Protocols:

Buffer Concentrations According to NEB for Cas12a :

Storage Buffer
500 mM NaCl
20 mM sodium acetate
0.1 mM EDTA
0.1 mM TCEP
50% Glycerol
pH 6 @ 25°C

Quantities:
1000 µL NaCl
67 µL NaOAc
2 µL EDTA
2 µL TCEP
6250 µL glycerol 80%
Adjust Ph accordingly to 6 and fill remainder to 10000 µL total

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Dilution of Primers to 100 µM:

Nanodrop Results:

Primer (A5): 1575 ng/µL
1.575 µg/µL
1.575 g/L

Molecular Weight Calculation:
GCA GTC AGG TGG TCT TAT GCC AAG AGG ACA GA
9928 daltons (g/M)

1.575/9928 = 158.64 µM Original

35 µL 158.64 µM
20.52 µL H_2O
= 100 µM Forward Primer (A5)

Primer (A6): 1406 ng/µL
1.406 µg/µL
1.406 g/L

Molecular Weight Calculation:
ACA TAT CCA TCA TCG TGC CTA ACA AAT CCC GT
9670 daltons (g/M)

1.406/9670 = 145.40 µM

35 µL 145.40 µM
15.89 µL H_2O
= 100 µM Forward Primer (A5)

Primer 5 (A5):
158.64 µM

Primer 6 (A6):
145.40 µM

(Original Concentration)

Mathematical Calculation for A5 & A6 Primer Dilution:

Method: First, we take an initial quantity of 35 µM of primer solution into a pipette before dilution. x is in µL and represents the additional volume of pure water that will have to be added for the desired concentration of primer solution to be reached. Note: this is using the dilution equation, where $M_1 * V_1 = M_2 * V_2$. Each primer has a different initial concentration (A5 is 158.642, A6 is 145.40), meaning that the amount of water required for each dilution is slightly different.

A5 Primer

$100\ \mu M * (x + 35) = 158.642 * (35)$
$100x + 3500 = 5552.47$
$100x = 2052.47,\ \therefore x \approx 20.52\ \mu L\ of\ H_2O$

A6 Primer

$100\ \mu M * (x + 35) = 145.40 * (35)$
$100x + 3500 = 5088.93$
$100x = 1588.93,\ \therefore x \approx 15.89\ \mu L\ of\ H_2O$

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

gRNA molecular weight and concentration
GGGUAAUUUCUACUAAGUGUAGAUACUGUGAACAUUAAUGAUAA
14 kDa = 14000 g/mol
125 ng/µL concentration of extracted/eluted RNA =

= 125 mg/L / (14000 g/mol)
= 125 / 1000 / 14000 mol/L
= 8.9 uM
= 8900 nM
6-9 uM of RNA for concentration of 85ng/µL to 125ng/µL of 42 bases.

Second Cas12a pilot test protocol: 10µL per reaction

Prepare cas12a-crRNA master mix by the followings: (for 4 tubes)

TABLE 1:

crRNA concentration	125 ng/µL	139.3 ng/µL	121 ng/µL	164 ng/µL	214 ng/µL	94.5 ng/µL
NEB 2.1 buffer	0.24 µl	=	=	=	=	=
crRNA (8.9 µM)	2.20 µl	1.974+ 0.226	2.27	1.68+0.52	1.29+0.91	2.91
cas12a (100 µM)	0.20 µl	0.20	0.20	0.20	0.20	0.20
Total	2.64 µl	2.64 µl	2.71 µl	2.64 µl	2.64 µl	3.35 µl
Per tube	0.66 µl	0.66 µl	0.66 µl	0.66 µl	0.66 µl	0.84 µl

1. Mix and incubate at room temperature for 10 minutes (Table 1)
2. Prepare the reaction by the following

The testing setup are as follows (for each concentration)
TABLE 2:

Control	Normal	RPA
No RPA No ssDNA 1.33 µL 10x NEB 2.1 buffer 1 µL ssDNA-FQ 7 µL UltraPure H2O 0.66 µL Enzyme master mix* *in crRNA 6 use 0.84 µL Total: 10 µL	1 µL 10x NEB 2.1 buffer 1 µL ssDNA-FQ 0.33 µL ssDNA template 7 µL UltraPure H2O 0.66 µL Enzyme master mix* *in crRNA 6 use 0.84 µL Total 10 µL	No water No buffer 8 µL RPA buffer (Twist-DX Liquid Basic master mix w/o template) 1 µL ssDNA-FQ 0.33 µL ssDNA template 0.66 µL Enzyme master mix* *in crRNA 6 use 0.84 µL Total: 10 µL

Numbers indicate which crRNA was used (these coordinate with the concentrations in the enzyme master mix table). Testing setup is as seen above.

