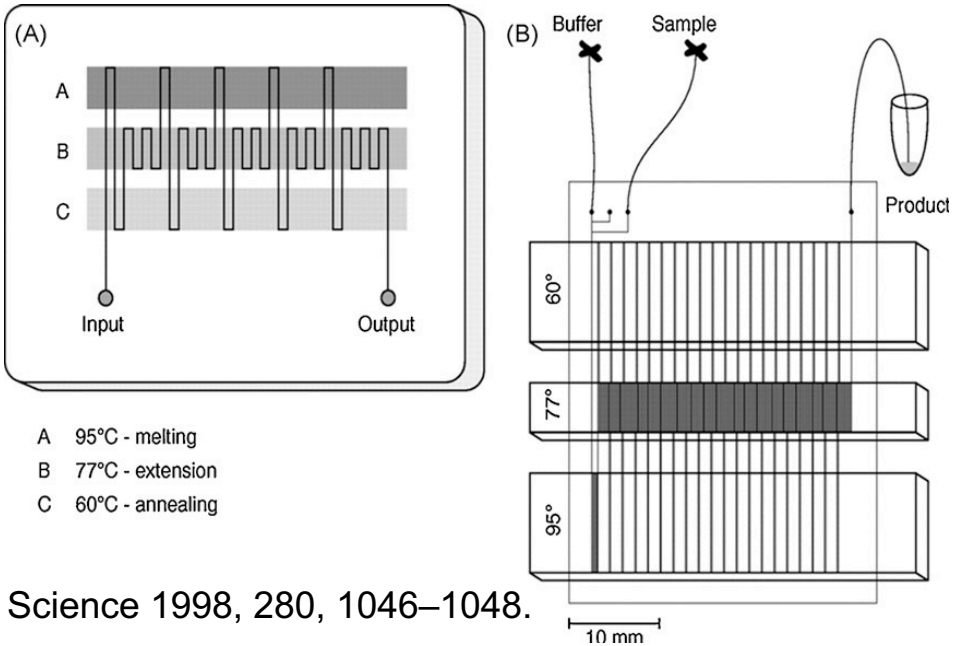
Polymerase Chain Reaction and Microfluidics

Approach 1
Microchamber devices



Micromachined DNA amplification chamber (after Northrup, et al., 1993).

Approach 2
Continuous flow devices



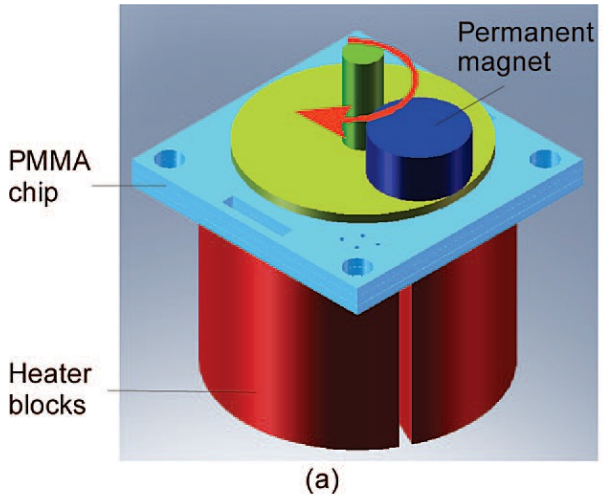
Science 1998, 280, 1046–1048.

⇨ Successive heating/cooling cycles of the reaction mixture in a micrometer-sized chamber

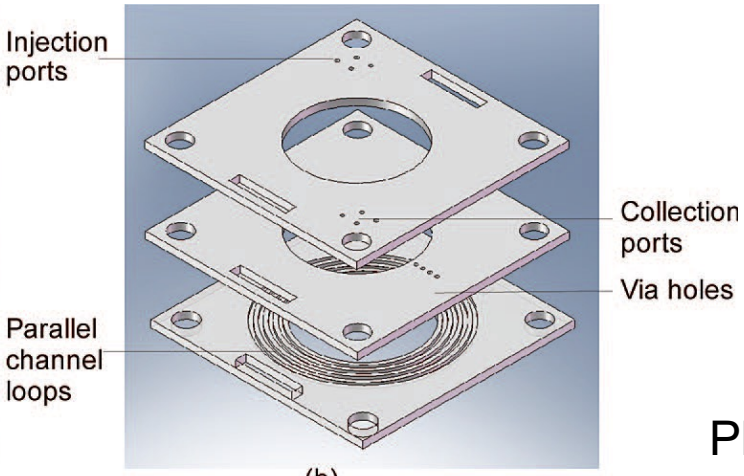
⇨ Reaction mixure continuously flowing in a serpentine channel between three zones at three given and well-defined temperatures

PCR in loops using magnetic actuation

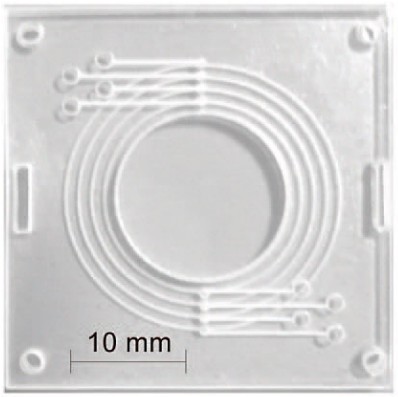
- Channels: circular loops
- Fluid actuation: ferrofluid plugs in solution + permanent magnet



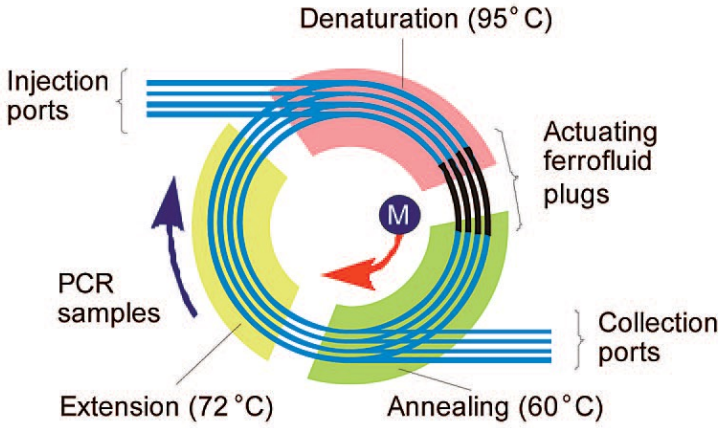
(a)



(b)



(c)



(d)

Chip 3 cm x 3 cm

4 independent channels (900 µm w; 400 µm h)
Length: 47-63 mm
⇨ 4 independent reactions in parallel

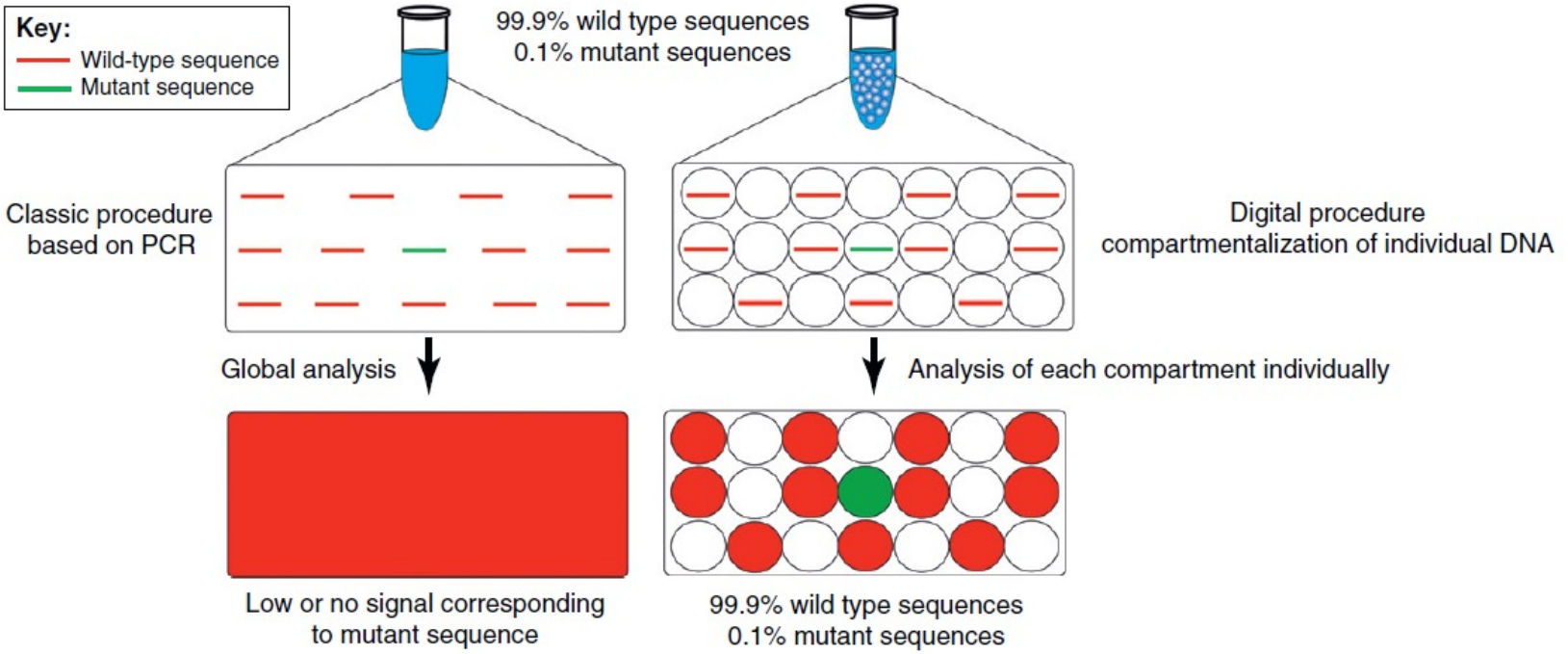
Plugs of ferrofluids in oil for actuation up to 7 mm/s

