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Evaluation of the RT-LAMP/CRISPR-Cas12 diagnostic method for SARS-COV-2

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ABSTRACT. Brazil is one of the countries that least performs detection tests for SARS-COV-2, even though diagnosing cases is the most effective way to control epidemics, which is crucial to guide public policies. From this perspective, this study aimed to evaluate the RT-LAMP/CRISPR-Cas12 detection method using synthetic and natural SARS-COV-2 sequences. A total of 84 reactions of RT-LAMP/CRISPR-Cas12 resulted in the colorimetric results: 55 reactions turned pink, 18 turned orange, and 11 turned yellow. This result showed that, in the RT-LAMP colorimetric criterion, most reactions (65.4%) were classified as negative, followed by inconclusive (21.4%), and a minority (13%) was classified as positive. The colorimetric results showed instabilities such as reaction sensitivity to heating and ambient temperatures at the reaction preparation site. The use of CRISPR/Cas12 proved unnecessary in this experiment and for the RT-LAMP methodology since its reagent is used only for the detection mix and for lateral flow strip analysis. The flow/detection strips in this experiment were ineffective for a retest as they were dependent on the Reporter reagent solution, and their result was not validated with the presence or absence of genetic material.

Keywords: Covid-19; Rna viral;test molecular.

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Introduction

Brazil is one of the countries that least tests for COVID-19, which increases the problem of underreporting (Teixeira & Lopes, 2021). However, carrying out mass testing with case tracking is the most effective way to control epidemics, constituting a critical approach to guide public policies through non-pharmacological methods and favoring the application or relaxation of social distancing or isolation measures.

There are two main types of COVID-19 tests: serological tests, which can detect antibodies produced by the human body in response to contact with the virus and detect viral antigens, and molecular tests, based on the detection of SARS-COV-2 genetic material (Diniz, Martins, Xavier, Silva, & Santos, 2020). Antigen and antibody tests are rapid techniques that can detect virus proteins or antibodies using naso/oropharynx, whole blood, serum, and plasma samples (BRASIL. Ministério da Saúde, 2021).

The molecular tests available for this purpose include reverse transcription loop-mediated isothermal amplification (RT-LAMP), which can amplify the nucleic acid under isothermal conditions of around 62ºC, generating high specificity by amplifying a specific gene of the human genome even with a single nucleotide region.

The RT-LAMP is composed of a set of LAMP reagents, salts, nucleotides, DNA polymerase (which will catalyze the synthesis of complementary strands), a primer set for amplification with six different primers designed to recognize specific regions of the target gene, direct inner primers (FIP), and retrograde inner primers (BIP), allowing the formation of the loop. Furthermore, outer primers play a key role in displacing the strand during the non-cyclic forward outer primer (F3) and reverse outer primer (B3) steps, whereas the two loop-loop (LF) and loop-back primer (LP) primers were designed to accelerate the amplification reaction by binding to specific sites accessed only by them (Augustine et al., 2020).

In addition to ensuring high amplification efficiency, this procedure is isothermal, i.e., no time is lost with temperature changes (Augustine et al., 2020). Its amplification does not require expensive equipment or laboratory infrastructure and shows lower prices and higher accessibility than other methods.

On the other hand, the Clustered Regularly Interspaced Short Palindromic Repeats system (CRISPR) occurs through an RNA (Ribonucleic Acid) sequence (called guide RNA) designed for target-sequence recognition. Through this process, the Cas protein recognizes the specific region that will be modified.

Mammoth Biosciences (2020) employed a SAR-COV-2 diagnostic methodology using CRISPR/Cas12 to increase the test's sensitivity and detect different coronavirus strains, accelerating the action time required for each reaction. In addition, CRISPR-Cas12 detection was combined with the RT-LAMP isothermal system in its amplification step.

From this perspective, this study aimed to evaluate the RT-LAMP/CRISPR-Cas12 methodology, its stability and sensitivity, using synthetic and natural SARS-COV-2 sequences.

Material and Methods

The experiments were carried out at the Laboratory of Biotechnology and Genetics of the Agricultural Sciences Campus (CCA) of the Federal University of Vale do São Francisco (UNIVASF).

