

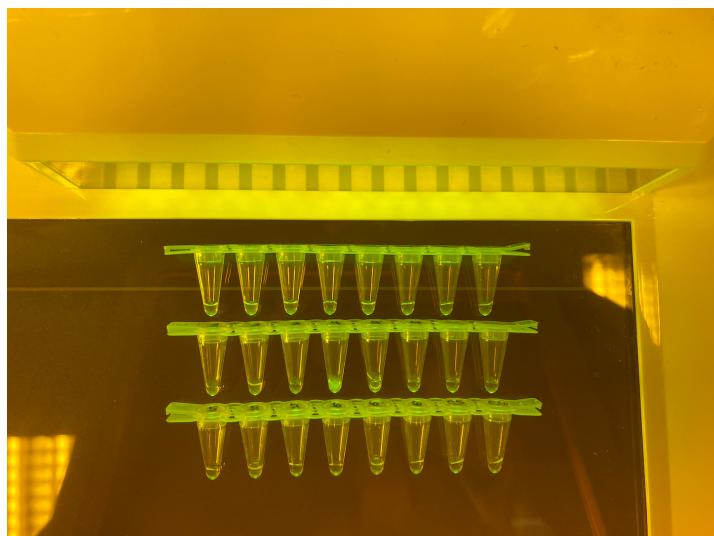
### Cas12a Assay Testing for optimized buffer (No RPA in the reaction)

In this experiment, we aimed to evaluate each crRNA's capacity for RPA amplification. For each crRNA, we conducted a controlled experiment with the buffer as the independent variable. We anticipated the RPA buffer to inhibit Cas12a activity. Upon recording our results, we found that crRNA 6 had a very poor result even under normal conditions. Meanwhile, crRNA 4, with the RPA buffer, had an extremely fluorescent result. crRNA 1, 2, 3, and 5 without the RPA buffer (normal), also had quite bright displays, even though their RPA solutions did not yield such significant results. Overall, despite the examples mentioned above, we found that the RPA did indeed inhibit Cas12a activity. We decided as a next step to retest crRNA 6 individually to determine whether or not the result was an error.

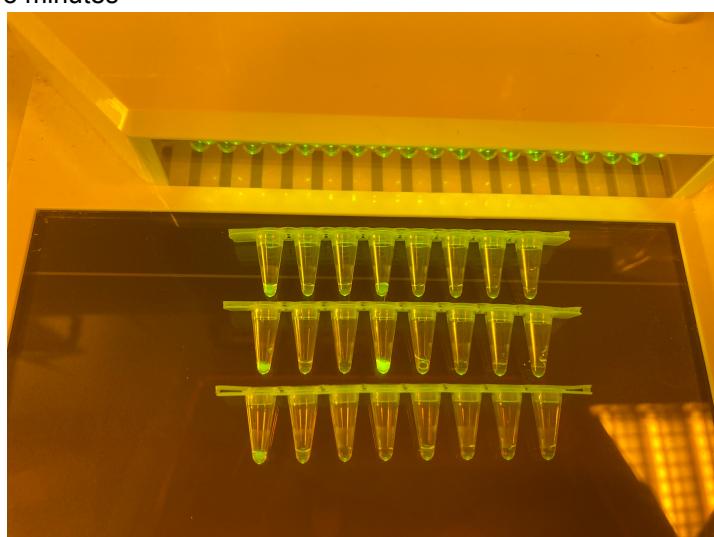
Layout:

Normal 1	RPA 1		Normal 2	RPA 2	Control 2		Control 1
Normal 3	RPA 3		Normal 4	RPA 4		Control 3	Control 4
Normal 5	RPA 5		Normal 6	RPA 6		Control 5	Control 6

