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Streamlining point-of-care assays

When designing diagnostic assays intended for point-of-care use, more attention should be given to simplicity of operation.

When it comes to assays for the detection of disease biomarkers, analytical performance typically makes the headline. An assay that misses a substantial percentage of samples positive for the biomarker or that singles out a fraction of samples with a concentration of the biomarker below the threshold will not be given much attention, at least not until those false negatives and false positives are reduced by the assay's developer. Designers of diagnostic assays for point-of-care use should also aim to make them robust (analytically and operationally) and, if feasible, inexpensive and rapid at providing the answer.

However, in the quest to make in vitro assays fast, cheap and sturdy, simplicity of operation often falls by the wayside, perhaps because of the perception that ease of use can be improved in many ways and at any stage of the assay's development. However, operational factors tend to impinge on other constraints: automation and portability may make assays easier to use, reduce the sample-to-answer time and increase throughput, but may require more expensive fabrication processes and instrumentation; reducing the number of reagents in the assay may make it cheaper, but may compromise robustness or analytical performance; using reagents that are shelf-stable may require retooling the assay's design, perhaps for a different biomarker; and minimizing the number of operation steps can increase the assay's robustness, but might involve the implementation of step-integration strategies involving the assay's biochemistry, materials, instrumentation or device components, which in turn can affect cost and analytical performance.

This issue of *Nature Biomedical Engineering* includes four Articles that describe strategies towards the simplification of the operation of diagnostic assays while navigating the constraints involved in their intended use in a point-of-care context. In one article, Aric Joneja and colleagues report an assay that leverages freeze-dried cell-free extracts to detect multiple respiratory viral pathogens in a single reaction. The assay takes advantage of the versatility of readout provided by the cell-free synthesis of reporter enzymes or peptides, and of the sensitivity and specificity of the target-specific splinted ligation of single-stranded DNA probes (the probes form a complex, or 'splint', with the complementary RNA target to be detected) to generate double-stranded protein-expression cassettes (Fig. 1). Most nucleic acid assays require instrumentation to control the temperature during target-amplification reactions (to maintain the optimal temperature for enzyme activity), as well as optics for the readout of the signal. Joneja and colleagues circumvented the need for amplification of the RNA target (and hence for target-specific amplification primers), and instead leveraged the cell-free expression of a colorimetric reporter and the optional ambient-temperature amplification of the ligated probe (via rolling-circle amplification of the expression cassette) for increased sensitivity. Therefore, the assay works at ambient temperature, and the presence of RNA targets can be detected colorimetrically or via a lateral-flow readout; hence, the detection of the target is visible to the naked eye and does not require specialized instrumentation (such as photometers for the detection of luminescence). To make it more amenable to point-of-care use, the assay would benefit from optimizations that bring its sensitivity to the level of the polymerase chain reaction (PCR) and that reduce the number of reaction steps to reach the simplicity of use of lateral-flow antigen tests.

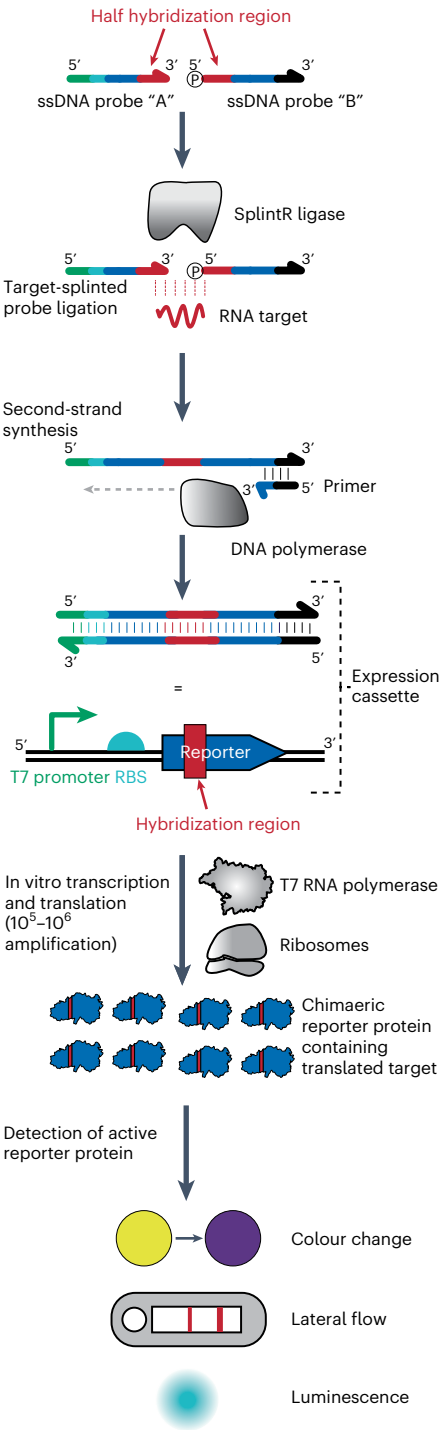

Fig. 1 | Workflow of an assay for the detection of viral RNAs at ambient temperature that does not require instrumentation. Single-stranded DNA probes (ssDNAs) for an RNA target that are ligated only in the presence of the target serve as a template for the DNA-polymerase-mediated synthesis of a cell-free expression cassette for a reporter enzyme or peptide that can be detected colorimetrically, via a lateral-flow device, or by measuring luminescence with a plate reader. The cassette encodes for a T7 promoter, a ribosome-binding site (RBS), a reporter protein and a T7 terminator. Reproduced with permission from the Article by Joneja and colleagues, Springer Nature Ltd.

