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ACS Synthetic Biology

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Research Article

Bio-SCAN: A CRISPR/dCas9-Based Lateral Flow Assay for Rapid, Specific, and Sensitive Detection of SARS-CoV-2

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Cite This: https://doi.org/10.1021/acssynbio.1c00499

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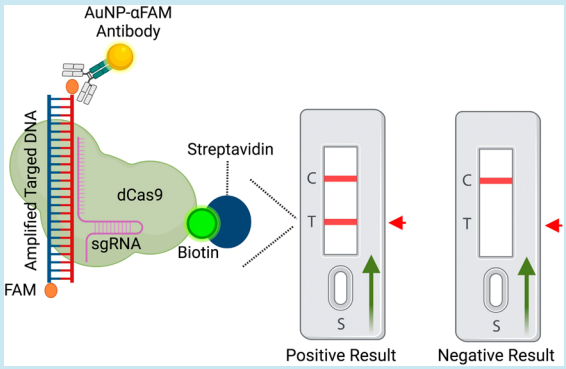
ABSTRACT: Simple, rapid, specific, and sensitive point-of-care detection methods are needed to contain the spread of SARS-CoV-2. CRISPR/Cas9-based lateral flow assays are emerging as a powerful alternative for COVID-19 diagnostics. Here, we developed Bio-SCAN (biotin-coupled specific CRISPR-based assay for nucleic acid detection) as an accurate pathogen detection platform that requires no sophisticated equipment or technical expertise. Bio-SCAN detects the SARS-CoV-2 genome in less than 1 h from sample collection to result. In the first step, the target nucleic acid sequence is isothermally amplified in 15 min via recombinase polymerase amplification before being precisely detected by biotin-labeled nuclease-dead SpCas9 (dCas9) on commercially available lateral flow strips. The resulting readout is visible to the naked eye. Compared to other CRISPR-Cas-based pathogen detection assays, Bio-SCAN requires no additional reporters, probes, enhancers, reagents, or sophisticated devices to interpret the results. Bio-SCAN is highly sensitive and successfully detected a clinically relevant level (4 copies/μL) of synthetic SARS-CoV-2 RNA genome. Similarly, Bio-SCAN showed 100% negative and 96% positive predictive agreement with RT-qPCR results when using clinical samples (86 nasopharyngeal swab samples). Furthermore, incorporating variant-specific sgRNAs in the detection reaction allowed Bio-SCAN to efficiently distinguish between the α, β, and δ SARS-CoV-2 variants. Also, our results confirmed that the Bio-SCAN reagents have a long shelf life and can be assembled locally in nonlaboratory and limited-resource settings. Furthermore, the Bio-SCAN platform is compatible with the nucleic acid quick extraction protocol. Our results highlight the potential of Bio-SCAN as a promising point-of-care diagnostic platform that can facilitate low-cost mass screening for SARS-CoV-2.



KEYWORDS: *COVID-19, SARS-CoV-2 variants, lateral flow assay, CRISPR-dCas9 and dCas9, nucleic acid detection, biotin labelling*

INTRODUCTION

Rapid, sensitive, and specific point-of-care (POC) testing procedures are required to contain and restrict the spread of *severe acute respiratory syndrome coronavirus* 2 (SARS-CoV-2).[1] SARS-CoV-2 threatens human lives, public healthcare systems, and most aspects of everyday life worldwide.[2] The first line of defense against pandemics is mass testing, tracking, and tracing to identify and isolate infected individuals, thus preventing further spread.[3] Currently, RT-qPCR is the gold standard method to detect SARS-CoV-2; however, it requires centralized, highly equipped laboratories operated by skilled technical staff.[4] SARS-CoV-2 testing capacity is limited by the number of samples per run, the cost, and the turnaround time of each test, as well as the sophisticated equipment and skilled personnel needed. As mass testing and early detection of SARS-CoV-2 will help isolate infected individuals before they spread the virus,[5] there is a pressing need to develop simple, nonlaboratory-based POC detection assays or at-home testing kits suitable for in-field deployment and mass testing. Such POC detection modules must meet the ASSURED (accurate, specific, sensitive, user-friendly, rapid, equipment-free, and deliverable to end users) guidelines set by the World Health Organization (WHO).[6] Additionally, these POC tests must be amenable to in-field deployment with clinically relevant specificity and sensitivity for use in communities, organizations, airports, and sport facilities.

Lateral flow assays (LFAs) are simple diagnostic methods for pathogen detection in nonlaboratory settings, as they are rapid, cost-effective, and user-friendly.[7,8] The first commercial application of LFAs, almost 50 years ago, was in pregnancy

Received: October 5, 2021

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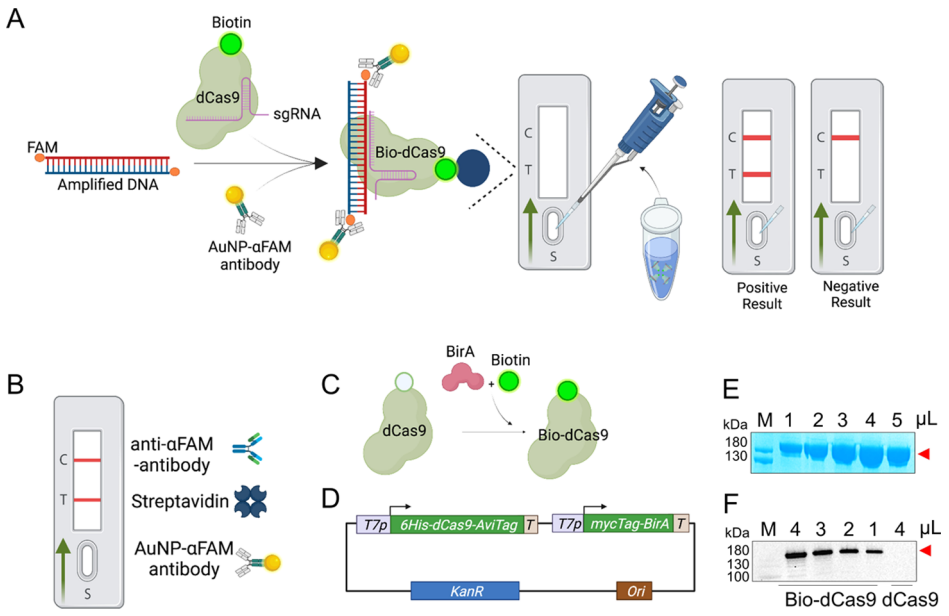

Figure 1. Schematic of the Bio-SCAN platform. (A) Assembly of Bio-SCAN components for detection nucleic acid. The dCas9 (Biotin-labeled, bio-dCas9)−sgRNA complex will precisely recognize the target DNA (FAM-labeled, FAM-DNA). The streptavidin-coated line (test line, T) captures the biotin-labeled Bio-dCas9−sgRNA-DNA complex. Once applied on the LFA strip, in the lateral flow, the FAM-DNA−bio-dCas9−sgRNA will accumulate gold (Au) nanoparticle-labeled−anti-FAM antibody (AuNP-αFAM antibody), thus resulting in the visual detection of the target nucleic acid at the test line. The control line (C) is imbedded with anti−anti FAM antibodies that accumulate the unbound residual AuNP−αFAM antibody and serve as a positive control. Bio-SCAN results interpretation. Appearance of two lines at the lower test (T) band and upper (C) band represents the presence of target nucleic acid (positive sample) and valid results. Appearance of only upper (C) band represents the absence of target nucleic acid (negative sample). If only lower (T) band appears on the strip, the test is invalid and must be repeated with the fresh reagent and new LFA strip. (B) Composition of the commercially available LFA strip. Sample pad, S, contains the AuNP−αFAM antibody. T line contains the streptavidin. C line contains the anti−anti FAM antibody. Absorbent pad facilitates liquid flow on the strip. Green arrows indicate the flow direction. The red arrowhead indicates the appearance of the positive test band on the LFA strip. (C) Experimental strategy of dCas9 biotinylation. Inside the bacterial cell, BirA enzymatically conjugates biotin to AviTag-fused dCas9 to produce Bio-dCas9. (D) Plasmid design for co-expressing *dCas9-AviTag* and *BirA*. Both *dCas9-AviTag* and *BirA* were individually cloned under a separate *T7p-lac* repression system on the same plasmid. DNA sequence coding for His (6×) residues was added to *dCas9-AviTag* for affinity-based purification. *KanR*, kanamycin resistance. *Ori*, origin of replication. (E) Confirmation of Bio-dCas9 production. Different amounts of the purified protein of the purified protein were separated on SDS-PAGE. The protein band of the expected size confirmed the production of Bio-dCas9. (F) Confirmation of biotinylation. Different amounts (μL) of the purified protein were separated on SDS-PAGE, blotted onto nitrocellulose membrane, and detected with αbiotin antibodies. Detection of the expected band size Bio-dCas9 with αbiotin antibodies confirmed the efficient conjugation of biotin to the AviTag fused with dCas9. dCas9 (without AviTag) was used as the control. The red arrowhead indicates the expected Bio-dCas9 protein. kDa, kilo Dalton. M, protein marker.

tests to detect human chorionic gonadotropin in urine; since then, LFAs have become one of the most reliable tools to detect disease markers for medical diagnostics.[9−13] The strong, noncovalent interaction between biotin and avidin is widely used to purify proteins and nucleic acids or isolate interacting partners.[14−19] Therefore, LFA strips were developed to detect proteins or nucleic acids by incorporating the biotin−avidin interaction.[20,21] Biotin−avidin-based LFAs have recently emerged as a convenient approach for the detection of pathogen-derived nucleic acids.[8] Strips are impregnated with avidin/streptavidin at the test line (test line, T) that specifically immobilizes biotin-labeled molecules present in the lateral flow, followed by visual detection.[22,23] However, direct use of biotin−streptavidin-based LFAs suffer from low sensitivity, nonspecific interactions, false detection, and the inability to differentiate correct amplicons from primer dimers. When considering the additional steps needed for amplification and hybridization, these approaches are not yet suitable for POC applications.[24,25] The extraordinary potential of LFAs has yet to be fully exploited for nucleic acid detection to meet the ASSURED criteria required to facilitate their effective use for point-of-care detection.

Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated protein (Cas) systems have been harnessed for nucleic acid-based diagnostics.[26,27] CRISPR diagnostics (CRISPR-Dx) relies on trans collateral activity of the type-V and -VI CRISPR-Cas systems triggered by specific target recognition.[28−33] In contrast to the trans activity-based CRISPR-Dx systems for nucleic acid detection, Cas9 and its catalytically inactive variant dCas9 have been recently shown to detect pathogen sequences directly or when coupled to an LFA using the binding activity of the Cas9 enzyme.[21,34−37] In the Cas9-mediated lateral flow nucleic acid assays, the visual output depends on incorporating FAM and biotin moieties in the detection complex.[21,38] However, these assays require complicated components such as AuNP-DNA (gold, aurum nanoparticle-DNA) probe, customized LFA strips, excess DNA to boost sensitivity, and unconventional reporters that complicate their use. Moreover, these materials are not easily commercially available and cannot be produced in low-resource settings. To simplify the Cas9- and LFA-based detection systems, we recently developed the Vigilant (VirD2-dCas9 guided and LFA-coupled nucleic acid test), which requires an extra FAM-labeled VirD2-dCas9 binding reporter

https://doi.org/10.1021/acssynbio.1c00499
ACS Synth. Biol. XXXX, XXX, XXX−XXX

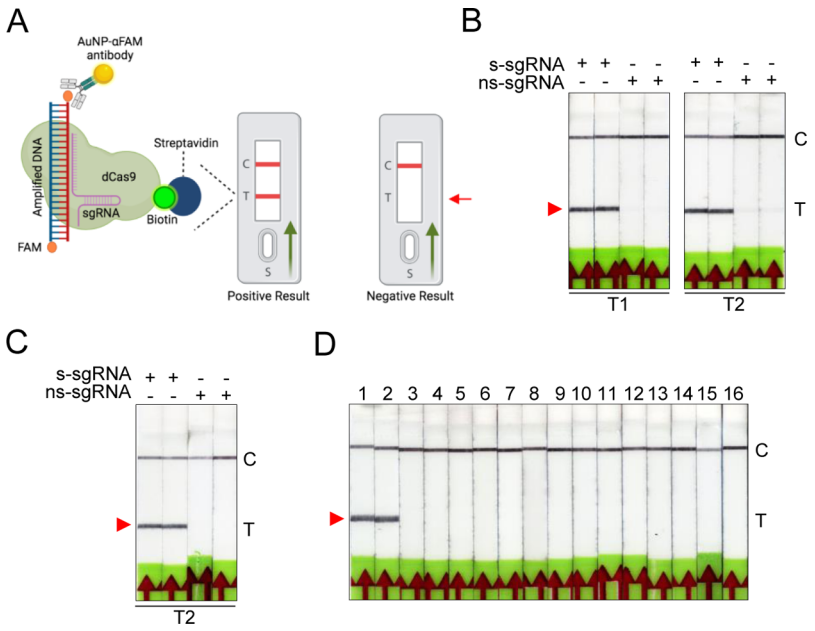

Figure 2. Validation of Bio-SCAN for target nucleic acid detection. (A) Proof of concept of the Bio-SCAN platform. DNA amplicons (FAM-labeled) recognized by biotin (green in bio-dCas9−sgRNA) will interact with streptavidin (blue) to the immobilized AuNP-αFAM antibody at the T (test line) line and will result in band appearance as a positive signature, while no such band will appear in the negative samples. Green arrows indicate the flow direction. The red arrowhead indicates the appearance of the positive test band on the LFA strip. (B) Detection of the target DNA with Bio-SCAN assay. PCR amplicons of two independent targets, T1 (Target 1) and T2 (Target 2), and FAM-labeled DNA (300 ng) were incubated with Bio-dCas9 (250 nM) and target-specific s-sgRNA (250 nM), and the sample was subjected to LFA strips. Nonspecific ns-sgRNA was used as the negative control. (C) Bio-dCas9−sgRNA RNP-based target detection. Bio-dCas9−sgRNA was preassembled as RNP and incubated with target DNA. Bio-dCas9−sgRNA RNP detected the FAM-labeled DNA within 3 min on lateral flow strips. (D) Specificity confirmation. The specificity experiment was conducted with specific s-sgRNA (lines 1 and 2), nonspecific sgRNA (lines 3 and 4), no sgRNA (lines 5 and 6), unlabeled target template (lines 7 and 8), no target template (lines 9 and 10), nonspecific target (lines 11 and 12), no Bio-dCas9 (lines 13 and 14), and unlabeled dCas9 (lines 15 and 16) for the Bio-SCAN platform. The red arrowhead indicates the expected band appearance at T (test line). C (control line).

and uses commercially available LFA strips for visual detection.[39] However, to expand the CRISPR-Cas-based LFA tool box, key advances and new concepts are needed to add simplicity and improve the specificity and sensitivity for the detection of nucleic acids that comply to the WHO ASSURED criteria.

Biotinylation is a common modification in living cells, whereby biotin−protein ligase covalently couples biotin to an amino acid or a carbohydrate moiety.[40] In molecular biology, biotin labeling, that is, proximity-dependent biotin identification (BioID and TurboID), is a well-established method to test protein−protein interactions or identify interacting partners in living cells.[15,17,41] Biotin can be added to any protein by chemically modifying primary amino groups ($-NH_2$) or enzymatic labeling with biotin, this latter method being generally preferred due to the resulting homogeneity and ease of labeling.[42,43] In *Escherichia coli*, biotin ligase (BirA) specifically attaches biotin to a 75-residue fragment of biotin carboxyl carrier protein (BCCP),[44] which was later optimized to a 14-amino acid sequence (AviTag) as the substrate for BirA.[45] The AviTag is currently used for protein purification, to trap and purify interacting proteins, and to determine protein−DNA interactions.[14,16,43,46]

Here, we combined the precision of SpCas9 nuclease-deactivated (dCas9) recognition with the power of the biotin−streptavidin interaction on a commercially available LFA to develop a cost-effective, simple, and portable nucleic acid detection protocol called Bio-SCAN (biotin-coupled specific CRISPR-based assay for nucleic acid detection). For Bio-SCAN execution, we produced and purified the biotin-labeled dCas9 (Bio-dCas9) from *E. coli*. In Bio-SCAN, the target nucleic acid sequence is isothermally amplified with FAM-labeled primers and then precisely recognized by Bio-dCas9, which immobilizes the AuNP−anti-FAM antibody complex at the test band for visual detection. Bio-SCAN requires no ancillary equipment or technical expertise and showed 100% negative and 96% positive predictive agreement with RT-qPCR assessment of 86 clinical nasopharyngeal swab samples. Importantly, the Bio-SCAN platform is adaptable for the detection of specific variants of SARS-CoV-2,[47] by simply redesigning the primers and sgRNA. Furthermore, Bio-SCAN reagents can be assembled locally for any emerging variant in limited-resource areas with an estimated cost of less than $10 per test. Our results demonstrate that Bio-SCAN is able to detect SARS-CoV-2 in clinical samples in less than 1 h from sample collection to result and complies with most criteria for POC use. Overall, the Bio-SCAN detection platform will facilitate rapid, sensitive, and specific mass screening of SARS-CoV-2 and potentially other infectious pathogens in non-laboratory and low-resource settings.

