Left: 1st primer set: Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control
Right: 2nd primer set: Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

SATURDAY, 9/21/2019

Agarose Gel Preparation:

a. Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
b. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading: (PCR post serial dilutions for ypo2088 Sept 17th)

1. 5 µl of samples from each PCRed diluted sample was added onto a piece of parafilm.
2. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
3. 1 µl of 500bp ladder was mixed with 5 µl of dye and loaded first followed by 15 µl of samples in the order: 1, 2, 3, 4, 5, 6, 7, 8, 9
4. The gels were left to run for 20 minutes

10µl final volume RPA for pcaA (2nd set of primers)

Protocal followed when doing HbcAg and IS481* RPAs:

a. Reaction mix in 1.5 mL tube:
    I. Primer A (10µM) - 4.8µL
    II. Primer B (10µM) - 4.8µL
    III. Rehydration Buffer - 59µL
    IV. dH2O - 16.4µL
b. Pipetted up and down after addition of each component in step 1
c. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
d. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
e. Added 1µL of template from each serial dilution in corresponding tube.
f. Added 0.5µL of 280mM magnesium acetate and mixed well to start the reaction.
g. Incubated at 38°C for 40 min using thermocycler

SUNDAY 9/22/2019

CRISPR of RPA samples of diluted is481* and HbcAg from above-said RPA run:

CRISPR reaction following NEB protocol:

1. Assemble the reaction at room temperature in the following order*:
    a. 20µl Nuclease-free water
    b. 3µl NEBuffer 2.1 Reaction Buffer (10x)
    c. 3µl 300nM gRNA
    d. 1µl 1 µM EnGen Lba Cas12a (Cpf1)
2. Pre-incubate for 10 minutes at 25^{0}C.
3. Add 3 µl of substrate DNA (30 µl final volume).
4. Vortex and pulse-spin in a microfuge.
5. Incubate at 37°C for 10 minutes.
6. Add 1.5 µl of 1uM FQ quencher
7. Incubate for 60 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

Result: No fluorescence was seen.

Serial Dilution of ypo2088, MP1:

Table 13

	A	B	C	D	E
1					
2	ypo2088 (MP1)				
3	**Serial Dilutions**	**Concentration (ng/µl)**	**From Previous Stock**	**dH2O**	**Remaining**
4		Highest Concentration (x ng/µl)	57.4		
5	1	57.4	25	0	14.11149826
6	2	25	10.88850174	14.11149826	15
7	3	10	10	15	12.5
8	4	5	12.5	12.5	20
9	5	1	5	20	22.5
10	6	0.1	2.5	22.5	22.5
11	7	0.01	2.5	22.5	22.5
12	8	0.001	2.5	22.5	
13	9	Negative Control (dH2O)	0	25	

Resuspension of RPA rev and fwd 2 for ypo2088 according to the order sheet.

10µl final volume RPA for ypo2088 (2nd set of primers)

a. Reaction mix in 1.5 mL tube:
   I. Primer A (10µM) - 4.8µL
   II. Primer B (10µM) - 4.8µL
   III. Rehydration Buffer - 59µL
   IV. dH2O - 16.4µL
b. Pipetted up and down after addition of each component in step 1
c. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
d. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
e. Added 1µL of template from each serial dilution in corresponding tube.
f. Added 0.5µL of 280mM magnesium acetate and mixed well to start the reaction.
g. Incubated at 38°C for 30 min using thermocycler

Gel Electrophoresis of RPA samples of diluted ypo2088 from above-said RPA run:

Agarose Gel Preparation:

I. Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
II. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

a. 5 µl of samples from each RPA diluted sample was added onto a piece of parafilm.
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.

c. 1 µl of 100bp ladder (molecular ruler) was loaded first (1 µl of ladder was mixed with 5 µl of purple dye), followed by 6 µl of samples in the order:
d. IS481: ladder, rpa with primer set 1 sample 1 - 9 corresponding to the dilutions from high to low concentrations, empty space, ladder, rpa with primer set 2 sample 1-9 correspondinG to dilutiosn from high to low conc.
e. HbcAg: ladder, rpa sample 1 - 9 corresponding to the dilutions from high to low concentrations, empty space, ladder, rpa with primer set 2 sample 1-9 correspondinG to dilutiosn from high to low conc.
f. The gels were left to run for 20 minutes

