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Production and Performance Assessment of a Severe Acute Respiratory Syndrome Coronavirus 2 Biomimetic in a Verification Program for Pandemic Readiness

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During the early stages of the 2019 coronavirus disease (COVID-19) pandemic in South Africa, one of many challenges included availability of control material for laboratory proficiency testing programs. Proficiency testing control material using live severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or RNA extracted from cell culture was either biohazardous or costly, particularly in resource-limited settings. This study reports the development and application of a noninfectious SARS-CoV-2 biomimetic *Mycobacterium smegmatis* strain that mimics a positive result in the GeneXpert SARS-CoV-2 Xpert Xpress cartridge. Nucleotide sequences located in genes encoding the RNA-dependent RNA polymerase, the nucleocapsid, and the envelope proteins were used. The resulting biomimetic strain was prepared as a positive proficiency testing control and distributed in South Africa for verification of laboratories before their testing of clinical specimens. Between April and December 2020, a total of 151 GeneXpert instruments with 2532 modules were verified to bring COVID-19 mass testing in South Africa online. An average concordance of 98.6% was noted in the entire laboratory network, allowing identification of false-positive/false-negative results and instrument errors. This noninfectious, easily scalable proficiency testing control material became available within 2 months after the start of the pandemic in South Africa and represents a useful approach to consider for other diseases and future pandemics. *(J Mol Diagn 2023, 25: 907—912; https://doi.org/10.1016/j.jmoldx.2023.08.007)*

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of 2019 coronavirus disease (COVID-19), was first described in December 2019, subsequently progressing to a global pandemic (Figure 1). It was initially designated as 2019 novel coronavirus but subsequently renamed.[1–6] It is a *β*-coronavirus with a single-stranded RNA genome of 29,903 nucleotides (Strain Wuhan-Hu-1; GenBank accession number MN908947.3, *https://www.ncbi.nlm.nih.gov/nuccore*). Early during the pandemic, it became evident that effective control required fast and accurate diagnosis, as the inability to rapidly identify infected/diseased individuals fueled further transmission. To detect the SARS-CoV-2 genome in clinical specimens requires nucleic acid amplification technologies. Sequence information and assay methods for early diagnostic assays were made publicly available to assist global health systems with the implementation of pandemic control measures.[7]

Global deployment of nucleic acid amplification technologies, particularly in resource-limited settings, was difficult at the time because of constraints on tools, materials, trained technicians, and laboratory resources.[8] The lack of robust, consistent proficiency testing (PT) controls to implement and verify the performance of these emerging SARS-CoV-2 diagnostics was a significant barrier to reliable mass testing. At the time, PT control materials were

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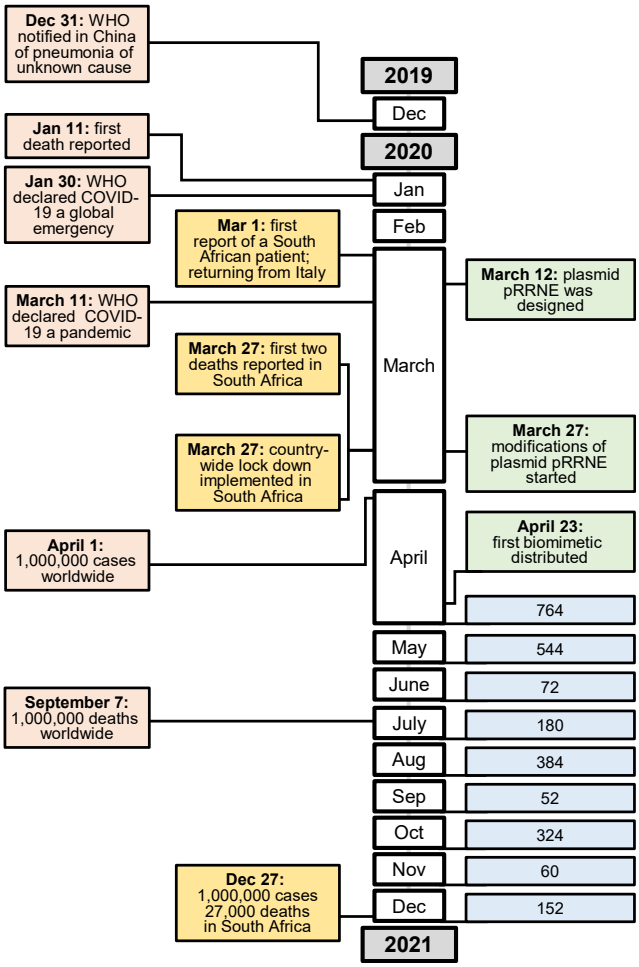