RESULTS AND DISCUSSION

Working Principle, Design, and Construction of Bio-SCAN. We aimed to develop a powerful and robust system to detect nucleic acids via LFA for efficient POC testing. We thus designed, built, and tested the Bio-SCAN module, which combines isothermal amplification of a target sequence with the advantages of the CRISPR-Cas9 system to bind and

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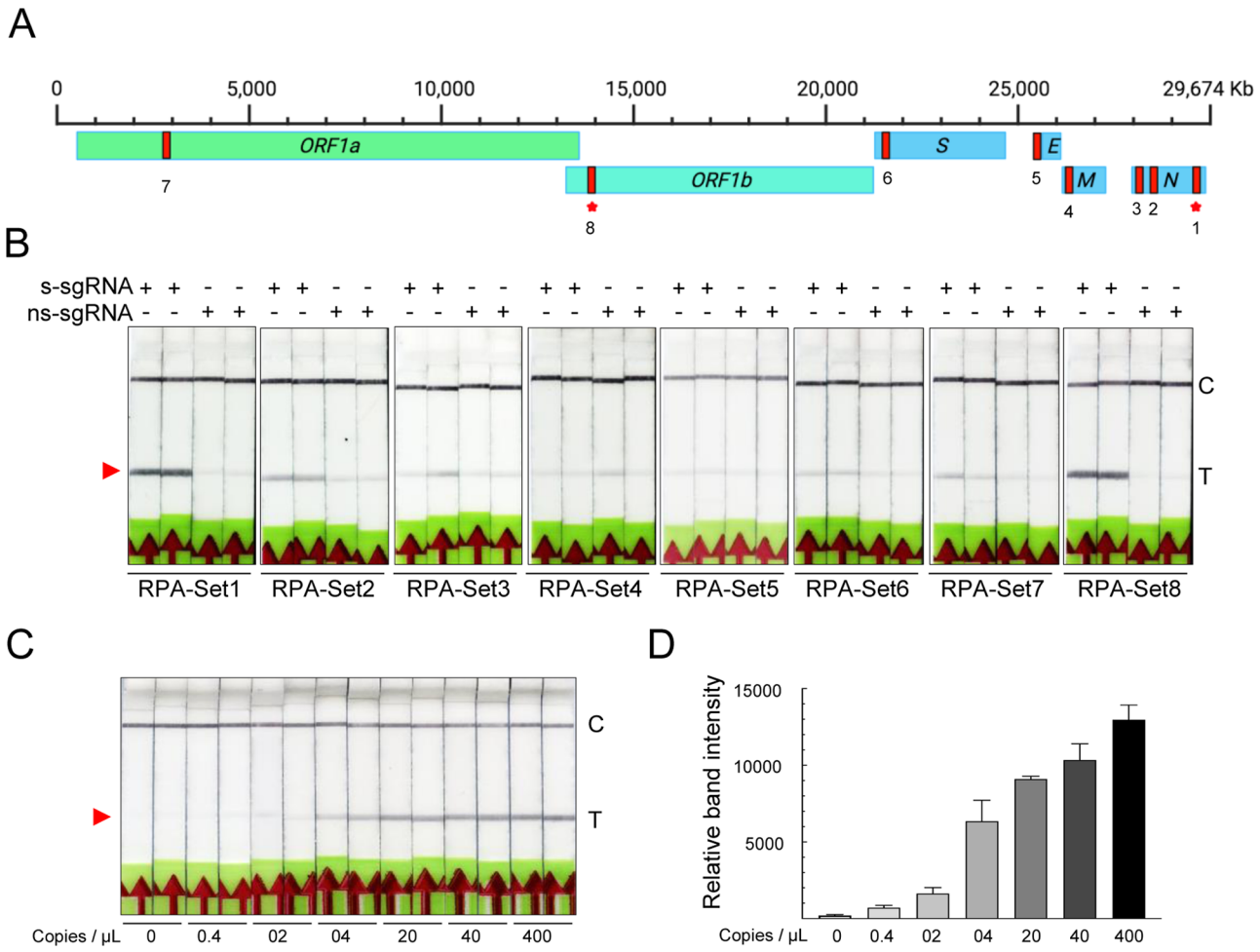

Figure 3. Bio-SCAN platform detected SARS-CoV-2 synthetic RNA with high sensitivity. (A) SARS-CoV-2 representative genome. Eight targets (red bars and numbered 1−8) were selected on the genome of SARS-CoV-2 for RT-RPA-coupled Bio-SCAN-based detection. Kb, kilo base. *ORF1a, ORF1b, S, Spike. E, envelope. M, membrane. N, nucleocapsid* genes of the SARS-CoV-2. (B) The target sequences were amplified via RT-RPA using 1000 copies of the synthetic SARS-CoV-2 genome with FAM-labeled primes. Each of the targets was subjected to Bio-SCAN assay with the respective sequence-specific s-sgRNA. A single ns-sgRNA was used as the control in all sets. Two sets RPA-Set1 and RPA-Set8 efficiently detected the synthetic SARS-CoV-2 genome. (C) Determination of the sensitivity level of the Bio-SCAN platform. Synthetic SARS-CoV-2 RNA was diluted to obtain 0, 0.4, 02, 04, 20, 40, and 400 copies/μL equal to 0, 10, 50, 100, 500, 1000, and 10,000 copies per reaction (25 μL reaction). The diluted samples were subjected to RT-RPA using FAM-labeled primers (optimized RPA-Set8 in B above) and followed by Bio-SCAN-based detection on LFA strips. Samples with 100 copies per reaction (04 copies/μL) were easily distinguishable from the sample containing no target RNA. (D) Results of the LOD experiment of the Bio-SCAN-based nucleic acid detection were converted via ImageJ software for statistical analysis and graphical representation. The error bar shows standard deviation. The red arrowhead indicates the expected band appearance at T (test line). C (control line). Green arrows indicate the flow direction.

precisely detect the pathogen nucleic acid sequence on commercially available streptavidin−biotin-based LFA strips. A short fragment from the genome of the pathogen of interest is then amplified by recombinase polymerase amplification (RPA) using customized FAM-labeled primers, followed by the scanning and recognition of pathogen sequences by an sgRNA−bio-dCas9 ribonucleoprotein (RNP) complex, yielding a band on LFA (Figure 1A).

For Bio-SCAN detection, the lateral flow strips contain four central regions: (1) the sample pad, which contains the gold nanoparticle (AuNP)−anti-FAM antibody complex; (2) the test line (T), a streptavidin-coated line that specifically immobilizes bio-dCas9 bound to a DNA complex for detection; (3) a control line containing anti-FAM antibodies; and (4) an absorbent pad for liquid flow through the strip (Figure 1B). Once the FAM-labeled target DNA complex containing sgRNA−bio-dCas9 is applied to the sample pad, the AuNP−anti-FAM antibody conjugates with FAM-labeled DNA. During lateral flow, streptavidin in the test line (T) immobilizes the bio-dCas9−DNA-FAM−anti-FAM−AuNP complex, which results in signal appearance at the (T) band, while the remaining unbound AuNP−anti-FAM antibody is detected at the control line (C). In negative samples, the absence of bio-dCas9−DNA-FAM−anti-FAM−AuNP complexes results in a single band at the control line (C) (Figure 1A).

For Bio-SCAN execution, we designed a single plasmid expressing dCas9-AviTag and BirA cassettes and directly purified the biotin-labeled dCas9 (Bio-dCas9) from *E. coli*. We selected the enzymatic biotinylation via BirA[48] as it produces homogeneous pools of biotinylated proteins compared to the random addition of biotin by chemical modification.[42,43] To produce and purify Bio-dCas9 from bacteria, the *dCas9* sequence was cloned in-frame with the *AviTag* sequence

https://doi.org/10.1021/acssynbio.1c00499
ACS Synth. Biol. XXXX, XXX, XXX−XXX

(Figure 1C). The two independent expression cassettes: *dCas9-AviTag* (coding for dCas9-AviTag) and *BirA* (coding for biotin ligase for AviTag biotinylation) each under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible T7 promoter were combined on a single plasmid and transformed into *E. coli* BL21 (DE3) cells (Figure 1D). Biotin was added to the medium and protein production was induced with IPTG, followed by purification of Bio-dCas9 using affinity purification and size-exclusion separation (Figure S1). Next, the purification of Bio-dCas9 and proper biotinylation of the protein was confirmed by SDS-PAGE and immunoblot analysis, respectively (Figure 1E,F). Our results confirm the efficient production and biotinylation of dCas9 inside the *E. coli* cells. Bio-Cas9 production also confirms that Bio-SCAN materials can be easily produced locally in a short time in low-resource settings.

Bio-SCAN Specifically Detects Target Nucleic Acids. To test the Bio-SCAN principle on specific nucleic acids (Figure 1), we first confirmed the activity of our designed sgRNAs on two different PCR-amplified DNA targets. The amplified PCR product was mixed with in vitro-produced sgRNAs (targeting a specific site in the amplicons) and catalytically active Cas9 enzyme. The digestion of the DNA fragments (as demonstrated by electrophoresis on agarose gels) confirmed the proper targeting activity of both sgRNAs (Figure S2).

Next, the target sequence was PCR-amplified with FAM-labeled primers and each FAM-labeled DNA (300 ng) was incubated with Bio-dCas9 and sgRNAs at 37 °C for 15 min. To validate the Bio-SCAN working principle (Figure 2A), the FAM-DNA−sgRNA−Bio-dCas9 complex was subjected to LFA. A nonspecific sgRNA ns-sgRNA was used as a negative control. Indeed, Bio-SCAN specifically detected the respective targets, as evidenced by the appearance of a band at the test line (T). At the same time, we observed no band in reactions with the ns-sgRNA, thus validating the working principle of our Bio-SCAN assay and its relevance for detecting nucleic acids (Figure 2B,C).

Cas9 complexation with sgRNA is a slow process[49] and may increase the time duration of the Bio-SCAN diagnostics. Accordingly, we tested a preassembled RNP (sgRNA−bio-dCas9 complex) to reduce the detection time. Compared to the 15 min incubation time (Figure 2B), a 3 min incubation of FAM-labeled DNA with a preassembled RNP was sufficient to successfully detect the target sequence on lateral flow strips (Figure 2C).

