POINT-OF-CARE DIAGNOSTICS

Sensitive detection of SARS-CoV-2 on paper

An assay leveraging strand-displacement reactions and enzymatic amplification for the recognition of viral RNA and implemented on origami paper allows for the fast colorimetric detection of SARS-CoV-2 variants, with single-nucleotide specificity.

Kaiyue Wu and Alexander A. Green

Antigen tests in a lateral-flow format are being widely used during the coronavirus disease 2019 (COVID-19) pandemic because they provide results fast, and are simple to use. However, antigen tests have lower sensitivity and specificity — and hence produce more false positives — than the gold-standard test based on quantitative PCR with reverse transcription (RT–qPCR)[1]. But RT–qPCR nucleic acid assays require trained personnel and expensive equipment to amplify the genetic material from the pathogen, and hence the tests are performed by centralized facilities and have slower turnaround times. A variety of recently developed alternative nucleic acid tests[2–8] are easier to perform and are less costly than RT–qPCR, and offer better sensitivity than lateral-flow assays. However, developing diagnostics that match the convenience of lateral-flow tests and the accuracy of RT–qPCR has been challenging. Writing in *Nature Biomedical Engineering*, Jinghong Li, Weimin Li, Ruijie Deng and colleagues now describe a paper-based nucleic acid assay for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that is fast (30 min from sample in to result out) and cheap (under one US dollar per test), and that can be read out by the naked eye[9]. The assay can detect as little as 400 copies per microlitre of viral RNA, and leverages highly specific nucleic acid interactions to identify SARS-CoV-2 variants of concern.

To create such a sensitive, fast and simple assay, Li and co-authors pursued a strategy that does not require nucleic acid amplification. The assay technology, which they named MARVE (short for multiplexed, nucleic-acid-amplification-free, single-nucleotide-resolved viral evolution), involves phenol red — a common pH indicator — that undergoes a dramatic change in colour (from red to yellow) as pH decreases. To couple a change in pH to the presence of viral RNA, the authors made clever use of the interactions of silver (Ag) ions with two components: a DNA-based probe, and the enzyme urease. Ag(I) ions, as with many metal cations, interact strongly with negatively charged DNA, forming C–Ag–C complexes when exposed to C:C mismatches within a DNA double helix[10]. At the same time, the urease enzyme, which hydrolyses urea to release the weak base ammonia, is strongly inhibited by free Ag(I) ions[11]. Therefore, to detect viral RNA, the authors designed the DNA probe so that Ag(I) is released on binding to a SARS-CoV-2 sequence (Fig. 1a). The released Ag(I) in turn suppresses urease activity, leading to lower pH conditions and a yellow readout. In the absence of viral RNA, Ag(I) is retained by the probe, and active urease breaks down urea to produce ammonia, which results in higher pH conditions and a red readout. The reactions can be performed in solution or on paper