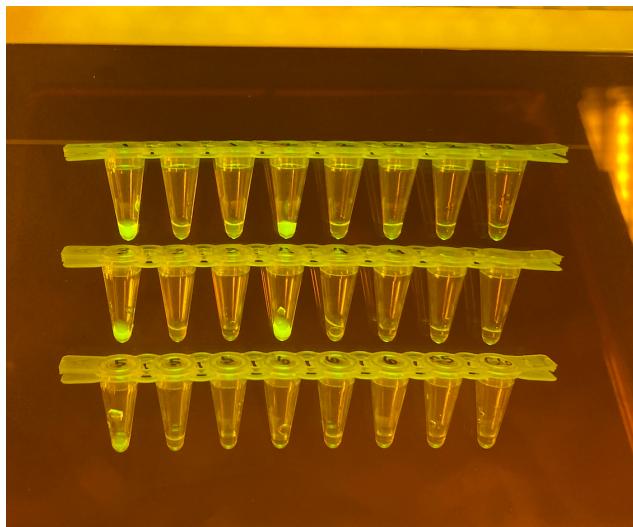
0 Minutes



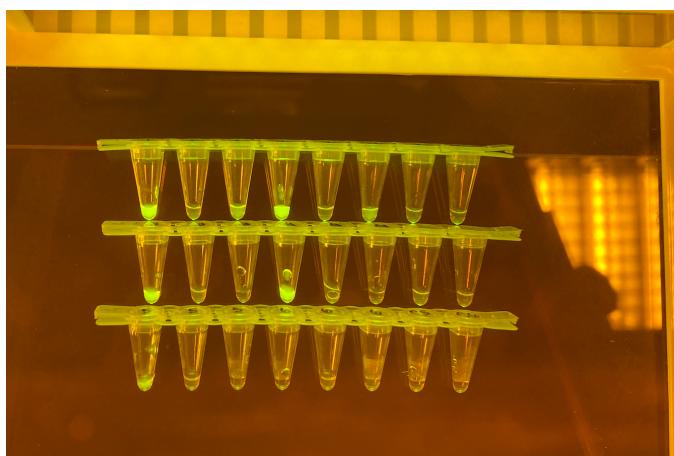
5 minutes



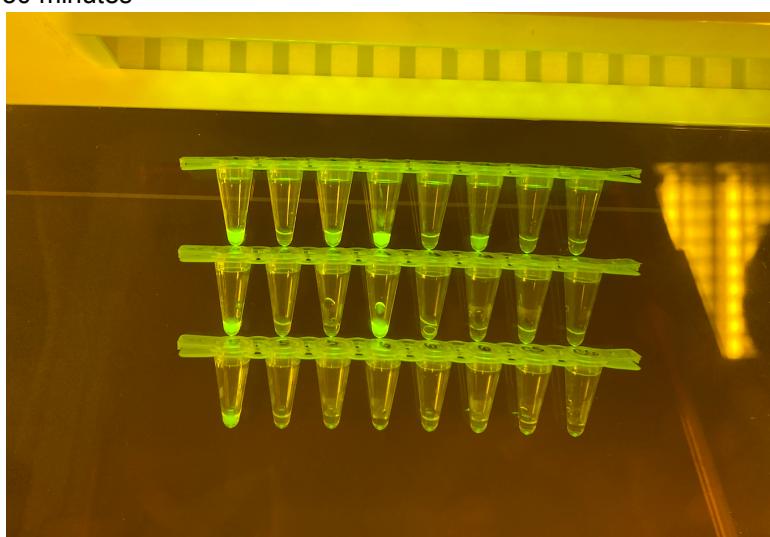
10 minutes



20 minutes



30 minutes



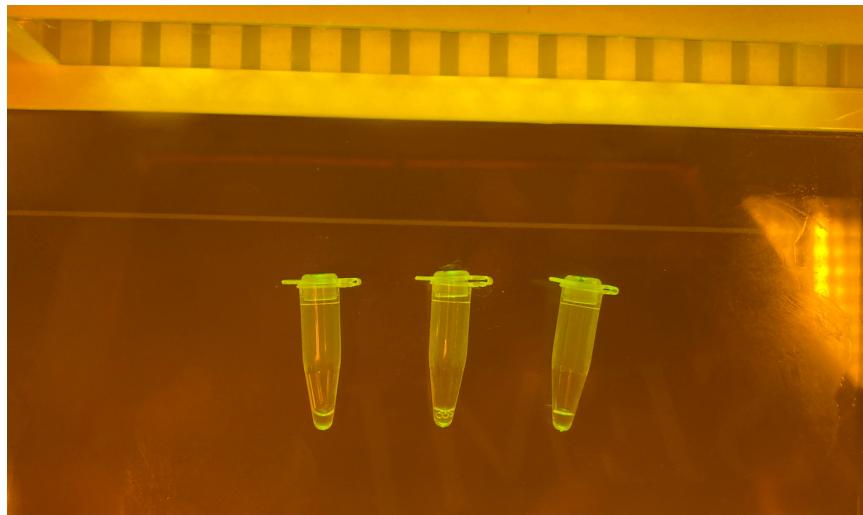
### Retesting crRNA-6 for background:

This experiment was to determine the viability of crRNA 6, following the results from our previous test of the RPA buffer with Cas12a. In our previous experiment, we found that even under normal conditions, crRNA 6 showed little to no activity. To check whether this was the result of an error, we individually tested crRNA 6 again. Our results matched those previously shown, thus confirming our suspicion that crRNA 6 was not fit to be used.

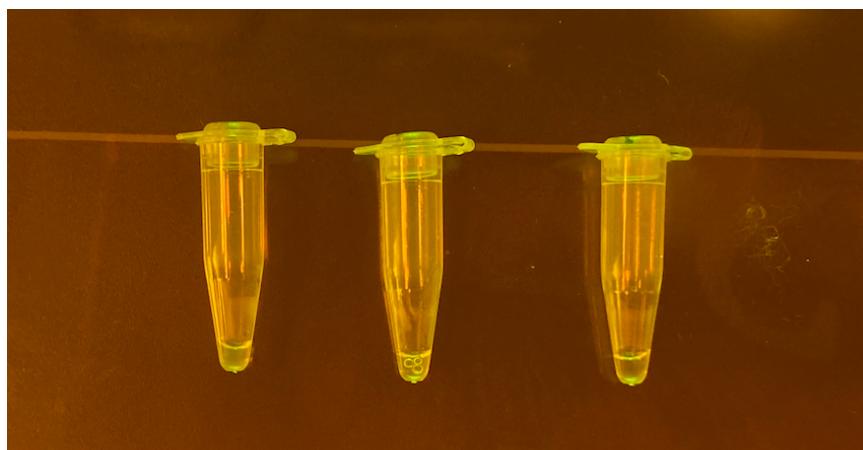
#### Layout

Normal	RPA	Control
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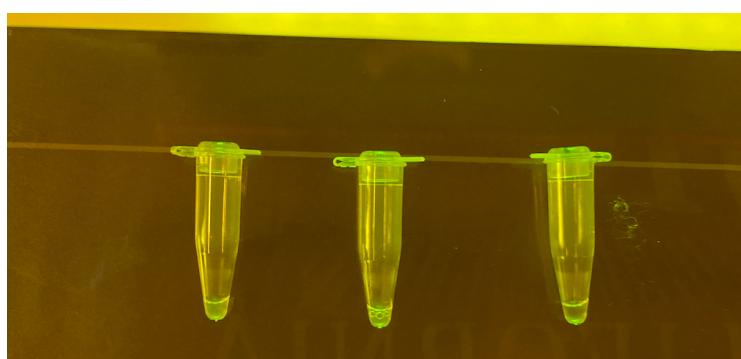
0 minutes



