DRUL for School: Opening Pre-K with safe, simple, sensitive saliva testing for SARS-CoV-2

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Abstract

To address the need for simple, safe, sensitive, and scalable SARS-CoV-2 tests, we validated and implemented a PCR test that uses a saliva collection kit use at home. Individuals self-collected 300 µl saliva in vials containing Darnell Rockefeller University Laboratory (DRUL) buffer and extracted RNA was assayed by RT-PCR (the DRUL saliva assay). The limit of detection was confirmed to be 1 viral copy/µl in 20 of 20 replicate extractions. Viral RNA was stable in DRUL buffer at room temperature up to seven days after sample collection, and safety studies demonstrated that DRUL buffer immediately inactivated virus at concentrations up to 2.75x10⁶ PFU/ml. Results from SARS-CoV-2 positive nasopharyngeal (NP) swab samples collected in viral transport media and assayed with a standard FDA Emergency Use Authorization (EUA) test were highly correlated with samples placed in DRUL buffer. Direct comparison of results from 162 individuals tested by FDA EUA oropharyngeal (OP) or NP swabs with co-collected saliva samples identified four otherwise unidentified positive cases in DRUL buffer. Over six months, we collected 3,724 samples from individuals ranging from 3 months to 92 years of age. This included collecting weekly samples over 10 weeks from teachers, children, and parents from a pre-school program, which allowed its safe reopening while at-risk pods were quarantined. In sum, we validated a simple, sensitive, stable, and safe PCR-based test using a self-collected saliva sample as a valuable tool for clinical diagnosis and screening at workplaces and schools.
Introduction

The SARS-CoV-2 pandemic has raged in the United States, with over 400,000 deaths by the end of Trump administration [1,2]. Mitigation of this tragedy has struggled alongside the lack of a uniform approach to testing, including mixed messages from the Centers for Disease Control and Prevention (CDC) [3]. These challenges were exacerbated by shortages of testing reagents and supplies [4–6]. Scalable, low cost, accessible testing, in symptomatic and asymptomatic individuals is critical to management of the pandemic. Workplaces and schools need workable strategies to test students, employees and families. Working mothers have been disproportionately affected by the need to care for children who are at home during school closures [7]. Testing is increasingly being used to supplement contact tracing efforts. Collecting, transporting and handling samples in buffer that inactivates virus may decrease exposure risk for healthcare providers and laboratory personnel [11].

Saliva testing is seen as an accessible and scalable means of testing, particularly in the school setting since it does not require technical expertise for collection. However, a wide range of tests have been developed, and those with low sensitivity pose an increased risk of reporting false negatives, which may give a false sense of security and decrease transmission mitigating behaviors. We developed an assay that simplifies sample collection and minimizes contact and exposure, using a kit for self-collection of saliva specimens. The DRUL buffer is based on the solution widely used in RNA extraction that contains [12]. Samples were assayed using a test developed using the CDC 2019-nCoV Real-Time PCR Diagnostic Panel primers and probes and RT-PCR [13] as authorized by
the NY State Clinical Laboratory Evaluation Program (CLEP) for use as a clinical diagnostic test. Here we report the results of our validation and initial implementation of this testing strategy.

Materials and Methods

Study subjects

Individuals voluntarily participated in sample collection for serial screening. They were provided with a sample collection kit and instructions (Supplemental Figure 1). Protocols for the collection of saliva samples were either approved by the Rockefeller University (RU) Institutional Review Board (IRB) and Biomedical Research Alliance of New York IRB or were deemed not to be human subjects research by the RU IRB. Where required, written or verbal consent was obtained from all volunteers.

Specimen collection and processing

Individuals were instructed to avoid eating or using oral cleansing agents for 30 minutes prior to collection of saliva (or their children's saliva) in a medicine cup, and then transfer 300 µl of saliva using a pre-calibrated plastic bulb into a vial containing 1200 ul of DRUL buffer (Table 1). Samples were stored and transported at room temperature.

