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Integration of PAper-based Nucleic acid testing mEthods into Microfluidic devices for improved biosensing Applications

Nucleic Acid Extraction and Multiplex Detection Methods

Nejra Omerović, Stefan Jarić, Kristina Živojević
BioSense Institute, Young Researchers

IPANEMA, Online Journal Club 5, July 22,2020, Online video meeting

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872662

LAMP Multiplex systems

When LAMP Singleplex is not helpful?

- Symptoms are not clear (and depend on circumstances)
- It is not clear which pathogen plays a role (e.g. air, water)
- Targets have very variable genomic sequences

LAMP Multiplex systems:

- Assimilation probe detection
- Combinatorial mixing
- Capillary multiplexing

- LAMP integrated with different systems, such as microfluidics, lateral flow stripe and capillaries has shown great potential for developing multiplex detection methods.
- Cell manipulations on small chips with dimensions of a few centimeters and in small reaction volumes.

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On-chip LAMP

Q-J. Zhou, L. Wang, J. Chen, R-N. Wang, Y-H. Shi, C-H. Li, D-M. Zhang, X-J. Yan, Y-J. Zhang, **Development and evaluation of a real-time fluorogenic loop-mediated isothermal amplification assay integrated on a microfluidic disc chip (on-chip LAMP) for rapid and simultaneous detection of ten pathogenic bacteria in aquatic animals**, *Journal of Microbiological Methods* 104 (2014), 26-35

- **The aim** was to develop on-chip LAMP for for multiplex detection of 10 pathogenic bacteria in aquaculture: *Nocardia seriolae, Pseudomonas putida, Streptococcus iniae, Vibrio alginolycus, Vibrio anguillarum, Vibrio fluvialis, Vibrio harveyi, Vibrio parahaemolyticus, Vibrio roteferianus, and Vibrio vulnificus.*
- 49 bacterial isolates from aquatic animals or products, or water environments were used.
- Genomic DNA was extracted from bacteria using the Universal Kit for Bacterial DNA Extraction (CapitalBio Co., Beijing, Chine).

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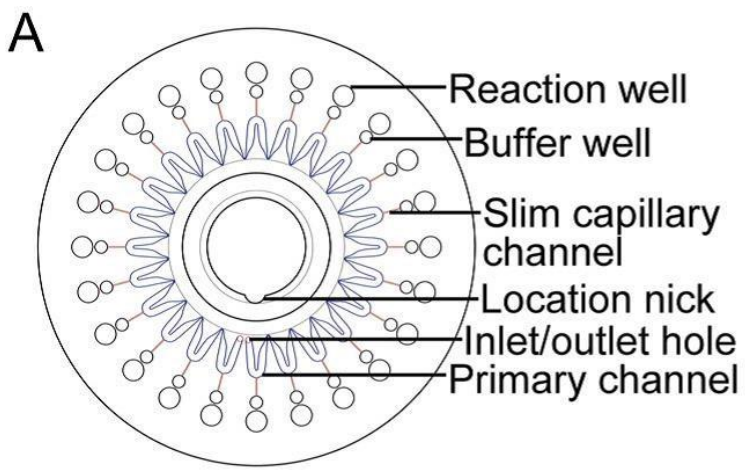
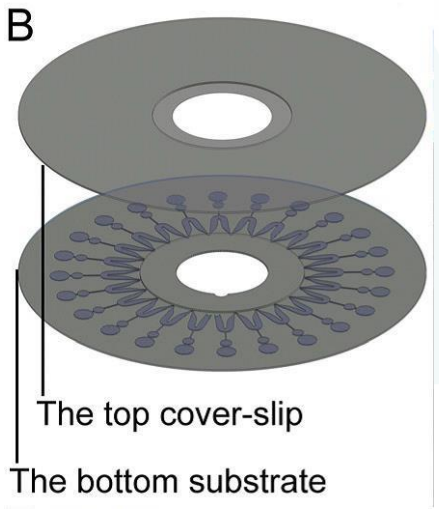
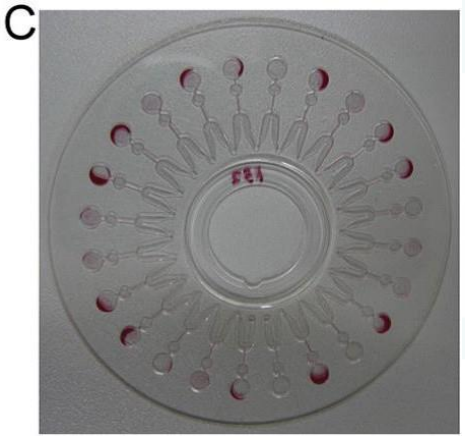
Chip fabrication

- Disc-type microfluidic chip – 62mm diameter and 0.6 mm thickness
- 24 reaction wells with 3 mm diameter; volume of each reaction well 1.414 µl, volume of the primary channel 48 µl
- Each reaction well closely connected with a buffer well. The buffer well connected with the primary channel via a slim capillary channel. When the chip was heated, the slim capillary channels were cut off by the thermal stress, thus isolating the reaction well to reduce possible contamination among reaction wells.
- Fabricated in polymethylmethacrylate (PMMA)
- Composed of two layers: the bottom substrate and the top cover-slip. The bottom substrate was firmly bonded with the disc cover-slip by stamping using an air press.







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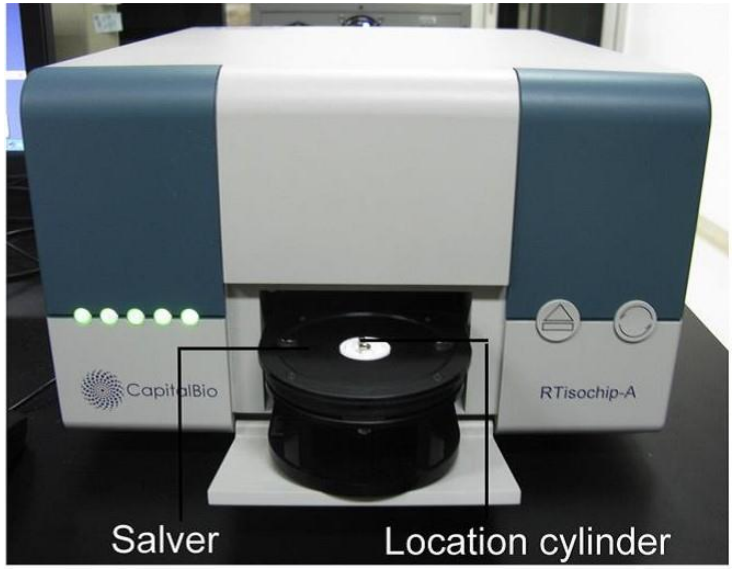
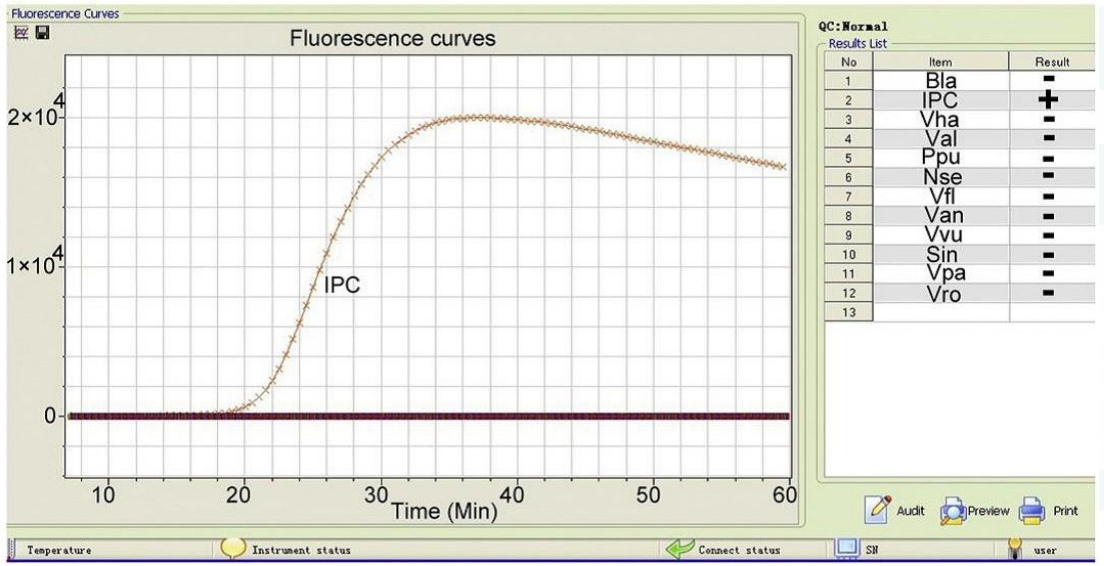
Experimental setup

- LAMP amplification platform and an imaging system were assembled together in a CapitalBio Rtisochip-A, consists of a temperature-controlled system and a real-time fluorescent acquisition unit.
- The detection results could be presented as real-time amplification plots following the reaction progress, or as positive (+) or negative (-).