Next, we assessed the specificity of Bio-SCAN using multiple controls. We obtained a band on the T line only when the specific sgRNA was used with FAM-labeled DNA and bio-dCas9 (Figure 2D). The absence of any discernible cross-reactivity in any of the control conditions clearly demonstrates that the Bio-SCAN is a viable module for nucleic acid detection.

We and other groups recently coupled CRISPR/Cas9 and (dCas9) with an RT-RPA-based LFA system to develop simple nucleic acid LFAs (NALFAs). However, the Vigilant platform requires an extra FAM-labeled ssDNA probe containing a VirD2 cutting and binding site, while CASLFA needs an additional custom-made AuNP-DNA probe as well as custom-made lateral flow strips. Similarly, other modules require additional soak DNA, extra probes or denaturation steps, and complex assembly for pathogen detection.[21,38,39] By contrast, Bio-SCAN requires no additional probes or DNA which significantly reduces the complexity of the system.

A recent report assessed a commercially available biotinylated dCas9 protein to detect the target DNA sequence.[36] However, the system requirements, such as the need for a very high concentration of soak DNA (2 μM, extra nonspecific DNA in the reaction tube), extended detection time (90 min), the appearance of the false test band on the LFA strips with nonspecific DNA or nonspecific sgRNA, the use of densitometry for result interpretation, and no direct use of the pathogen RNA coupled with RT-RPA, precluded the potential use of a biotin-based system for quick diagnostics of SARS-CoV-2. As no information of the biotinylation procedure is provided, the false-positive results were attributed to the biotin-labeled dCas9 nonspecifically binding to LFA strips and the target sequence.[36] Homogeneous distribution and appropriate biotinylation of proteins are essential for biotin−streptavidin specific interaction.[43] To avoid false interaction, in Bio-SCAN, we used the BirA-based targeted biotinylation of AviTag (14 amino acids) fused to dCas9 over chemical methods of random biotinylation. Our results confirmed that AviTag-based biotinylated dCas9 did not result in any false-positives. Our developed modules require no additional probes or DNA, which validate the use of Bio-SCAN as a user-friendly POC diagnostic modality.

Bio-SCAN Detects Synthetic SARS-CoV-2 Samples. Next, we tested whether Bio-SCAN can detect synthetic SARS-CoV-2 RNA. To this end, we designed multiple RPA primers covering the SARS-CoV-2 genome (Figure 3A) to optimize target sequences' isothermal amplification.[50] FAM-labeled RT-RPA primers for *ORF1a*, *ORF1b*, the *S*, *E*, *M* genes, or the 3′ and 5′ end of the *N* gene were custom-synthesized, and sgRNAs targeting the respective sites were produced by in vitro transcription. We first used synthetic SARS-CoV-2 RNA (10,000 copies/reaction) as a template for RT-RPA with FAM-labeled primer sets. Out of eight RPA primer sets, two sets along their respective sgRNAs designed against *ORF1b* (RPA primer set 8) or the 3′ end of the *N* gene (RPA primer set 1) efficiently detected the synthetic SARS-CoV-2 RNA (Figure 3B,C). Differential target amplification in isothermal reactions typically stems from unpredictable secondary structures in the target RNA, secondary structure in the primer ssDNA, primer dimerization, primer hybridization to each other, and improper hybridization of the primer to the RNA at low temperature (RT-RPA working temperature 37−42 °C) and has been previously observed for RPA-mediated amplification of SARS-CoV-2.[50,51]

A test with high sensitivity is vital to identify individuals at the early stages of infection and limit the spread of pathogens. To determine the sensitivity of Bio-SCAN, we tested multiple dilutions (0, 0.4, 02, 04, 20, 40, and 400 copies/μL equal to 0, 10, 50, 100, 500, 1000, and 10,000 copies in a 25 μL reaction) of the synthetic SARS-CoV-2 RNA. Our results revealed that Bio-SCAN successfully detects SARS-CoV-2 synthetic RNA down to 100 copies in a 25 μL reaction (or 4 copies/μL) (Figures 3C,D and S3). Most of the SARS-CoV-2 detection modules consider 100 copies/μL as the reliable detection limit.[52] In addition, infectious transmission requires over 1000 copies/μL of SARS-CoV-2.[53] The predicted limit of detection (LOD) (4 copies/μL, or 100 copies/reaction) of Bio-SCAN indicates the high sensitivity of our detection modules and can be applied for SARS-CoV-2 detection in clinical samples.

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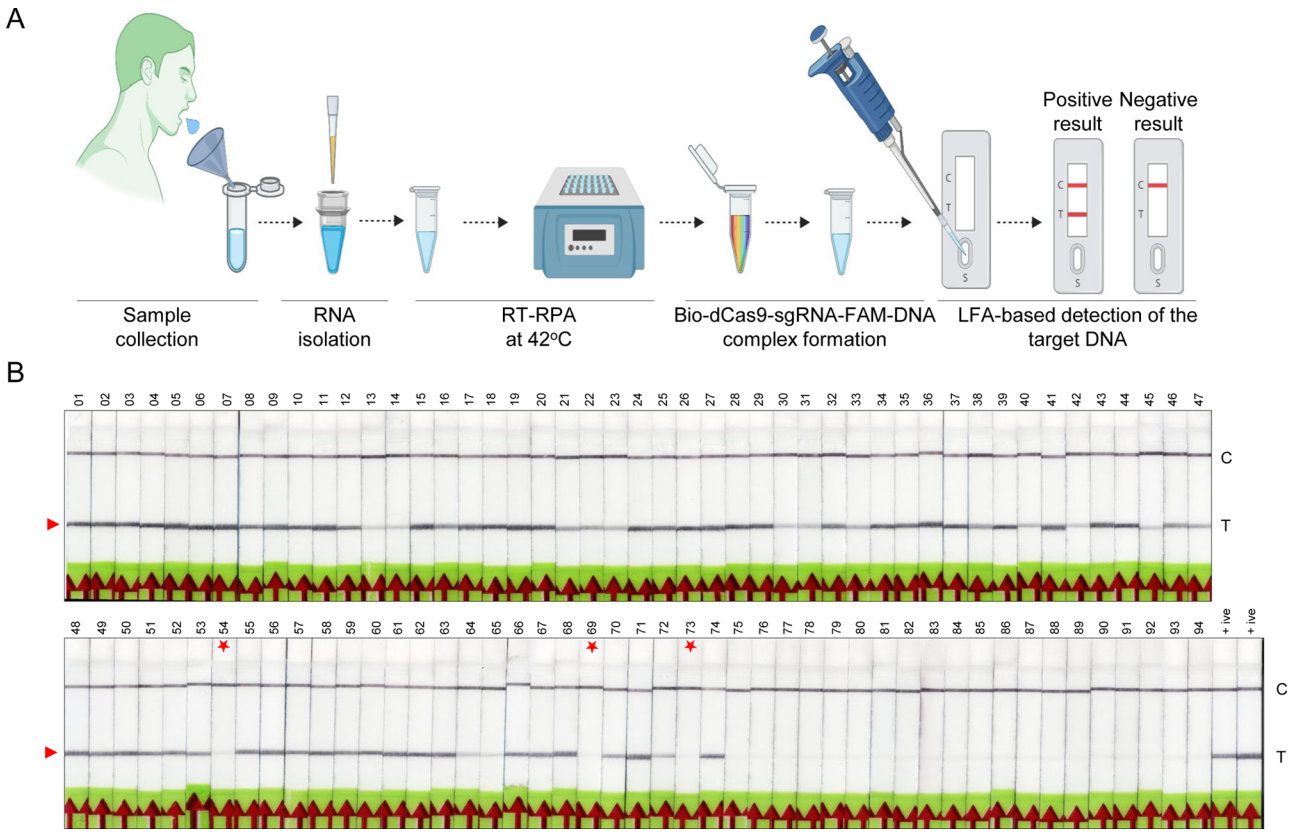

Figure 4. Validation of the Bio-SCAN platform for SARS-CoV-2 detection in clinical samples. (A) Depiction of the Bio-SCAN-based detection of SARS-CoV-2 in clinical samples. Samples were collected from SARS-CoV-2-infected individuals and total RNA was isolated. The extracted RNA was subjected to RT-RPA (isothermal amplification) using FAM-labeled primers. The RT-RPA-amplified target DNAs were detected on LFA strips using the Bio-SCAN platform for the presence (both lines, T and C) or absence (only C line). (B) SARS-CoV-2 detection in clinical samples. RPA-Set8 along with its specific sgRNA was used to validate the Bio-SCAN for SARS-CoV-2 (*ORF1b*) detection in 94 clinical samples. Samples (54, 69, and 73, not detected) are indicated with red stars. The red arrowhead indicates the expected band appearance at T (test line). C (control line). Green arrows indicate the flow direction.

