Paolo Dametto
August 2013

DNA amplification and detection using a pH-sensing system:

implications for current analytical technologies and Point-of-Care nucleic acid tests

Outline

1. Point-of-care (POC) diagnostics
   - Developing world
     - General considerations, clinical needs and requirements
       - **Tubercolosis**
       - **HIV**

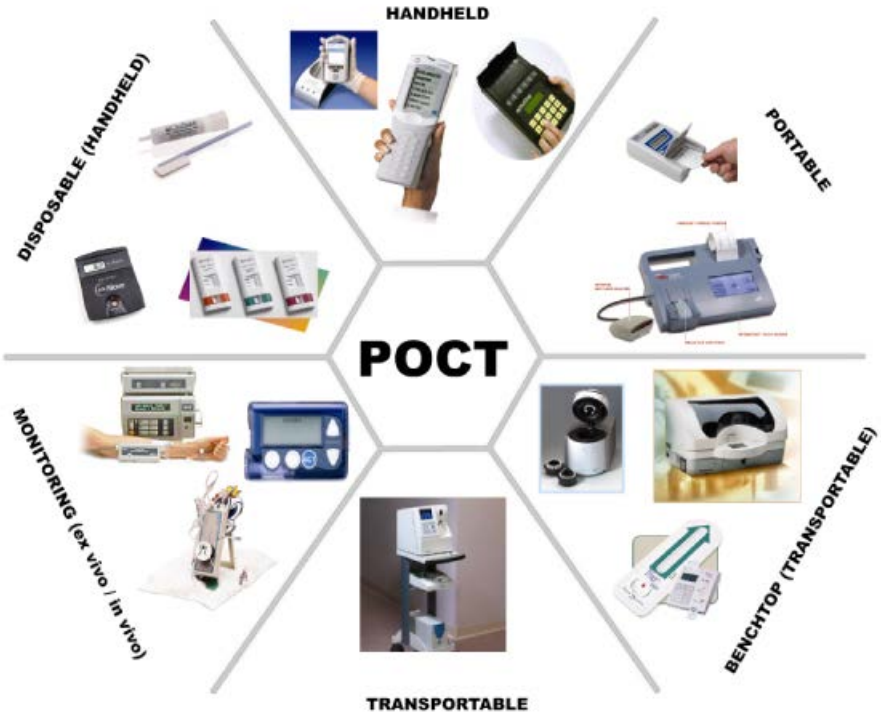
2. POC Nucleic Acid Tests (NAT)
   - Loop mediated isothermal amplification (LAMP)
   - Simultaneous DNA amplification and detection using a pH-sensing semiconductor system

POC: definition

- The location at which patient services are delivered

POC tests:

- Point-of-care testing (POCT) is defined as **medical testing** at or near the site of patient care
  - blood glucose testing
  - blood gas and electrolytes analysis
  - rapid coagulation testing
  - rapid cardiac markers diagnostics
  - abuse of drugs screening
  - urine strips testing
  - pregnancy testing
  - fecal occult blood analysis
  - food pathogens screening
  - hemoglobin diagnostics
  - **infectious disease testing**
  - cholesterol screening



Gerald J. Kost: Principles and Practice of Point-of-Care Testing, 2002

POCT: benefits

- More rapid decision making and triage
- Reduce operating times
- Reduce postoperative care time
- Reduce emergency room time
- Reduce number of outpatient clinic visits
- Reduce number of hospital beds required
- Ensure optimal use of professional time

- A reduction in morbidity and mortality has been associated with goal-directed therapy techniques when used in conjunction with POCT

- **POCT + electronic medical record =** results can be shared instantaneously with all members of the medical team through the software interface enhancing communication by decreasing turnaround time

Gerald J. Kost: Principles and Practice of Point-of-Care Testing

Disease burden

- Disability-adjusted life year: accounts for the years of life lost due to mortality and disability as a consequence of the incidence of disease
  - **Burden of disease**
    - Developed world: ischemic heart disease, cerebrovascular disease and diabetes
    - Developing world: infectious diseases (Malaria, HIV, TB, ARIs, STI)

- In the absence of dignostic tests, diseases are treated based on clinical symptoms and local prevalence of disease:

✓ Capture most patients requiring treatment
(e.g. Malaria = Sensitivity 88% at a specificity of 66%)

! Treats patients who do not require treatment
  - ! Patients are not treated for their disease
  - ! High mortality rates in patients mistreated (e.g. Malaria)
  - ! May accelerate drug resistance (e.g. Malaria and Chloroquine)

(Yager P et al, Ann. Rev.Biom. Eng. 2008)

Tubercolosis

- 2 billion people currently affected with TB
- 9 million people developing TB in 2011
- 1.6 million dying of TB each year *(Font: CDC)*

Major cause of mortality in HIV-infected people, hence in sub-Saharan Africa, where HIV is endemic

Sputum smear microscopy is the main method for TB diagnosis, particularly in low-resources settings. But:

! Lacks the desired sensitivity

! Specimen collection and processing both affect sensitivity *(Steingart KR et al, Lancet Infect. Dis. 2006)*

! Intrinsic limitations of performing microscopy

TB management calls for the use and development of new diagnostic technologies:

✓ An alternative or improvement to sputum smear microscopy to detect active TB

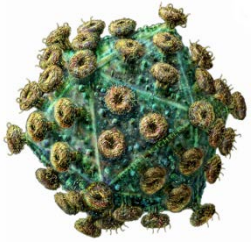
✓ An accelerated approach to current culture approaches (wider use and faster return of results)

✓ Tests that are not compromised by the HIV status

✓ A cost-effective drug-resistance test

✓ A screening tool for latent infection in asymptomatic patients *(Yager P et al, Ann. Rev.Biom. Eng. 2008)*

HIV



- 34.2 million people living with HIV around the world *(Font: CDC)*
- 2.5 million new cases of HIV in 2011 (3.1 in 2001) *(Font: UNAIDS, Geneve)*
- 2.9 million dying with AIDS in 2006, of which 2.1 in sub-Saharan Africa *(Font: UNAIDS, Geneve)*
- 1.8 million dying with AIDS in 2010 *(Font: CDC)*

Diagnostic tests	Biomarker	Clinical applications	Available platforms	Specimen requirements	Cost per test (USD)
Rapid HIV tests	IgG	Adult screening, antenatal screening as part of PMCT	RDT, agglutination	Whole blood, sera, or plasma	0.60–4.00
HIV EIA	IgG	High-throughput screening	EIA plates	Whole blood, sera, or plasma	0.27–3.00
HIV EIA	IgG/p24 combination	High-throughput screening	EIA plates	Whole blood, sera, or plasma	0.35–1.50
Confirmatory tests	IgG	Screening confirmation	IFA, LIA, Western blots	Whole blood, sera, or plasma	10.97–15.50
Molecular NAAT HIV test	DNA	Infant diagnosis, early acute phase HIV diagnosis	Isothermal amplification, PCR	Whole blood	10.00–30.00
Antigen detection	p24	Infant diagnosis, early acute phase HIV diagnosis	EIA	Plasma, dry plasma spots	10.00
Viral load	RNA	Infant diagnosis, early acute phase HIV diagnosis, HIV therapy monitoring	Isothermal amplification, PCR, branched DNA	Plasma (requires cold chain preservation), dry plasma spots (with higher limits of detection)	17.00–87.00
Viral load	Reverse-transcriptase activity	Infant diagnosis, early acute phase HIV diagnosis, HIV therapy monitoring	Colorimetric assay	Plasma	13.00–15.00
CD4 counts	CD4 cells	CD4 levels used to stage patients for ART therapy and monitor response to therapy; for children CD4 is required	(*a*) Flow cytometry, (*b*) Dedicated cytometry, (*c*) Manual affinity enrichment of CD4 with light microscopy for CD4 count, EIA	Whole blood and requires cold chain preservation if test cannot be performed immediately	(*a*) 1.00–25.00 (*b*) 2.26–20.00 (*c*) 3.00–8.00