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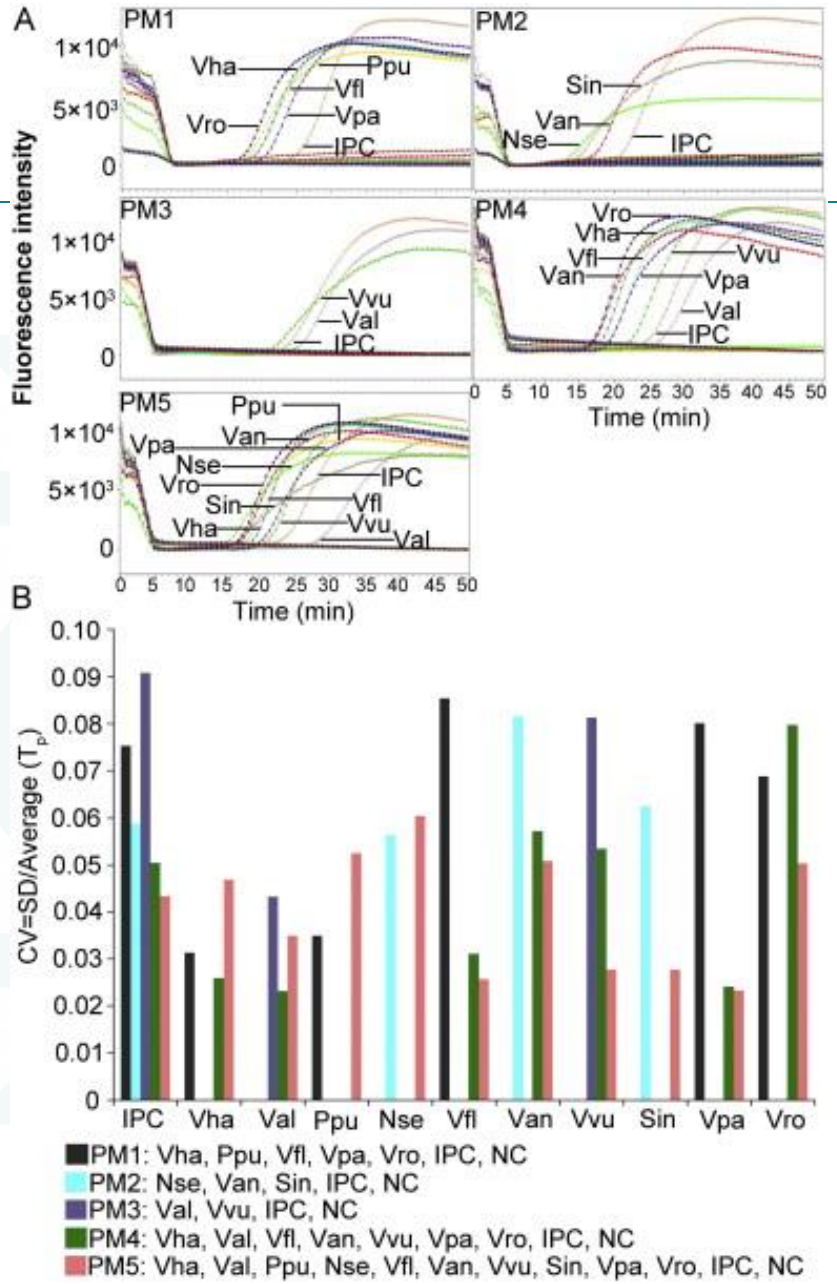
Parallel detection of multiple bacteria

- The DNA of 10 bacteria with desired concentrations (10-fold higher than the LOD of on-chip LAMP for each bacterium) was mixed randomly to form 5 combinations (PM1, PM2, PM3, PM4, PM5). All experiments were performed in triplicate.
- Among the five tested combinations, the expected positive signals were observed as typical sigmoidal amplification curves.
- The time to amplification for all bacteria varied between 15 and 30 min.
- Little variation and good reproducibility among replicates for each bacterium (CV<0.10) indicated good reproducibility in parallel detection of multiple bacteria.



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Integrated micro-LAMP (iμLAMP)

X. Feng, H. Chen, L. Xu, X. Jiang, W. Wu, J. Kong, **A portable and integrated nucleic acid amplification microfluidic chip for identifying bacteria**, *Lab Chip* 12 (2012), 1495

- **The aim** was to develop a portable integrated microchip of LAMP (iμLAMP), with ability to perform rapid DNA release, exponential signal amplification and naked-eye result read-out in single or multiplex format.
- Three kinds of genetically engineered *Escherichia Coli* (GE-*E. Coli*) were used.
- The amplification was performed at 65 °C in a laboratory water bath for 45 min. The detection result was directly determined by naked eye according to the presence of a green color in the reaction chambers.

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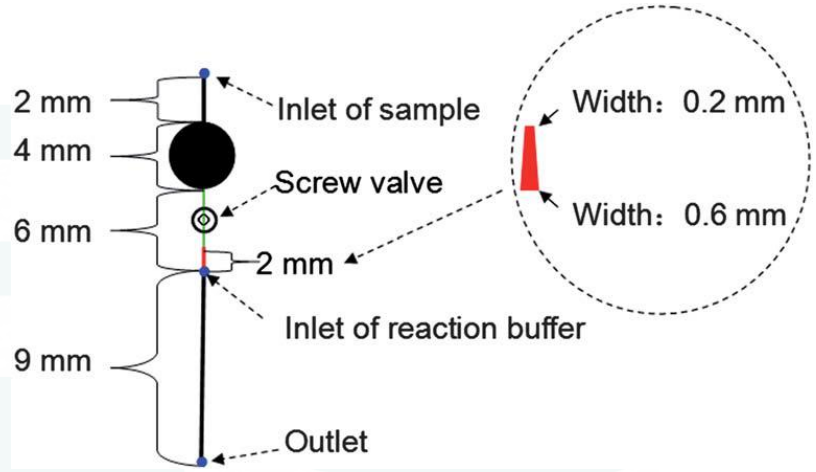
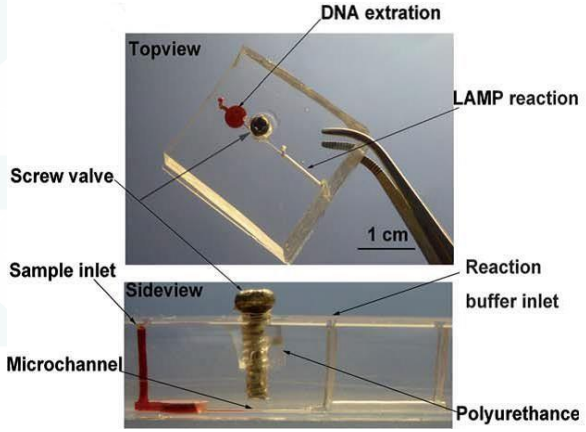
Chip design and fabrication

- The micro-pattern (PMMA; through mechanical microfabrication) was composed of a DNA release chamber, a screw valve controlled channel, gradient bridges, LAMP reaction chamber (length, 9 mm; width, 0.6 mm; height, 0.8 mm) with a volume of 5 mL.
- Multiplexed format is composed of multiple LAMP reaction chambers.
- PDMS replicas were produced by soft lithography.
- The replica was sealed with a microscope glass slide by air plasma to form leak-proof microchannels.





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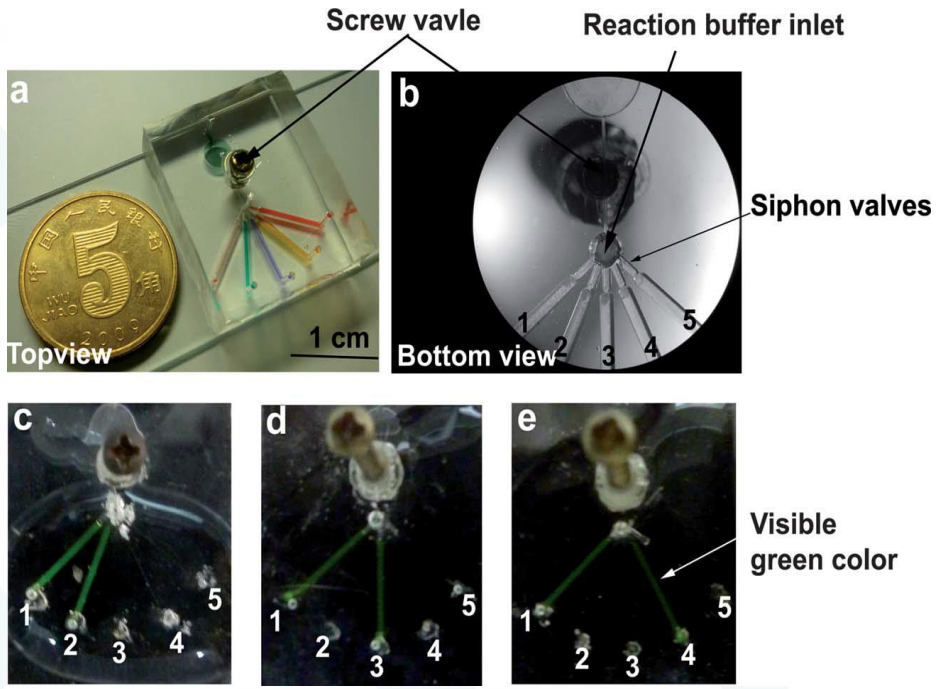
Parallel detection of multiple bacteria

- Reaction chambers were connected to capillary siphon valve channels extending from the LAMP reaction buffer inlet. These siphon valves with low-mass-transfer coefficient were designed to prevent the crosstalk of primers among different reaction chambers.
- Each chamber was pre-coated with specific LAMP primers.
- It was observed that each target only triggered the LAMP signal in its corresponding micro-chamber and no cross-activity appeared.
- This iμLAMP chip could be applied for the detection of multiple pathogens.