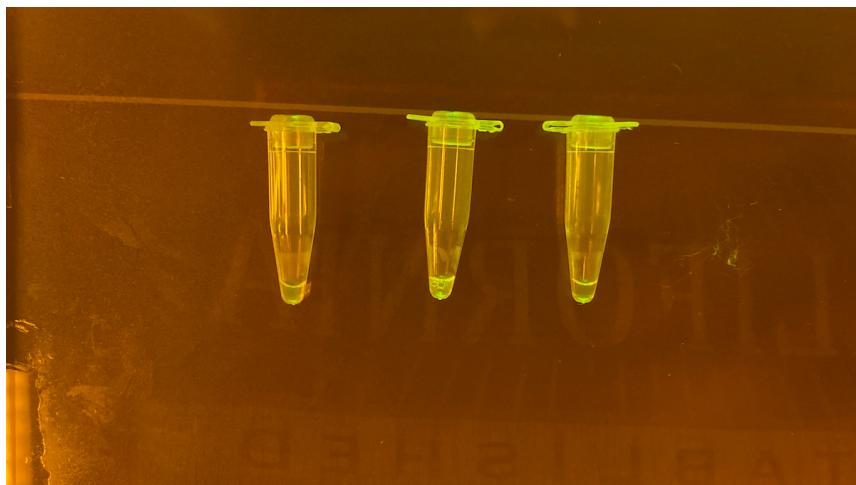
5 minutes



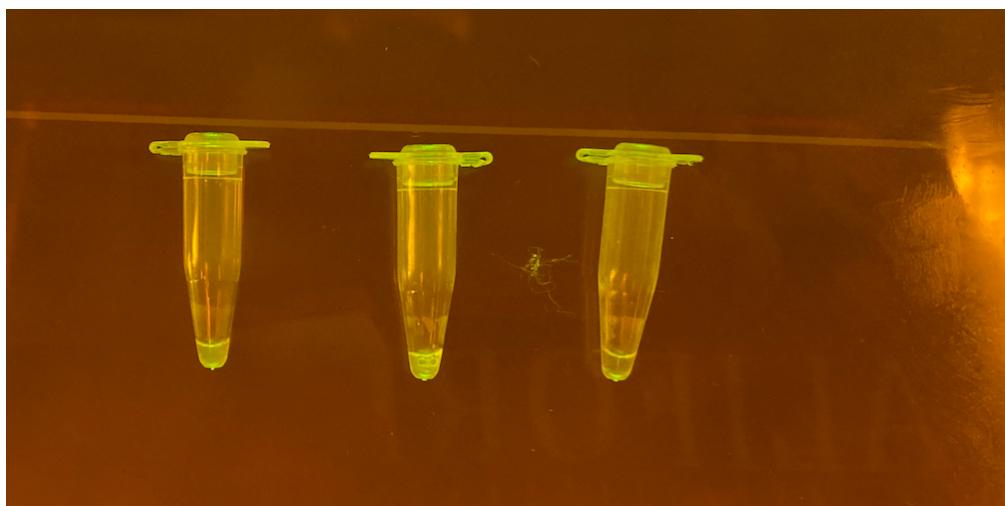
10 minutes



20 minutes



30 minutes



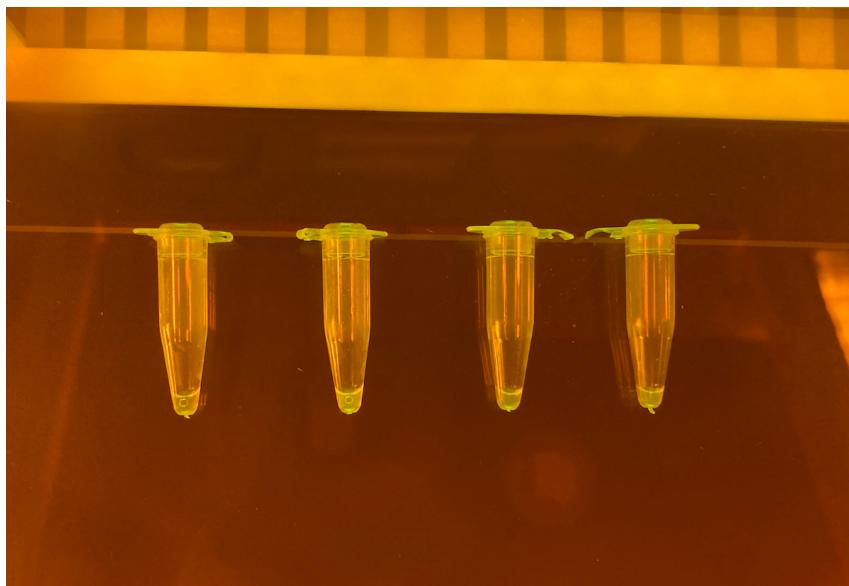
### NEB vs RPA experiment

This experiment compared the results using NEB 2.1 buffer with RPA buffer. We expected to have no results for the controls, and a bright result from the experiments. Our goal was to compare the brightness of the RPA and the NEB to see the difference. Our results were as expected: after 20 minutes, the controls for both NEB and RPA had very dull results, showing a lower reaction. Meanwhile, the NEB was slightly brighter than the RPA, but they both had extremely visible results.