SARS-CoV-2 assay

In early experiments, RNA was extracted using a modified phenol-chloroform extraction method. 80 µl of 3M sodium acetate, pH 5.5 was added to 800 µl of sample plus buffer and mixed. Then 800 µl of acid-phenol:chloroform pH 4.5 (with IAA, 125:24:1, Ambion,
Cat# 9720) was added and mixed. Samples were centrifuged at 12,000 x g for 10 minutes at 4°C after which the aqueous phase (750 µl) was placed into a new tube. 750 µl of OmiPur chloroform: Iso-Amyl Alcohol (Calbiochem, Cat# 3155) was added, mixed, then centrifuged at 12,000 x g for 10 minutes at 4°C. The aqueous phase (550 µl) was placed into a new tube to which 2 µl GlycoBlue (Invitrogen, Cat# AM9515) was added and mixed. 550 µl of ice cold 100% isopropanol was then added and incubated for 15 minutes at -80°C or overnight at -20°C. Samples were centrifuged at 20,000 x g for 20 minutes at 4°C and supernatant removed without disturbing the pellet. 1 ml of cold 75% ethanol was added to the pellet and centrifuged at 20,000 x g for 5 minutes at 4°C. The supernatant was removed and the pellet dried at room temperature for 10 minutes and resuspended in 35 µl of nuclease-free water. In later experiments, RNA was extracted using a column extraction method with a commercial kit (Qiagen, QIAamp DSP Viral RNA Mini Kit, Cat# 61904) according to the manufacturer's instructions. RNA was eluted in 35 µl of nuclease-free water.

cDNA was amplified using TaqPath 1 Step RT-PCR (Life Tech, Cat# A15300) with CDC validated primers and probes (IDT, CDC Emergency Use Authorization Kit) using the Bio-Rad CFX96 C1000 Touch Real-Time PCR Detection System. Samples were considered interpretable if the housekeeping control (RNase P) cycle threshold (Ct) was less than 40 and viral RNA was considered detected with both viral primers/probes (N1 and N2) at Ct <40.
To determine the limit of detection (LOD) of the DRUL saliva assay, contrived clinical specimens (found to be viral-free in the absence of synthetic RNA) were made by spiking in known amounts of quantitative synthetic RNA from SARS-Related Coronavirus 2 (BEI Resources, Cat# NR-52358) into 300 µl of saliva added to indicated amounts of DRUL buffer. Saliva collected from normal volunteers previously determined to be negative for SARS-CoV-2 was pooled and spiked with DRUL buffer containing synthetic SARS-CoV-2 RNA (BEI Resources, Cat # 52358).

To assess sensitivity and specificity of the DRUL saliva assay, thirty NP swab samples were obtained from New York City Public Health Laboratory (NYC PHL). The NP swabs were collected using standard methods by a provider and placed in 3 ml of VTM, and 300 µl of the VTM was added to 1200 µl DRUL buffer at NYC PHL and then transported to the Darnell laboratory for testing.

To determine the ability of DRUL buffer to inactivate virus, Huh-7.5 cells were plated at 1.67x10^5 per well in each well of 6 well plates and allowed to adhere overnight. Human coronavirus 229E (3.66 x 10^6 PFU/ml) was used as a surrogate for SARS-CoV-2. Mixtures of DRUL buffer and virus at volume ratios of 1:2, 1:3, 1:4, 1:5, 1:6 and 1:10 were incubated overnight and added to the Huh-7.5 cells the following morning. The viability of the Huh-7.5 cells was assessed after 3 and 5 days of incubation, yielding approximate TCID_{50} values. The TCID_{50} was calculated as the concentration of virus that when diluted in a defined concentration of DRUL buffer led to 50% viability of Huh-7.5 cells on day 3 post inoculation.
To measure viral RNA stability in DRUL buffer, specified concentrations of human coronavirus 229E were incubated with saliva and DRUL buffer and assayed for presence of viral RNA after overnight incubation or after seven days at room temperature and at 0°C, 25°C, and 38°C for seven days. cDNA was amplified using iScript Reverse Transcription Supermix (BioRad, Cat#1708841) and two primer sets for human coronavirus 229E, set 1: forward-TGAAGATGCTTGTACTGTGGCT and reverse-CTGTGTCATGTTGCTCATGGGG, set 2 forward-AGATGCTTGTACTGTTGCTTCT and reverse-GTCATGTTGCTCATGGGGAG (IDT, custom) from 5' to 3' [8] and FASTSTART Universal SYBR Green Master Mix (Millipore Sigma, Cat # 4913914001) using the Bio-Rad CFX96 C1000 Touch Real-Time PCR Detection System. Samples were considered interpretable if the house keeping control (beta-actin) Ct was <40 and viral RNA was considered detected if Ct for both viral primers were <40.