3 T°C zones
⇨ 3 steps in the PCR
⇨ One cycle = 1 turn

25 cycles ⇔ 13 min
vs. 2h in a thermal cycler

Sun et al. 2008

Droplet microfluidics for single cell/molecule Intermezzo



- **Poisson's law**

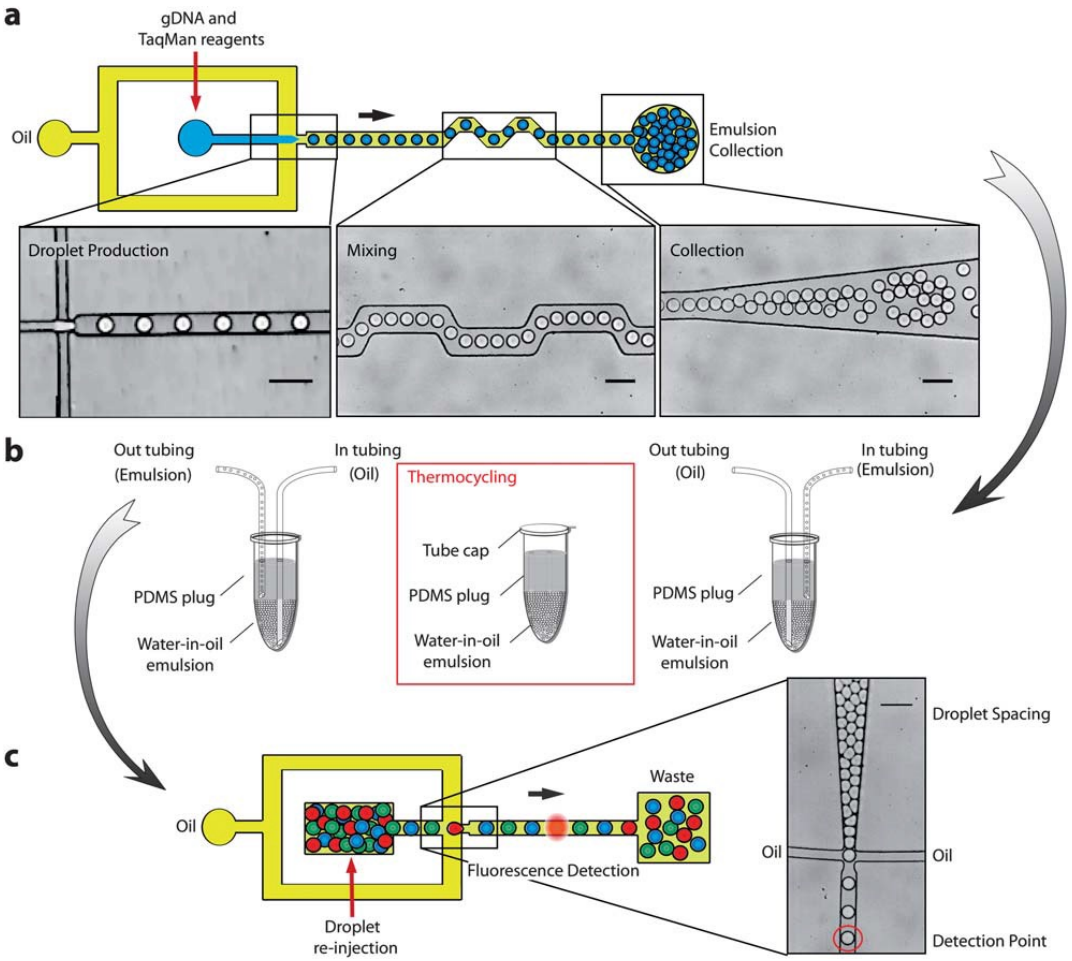
$$p_k = \frac{\lambda^k e^{-\lambda}}{k!}$$

Probability (p_k) that a compartment includes k entity, with k being 0, 1 or or more, if λ is the average number of entities expected per compartment.

Taly et al., 2012.

Droplet PCR for single mutation detection

- Presence of **circulating DNA in body fluids** released by cells
- Tumor cells: release of DNA containing specific mutations (< 1%)
  - ⇨ Mutated DNA analysis for **diagnosis, prognosis and patient follow-up**



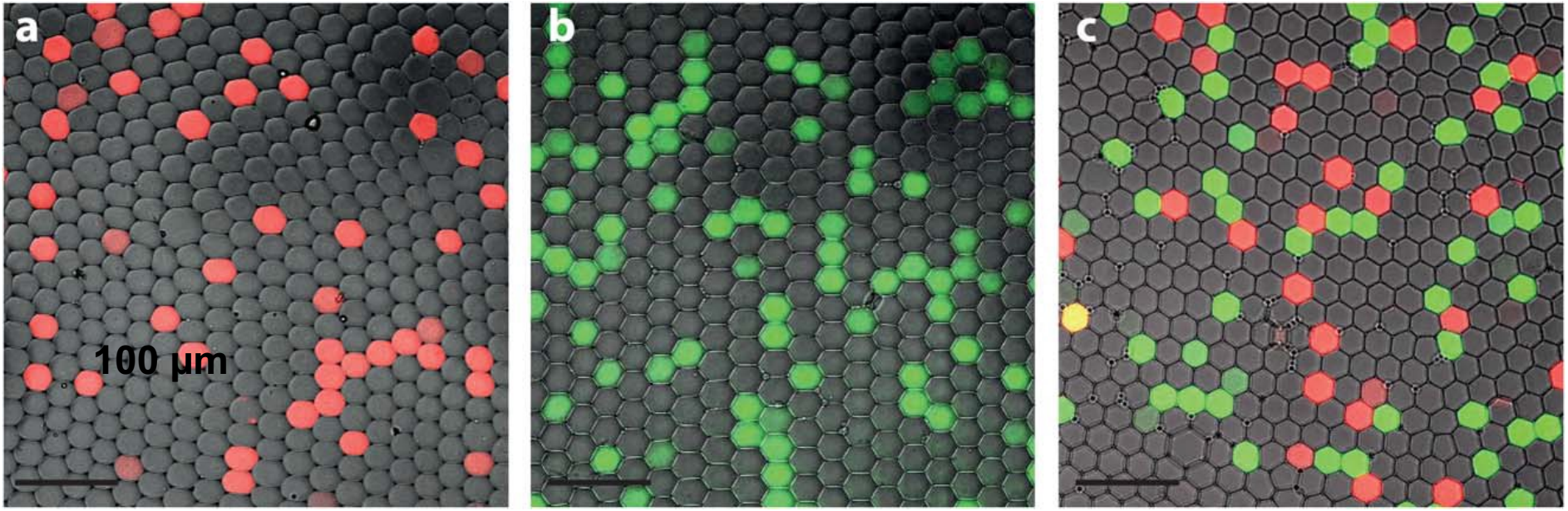
- LOC: droplet microfluidic platform for **compartmentalization of the sample** and highly parallelized analysis (>1000 droplets of ~pL)
- Each droplet (~pL): **one genome equivalent** + 2 probes for amplification of DNA with or without any mutation
- One droplet: one **microreactor** for DNA amplification (off chip)
- **Readout**: fluorescence
  - ⇨ Green = mutated DNA
  - ⇨ Red = wild-type DNA

Pekin et al., Lab Chip, 2011.

Droplet PCR for single mutation detection

- Focus on one specific **oncogene *KRAS*** that can present **> 6 mutations**
- Mutation pattern ⇔ “type” of cancer, patient resistance to specific drugs
- Validation of the technology on one specific mutation of *KRAS*

Wild-type DNA **Mutated DNA** **Mixed DNA (50:50)**



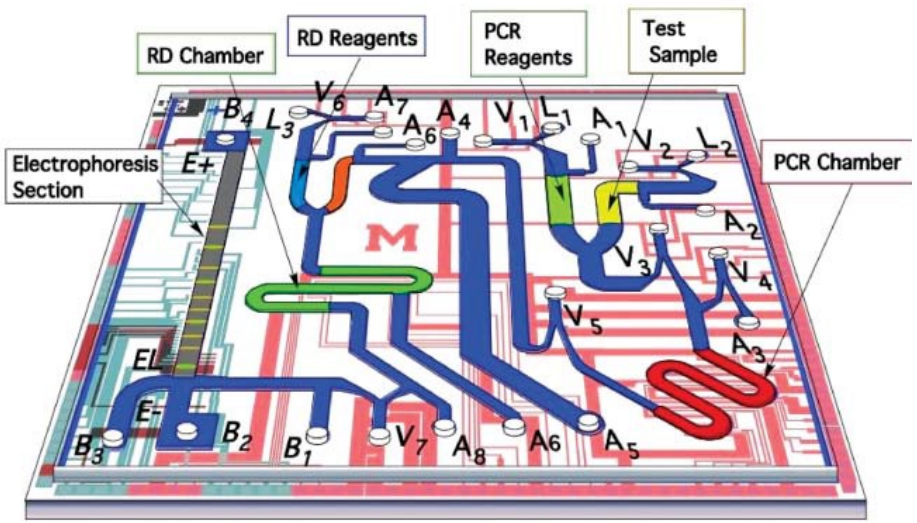
- Validation of the technology for the detection of mutations with a frequency < 1:200,000
- *Next step*: multiplexed detection of up to 6 mutations with color encoding of droplets containing specific probes for specific mutations

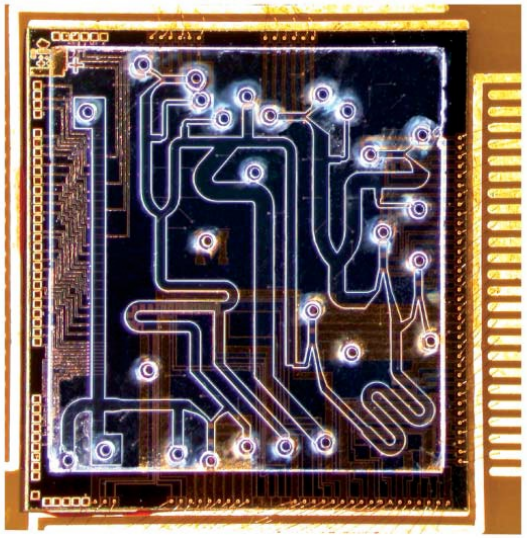
Pekin et al., Lab Chip, 2011.