- All current HIV-related tests required sophisticated laboratory infrastracture
- Additional diagnostic tools for opportunistic diseases in HIV-endemic area are needed to minimize the incidence of drug-related toxicity and restraining arises of drug resistance
- **Easier-to-use POC HIV diagnostic, POC CD4 and HIV viral load tests, and POC speciment handling, storage, and transportation systems are all needed for successful antiretroviral therapy rollout to low-resource settings**

(Yager P et al, Ann. Rev.Biom. Eng. 2008)

There is also an urgent necessity for new POC diagnostics concerning:

- Malaria
- Sexually transmitted infections
- Blood transfusions
- Neglected disease
- Drug resistance

Soil transmitted helminths
Snail fever (schistosomiasis)
Elephantiasis (Lymphatic filariasis)
Trachoma
River blindness (onchocerciasis)
Kala-azar black fever
Chagas disease (American trypanosomiasis)
Leprosy
African sleeping sickness (Human African Trypanosomiasis)
Guinea-worm (dracunculiasis)
Buruli ulcer

Improved and appropriate diagnostic could lead to a large reduction of disease burden resulting from the major causes of disease in low-resource settings

Urdea M et al. Nature 2006

Table 5 Laboratory structure constraints in low-resource settings informing product attributes

Laboratory infrastructure constraints in low-resource settings	Implications on point-of-care diagnostic product attributes
A wide disparity of laboratory facilities and capacities within a country and among countries	Careful consideration for the final user of the test is required.
Poor or nonexistent external quality control and laboratory accreditation systems	The test should be reproducible and provide clear and easy to interpret internal and process controls.
Unreliable procurement system leading to stock outs of key laboratory supplies	The test should require as few external reagents and supplies as possible.
Unreliable quality of reagents and supplies procured through national channels	The test should require as few external reagents and supplies as possible.
Lack of basic essential equipment	The test should require as little instrumentation as possible or provide its own instrumentation.
Lack of laboratory consumables	No assumptions should be made regarding supplies for specimen collection, storage, and handling.
Unreliable water supply and quality	This is extremely variable in different regions and seasons, and a device should not require external water if high quality is needed.
Unreliable power supply and quality	This is often tied to water supply. Devices requiring external power should account for long periods of time without network electricity supply and high variability as well as frequency of surges from the network electricity supply.
Inconsistent refrigeration capacity	This is associated with unreliable power supply. A test should be able to withstand large fluctuations in temperatures (from 40°C to 10°C) during transportation as well as sustained storage at 30°C.
Insufficiently skilled staff	The test should be easy to use and interpret.
Limited training opportunities	Any training requirements should be given special consideration for the introduction strategy.
Limited access to distributors' service maintenance staff	Any device should be robust with over 1 year half-life.
Poor waste-management facilities	The environmental impact of disposable, chemical reagents, and biohazardous materials should be considered.

Nucleic acid testing (NAT)

- Nucleic acid testing for infectious diseases at the point of care is beginning to enter clinical practice in developed and developing countries

- POC NAT in the developing world focuses on diagnosis and management of endemic infectious diseases (TB, HIV…)

  - Limited financial resources > cannot implement well-established, yet complex commercially available NAT systems

    - POC NAT can provide access to much-needed diagnostic methods in low-resource, high disease-burden areas, but new devices should be:

      - Affordable
      - Robust
      - Easy-to-use by minimally trained personnel
      - Ready-to-use reagents
      - Maintenance-free instrumentation
      - Sensitive and specific

(Boyle DS et al, Cell 2011)

NAT: sample preparation

- Lengthy processes that are often manually performed. In clinical laboratories, it is typically automated on large instruments

- Ideally, sample preparation should be integrated and coupled with amplification and detection in an inexpensive, automated, miniaturize, closed system format > development of microfluidic devices > not yet commercially available

1. Lysis (through chemical or enzimatic means)

2. Solid phase extraction (SPE) of nucleic acids:
   - SPE must be rigorous to avoid inhbition of polymerases used in subsequent amplification reactions

(Boyle DS et al, Cell 2011)

NAT: amplification

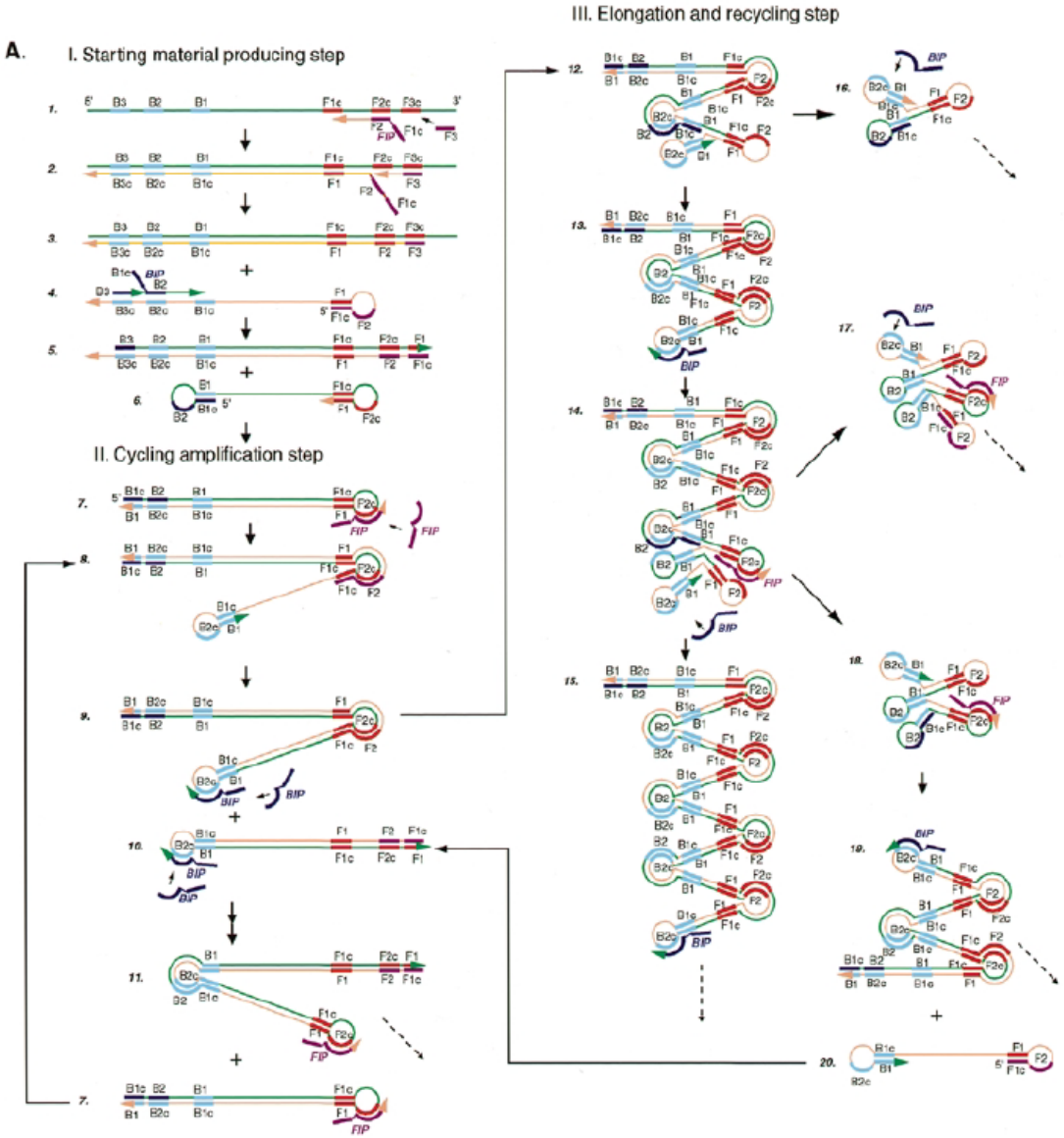
- PCR

- **Isothermal amplification**: a single reaction temperature
  - Less complex and less expensive instrumentation (a water bath)