Figure 1 Timeline of the 2019 coronavirus disease (COVID-19) pandemic. The first column in pink indicates the worldwide progression of clinical cases and response from the World Health Organization (WHO). The second column in orange indicates the progression of clinical cases in South Africa and the response from the South African National Department of Health. The third column shows the dates from December 2019 to December 2020 (not to scale). The fourth column shows the progress of plasmid and strain development in green, and the number of GeneXpert modules verified in each month between April and December 2020 in blue. This illustrates the rapid development of biomimicry approaches to enable rollout of mass COVID-19 testing.

either clinical specimens with a confirmed positive result or purified viral RNA from cell culture. This was problematic and costly because of inherent biohazard concerns, instability of RNA, the need for cold chain storage, and/or limited availability.

We previously reported the use of modified, noninfectious derivative strains of *Mycobacterium smegmatis* for use in instrument verification and external quality assurance in tuberculosis nucleic acid amplification technology diagnostics programs, which faced similar challenges with respect to biologically safe PT controls.[9] These biomimetic strains were able to mimic positive tuberculosis diagnosis on two distinct platforms: GeneXpert technology (Cepheid, Sunnyvale, CA) and the Hain Lifescience line probe assay (Hain Lifescience, Nehren, Germany). In addition, this approach also demonstrated utility for detection of a *Staphylococcus aureus* target sequence on the GeneXpert platform. On the basis of the sequence of genome targets available during 2020 at the time of the COVID-19 outbreak, this biomimetic approach was adapted to generate noninfectious PT control material that was easily scalable in South Africa. It was used by SmartSpot Quality (Pty) Ltd. (Johannesburg, South Africa) to generate PT controls in its verification procedure, which was implemented in 2020 to verify the competency of laboratories to test for SARS-CoV-2. This approach can easily be adapted for a variety of established or emerging diseases, and it holds promise for future pandemics because of its rapid design and production capabilities.

Materials and Methods

Culture Conditions

All strains and plasmids used and generated in this study are listed in Table 1.[10,11] Strain *Escherichia coli* DH5α was grown at 37°C shaking in standard lysogeny broth or on Luria agar, supplemented with 50 μg/mL of kanamycin where applicable. *M. smegmatis* strains were grown at 37°C shaking in Difco Middlebrook 7H9 liquid medium (Becton, Dickinson and Company, Sparks, MD) supplemented with 0.085% NaCl, 0.2% glucose, 0.2% glycerol, and 0.05% Tween-80 or on Difco Middlebrook 7H10 solid medium (Becton, Dickinson and Company) supplemented with 0.085% NaCl, 0.2% glucose, and 0.5% glycerol. Kanamycin was used at 25 μg/mL.

Design of Shuttle Vectors and Biomimetic Strains

The complete genomic organization of SARS-CoV-2 is shown in Figure 2A, and the selected target regions for generating the biomimetic strain are shown in Figure 2B. Plasmid pRRNE was purchased commercially and carried the target sequences for the regions in the SARS-CoV-2, internal to the genes encoding the RNA-dependent RNA polymerase (RdRP1 and RdRP2), the nucleoprotein (N1-N3-N2), and the envelope protein (E). These sequences were made publicly available in January 2020.[12] pRRNE-ori was introduced into *M. smegmatis*, in combination with a mycobacterial origin of replication by standard electroporation method,[10] to yield strain mc^{2}155-rrne as a positive PT control. *M. smegmatis* strain mc^{2}155-SARSneg, lacking the target sequences for the SARS-CoV-2 cartridge, was used as the PT negative control.