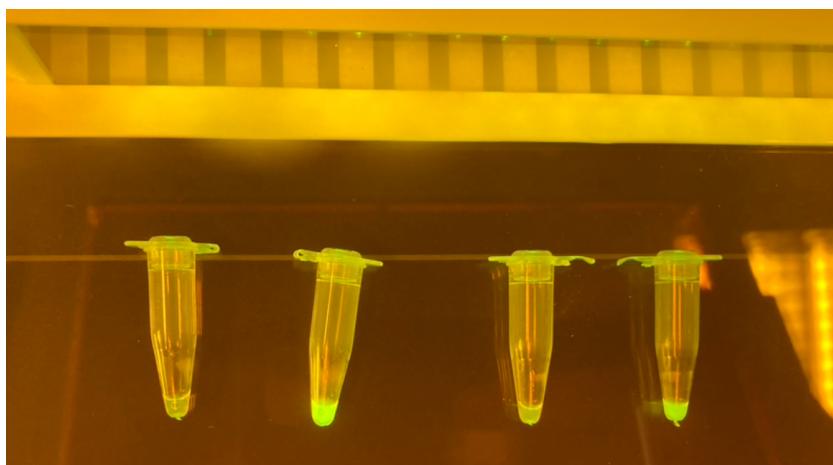
Layout:

Control NEB	Experiment NEB	Control RPA	Experiment RPA
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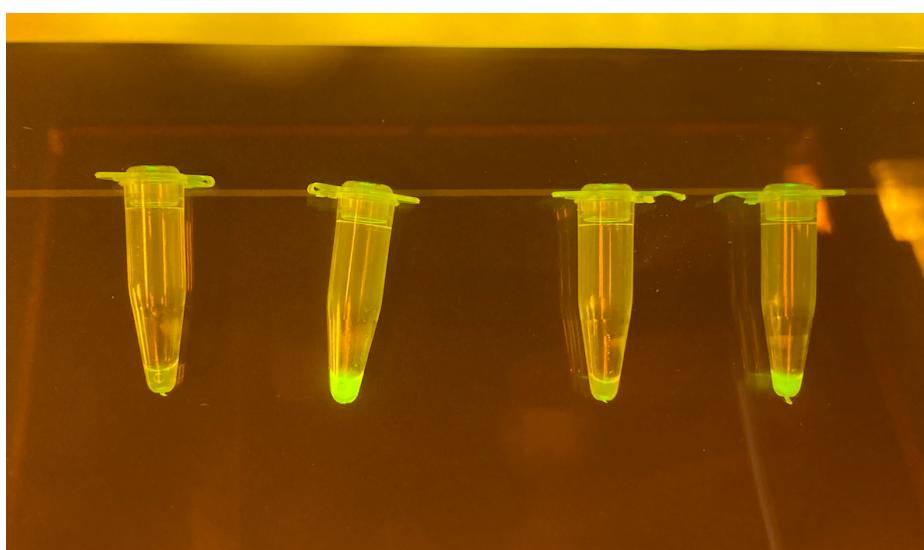
0 minutes



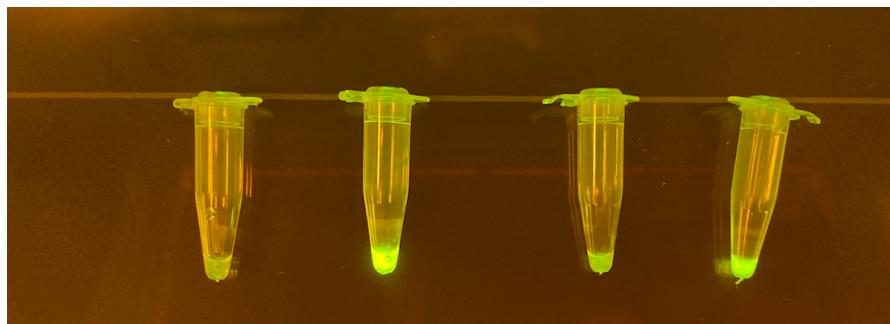
5 minutes



10 minutes



20 minutes



20 minutes

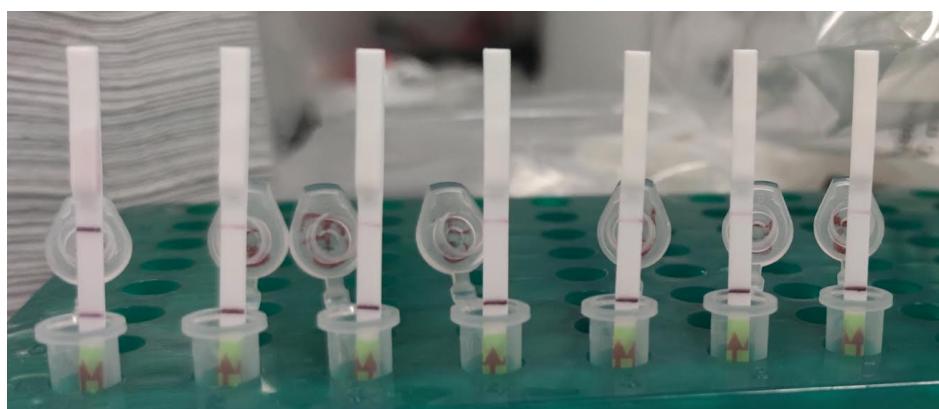


#### Lateral Flow Test – Cas12a Concentration Experiment Protocol.

In this experiment, we wanted to determine the minimum concentration of Cas12a required to show a result. The highest concentration (G1) had a very clear result showing that it was possible to simply add a large amount of Cas12a to show a clear result. In addition to this, we found that all of the tubes showed a positive result, even the controls (which had a very faint positive band). This led us to believe that there was possibly biotin overflow or oversaturation of gold particles in the LFA strips. For our next steps, we decided to troubleshoot the lateral flow assay, to get more accurate results. We decided to further test for biotin degradation and gold nanoparticle oversaturation.