EXAMPLES OF INTEGRATED PLATFORMS

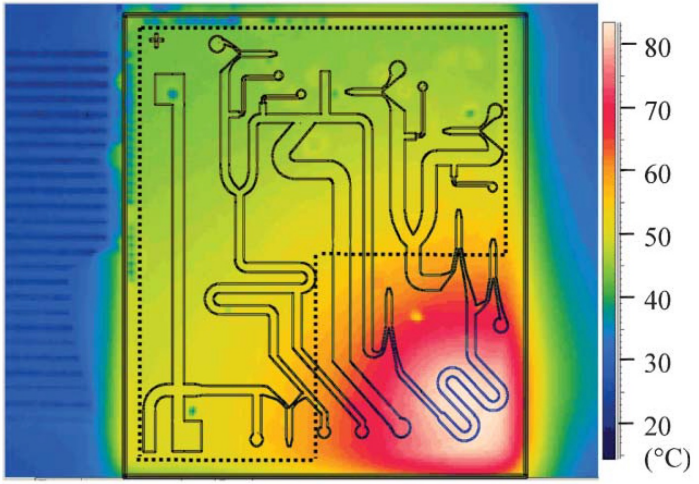
Influenza and gene analysis

- **Genetic analysis - Sample-in/answer-out integrated device**





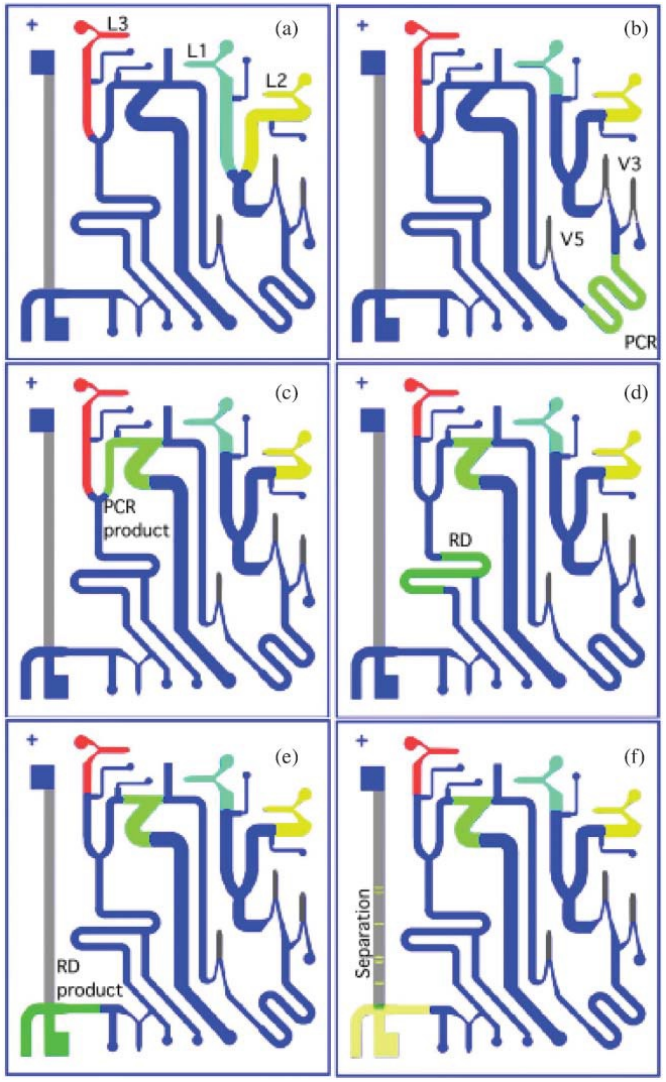
- Two microreactors (nL volume): PCR and restriction digestion reaction
- One module for gel electrophoresis separation
- Glass-silicon device
- Device integrating temperature control (heaters & sensors), and wax-based valves (electronically addressable)
- Samples: influenza viral strain (A/LA/1/87) subtyping, human and mouse DNA



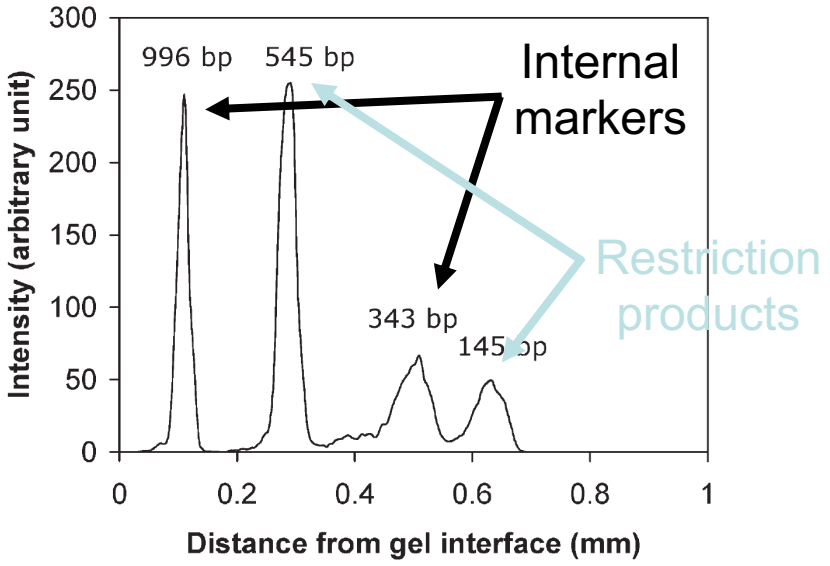
Lab Chip, 2005, 5, 1024–1032 DOI: 10.1039/B505994A

Influenza and gene analysis

- **Genetic analysis - Sample-in/answer-out integrated device**



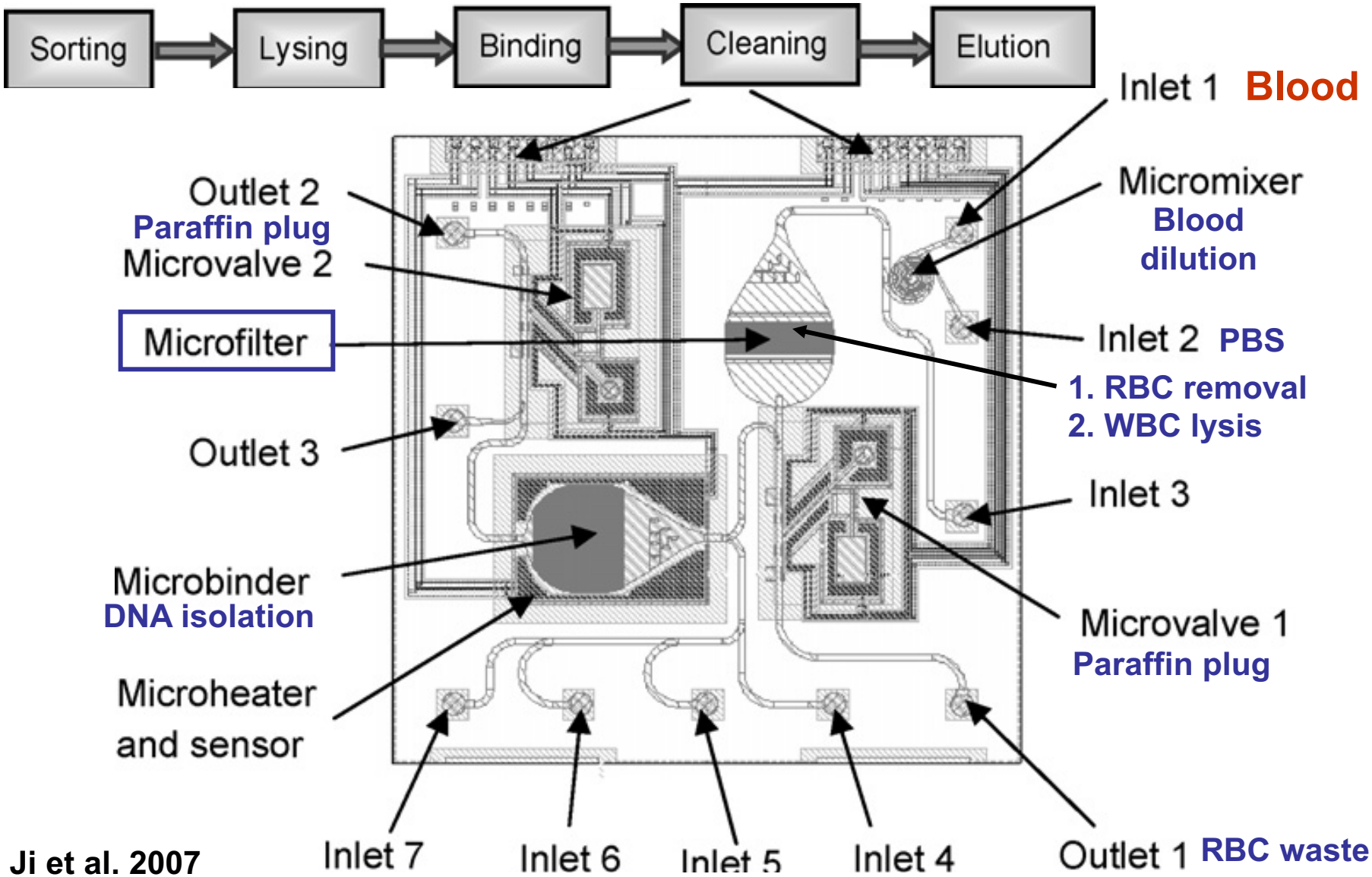
- Sample and reagents loading (L1, L2, L3)
- Mixing of sample and reagents for PCR
- PCR amplification reaction
- Mixing of products with restriction enzymes
- Restriction digestion (RD) reaction
- Electrophoretic separation of the RD products



- Fully on-chip analysis of influenza viral strain (A/LA/1/87)

