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Development of a Saliva-Based Lateral Flow Assay for SARS-Cov-2 with the Potential to Quantify Viral Load

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Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is one of the deadliest virus in the last 50 years, with the USA having the highest reported deaths and cases at over 1.1 million and over 100 million, respectively, as of June 1, 2023. Identifying infected people remains the primary method of stopping the spread of the virus, either using real-time, quantitative polymerase chain reaction instruments or at-home lateral flow assay (LFA) antigen tests. Herein we describe a simple four step at-home SARS-CoV-2 LFA test that provides three advantages over current LFAs. The test employs 1) saliva sampling, 2) three antibodies to bind the virus to the LFA Test Line, and 3) a smartphone to quantify the reflectance of the Test Line in terms of Ct values. The use of saliva samples eliminates the pain and fear of nasopharyngeal sampling, especially for children. The use of three antibodies yielded 100% correct sensitivity, specificity, predicted positive and predicted negative for samples with Ct values of 29 and below. The use of a smartphone to measure reflectance allowed calculating the Ct values for 16 samples with an average error and standard deviation of 0.58±0.43 for samples with Ct values below 26. The smartphone also adds the capability of sharing the results to track and slow the spread of the virus. The combination lateral flow assay and smartphone quantitation is well suited for identifying, quantifying, and slowing the spread of future viruses.

Keywords: SARS-CoV-2; Viral load; Saliva sampling

Introduction

In just over three years since the initial detection of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the USA, more than 1.1 million people have died and over 100 million cases have been reported, far more than any other country in the world [1]. A major component to stopping the spread of the disease was the development of real-time, quantitative polymerase chain reaction methods (PCR) used to identify infected people. PCR employs nucleic acid primers to double the amount of SARS-CoV-2 ribonucleic acid in an oral fluid or nasopharyngeal sample through each temperature cycle. The cycles are repeated until a fluorescent dye attached to the primers increases sufficiently to be detected, otherwise known as the cycles-to-threshold of detection (Ct). The Ct value is an indication of the concentration of the virus in the sample. Specific primers used to replicate the SARS-CoV-2 genome, identified in January 2020 [2], were incorporated into existing PCRs, and widespread testing began in March 2020 [3]. Tests sites were set up initially at hospitals, and later at many drive-through venues. By July 2020, the Center for Disease Control (CDC) established a national tracking database for all PCR test results [4]. A significant amount of testing was driven by employers who wanted healthy, non-contagious workers. Unfortunately, PCR has several limitations, such as the use of expensive primers, and the need for highly trained technicians and well-equipped laboratories. More importantly, the test takes 2-6 hours to perform and 2-5 days to provide results, due to limited PCR instrument availability and sample transport to labs. During the delay contagious people spread the virus to others [5]. In order to overcome these limitations, we and others developed at-home tests. Such tests would eliminate drive-through lines and the wait for results, and they cost much less than PCR tests. Our test, like most, employed antibodies to bind the virus at the Test Line of a lateral flow assay (LFA) [6]. However, there are two important differences. Saliva was used as the sample medium, and three antibodies were used to bind to the virus. The former proved useful in the testing of children, and the latter provided greater sensitivity compared to other LFA at-home tests. Here we describe the development of the LFA, Food and Drug Administration (FDA) test results required for emergency use authorization, and the use of a smartphone to quantify the Test Line in terms of Ct values.

Materials and Methods

Materials

All reagents, such as HPLC water and pH 7 buffers, were obtained from Sigma-Aldrich (Allentown, PA), while COVID-19 antibodies were obtained from Meridian Bioscience (Cincinnati, OH). Heat inactivated SARS-Related Coronavirus 2, Isolate USA-WA1/2020, was obtained from BEI Resources (NR-52286, Lot: 70037779, Bethesda, MD). De-identified pooled saliva samples, with and without the SARS-CoV-2 virus for product development were obtained from Lee Biosolutions (Maryland Heights, MO). De-identified pooled saliva samples with the SARS-CoV-2 virus for quantitation were obtained from Boca Biolistics (Pompano Beach, FL). Viruses, bacteria, common drugs, and human blood were purchased from BEI Resources, American Type Culture Collection (ATCC, Manassas, Virginia), Hardy Diagnostics (Santa Maria, California), ZeptoMatrix (Buffalo, New York), and the University of Rhode Island (Kingston, RI) as indicated in Table 1. Microbial interferents, listed in Table 2, were obtained from Lee Biosolutions. All pathogenic samples were received in 2 mL plastic vials, and all sample preparations were performed in a Biosafety Level 2 cabinet following standard safety procedures, using non-cotton swabs, 1 mL plastic centrifuge tubes and a manual pipetter for sample and

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Table 1: Sources for viruses, bacteria, medications, and blood used in this study.