3. Incubate at 37 °C for 30 minutes, recording images at 0 mins, 5 mins, 10 mins, 20 mins, 30 mins

Layout in 8x3 PCR tubes
TABLE 3:

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

Retesting crRNA-6 for background:

The three tubes are arranged in the following order, with the same quantities as the previous experiment.

TABLE 4:

Control No RPA No ssDNA 1.33 µL 10x NEB 2.1 buffer 1 µL ssDNA-FQ 7 µL UltraPure H2O 0.84 µL crRNA-6 Total: 10 µL	Normal 1 µL 10x NEB 2.1 buffer 1 µL ssDNA-FQ 0.33 µL ssDNA template 7 µL UltraPure H2O 0.84 µL crRNA-6 Total 10 µL	RPA No water No buffer 8 µL RPA buffer (Twist-DX Liquid Basic master mix w/o template) 1 µL ssDNA-FQ 0.33 µL ssDNA template 0.84 µL crRNA-6 Total: 10 µL

Follow the same procedure as the previous experiment

Comparing results with NEB 2.1 Buffer and RPA

Prepare Enzyme Master Mix
0.24 µL NEB 2.1
1.29 µL crRNA 5
0.91 µL UltraPure H2O
0.20 µL Cas12a 100 µM

Cas12a Master Mix
2 µL 10x buffer
2 µL ssDNA DQ
12.68 µL UltraPure H2O
0.66 µL Enzyme Master Mix

Master Mix	0.66 µL	0.66 µL	0.66 µL	0.66 µL
100 µM FQ	0.8 µL	0.8 µL	0.8 µL	0.8 µL
RPA	2 µL	2 µL	8 µL	8 µL
NEB 2.1	6 µL	6 µL	0 µL	0 µL
Total	9.46 µL	9.46 µL	9.46 µL	9.46 µL

Lateral Flow Test – Cas12a Concentration Experiment Protocol.
The purpose of this experiment is to determine the least amount of Cas12a enzyme required for a visible result. The Moles of Cas-RNA indicate the final Molarity within the full generation tube.

RPA mixture protocol from the TwistDX Liquid Basic protocol with their exact quantities.

Total: 30 µL (4 µL per tube)
15 µL 2x reaction
.54 µL dntps
3 µL 10x Basic E-Mix
1.5 µL 20x core reaction
2.58 µL Ultra-Pure H_2O
1.44 µL forward primer
1.44 µL reverse primer

Put this in after pipetting 4 µL for Control without template.
2.6 µL template
1.3 µL MgOAc
Note: Add 0.173 µL MgOAc separately for CT

TABLE 5:

Generation:	Reaction ($\sqrt{10}$Factor Dilution):	Dilution (-D):
G0 (Initial 26µL Cas12a + crRNA mix)	2.6µL NEB 2.1 1x 2.6µL Cas12a enzyme 10µM 1.7µL crRNA–5 (RNA protocol) 19.1µL H_2O	n/a
G1	10µL G0 (500nM Cas-RNA) 4µL RPA mix 1µL Biotin-FAM (10µM) 5µL NEB 2.1 1x	5µL G0 (316 nM Cas-RNA) 10.8µL NEB 2.1 1x
G2	10µL G1-D (158nM Cas-RNA) 4µL RPA mix	5µL G1-D (100 nM Cas-RNA) 10.8µL NEB 2.1 1x

	1µL Biotin-FAM (10µM) 5µL NEB 2.1 1x	
G3	10µL G2-D (50nM Cas-RNA) 4µL RPA mix 1µL Biotin-FAM (10µM) 5µL NEB 2.1 1x	5µL G2-D (31.6 nM Cas-RNA) 10.8µL NEB 2.1 1x
G4	10µL G3-D (15.8nM Cas-RNA) 4µL RPA mix 1µL Biotin-FAM (10µM) 5µL NEB 2.1 1x	5µL G3-D (10 nM Cas-RNA) 10.8µL NEB 2.1 1x
G5	10µL G4-D (5nM Cas-RNA) 4µL RPA mix 1µL Biotin-FAM (10µM) 5µL NEB 2.1 1x	5µL G4-D (3.16 nM Cas-RNA) 10.8µL NEB 2.1 1x
CT (Control – Without Template)	10µL G0 (500 nM Cas-RNA) 4µL no Template RPA mix 1µL Biotin-FAM (10µM) 5µL NEB 2.1 1x	n/a
CC (Control – Without Cas12a)	15µL NEB 2.1 1x 4µL RPA mix 1µL Biotin-FAM (10µM)	n/a

Procedure:
Label 13 tubes according to the table above (Table 5), in spots with n/a do not use a tube. (ex. G1, G2-D, etc.)
Fill each tube accordingly, dilution tubes are used as listed in following generations
Incubate the non-dilution generation tubes at room temperature for 25 minutes
Add 80 µL Milenia Assay Buffer, or Modified TBS Buffer to each tube
Add Lateral Flow Assay Stick with sample region into the solution
Let sit for 5 minutes
Record results

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.