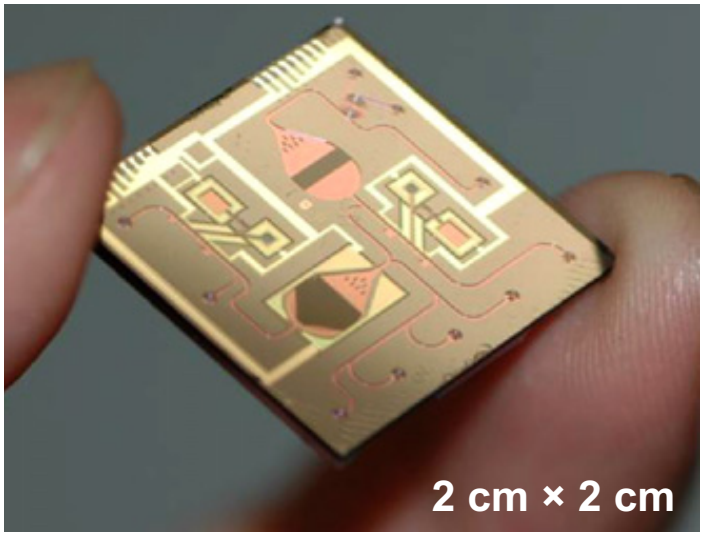
DNA Analysis on a Silicon Chip

Integrated platform for DNA isolation and purification from blood samples

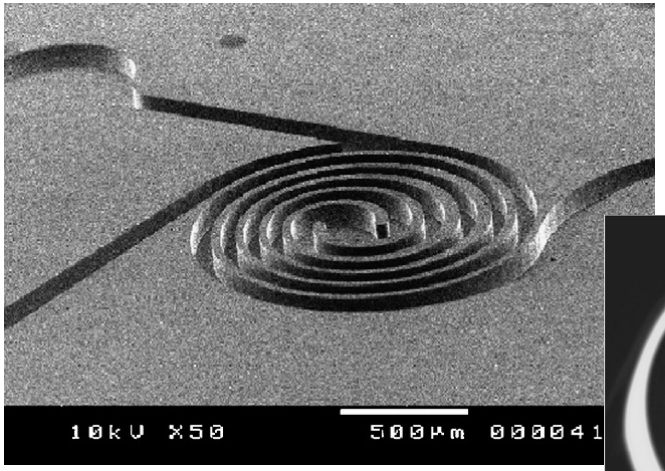


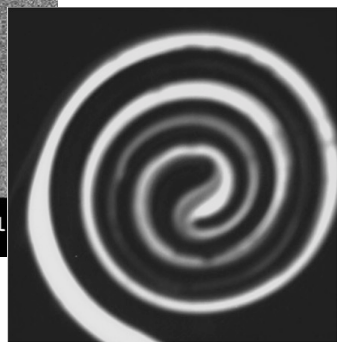
Ji et al. 2007

DNA Analysis on a Silicon Chip

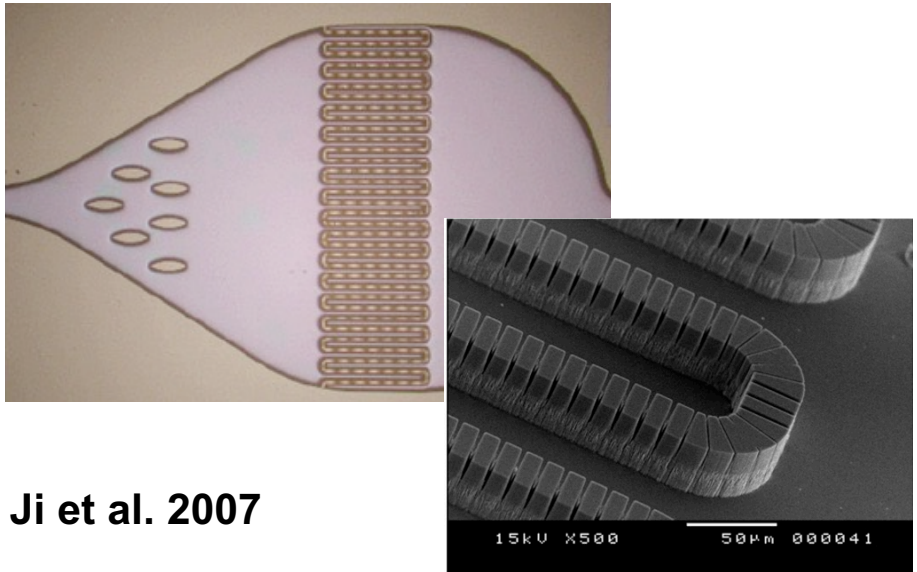


Blood **dilution** with PDMS (**mixer**)





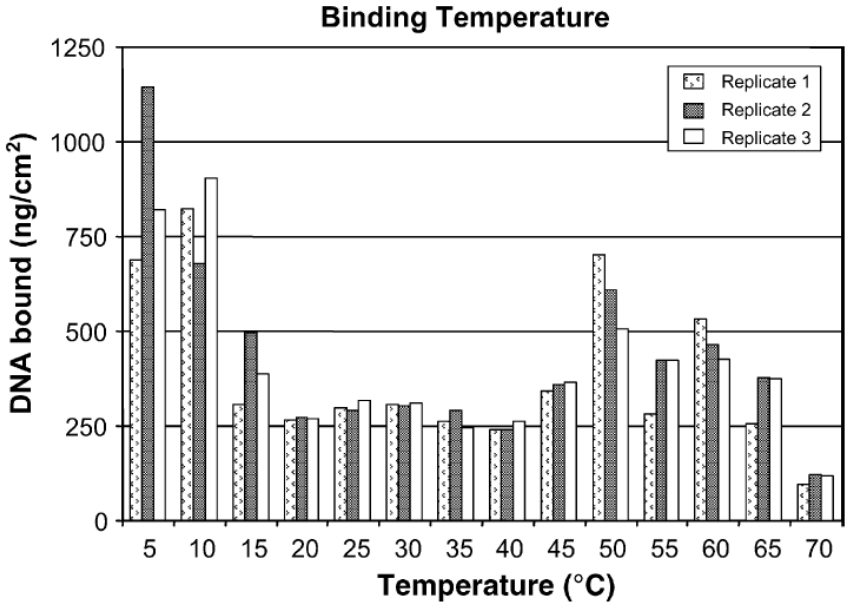
Blood **filtration**: removal of RBCs/ trapping of WBCs

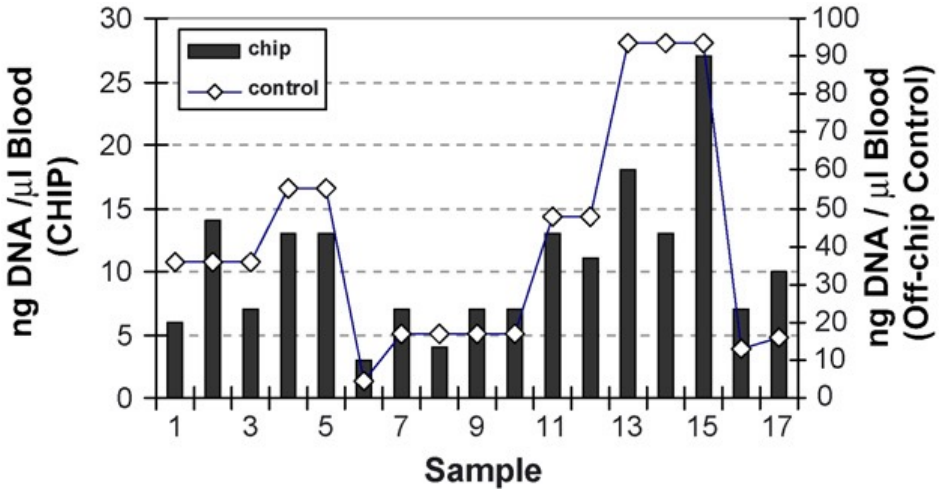


Ji et al. 2007

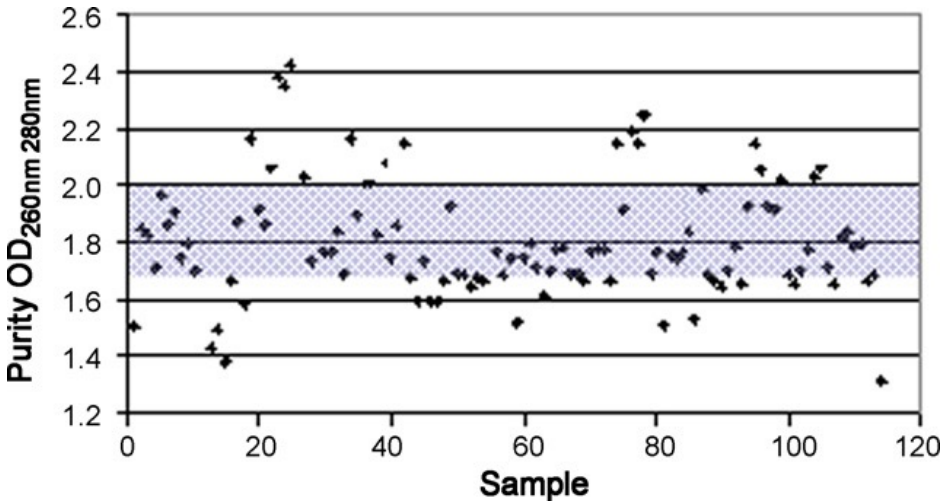
- WBCs **lysis**: release of WBC nuclei
- Reversible **binding** of nucleic acids on a silica-like surface (in presence of chaotropic salts, e.g., guanidine hydrochloride)
- **Wash** with ethanol (else PCR inhibition)
- Release of DNA

DNA Analysis on a Silicon Chip





Comparison of **DNA extraction yield** using a microchip or a conventional method.

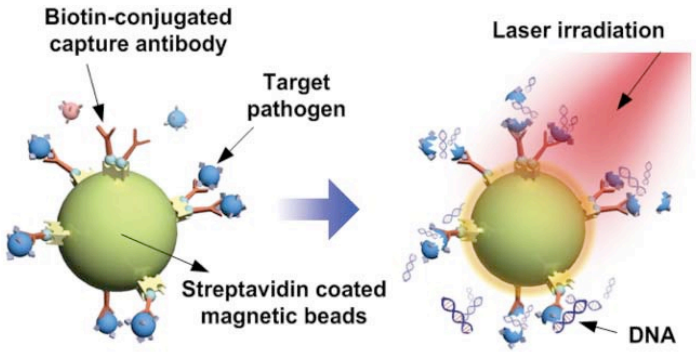


Purity of DNA extracted from human blood using a microchip
DNA purity ⇔ in the 1.7-2.0 range

Ji et al. 2007

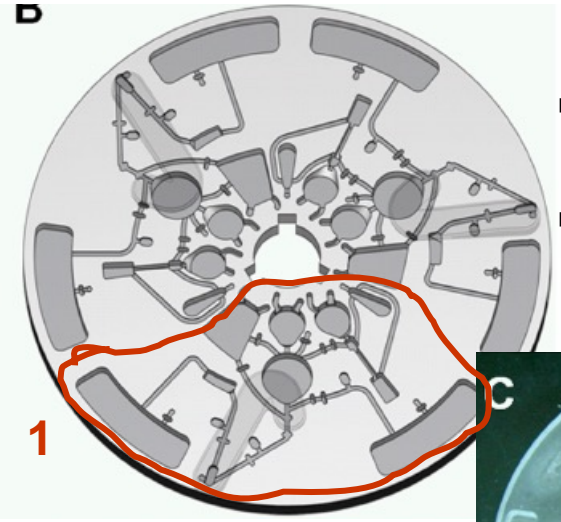
Pathogen specific DNA Extraction

Goal: genetic analysis of **pathogens** on a highly integrated fluidic platform.