Assay	Reaction temperature (°C)[a]	Reaction duration (min)[a]	Multiplex[b]	Rapid detection formats[c]	Target	Amplification product
Methods based on RNA transcription						
NASBA	41[d]	105	Y	RTF, NALF	RNA (DNA)	RNA, DNA
TMA	60[d]	140	Y	RTF	RNA (DNA)	RNA, DNA
SMART	41[d]	180	N/A	RTF	RNA, DNA	RNA
Methods based on DNA replication with enzymatic duplex melting/primer annealing						
HDA	65	75-90	Y	RTF, NALF	DNA[e]	DNA
RPA	30-42	20	Y	RTF, NALF	DNA[e]	DNA
Methods based on DNA polymerase-mediated strand displacement from linear or circular targets						
LAMP	60-65[d]	60-90	N/A	RTF, NALF, RTT, TE	DNA[e]	DNA
~~SPA~~	~~35~~	~~60~~	~~N/A~~	~~RTF, NALF~~	~~DNA~~	~~DNA~~
SMART-AMP	60	45	N/A	RTF	DNA[e]	DNA
RCA	65	60	N/A	RTF	DNA[e]	DNA
RAM	63[d]	120-180	N/A	RTF	DNA[e]	DNA
Methods based on polymerase extension/strand displacement, plus a single strand cutting event						
SDA	37	120	Y	RTF, NALF	DNA[e]	DNA
NEAR	55	10	Y	RTF, NALF	DNA[e]	DNA
NEMA	65	30	N/A	NALF	DNA	DNA
ICA	60	60	N/A	RTF	DNA	DNA
EXPAR	55	10-20	Y	RTF, NALF	DNA	DNA
BAD AMP	40	40	N/A	RTF	DNA	DNA
PG-RCA	60	60-120	N/A	RTF	DNA	DNA

(Boyle DS et al, Cell 2011)

Loop-mediated isothermal amplification of DNA (LAMP)



http://loopamp.eiken.co.jp/e/lamp/anim.html

Conditions

- 4 primers, 1 h reaction at 65°C
- From 6 copies of DNA to 10^9 in < 1 h
- The final products are a mixture of stem-loop DNAs with various stem length and couluiflower-like structures with multiple loops
- 4 primers ensure high specificity

Advantages

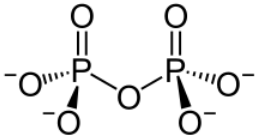
- High efficiency under isothermal condition
- Detection limit comparable to PCR
- Highly specific for the target sequence
- Simple and easy to perform once the 4 primers are available
- Can amplify RNA sequences (in combination with reverse transcription)

(Notomi T et al, Nucleic Acids Research 2000)

NAT: detection

Assay	Reaction temperature (°C)[a]	Reaction duration (min)[a]	Multiplex[b]	Rapid detection formats[c]	Target	Amplification product
Methods based on RNA transcription						
NASBA	41[d]	105	Y	RTF, NALF	RNA (DNA)	RNA, DNA
TMA	60[d]	140	Y	RTF	RNA (DNA)	RNA, DNA
SMART	41[d]	180	N/A	RTF	RNA, DNA	RNA
Methods based on DNA replication with enzymatic duplex melting/primer annealing						
HDA	65	75-90	Y	RTF, NALF	DNA[e]	DNA
RPA	30-42	20	Y	RTF, NALF	DNA[e]	DNA
Methods based on DNA-polymerase-mediated strand displacement from linear or circular targets						
LAMP	60-65[d]	60-90	N/A	RTF, NALF, RTT, TE	DNA[e]	DNA
CPA	65	60	N/A	RTF, NALF	DNA	DNA
SMART-AMP	60	45	N/A	RTF	DNA[e]	DNA
RCA	65	60	N/A	RTF	DNA[e]	DNA
RAM	63[d]	120-180	N/A	RTF	DNA[e]	DNA
Methods based on polymerase extension/strand displacement, plus a single strand cutting event						
SDA	37	120	Y	RTF, NALF	DNA[e]	DNA
NEAR	55	10	Y	RTF, NALF	DNA[e]	DNA
NEMA	65	30	N/A	NALF	DNA	DNA
ICA	60	60	N/A	RTF	DNA	DNA
EXPAR	55	10-20	Y	RTF, NALF	DNA	DNA
BAD AMP	40	40	N/A	RTF	DNA	DNA
PG-RCA	60	60-120	N/A	RTF	DNA	DNA

Pyrophosphate ions, a byproduct of DNA synthesis, precipitate in the presence of metal cations, which leads to an increase in turbidity



− +

template
(*Salmonella* spp.)

- RTT: real-time turbidity

5000 $

- TE: turbidity end-point

1100 $

Applications

- **Tubercolosis**
  - Rapid diagnosis and drug susceptibility testing can limit the spread of TB and facilitate effective treatment

GeneXpert can concentrate the pathogen, extract the genomic DNA and perform PCR assay targeting specific genes for TB diagnosis and detection of rifampicin resistance.

- **HIV viral-load monitoring**
  - Routinely performed in developed country to identify virological treatment failure.
  - WHO recognizes that increased access to viral-load monitoring in low-resource settings can help prevent emergence of drug-resistance HIV strains and patient health decline

Several African countries are utilizing viral-load monitoring from dried blood spots sent to centralized laboratories > logistic challenges and cannot reach all patients

- **Infant HIV diagnosis**
- **Group B streptococcus (GBS)**
- **Nosocomial infections; e.g. Methicillin-resistant *S. aureus* (MRSA)**

(Boyle DS et al, Cell 2011)

POC NAT: conclusions

- The progress in developing POC-compatible NAT technologies has increased dramatically in the past 5 years, driven by applications that require rapid turnaround in developed countries and by expanding markets related to endemic diseases in the developing world
  - POC NAT is possible: the GeneXpert, originally developed to detect biothreat agents, has successfully crossed over to clinical diagnostic applications. After receiving FDA clearance for the GBS assay, it is now used to detect nosocomial infections, including MRSA.
    Through the MTB/RIF test, it can meet crucial needs that are related to infectious diseases diagnosis in the developing world.