Result:

ypo2088 primer set 2 RPA.tif

Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

MONDAY, 9/23/2019

- CRISPR cas 12a on optimized RPA samples out of the serial

10µl final volume RPA for ypo2088 (1st & 2nd set of primers)

Reaction mix was done in 2 different 1.5mL eppendorf tube:

1. Primer A (10µM) - 4.8µL
2. Primer B (10µM) - 4.8µL
3. Rehydration Buffer - 59µL
4. dH2O - 16.4µL

I. Pipetted up and down after addition of each component in step 1
II. The 8.5reaction mix (for ypo (for fwd primer 1 and rev 1 primer) was added to the 9 freezed dried reaction (including Negative control) . Pipette up and down to mix.
III. Repeat step 3 for ypo with primers set 2 (fwd primer 2 and rev primer 2)
IV. Added 1µL of template from each serial dilution to both set corresponding tubes.
V. Added 0.5µL of 280mM magnesium acetate and mixed well to start the reaction.
VI. Incubated at 38℃ for 40 min using thermocycler

Cas12 Cleavage with ypo 2088 gene(serial dilutions) :

1. Making 125nM gRNA: 0.2 µl of 1000 µM gRNA and 1.58 ml of water were mixed
2. Making 100nM CRISPR LbCas12a: 1 µl of 100 µM Cas12 and 999 µl of water

3. 1 µl of each solution were used to make 2 µl 62.5nM gRNA : 50nM Cas12a solution and added to approximately 18 µl of Cra DNA solution.
4. The same solution was made with 1 µl each of undiluted Cas12a and gRNA solutions (gRNA: 1000 µM; CAS12a: 100 µM).
5. The solutions were left to set for 1.5 hours in 37°C incubator.

- LAMP on ypo

Preparation of LAMP Primer Mix ypo2088

1. Dilute 100µM FIP, BIP, F3, B3 primers to be 10µM following last year's LAMP optimization.
2. Mix them in one ependorff tube.
3. Pipetted up and down to mix

Preparation of LAMP Primer Mix stock (total volume 100µl) for HBcAg and IS481

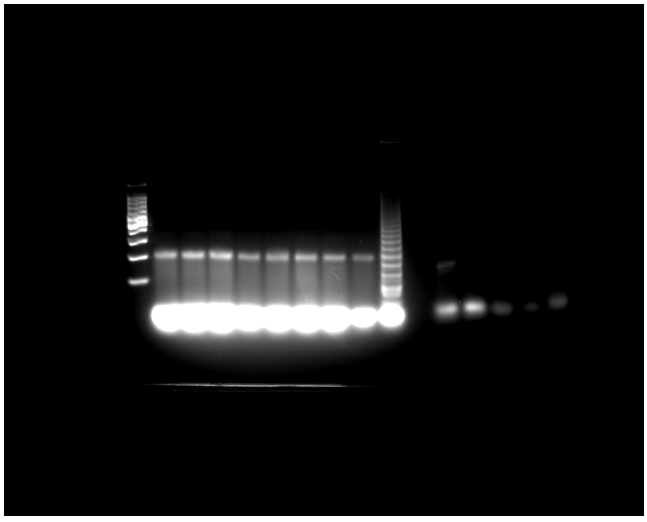
1. Add 4µl of FIP (100µM), 4µl of BIP (100µM), 1µl of F3 (100µM),, 1µl of B3 (100µM), and 45µl of dH2O in the ependorff tube.
2. Pipetted up and down to mix.
3. Vortex the primer mix.

LAMP reaction for HBcAg and IS481*post serial dilution samples

1. Add 15µl of isothermal master mix to each tube (total 9 tubes for every gene).
2. Add 5µl of the primer mix to each tube.
3. Add 5µl of the dilution sample to each tube (8 dilution samples + 1 negative control each gene).
4. Incubate at 65C for 20 minutes.