TABLE 6:

Tube	Biotin volume (µL) (10x)	1x NEB 2.1 Buffer volume (µL)	Concentrations (Based off of Cas12a Concentration Experiment):

1	2 µL	18 µL	2x
2 - two-fold dilution of tube 1	1 µL	19 µL	1x
3 - two-fold dilution of tube 2	0.5 µL	19.5 µL	0.5x
4 - two-fold dilution of tube 3	0.25 µL	19.75 µL	0.25x

Procedure
Mix the amounts listed in Table 6 into four tubes
Incubate at room temperature for 20 minutes
Once incubated, add 80 µL of milenia assay buffer into each tube
Immerse lateral flow assay dipstick's sample region into solution
Let sit for 5-10 minutes
Record the results

Control Lateral Flow Assay Test

In this test we only used the NEB 2.1 1x buffer and the dipstick assay buffer to test the control with our lateral flow assay dipsticks

20 µL NEB 2.1 1x
80 µL dipstick assay buffer

Lateral Flow Assay Dipstick Gold-Nanoparticle Quantity Assessment

The goal of this experiment is to determine how much of the gold nanoparticles we have to cut in order to optimize the LFA results.

Amounts of reagents

For 4 tubes:
1 µL Biotin 10x
19 µL NEB 2.1 1x
80 µL Assay buffer

TABLE 7:

Lengths:
0mm Cut-Off Control
8mm Cut-Off
10mm Cut-Off
12mm Cut-Off
14mm Cut-Off

Procedure
Cut off the lateral flow assay dipsticks testing region at the lengths detailed in table 7
Mix the biotin, NEB 2.1 buffer, and assay buffer into each tube (amounts listed above)
Insert the cut lateral flow assay dipsticks into each tube
Let sit for 5-10 minutes and record results

Viscosity Experiment For Poly(ethylene glycol)

We conducted this test to determine what the right viscosity is for a controlled experiment, while using the correct biotin amounts. After inspecting Milenia protocols, we have reduced the amount of Biotin used from 10 pmol to 5 pmol.
The mix ratio concentration ratio was altered because this is designed for 1 µM of biotin at 1 pmol instead of the previous 10 pmol, in a 10 µL total reaction.

Cas-RNA Mix
2.5 µL NEB 2.1 10x
2.5 µL Cas12a enzyme 10x
1.63 µL crRNA–5 (RNA protocol)
18.38 µL H_2O

RPA Mix (Makes 20 µL)
10 µL 2x reaction
.36 µL dntps
2 µL 10x Basic E-Mix
1 µL 20x core reaction
1.72 µLH_2O
0.96 µL forward primer
0.96 µL reverse primer

Take out 8 µL of RPA for this protocol
0.8 µL template
0.4 µL MgOAc

TBS Buffer:
4% PEG TBS Buffer :
0.4 (g) PEG 8000 Weight
1 (ml) TBS Buffer
Fill up until 10 ml

5% PEG TBS Buffer :
0.5 (g) PEG 8000 Weight
1 (ml) TBS Buffer
Fill up until 10 ml

	Controlled Positive:	Controlled Negative:
4% PEG:	5 µL (500nM Cas-RNA) 2 µL RPA mix 1 µL Biotin-FAM (1µM) 2 µL NEB 2.1 1x	5 µL (500nM Cas-RNA) 1 µL Biotin-FAM (1µM) 4 µL NEB 2.1 1x
5% PEG:	5 µL (500nM Cas-RNA) 2 µL RPA mix 1 µL Biotin-FAM (1µM) 2 µL NEB 2.1 1x	5 µL (500nM Cas-RNA) 1 µL Biotin-FAM (1µM) 4 µL NEB 2.1 1x

Procedure:

For the RPA mix, acquire the reagents listed above, as well as these two:
0.8 µL template
0.4 µL MgOAc

Centrifuge all (except the template and MgOAc) after they have thawed completely.
Pipette all but the last two into a tube in their respective amounts, in no particular order.
After this, run the tube through a centrifuge, making sure to balance weight.
Pipette out 8 µL of this solution into another tube.
Add 0.8 µL of the template directly into this tube.
Add 0.4 µL of MgOAc, but do not place it directly into the tube. Instead, pipette it into the cap.
Immediately after closing the cap, run the tube through a centrifuge, again making sure to balance.
Incubate at 37 degrees Celsius for at least 30 minutes.

For the Cas-RNA mix, acquire the reagents listed above.

Centrifuge all after they have thawed completely.
Pipette all into a tube in their respective amounts, making sure to place the cas12a enzyme itself in last.
Run through the centrifuge again.
Incubate 10 minutes at room temperature.
Once incubated, add the stated amounts of assay buffer to the different samples (PEG is % by volume).
Spin down, and place the dipsticks into the solutions.

Document results.

