# Perspective

# Towards a Future of Rapid, Low-Cost, Multiplexed Detection of Antimicrobial Resistance Markers for Tuberculosis and Other Pathogens

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By 2050, antimicrobial resistance (AMR)[3] will put an estimated 10 million lives at risk each year *(1)*. The discovery of new antibiotics has slowed in recent years, and the prospect of a return to the preantibiotic era, in which simple medical procedures become life-threatening, has triggered various efforts to improve antimicrobial stewardship to halt the development of further AMR.

A key element of antimicrobial stewardship is the use of diagnostic procedures that inform antibiotic prescribing. This is typically achieved using phenotypic drug susceptibility testing by microbiological culture, the gold standard method for evaluating the drug susceptibility profile of a given clinical isolate. However, this is time-consuming, usually taking days. Molecular tests, in which nucleic acid sequences of a specific pathogen, strain, or drug resistance-conferring mutation are detected, are significantly faster, typically yielding results in hours.

Nucleic acid detection tests are already used to guide antibiotic usage in laboratory settings for well-characterized resistance-associated mutations. For example, the Cepheid Xpert MTB/RIF nucleic acid amplification test detects the presence of genes specific to *Mycobacterium tuberculosis*, in addition to rifampicin resistance-conferring mutations in the *rpoB* gene *(2)*. Such diagnostic tests can provide a rapid guide for clinicians when prescribing antibiotics. However, the multiplexing capabilities of multicolor fluorophore-based diagnostics are limited by the need for fluorophores with sufficient spectral resolution that can be used in a homogenous reaction. Base calling resolution also limits the use of molecular diagnostics for the detection of single-nucleotide polymorphisms (SNPs), which is required for the detection of AMR in some pathogens, such as *M. tuberculosis*.

Next-generation sequencing technologies are not limited by multiplexing capabilities and base calling resolution and have been used to diagnose infections and identify AMR. The time requirements and cost of next-generation sequencing have fallen markedly in recent years, increasing their clinical utility. In 2017, Votintseva et al. applied PCR followed by sequencing using the MinION (Oxford Nanopore Technologies) to patient specimens, which yielded full drug susceptibility results for *M. tuberculosis* BCG strain in <12.5 h *(3)*. However, this technique is currently low-throughput, requires substantial hands-on time, and is not yet affordable to most users.

A recent publication in *Nature Biotechnology* by Hassibi et al. describes the development of a semiconductor biochip that can identify, quantify, and genotype pathogens *(4)*. The device allows for multiplexed real-time quantification of PCR amplification and SNP base-calling resolution using melting-curve analysis in <2 h, although this does not include sample preparation or nucleic acid extraction time. A workflow is shown in Fig. 1A.

A miniaturized, single-use complementary metal oxide semiconductor (CMOS) chip was designed with 1024 pixels, each of which can be functionalized with a different probe sequence. Fluorescence readout was performed on-chip, with a photodetector and readout circuitry for each pixel, and a thin-film emission filter was incorporated between the DNA probes and the photodetector. Excitation came from an external light source. Passive heating elements were patterned along the chip and cooling was achieved with a heat sink capable of heating and cooling the sample solution at a rate of 4 °C/s with an accuracy of ±0.3 °C, controlled with temperature sensors. Although on-chip PCR has been previously demonstrated, as well as real-time quantitative PCR in microfluidic devices, here the combination of thermocycling and fluorescence readout allowed on-chip, real-time, quantitative PCR.

PCR was performed and monitored in the 40 μL flow-through chamber using quencher-modified primers and on-chip temperature cycling. Sensor pixels were functionalized with fluorescently labeled DNA probes. The hybridization of the resulting quencher-labeled amplicons to the fluorescent probes reduced the measured