Layout:

G1	G2	G3	G4	G5	Control No Template	Control No Cas12a
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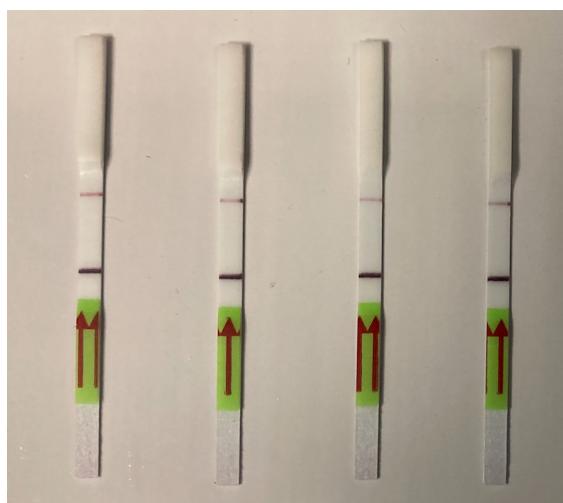
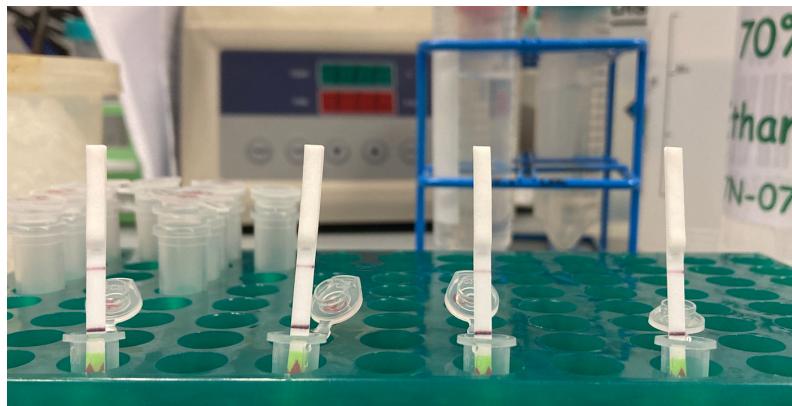


### Testing for degradation of Biotin:

The purpose of this experiment was to determine if the false-positive results from the two-stage Lateral Flow Assay were a result of biotin degradation or biotin overflow. We found that the results were quite strange. While there was a slight gradient from the highest concentration to the lowest concentration, the 0.25  $\mu$ L tube still had a rather defined result, almost the same as the 2  $\mu$ L tube. To move forward, we decided to run more tests on the Lateral Flow Assays, to troubleshoot such strong results.

Layout:

2x Cas12a (2 $\mu$ L Biotin)	1x Cas12a (1 $\mu$ L Biotin)	0.5x Cas12a (0.5 $\mu$ L Biotin)	0.25x Cas12a (0.25 $\mu$ L Biotin)
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### Lateral Flow Assay Dipstick Gold-Nanoparticle Quantity Assessment

In this experiment, we attempted to reduce the saturation of gold nanoparticles by physically cutting the testing area of the lateral flow assay dipsticks. We cut each stick in precisely uniform intervals, to further test the dipsticks. While this method was crude, it aided us in finding our final solution to the problem. The results matched the expected outcome, as the sticks got shorter, the result became fainter and fainter. Thus, this solution helped in reducing the oversaturation of gold particles. Despite this positive outcome, we found this solution extremely crude and unwieldy so we continued to look for better answers to the problem of oversaturation.

Layout (L-R):

Lengths:

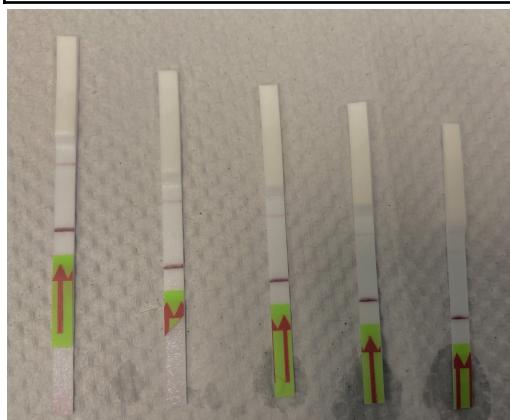
0mm Cut-Off Control

8mm Cut-Off

10mm Cut-Off

12mm Cut-Off

14mm Cut-Off



**Viscosity Experiment For Poly(ethylene glycol)**

This experiment was our final attempt to optimize the lateral flow assay sticks. We decided to increase the viscosity of the assay buffer so that the results would show at a slower rate, and avoid overflowing the sticks. We found that this solution was not only more effective, but also more sophisticated and precise than cutting the dipsticks. By using a 4% concentration of PEG in the assay buffer, the dipstick produced an extremely visible reaction. With the controlled negative showing very little in the band, and the controlled positive showing a very visible band. Overall, this experiment was a success and allowed us to proceed with an optimized assay buffer for our lateral flow assay.