Viscosity and Biotin Confirmation With ssDNA instead of RPA:

RPA Dummy Mix:

25 µL 2x reaction
0.9 µL dNTPs
5 µL 10x Basic E-Mix
2.5 µL 20x Core reaction
4.3 µL H2O
2.4 µL Forward Primer
2.4 µL Reverse Primer

2.5 µL MgOAc

Cas-RNA Mix
1.1 µL NEB 2.1 1x
1.1 µL Cas12a enzyme 10x
0.73 µL crRNA–5 (RNA protocol)
8.07 µL H_2O

TABLE 8:

Positive Control:	Negative Control:	Control:
5 µL Cas-RNA Mix 0.33 ssDNA Template (1.52 µM) 1 µL Biotin (1 µM) 1.67 µL RPA Mix 2 µL NEB 2.1	5 µL Cas-RNA Mix 1 µL Biotin (1 µM) 2 µL RPA Dummy Mix 2 µL NEB 2.1	1 µL Biotin 9 µL NEB 2.1 1x

Procedure:

Prepare RPA mix using quantities given above (Note: add MgOAc last, following the same procedure as previous viscosity experiment) before incubation.
Prepare Cas-RNA mix using quantities given above (Cas12a enzyme last) before incubation.
Prepare 3 tubes, corresponding to each of the groups.
Add the reagents listed above for each tube in their respective quantities.
Spin down, and place the sample region of the Lateral Flow Assay into the solution, and record the results

Prepare the tubes using the amounts in Table 8
Record results

RPA - Cas Ratio Optimization:

Cas-RNA Mix:
0.3 µL Cas12a Enzyme 100µM
1.95 µL crRNA-5
0.2 µL NEB 2.1 10x

(RPA Mastermix)
15 µL 2x reaction
0.60 µL dNTPs
3 µL 10x Basic E-Mix
1.5 µL 20x Core reaction
2.52 µL H2O
1.44 µL forward primer (0.32 µM)
1.44 µL reverse primer (0.32 µM)
3 µL Template

Note: Pipette MgOAc On Tube Lid Right Before Use
1.5 µL MgOAc

For Us
35 Total RPA

33.25 Master Mix
1.75 20x Core Reaction Mix

TABLE 9:

Tube Labels	1	2	3	4	5	6
RPA µL:	1 µL RPA Mix	2 µL RPA Mix	4 µL RPA Mix	6 µL RPA Mix	8 µL RPA Mix	9 µL RPA Mix
Cas Mix µL:	0.4 µL Cas Mix 7.8 µL NEB 2.1	0.4 µL Cas Mix 6.8 µL NEB 2.1	0.4 µL Cas Mix 4.8 µL NEB 2.1	0.4 µL Cas Mix 2.8 µL NEB 2.1	0.4 µL Cas Mix 0.8 µL NEB 2.1	0.4 µL Cas Mix
ssDNA FQ	0.8 µL FQ	=	=	=	=	=

Pipette these reagents into a tube as RPA Mastermix
- 15 µL 2x reaction
- 0.60 µL dNTPs
- 3 µL 10x Basic E-Mix
- 1.5 µL 20x Core reaction
- 2.52 µL Hle2O
- 1.44 µL forward primer (0.32 µM) (primer 5) (Swap primer according to crRNA)
- 1.44 µL reverse primer (0.32 µM) (primer 6) (Swap primer according to crRNA)

3 µL Template
1.5 µL MgOAc (add last, pipette onto lid before closing)

Spin down, mix, incubate at 37º C

Prepare following Cas-RNA mix

0.3 µL CRISPR Cas12a enzyme (100 µM)
1.95 µL crRNA-5 (214 ng/µL) (Swap to RNA of choice)
0.2 µL NEB 2.1 (10x concentration)

Let incubate for 10 min at room temperature (for Cas-RNA complex formation)

Prepare 6 tubes with the reagents listed in the table above.

Let reagents incubate at 37º C

Take tubes out and inspect fluorescent results at intervals of 0 minutes, 5 minutes, 10 minutes, 20, 30, 60, 75

Troubleshooting high fluorescence:

The goal of this experiment is to replicate the previous ratio experiment's best ratio and determine if there were any errors. A biotin tube sample was also included to determine if the Lateral Flow would work properly. This experiment was just to see if this bright result was normal.

A negative RPA reaction was included to see if the RPA mix 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.

The results were very bright at 5 minutes, and 10 minutes, telling us that there wasn't anything wrong with the previous experiment and that this was the optimal concentration ratio. (NOTE: in the results of this experiment, the Negative RPA tubes were on the left, and the Positive RPA tubes were on the right.