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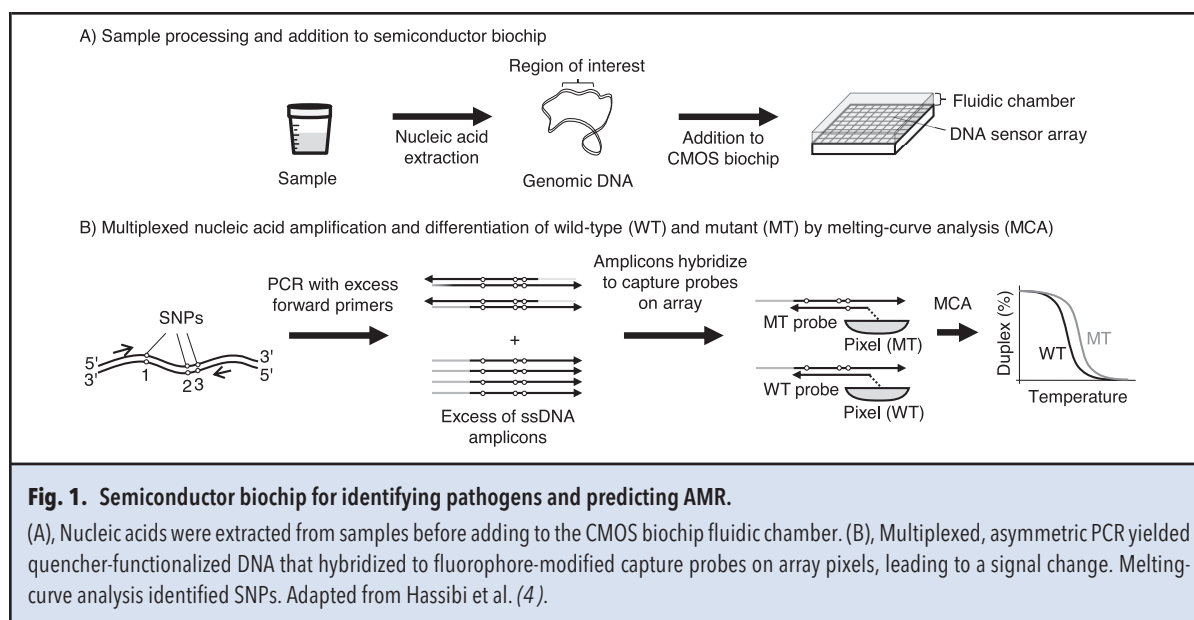† B.S. Miller and H.D. Gliddon contributed equally to this work.
[3] Nonstandard abbreviations: AMR, antimicrobial resistance; SNP, single-nucleotide polymorphism; CMOS, complementary metal oxide semiconductor.

**Fig. 1. Semiconductor biochip for identifying pathogens and predicting AMR.**

(A), Nucleic acids were extracted from samples before adding to the CMOS biochip fluidic chamber. (B), Multiplexed, asymmetric PCR yielded quencher-functionalized DNA that hybridized to fluorophore-modified capture probes on array pixels, leading to a signal change. Melting-curve analysis identified SNPs. Adapted from Hassibi et al. *(4)*.

fluorescence, allowing real-time quantification of amplicon production, as shown in Fig. 1B.

Here, the inverse fluorescence technique used quenchers that do not change the signal unless probe and amplicon are hybridized, because of the required proximity of the quencher to the fluorophore, allowing for real-time measurements. In addition, the sensitivity is improved by both the proximity of the fluorophores to the photodetector and the use of a dual fluorophore, enlarging the Stokes shift.

Asymmetric PCR generated excess single-stranded DNA amplicons, which hybridized to capture probes on the biosensor pixels. This also allowed the PCR reaction to be monitored by depletion of the forward primer, independently of amplicon binding. Similar assays are regularly performed in solution; however, in this surface-based assay, the high level of spatial multiplexing, constrained only by the number of pixels, allows for multiple targets to be measured simultaneously, as well as robust background subtraction and on-chip replicates.

Although in this context, real-time monitoring of amplification is not crucial, temporal measurements are necessary for melting-curve analysis, used here to detect SNPs. Differences in the melting curves were measured during heating from 55 to 95 °C, achieving SNP resolution by measuring fluorescence every two minutes. This meant that clinically important SNPs, such as the rifampicin resistance-conferring S531L mutation in the *rpoB* gene of *M. tuberculosis*, could be detected.

The clinical utility of the biochip was demonstrated in 2 phases. In the first phase, 6 respiratory tract pathogens (4 RNA viruses and 2 DNA viruses) were detected. Using reverse transcription PCR, followed by a hybrid-ization phase and then melting-curve analysis, the system was able to accurately quantify the microbial load of a sample. For influenza A, the dynamic range between 100 and 100 000 copies/μL was linear. In addition, 11 blinded clinical samples were tested using the biochip and a commercially available system, with concordance between the two. The second phase of clinical testing involved the detection of 54 drug resistance-conferring mutations in 6 genes in the *M. tuberculosis* genome, confirmed by next-generation sequencing.