Virus	Source	Bacteria	Source
Adenovirus	BEI, cell preparation	Streptococcus mutans	ATCC
Respiratory syncytial virus	ATCC	Streptococcus pneumoniae	ATCC
Haemophilus influenzae	Hardy Diagnostics	Streptococcus pyogenes	ATCC
Human Metapneumovirus	BEI, Inactive cell lysate	Mycoplasma pneumoniae	ATCC
Enterovirus	BEI, cell preparation	Chlamydia pneumoniae	ATCC
Rhinovirus	BEI, Inactive cell lysate	Legionella pneumophila	ATCC
Influenza A	BEI, Inactive cell lysate	Candida albicans	ATCC
Influenza B	BEI, Inactive cell lysate	Mycobacterium tuberculosis	Univ. Rhode Island
Human coronavirus 229E	ZeptoMetrix	Interferents	Source
Human coronavirus OC43	ZeptoMetrix	Acetaminophen	Sigma-Aldrich
Human coronavirus NL63	ZeptoMetrix	Amoxicillin	Sigma-Aldrich
MERS	BEI, Inactive cell lysate	Aspirin	Sigma-Aldrich
SARS-Related Coronavirus 2	BEI, Heat inactivated (Isolate USA-WA1/2020)	Caffeine	Sigma-Aldrich
		Human Blood, EDTA	Lee Biosolutions

Table 2: COVID-19 Saliva Antigen At-Home Test limit of detection.

(SARS-CoV-2), isolate USA-WA1/2020, 1.6 × 10^5 $TCID_{50}$ per mL				
Dilution	1/10	1/100	1/200	1/400
Conc.	1.6 x 10^4	1.6 x 10^3	8 x 10^2	4 x 10^2
5 replicates	100% (5/5)	100% (5/5)	60% (3/5)	0% (0/5)
20 replicates	100% (20/20)	100% (20/20)	50% (10/20)	0% (0/20)

buffer transfer, all from VWR (Bridgeport, NJ). The COVID-19 Saliva Antigen At-Home Test consisted of a polyester flock swab (Guilford, ME), a 2 mL plastic vial containing 0.5 mL of buffer, a 1 mL disposable plastic pipette (Franklin, MA), and an RTA designed lateral flow assay cassette (Figure 1A). The LFA cassettes were of standard construction (nanoComposix, San Diego, CA), consisting of 1) a sample pad, 2) a conjugate pad containing probes, 3) a Test Line functionalized with mouse monoclonal SARS-CoV-2 nucleocapsid and spike protein antibodies as capture antibodies, 4) a Control Line functionalized with goat anti-mouse IgG antibodies, and 5) a wicking pad, all on 6) a nitrocellulose support, and 7) enclosed in a plastic cassette containing a sample addition port and a viewing section. The conjugate probes consisted of synthesized gold nanoparticles [7], coated with a dye, as a reporter molecule, and were also functionalized with the three different antibodies as the Test Line [8].

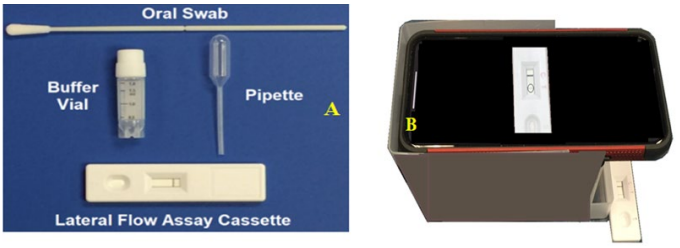

Figure 1: Photographs of A) RTA's COVID-19 Saliva Antigen At-Home Test, and B) a smartphone on a cassette holder.