Cas Mix (22 µL Total):
2.2 µL NEB 2.1 10x
2.2 µL Cas12a enzyme 10x
1.46 µL crRNA–5 (RNA protocol)
16.14 µL H_2O

RPA Mix:
5 µL 2x reaction
0.18 µL dNTPs
1 µL 10x Basic E-Mix
0.5 µL MgOAc
0.84 µL H2O
0.32 µL forward primer (10 µM)

0.32 µL reverse primer (10 µM)
1 µL Template
0.5 µL Core Reaction Mix

TABLE 10:

Negative RPA Reaction (5 µL ice)		Positive RPA Reaction (5 µL)	
FQ: 2 µL Negative RPA 5 µL Cas Mix 2 µL NEB 2.1 1x 0.8 FQ (100 µM) Label: (no RPA FQ)	Lateral Flow (Biotin): 2 µL Negative RPA 5 µL Cas Mix 2 µL NEB 2.1 1x 1 µL Biotin (1 µM) Label: (no RPA Bio)	FQ: 2 µL Positive RPA 5 µL Cas Mix 2 µL NEB 2.1 1x 0.8 FQ (100 µM) Label: (RPA FQ)	Lateral Flow (Biotin): 2 µL Positive RPA 5 µL Cas Mix 2 µL NEB 2.1 1x 1 µL Biotin (1 µM) Label: (RPA Bio)

Prepare RPA Mix according to quantities above:

- 5 µL 2x reaction
- 0.18 µL dNTPs
- 1 µL 10x Basic E-Mix
- 0.5 µL MgOAc
- 0.84 µL H2O
- 0.32 µL forward primer (10 µM)
- 0.32 µL reverse primer (10 µM)
- 1 µL Template
- 0.5 µL Core Reaction Mix

Pipette out 5 µL of RPA Mix, keep separate amount on ice, Incubate the rest at 37º C for 20 minutes
While RPA Mix is incubating, prepare Cas Mix according to quantities above:

- 2.2 µL NEB 2.1 1x
- 2.2 µL Cas12a enzyme 10x
- 1.46 µL crRNA–5 (RNA protocol)
- 16.14 µL H_2O

Let incubate at room temperature for 6-10 minutes
Prepare 4 tubes according to the quantities in Table 9 and label accordingly
Incubate 4 reaction tubes at 37º C, regularly take out tubes until the Fluorescent signal has reached desired brightness
Add 90 µL 4% PEG TBS buffer (see above for how-to-make) into biotin reaction tubes, mix, then add Lateral Flow Strip
Record the results.

Asymmetric RPA Experiment:

The goal of this experiment is to see if we can increase the yield of ssDNA and thus increase reaction result brightness without increasing Cas enzyme usage.

Cas Mix (44 µL Total):
4.4 µL NEB 2.1 10x
2.2 µL Cas12a enzyme 10x

1.96 µL crRNA–5 (RNA protocol)
35.44 µL H_2O

Forward Primer is A5
Reverse Primer is A6

RPA Mix:
30 µL 2x reaction
2.16 µL dntps
6 µL 10x Basic E-Mix
3 µL MgOAc
6 µL H_2O
6 µL Template

TABLE 11: (0.32 µM primers nature protocol)

Concentrations:	Forward Primer (Individual Tube):	Reverse Primer (Individual Tube):
Concentration 6.4 µM:	8.86 µL RPA Master Mix 0.64 µL 100 µM Forward Primer	8.86 µL RPA Master Mix 0.64 µL 100 µM Reverse Primer
Add 2.56 µL H_2O to RPA Master Mix tube – Adjust for primer quantity loss in following dilutions		
Concentration 0.64 µM:	9 µL RPA Master Mix 1 µL 6.4 µM Forward Primer Mix	9 µL RPA Master Mix 1 µL 6.4 µM Reverse Primer Mix
Concentration 0.064 µM:	4.5 µL RPA Master Mix 0.5 µL 0.64 µM Forward Primer Mix	4.5 µL RPA Master Mix 0.5 µL 0.64 µM Reverse Primer Mix

TABLE 12: (RPA Master Mix Post Water Dilution)

Target Concentrations & Ratio:	Forward Primer Quantity & Molarity:	Reverse Primer Quantity & Molarity:	20x Core Reaction (To Lid):
0.32 µM F + 0.32 µM R	2.376 µL 0.64 µM	2.376 µL 0.64 µM	0.25 µL
3.2 µM F + 0.32 µM R	2.376 µL 6.4 µM	2.376 µL 0.64 µM	0.25 µL
0.32 µM F + 3.2 µM R	2.376 µL 0.64 µM	2.376 µL 6.4 µM	0.25 µL
3.2 µM F + 3.2 µM R	2.376 µL 6.4 µM	2.376 µL 6.4 µM	0.25 µL
0.32 µM F + 0.032 µM R	2.376 µL 0.64 µM	2.376 µL 0.064 µM	0.25 µL

0.032 µM F + 0.32 µM R	2.376 µL 0.064 µM	2.376 µL 0.64 µM	0.25 µL
0.032 µM F + 0.032 µM R	2.376 µL 0.064 µM	2.376 µL 0.064 µM	0.25 µL
0 µM F + 0 µM R	4.75 µL RPA Master Mix		0.25 µL

Prepare RPA Master Mix (centrifuge all components before pipetting in):

- 30 µL 2x reaction
- 2.16 µL dntps
- 6 µL 10x Basic E-Mix
- 3 µL MgOAc
- 6 µL H_2O
- 6 µL Template
- Centrifuge mixture.