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Multiplexed On-chip detection

Preview:

1. Dou et al. *"A paper/polymer hybrid CD-like microfluidic SpinChip integrated with DNA-functionalized graphene oxide nanosensors for multiplex qLAMP detection"*
2. Melaine et al. *"Selective and High Dynamic Range Assay Format for Multiplex Detection of Pathogenic Pseudomonas aeruginosa, Salmonella typhimurium, and Legionella pneumophila RNAs Using Surface Plasmon Resonance Imaging"*
3. Labroo et al. *"Graphene nano-ink biosensor arrays on a microfluidic paper for multiplexed detection of metabolites"*

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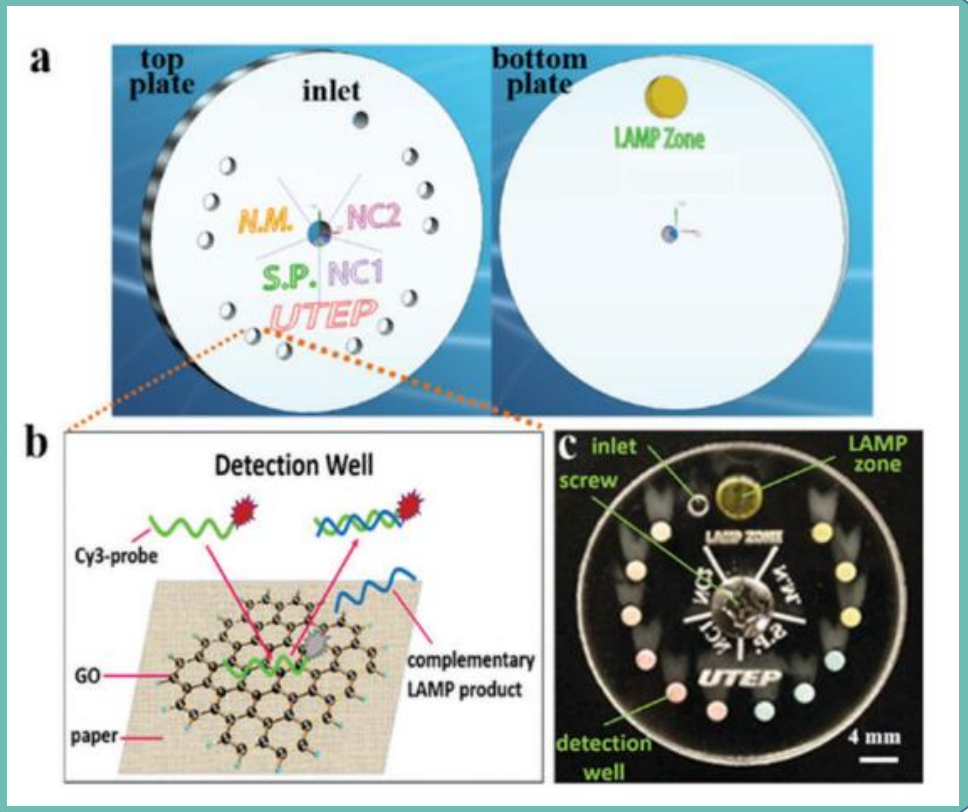
- Hybrid microfluidic SpinChip integrated with both LAMP amplification and GO-based nanosensor detection
- Multiplex detection of *Neisseria meningitidis* (*N. meningitidis*) and *Streptococcus pneumoniae* (*S. pneumoniae*)
- Materials: PMMA disks, porous chromatography paper (Whatman#1) and graphene-oxide functionalized with ssDNA strands labeled with Cy3 dyes
- Design: two PMMA disks screwed on the center (alowing rotations), microzones (sampling wells with paper-suported ssDNA-functionalized GO nanosensors - top disk, and LAMP zone – bottom disk)



M. Dou et al., *A paper/polymer hybrid CD-like microfluidic SpinChip integrated with DNA-functionalized graphene oxide nanosensors for multiplex qLAMP detection*, Chem. Commun. 2017, **53**, 10886-10889

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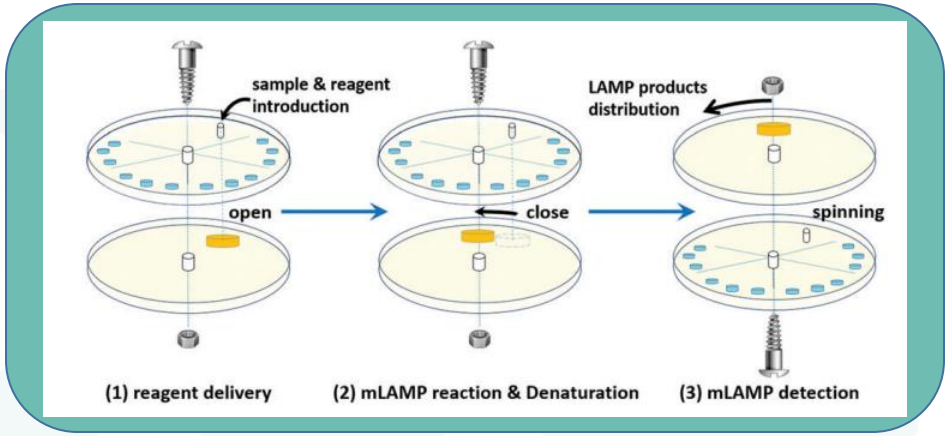
- Principle of detection:
  1. Reagent/sample introduction – LAMP zone – SpinChip open
  2. mLAMP reactions (63 °C and 95 °C for reaction termination and product denaturation) - SpinChip closed
  3. Rotations to the microzones – detection of LAMP products (qLAMP) - quenched fluorescence recovery



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Optimizations of ssDNA probe concentrations (1 µM chosen) for better fluorecsence intensity after recovery, higher recovery rate and higher net fluorescence recovery

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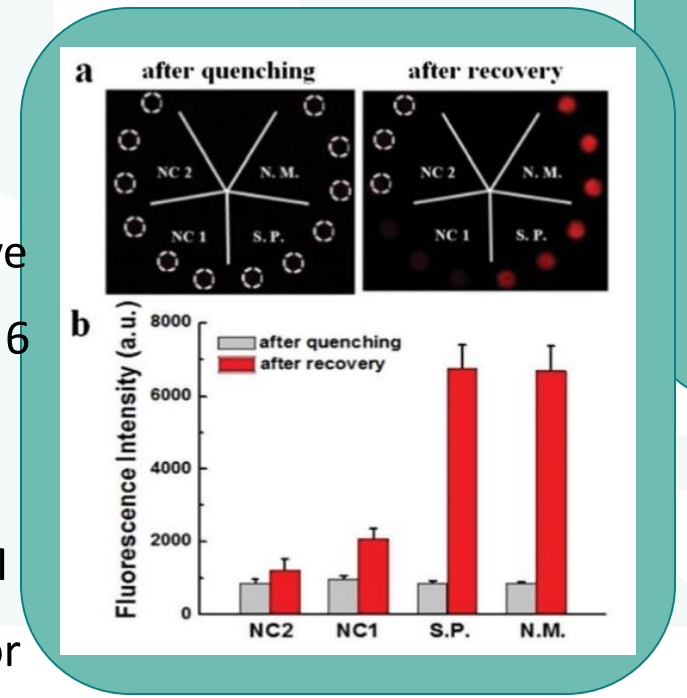
Results:

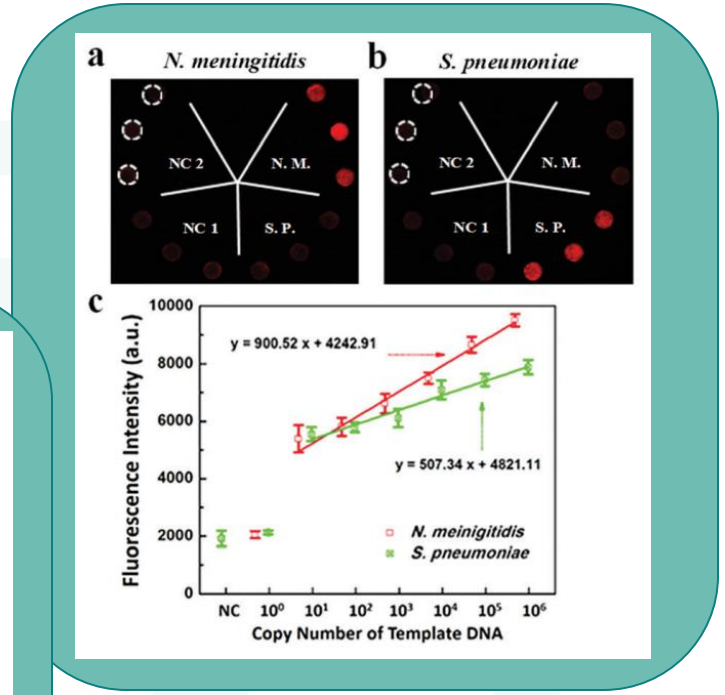
a) **mLAMP reactions (Fig. 1):** succesful multiplex LAMP reaction for both *N. meningitidis* and *S. pneumoniae,* higher fl. intensity than for negative controls. Confirmation by fragment analysis.

b) **Specificity tests (Fig. 2a and b):** 8.1x and 6.6x higher net recovery fl. intensities to corresponding probes than to non-corresponding probes for NM and SP, respectively.

c) **Quantitative analysis (Fig. 2c):** calibration curve of fl. intensities vs. initial number of template DNA copies. Linear relationship from 6 - $6x10^5$ and 12 – $1.2x10^6$ copies per assay for NM and SP, respectively. LODs for NM and SP are 6 and 12 copies per assay, respectively.

d) **Additional measurements:** quantification of mLAMP amplicons by using various diluted NM and SP LAMP products. GO nanosensors detected as low as 80 ng μl^{-1} and 87.5 ng μl^{-1} for NM and SP, respectively.