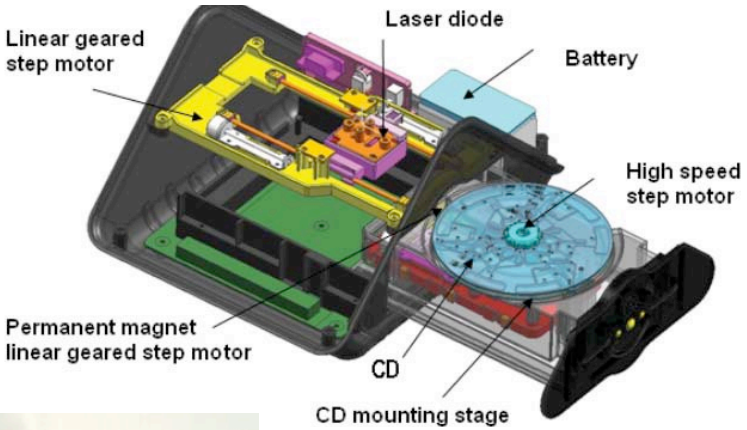


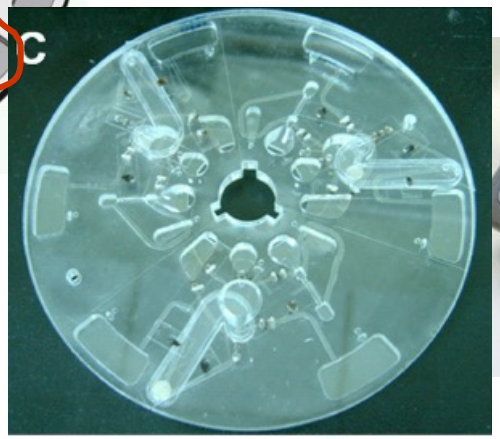
Principle:

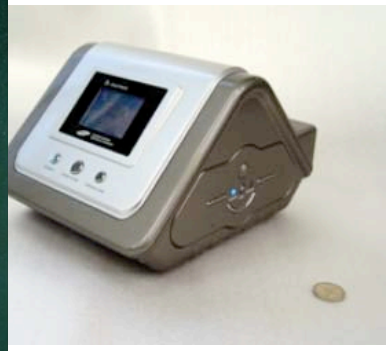
- Specific capture of pathogens from blood,
- Lysis of pathogens
- DNA isolation for PCR and on-chip analysis



- CD-shaped microfluidic system
- Wax-based valves (laser actuation)





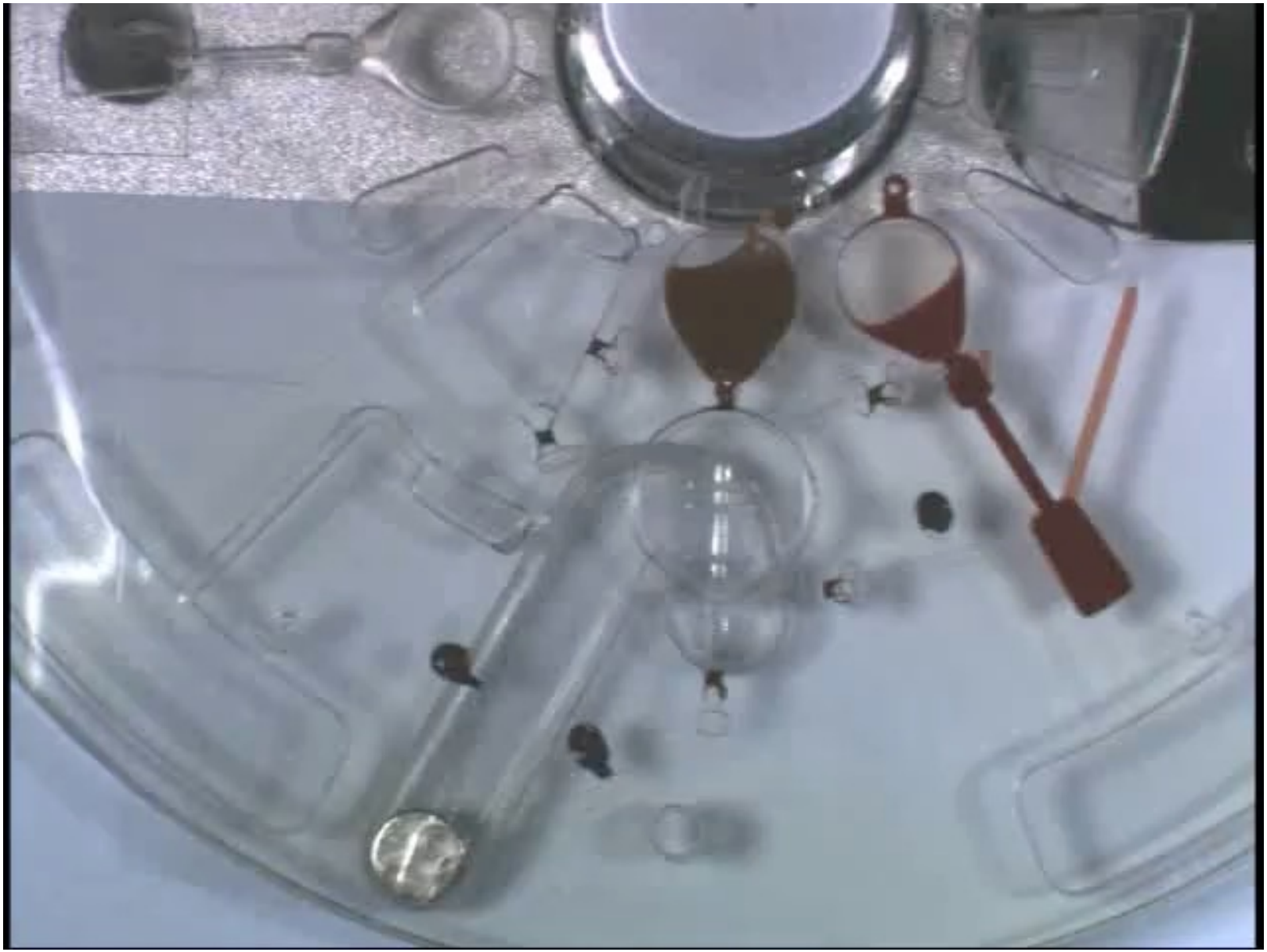


4 independent analysis devices

Dedicated **platform** for automated laser and magnet-based operation

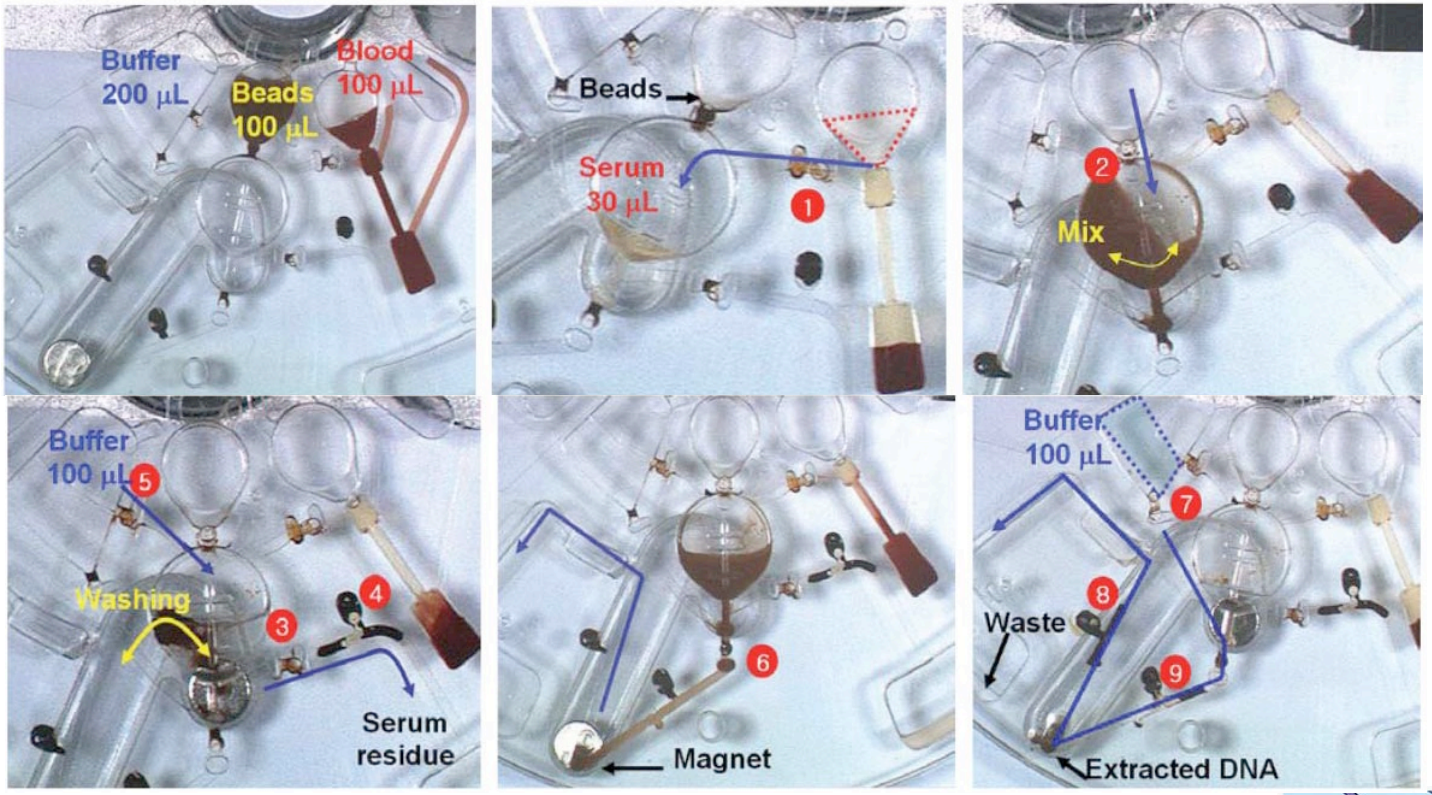
Cho et al., 2007

Pathogen specific DNA Extraction



Cho et al., 2007, https://doi.org/10.1039/B616115D

Pathogen specific DNA Extraction



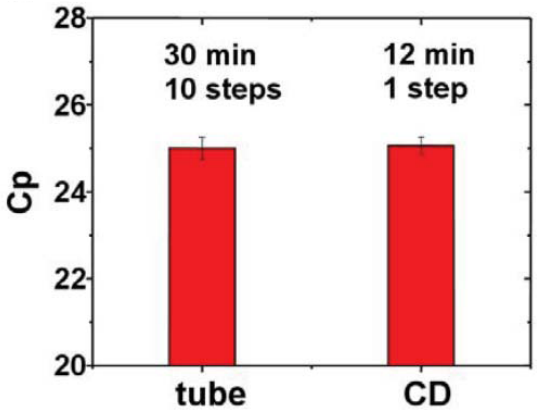
1. Blood → Serum

2. Addition of the particles for trapping

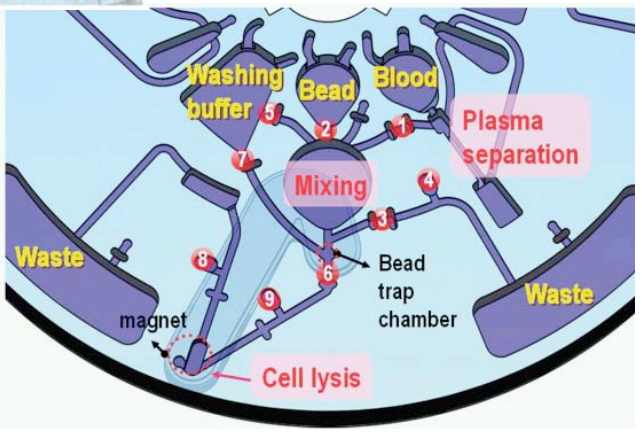
3. Wash

4. Isolation of the particles

5. Pathogen lysis and DNA isolation



Extraction yield and duration:
Conventional approach
vs.
Integrated/automated CD microfluidic platform