Prepare a 6.4 µM primer concentration mix (take two tubes, one for forward primer and one for reverse), with the quantities given in Table 10.
Add 2.56 µL of H2O to the RPA Master Mix (ONLY DO THIS AFTER STEP 2).
Prepare 0.64µM primer concentration mix (take two tubes, one for forward primer and one for reverse) using the reagent quantities given in Table 11.
Prepare 0.064µM primer concentration mix (take two tubes, one for forward primer and one for reverse) using the reagent quantities given in Table 11.
Prepare 8 PCR tubes, using the reagent types, quantities, and instructions that are given in Table 12, with each tube representing a specific row in the table.
Close the lids, centrifuge all the tubes, and incubate for 20 minutes.
During the 20 minutes, prepare Cas-Mix (centrifuge all components before putting in). After preparation, incubate for 10 minutes.

- 4.4 µL NEB 2.1 10x
- 2.2 µL Cas12a enzyme 10x
- 1.96 µL crRNA–5 (RNA protocol)
- 35.44 µL H_2O

After both incubations are complete, pipette out 2.8 µL of solution per PCR tube, and dispose of the 2.8 µL. Only about 2.2 µL of solution should be remaining in each tube.
To the solution remaining in each tube, add:

- 5 µL Cas-Mix
- 0.8 µL fluorescent quencher
- 2 µL NEB 2.1 1x

Put the final tubes into the PCR machine, and every 5 minutes remove the tubes and compare brightness, recording results.

Hypothesis Testing for result variation

The purpose of this test is confirmation for tubes 2 and 3, 1 is used as a benchmark, 4 is used as a control.

RPA duration reduced 2.5 fold. 10 minutes

Table 13:

Concentrations:	Forward Primer (Individual Tube):	Reverse Primer (Individual Tube):
Concentration 6.4 µM:	8.86 µL RPA Master Mix 0.64 µL 100 µM Forward Primer	8.86 µL RPA Master Mix 0.64 µL 100 µM Reverse Primer
Add 2.56 µL H_2O to RPA Master Mix tube – Adjust for primer quantity loss in following dilutions		
Concentration 0.64 µM:	9 µL RPA Master Mix 1 µL 6.4 µM Forward Primer Mix	9 µL RPA Master Mix 1 µL 6.4 µM Reverse Primer Mix

Table 14:

Target Concentrations & Ratio:	Forward Primer Quantity & Molarity:	Reverse Primer Quantity & Molarity:	20x Core Reaction (To Lid):
0.32 µM F + 0.32 µM R	2.376 µL 0.64 µM	2.376 µL 0.64 µM	0.25 µL
3.2 µM F + 0.32 µM R	2.376 µL 6.4 µM	2.376 µL 0.64 µM	0.25 µL
0.32 µM F + 3.2 µM R	2.376 µL 0.64 µM	2.376 µL 6.4 µM	0.25 µL
0 µM F + 0 µM R	4.75 µL RPA Master Mix		0.25 µL

Prepare RPA Master Mix (centrifuge all components before pipetting in, and centrifuge solution after all have been added):

- 25 µL 2x reaction
- 1.8 µL dntps
- 5 µL 10x Basic E-Mix
- 2.5 µL MgOAc
- 5 µL H_2O
- 5 µL Template

Prepare 6.4 µM primer concentration mix (procure two tubes, one for forward primer and one for reverse), with the quantities given in Table 13.
Add 2.56 µL of H2O to the RPA Master Mix (ONLY DO THIS AFTER STEP 2).
Prepare 0.64µM primer concentration mix (take two tubes, one for forward primer and one for reverse) with the quantities given in Table 13.
Prepare 4 PCR tubes, using the reagent types, quantities, and instructions that are given in Table 14, with each tube representing a specific row in the table.
Close the lids, centrifuge all the tubes, and incubate for 10 minutes.
During the 10 minutes, prepare Cas-Mix (centrifuge all components before putting in). After preparation, incubate for 10 minutes.

- 2.0 µL NEB 2.1 10x

1.0 µL Cas12a enzyme 10x
0.89 µL crRNA–5 (RNA protocol)
16 µL H_2O

After both incubations are complete, pipette out 2.8 µL of RPA reaction per tube, and dispose of the 2.8 µL. Only 2.2 µL of solution should be remaining in each tube.
To the solution remaining in each tube, add:

5 µL Cas-Mix
0.8 µL fluorescent quencher
2 µL NEB 2.1 1x

Put the final tubes into the PCR machine, and every 5 minutes take the amount and compare brightness.