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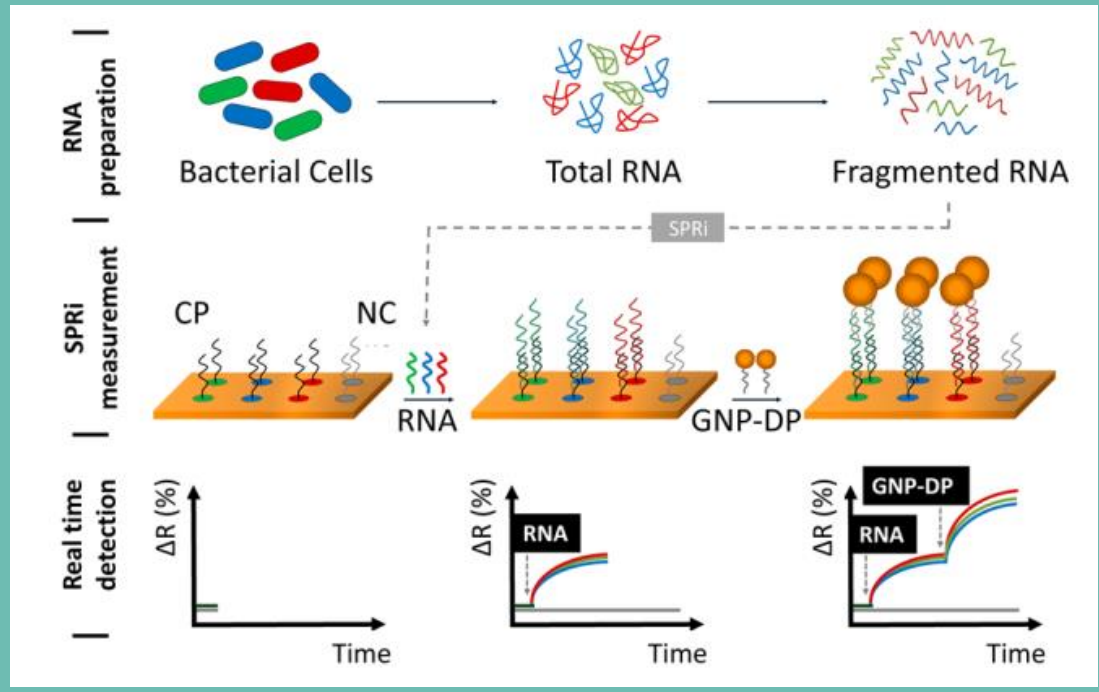
- Multiplex detection of specific 16S rRNA region in *P. aeruginosa*, *S. typhimurium* and *L. pneumophila* with the SPR imaging (SPRi) detection principle.
- Materials: gold-coated prisms with thiolated oligonucleotide probe (collection probes, CPs and negative controls, NCs) monolayers, gold nanoparticles (GNPs) functionalized with detection probes (DPs), extracted and fragmented RNAs from PA, ST and LP species.
- Measurements: scanning-angle SPRi instrument (800 nm LED source, CCD camera, and microfluidic flow cell).
- Resulted signals: reflectivity difference (ΔR, %) from buffer w/o RNAs and buffer w/ RNAs; surface regenerations with 50 mM NaOH.



F. Melaine et al., *Selective and High Dynamic Range Assay Format for Multiplex Detection of Pathogenic Pseudomonas aeruginosa, Salmonella typhimurium, and Legionella pneumophila RNAs Using Surface Plasmon Resonance Imaging,* Anal. Chem. 2017, **89**, 7802-7807

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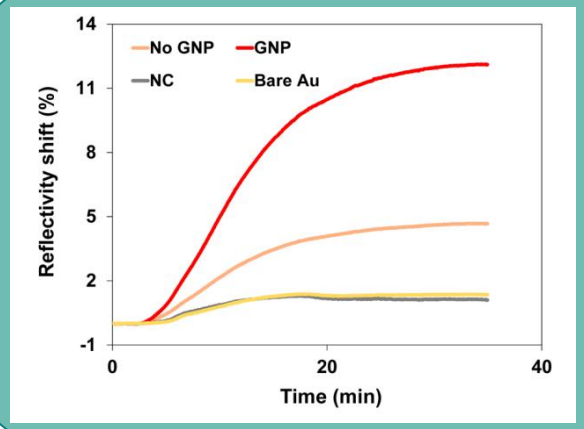
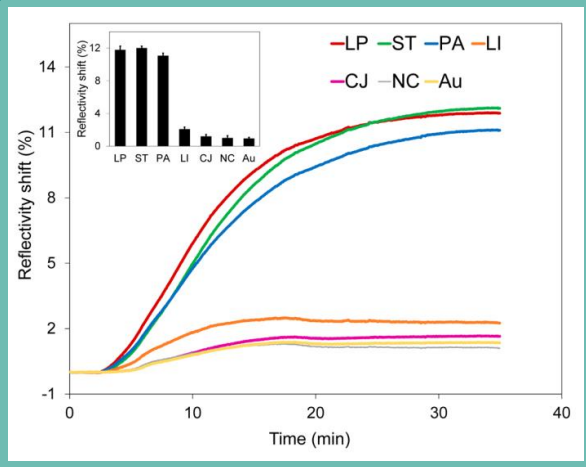
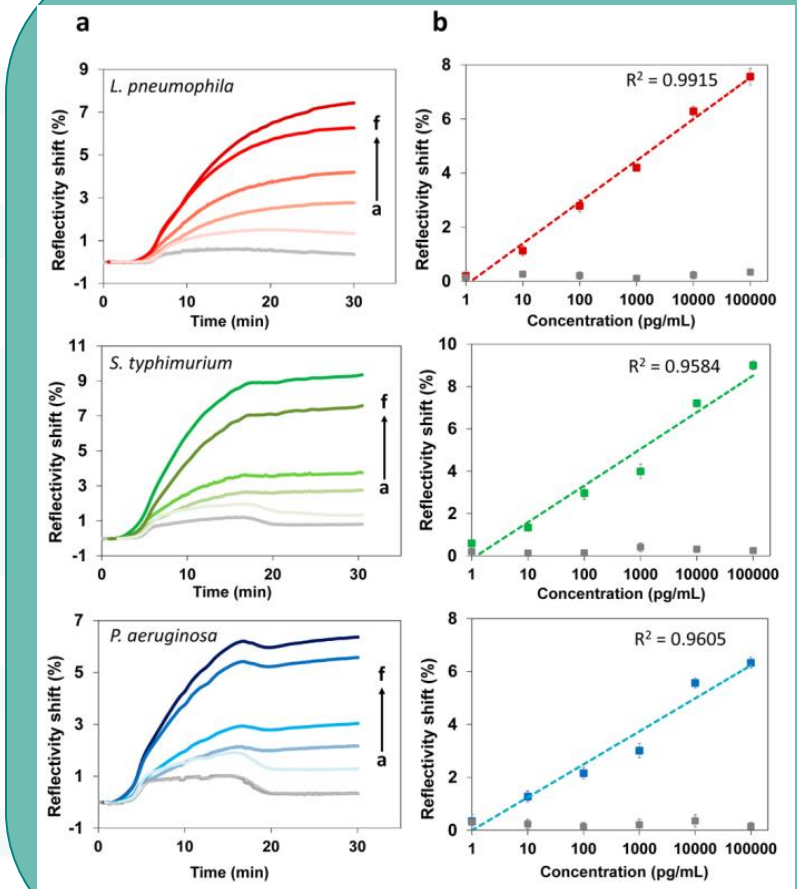
Results:

1. **Signal amplification with GNPs** (Fig. 1): DP introduction increased SPR signal by ~4.6%, while GNP+DP introduction increased SPR signal by ~12.01%
2. **Multiplex detection** (Fig. 2a): various RNA concentrations (1, 10, 100, 1000, 10000 and 100000 pg ml^{-1}). For the highest concentration, percentage reflectivity changes were 0.31, 0.25 and 0.21% ng^{-1}, with respect to the 0.027% ng^{-1} for NC. Calibration curves (Fig. 2b): linear relationships between total reflectivity change and RNA concentrations. The LOD was 10 pg ml^{-1}.
3. **Specificity tests** (Fig. 3): Samples with 0.1 µg ml-1 RNA of five different species: PA, ST, LP, *C. jejuni* and *L. israelensis*.