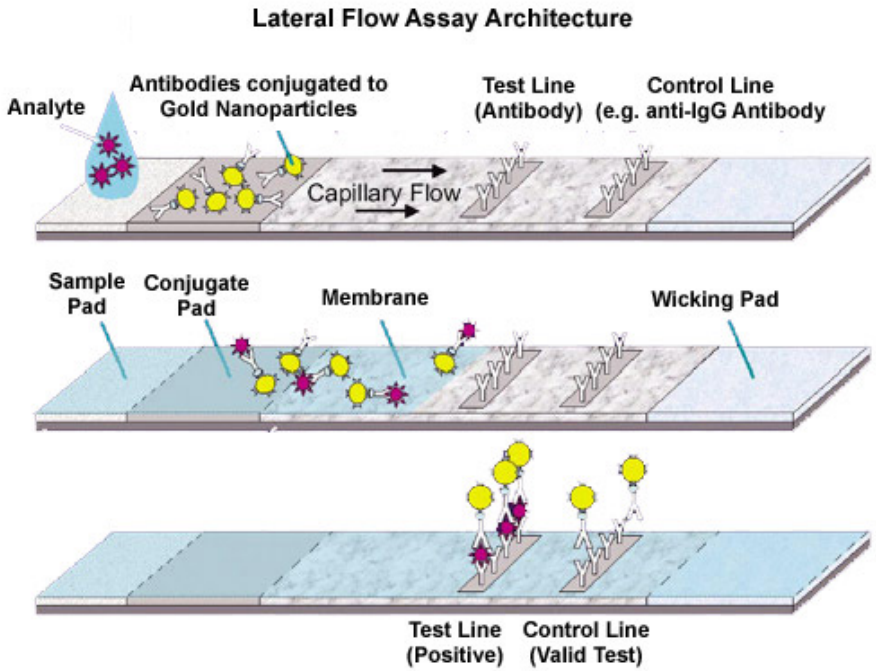
Cho et al., 2007

Lateral flow assay

Pregnancy test

Detection in urine of specific hormones
(hCG or human chorionic gonadotropin)

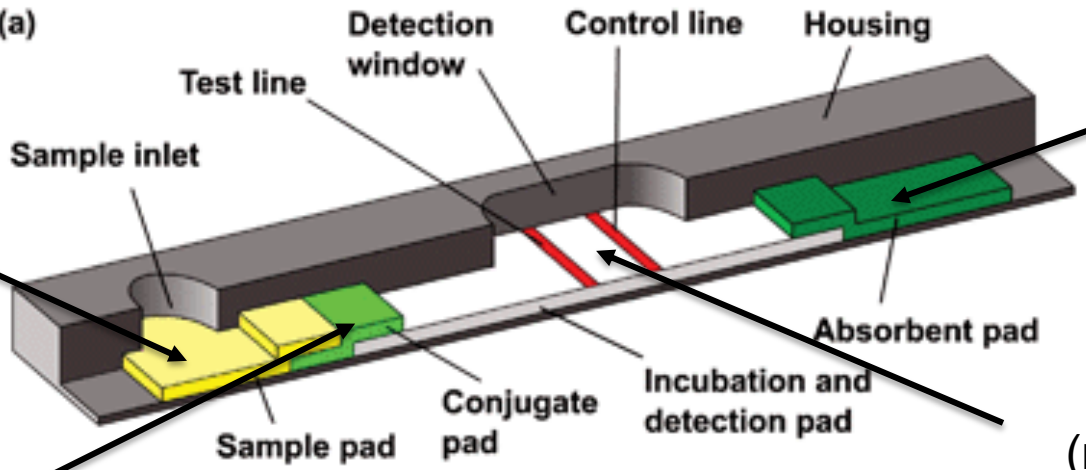
Analysis principle: lateral flow immunoassay
Detection: color change (gold nanoparticles)



- Reagents / recognition agents coupled to GNP pre-loaded on the strip
- Progression of the sample on the strip by capillary action
- ***Different zones***: sample zone, conjugate zone, reaction zone, wick.

www.cytodiagnostics.com

Lateral flow-assay

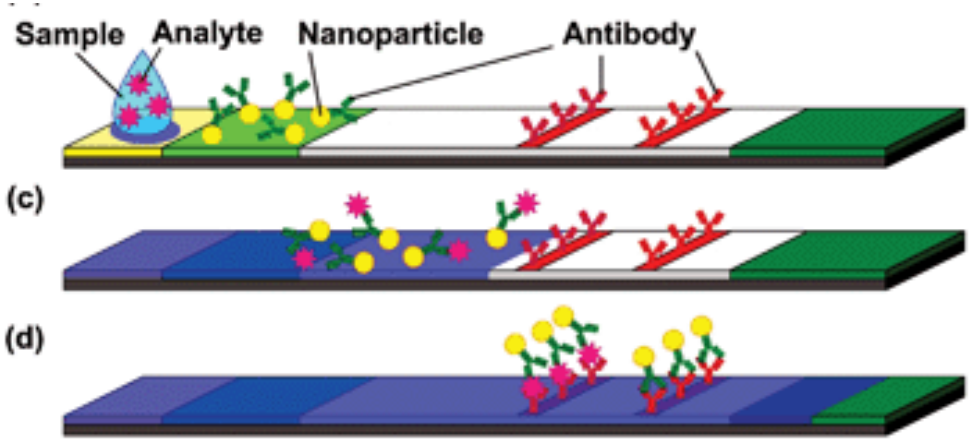


Sample pad (+ filtering)

Absorbent pad (liquid actuation; capillary action)

Incubation and detection pad (recognition assay; detection lines)

Control pad (recognition agents; detection agents; control entities)



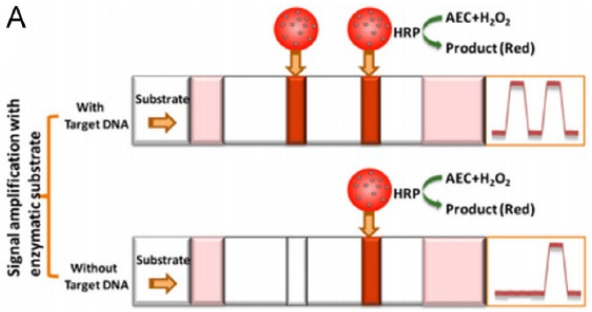
Analysis:

- Sandwich immunoassay
- Antibody immobilized on the detection lines and coupled to detection agents)

Posthuma-Trumpie et al., 2009

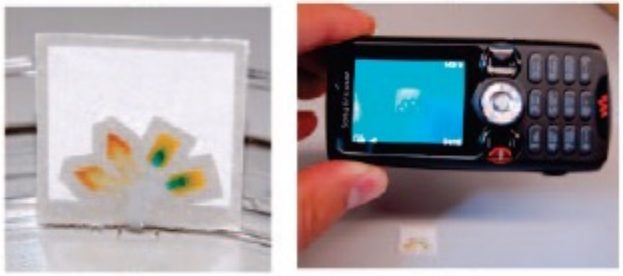
Limitations of paper-based devices (LFA)

- **Sensitivity of the assay**
  - Need for a signal amplification approach (*e.g.*, enzymatic amplification)



He et al., 2011

- **Quantification?**
  - Electrochemistry: yes
  - Optical readout: need for an external reader? Or use of a Smartphone (Telemedicine)



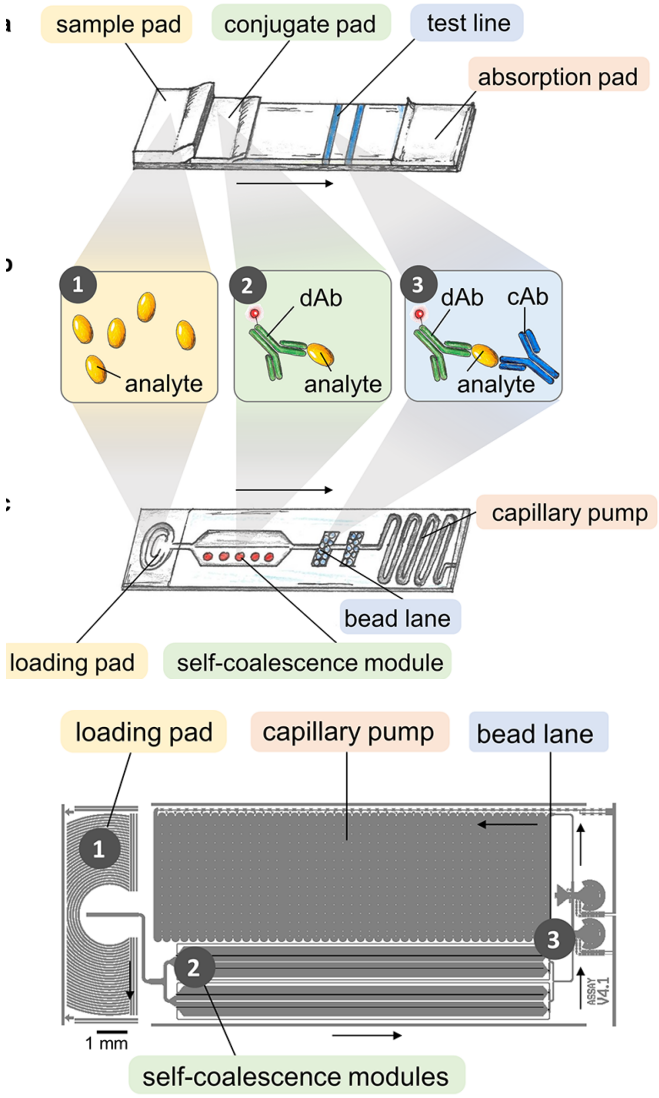
Martinez et al., 2008

- **Complexity of the process**: limited to 1-2 steps

- **Initial sample?**
  - **Liquid**…. So limited to body fluids (urine, blood, saliva, tears, etc..)
  - Paper = **filtering** of cells and debris/contamination!!!