Layout:

Control Negative

Control Positive



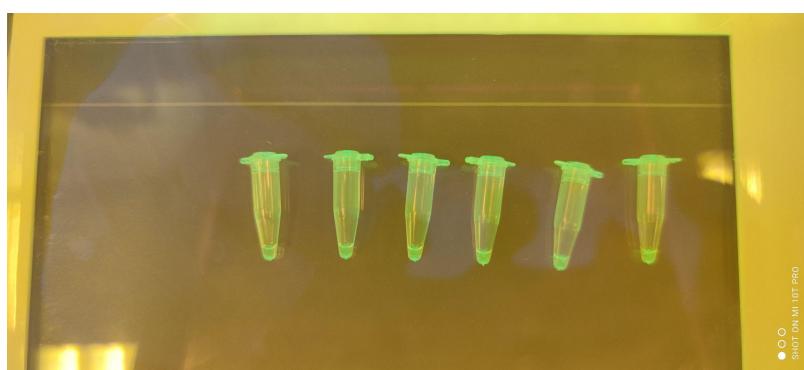
### RPA - Cas Ratio Optimization:

This experiment was to test the ratios between RPA and Cas12a to determine the optimal ratio for our system. We tested different ratios, expecting some variation in the different ratios. However, due to the incredibly high fluorescence of the results, it was much more difficult to determine which ratio was the best. To move forward, we decided to troubleshoot this brightness by testing if it was an error, or the true display of the reaction.

### Layout:

Tube Labels	1	2	3	4	5	6
RPA $\mu$ L:	1 $\mu$ L RPA Mix	2 $\mu$ L RPA Mix	4 $\mu$ L RPA Mix	6 $\mu$ L RPA Mix	8 $\mu$ L RPA Mix	9 $\mu$ L RPA Mix
Cas Mix $\mu$ L:	0.4 $\mu$ L Cas Mix 7.8 $\mu$ L NEB 2.1	0.4 $\mu$ L Cas Mix 6.8 $\mu$ L NEB 2.1	0.4 $\mu$ L Cas Mix 4.8 $\mu$ L NEB 2.1	0.4 $\mu$ L Cas Mix 2.8 $\mu$ L NEB 2.1	0.4 $\mu$ L Cas Mix 0.8 $\mu$ L NEB 2.1	0.4 $\mu$ L Cas Mix

0 minutes



5 minutes



### Troubleshooting High Fluorescence:

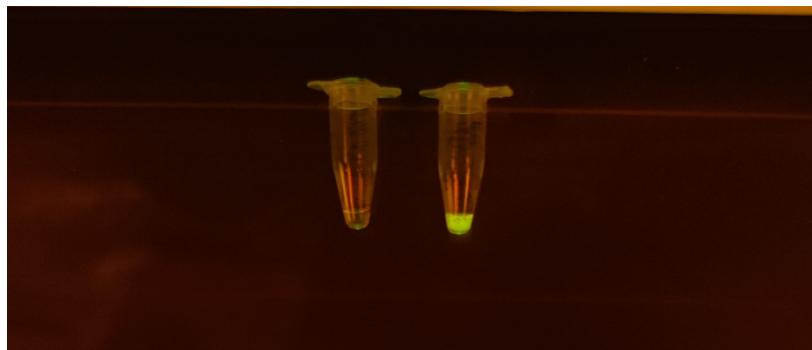
After the incredibly high fluorescence of our previous experiment, we decided to test the fluorescence of the reaction. A negative RPA reaction was included to see if the RPA was the issue, and we readjusted the quantity of Cas enzyme without changing the concentration to see if the low quantity but high concentration of Cas12a was creating a higher-than-normal yield of Cas-crRNA complex, causing the bright result at 5 minutes for the previous ratio experiment. We found that the results were of the same brightness as the previous experiment, showing that this was the optimal ratio.

Layout (for both PCR tubes and Lateral Flow Assay):

Control Negative	Control Positive
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10 minutes:

Fluorescence:



Assay:



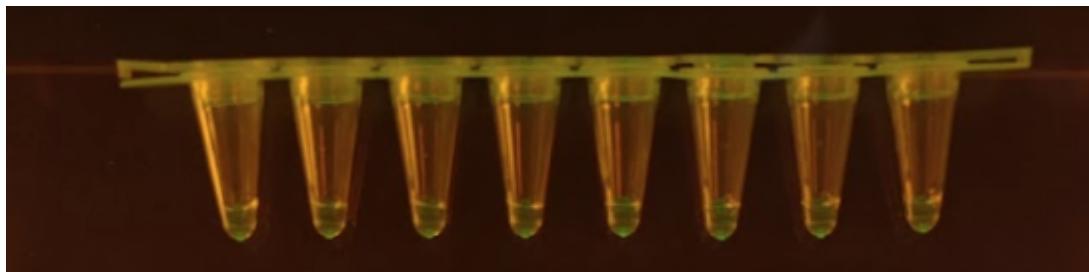
#### Asymmetric RPA Experiment:

In this experiment, we attempted to increase the yield of ssDNA without increasing the use of the Cas12a enzyme. The reason behind our desire to increase yield while decreasing Cas enzyme usage lies in improving our product(kit)'s cost, as we aim to reduce cost and ensure that net profit for our target consumers does not decrease. To do this, we tested different ratios of forward and reverse primers to find the best ratio of asymmetry. From our results, we found that the 0.32F and 0.032R tube had the best result, and generally, the lower concentration of forward and reverse primers had a better result than the higher concentrations.