Samples and Reactions

Natural/positive SARS-COV-2 RNA samples from patients were kindly provided by the COVID-19 Petrolina Molecular Diagnosis Laboratory, located at the Central Campus of the Federal University of Vale do São Francisco (UNIVASF), coordinated by Research Group on Infectious and Neglected Diseases.

The reactions employed two types of positive samples: inactivated natural positives from patients and synthetic positive samples. Ten positive samples confirmed by the COVID-19 Petrolina Molecular Diagnostics Laboratory were used in this study. The samples were numbered from 1 to 10 and quantified by the standard RT-PCRq method. The higher the quantification value (Cq_ORF), the lower the viral load identified in the sample (Table 1).

Table 1. RNA quantification by the RT-PCRq method for natural positive samples.

Sample	RNA (ngμL^{-1})	Cq_ORF
1	1.80	18.15
2	-0.80	29.50
3	1.40	34.19
4	-0.50	17.75
5	2.50	18.49
6	-0.50	21.87
7	3.70	22.19
8	-0.80	27.77
9	-0.10	22.70
10	0.00	32.29

Source: Adapted from the Research Group on Infectious and Neglected Diseases(Grupo de Pesquisa em Doenças Infecciosas e Negligenciadas, 2021).

The following sequences were used for the synthetic positive samples: E (2019-Ncov E-gene), N (2019-nCov-Ngene), and P (RNase P gRNA Sample control), in addition to the primers described at Mammoth Biosciences (2020).

Preparation of CRISPR/Cas12 reaction

The reactions were adapted from Brought et al. (2020). Three reactions were used in this procedure, one for each viral gene (N and E), in addition to a control reaction for the human gene (RNaseP). A gRNA mix (one for each sample) was prepared containing 14.5 uL of ultrapure water, 1X NEBuffer 2.1, 50 nM of LbCas12 enzyme, and 62.5 nM of the encoded gene (gRNA) from each sample (E, N, and RNaseP). This mixture was incubated for ten minutes at 37°C. The final step of this procedure consisted of adding 500 nM of the Biotin-labeled Reporter solution to the final solution.

Isothermal loop mediated amplification (RT - LAMP)

Three reactions, one for each gene (N, E and RNase P) were prepared with the primers and the synthetic control sequence of nCoV-19. For this, each reaction used 7.5 units/rxn of NEB WarmStart® solution, 10X primer mix (0.2 uM F3, 0.2 uM B3, 1.6 uM FIP, 1.6 uM BIP, 0.8 uM LF, and 0.8 uM LB), 10X Isothermal Amplification Buffer (NEB), 100 mM MgSO4 (NEB), eight units/rxn of Bst 2.0 polymerase (NEB), and 10X Isothermal Amplification Buffer (NEB). Also, 2 uL of synthetic RNA or the positive sample was added for each gene at the end of the procedure. Then, the solution was incubated at 62°C for 30 minutes.

Detection reaction

According to the adapted protocol by Broughton et al. (2020), 2uL of the RT-LAMP reaction was combined with 18ul of the CRISPR/CAS12 reaction, then receiving 80 uL of the appropriate buffer. After that, the final reaction of each gene was incubated for 10 minutes at 37°C. After incubation, the lateral flow strip (Milenia HybriDetect, 2019) was inserted directly into the reaction and rested for two minutes at room temperature, after which the result was observed.

Gene-free detection reaction

Four total gene-free reactions were carried out to test the lateral flow detection strips, split into 100 uL of milli-Q® water , 100 µL of 10X Buffer, 99 uL of milli-Q® water + 1 uL Reporter solution, and 99 uL of 10X Buffer + 1 uL Reporter solution.

Reporter solution

The following tests were performed to understand the influence of the Reporter solution on the detection strips: CRISPR/Cas 12 reactions with the Reporter solution (Nr, Er, Pr, and Br) and CRISPR/Cas12 reactions only with the buffer solution in the absence of the Reporter solution (Nt, Et, Pt, and Bt).

Test interpretation

Two dashes or C (control) and T (test) lines can be seen in the lateral flow strip. The line closest to the sample contact block is the control line, whereas the line that appears furthest from the sample contact (and closest to the arrows) is the test line. Positivity or negativity was observed for each gene tested.