Final Engineering Success

The point of this experiment is to put everything together for one final mixture protocol.

RPA Master Mix:
30 µL 2x reaction
2.16 µL dntps
6 µL 10x Basic E-Mix
3 µL MgOAc
6 µL H_2O
1.92 µL (0.32 µM Final Concentration) Forward Primer
1.92 µL (0.32 µM Final Concentration) Reverse Primer
(Not including template, or 20x Core Reaction Mix)
(Made for 5 RPA reactions)

Table 17: Lysis Buffer Solutions

1. Bacteria Negative Control	2. Bacteria Positive Control (With Gene)	3. Plasmid Positive Control	4. Lysis Buffer Only	5. Vibrio Genome Positive Control
50 µL Lysis Buffer Dipstick swab of colonies	50 µL Lysis Buffer Dipstick swab of colonies	50 µL Lysis Buffer 5.55 µL Gene Plasmid	50 µL Lysis Buffer	50 µL Lysis Buffer 5.55 µL Vibrio Genome

Table 18: RPA reaction

1. Bacteria Negative Control	2. Bacteria Positive Control (With Gene)	3. Plasmid Positive Control	4. Lysis Buffer Only	5. Vibrio Genome Positive Control
8.5 µL RPA Master Mix	8.5 µL RPA Master Mix	8.5 µL RPA Master Mix	8.5 µL RPA Master Mix	8.5 µL RPA Master Mix

0.5 µL 20x Core Reaction Mix	0.5 µL 20x Core Reaction Mix	0.5 µL 20x Core Reaction Mix	0.5 µL 20x Core Reaction Mix	0.5 µL 20x Core Reaction Mix

Prepare RPA master mix:

- 30 µL 2x reaction
- 2.16 µL dntps
- 6 µL 10x Basic E-Mix
- 3 µL MgOAc
- 6 µL H_2O
- 1.92 µL (0.32 µM Final Concentration) Forward Primer (Table 14)
- 1.92 µL (0.32 µM Final Concentration) Reverse Primer (Table 14)

Prepare Lysis Buffer Solutions According to Table 17
Prepare RPA solutions according to Table 18
For each solution reaction:

- Dip cellulose dipstick into respective lysis solution three times
- Dip cellulose dipstick into a wash buffer three times
- Dip cellulose dipstick into respective RPA reaction solution 15 times

Incubate at 37° C for 20 minutes
Prepare Cas Mix:

- 3.0 µL NEB 2.1 10x
- 1.5 µL Cas12a enzyme 10x
- 1.335 µL crRNA–5 (RNA protocol)
- 24 µL H_2O

Mix thoroughly and incubate for 10 minutes at room temperature
Prepare 5 tubes, label properly for each corresponding experimental group, and add the following to each :

- 5 µL Cas-Mix
- 0.8 µL fluorescent quencher
- 2 µL NEB 2.1 1x
- 2 µL of its respective RPA Solution

Incubate for 20 minutes at 37° C

Proof of Concept Kit Implementation:

The point of this experiment is to test whether our kit is capable of detecting real Vibrio genomic DNA isolated from Vibrio bacteria.

RPA Master Mix:
30 µL 2x reaction
2.16 µL dntps
6 µL 10x Basic E-Mix
3 µL MgOAc
6 µL H_2O
1.92 µL (0.32 µM Final Concentration) Forward Primer

1.92 µL (0.32 µM Final Concentration) Reverse Primer
(Not including template, or 20x Core Reaction Mix)
(Made for 5 RPA reactions)

Table 17: Lysis Buffer Solutions

1. Vibrio Colony 1 Positive Control	2. Bacteria Positive Control (With Gene)
50 µL Lysis Buffer 20 mL Tris Wash Buffer Dipstick swab of colonies	50 µL Lysis Buffer 20 mL Tris Wash Buffer Dipstick swab of colonies

Table 18: RPA reaction

1. Vibrio Colony 1 Positive Control	2. Vibrio Colony 1Negative Control
Dipstick Sequence for Colony 1 8.5 µL RPA Master Mix 0.5 µL 20x Core Reaction Mix	Dipstick Sequence for Random Bacteria 8.5 µL RPA Master Mix 0.5 µL 20x Core Reaction Mix

Table 19: LFA and FQ

1. Vibrio Colony 1 Positive Control Fluorescent Quencher	2. Random Colony Negative Control Fluorescent Quencher	1. Vibrio Genome Colony 1 Positive Control Lateral Flow Assay	2. Random Colony Negative Control Lateral Flow Assay
5 µL Cas-Mix 0.8 µL fluorescent quencher 2 µL NEB 2.1 1x 2 µL RPA Solution	5 µL Cas-Mix 0.8 µL fluorescent quencher 2 µL NEB 2.1 1x 2 µL RPA Solution	5 µL Cas-Mix 1 µL Biotin Labelled Reporter 2 µL NEB 2.1 1x 2 µL RPA Solution	5 µL Cas-Mix 1 µL Biotin Labelled Reporter 2 µL NEB 2.1 1x 2 µL RPA Solution