Distribution of PT Controls

Before distribution, PT controls were validated at a central laboratory. They were tested on 2 independent days by two separate individuals to ensure consistency of the material. The amounts of biomimetic material included in a positive PT control were adjusted to yield cycle threshold (C_T)

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Table 1 Plasmids and Bacterial Strains Generated or Used in this Study

Plasmid name	Description	Reference
pRRNE*	Commercial plasmid bearing targets for RdRP1, RdRP2, N protein, and E protein of SARS-CoV-2, *aph*	Thermo Fisher Scientific, GeneArt GmbH (Regensburg, Germany)
pYUB12	*Escherichia coli* and mycobacterial shuttle vector, oriM from *Mycobacterium fortuitum* plasmid pAL5000, *aph*	10,11
pRRNE-ori	pRRNE with the mycobacterial origin of replication of plasmid pAL5000; targets for RdRP1, RdRP2, N protein, and E protein of SARS-CoV-2, *aph*	This work
Strains	**Description**	**Reference**
Escherichia coli		
DH5-α	*fhuA2 lac(del)U169 phoA glnV44 Φ80′ lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Promega (Madison, WI)
Mycobacterium smegmatis		
mc^2155	*ept*-1; High-frequency transformation mutant of *M. smegmatis* ATCC 607 (Manassas, VA)	10
mc^2155-rrne	Derivative of mc^2155 bearing shuttle plasmid pRRNE-ori; targets for RdRP1, RdRP2, N protein, and E protein of SARS-CoV-2, Kan	This work
mc^2155-SARSneg	Derivative of mc^2155 bearing an integrating plasmid with no SARS-CoV-2 targets, Kan	This work

*Targets for RdRP1, RdRP2, the N protein (N1-N3-N2), and E protein were all included on the plasmid; however, the GeneXpert SARS-CoV-2 Xpert Xpress cartridge tests only for N2 and E.

E, envelope protein; Kan, kanamycin; N, nucleoprotein; RdRP, RNA-dependent RNA polymerase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

values of 35 ± 2 for target E and 38 ± 2 for target N2. Verification panels contained two PT controls each, one positive and one negative (Figure 3). One verification panel was distributed to each GeneXpert instrument in laboratories enrolled in the SmartSpot Quality SARS-CoV-2 verification program.

Data Collection and Analysis

Each affiliated laboratory exported the test results from its GeneXpert instrument via a cloud-based interface to SmartSpot Quality (Pty) Ltd. (Figure 3). The results were auto-analyzed on SSQmonitor, and verification reports were released in real time. Where results were not concordant, reports were held back for review before report finalization. Where warranted, C_T values were analyzed to facilitate accurate result scoring and to identify and address potential root causes for incorrect results.

Results

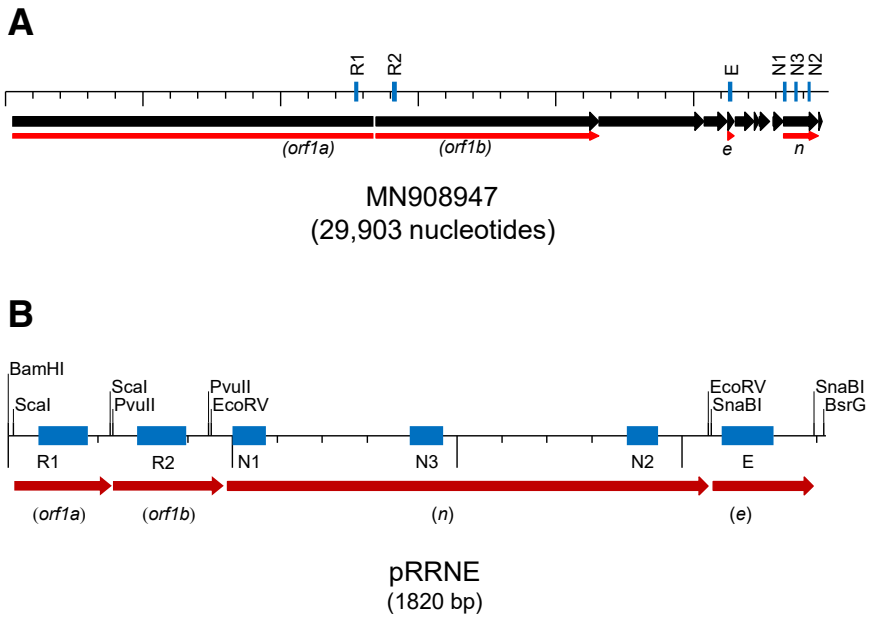
Test Result of SARS-CoV-2 Xpert Xpress Analysis