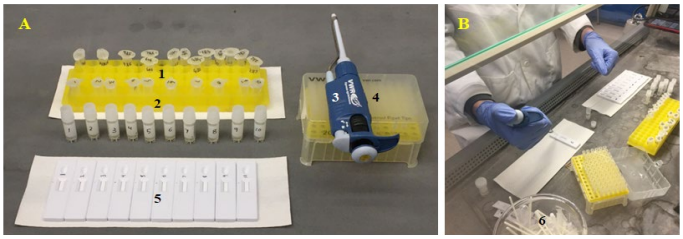

Figure 2: Photographs of A) the set up to measure "unknown" saliva samples (10 shown) using RTA's COVID-19 Saliva Antigen Test kit and B) Lab technician measuring the 10th sample. 1) Ten 0.5 mL saliva samples in 1 mL centrifuge tubes, 2) ten vials of 0.5 mL buffer, 3) auto-pipetter, 4) box of pipettes, 5) ten cassettes, and 6) used swabs to be bagged and autoclaved.

Methods

The COVID-19 Saliva Antigen At-Home Test procedure was initially developed by varying the following parameters: sample volume to buffer volume, mixing time, wait time, number of microL added to the cassette and time to detect a visible Test Line. This procedure was performed using 5 purchased SARS-CoV-2 positive saliva samples and 5 pooled, de-identified saliva samples (Lee Bio Solutions), measured by a minimally trained lab technician in a randomized and blind fashion using RTA's Test kit (Figure 1A). The samples in 1 mL plastic centrifuge tubes were measured according to the following steps: 1) a sterile swab was immersed and swirled in a tube with a sample for 30 seconds (Figure 2A); 2) the swab was then immersed and swirled in a vial containing 0.5 mL extraction buffer for 30 seconds; 3) the swab was pressed along the edge of the vial walls to release the solution into the vial; 4) the vial was capped, shaken, and allowed to sit and incubate for 5 minutes at room temperature; 5) 100 microL of sample were drawn from the vial using an auto-pipetter with disposable tips and dispensed into the sample well of a LFA cassette (Figure 2B); and 6) 10 minutes after deposition, the lab technician was asked if the test was positive or negative (2 lines or 1 line). The lab technician correctly identified the 5 positive and 5 negative sample. Measurements of SARS-CoV-2 on cassettes were initially performed visually, identifing results as positive or negative based on visible lines at the Test and Control Lines or no visible line at the Test Line with a visible line at the Control Line, respectively. After full development of the metod, additional samples were measured using an App (RTA) on a smartphone and a cassette holder to quantify the LFA Test Line Reflectance in terms of a Ct value (Figure 1B).

Results

LFA performance statistics

Once the LFA procedure was optimized, the limit of detection

(LOD), cross-reactivity, interference, and clinical evaluation were performed. The LOD, also known as the analytical sensitivity, was established by measuring the heat inactivated SARS-Related Coronavirus 2, Isolate USA-WA1/2020, at the concentration of 1.6x105 TCID50 per mL, followed by dilution in pooled, de-identified saliva by factors of 10, 100, 200, and 400 on 5 and 20 replicate LFA cassettes (Table 2). In accordance with FDA, the 100% detection, defined as a visible Test Line for 20 tested samples, yielded an LOD of 1.6x103 TCID50 per mL. The cross-reactivity, also known as the analytical specificity, was established by measuring 12 viruses, which included 3 non-human pathogenic coronaviruses, and 7 bacteria at the purchased concentrations using the LFA cassettes. None of pathogens produced a visible Test Line, confirming no cross-reactivity for these FDA suggested pathogens (Table 3). Similarly, microbial interference was tested by adding a virus, bacteria, biologicals, or drugs to a saliva sample containing SARS-CoV-2 at 1.6x103 TCID50 per mL and measuring each 3 times. None of these FDA suggested potential interferents at relevant concentrations interfered with the expected visible Test Line indicating a positive SARS-CoV-2 detection (Table 4). An evaluation of the LFA cassettes, in terms of true positives, true negatives, false positives and false negatives, was performed by measuring 50 true negative saliva samples and 38 true positive samples. The true negative samples were de-identified pooled saliva, while the true positive samples contained SASR-CoV-2 with Ct values ranging from 11.6 to 32.5 (Lee Biosolutions and Boca Biolistics). All samples were tested with the LFA cassettes 3 times by RTA lab personnel. 100% of the true negative samples did not produce a visible Test Line, while 29 of the 38 true positive samples (76.32%) did produce a visible Test Line. All 9 true positive samples that incorrectly tested negative had Ct values from 30-32.5 (Table 5). Several studies suggested that the ideal antigen test would detect the virus 100% of the time at Ct values below 30, when people are most contagious. The COVID-19 Saliva Antigen At-Home Test met this criterion by identifying all samples with Ct values of 29 and below as positive (Figure 3 and Table 6).