- Isothermal amplification methods require less-stringent sample preparation, and can facilitate rapid, sensitive, and specific target amplification via single-temperature incubation, which reduces system complexity and cost compared to PCR-based methods

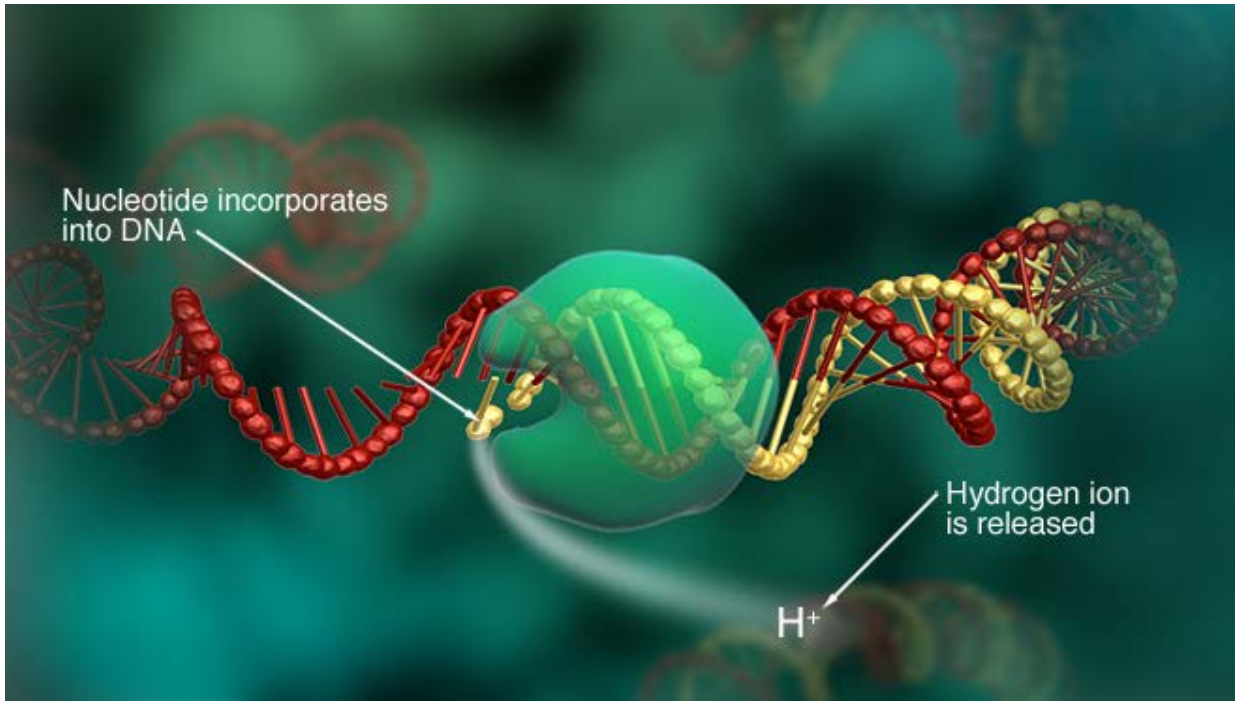
- Sustained implementation of POC NAT requires that new technologies **truly address the needs of the targeted settings**, which is especially true in developing countries
  - Appropriate integration of multiple crucial components in a robust, user-friendly format, while minimizing complexity and cost.

(Boyle DS et al, Cell 2011)

Simultaneous DNA amplification and detection using a pH-sensing semiconductor system

Christofer Toumazou[1,2], Leila M Shepherd[1], Samuel C Reed[1], Ginny I Chen[1], Alpesh Patel[1,3], David M Garner[1,3], Chan-Ju A Wang[1,3], Chung-Pei Ou[1], Krishna Amin-Desai[1], Panteleimon Athanasiou[1], Hua Bai[1], Ines M Q Brizido[1], Benjamin Caldwell[1], Daniel Coomber-Alford[1], Pantelis Georgiou[2], Karen S Jordan[1], John C Joyce[1], Maurizio La Mura[1], Daniel Morley[1], Sreekala Sathyavruthan[1], Sara Temelso[1], Risha E Thomas[1] & Linglan Zhang[1]

[1]DNA Electronics Ltd., London, UK. [2]Department of Electrical and Electronic Engineering, Imperial College London, South Kensington Campus, London, UK. [3]These authors contributed equally to this work. Correspondence should be addressed to C.T. (chris.toumazou@dnae.co.uk) or L.M.S. (leila.shepherd@gmail.com).



Overview

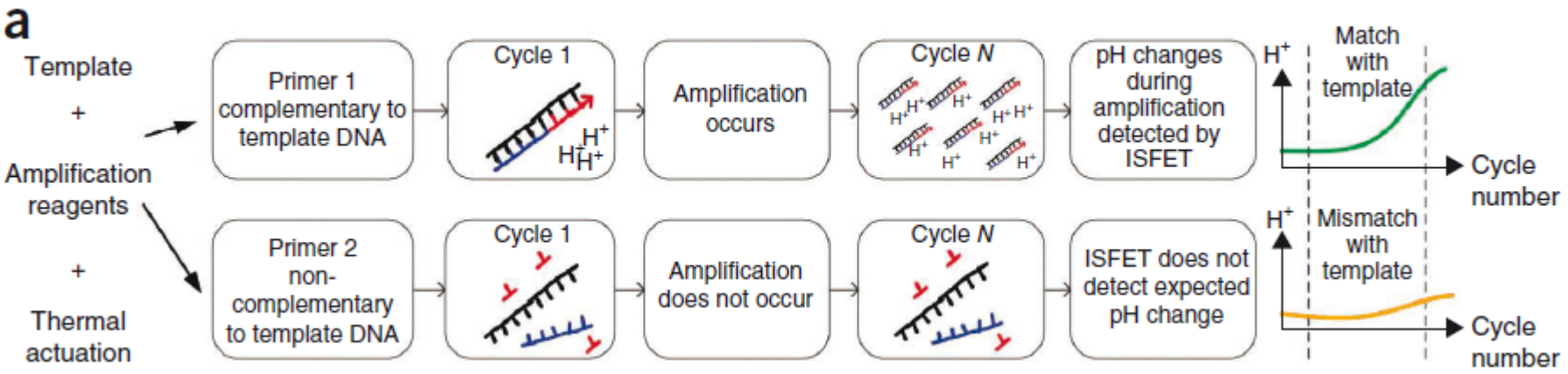
Current technologies for RT amplification and detection of nucleic acids require:

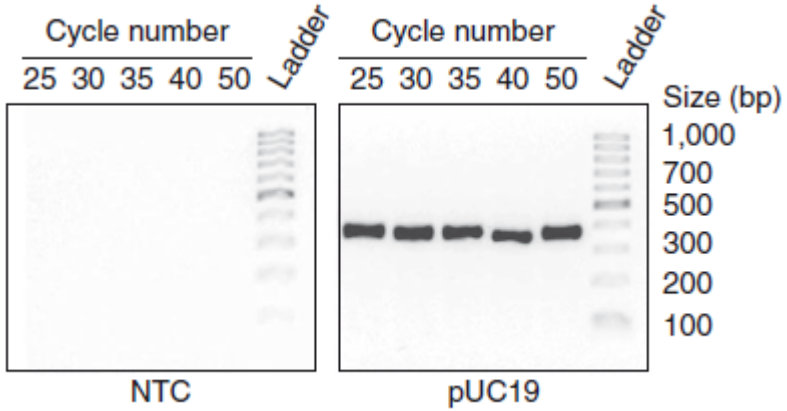
- A thermal cycler
- Precision optics for detection
- Fluorescently labeled sequence-specific probes or fluorescent dyes for labeling DNA

Using complementary metal-oxide semiconductor (CMOS) technology integrated with ion-sensor field effect transistors (ISFET), they developed a device that has thermal actuation integrated with pH-sensing technologies

Low-cost and disposable thermocycling platform, which is able to amplify and detect nucleic acid under dynamic pH changes

Proof of principle: a change in pH as a readout for DNA amplification?