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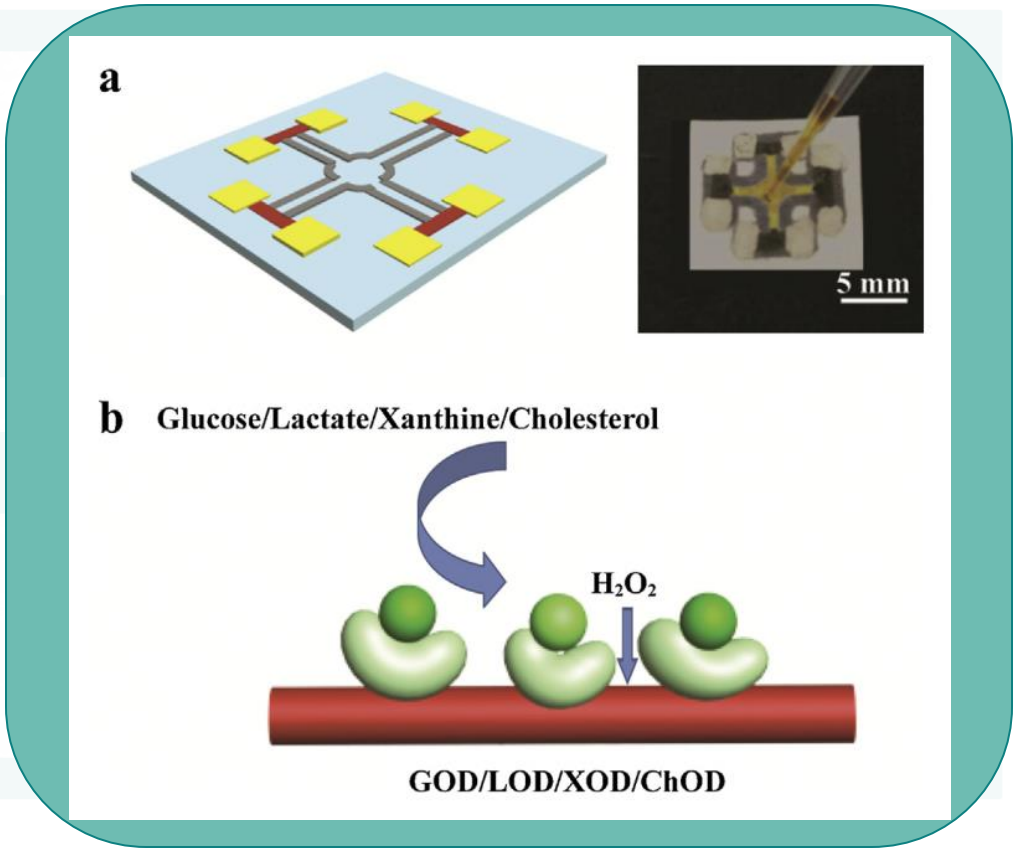
3. Graphene nano-ink biosensor arrays on a microfluidic paper for multiplexed detection of metabolites

- Simultaneous detection of several metabolites – glucose, lactate, xanthine and cholesterol – with graphene ink-based biosensor arrays on microfluidic paper.
- Materials: graphene nano-platelets functionalized with enzymes (glucose oxidase (GOD), lactate oxidase (LOD), xanthine oxidase (XOD) and cholesterol oxidase (ChOD)), solid ink for microfluidic channels and regular paper
- Design of chip: four enzyme-graphene ink electrodes with a pair of silver terminals were printed on a paper. Four microfluidic channels reach graphene electrodes on one end and sample well in the center of the chip on other end.
- Mechanism of reaction: Enzymes on graphene ink induce oxidation process, releasing H_2O_2 which is detected by the current change.



P. Labroo, Y. Cui, *Graphene nano-ink biosensor arrays on a microfluidic paper for multiplexed detection of metabolites,* Anal. Chim. Acta 2014, **813**, 90-96

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3. Graphene nano-ink biosensor arrays on a microfluidic paper for multiplexed detection of metabolites

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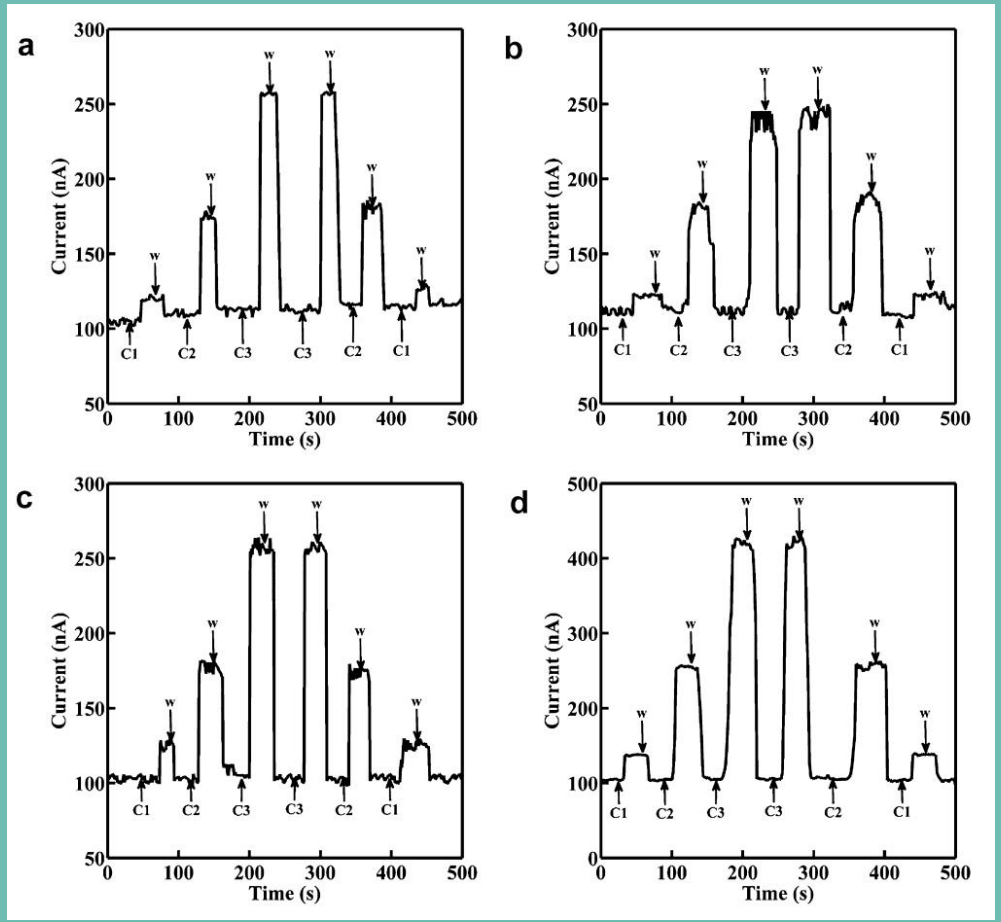
Results:

1. **Ink preparation:** various enzyme concentrations to determine best sensitivity of the chip (2 mg μl^{-1}).
2. **Time-dependent measurements** (Fig. 1): various metabolite concentrations (1 (C1), 5 (C2) and 10 (C3) µM) (w – washing step). Response time: ~20 s. Total measurement time was less than 2 min.



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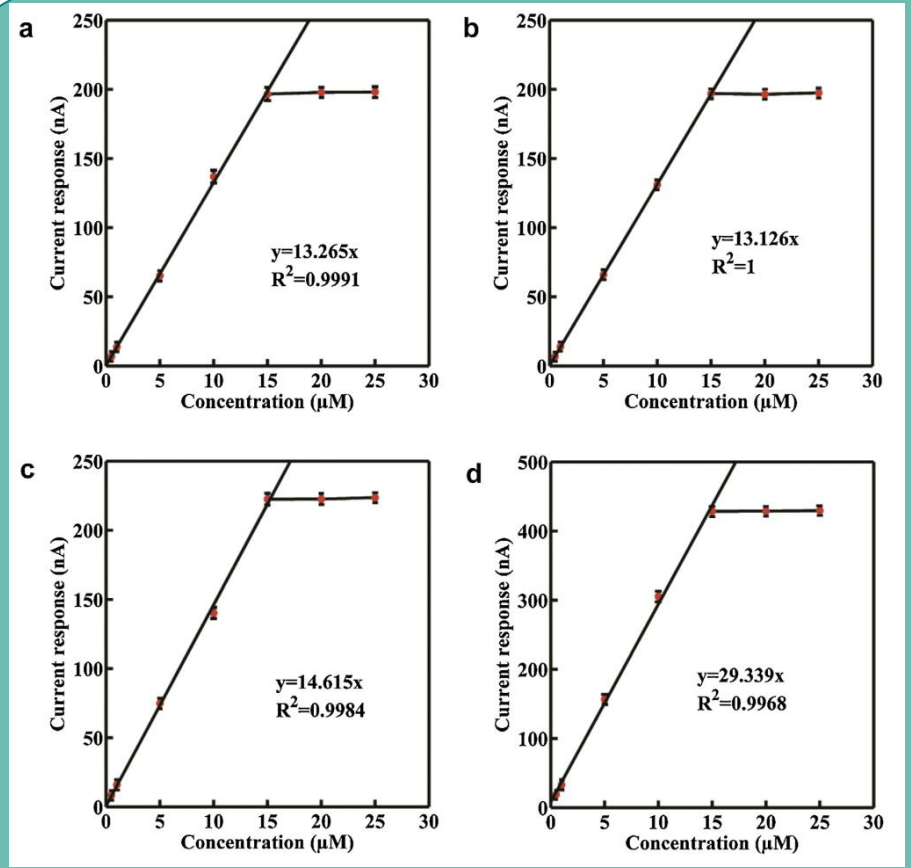
3. **Calibration curve measurements** (Fig. 2): linear slopes of 13.3, 13.1, 14.6 and 29.3 nA μM^{-1} for glucose, lactate, xanthine and cholesterol, respectively. Detection limit of 0.3 µM.
4. **Performances of the sensor**: wetting cycles measurements to determine sensor stability – after 25th – 30th cycle, sensor retained ≥50% of its initial activity. Continuous measurements during 20 days (each alternate day) on room temperature (retained 50%) and 4 °C (retained 70%) (for glucose, similar for other metabolites).
5. **Real-sample measurements**: human blood diluted with PBS. Comparison between enzyme-graphene ink biosensor and screen-printed biosensor