Clinical Validation of Bio-SCAN for Detection of SARS-CoV-2 in Clinical Samples. As our Bio-SCAN detection module exhibited sufficient specificity and sensitivity levels on synthetic samples, we next turned to clinical samples to assess its suitability for use as a POC test (Figure 4A). Accordingly, samples were collected from 12 patients infected with SARS-CoV-2 and 2 noninfected individuals. RNA was isolated and samples were confirmed for the presence of SARS-CoV-2 by qPCR (Figure S4A). Next, the same RNA samples were subjected to RT-RPA using our optimized reaction conditions using SARS-CoV-2-specific sgRNA and primers. Bio-SCAN efficiently detected the SARS-CoV-2 in 11 out of 12 patient samples when using the *N* gene-specific sgRNA and RT-RPA primer set (Figure S4B). Bio-SCAN did not detect SARS-CoV-2 in the uninfected clinical samples. SARS-CoV-2 synthetic RNA was used as a positive control. Human *RNaseP*-specific sgRNA and primer set was used as the experiment internal control. Bio-SCAN properly detected the *RNaseP* sequence in all the clinical samples (Figure S4C), confirming the integrity of the extracted RNA in the samples. We then repeated the assay on the same samples with the optimized sgRNA and RT-RPA primer set designed to the *ORF1b* sequence: in this case, Bio-SCAN identified all 12 out of 12 positive samples, while none of the negative samples showed a band at the T line on the LFA strip (Figure S4D). Detection of all (100%) of the clinical samples clearly confirm the efficacy of *ORF1b* targeting sgRNA and RT-RPA primer set for Bio-SCAN-based detection of SARS-CoV-2 in clinical samples. Compared to *N* gene targeting sgRNA and RT-RPA primer set, we selected *ORF1b*-targeting sgRNA and RT-RPA primers for the following experiment.

To validate the Bio-SCAN module for mass testing, a set of 94 nasopharyngeal clinical samples, including 20 samples from uninfected individuals, were subjected to the Bio-SCAN protocol for SARS-CoV-2 detection. SARS-CoV-2 synthetic RNA (1000 copies/reaction) was used as the positive control in the Bio-SCAN-based LFA detection assay. Using *ORF1b*-targeting sgRNA and RT-RPA primers, Bio-SCAN showed 100% negative and 96% positive predictive agreement (Figure 4B) with RT-qPCR-assessed 94 clinical samples (Figure S5). The RT-qPCR and RT-RPA-based Bio-SCAN cannot be compared directly as each procedure uses completely different molecular approaches and reagents and has particular advantages in terms of application and simplicity. The 96% detection ability of Bio-SCAN confirmed the reliability of our module for SARS-CoV-2 detection at POC settings. In fact, clinical studies demonstrated that SARS-CoV-2 transmission and infectivity reduce drastically with the low viral titer and an analysis of 754 clinical samples showed that SARS-CoV-2 titer remained at a Ct value of 28.18 in the first week, dropping to a Ct value of 30.65 in the second week (up to day 14).[53] Notably, SARS-CoV-2 dynamic models for reducing virus transmission rank fast and frequent testing higher than test sensitivity, with an estimation of 100 copies/μL virus titer

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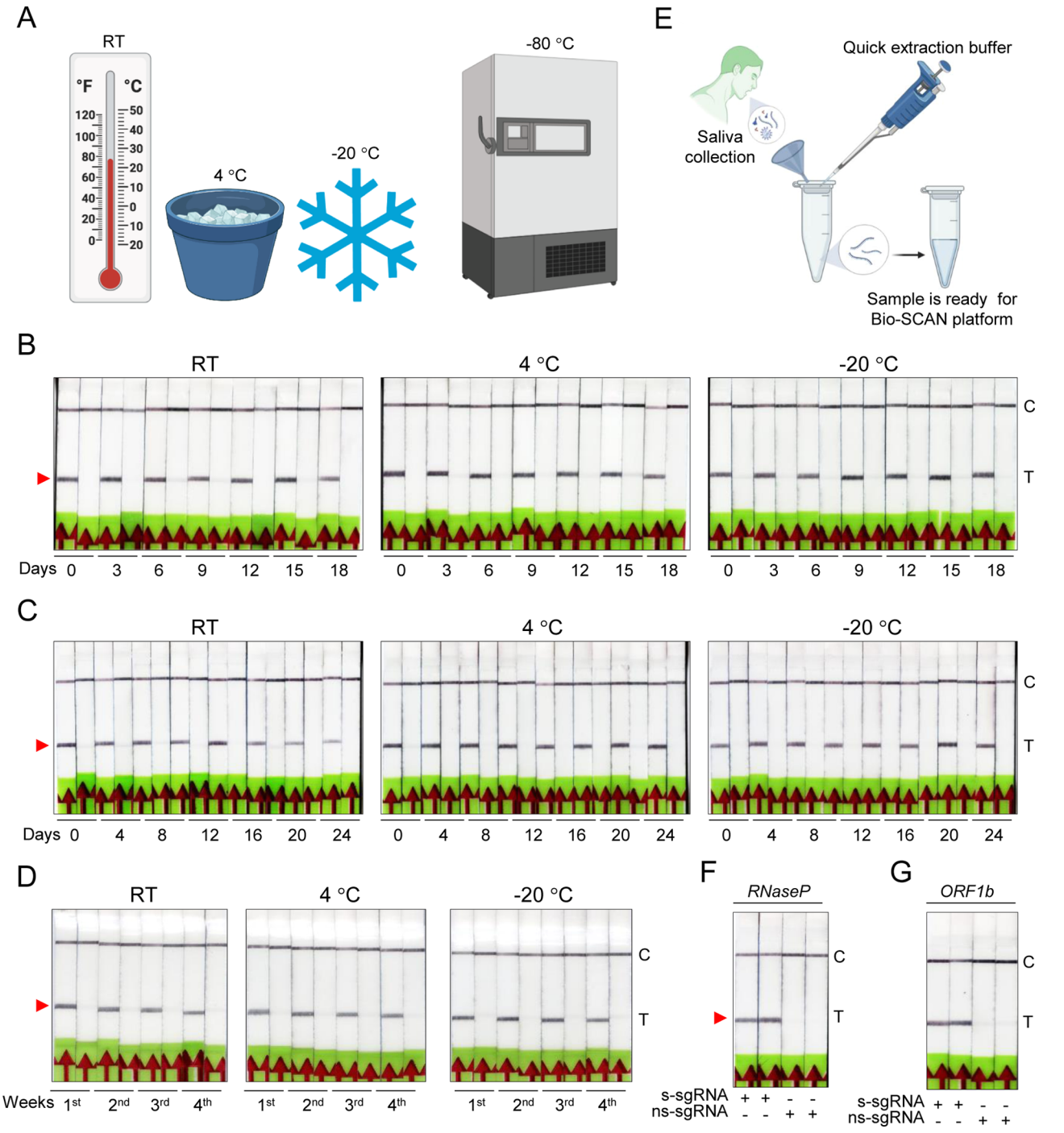

Figure 5. Storage compatibility of Bio-SCAN reagents for the POC setup. (A) Storage requirements for diagnostic reagents. Room temperature 23 °C (RT) and 4 °C are the preferred storage conditions for field-based POC testing. Reagents with −20 and −80 °C storage requirement are avoided in POC testing. (B) Stability evaluation of the Bio-dCas9. Purified Bio-dCas9 was stored under different temperature conditions (room temperature RT, 4 °C, and −20 °C) and used for detection of the target DNA with a 3 day time duration. (C) Stability evaluation of the Bio-dCas9−sgRNA complex. Bio-dCas9 was complexed with sgRNAs and stored under different temperature conditions (room temperature RT, 4, and −20 °C) and used for detection of the target DNA with a 4 day time duration. Freeze-drying for Bio-SCAN reagent storage. Bio-dCas9−sgRNA (RNP complex) was freeze-dried and stored under different temperature conditions. (D) Stability evaluation of the Bio-dCas9−sgRNA freeze-dried complex. Bio-dCas9 was complexed with sgRNAs that were freeze-dried and stored under different temperature conditions (room temperature RT, 4, −20 °C) and used for detection of the target DNA with a week duration and followed for 4 weeks. In all experiments, in (B,C,E), the SARS-CoV-2 synthetic RNA (1000 copies/reaction) was used. (E) Nucleic acid quick extraction. Saliva samples were collected and mixed with quick extraction buffer. The extracted nucleic acid was used directly in the Bio-SCAN-based detection. (F) Compatibility of the Bio-SCAN platform with the nucleic acid quick extraction protocol. Human *RNasP* was selected as a proof of concept. Total RNA was isolated from saliva, and *RNasP* was detected using Bio-SCAN protocol. (G) Detection of SARS-CoV-2 RNA spiked into saliva samples. SARS-CoV-2 RNA (10,000 copies) was spiked in 50 μL of saliva and the sample was treated with a nucleic acid quick extraction buffer. Total RNA (4 μL) was subjected to Bio-SCAN for SARS-CoV-2 (*ORF1a*) detection. The red arrowhead indicates the expected band appearance at T (test line). C (control line). A nonspecific ns-sgRNA was used as the control in all experiments. Green arrows indicate the flow direction.