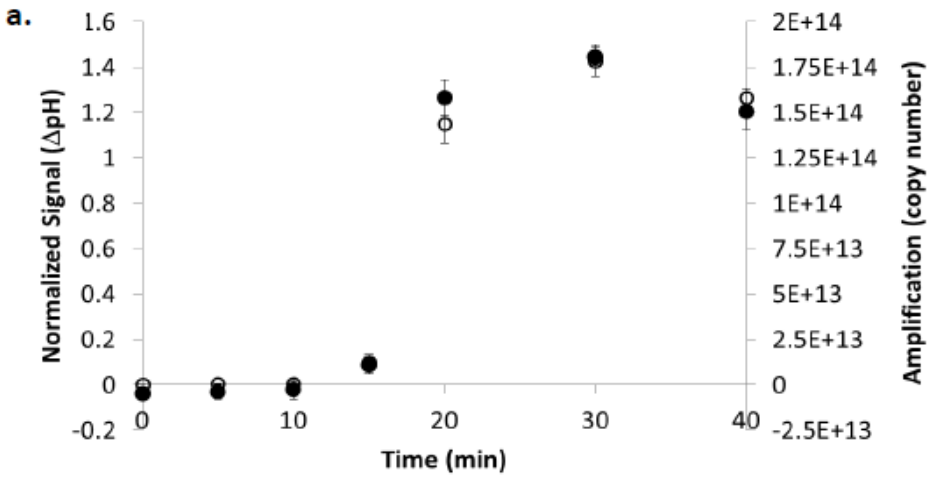


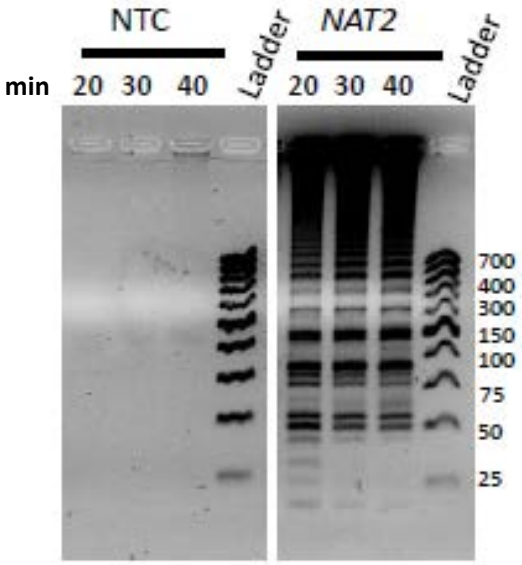


The change in pH was not due to nonspecific amplification

Proof of principle: a change in pH as a readout for DNA amplification? **YES**

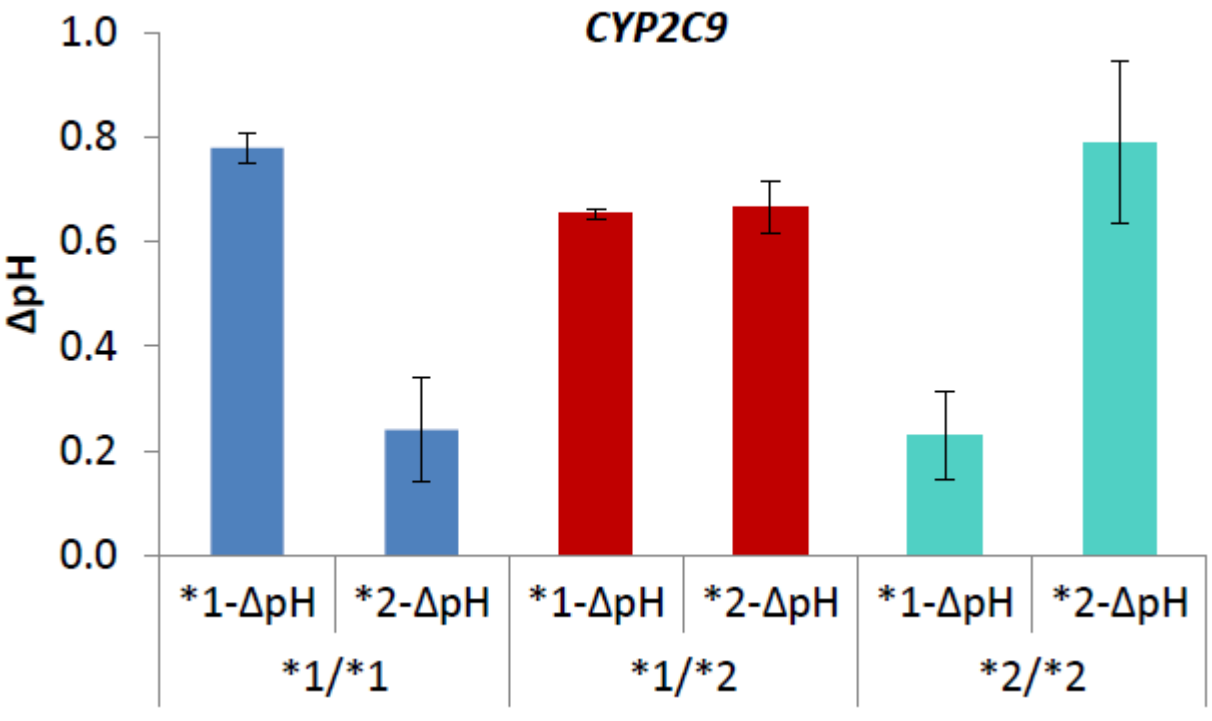
Tube-based pH-LAMP amplification of pUC19





pH-PCR can discriminate different SNP genotypes? **YES**

CYP2C9 gene encodes one of the cytochrome P450, a major drug metabolizing enzyme

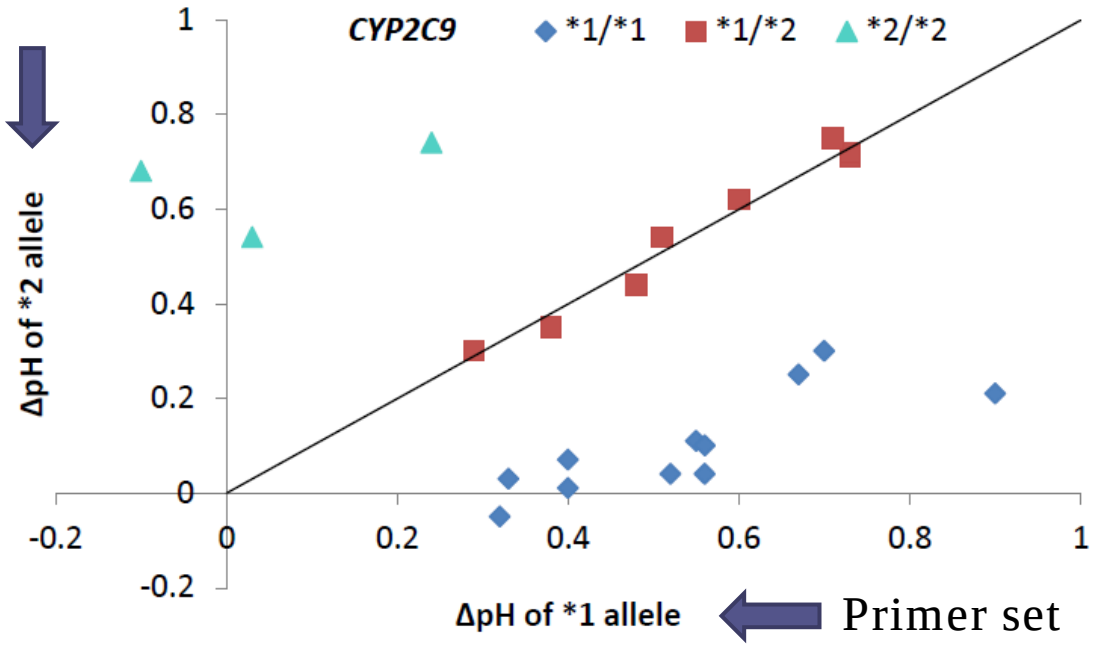


CYP2C9*1 (C430)
CYP2C9*2 (C430T)