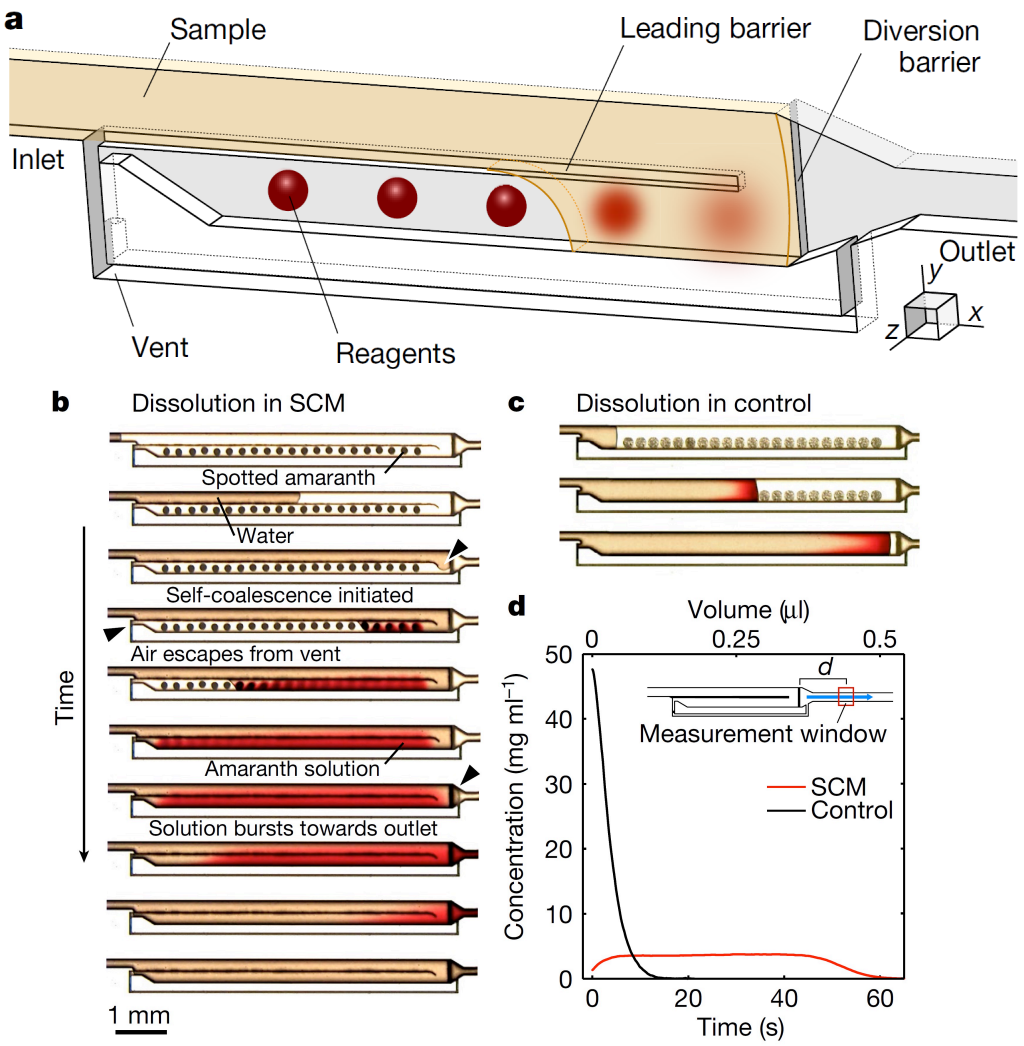
Capillary-driven (sandwich) immunoassay

Detection of a cardiac marker (Troponin I) in human serum



Hemmig et al., Anal Chem, 2020

Self-coalescence module



Gökçe et al., Nature, 2019

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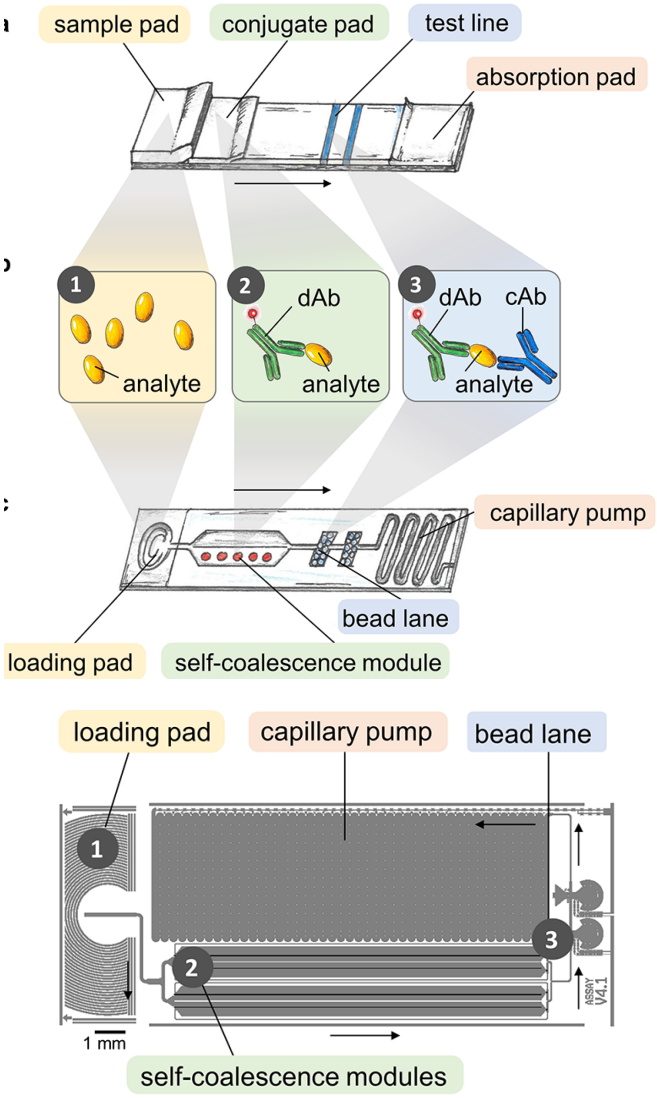
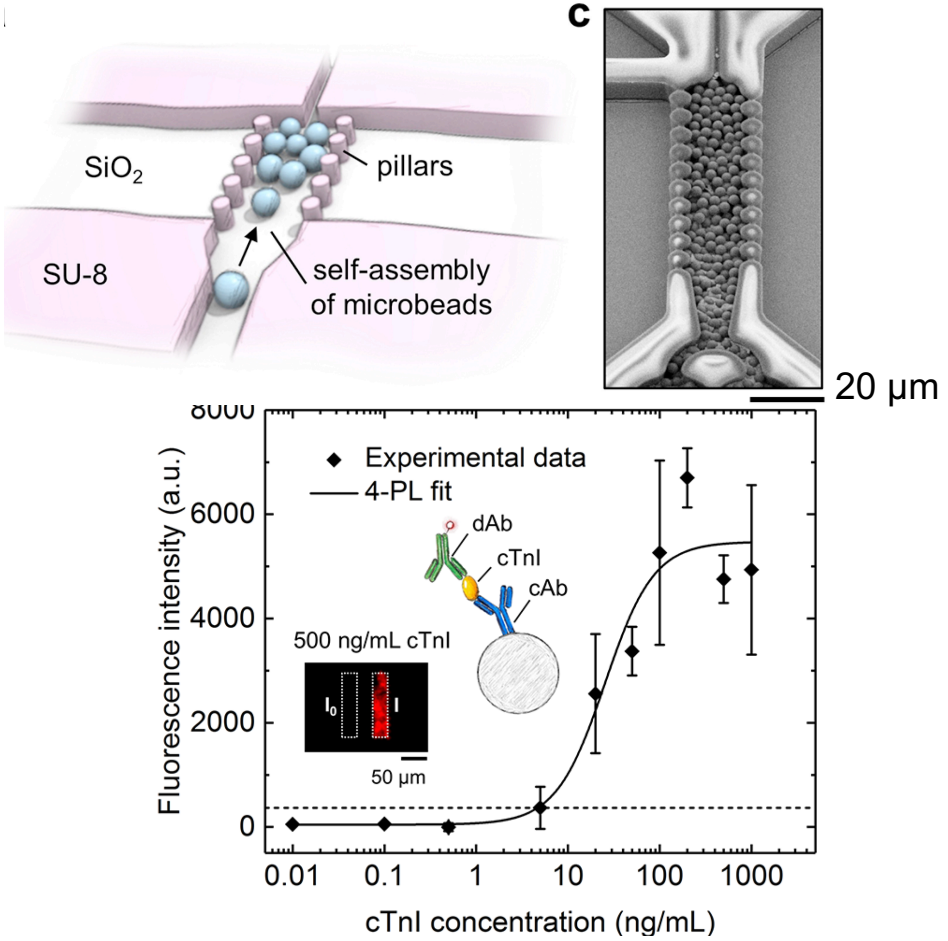


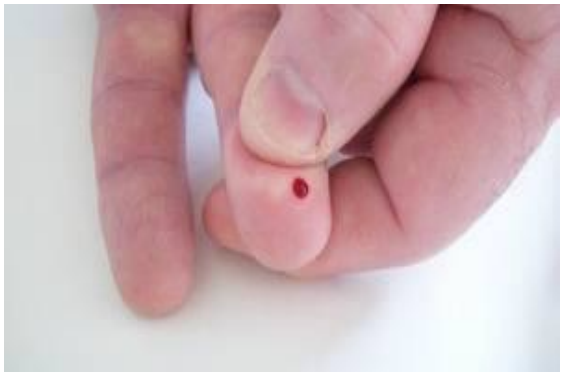


Figures of merits

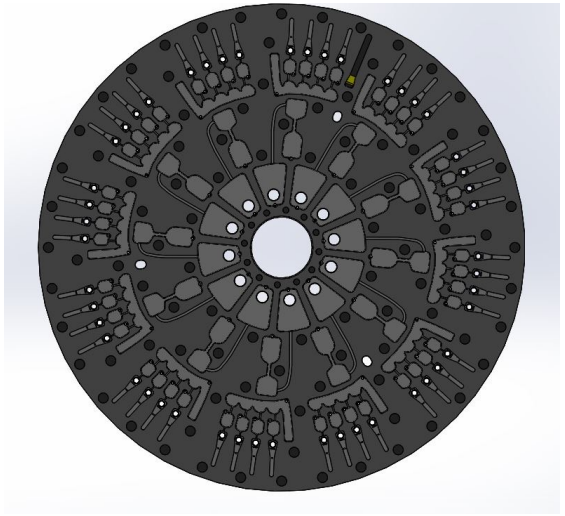
- 1 µL of sample
- 4 ng of Troponin I
- Assay time: 25 min

Hemmig et al., Anal Chem, 2020

MammoAlert™ for breast cancer screening



- CD-shaped microfluidic platform
- Simultaneous analysis of 4 specific biomarkers: higher detection accuracy.
- Disposable platform pre-loaded with all reagents (antibody-conjugated beads (capture), fluorescently labeled antibodies (detection)).
- Cloud-based analytics, storage and sharing of reports.