Table 3: COVID-19 Saliva Antigen At-Home Test cross-reactivity data.

Virus/Bacteria	Source	Concentration	Result
Adenovirus	BEI, cell preparation	2.5 x 10^7 $TCID_{50}$/ml	Negative
Respiratory syncytial virus	ATCC	4 x 10^5 $TCID_{50}$/ml	Negative
Haemophilus influenzae	Hardy Diagnostics	3 x 10^6 $TCID_{50}$/ml	Negative
Human Metapneumovirus	BEI, Inactive cell lysate	5 x 10^5 $TCID_{50}$/ml	Negative
Enterovirus	BEI, cell preparation	2.4 x 10^5 $TCID_{50}$/ml	Negative
Rhinovirus	BEI, Inactive cell lysate	2 x 10^6 $TCID_{50}$/ml	Negative
Influenza A	BEI, Inactive cell lysate	6 x 10^5 $CEID_{50}$/ml	Negative
Influenza B	BEI, Inactive cell lysate	5.3 x 10^4 $CEID_{50}$/ml	Negative
Human coronavirus 229E	ZeptoMetrix	1 x 10^5 $TCID_{50}$/ml	Negative
Human coronavirus OC43	ZeptoMetrix	1 x 10^5 $TCID_{50}$/ml	Negative
Human coronavirus NL63	ZeptoMetrix	1 x 10^5 $TCID_{50}$/ml	Negative
MERS	BEI, Inactive cell lysate	8.9 x 10^5 $TCID_{50}$/ml	Negative
Streptococcus pneumoniae	ATCC	5 x 10^6 cells/ml	Negative
Streptococcus pyogenes	ATCC	8 x 10^5 cells/ml	Negative
Mycoplasma pneumoniae	ATCC	3.2 x 10^6 cells/ml	Negative
Chlamydia pneumoniae	ATCC	7.5 x 10^7 cells/ml	Negative
Legionella pneumophila	ATCC	5 x 10^5 cells/ml	Negative
Mycobacterium tuberculosis	Univ. Rhode Island	6.3 x 10^6 cells/ml	Negative
Candida albicans	ATCC	4 x 10^6 cells/ml	Negative

$TCID_{50}$ = 50% Tissue Culture Infectious Dose, $CEID_{50}$ = 50% Chicken Embryo Infectious Dose

Table 4: COVID-19 Saliva Antigen At-Home Test interferent data.

Interferent	Concentration	SARS-CoV-2 Concentration	Result, n = 3
Influenza A	6x105 CEID50 /ml	1.6x103 TCID50 per mL	Positive
Streptococcus pneumoniae	5x106 cells /ml	1.6x103 TCID50 per mL	Positive
Streptococcus mutans	1x107 cells /ml	1.6x103 TCID50 per mL	Positive
Human Blood, EDTA	5% v/v	1.6x103 TCID50 per mL	Positive
Acetaminophen	1 mg/ml	1.6x103 TCID50 per mL	Positive
Aspirin	1mg/ml	1.6x103 TCID50 per mL	Positive
Caffeine	5mg/ml	1.6x103 TCID50 per mL	Positive
Amoxicillin	5 mg/ml	1.6x103 TCID50 per mL	Positive

Table 5: COVID-19 Saliva Antigen At-Home Test sensitivity, specificity, predicted positive, and predicted negative percents are provided for Ct values 11.6 to 32.5 and 11.6 to 30.