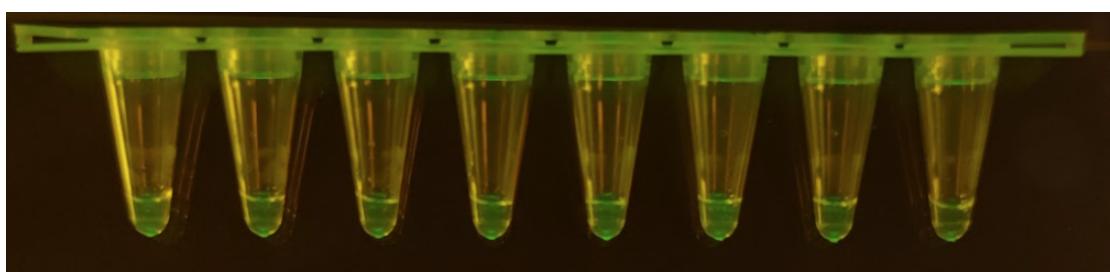
Layout:

0.32 $\mu$ M F 0.32 $\mu$ M R	3.2 $\mu$ M F 0.32 $\mu$ M R	0.32 $\mu$ M F 3.2 $\mu$ M R	3.2 $\mu$ M F + 3.2 $\mu$ M R	0.32 $\mu$ M F 0.032 $\mu$ M R	0.032 $\mu$ M F 0.32 $\mu$ M R	0.032 $\mu$ M F 0.032 $\mu$ M R	0 $\mu$ M F 0 $\mu$ M R
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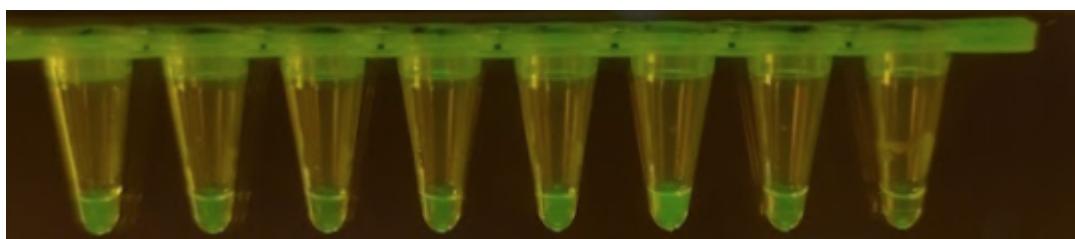
0 minutes



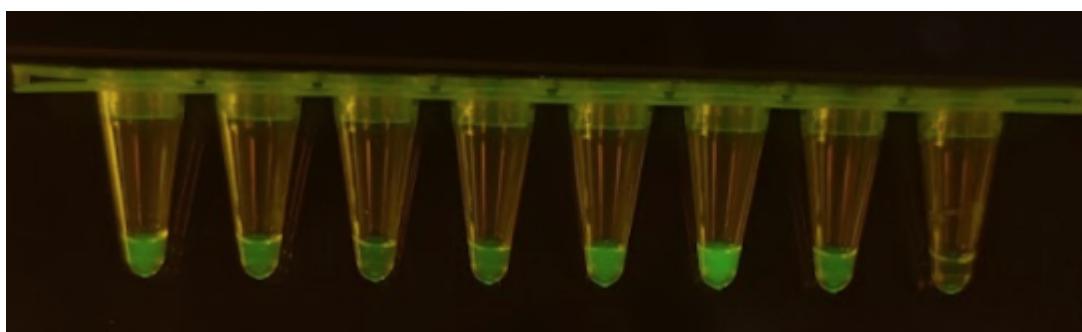
5 minutes



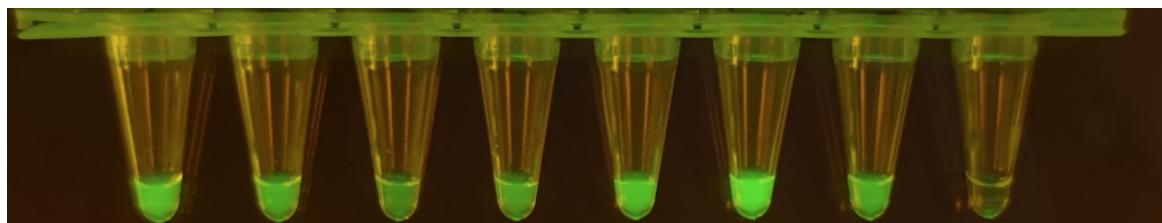
10 minutes



25 minutes



35 minutes

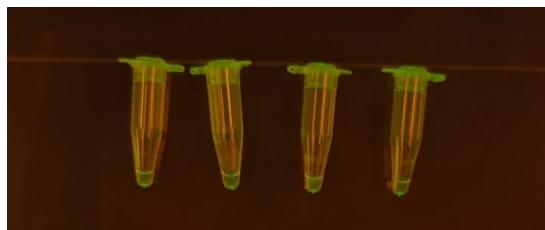


#### Hypothesis Testing for result variation Experiment:

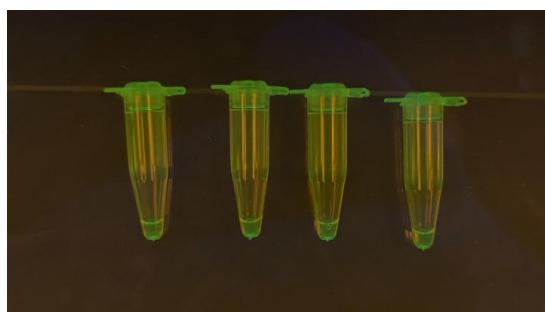
Due to the unexpected results for primer asymmetry experiment for tube 3, with ratio of 1:10 forward to reverse primers, we have carefully examined the processes and have come to a conclusion that longer RPA duration with higher concentrations of primer result in more completed RPA with more duplex strands. Since PAM-less duplexes cannot activate CRISPR Cas12a-gRNA complexes, lower RPA completion is desired. Therefore this experiment is designed with lower RPA incubation duration in order to reduce RPA completion. The results of the experiment indicate that this is the case, as we retested for the highest concentrations of primers (any ratio involving 3.2  $\mu$ M primers) with 10 minutes duration of RPA and got significantly brighter results than the previous experiment at 20 minutes duration RPA.