Metabolite	Graphene ink sensor (µM)	Screen-printed sensor (µM)
Glucose	4094.28	4106.18
Lactate	1041.22	1021.09
Cholesterol	5041.66	5051.88
Xanthine	1.12	1.02



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Silicon-Based Nucleic Acid Extraction and Purification

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PoC microfluidics-based diagnostics

Sample preparation NA isolation → **Amplification** → **Detection**

- Prior to amplification, NAs must be extracted from biological matrices and separated from membrane lipids, proteins, polysaccharides and other contaminants.
- Extraction determines the success of all the subsequent steps in the process.
- One method to **improve the sensitivity** of multiplex detection is **NA extraction/purification**.
- High quality NA extraction is needed for multiplex testing as the end-results depend on the NA quality.

Silicon-based extraction:

- Silica beads
- Silicon micropillars
- Silica-based glass fiber membrane
- Silica-coated magnetic beads etc.

Steps in NA extraction process:

1. Cell disruption (mechanical/chemical)
2. Removal of membrane lipids, proteins etc.
3. NA purification/binding from bulk
4. NA concentration

Image source: https://pixabay.com/illustrations/dna-matrix-genetics-control-3888228/
https://commons.wikimedia.org/wiki/File:White_Silica_Gel.jpg

Silicon-Based Methods

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- Silicon-based materials are common matrices for solid phase extraction (SPE) of NA that can be incorporated into microchips.
- NAs exhibit high binding affinity towards silica (adsorption) in the presence of high salt (chaotrope) concentration and under certain pH conditions followed by washing and elution steps.

Boom Method

Sample preparation (lysing/ homogenizing)

➢ Using a detergent and protein degrading enzymes

NA binding in the presence of a chaotrope

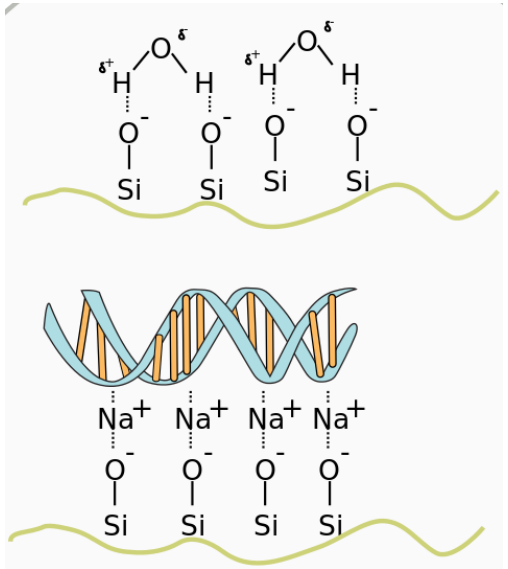
➢ Salt counterions form a salt bridge between silica and NA
➢ Chaotrope disrupts the shell of hydration around NA (salting out effect)

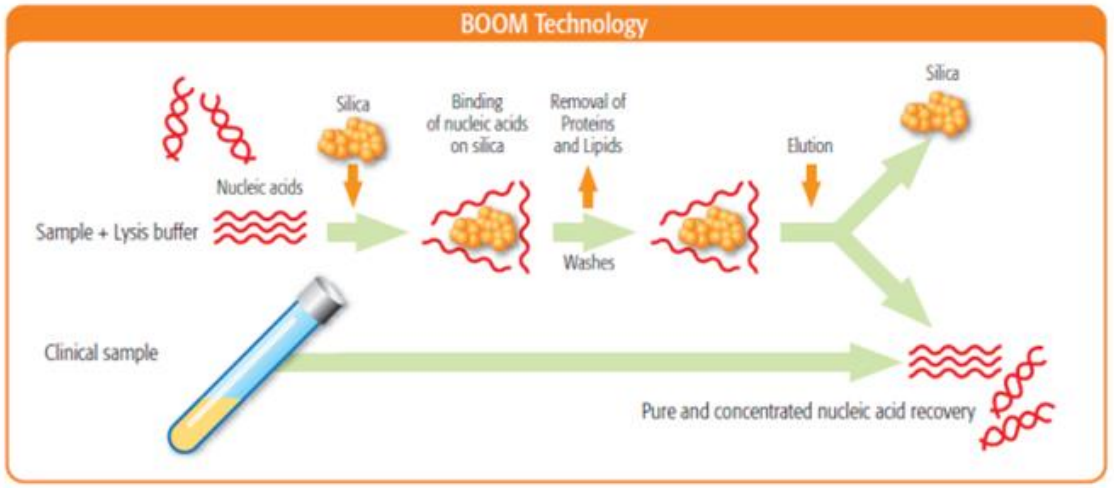
Washing

➢ Removal of contaminants while NA is bound to silica

NA Elution

➢ Low salt concentration or water

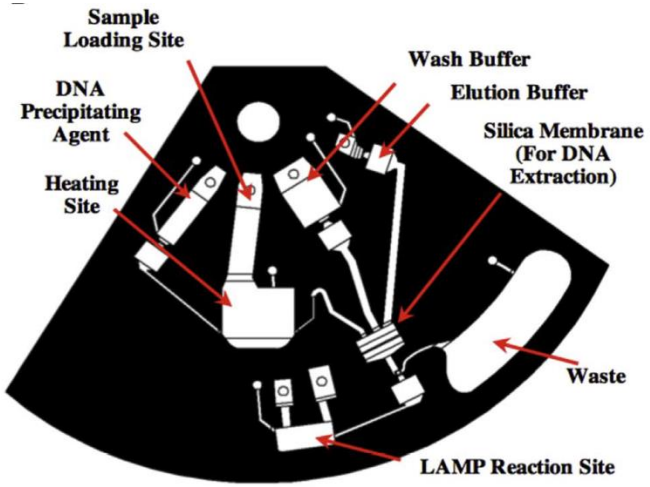




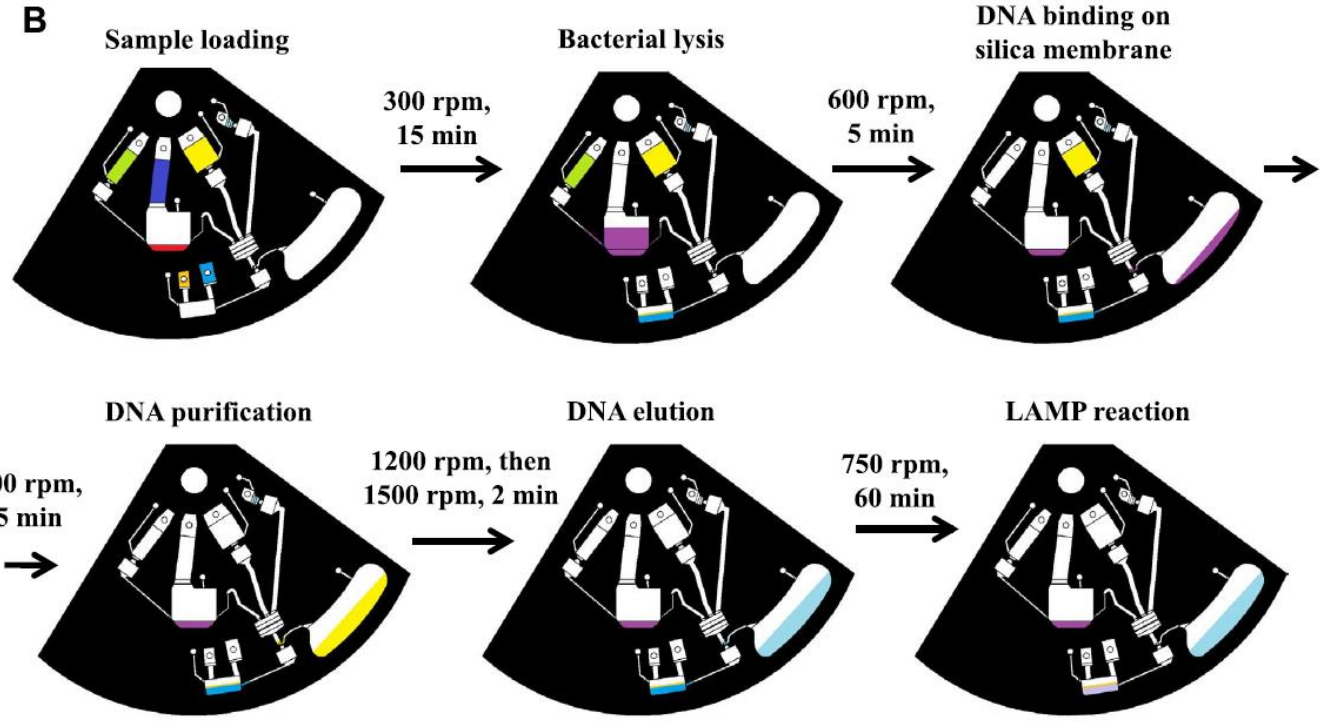
https://en.wikipedia.org/wiki/Spin_column-based_nucleic_acid_purification#/media/File:Qiagen_Mini_Spin_Column.svg