LFA Statistics	Sensitivity	Specificity	Predicted Positive	Predicted Negative
	a/(a+c)	d/(d+b)	a/(a+b)	d/(d+c)
Ct =13-33	29/(29+9)	50/(50+0)	29/(29+0)	50/(50+9)
a=29, b=0, c=9, d=50	76.32%	100.00%	100.00%	84.75%
Ct =13-29	29/(29+0)	50/(50+0)	29/(29+0)	50/(50+0)
a=29, b=0, c=0, d=50	100.00%	100.00%	100.00%	100.00%

Table 6: List of true positive and false negative results for 38 samples as determined by RTA's COVID-19 Saliva Antigen At-Home Test (actual Ct values shown).

Positive Samples	PCR Ct value	LFA Test	Positive Samples	PCR Ct value	LFA Test
1	21.2	Positive	20	13.1	Positive
2	22.5	Positive	21	15.3	Positive
3	19.8	Positive	22	16.9	Positive
4	23	Positive	23	20.4	Positive
5	16.2	Positive	24	22.8	Positive
6	22.6	Positive	25	24.1	Positive
7	12.2	Positive	26	31.5	Negative
8	17.1	Positive	27	30.5	Negative
9	24.5	Positive	28	32.5	Negative
10	13.7	Positive	29	28	Positive
11	15.3	Positive	30	25	Positive
12	32	Negative	31	13	Positive
13	19.2	Positive	32	21	Positive
14	24.4	Positive	33	32	Negative
15	22.1	Positive	34	30	Positive
16	30	Negative	35	28	Positive
17	17.5	Positive	36	31	Negative
18	17.7	Positive	37	32	Negative
19	11.6	Positive	38	32	Negative

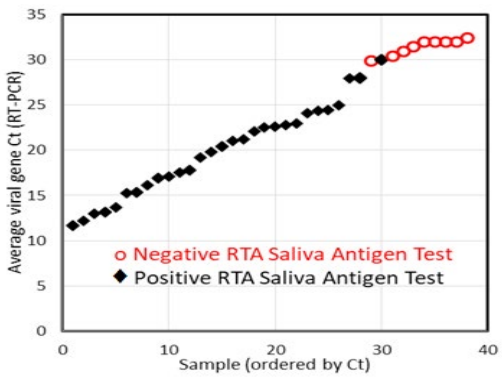

Figure 3: Plot showing true positive (♦) and false negative (o) results for RTA's COVID-19 Saliva Antigen At-Home Test for sample Ct values as a function of samples tested in increasing Ct values.

LFA Quantitation

While analyzing the 38 samples described above it became clear that the Test Line intensity decreased with lower viral loads, i.e. increasing Ct values. In an effort to quantify this intensity several spectroscopic methods were investigated, such as surface-enhanced Raman spectroscopy and both transmission and reflectance visible spectroscopy [8]. Since reflectance showed an inverse relationship to the Test Line intensity, measurement of reflectance using a smartphone was tried, as it would be ideal for at-home tests. During preliminary measurements it was found that a stand was required to fix the smartphone camera above the cassette Test Line for reproducible alignment (Figure 1B). A basic smartphone App was written using the built in camera to 1) magnify the Test Line image, and 2) measure the Test Line reflectance. Four saliva samples covering most of the Ct range, specifically Ct values of 12.33, 16.35, 19.91, and 24.53, were purchased, and the reflectance measured to establish a correspondence to the Ct values. The samples were prepared and added to cassettes as described previously. For each sample, the reflectance was measured at 10 minutes after it was added to the cassette sample port using the smartphone camera. In addition, a saliva sample, devoid of SARS-CoV-2, was also measured to represent a Test Line containing no probes and the upper limit of reflectance. The Ct 12.33 was used to represent a Test Line saturated with probes and the lower limit of reflectance. The Test Line reflectances for these two samples were quantified using the smartphone App as 208 and 50, respectively. There is a clear decrease in visible intensity of the Test Lines for the 5 samples as the viral load decreases (Figure 4A). A plot of the reflectance as a function of Ct values for the 4 samples and the blank saliva sample was fit with the Avrami equation (Figure 4B, Equation 1) [9], used to describe a limited system that has initial exponential growth followed by exponential decay. For the present system, an exponential increase in antibody binding is expected at the Test Line as the sample concentration increases, followed by an exponential decrease as all the bonding sites become occupied.