https://doi.org/10.1021/acssynbio.1c00499
ACS Synth. Biol. XXXX, XXX, XXX−XXX

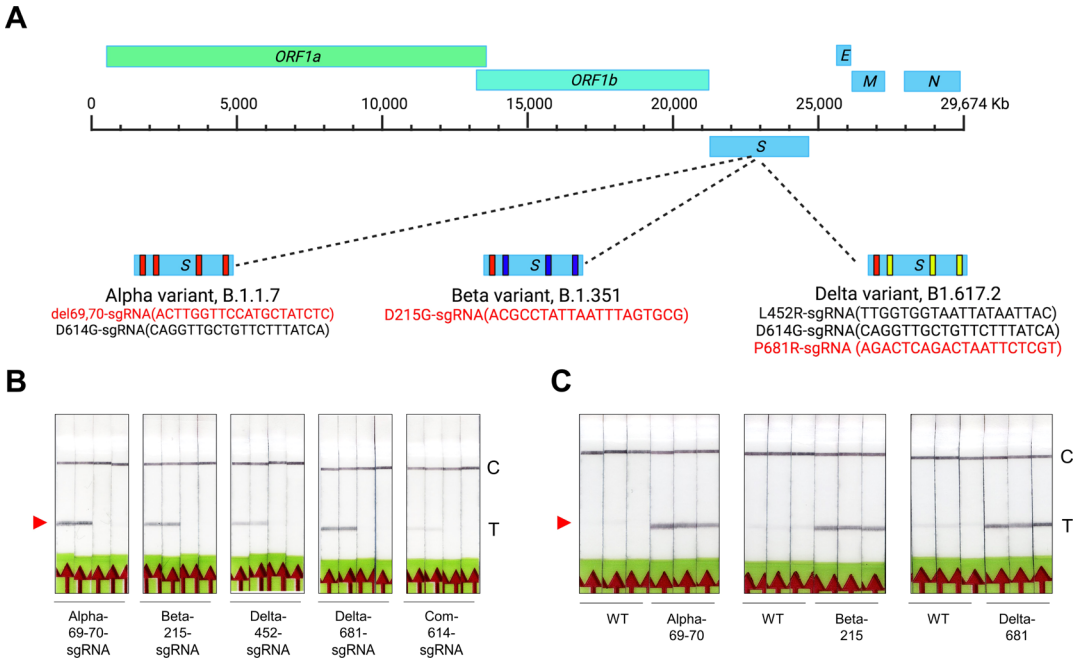

Figure 6. Bio-SCAN efficiently detected variants of SARS-CoV2. (A) Representative genome of α, β, and δ variants of SARS-CoV-2. The *spike* gene sequence of the α, β, and δ variants was selected for sgRNA design. Sequences of the sgRNAs designed for each variant detection are presented under each variant. sgRNAs del69-70-sgRNA (α), D215G-sgRNA (β), and P681R-sgRNA (δ) efficiently detecting the variant sequence are shown in red. (B) Confirmation of sgRNAs for precise detection of the SARS-CoV-2 variant sequence. FAM-labeled primers were used to PCR-amplify the variant sequence-specific target flanking DNA. The PCR-amplified FAM-labeled DNA fragment was subjected to Bio-SCAN-based detection. (C) SARS-CoV-2 variant detection via Bio-SCAN. Variant-specific in vitro-transcribed RNA was used as a template in the RT-RPA-based amplification of the target sequence. The RT-RPA-amplified FAM-labeled fragments were subjected to Bio-SCAN-based detection. SARS-CoV-2 synthetic RNA was used as the control with each variant. The red arrowhead indicates the specific detection of each variant (band appearance at T, test line) in C (control line). Green arrows indicate the flow direction.

being sufficient for reliable detection.[54] Taken together, our results show that Bio-SCAN represents a highly specific and sensitive bio-dCas9-based LFA protocol that could be adopted for rapid POC testing.

Bio-SCAN Reagents Are Compatible with POC Storage Conditions. Maintenance of cold chain transportation and storage conditions of detection reagents are major concerns for POC diagnostics. To test whether Bio-SCAN reagents require cold storage (Figure 5A), we tested their stability under multiple conditions. Specifically, we stored Bio-dCas9 protein at room temperature and 4 and −20 °C and performed Bio-SCAN assay every 3 days for 18 days. As shown in Figure 5B, Bio-dCas9 remained active when stored at room temperature, at 4 °C, and at −20 °C up to 18 days without compromising the detection of nucleic acids (Figure 5B).

As RNA molecules are prone to degradation, we used sgRNAs from a stock that had been stored at −80 °C for all the abovementioned experiments, but deep freezers may not be available at POC locations. When complexed with Cas9, sgRNAs have recently been reported to be protected from nucleases and remain stable for a longer time. Accordingly, we determined whether storing Bio-dCas9−sgRNA (RNP) complexes increased the shelf life of sgRNAs and Bio-SCAN reagents under storage conditions compatible with POC diagnostics. RNP (Bio-dCas9 with sgRNA) was stored at room temperature, 4 °C, or −20 °C. Detection of SARS-CoV-2 RNA on lateral flow strips indicated that the Bio-dCas9−sgRNA complex remained active up to 24 days without compromising sensitivity (Figure 5C). Overall, the Bio-SCAN reagents remained active for longer time, and only a slight decrease in the band intensity at the T line was observed when Bio-dCas9 or the RNP complex was stored at room temperature.

Lyophilization preserves the activity of proteins and RNP complexes compared to liquid-phase storage. We therefore also tested the stability of lyophilized Bio-dCas9 complexed with the sgRNA, followed by storage at room temperature, 4 °C, or −20 °C and performed Bio-SCAN assay every week. As shown in Figure 5D, the RNP complex remained stable for over 1 month when stored at room temperature, 4 °C, and −20 °C. We previously showed that the Vigilant assay reagents remained active for 1 week when stored at room temperature;[39] by comparison, Bio-SCAN reagents remained active for more than 18 days and the lyophilized reagents can be stored for at least 1 month at room temperature. These results demonstrated the extended shelf life of Bio-SCAN reagents and hence endorse the feasibility of implementing the Bio-SCAN platform for pathogen detection at POC locations.

Bio-SCAN Is Compatible with the Quick Extraction Protocol. Nucleic acid extraction from collected samples via conventional methods is a bottleneck in POC diagnosis. Recent studies reported simpler methods of cell membrane lysis by chemical or physical methods[55,56] that were compatible with RPA for the identification of a target sequence.[57] We sought to assess the potential of Bio-SCAN for the detection of target sequence amplified via RT-RPA from nucleic acids isolated by quick extraction methods. As a proof of concept, we selected human *RNAseP* as the target sequence for Bio-SCAN detection using the quick extraction protocol. Saliva samples were collected and treated with a quick extraction buffer and

https://doi.org/10.1021/acssynbio.1c00499
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heated to 95 °C to release total RNA. The extracted samples (4 μL) were then subjected to RT-RPA using human *RNAseP*-specific FAM-labeled primers. After the completion of RT-RPA, we used 5 μL of the RT-RPA product for Bio-SCAN-based detection of human *RNAseP*, which showed a band at the T line when using a specific sgRNA but not a nonspecific sgRNA (Figure 5F). We also spiked synthetic SARS-CoV-2 RNA (10,000 copies/μL) in human saliva samples and extracted total RNA with our quick extraction protocol, and the samples were subjected to RT-RPA and Bio-SCAN protocol for SARS-CoV-2 target detection using the *ORF1b*-specific sgRNA and primer set. Bio-SCAN efficiently detected the SARS-CoV-2 genome when spiked into human saliva samples (Figure 5G). Our results demonstrated that the Bio-SCAN protocol is compatible with quick extraction methods and can detect target nucleic acids amplified from unextracted samples. These results show that the Bio-SCAN platform fulfills most of the pathogen diagnostic requirements for POC setups and can be adopted under low-resource conditions.

Mass testing, tracking, tracing, and isolation of infected individuals is instrumental to limit pandemics,[3] but bulk testing at POC locations requires affordable methods. Bio-SCAN can be assembled locally for less than $10 per test (Figure S6). None of the current diagnostic methods are amenable to mass testing, particularly in POC settings, as these methods require centralized highly equipped laboratories and skilled technical staff;[4] they also do not fulfill WHO-recommended ASSURED guidelines for in-field POC detection.[6] The existing methods are also generally not affordable for mass testing. We believe that the Bio-SCAN platform has potential for rapid mass screening at low cost, with good shelf life for the needed reagents, and is compatible with quick RNA extraction protocols. These features make the Bio-SCAN platform a suitable candidate for POC diagnosis of pandemic pathogens.

Bio-SCAN Efficiently Detects Specific Variants of SARS-CoV-2. The emergence of the new SARS-CoV-2 variants severely impacted the healthcare systems worldwide. Indeed, new SARS-CoV-2 variants are more infectious, more contagious, and more replicative, raising a global concern over the efficacy of available vaccines. The WHO has given these variants of concern simple and unique identifiers: α (originally named B.1.1.7, first isolated in the UK), β (B.1.351, first isolated in South Africa), gamma (P.1, first isolated in Brazil and Japan), and δ (B1.617.2, first isolated in India). A detection method should be able to identify infected individuals regardless of the variant in question. However, identifying the specific variant infecting an individual would provide important information for public health measures.