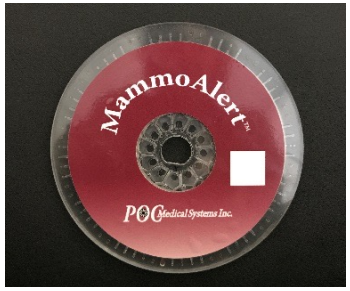


- **MammoAlert** ™ 1st product for breast cancer screening
- **Portable device**
- **Inexpensive**
- **Painless:** Just a finger prick for sample.
- **Fast:** Results in less than 20 minutes.

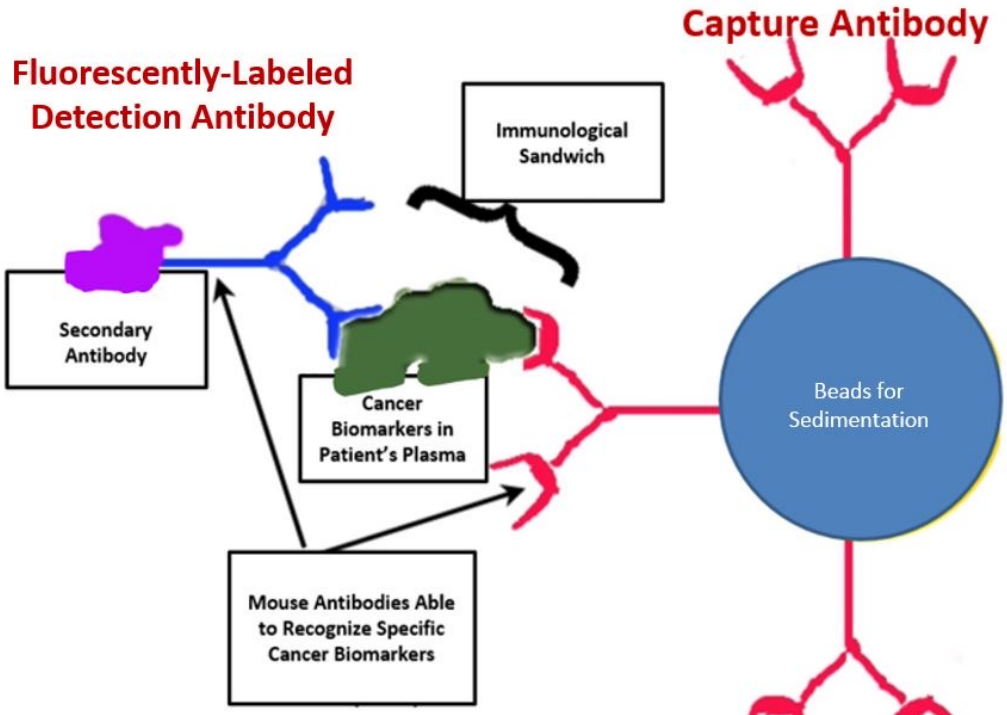
http://www.pocmedicalsystems.com/mammoalert/

Courtesy Luc Bousse

MammoAlert™ for breast cancer screening



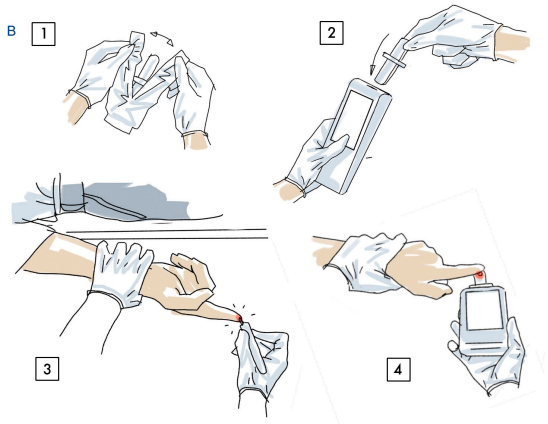
- Four Bead-Based Sandwich Immunoassays



http://www.pocmedicalsystems.com/mammoalert/

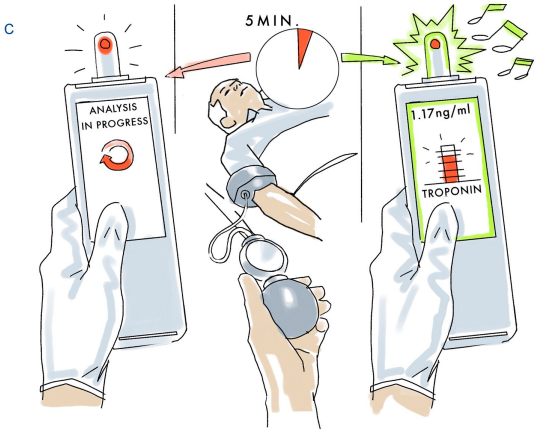
Courtesy Luc Bousse

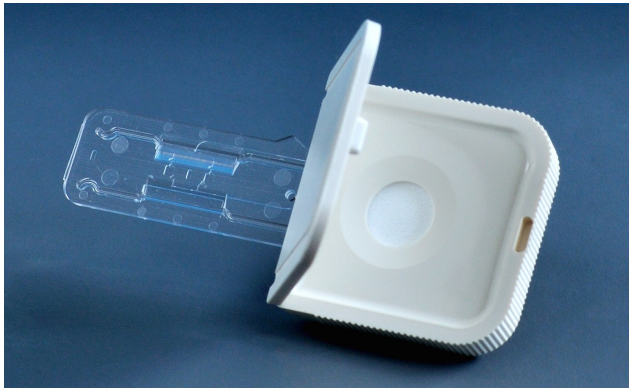
Early diagnosis of heart failure
Minicare @ Philips



Motivation

Detection of a cardiac marker (cardiac troponin I) from a finger-blood prick sample with a turnaround time of 5 min



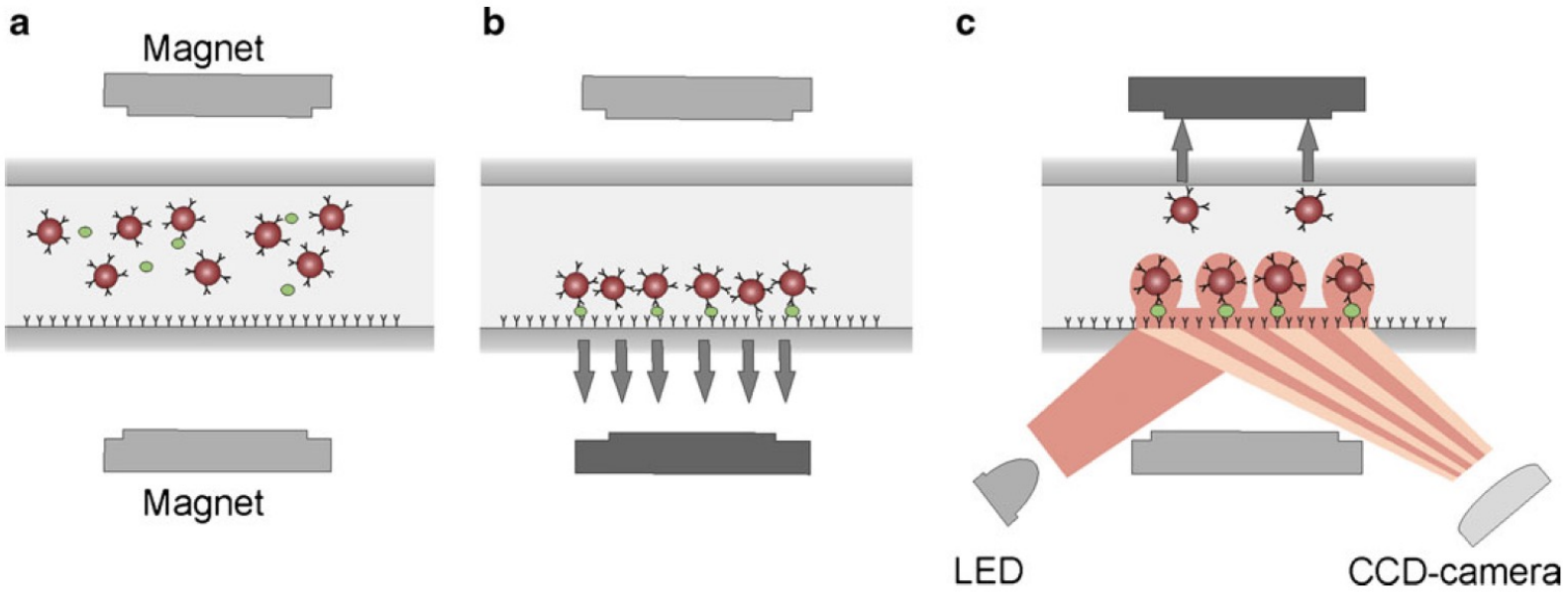


http://www.business-sites.philips.com

Early diagnosis of heart failure
Minicare @ Philips

Principle

- Capture of the biomarker using magnetic beads (500 nm) functionalized with specific antibody
- Sandwich immunoassay: aggregation of the beads with biomarker on an antibody-coated surface (magnetic actuation)
- Optical detection (total internal reflection)



- Limit of detection: 0.03 ng/mL

Conclusions

- Analysis of **biomolecules** (nucleic acids, proteins) from crude samples
- **Sample preparation** = essential step before actual analysis
- Implementable on chip for fully integrated protocol
  - eventual staining (fluorescence detection)
  - removal of “large” contamination
  - sample concentration
  - sample biochemical processing
- Different approaches possible for each step
- **Trends**
  - Start: “simple” (bio)molecule separation
  - Sample-in-answer-out integrated platforms
  - Point-of-care devices – integration; user-friendliness; fast analysis
  - Single cell comprehensive molecular analysis - -omics/multi-omics
  - Combination with cell culture devices / organ-on-chip models