Results

DRUL saliva assay validation

To establish the LOD of the DRUL saliva assay, simulated specimen matrix was made using 5 pooled saliva samples and DRUL buffer spiked with 10-fold serial dilutions of synthetic SARS-CoV-2 RNA. Samples were extracted using a phenol-chloroform or column-based method. Dilutions were tested in triplicate at each concentration of viral RNA. The LOD was determined to be 1 copy/µl with both extraction methods (Figure 1, A and B). The LOD was confirmed with 20 replicates, each spiked with 2, 1, and 0.2 copies/µl of synthetic RNA, using both extraction methods (Figure 1, C and D) and with
20 separate extractions, each spiked with 2, 1, and 0.2 copies/µl of synthetic RNA using the column-based extraction method (Figure 1E).

Given a paucity of positive samples to use for a clinical evaluation study, we created simulated positive samples representing various viral concentrations. Synthetic viral RNA was spiked into five individual specimen matrices at 2, 4, 6, 8, 10, and 100 times the confirmed LOD (1 copy/µl) to simulate a range of viral load. RNA was extracted using the phenol or column-based method. In addition, 10 negative specimen matrices were assayed. As expected, mean Cts decreased with increasing viral RNA concentrations using both extraction methods (Figure 2, A and B). RNase P Cts was <40 and Cts were similar in all samples.

To compare the DRUL saliva assay with a clinically validated platform, we obtained 30 NP swab samples that had tested positive with a wide range of Cts (17.3 to 39.5 on the N2 target) on the Cepheid Xpert Xpress SARS-CoV-2 assay [14]. We detected 30/30 positives (100% sensitivity), and comparison of the Ct values of the N2 target on both platforms revealed that they were highly correlated (Figure 3, Pearson correlation, R²=0.96). These results indicate that the DRUL saliva assay performed with high specificity, and with quantitative results that were concordant over a wide range (4.8 x 10^6-fold) of detectable viral RNA in clinical samples, as confirmed by comparison to the Cepheid Xpert Xpress SARS-CoV-2 assay.

To further investigate the sensitivity and specificity of the DRUL saliva assay, we compared 63 OP swab results collected from RU Occupational Health Services, which
were tested at commercial laboratory and with co-collected self-collected saliva samples in DRUL buffer. The majority of co-collected specimens (57/63, 90.5%) were negative by both assays (Table 2). Of the remaining six specimens, SARS-CoV-2 RNA was detected in the saliva specimen in three participants. Of these three, two of the co-collected OP specimens were negative and one was indeterminate by the Commercial Laboratory A test. These individuals were symptomatic. Three additional participants were negative by OP swab, and the saliva test was invalid. RNaseP target was not detected, which we most commonly found correlated with insufficient saliva specimen, although in some cases inhibitors may have been present.

In a second study to assess sensitivity and specificity, we compared 99 NP swab samples collected by healthcare providers at New York City Health and Hospital – Elmhurst (tested at Commercial Laboratory B) with co-collected, self-collected saliva samples in DRUL buffer (Table 3). All samples but one were negative by both assays. The DRUL saliva assay identified one positive sample of the 99 which was negative by NP swab in an asymptomatic individual. At the time these experiments were done, the turnaround time for results from paired samples in Commercial Laboratory B was three to five days, while results from the DRUL saliva assay were generally available the next day, including the one positive sample. These studies taken together suggest similar, if not higher sensitivity of the DRUL saliva assay than commonly accepted viral assays using OP or NP swabs.

To assess the stability of viral RNA in DRUL buffer, we titrated concentrations of human coronavirus 229E into saliva and DRUL buffer and compared Ct values of samples incubated overnight or after 7 days at room temperature. There was no significant
difference between the Ct values of samples incubated overnight and those incubated for 7 days (Figure 4A and B). We further assessed the stability of viral RNA incubated at 0°C, 25°C, and 38°C for 7 days, to mimic potential temperature ranges during sample transport and did not find a significant difference in Ct values (Figure 4C).