Sample source:
Purified genomic DNA

pH-PCR can discriminate different SNP genotypes? **YES**

CYP2C9 gene encodes one of the cytochrome P450, a major drug metabolizing enzyme

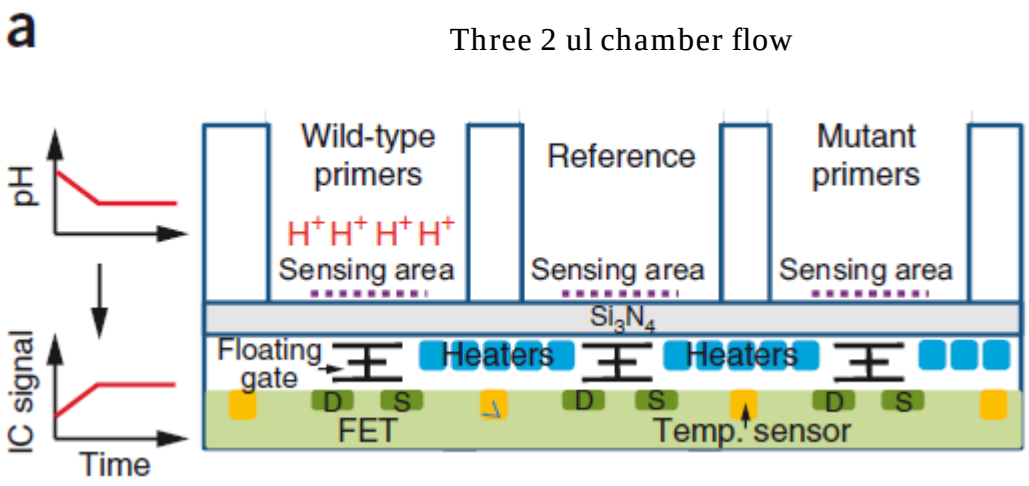


CYP2C9*1 (C430)
CYP2C9*2 (C430T)

Sample source:
Crude saliva

TaqMan genotyping assay confirmed the genotypes of all saliva samples

A system-on-chip for label-free analysis of nucleic acids

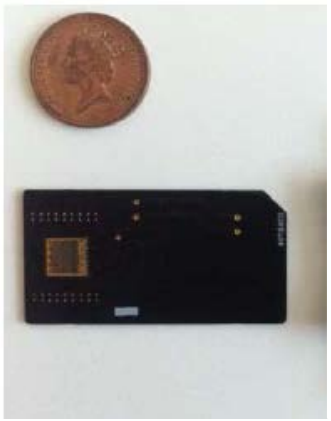


IC: integrated circuit

- ISFET: Floating gate + Source + Drain
- Temperature sensors
- Heater elements
- Silicon nitride sensing surface

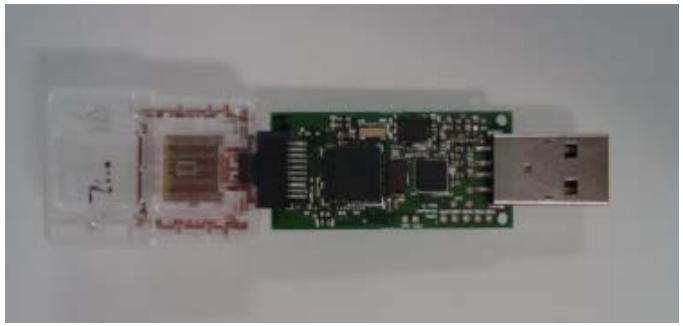
Process

1. H^+ accumulate at the Si_3N_4 surface
2. The charge distribution of the ISFET (between S and D) increases
3. The IC output signal voltage increases



4.8 mm x 5.5 mm
120000 transistors
Mounted on a test card

Analyzer board (20g)
Provides power
Microcontroller interface

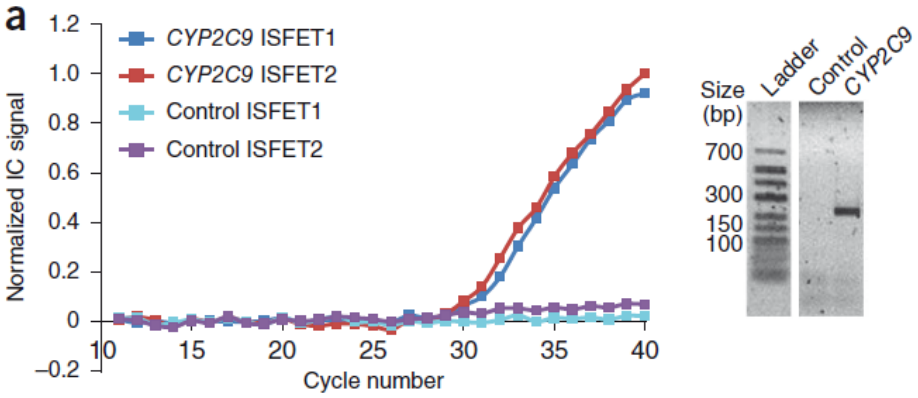


Perform both thermal cycling or isothermal amplification

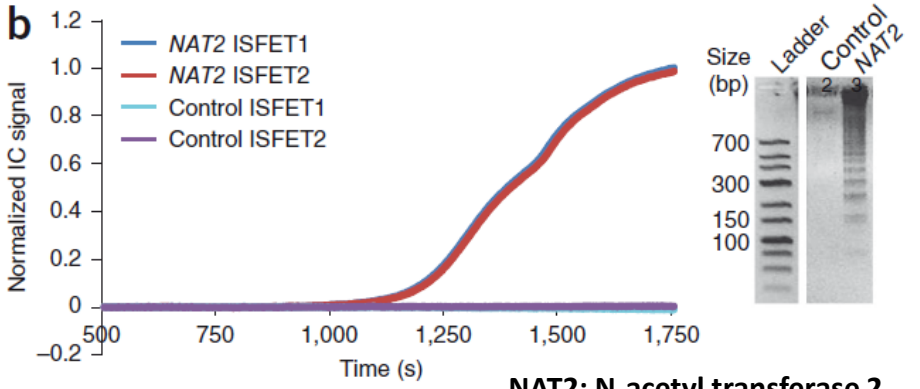
On-chip real-time pH-sensitive amplification