The results were interpreted in five different ways (Table 2), one being positive, two possible positives, one negative, and one test failure. The positive is given when the E and N genes have + results and the RNAseP (Positive Test Control) is either – or + . Possible positives occur when one gene returns + and the other - regardless of whether it is E or N and the RNAseP is – or +. The negative result occurs when the E and N genes present stripes – and RNAseP +. Finally, the test fails when all samples test negative (-).

Table 2. Interpreting the results of the three genes for detection

	Gene- N	Gene-E	RNAseP	Interpretation
1	+	+	+/-	SARS-COV-2 positive
2	+	-	+/-	Presumptive SARS-COV-2
3	-	+	+/-	Presumptive SARS-COV-2
4	-	-	+	SARS-COV-2 negative
5	-	-	-	Test failed

Source: Mammoth Biosciences (2020).

Results and discussion

In total, 78 RT-LAMP tests were performed with 84 total reactions using the same samples. The results were analyzed for colorimetry and flow strip detection.

Three color criteria were used for the colorimetric evaluation: orange, pink, and yellow, with pink meaning a negative result for the presence of SARS-COV-2, yellow meaning a positive result, and orange meaning either a false negative or positive result or also a low viral load.

The 84 RT-LAMP reactions were subdivided into 55 reactions in pink, 18 orange, and 11 yellow. This result showed that, according to the RT-LAMP colorimetric criterion, most reactions (65.4%) would be classified as negative, followed by inconclusive (21.4%), and the minority (13%) would be classified as positive.

The tests carried out in this study counted on synthetic samples, blank/gene absence, and positive results quantified with low, medium, and high viral loads. These data were provided by the COVID-19 Petrolina Diagnostic Laboratory.

During the analysis, no repetition of colors was observed for a specific gene (N, E, or RNAseP), and the resulting coloration was random at each test. The distribution of this parameter consisted of 27 reactions of the E gene, three of which were yellow, 17 pink, and seven orange. For the N gene, we had 31 reactions, five of which were yellow, 20 pink, and six orange ones. The RNAseP gene returned 19 reactions, 12 of which were pink, three yellow, and four orange. Finally, the gene-free reactions used as a negative control returned seven reactions, six pink and one orange (Table 3).

Table 3: Distribution of the reactions regarding the RT-LAMP colorimetric analysis of per gene

Color / Gene	N	E	RNAseP	Absence
Yellow	5 (16.1%)	3(11.1%)	3 (15.7%)	0
Pink	20 (64.5%)	17 (62.9%)	12 (63.1%)	6 (85.7%)
Orange	6 (19.3%)	7 (25.9%)	4 (21%)	1 (14.3%)
TOTAL (84)	31	27	19	7

Source: The authors.

Figure 1 represents four reactions for the detection of SARS-COV-2, two for the E and N virus genes, one to detect the presence of the human gene (RNAseP), and the fourth reaction with a gene-free negative control solution (B). The results of the RT-LAMP colorimetric test indicated the reactions that contained the N and E genes with orange staining, interpreted as of low viral load or false positive/negative, whereas the RnaseP and blank reactions resulted in pink staining, i.e., negative results.

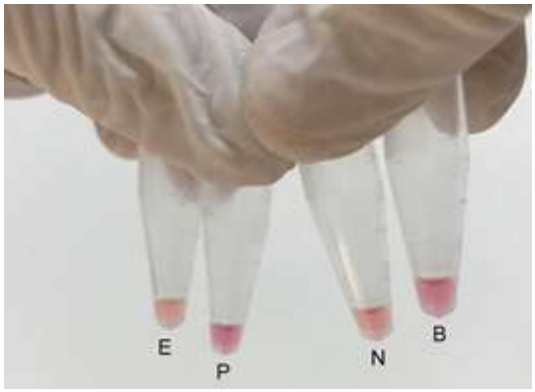

Figure 1. E, RNAseP, N, and B for RT-LAMP reactions in the colorimetric analysis showing shades of pink and orange. Source: The authors.

Nine reactions were used for the first test of natural positive samples, distributed as follows: genes E1, E2, EB, N1, N2, NB, P1, P2, and PB, with the reactions ending with 1 and 2 containing different positive samples, whereas those marked with b (blank) corresponded to the negative control, i.e., absence of genetic material (Figure 2).

The reactions were colored in orange shades closer to the desired yellow shade to confirm the positive result. The result showed that the presence of positive samples could change the color in the colorimetric method.