To evaluate the effect of DRUL buffer on viral infectivity, we used human coronavirus 229E as a surrogate for SARS-CoV-2. We assessed the viability of Huh-7.5 cells, a well characterized, adult hepatocellular carcinoma cell line, after exposure to various dilutions of coronavirus (stock 3.66 x 10^6 PFU/ml) in DRUL buffer. Huh-7.5 cell survival indicates that the virus was inactivated by the DRUL buffer, and cells remained viable after exposure of stock virus diluted with DRUL buffer at ratios of 1:4 (DRUL:virus), indicating that DRUL buffer completely inactivates virus at 2.75 x 10^6 PFU/ml (Figure 5A and 5B). At DRUL to virus ratio of 1:5 (3.41 x 10^6 PFU/ml), approximately half the Huh-7.5 cells were lysed at day 5 indicating viral survival, and this value was taken as a conservative estimate of the TCID_{50}. We compared this with the AVL buffer, a component of the QIAamp Viral RNA kit that the CDC determined to inactivate virus. [7] AVL buffer inactivated virus at a buffer to virus ratio of 1:3 (2.44 x 10^6 PFU/ml) but not at 1:4, indicating that DRUL buffer inactivates virus at a level comparable to AVL buffer (Figure 5A and B). In control samples, cell lysis occurred when we exposed Huh-7.5 cells to as little as 4 PFU/ml human coronavirus E229 without DRUL buffer, and no cell lysis occurred when DRUL buffer was added without virus.

To determine the minimum incubation time required for the DRUL buffer to inactivate virus, we incubated DRUL buffer and virus at a ratio of 1:4 for 60 minutes, 10 minutes
and 10 seconds before incubating with Huh-7.5 cells. We found that 100% of the Huh-7.5 cells were viable at 3 and 5 days after incubation with virus exposed to DRUL buffer for as little as 10 seconds (Figure 4C). Taken together these results indicate that DRUL buffer nearly instantly inactivates live, high titer coronavirus.

Clinical use of the DRUL saliva assay

These validation data were submitted to New York State CLEP and the DRUL saliva assay was subsequently authorized for use as a clinical diagnostic test and are currently pending final approval. The assay was used in 3,724 samples between May and October of 2020 from individuals who ranged in age from 3 months to 92 years. We began with testing symptomatic employees and asymptomatic essential employees coming onto the RU campus.

In July of 2020, the RU Child and Family Center (CFC) for children of employees between the ages of three months and five years reopened on a pilot basis, enrolling 58 children in July and August of 2020, then 87 children starting in September. Each child, teacher and staff member was tested weekly, and parents were also offered testing. 2117 kits were distributed over 12 weeks (Supplemental Table 1), which were typically taken home, where saliva was collected and added to DRUL buffer with a plastic bulb syringe. Electronic sample submission forms linked to a personalized registration data were completed for each sample, and tubes returned to RU the following day.

Over these 12 weeks, only one asymptomatic parent tested positive. The parent was isolated, the child (a contact) was quarantined, and the classroom closed. Overall, 26
children school days were missed (number of children in room x number of days classroom closed or school days missed; Table 4). All other tests among the children, teachers, staff, and parents were negative, allowing these rooms to remain open, consistent with (or more conservative than) NYS/NYC DOE school guidance. There were three additional room closures due to symptomatic (as defined by CDC guidelines) children or teachers who tested negative, resulting in 46 children school days missed. There were 72 missed children school days out of 4205 (1.7%) over the course of 12 weeks.

Discussion

Here, we report the validation of the DRUL saliva assay for SARS CoV-2 molecular testing as performed at RU. This assay was easy to administer, using a self-collection kit that could be performed at home by adults or by older children under adult supervision. RT-PCR assays, using either traditional phenol-chloroform or column-based extraction methods revealed that the assay was extremely sensitive, with a LOD of 1 copy/µl of viral RNA, and was found to perform nearly identically to a clinical platform (Cepheid Xpert Xpress SARS-CoV-2 assay). Moreover, the assay was found to be at least as sensitive as OP and NP swabs assessed by commercial laboratories using FDA approved molecular tests.

The DRUL saliva assay was developed with the goal of overcoming the early obstacles to widespread SARS-CoV-2 testing, such as shortages of reagents and specialized supplies, healthcare provider access, and PPE for healthcare providers. This method also
limits potential exposure during transit and of laboratory personnel during performance of
the assay. The TCID$_{50}$ of virus (~2.64 x 10$^6$ PFU/ml) diluted 1:4 (v:v) in DRUL buffer was
found to compare favorably to commercial inactivation buffers (Qiagen’s AVL buffer,
Figure 5 or the SDNA-1000 saliva collection device with an estimated TCID$_{50}$ of ~1 x 10$^4$
PFU/ml used by Rutgers Clinical Genomics Laboratory TaqPath SARS-CoV-2 Assay) [13,15].