The Cas9−sgRNA complex specifically recognizes its target DNA sequence via sgRNA complementarity and the presence of a protospacer adjacent motif (PAM, NGG sequence). The lack of the PAM or mismatches between the sgRNA and its complementary target sequence (target seed sequence) preceding the PAM is not tolerated by Cas9−sgRNA complexes, providing the means to efficiently differentiate between SARS-CoV-2 variants with up to single base resolution. To test the feasibility of detecting SARS-CoV-2 variants via Bio-SCAN, we selected the mutations present in the spike polypeptide that are responsible for increasing infectivity and the global spread of COVID-19. We then designed five sgRNAs with specific focus on PAM or seed region of the target sequence of the prevalent variants: α (deletions of two amino acid residues 69 and 70), β (D215G), δ (L452R, P681R), and D614G (common to α, β, and δ variants) (Figure 6A). α-del 69,70-sgRNA has six nucleotides difference in the protospacer seed sequence with the wild type. β-D215G and L452R mutations have new PAMs for β-215-sgRNA and δ-452-sgRNA, respectively, which are absent in the wild type. D614G, which is common in α, β, and δ variants, has new PAM and one nucleotide difference with the wild-type sequence in the seed region. P681R has one nucleotide difference in the sgRNA-binding seed sequence compared to the wild strain isolated first in Wuhan, China. First, we confirmed the efficiency of the designed sgRNAs via the Bio-SCAN platform to detect variant-specific sequences in FAM-labeled PCR-amplified *S* gene DNA containing the variant-specific mutations. Our results demonstrated that out of five sgRNAs, three, α69-70-sgRNA, β-215-sgRNA, and δ-681-sgRNA, efficiently detected variant sequences compared to δ-452-sgRNA and Com-614-sgRNA (Figure 6B). The wild-type SARS-CoV-2 sequence was used as the control. We next employed Bio-SCAN to identify the SARS-CoV-2 variants using in vitro-transcribed synthetic RNA containing the variant target sequence as a template for RT-RPA. As shown in Figure 6C, Bio-SCAN detected each variant sequence specifically without any discernible cross-reactivity as evidenced by the lack of a band at the T line when using a synthetic wild-type SARS-CoV-2 RNA sequence as the control. These results highlight the potential of adopting Bio-SCAN for the detection of current and potentially emerging SARS-CoV-2 variants. The three-nucleotide PAM (NGG) and proximity of the seed sequence to PAM are well-defined for Cas9, and incorporating the mutated target sequence corresponding to each variant in the sgRNA seed sequence or PAM is easier for Cas9 than for Cas12 or Cas13.[37,58,59] Taken together, these results show that the Bio-SCAN platform can be used to predict the emerging trends and prevalence of specific variant(s) in a specific sample collection from a given population or region. Moreover, our simplified verification procedure would allow local administration authorities to mitigate the spread of virulent variants by ordering or lifting containment.

Bio-SCAN represents a simple diagnostic protocol that requires heating blocks and consumables such as tubes, strips, pipets, and tips. We anticipate that Bio-SCAN can be run on a simple device equipped with a sample pad, microfluidic chip, isothermal block for RT-RPA, and lateral flow strip to perform the entire reaction and interpret the results automatically. Another bottleneck is laboratory-based RNA isolation. We showed that Bio-SCAN efficiently detects human *RNaseP* and SARS-CoV-2 RNA spiked-in saliva samples and isolated with a quick extraction buffer. While we have no access to untreated clinical saliva samples, coupling of Bio-SCAN with clinical saliva samples and quick RNA extraction protocols will convert Bio-SCAN into a quick and robust POC diagnostic platform.

CONCLUSIONS

Bio-SCAN fulfills the basic features of the WHO-recommended ASSURED guidelines and complies with most requirements for a POC diagnostic method, with results readable by the naked eye. Most Bio-SCAN reagents can be assembled locally in resource-limited settings at low cost, thus overcoming the disruption of supply chains caused by pandemics. Notably, Bio-SCAN detected SARS-CoV-2 with a clinically acceptable sensitivity that validates its potential for mass screening in nonlaboratory POC settings. The RNA genome of SARS-CoV-2 is prone to mutations, as attested by

https://doi.org/10.1021/acssynbio.1c00499
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the multiple variants in circulation, which pose a severe risk to global public health. Bio-SCAN can precisely detect specific variants, thus allowing the prediction of trends or the identification of the prevalent SARS-CoV-2 variant(s) upon mass screening. In addition, Bio-SCAN can be quickly adapted for newly emerging variants. Taken together, Bio-SCAN is an accurate, sensitive, and powerful technology to detect pathogen-derived nucleic acids specifically and quickly *en masse* in nonlaboratory settings.

METHODS/EXPERIMENTAL SECTION

Plasmid Construction. Nuclease-dead Cas9 (dCas9) was used for target recognition, and AviTag-biotin was used to immobilize the target nucleoprotein complex on lateral flow strips for visual detection via the Bio-SCAN protocol. A DNA fragment (*AgeI-AviTag-T7-Terminator-spacer-T7-Promoter-LacO-BirA-XhoI*) was synthesized as a g-block from Integrated DNA Technologies (IDT) to place the coding sequence of the *AviTag* in-frame and downstream of the *dCas9* sequence and to add the *BirA* (for biotinylation of the AviTag) expression cassette on a single plasmid. The g-block was PCR-amplified with specific primers; the resulting PCR amplicon was digested with AgeI and XhoI restriction enzymes and cloned into pET28a-dCas9 linearized with the same restriction enzymes. The resulting plasmid pET28a-dCas9-AviTag-BirA was confirmed by restriction enzyme digestion and Sanger sequencing. pET28a-dCas9-AviTag-BirA drives the expression of both *dCas9-AviTag* and *BirA* individually from individual T7 promoters. The sequence of pET28a-dCas9-AviTag-BirA is provided in the Supporting Information; all primers used in this study are provided in Table S1.

Protein Purification. *E. coli* strain BL21 (DE3) (NEB) was used for the production and biotinylation of dCas9-AviTag. Briefly, the pET28a-dCas9-AviTag-BirA plasmid was introduced into BL21 (DE3) cells. Cells were grown in 4 L of 2XYT medium containing 50 mg/L kanamycin sulfate at 37 °C until the cultures reached an OD_{600} of 0.7. Biotin (100 μM, 22.4 mg/L) was then added, and dCas9-AviTag production was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG, 120 mg/L) for 16 h at 18 °C. The cell pellet was collected by centrifugation at 6000*g* for 20 min and resuspended in 3 mL per 1 g of wet cells in lysis buffer (50 mM Tris−HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.1% [v/v] NP-40, 1 mM PMSF, 5% [v/v] glycerol, and EDTA-free protease inhibitor cocktail tablet/50 mL [Roche, UK]). Cells were lysed enzymatically by adding 2 mg/mL lysozyme, followed by mechanical lysis by sonication (5 s on, 10 s off) for 4 min. The lysate was clarified by centrifugation at 18,000*g* for 15 min. The biotinylated dCas9-AviTag protein was purified on an AKTA Pure system with HisTrap HP 5 mL affinity columns (GE Healthcare) with Buffer A (50 mM Tris−HCl pH 8.0, 300 mM NaCl, and 20 mM imidazole) and Buffer B (50 mM Tris−HCl pH 8.0, 300 mM NaCl, and 300 mM imidazole). The peak fractions were pooled and concentrated to 1 mL and loaded onto the Acta Pure system with a HiLoad Superdex 16/600 200 pg gel filtration column (GE Healthcare) pre-equilibrated with gel filtration buffer (20 mM Tris−HCl pH 7.5, 200 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10% [v/v] glycerol). Protein concentration was determined on a Nanodrop spectrophotometer, and the eluted fractions were collected, concentrated, flash-frozen, and stored at 80 °C.

Confirmation of dCas9-AviTag Biotinylation by Immunoblot. Purified dCas9-AviTag (0.148 μM) was diluted 20 times, and different volumes (1.75; 2.5; 5; and 10 μL) was mixed with 2× loading dye and heated at 95 °C for 3 min. The proteins were then separated on 10% NuPAGE (Invitrogen) for 1 h in a running chamber placed on ice. Next, the proteins were transferred to a nitrocellulose membrane for 1 h in a protein transfer chamber at 4 °C. The membrane was incubated in a blocking buffer for 1 h at room temperature before immunodetection with anti-biotin (mouse) primary antibody (dilution 1:1000; Cat. no. SC-53179, Santa Cruz Biotechnology) and anti-mouse secondary antibody (dilution 1:2000; Cat. no. A3688, Sigma) and detected with chemiluminescent ECL solution (BioRad) using a Gel Doc XR system (BioRad). The signals were photographed with colorimetric settings as the control for size determination of the detected proteins.

In Vitro Transcription of sgRNAs. For sgRNA expression, DNA fragments (T7-promoter-20-nt target recognition and Cas9 binding scaffold) were synthesized as g-blocks (IDT). g-blocks were PCR-amplified and 1 μg of DNA was subjected to in vitro transcription using a HiScribe T7 Quick, high-yield RNA synthesis kit (E2050S, NEB) following the manufacturer's instructions. Briefly, 10 μL of NTP buffer mix, 2 μL of T7 RNA polymerase mix, and 1 μL of RNase Out (100000840, Invitrogen) were mixed with 1 μg of DNA and DEPC-treated water in a total reaction volume of 30 μL. The reaction mixture was incubated at 37 °C for 8 h. In vitro-transcribed RNA was purified using a Direct-zol RNA MiniPrep Kit (Zymo Research). Production of the proper-sized sgRNA fragments was confirmed on a 2% agarose gel run in 1× Tris borate EDTA (TBE) buffer. The sequences of g-blocks (pT7-sgRNAs) and primers used for PCR amplification are provided in Table S1.