Control: no-template reaction
Control for any nonspecific amplification

On-chip pH-PCR



On-chip pH-LAMP



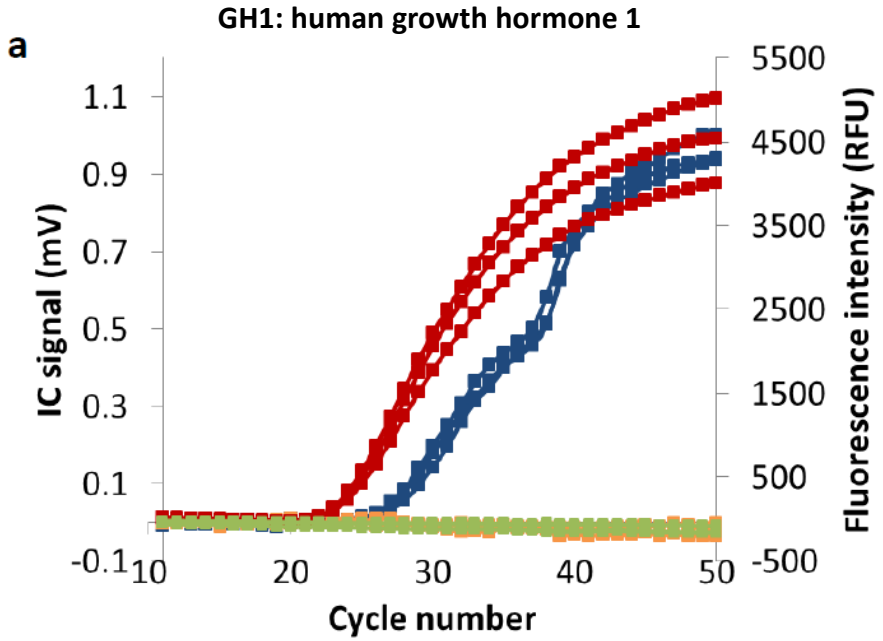
NAT2: N-acetyl transferase 2

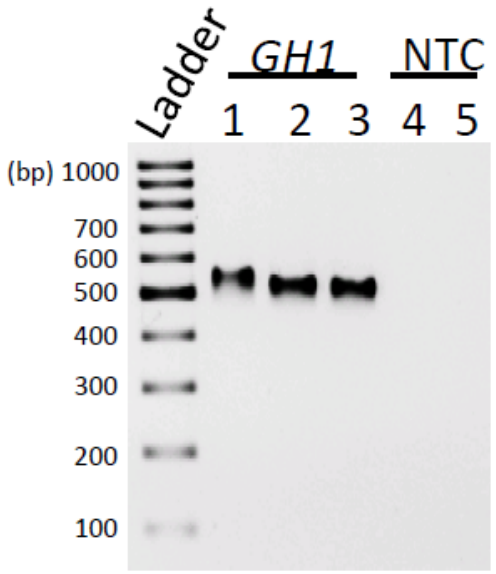
Standard two-temperature PCR protocol

- 95°C and 66°C, ramp rate 3°C s^{-1}

Stable temperature at 66°C

Comparision between fluorescence-based PCR and pH-sensing semiconductor-based PCR

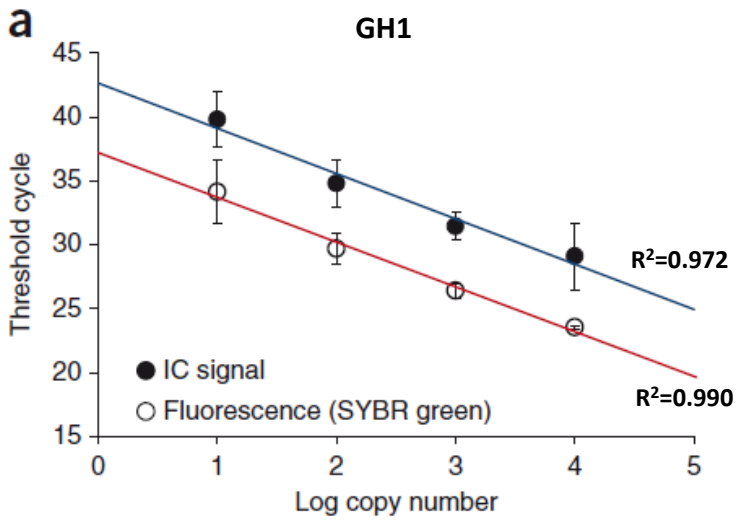




- ✓ The amplification curves of the integrated circuit signal measured by changes in voltage were highly comparable to the fluorescence signals detected by the conventional real-time PCR apparatus

- Red lines: fluorescent qPCR
- Blue lines: on-chip pH-PCR
- Green lines: NTC qPCR
- Orange lines: NTC pH-PCR

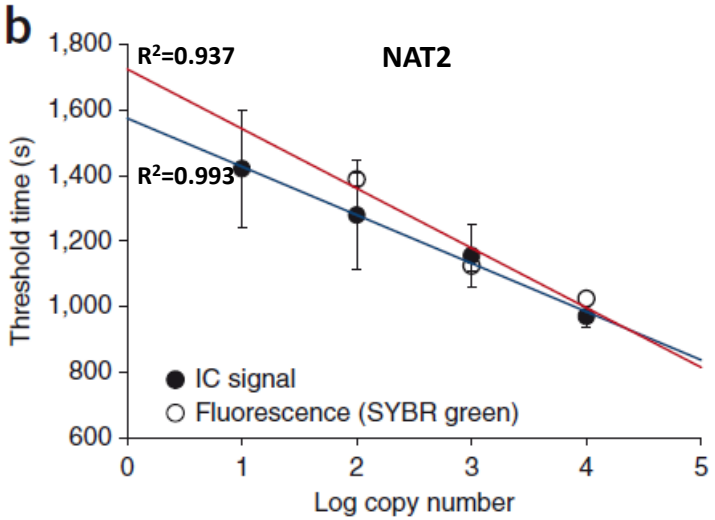
Evaluation of integrated circuit sensitivity



- pH-PCR vs Syber green qPCR

10 fold serial dilution of haploid genome per chamber from purified human genomic DNA

- Efficiency
  - 91.57% pH-PCR
  - 93.07% qPCR
- They both detected as few as 10 copies of genomic DNA



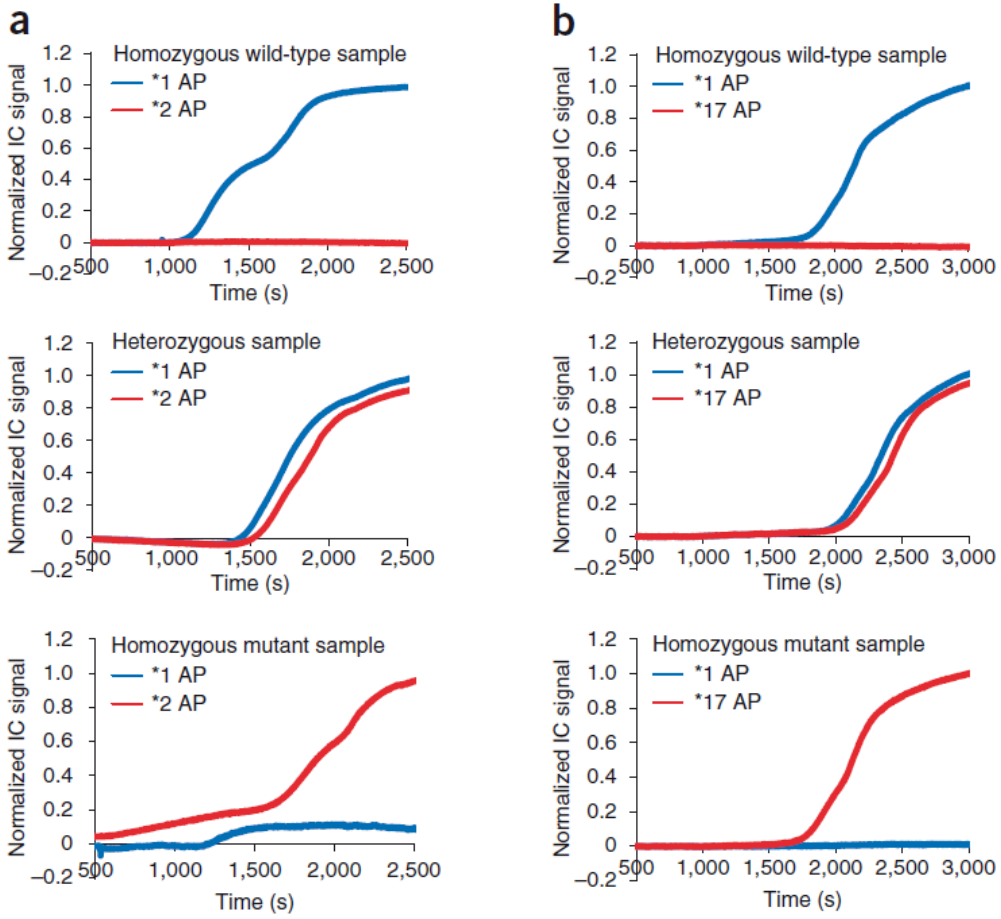
- pH-LAMP vs Syber green qLAMP

10 fold serial dilution of haploid genome per chamber from purified human genomic DNA