$$Ct = 208\text{-}157(\exp[-0.014x(Reflectance\text{-}12)^2]) \quad (1)$$

Next, a sample set of 16 "unknowns" were prepared by diluting each of the 4 purchased samples 4 times in de-identified, pooled saliva by 50% (Figure 5). This dilution factor was chosen to mimic the factor of 2 nucleic acid replication achieved from one PCR cycle to the next. As before, the samples were prepared, added to cassettes, and the reflectance measured at the Test Line, but this time the smartphone was also used to calculate the Ct values using Equation 1. However, the calculated Ct values did not match the Ct values based on 50% dilution (Figure 6A, Table 7). Notably, the intensity of the purchased Ct sample with a value of 19.91 should equal 23.91 after 4 dilutions, but its Test Line is lighter than the purchased sample with a Ct value of 24.53. In an effort to correct this discrepancy, the Ct gaps between the original samples were used to correct all of the diluted sample Ct values by a multiplication factor of 1.086 (Figure 6B and Table 7). The correction factor indicates that the replication for each cycle was not a factor of 2.0, but 1.84 (2.0/1.086). This is not unusual, as polymerase chain reaction replications are not 100% efficient, as occasional sequencing errors occur during each cycle [10, 11]. Reordering the sample cassettes based on this correction shows a continual visible decrease in the Test Line intensity from the Ct 12.33 sample to the blank saliva sample (Figure 7).

Discussion

The described saliva-based at-home SARS-CoV-2 test met the FDA

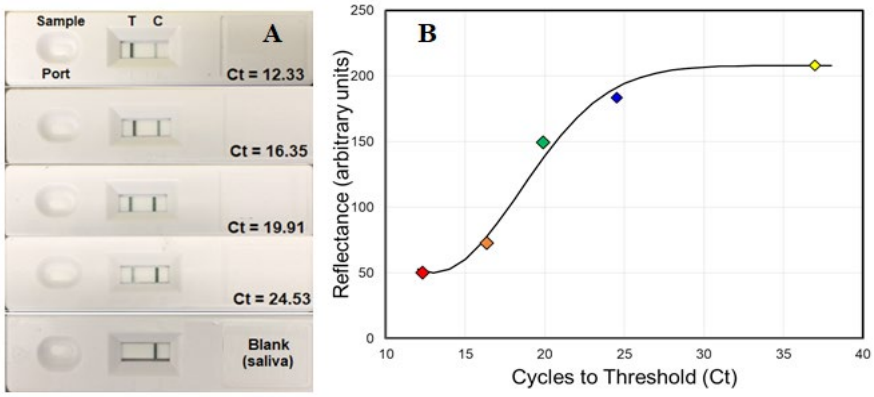

Figure 4: A) Photograph of 5 cassettes. First 4 cassettes prepared using 4 purchased Sars-CoV-2 samples with Ct values as indicated, and 1 cassette prepared using purchased de-identified saliva without the virus. B) Plot of reflectance values of the LFA Test Line for 4 samples and a saliva blank (diamonds) fit with Equation 1 shown as a black line. See text for measurement conditions.

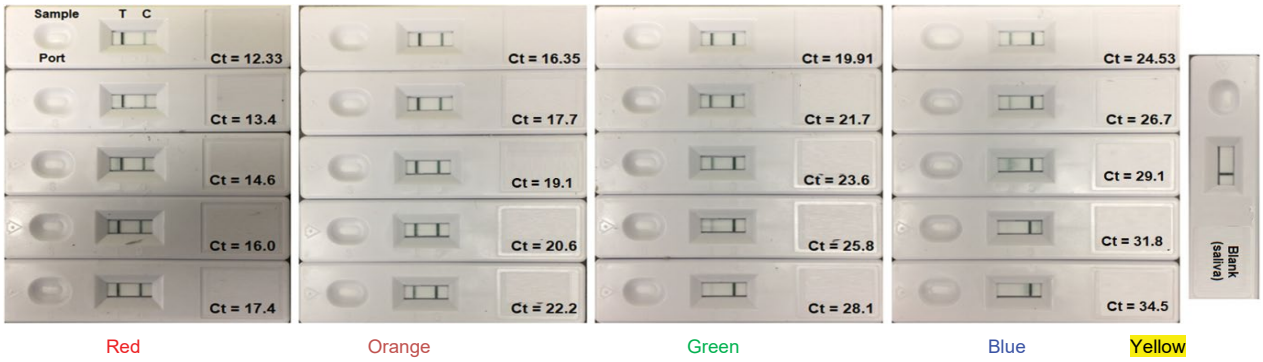

Figure 5: Photograph of 21 cassettes. Four cassettes across the top prepared using 4 purchased COVID-19 samples with Ct values as indicated, 4 successive cassettes of diluted samples below each purchased sample, and 1 cassette of a sample prepared using purchased de-identified saliva without the virus. Ct values calculated using the measured reflectance at the Test Line (T, left side of the viewing port) and the Equation 1 are shown on each cassette. See text for conditions.