https://www.biomerieux-diagnostics.com/sites/clinic/files/boom-technology.jpg

Silica Membrane

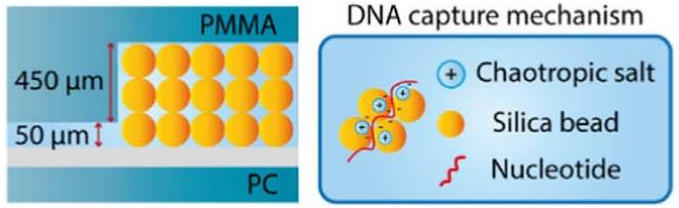


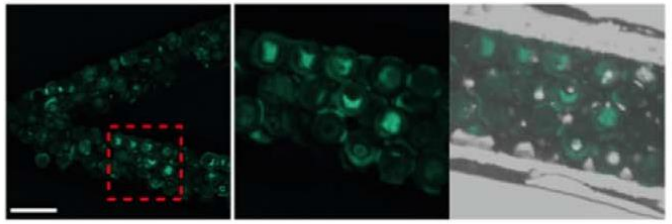
- **Centrifugal force** actuates the release of samples and reagents from compartments within the microfluidic platform.
- **Silica membrane** made of silica gel employed for DNA extraction.
- LAMP amplification and fluorescent detection of *Mycobacterium tuberculosis* and *Acinetobacter baumanii* within 2h.



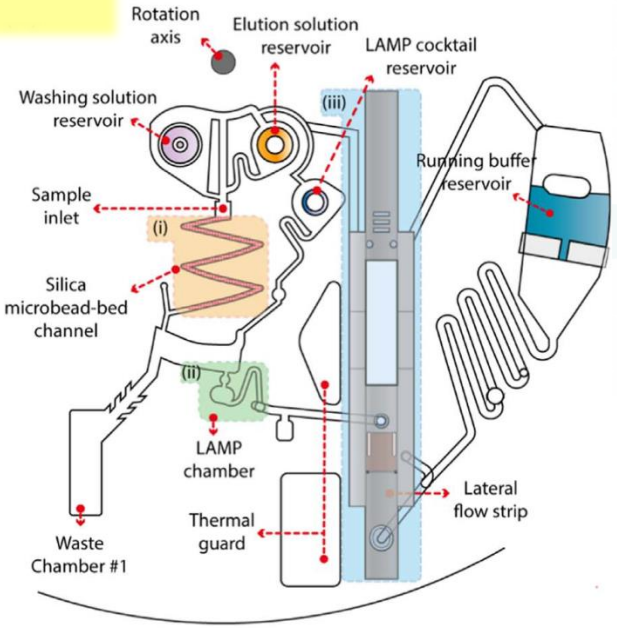
Loo, J. F. C. *et al*. Sample-to-answer on molecular diagnosis of bacterial infection using integrated lab-on-a-disc. *Biosens. Bioelectron.* **93**, 212–219 (2017).

Silica Beads





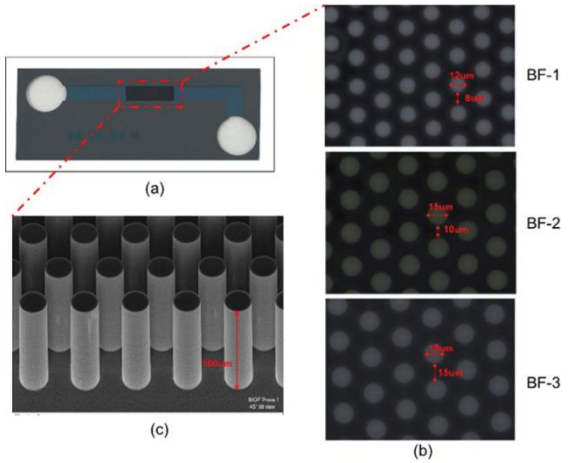
Solid phase DNA extraction

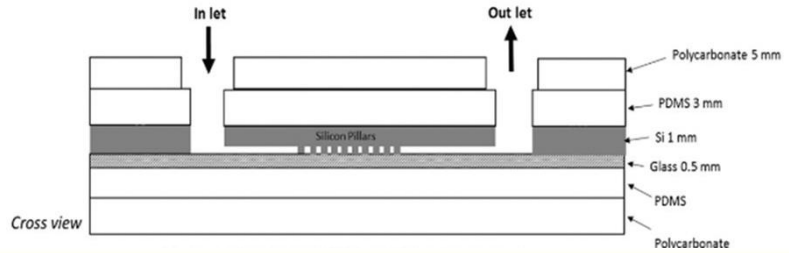


- Integrated rotary microfluidic system with a **glass microbead based DNA extraction**, LAMP amplification and colorimetric lateral flow strip based detection for food-borne bacterial pathogen detection.
- Monoplex *Salmonella Typhimurium* as well as **multiplex** *Salmonella Typhimurium* and *Vibrio parahaemolyticus* were analyzed with a LOD of 50 CFU in 80 min.

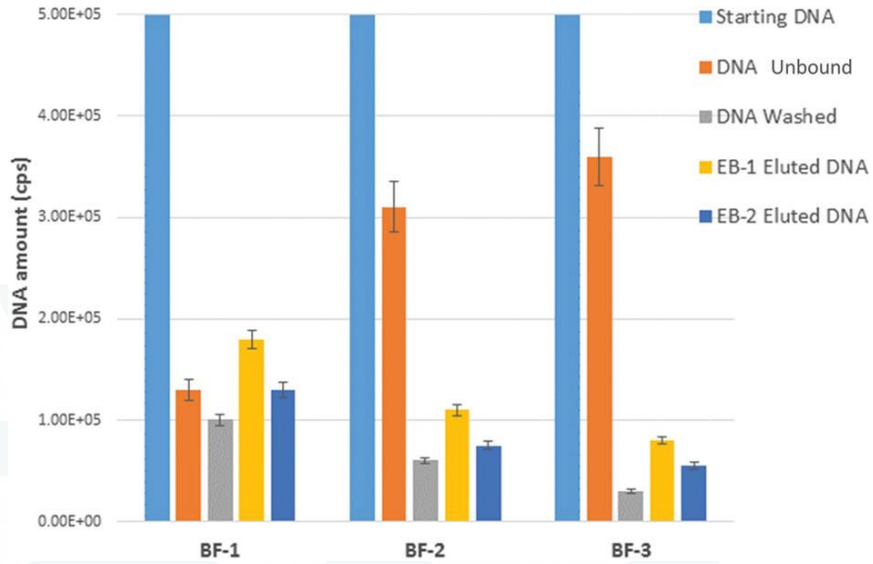
Park, B. H. *et al*. An integrated rotary microfluidic system with DNA extraction, loop-mediated isothermal amplification, and lateral flow strip based detection for point-of-care pathogen diagnostics. *Biosens. Bioelectron.* **91**, 334–340 (2017).