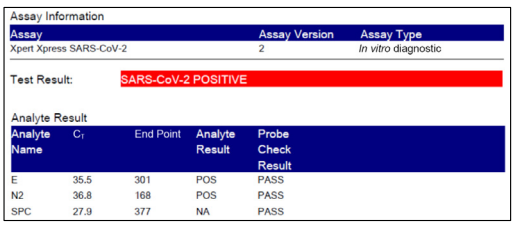
PT controls were validated in the SARS-CoV-2 Xpert Xpress cartridge (GeneXpert Dx software version 4.7b) before launching the control in testing laboratories. They yielded the expected result of positive (Figure 2C), based on the presence of the E and N2 targets at the correct range of C_T values 35 ± 2 for target E, and 38 ± 2 for target N2. PT negative controls with C_T values of 0.0 for both E and N2 yielded the expected result of negative (Figure 2D).

Distribution of PT Control Panels

Verification panels were shipped to laboratories to bring GeneXpert modules online for testing, where one module is the unit in a GeneXpert machine that can test one cartridge at a time (Figure 3). The large demand for SARS-CoV-2 testing and reagents, together with lockdown regulations, limited access to resources, which were accordingly prioritized for patient testing. To minimize the number of cartridges required to verify a laboratory for SARS-CoV-2 testing, machines that were already enrolled for PT programs relating to other diseases, such as tuberculosis, received only one positive and one negative PT control for SARS-CoV-2 verification. Sixty-five laboratories across South Africa were enrolled in the verification program with a total of 151 instruments, which effectively comprised 2532 modules. A total of 702 controls were distributed, from which laboratories tested 349 positive PT controls and 350 negative PT controls. The data from these controls are reported in Table 2. Discrepancies in the numbers of PT controls submitted indicate that some laboratories did not test both PT controls (positive and negative) supplied within the verification panel. This was attributed to reagent shortages, human error in processing the PT control/s, and/or human error in submitting both results.

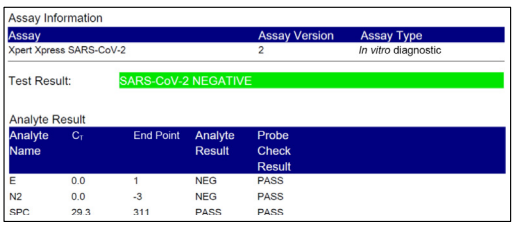
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C_T values in the correct range for E and N2

Expected positive result



No C_T values for N2 and E

Expected negative result

Figure 2 Nucleotide sequence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; GenBank; *https://www.ncbi.nlm.nih.gov/nuccore/MN908947.3*; accession number MN908947.3) and representative positive (POS) and negative (NEG) test results from the SARS-CoV-2 Xpert Xpress cartridge version, to illustrate the presence or absence of cycle threshold (C_T) values in the correct range. **A:** Genomic sequence of the entire single-stranded RNA coding molecule. Genes containing target sequence: *orf1a*, N-terminal coding region of RNA-dependent RNA polymerase; *orf1b*, C-terminal coding region of RNA-dependent RNA polymerase; *e*, gene encoding envelope protein; *n*, gene encoding nucleocapsid protein. Black, annotated genes; red, the genes in which target sequences are located; blue, the target locations published by Corman et al.[12] **B:** The sequence designed and purchased for this study, based on the targets indicated in **A**. Unique restriction enzyme recognition sequences were included, flanking each target. **C:** Positive result for the biomimetic positive proficiency testing control. **D:** Negative result for the negative proficiency testing control. NA, not applicable; SPC, sample processing control.