Prepare RPA master mix:

- 30 µL 2x reaction
- 2.16 µL dntps
- 6 µL 10x Basic E-Mix
- 3 µL MgOAc
- 6 µL H_2O
- 1.92 µL (0.32 µM Final Concentration) Forward Primer
- 1.92 µL (0.32 µM Final Concentration) Reverse Primer

Prepare Lysis Buffer Solutions According to Table 17
Prepare RPA solutions according to Table 18

- For each solution reaction perform the following actions in order:
  - Dip cellulose dipstick into respective lysis solutions three times
  - Dip cellulose dipstick into respective wash buffer three times
  - Dip cellulose dipstick into respective RPA reaction solution 15 times

Incubate each RPA reaction solution at 37° C for 20 minutes

Prepare Cas Mix:

- 3.0 µL NEB 2.1 10x
- 1.5 µL Cas12a enzyme 10x
- 1.335 µL crRNA–5 (RNA protocol)
- 24 µL H_2O

Mix thoroughly and incubate for 10 minutes at room temperature

Prepare 5 tubes, label properly for each corresponding solution, and add the following:

- 5 µL Cas-Mix
- 0.8 µL fluorescent quencher
- 2 µL NEB 2.1 1x
- 2 µL RPA reaction Solution

Incubate for 20 minutes at 37° C

Proof of Concept Limits of Detection:

The point of this experiment is to determine the target quantity detection limits of our kit.

Target Serial Dilutions (Template DNA):

Base Solution is 1.7*10^10 Template Copies per µL

Table 15:

$5 * 10^7$ /µL Copies	$5 * 10^6$ /µL Copies	$5 * 10^5$ /µL Copies	$5 * 10^4$ /µL Copies	$5 * 10^3$ /µL Copies	$5 * 10^2$ /µL Copies	$5 * 10^1$ /µL Copies	$5 * 10^0$ /µL Copies
1 µL of $1.7 * 10^{10}$ solution 339 µL H_2O	10 µL of $5 * 10^7$ /µL solution 90 µL H_2O	10 µL of $5 * 10^6$ /µL solution 90 µL H_2O	10 µL of $5 * 10^5$ /µL solution 90 µL H_2O	10 µL of $5 * 10^4$ /µL solution 90 µL H_2O	10 µL of $5 * 10^3$ /µL solution 90 µL H_2O	10 µL of $5 * 10^2$ /µL solution 90 µL H_2O	10 µL of $5 * 10^1$ /µL solution 90 µL H_2O

Table 16: (Individual Tube Reagents and Quantities with Correlating Final RPA Template Copy Counts)

1	2	3	4	5	6	7	Control
1 µL of $5 * 10^6$ /µL solution 0.5 µL 20x Core Reaction Mix	1 µL of $5 * 10^5$ /µL solution 0.5 µL 20x Core Reaction Mix	1 µL of $5 * 10^4$ /µL solution 0.5 µL 20x Core Reaction Mix	1 µL of $5 * 10^3$ /µL solution 0.5 µL 20x Core Reaction Mix	1 µL of $5 * 10^2$ /µL solution 0.5 µL 20x Core Reaction Mix	1 µL of $5 * 10^1$ /µL solution 0.5 µL 20x Core Reaction Mix	1 µL of $5 * 10^0$ /µL solution 0.5 µL 20x Core Reaction Mix	1 µL H_2O 0.5 µL 20x Core Reaction Mix 8.5 µL

8.5 µL RPA	8.5 µL RPA	8.5 µL RPA	8.5 µL RPA	8.5 µL RPA	8.5 µL RPA	8.5 µL RPA	RPA

Prepare 80 µL RPA MasterMix:

- 40 µL 2x reaction
- 2.88 µL dntps
- 8 µL 10x Basic E-Mix
- 4 µL MgOAc
- 2.56 µL Forward Primer
- 2.56 µL Reverse Primer
- 8 µL Water

Centrifuge RPA Master mix and mix thoroughly
Prepare Template Dilutions according to Table 15
Centrifuge template dilution tubes and mix thoroughly
Incubate for 22 Minutes
While incubating, prepare Cas Mastermix

- 5 µL NEB 2.1 10x
- 2.5 µL Cas12a enzyme 10x
- 2.225 µL crRNA–5 (RNA protocol)
- 40 µL H_2O

Centrifuge Cas Mastermix for thorough mixing, and incubate for 10 minutes
Prepare 8 tubes, label properly for each corresponding solution, and add the following:

- 5 µL Cas-Mix
- 0.8 µL fluorescent quencher
- 2 µL NEB 2.1 1x
- 2 µL RPA Solution

Incubate for 20 minutes at 37° C