Silicon Micropillars



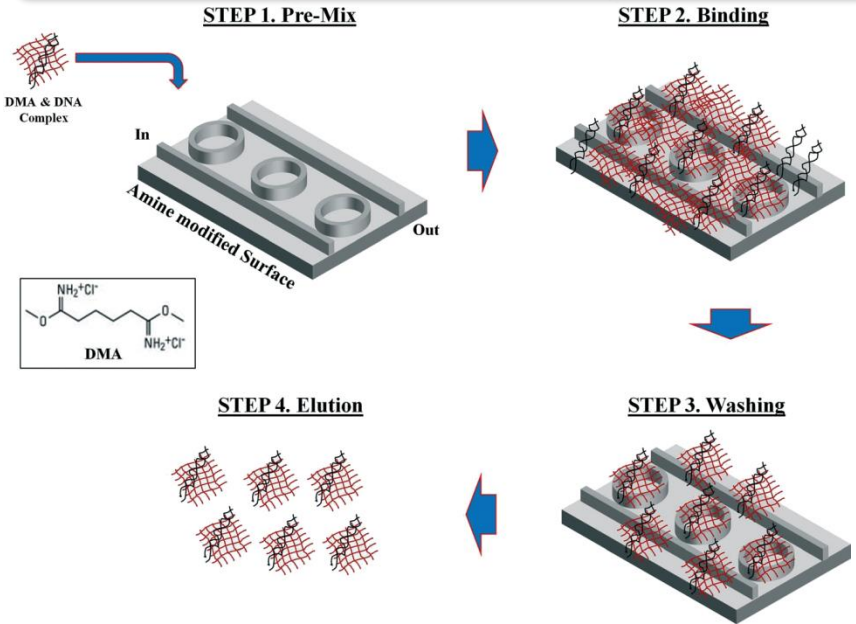


- Microfluidic biofilter devices based on a **silicon micropillar extraction** method of DNA from Hepatitis B Virus (HBV).
- DNA binding and the elution efficiency strictly depend on the dimensions of micropillars and increase proportionally with the surface/volume ratio.
- The extraction efficiency reaches about 40% in the case of the device exhibiting the highest SVR value (BF-1). This value is about 16% higher than that measured with a commercial kit, under the same experimental conditions.



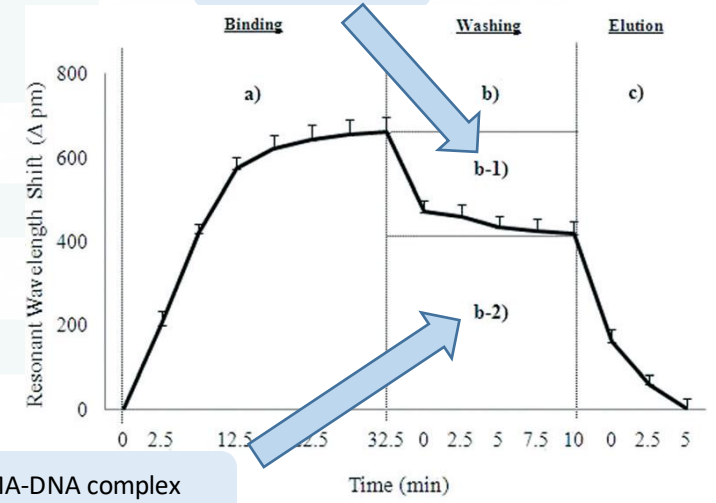
Petralia, S., Sciuto, E. L. & Conoci, S. A novel miniaturized biofilter based on silicon micropillars for nucleic acid extraction. *Analyst* **142**, 140–146 (2017).

Silicon-based microring resonators + non-chaotropic agent



- SPE based method using **DMA (dimethyladipimidate)** as a **non-chaotropic reagent** for DNA isolation.
- DMA forms reversible cross-linking structures providing high SVR for DNA capture.
- Silicon-based microring resonators (refractive index-based biosensor) for real-time monitoring of the reaction.

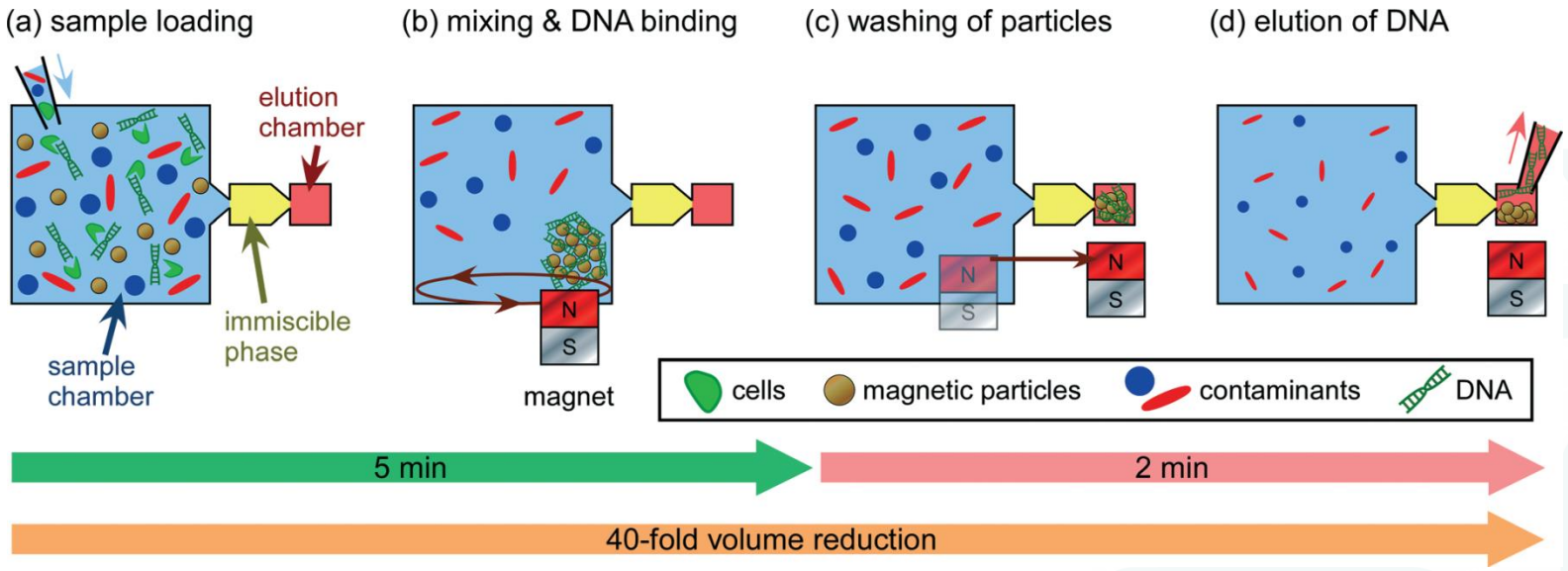
Non-specific binding of DNA to DMA

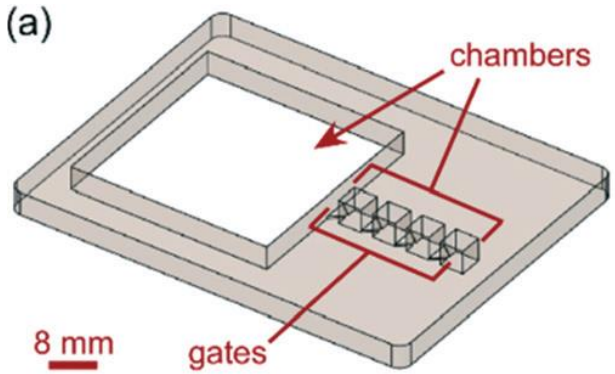


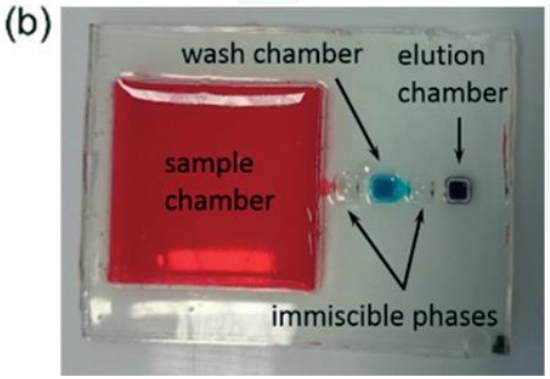
DMA-DNA complex remained on the surface

Shin, Y., Perera, A. P., Wong, C. C. & Park, M. K. Solid phase nucleic acid extraction technique in a microfluidic chip using a novel non-chaotropic agent: Dimethyl adipimidate. *Lab Chip* **14**, 359–368 (2014).

Silica coated magnetic beads







- Direct on-chip processing of samples for *Helicobacter pylori* DNA extraction and pre-concentration.
- Silica coated superparamagnetic particles (PMPs) capture the DNA followed by its washing and elution in separate chambers.
- Chambers are filled with alternating aqueous and oil phases that form “virtual walls” allowing PMPs to pass through and leave the contaminants behind.
- 7 min process with a 40-fold reduction in working volume from crude biological samples.

Mosley, O. *et al*. Sample introduction interface for on-chip nucleic acid-based analysis of: Helicobacter pylori from stool samples. *Lab Chip* **16**, 2108–2115 (2016).

Reference:

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F. Melaine et al., *Selective and High Dynamic Range Assay Format for Multiplex Detection of Pathogenic Pseudomonas aeruginosa, Salmonella typhimurium, and Legionella pneumophila RNAs Using Surface Plasmon Resonance Imaging,* Anal. Chem. 2017, **89**, 7802-7807

P. Labroo, Y. Cui, *Graphene nano-ink biosensor arrays on a microfluidic paper for multiplexed detection of metabolites,* Anal. Chim. Acta 2014, **813**, 90-96

Loo, J. F. C. et al. *Sample-to-answer on molecular diagnosis of bacterial infection using integrated lab-on-a-disc*. Biosens. Bioelectron. **93**, 212–219 (2017).

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X. Feng, H. Chen, L. Xu, X. Jiang, W. Wu, J. Kong, *A portable and integrated nucleic acid amplification microfluidic chip for identifying bacteria*, Lab Chip **12** (2012), 1495

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Thank you for your attention!