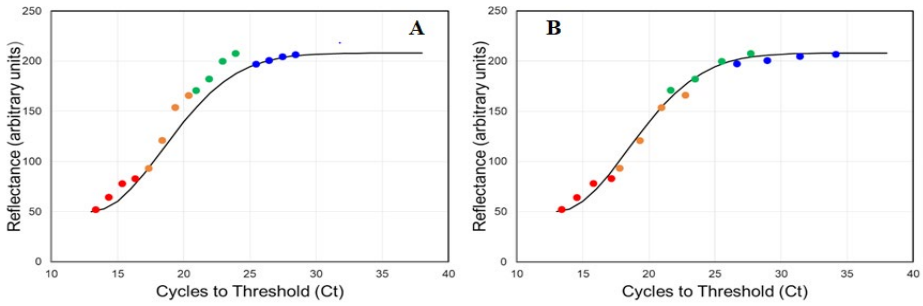

Figure 6A: Plot of reflectance values of the LFA Test Line for the 16 diluted samples calculated using Equation 1. Diluted samples are represented as red, orange, green, and blue circles, prepared from Ct values of 12.33, 16.35, 19.91, and 24.53, respectively. B) Plot of the same samples, but the Ct values corrected by multiplying by 1.086. Equation 1 is shown as a black line in both figures. See text for measurement conditions.

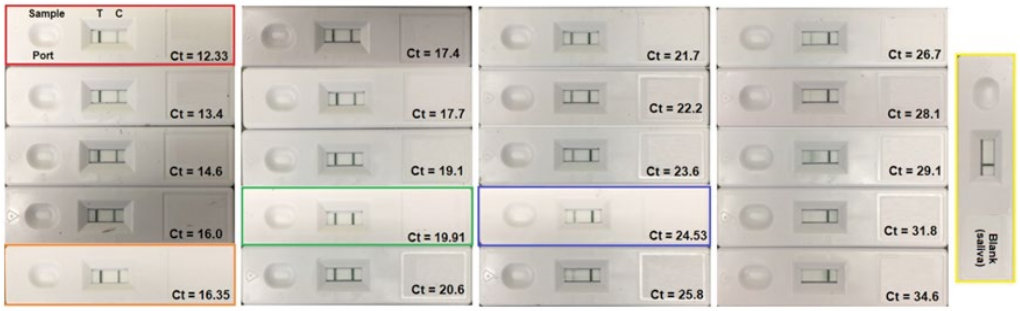

Figure 7: Photograph of 21 cassettes reordered based on calculated Ct values from measured reflectance and blank saliva sample. Continuous loss of intensity (increasing reflectance and corresponding Ct values) of Test Line (T, left side of the viewing port) is apparent. Original cassettes for samples with Ct values of 12.33, 16.35, 19.91, and 24.53, are red, orange, green and blue framed, respectively. See text for measurement conditions.

Table 7: Comparisons of Ct values for prepared samples by dilution, predicted reflectance for prepared samples, and measured reflection for prepared samples. Corrected predicted Ct values (x1.086), Calculated Ct values based on Equation 1, and Ct error (Corrected minus Calculated Ct values) are listed. Average error (absolute) and standard deviation between these values are also provided.