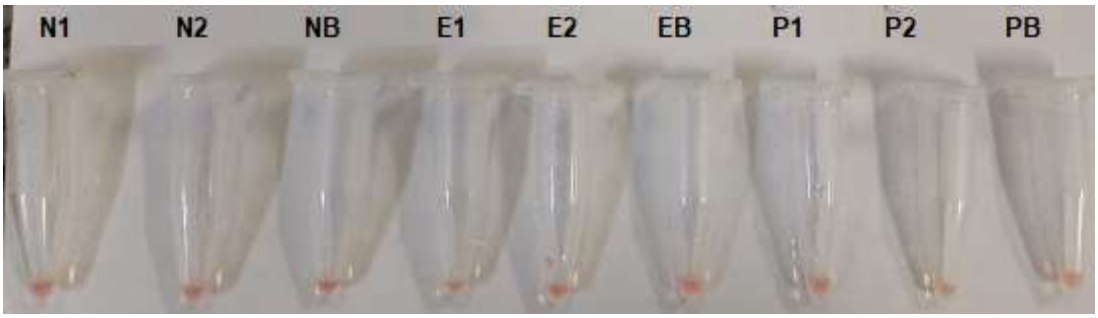

Figure 2. Reactions for genes E1, E2, EB, N1, N2, NB, P1, P2, and PB in orange staining for RT-LAMP. Source: The authors.

The tests with synthetic and positive samples were distributed as follows: N positive sample (Na), N synthetic sample (Ns), E positive sample (Ea), E synthetic sample (Es), P positive sample (Pa), P synthetic sample (Ps), and the negative control with water (B).

The N gene reactions that tested positive in the colorimetric analysis turned yellow for the synthetic gene, whereas inconclusive results turned orange for the positive sample (Na). The E gene was yellow for the positive sample and pink for the synthetic sample. Gene P tested negative for both samples, which were pink. The negative control turned orange, perhaps showing possible contamination of the samples (Figure 3).

Reactions Nt, Nr, Et, Er, Pt, Pr, Bt, and Br were used to evaluate the influence of the reporter solution, changing only regarding the absence (t) or presence of the reporter solution (r). For the RT colorimetric method, it was not possible to notice any changes in the test, with most samples turning yellow in the yellow

colorimetric analysis, e.g., Nt, Nr, Et, and Er, and the orange Pt followed by Pr, Bt, and Br in light pink shades. The modification of the original protocol with twice the standard time (20 min) at 37ºC showed that the amplified N and E genes reached the desired positive color shade. However, the P gene maintained a negative result (Figure 4).

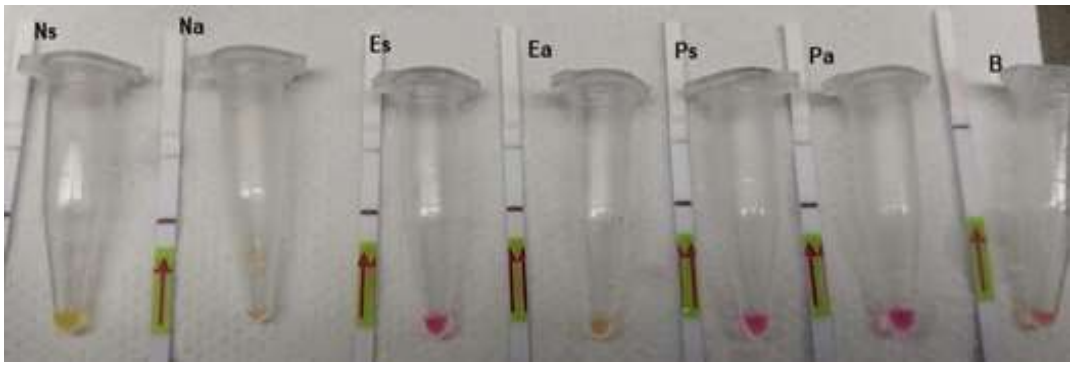

Figure 3. Result of the analysis of synthetic (s) and positive genes for colorimetric RT-LAMP with shades of yellow, pink, and orange. The lateral flow strips show positive results for all reactions except the blank one (B). Source: The authors.