Melting-curve analysis allowed for key AMR-conferring SNPs to be detected. A different assay was used for *M. tuberculosis* melting-curve analysis, in which quencher-labeled nucleotides were added to the PCR master-mix. The authors noted that although this reduced PCR efficiency, the increased number of quenchers per amplicon increased the measured signal, improving resolution. However, the amplification step was performed off-chip, requiring purification before adding to the flow chamber for melting-curve analysis. This increased assay time and complexity.

The authors state that one of the limitations of melting-curve analysis is a difficulty in identifying mutations present at <20% in a background of wild-type sequences. This is particularly important in the context of tuberculosis, for which detection of drug-resistant minority variants is required for the correct treatment of patients. In addition, AMR of many pathogens is not always conferred by small changes in the genome such as SNPs, but by larger scale events such as horizontal gene transfer of plasmids. In many of these cases, single-base resolution is less important than the detection of these specific gene sequences. Furthermore, a pathogen's geno-

type is not always concordant with its phenotype, although for *M. tuberculosis*, recent evidence confirms they are correlated *(5)*.

An important limitation of this early prototype, in its current form, is the requirement for nucleic acid extraction to be performed off-chip, which restricts its potential for near-patient testing. Second, the time between sample in and result out remains lengthy. The additional 2 h amplification time on top of nucleic acid extraction time makes this approach only marginally faster than some next-generation sequencing workflows (i.e., by approximately 2 to 4 h). In the future, this additional time requirement could be shortened by a faster off-chip amplification, for example, isothermal amplification, combined with nucleic acid extraction. Incorporation into a microfluidic system could allow extraction, amplification, and melting-curve analysis on-chip with no user input. Moreover, using brighter fluorophores could shorten the amplification step, since greater analytical sensitivity could be achieved because fewer binding events would generate the same fluorescence reduction. Third, this device is suited to laboratory settings but may not be immediately transferrable to near-patient testing because it requires a power source, readout device, and external light source. In addition, nucleic acid amplification is susceptible to issues such as contamination and sample quality that would require trained personnel to oversee. The equipment required could be minimized via a smartphone-connected device containing the light source or a battery-powered standalone device. This would provide power, data analysis, and geolocation information, allowing for geospatial tracking of disease mutations during outbreaks.

Hassibi et al. acknowledge that, unlike sequencing approaches, this assay is hypothesis-driven, meaning the sequence(s) of interest must be known in advance. Next-generation sequencing technologies can detect novel mutations while simultaneously aiding surveillance and epidemiological studies. The rapidly falling costs and time requirements of sequencing technologies make them a major competitor to molecular diagnostics described here. Nevertheless, the advantages of this system include the low-cost of CMOS bulk manufacturing, small size, and low PCR reagent costs, although no estimated cost is given in the paper. Furthermore, this hypothesis-driven approach does not require bioinformatics expertise to interpret the results.

This promising prototype device presented by Hassibi et al. is currently suitable for incorporation into a laboratory workflow, although it would still require development to be used for near-patient testing. If successful, it has the potential to substantially decrease complexity, sample volume and user time, as well as improve assay time by virtue of its highly multiplexed format and suitability for different pathogens.

## References

1. O'Neill J. Tackling drug-resistant infections globally. Review of Antimicrobial Resistance 2016.
2. Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, et al. The new Xpert MTB/RIF ultra: improving detection of Mycobacterium tuberculosis and resistance to Rifampin in an assay suitable for point-of-care testing. MBio 2017;8.
3. Votintseva AA, Bradley P, Pankhurst L, Del Ojo Elias C, Loose M, Nilgiriwala K, et al. Same-day diagnostic and surveillance data for tuberculosis via whole-genome sequencing of direct respiratory samples. J Clin Microbiol 2017;55:1285–98.
4. Hassibi A, Manickam A, Singh R, Bolouki S, Sinha R, Jirage KB, et al. Multiplexed identification, quantification and genotyping of infectious agents using a semiconductor biochip. Nat Biotechnol 2018;36:738–45.
5. The CRyPTIC Consortium and the 100,000 Genomes Project. Prediction of Susceptibility to First-Line Tuberculosis Drugs by DNA Sequencing. N Engl J Med 2018;379:1403–15.