- They both detected as few as 10 copies of genomic DNA, although qLAMP did not consistently amplify NAT2 at 10 copies

Genotyping: potential clinical utility

CYP2C19*1
CYP2C19***2*** (81G>A): poor metabolism of clopidogrel (Plavix)
CYP2C19***17*** (806C>T): ultrametabolsim of clopidrogel (Plavix)

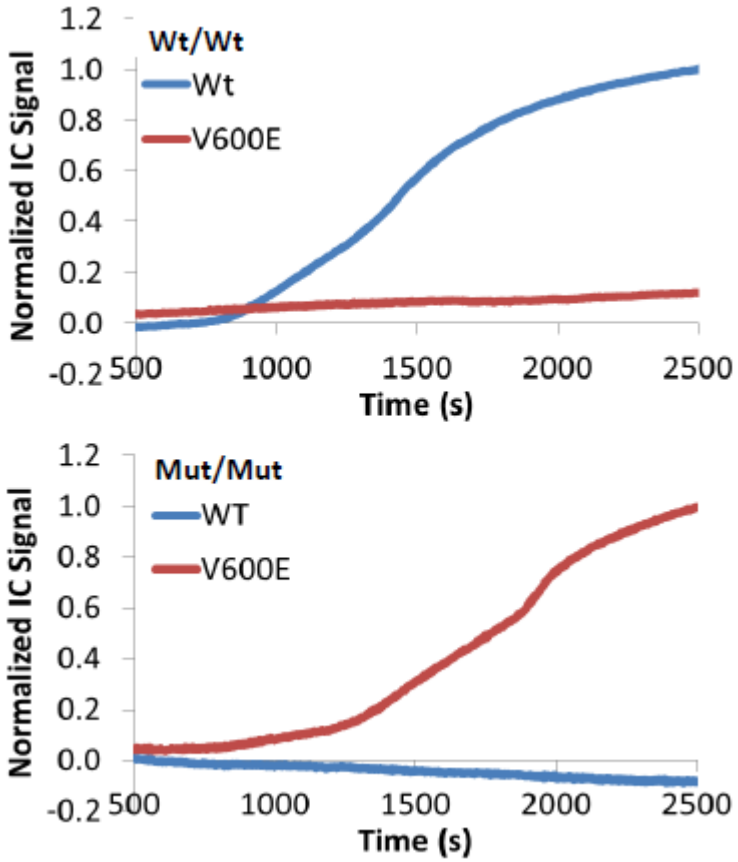


Parameters

- pH-LAMP
- Samples: crude saliva
  - 30 samples CYP2C19*2 (29 corrected identified)
  - 32 samples CYP2C19*17 (30 corrected identified)

Genotyping: potential clinical utility

BRAF (encodes serine/threonine-protein kinase B-Raf)
BRAF (V600E, found in many human cancers)



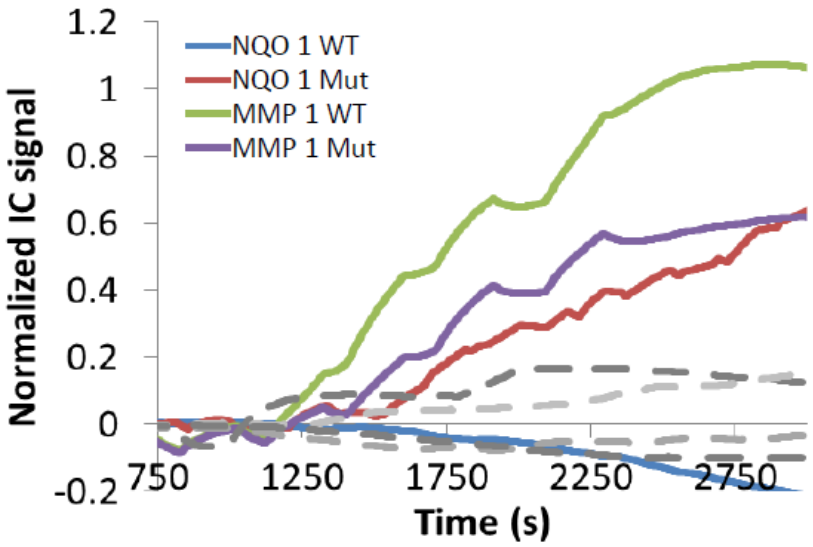
Parameters

- pH-LAMP
- Samples: purified genomic DNA
  - No statistics

Scalability of the system: multiplex analysis

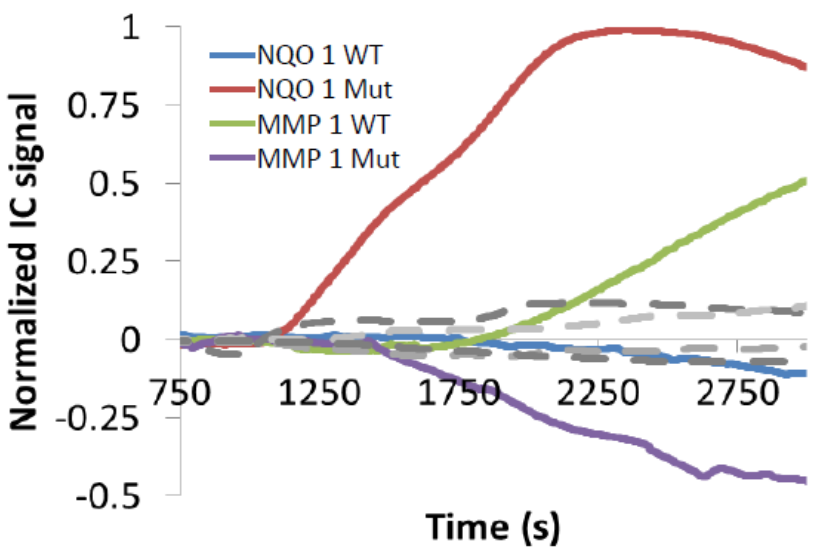
- 4 single chips were serially connected, 12-chamber flow (no chip redisign)
- They interrogated two known biomarkers of aging: NAD(P)H dehdrogenase (NQO1) and Matrix Metalloproteinase-1 (MMP1)

Sample 1



NQO1
MMP

Sample 2



NQO1
MMP

Discussion

- The amplification curves are comparable between fluorescence-based qPCR and on-chip pH-PCR
- The low intra- and interchip coefficients of variation observed demonstrate a high reproducibility of the chip
  - technical variability can be minimized by integrating seamless sample preparation and delivery to the chip as well as automated rather than manual assembly of the platform
- Reaction volume (currently 2 ul) can be further decreased, leading to greater sensitivity, less contamination, lower cost and greater throughput, hence promoting the development of portable and scalable platforms for amplification and detection of nucleic acid
- The scalability of the CMOS platform, and the ability to quantitate, means that one can create chips with large sensor arrays to provide high-density, multiplexed molecular analysis platforms based on real-time detection of pH.

We envision that with the appropriate fluidic interfaces, this CMOS architecture can be used to transfer any current analytical techniques, including sequencing, microarrays and 'digital PCR' into fast, integrated analysis platforms

- **The technology opens the door for development of a portable, affordable POC platform for NAT, which can move personalized medicine to the clinic and overcome issues such as turnaround time associated with laboratory-based tests**

Thanks for your attention!!