Results of PT Testing

The verification panels yielded 99.1% (346/349) correct positive results and 98.0% (343/350) correct negative results, with an average concordance of 98.6% (Table 2). The incorrect results submitted for the negative PT controls were as follows: 1.7% (6/350) reported as positive and 0.3% (1/350) reported as presumptive positive (a C_T value was obtained for analyte E and no C_T value was obtained for N2). The incorrect results submitted for the positive PT controls were as follows: 0.9% (3/349) reported as negative and 0.3% (1/349) reported as an instrument error.

Assessment of Laboratory Competency

To assess the proficiency of testing facilities, a GeneXpert system was deemed fit for purpose provided that all modules that were tested with the panel passed verification. If any module did not pass verification, a repeated PT control was tested.

Discussion

In this work, the utility of using a noninfectious biomimetic to generate biologically safe COVID-19 diagnostic PT controls, and integrating these into verification of a molecular diagnostic assay, was demonstrated. The resulting biomimetics were robust and easily scalable as PT control material for laboratories across an entire country. This approach enabled activation of 2532 GeneXpert modules for immediate SARS-CoV-2 mass testing. The application of this technology for SARS-CoV-2 can be applied to other diagnostic platforms, based on nucleic acid amplification

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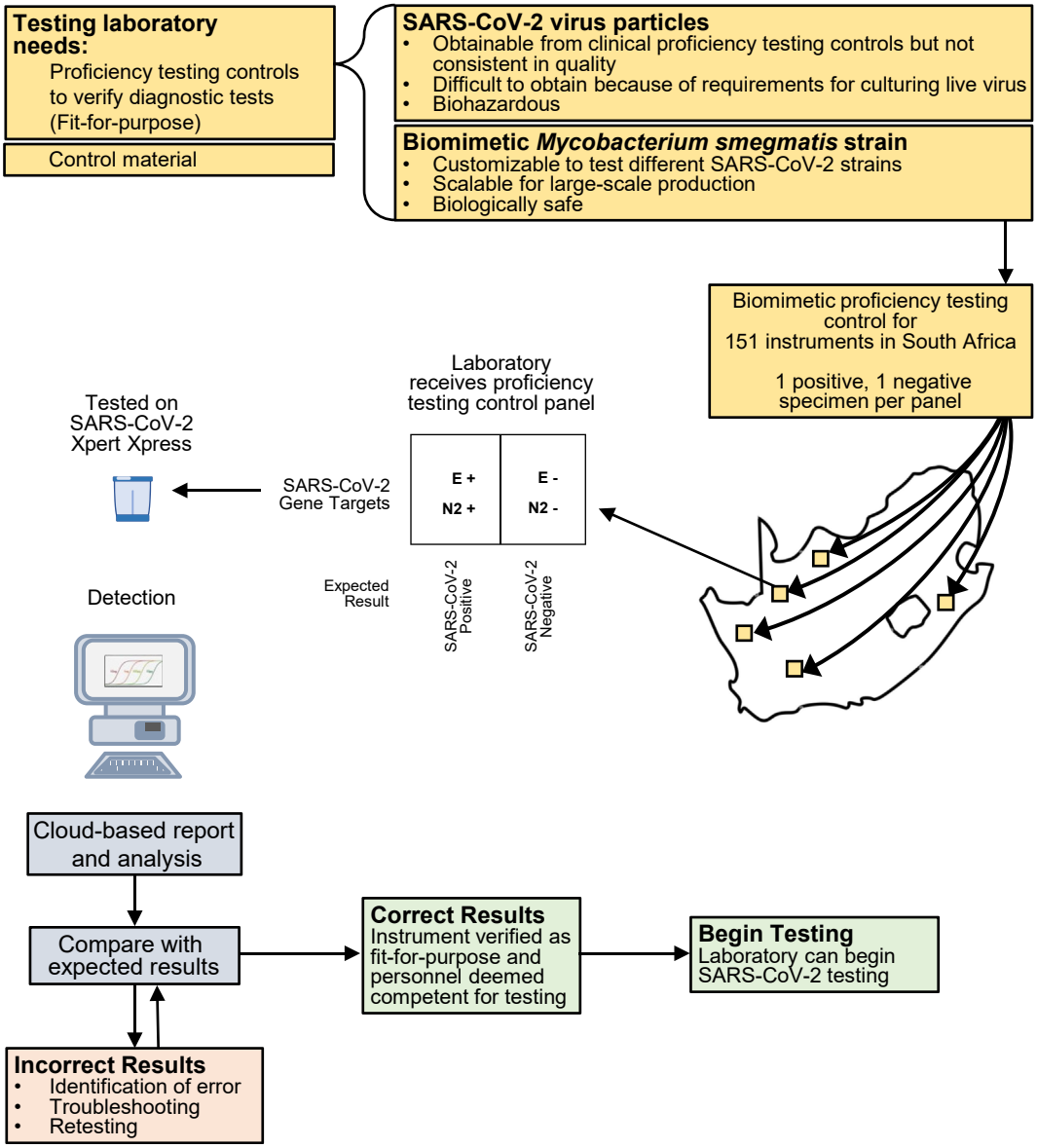