Sample Number	Prepared Ct Values	Eq 1 Predicted Reflectance	Measured Reflectance	Corrected Ct Values	Calculated Ct Values	Ct Error Corr-Calc
1	12.326	51.22	50	12.3	12.3	
2	13.326	50.29	52.33	13.4	14.1	-0.7
3	14.326	54.65	64.33	14.5	15.2	-0.7
4	15.326	63.88	78	15.8	16.3	-0.5
5	16.326	77.09	83	17.1	16.8	0.3
6	16.35	77.44	72.67	16.4	16.4	
7	17.35	93.46	93.33	17.8	17.5	0.3
8	18.35	110.87	121	19.3	19	0.3
9	19.35	128.39	154	20.9	20.9	0
10	20.35	144.93	166	22.7	21.9	0.8
11	19.91	137.83	149.33	19.9	19.9	
12	20.91	153.31	171	21.6	22.3	-0.7
13	21.91	166.9	182.33	23.5	23.5	0
14	22.91	178.14	200	25.5	27	-1.5
15	23.91	187.04	207.67	27.7	35	-7.3
16	24.53	191.51	183.33	24.5	24.5	
17	25.53	197.05	197.33	26.6	25.6	1
18	26.53	200.97	200.67	28.9	26.6	2.3
19	27.53	203.64	204.67	31.4	29	2.4
20	28.53	205.38	206.67	34.1	30	4.1
Saliva Blank	37	208	208	37.1	"Average Error:"	1.4
					"Standard Deviation:"	1.9

EUA requirements for sensitivity, cross-reactivity, and interference using purchased saliva samples containing the virus at known Ct values. Furthermore, the statistical measurements of sensitivity, specificity, predicted positive and predicted negative were all 100% correct for samples with Ct values of 29 and below. However, a clinical study was not performed using the kit, which would have included 1) collecting a saliva sample using a swab or passive drool, 2) mixing the saliva sample with buffer, 3) transferring the sample to a cassette, and 4) visually determining if a line appeared or not at the Test Line between 15 and 20 minutes. A preliminary laboratory study of saliva samples with known Ct values ranging from 12 to 38 Ct values was successfully used to establish a relationship between the smartphone measured reflectance at the LFA Test Line and the Ct values. However, as presented in Table 7, there remains a minor discrepancy between the corrected Ct values and the calculated Ct values with an average difference of 1.4. There are three potential sources of error, 1) preparation of the diluted samples, 2) the accuracy at which the smartphone camera is aligned with the Test Line, and 3) the probe to antibody binding distribution at the Test Line. Nevertheless, this error is quite small in terms of making decisions regarding the level of a person's infection and contagious levels. In contrast, the standard deviation of 1.9 is somewhat high, although it is skewed by Sample 15, the fourth dilution of the Ct 19.9 sample, with a "corrected minus calculated Ct value" of -7.3. Removing this one sample improves both the average error and standard deviation to 1.05±1.12. Furthermore, the error increases significantly above Ct 26 as the reflectance approaches 200, 95% of the measureable reflectance limit of 208 ([208-200]/[208-50]). Eliminating Samples 15, 18, 19, and 20 yields an average difference and standard deviation of 0.58±0.43 for the more critical diagnostic regions: highly contagious, Ct < 21, and contagious, Ct 21 to Ct 27 [12].

Conclusion

A simple four step at-home SARS-CoV-2 test was developed and successfully used to perform FDA EUA required laboratory measurements for lateral flow assay based kits. The test employed saliva, which could prove useful for testing children. The test also employed three antibodies to bind to the virus, which provided excellent sensitivity and selectivity, and should also allow detecting future variants. The ability of measuring the reflectance of the LFA Test Line for a saliva sample using a smartphone to calculate Ct values, and estimate the level of SARS-CoV-2 infection was also demonstrated. The relationship between the measure reflectance and Ct values followed the Avrami equation, which has been previously demonstrated for PCR plots of sample fluorescence as a function of Ct values [13]. While the upper Ct limit of 26-27 was determined for quantitative measurements, an upper limit for true positives was found to be 29-31 for 38 purchased saliva samples. The small scatter in the data from the Avrami curve demonstrates that the performance of the LFAs were very consistent in terms of probe-to-antigen binding at the conjugate pad and antigen-to-antibody binding at the Test Line, as well as flow across the LFAs. The consistency and quantitative nature of the data also suggests the LFAs could possibly be used to indicate the level of infection [12-16]. Furthermore, the use of the smartphone App and LFAs could be used at-home by individuals to estimate their infection and contagious levels, self-isolate as necessary, and anonymously share their data with health agencies, such as the CDC, to better track and limit outbreaks. The combination lateral flow assay and smartphone quantitation is well suited for identifying, quantifying, and quarantining to slow the spread of future viruses.

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