Such solutions have a health hazard label that grades them as less toxic than household
bleach. DRUL buffer kits are distributed with appropriate cautions and instructions on
what to do in case of a spill or contact. To further minimize risk, we have recently
succeeded in decreasing the required volume of DRUL from 1200 µl to 300 µl with similar
results (unpublished data).

The DRUL saliva assay was used for testing symptomatic individuals and screening
asymptomatic essential employees on the RU campus over the course of 6 months. It
was easy to use across a variety of ages and individuals with varied backgrounds. During
this time the assay was used to aid in the reopening of a childcare center that enrolled
children as young as three months old. The use of the test minimized the number of days
a classroom closed and allowed the rest of the center to remain open safely. With testing,
98.7% student attendance was possible, along with reassurance that both they and their
teachers had undetectable viral RNA on a weekly basis. As SARS-CoV-2 infection remain
a significant clinical issue, the DRUL saliva test offers a simple, safe, and cost-effective
method for use as part of highly scalable “back to work/school” strategies.
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Tables

Table 1. DRUL buffer reagents.

| Reagent                                      | Amount needed | Purpose                               | Manufacturer            | Catalog #         |
|----------------------------------------------|---------------|---------------------------------------|-------------------------|-------------------|
| 5M Guanidine Thiocyanate                     | 59.08 g       | Protein denaturing agent, Isolation of RNA | Fisher BioReagents      | BP221-1           |
| 0.5% Sarkosyl                                | 5 ml of 10% Sarkosyl | Cell lysis, detergent                | Fisher BioReagents      | BP234-500         |
| 25mM Sodium Acetate (3M), pH 5.5             | 0.83 ml       | Precipitation of RNA                  | Invitrogen              | AM9740            |
| Nuclease-free Water                          | Bring up to 100 ml | Dilution                             | Ambion                  | AM9932            |

Table 2. Comparison of SARS-CoV-2 DRUL assay with commercially tested OP swabs.

| Commercial Lab A-OP swab | Darnell Lab-Saliva |
|--------------------------|--------------------|
|                          | Positive | Negative | Invalid |
| Positive                 | 0        | 0        | 0       |
| Negative                 | 2        | 57       | 3       |
| Inconclusive             | 1        | 0        | 0       |
Table 3. Comparison of SARS-CoV-2 DRUL assay with commercially tested NP swabs.

|                  | Darnell Lab-Saliva |
|------------------|--------------------|
|                  | Positive | Negative |
| Commercial Lab B-| Positive | 0        | 0          |
| NP swab          | Negative  | 1        | 98         |

Table 4. Child and Family Center closures.

| Closure event | Cause                | Total days closed (no.) | School days closed (no.) | Children school days missed (no.) |
|---------------|----------------------|-------------------------|--------------------------|-----------------------------------|
| 1             | symptomatic child    | 6                       | 4                        | 28                                |
| 2             | positive parent      | 6                       | 4                        | 26*                               |
| 3             | symptomatic teacher  | 3                       | 1                        | 10                                |
| 4             | symptomatic child    | 4                       | 2                        | 8                                 |

*includes 14-day quarantine by child of the positive parent
Figures

Figure 1. Limit of detection (LOD).

Determination of LOD with (A) phenol and (B) column-based extraction methods and confirmation of LOD using (C) phenol and (D) column-based extraction methods in 20 replicates and (E) column-based extraction method in 20 separate extractions from unique saliva samples. Black bars=N1 primer, open bars=N2 primer, gray bars=RNase P primer. Error bars=1 standard deviation.

Figure 2. Assay performance with two extraction methods.

Specimen matrix spiked with specified concentration of synthetic RNA and extracted using A. phenol and B. column-based methods. Black bars=N1 primer, open bars=N2 primer, gray bars=RNase P primer. Error bars=1 standard deviation.

Figure 3. Correlation of NP samples on Cepheid Xpert Xpress SARS-Cov-2 platform versus phenol extraction in DRUL buffer.