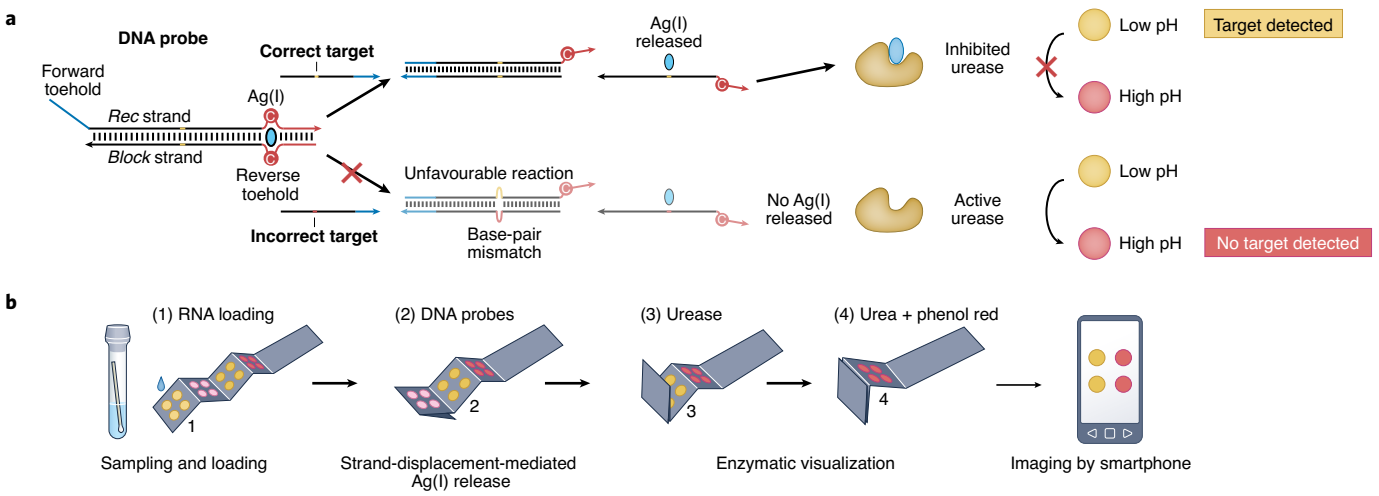

Fig. 1 | Colorimetric sensitive detection of viral RNA without the need for nucleic acid amplification. a, A DNA-toehold exchange probe selectively interacts with the viral RNA target, releasing Ag(I) ions to inhibit urease hydrolysis and establishing the low-pH conditions that generate a yellow readout. An incorrect viral RNA containing a point mutation cannot activate the probe, which prevents the release of Ag(I), leading to active urease hydrolysis and to the high-pH conditions that generate a red readout. **b**, Integration of the reactions of the assay into origami paper. The reaction steps occur sequentially as layers of paper are folded into contact with each other, enabling reaction intermediates to be transferred between layers. The readout can be analysed via a smartphone. Panel **b** adapted with permission from ref. [9], Springer Nature Ltd.

substrates, hence providing colorimetric test results that can be easily seen by eye and analysed via a smartphone.

The DNA-toehold exchange probe[12] used in Li and co-authors' assay is central to its specificity. The probe consists of two partially complementary strands, referred to as *Rec* and *Block*, that are initially hybridized to one another. The *Rec* strand in the probe complex has a single-stranded region (known as a forward toehold) to enable it to bind to the viral RNA. A duplex region (the reverse toehold) is present at the other end of the probe, and features the C:C mismatch for the binding of Ag(I) (Fig. 1a). When the target viral RNA binds to the probe, the *Block* strand is displaced, causing the disruption of the reverse toehold duplex and the release of Ag(I). Importantly, the sequences of the forward and reverse toeholds are carefully programmed such that the displacement of the *Block* strand is favoured only with a viral RNA that is perfectly complementary to the recognition region of the *Rec* strand. An RNA with a single point mutation sets a mismatch that prevents the displacement reaction from proceeding[12]. By designing the probes to identify different target RNAs of interest, the authors demonstrate that MARVE can detect three coronaviruses (SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2) and seven influenza subtypes. In addition, they devised a paper-origami strategy for the portable deployment of the assay. The reaction steps are divided across multiple paper layers, and are carried out sequentially by simply folding the paper to enable reaction intermediates to flow from one layer to the next (Fig. 1b). By allowing for multiple sample-loading sites, the origami paper also permits the simultaneous detection of multiple viruses, as well as smartphone-based image processing.

Moreover, Li and colleagues adapted MARVE for the detection of SARS-CoV-2 variants. They implemented a pipeline for probe design, and established arrayed reactions for detecting SARS-CoV-2 and to distinguish four different variants of it within a single origami paper. This multiplexed MARVE assay, which was developed in only 18 days, also provides the results, visible by eye or a smartphone, in 30 min. Notably, it can process RNA samples coming straight out of a rapid thermal-lysis step, without the need for target amplification or enrichment, thus reducing assay steps and the time to result. Validated with clinical throat-swab samples, the assay showed 100% concordance with the results obtained by RT–qPCR and sequencing.

Overall, MARVE combines the specificity of nucleic acid tests with assay times and costs that approach those of lateral-flow tests. Central to these features is the Ag(I)/urease-mediated detection scheme, which requires only a single enzyme and a single DNA probe and avoids a nucleic-acid-amplification step that would otherwise increase the time, cost and complexity of the assay. The reactions in MARVE are performed at room temperature, obviating the need for a heat block (except for the thermal-lysis step). Moreover, MARVE assays are fast to develop, because only a single probe needs to be designed for each target-RNA sequence of interest; instead, for existing assays with comparable specificity, amplification primers and probes must be typically developed[8,13]. The simplicity and accuracy of MARVE suggests that the assays could be used for home-based testing, and serve as a complement to next-generation sequencing by using them to quickly interrogate epidemiological patterns of sequence-confirmed variants. An affordable and easy-to-develop diagnostic with single-nucleotide specificity could also provide more personalized treatment information for patients, and enable tests that are better tailored to the specific needs of a community, especially in low-resource settings.

Although origami paper can stabilize urease activity at 4 °C for about a month, preservation of the reactions for extended periods at room temperature or above it would improve the field-readiness of the assay and eliminate the need for costly cold-chain distribution and refrigeration of the test kits. A detection limit of 400 RNA copies per microlitre is superior to the typical detection limits of lateral-flow assays[8], but it is insufficient for the detection of all active infections and to match the sensitivity of RT–qPCR[14]. MARVE could be streamlined to eliminate the need for origami-folding steps, to match the ease of use of lateral-flow assays. For instance, control strategies for paper microfluidics[15] could be integrated with MARVE to develop a diagnostic that goes from RNA sample to multiplexed result without the need for user intervention. ❐

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Competing interests

A.A.G. is a co-founder of En Carta Diagnostics, Inc. K.W. declares no competing interests.