Figure 3 Distribution of proficiency testing controls for laboratory verification and analysis of test results. Verification panels were shipped to laboratories in South Africa on demand. Tests were performed on GeneXpert modules for detection in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Xpert Xpress cartridge. Incorrect results required assessment and troubleshooting to ensure that the source of errors was identified and corrected before bringing a laboratory online. E, envelope protein; N2, nucleoprotein.

technology approaches. However, conditions that ensure lysis of the bacterial biomimetic cell wall may require some optimization on other platforms. Given the robust results obtained in this study, this issue did not emerge in the GeneXpert platform.

In the field, SARS-CoV-2 Xpert Xpress performed well in side-by-side comparisons with other diagnostic platforms to detect the virus on clinical samples.[13,14] This is likely because of the high reliability of the SARS-CoV-2 Xpert Xpress assay, where the reverse transcription and PCR steps

Table 2 Test Result of Xpert Xpress SARS-CoV-2 Analysis

			Incorrect, *n* (%)			
Variable	PT controls tested, *N*	Correct, *n* (%)	Positive	Presumptive*	Negative	Instrument error
Positive PT control	349	346 (99.1)	NA	0 (0.0)	3 (0.9)	1 (0.3)
Negative PT control	350	343 (98.0)	6 (1.7)	1 (0.3)	NA	0 (0.0)

*A presumptive positive result indicates a test that only flagged positive for the *e* gene, which was indicative of any β-coronavirus, not only SARS-CoV-2.
NA, not applicable; PT, proficiency testing; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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are integrated and robust within a single cartridge. In this context, the biomimetic PT controls reported herein are DNA based and, as such, do not control for efficiency of the reverse transcription step.

A critical factor in the ease of implementation in the rollout of diagnostic capability in South Africa was that GeneXpert technology already had a large presence because of its extensive use for tuberculosis diagnosis.[15] For instrument verification, it was shown that the results obtained from the biomimetic *M. smegmatis* SARS-CoV-2 PT control material reliably mimic those expected from corresponding clinical specimens, based on the presence/absence of the targets included in the material. This approach can be easily adapted for emerging pathogens and future pandemics and negates the requirement to handle large-scale infectious specimens in PT programs. Given the increased regulation on growing pathogens in laboratories, this approach holds promise for bolstering expanded rollout of molecular diagnostics.

Author Contributions

E.E.M. and B.D.K. conceived the study and designed the biomimetic strain; E.E.M. performed laboratory procedures to generate mc^{2}155-rrne and mc^{2}155-SARSneg; and A.E.R. and D.E.S. produced proficiency testing control panels and led the distribution thereof, the data collection, and the data analysis of the study. All authors wrote the manuscript.

Disclosure Statement

B.D.K. and E.E.M. have filed a patent on this work on which there are no embargo restrictions (patent number US10774391B2) and receive a benefit share from SmartSpot Quality (Pty) Limited proceeds. D.E.S. and A.E.R. are employed by SmartSpot Quality (Pty) Limited.

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