Confirmation of the sgRNA Activity by In Vitro Digestion of Target DNA. Target DNA fragments were PCR-amplified with specific primer sets (Table S1). Each target amplicon (300 ng) was then incubated at 37 °C with 250 nM sgRNA and 250 nM Cas9 for 30 min. dCas9 alone was used as a control. Samples were treated with 1 μL of proteinase K for 30 min at 40 °C and heated to 95 °C for 10 min to stop the reactions. The DNA fragments were separated on 2% agarose gels. Sequence primers used for PCR amplification are provided in Table S1.

Target DNA (PCR Product) Detection via Bio-SCAN Assay. Target DNA was amplified by PCR using FAM-labeled primers (Table S1). The reporter complex (final concentration of 100 ng of target DNA, 250 nM each of dCas9-bio or dCas9 [control] and sgRNA) was prepared by combining 8 μL of 0.15 μM stock of dCas9-bio or dCas9 (control), 2.5 μL of 5 μM sgRNA, and 5 μL of 10× NEB Buffer 3.1 in a 50 μL reaction, with ultrapure water up to 50 μL. The reaction mixture was incubated at 37 °C for 5 min followed by 2 min at 60 °C. Next, 50 μL of dip stick assay buffer (HybriDetect Milenia biotec) was added to each tube, mixed, and briefly centrifuged to collect the reagents at the bottom of the tube. HybriDetect Dipsticks equilibrated to room temperature were placed into each tube. Lateral flow strips were removed from the tube as soon as the control band appeared and the result was called within 2 min thereafter. Images of the strips were taken within 5 min after the beginning of the LFA. Interpretation of lateral flow results was performed as follows: two bands, one close to the sample pad (test band) and one at

https://doi.org/10.1021/acssynbio.1c00499
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the top (control band) indicated a positive result; a single band close to the top of the strip (control band) represented a negative result; and only a single band (test band) indicated an invalid result.

Coupling of Bio-SCAN Detection Assay with RT-RPA (on Synthetic SARS-CoV-2 Genome). RT-RPA was performed using the TwistAmp basic kit following the manufacturer's instructions. Briefly, a well-mixed 47.5 μL reaction mixture (1 μL of 25 μM FAM-labeled forward and 1 μL of 25 μM unlabeled reverse primers, 29.5 μL of rehydration buffer, 0.5 μL of SuperScript IV reverse transcriptase, 0.5 μL of RNase H, 0.5 μL of RNase Out, and 10.2 μL of H_20) was added to the lyophilized RPA reaction components (TwistAmp Basic) and homogenized by pipetting and vortexing. The mixture was then divided into two tubes (for two replicates per sample). Magnesium acetate (2 μL of 280 mM) was added to each tube and mixed. Next, 4 μL (1000 copies/reaction of synthetic SARS-CoV-2 genome) or 4 μL of clinical samples was added to each tube. Isothermal amplification was performed at 42 °C for 15 min. To confirm DNA isothermal amplification, 10 μL of each reaction was purified using a QIAquick PCR purification kit and separated on a 1.5% agarose gel.

For detection, 5 μL of the RPA product was incubated with 250 nM either Bio-dCas9 or dCas9 (control) and sgRNA (8 μL of 0.15 μM stock of dCas9-bio or dCas9 [control], 2.5 μL of 5 μM sgRNA, 5 μL 10× NEB Buffer 3.1 in a 50 μL reaction, and ultrapure water up to 50 μL). The reaction mixture was incubated at 37 °C for 5 min, followed by 2 min at 60 °C. Next, 50 μL of dip stick assay buffer (HybriDetect Milenia biotec) was added to each tube, mixed, and briefly centrifuged to collect the reagents at the bottom of the tube. LFA was then performed as described above with the same interpretation of results within 2 min with visual observation.

LOD Determination of the Bio-SCAN Assay. SARS-CoV-2 RNA (synthetic RNA from IDT) was diluted to final concentrations corresponding to 0, 0.4, 02, 04, 20, 40, and 400 copies/μL equal to 0, 10, 50, 100, 500, 1000, and 10,000 copies/reaction. Each RNA sample (4 μL) was added to RT-RPA reactions. Nuclease-free water was used as the negative control. Following the RT-RPA reaction, 5 μL of the product was incubated with 250 nM sgRNA and 250 nM bio-dCas9 in 1× NEB Buffer 3.1 and nuclease-free water in a 50 μL final volume. The reaction mixture was incubated at 37 °C for 5 min, followed by 2 min 60 °C. Next, 50 μL of dip stick assay buffer (HybriDetect Milenia biotec) was added to each tube, mixed, and briefly centrifuged to collect the reagents at the bottom of the tube. LFA was then performed as described above with the same interpretation of results. The detection limit was set as the number of RNA copies that could be successfully detected within 2 min of the LFA in all three replicates.

Validation of the Bio-SCAN Protocol with SARS-CoV-2 Clinical Samples. RNA samples from SARS-CoV-2 real-time reverse transcription PCR (RT-PCR)-positive (106 clinical samples) individuals were used for evaluation of our Bio-SCAN. RNA (4 μL) was added to the RT-RPA reaction, and 5 μL of the amplified product was used for detection in the next step.

SARS-CoV-2 Variant Identification in Clinical Samples. For a variant-specific sequence, gblock of the *S* gene containing α (deletions of residues 69 and 70), β (D215G), δ (P481R), δ (L452R), and D614G (common to α, β, and δ variants) with a T7 promoter was custom-synthesized from IDT. The gblock DNA fragment was PCR-amplified and 1 μg of DNA was subjected to in vitro transcription using a HiScribe T7 Quick, high-yield RNA synthesis Kit (E2050S, NEB) following the manufacturer's instructions. Briefly, 10 μL of NTP buffer mix, 2 μL of T7 RNA polymerase mix, and 1 μL of RNase Out (100000840, Invitrogen) were mixed with 1 μg of DNA and DEPC-treated water in a total reaction volume of 30 μL. The reaction mixture was incubated at 37 °C for 8 h. In vitro-transcribed RNA was purified using a Direct-zol RNA MiniPrep Kit (Zymo Research). Production of the proper-sized sgRNA fragments was confirmed on a 2% agarose gel run in 1× Tris borate EDTA (TBE) buffer. The sequences of gblocks (pT7-*S* gene, variant) and primers used for PCR amplification are provided in Table S1. We next employed Bio-SCAN to identify the SARS-CoV-2 variant.

RT-PCR for Detection of Positive SARS-CoV2 RNA Samples. RT-PCR was conducted using the oligonucleotide primer/probe (Integrated DNA Technologies, catalog #10006606) and a Superscript III one-step RT-PCR system with Platinum Taq Polymerase (catalog #12574-026) as per the manufacturer's protocol on the extracted RNA sample. The RNA sample for SARS nCoV 2019 considered as positive when the cycle threshold (Ct) value for both *E* and *N* genes was ≤36 and negative when the Ct value was more than >36 for both genes.

Rapid RNA Extraction for Bio-SCAN-Based Target Detection. Saliva, 50 μL, was mixed with 50 μL of Quick Extract DNA extract solution (QE09050, Lucigen). The mixture was incubated at 95 °C to release RNA and deactivate the nucleases and proteases. The sample, 4 μL, was subsequently used for Bio-SCAN-based detection. For SARS-CoV-2 spiking in saliva, 10,000 copies of the synthetic RNA was mixed into 50 and 50 μL of Quick Extract DNA extract solution. The sample, 4 μL, was used for Bio-SCAN-based SARS-CoV-2 RNA detection.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00499.

Purification of Bio-dCas9; sgRNA activity confirmation; LOD confirmation for Bio-SCAN; Bio-SCAN-based detection of SARS-CoV-2 in clinical samples; RT-qPCR analysis for SARS-CoV-2 detection in clinical samples; Bio-SCAN diagnostics price estimation; and primers and nucleic acid used in this study (PDF)

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https://doi.org/10.1021/acssynbio.1c00499
ACS Synth. Biol. XXXX, XXX, XXX−XXX

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Author Contributions

M.M. and Z.A. conceived the research. M.M. and Z.A. designed the research. Z.A. and E.S. built and performed the research. M.T. and S.H. generated and contributed reagents. A.M., T.M., R.A., F.S.A., M.A., A.M., Z.A., and E.S. contributed and conducted the clinical validation. M.M. and Z.A. wrote the paper with input from all authors.

Funding

This work was supported, in part, by the Smart Health Initiative at KAUST and the IAF grant from the KAUST IED to M.M.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to thank F.S.A. and her team at the Department of Infection and Immunity, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia, for providing RNA from SARS-CoV-2 clinical samples. We also thank members of the genome engineering and synthetic biology laboratory for insightful discussions and technical support.

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