Editorial

Minimizing the steps in a workflow or automating it makes the workflow simpler to operate. Bo Zhang, Hongjie Dai, Jing Yuan, Xingyu Jiang, Meijie Tang and co-authors followed both strategies. They report a portable and fully automated device housing a microarray-based assay for the discrimination of known variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) — the virus causing COVID-19 — with single-RNA-copy sensitivity and single-nucleotide specificity. The assay involves three steps: thermal lysis for releasing viral RNA; recombinase polymerase amplification of the target RNA in one pot; and the hybridization of the amplified target RNA, which is bound to a streptavidin-labelled fluorophore (via a biotinylated forward primer), with DNA probes immobilized on a plasmonic gold substrate, for enhancing the resulting near-infrared fluorescence. The authors report that, in nasopharyngeal swabs from 1,034 individuals, the assay detected the virus with 98.8% sensitivity and 100% specificity, and discriminated 12 SARS-CoV-2 lineages with 97.6% concordance with genome sequencing. The device is the size of a toolbox, automates the entire swab-to-result process (which takes 2 hours), and was deployed at a clinical site for SARS-CoV-2 surveillance.

Assays for detecting microRNAs — non-coding short RNAs involved in the post-transcriptional repression of messenger RNA — in biofluids accurately and inexpensively could provide another means for the diagnosis and monitoring of cancers from blood samples. Yong Zeng and co-authors describe a particularly simple assay for the detection of microRNAs in plasma: it involves a single step (integrating reagent mixing, amplification reactions and fluorescence detection) and is done in a single reaction tube at constant temperature. The assay leverages the *cis*-cleavage activity of Cas12a to enable exponential rolling-circle amplification of target sequences, and the *trans*-cleavage activity of Cas12 for the detection of the target sequences and for the amplification of the fluorescence signal. In plasma from patients with pancreatic ductal adenocarcinoma, the assay detected microRNAs (one at a time, as the assay does not allow for the detection of multiple targets in one pot) at femtomolar concentrations with single-nucleotide specificity, with sample-to-answer times ranging from 20 minutes to 3 hours. Notably, it does not require specialized instrumentation; the authors show that the detection of fluorescence can be done with a photometer, a smartphone or a lateral-flow device.

The least invasive and most convenient and general way to detect cancers early is to look for genomic alterations by sequencing circulating cell-free DNA in a sample of blood. The typical abundance of mutations in cell-free DNA is typically within 0.01–0.1%, and these fractions are too low for DNA-enrichment techniques — that is, techniques for increasing the abundance of the genomic regions to be sequenced — to bring them to the levels needed by common sequencing technologies (next-generation sequencing, Sanger sequencing and droplet digital PCR). Meiping Zhao, Tongbo Wu, Xianjin Xiao and co-authors leveraged the non-specific endonuclease deoxyribonuclease (DNase) to efficiently enrich mutations with an abundance level lower than 0.1% in clinical samples. When a high concentration of DNase is complexed with single-stranded phosphorothioated DNA (whose binding affinity for DNase is higher than that of other DNA strands), the DNase preferentially digests the strands perfectly matched with the phosphorothioated DNA. Hence, by designing complexes against any wild-type DNA sequences, all mutations within the target-binding region of the phosphorothioated DNA can be enriched. The method is easier to implement than other DNA-enrichment techniques, does not require precise temperature control, and allowed the authors to identify clinical samples with specific mutations in driver genes that would not have otherwise been picked out.

Simplicity at the point of care also involves convenience of sampling. For example, for continuous monitoring, dermal interstitial fluid would be easier to sample than blood. Yet, as argued by Jason Heikenfeld, Hyongsok Tom Soh and colleagues in a Perspective also included in this issue, for diagnostic purposes interstitial fluid is unfortunately not a good proxy for blood — particularly when assessing the concentration of large solutes, as they partition asymmetrically between the two fluid compartments. But dermal interstitial fluid can still be useful for the continuous sensing of small-molecule analytes (by sampling nanolitre volumes of interstitial fluid, or via wearable sensors), and for monitoring the immune system (cytokines in dermal interstitial fluid can be detected earlier than in blood).

Balancing all the design and operational constraints of a diagnostic assay is difficult, particularly when the assay's throughput requirements are demanding, and when it has to detect multiple biomarkers or to integrate more than one type of sample. This is the sort of streamlining work that falls on the biomedical engineer's turf.

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