0.32 $\mu$ M F + 0.32 $\mu$ M R	3.2 $\mu$ M F + 0.32 $\mu$ M R	0.32 $\mu$ M F + 3.2 $\mu$ M R	0 $\mu$ M F + 0 $\mu$ M R
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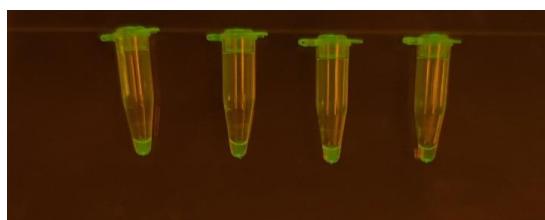
0 Minutes



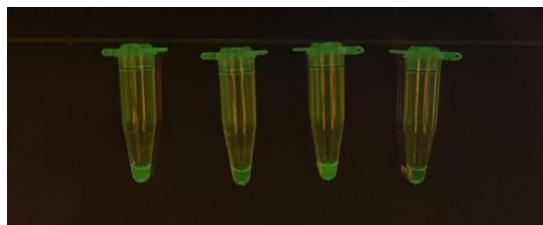
5 minutes



10 minutes



20 minutes



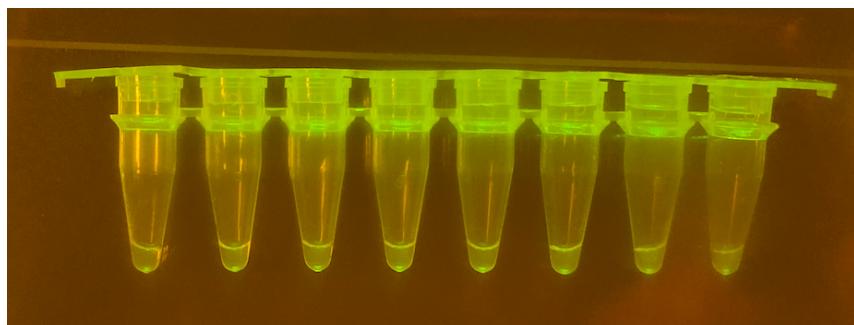
### Proof of Concept: Detection Limits

One way to test the sensitivity of our system was by testing the limits of detection. To do this, we ran our detection system with different concentrations of template in the same amount. From this, we were able to see a clear gradient of the detection system, where it was able to detect at all levels. Overall, these results were quite good, and indicate a successful experiment with excellent results.

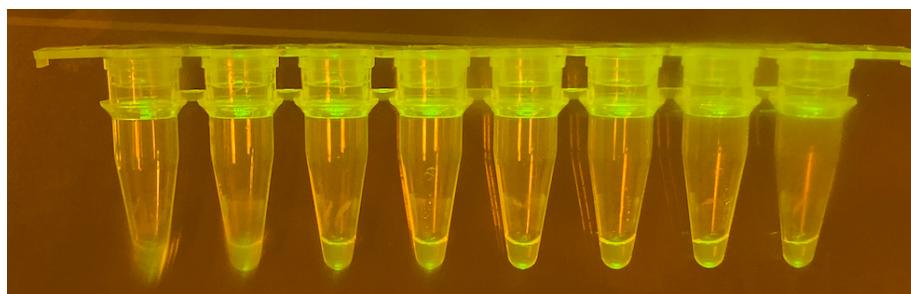
Layout:

$5 * 10^6/\mu\text{L}$ Copies	$5 * 10^5/\mu\text{L}$ Copies	$5 * 10^4/\mu\text{L}$ Copies	$5 * 10^3/\mu\text{L}$ Copies	$5 * 10^2/\mu\text{L}$ Copies	$5 * 10^1/\mu\text{L}$ Copies	$5 * 10^0/\mu\text{L}$ Copies	No template (control)
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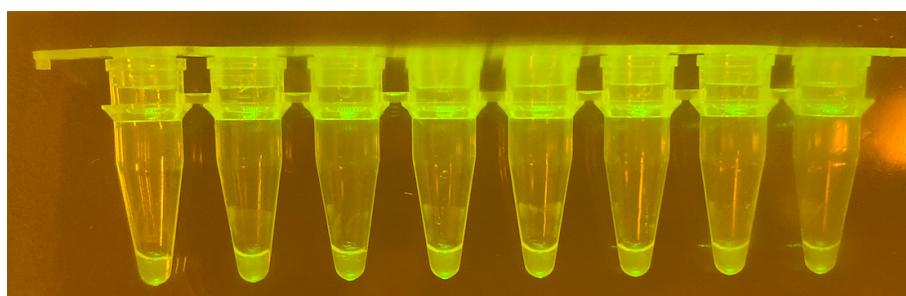
0 minutes:



5 minutes:



10 minutes:



20 minutes:



30 minutes