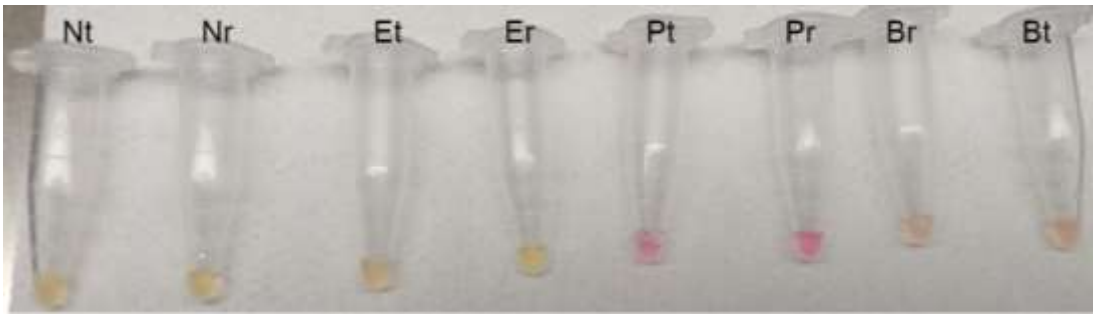

Figure 4. Reactions for the RT-LAMP colorimetric analysis showing most samples in yellow, i.e., positive for SARS-COV-2. Source: The authors.

The HybriDetect Lateral Flow detection/run strip was used to determine whether it corroborated the colorimetric results for both reactions with synthetic and natural positive samples. Sixty-six flow strips were used, with 18 reactions with the E gene, in which 13 were positive, four negative, and one negative for the Et gene in the absence of the reporter substance and the presence of the gene. A total of 23 strips were used for the N gene, 18 of which were positive, four negative, and another negative for Nt. The RNAseP gene had 14 strips, with 11 positive results, two negative results, and one negative for RNAsePt. Reactions with no gene had eight strips with four positive results, three negative results, and negative results for the blank samples.

The test performed included the standard reactions of the N, E, and RNAseP genes and the gene-free (blank). The flow strips tested positive, with the presence of two lines for all samples, including the gene-free sample (blank). Since the strips tested positive for all samples, a hypothesis was raised referring to the contamination of the blank reaction (gene-free) or the inefficiency of the flow-detection strips (Figure 5).

Figure 5. Lateral flow strips for the N, RNAseP, and E genes. The blank sample shows positive results (presence of two lines, the closest to the sample detection arrow and the second closest to the clearer control strip). Source: The authors.

Moreover, it was also tested whether the presence of the reporter solution in the reaction influenced the positivity of the strip. Four gene-free reactions containing only water or buffer showed positive results for the samples that contained the reporter solution, and negative for the blank solutions/absence of the reporter. This procedure highlighted the influence of the reporter solution on the positivity of the strips regardless of the presence or absence of genes (Figure 6). It was observed that the strips were valid by the presence of a second line (furthest from the arrow) in all samples tested.

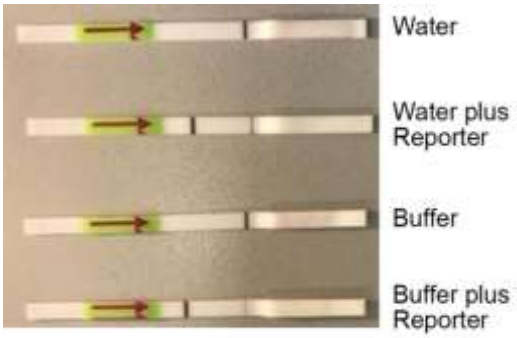

Figure 6. Result of the flow/detection strip for the test with gene-free solutions, with the presence or absence of the reporter solution (presence or absence of lines, the closest to the sample detection arrow, and the second clearest strip control). Source: The authors.

The analyzed samples corresponding to N1, N2, NB, E1, E2, EB, P1, P2, and PB contained synthetic and actual positive samples (terminations 1 and 2). In the analysis for the flow detection strips, samples N1, E1, P1, P2, and PB were positive, with two lines, and samples N2, Nb, E2, and EB tested negative, with the presence of only the control line of the test (Figure 7).