Figure 4. Stability of viral RNA.

Stability of RNA assessed with (A) primer set 1 and (B) primer set 2 in DRUL buffer. Black bars=overnight incubation, open bars=after 7 days of incubation. C. Stability assessed at 0°C (black bars), 25°C (open bars), and 38°C (gray bars) and saliva alone (striped bars). Error bars=1 standard deviation.

Figure 5. Inactivation of virus in DRUL buffer.

Huh-7.5 cell lysis assessed at day 3 (A) and day 5 (B) after incubation with DRUL (black bars) or AVL buffer (open bars) and human coronavirus E229 at buffer:virus volume ratios ranging from 1:2 to 1:10. Insert table shows viral concentration at each ratio. C. Huh-7.5
cell lysis assessed at days 3 (black bars) and 5 (open bars) after incubation with human coronavirus E229 exposed to DRUL buffer for 60 minutes, 10 minutes, or 10 seconds.
Supplemental Figure 1. Instructions for self-saliva collection included with the kit.

**Instructions for at home saliva collection**

**Contents of the kit:**
- paper medicine cup
- small tube with solution in the tube
- dropper for transferring saliva
- biohazard bag
- shipping container

**Items that you need but are not in the kit:**
- tissues
- tape

**Steps for collecting sample and transferring to buffer:**

1. Please do not eat, drink, brush your teeth, or smoke for at least 1 hour before collection; ideally do this collection first thing in the morning when you wake up.

2. Collect items needed and open the kit.

3. Cough 2-3 times, and collect saliva in your mouth and spit into the medicine cup.

4. Open the small tube with solution in it. **Be careful not to spill, swallow or inhale the solution. Do not leave the small tube uncapped for longer than 10 minutes.** If you spill the solution on yourself, immediately wash with water and call your doctor.

5. Squeeze the dropper and draw up saliva to the line shown by the arrow (try to avoid bubbles) and transfer that amount of saliva from the medicine cup into the small tube. Repeat this step, so that together you add a total of 2 droppers worth of saliva (approximately 0.3 ml) to the tube.

6. Cap the small tube; make sure the cap is fully closed.

7. Invert/turn the small tube up and down at least 10 times to mix the saliva with the solution.

8. Use tissues to wipe the outside of the tube if any saliva spills. Wash your hands with soap and water.

9. Print your name and date of birth clearly on the tube (if there is a sticker provided with this information, confirm that it is correct and place it on the tube).

10. Tape the tube to seal the seam between the tube and the cap.

11. Place the small tube into the biohazard bag and seal. Leave the absorbent material in the biohazard bag with the tube.

12. Place the biohazard bag in between the bubble wrap in the cardboard box.

13. Close the cardboard box and put it into the FEDEX Clinical Pak, seal shut and return or follow other delivery instructions if given.

**Place the dropper and the medicine cup in the trash.**
Figure 1

A. 

B. 

C. 

D. 

E.
Figure 2

A

![Bar chart showing cycle threshold vs. copies of RNA/ul for N1, N2, and RP replicates for different dilutions ranging from 100X to 0 copies of RNA/ul.]

B

![Bar chart showing cycle threshold vs. copies of RNA/ul for N1, N2, and RP replicates for different dilutions ranging from 100X to 0 copies of RNA/ul.]

The bars represent the mean cycle threshold with standard deviation for each replicate across the different dilutions.

The x-axis indicates the number of copies of RNA/ul, ranging from 100X to 0, and the y-axis shows the cycle threshold.
Figure 3

$R^2 = 0.9553$
Figure 4

A.

B.

C.

D.

E.

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W.

X.

Y.

Z.
Figure 5

A.  

Huh7.5 cell viability (%)  

| Dilution Factor (Buffer:Virus) | Conc. assayed (x10^6 PFU/ml) |
|-------------------------------|-----------------------------|
| 2                             | 1.83                        |
| 3                             | 2.44                        |
| 4                             | 2.75                        |
| 5                             | 2.93                        |
| 6                             | 3.05                        |
| 10                            | 3.29                        |

Buffer:299E

B.  

Huh7.5 cell viability (%)  

Day 3  
Day 5  

Buffer:299E

C.  

Huh7.5 cell viability (%)  

60 min  
10 min  
10 sec  
100 PFU  
10 PFU  
DRUL  
no DRUL  
no virus