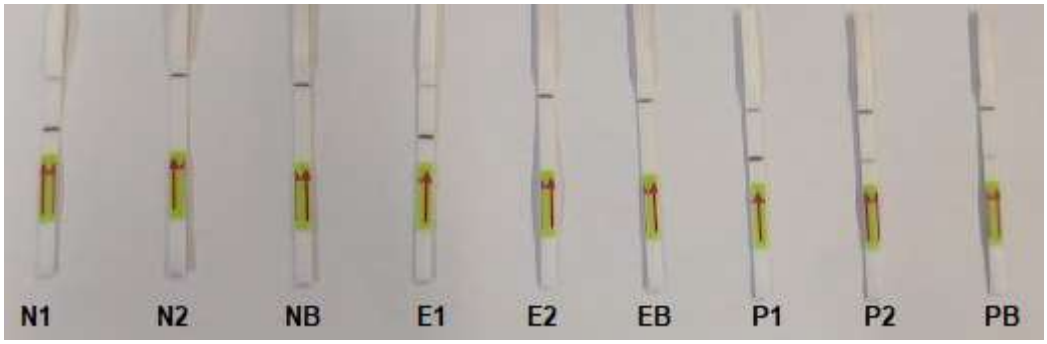

Figure 7. Samples E1, EB, N1, NB, P1, and PB for the lateral flow strips with positive and negative results. Source: The authors.

The flow strips used showed important changes and inconsistencies. All reactions that contained only the buffer solution or only samples, whether synthetic or natural, with the absence of the reporter solution, returned negative results, with a single line on the strip since the reactions that contained the reporter solution tested positive, showing two lines on the strip. The presence of the reporter solution in this experiment made the strips positive regardless of the presence or absence of genes.

The RT-LAMP methodology has high specificity for amplifying a specific gene and efficiency for not requiring thermal change. It has a lower amplification than PCR and is widely used for enabling analysis with the naked eye due to the color change throughout the process (Parida, Sannarangaiah, Dash, Rao, & Morita, 2008).

RT-LAMP allows the release of results on a large scale, even with several hundred samples, with a technique that can be implemented in developing countries. It also enables the combination of several target sequences in the same reaction, providing a valuable diagnosis for different types of pathogens or even the detection of variants of the same virus within a single reaction. When standardized, the RT-LAMP technique can be used to monitor potential new pandemics and viral spread waves (Ludwig et al., 2021).

The disadvantages of using RT-LAMP are associated with the need for many primers, the availability of reagents, and their sensitivity to external factors, e.g., the influence of ambient temperature and the incubation period, leading to changes in the colorimetric result of the test samples analyzed.

CRISPR/Cas 12 for diagnosing SARS-COV-2 proved effective due to its capacity for cellular programming and direct detection of viral genome sequences. In addition, this technique provides increased sensitivity and

detection speed, acting as a catalyst. Also, for taking place in a single container, the CRISPR solution can be used to detect SARS-COV-2 and other viral genomes (Liu et al., 2021).

With regard to the flow and detection strips, the manufacturer itself, in a booklet (https://www.milenia-biotec.com/uploads/2019/07/Confusion-T-and-C-Line_final.pdf), points out the possible misinterpretation of the flow bands, which tends to confuse the understanding of the control and test band/lines. This confusion is due to the existence of two possible modalities, HybriDetect Instructions and SHERLOCK-/DETECTR, each featuring a different detection system and causing lines to be interpreted in reverse in tests.

The inconsistency of the results presented by the flow strip in this study seems to be associated with the use or not of the reporter solution, which, even at low concentrations, was sufficient to make the lateral flow strips positive regardless of the presence of viral nucleic acid.

Conclusion

The RT-LAMP colorimetric method is a more effective and efficient test than the conventional ones already used. Moreover, its result can be released quickly, depending on the number of samples under analysis.

The colorimetry results showed instabilities such as the sensitivity of the reactions to the heating and room temperatures at the place of preparation of the reactions. Therefore, it is ideal to use the RT-LAMP together with another control model in order to avoid false positives and negatives.

CRISPR/Cas12 proved unnecessary in this experiment for the RT-LAMP methodology since its reagent is used only for the detection mix and for analysis of the lateral flow strip.

The flow/detection strips in this experiment proved ineffective for a control test since it depended on the Reporter reagent solution, and the presence or absence of the genes did not validate its result.

Acknowledgments

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