- Gel visualization
    a. 5 µl of samples from each RPA diluted sample+ CRISPR cas 12a was added onto a piece of parafilm.
    b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
    c. 1 µl of 100bp ladder (molecular ruler) was loaded first (1 µl of ladder was mixed with 5 µl of purple dye), followed by 6 µl of samples in the order: ypo gene: ladder, empty space, lamp primers 1-8, negative control and rpa with primer set 1 sample 1 - 5 corresponding to the dilutions from high to low concentrations.

lamp ypo, rpa+crispr (1).tif



Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

RPA and CRISPR on HbcAg MP1 samples (1st and 2nd set of primers):

RPA:

1. reaction mix in 2 different 1.5 eppendorf tube:
    a. Primer A (10μM) - 2.4μL
    b. Primer B (10μM) - 2.4μL
    c. Rehydration Buffer - 29.5μL
    d. dH2O - 8.5μL
    e. sample - 5μL
2. Pipetted up and down after addition of each component in step 1
3. Added 2.5μL of 280mM magnesium acetate and mixed well to start the reaction.
4. Incubated at 38℃ for 30 min using thermocycler

CRISPR following NEB protocol:

1. Assemble the reaction at room temperature in the following order*:
    a. 20μl Nuclease-free water
    b. 3μl NEBuffer 2.1 Reaction Buffer (10x)
    c. 3μl 300nM gRNA
    d. 1μl 1 μM EnGen Lba Cas12a (Cpf1)
2. Pre-incubate for 10 minutes at 25^{o}C.
3. Added 3 μl of substrate DNA (30 μl final volume).
4. Vortex and pulse-spin in a microfuge.
5. Incubate at 37°C for 10 minutes.
6. Added 1.5 μl of 1uM FQ quencher
7. Incubated for 60 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

Results: No fluorescence can be seen.

TUESDAY, 9/24/2019

Overnight PCR for IS481* mp2 post serial dilution samples:

a. Added 20 μl PCR Mastermix to each PCR tube (not enough mastermix was left for the negative control/ just added what was left around 10ul)
b. Added 1 μl of 10 μM forward primers to each tube.
c. Added 1 μl of 10 μM reverse primers to each tube.
d. Added 2ul of each dilution to each tube. None was added to negative control.
e. Water was added to reach a total volume ot 40 μl (16 μl for each tube with DNA and 18 μl for negative control)
f. PCR temperatures were set on machine according to BioRad protocol and 35 cycles were done.
g. PCR was left overnight.

50μl final volume RPA for pcaA (1st set of primers)

Protocal followed when doing HbcAg and IS481* RPAs:

a. Reaction mix in 1.5 mL tube:
    I. Primer A (10μM) - 21.6μL
    II. Primer B (10μM) - 21.6μL
    III. Rehydration Buffer - 265.5 μL
    IV. dH2O - 118.8 μL
b. Pipetted up and down after addition of each component in step 1
c. Splitted the reaction mix in nine (47.5μL) and added each to 1 freeze dried reaction. Pipetted up and down to mix.
d. Added 1ul of DNA (with buffer) into each tube

e. Added 2.5µL of 280mM magnesium acetate and mixed well to start the reaction.
f. Incubated at 39℃ for 20 min using thermocycler

10µl final volume RPA for HbcAg (done with 2 sets of RPA primers) and pcaA (2nd set)

Protocal followed when doing HbcAg and IS481* RPAs:

1. Reaction mix in 1.5 mL tube: (2 tubes, 1 for each primer pair)
    a. Primer A (10µM) - 4.8µL
    b. Primer B (10µM) - 4.8µL
    c. Rehydration Buffer - 59µL
    d. dH2O - 16.4µL
2. Pipetted up and down after addition of each component in step 1
3. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
5. Added 1µL of template from each serial dilution in corresponding tube.
6. Added 0.5µL of 280mM magnesium acetate and mixed well to start the reaction.
7. Incubated at 38℃ for 30 min using thermocycler

Gel Electrophoresis of RPA samples of diluted HbcAg, IS481 and pcaA from above-said RPA run:

Agarose Gel Preparation:

I. Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
II. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

a. 5 µl of samples from each RPA diluted sample was added onto a piece of parafilm.
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
c. 5 µl of 100bp ladder (molecular ruler) was loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 6 µl of samples in the order:
d. HbcAg: space, ladder, rpa sample 1 - 9 corresponding to the dilutions from high to low concentrations, empty space, ladder, rpa with primer set 2 sample 1-9 correspondinG to dilutiosn from high to low conc.
e. The gels were left to run for 20 minutes

Serial Dilution for pcaA (Using Miniprep 1-A)

Table 14

	A	B	C	D
1	Pcaa			
2	**Serial Dilutions**	**Concentration (ng/µl)**	**From Previous Stock**	**dH2O**
3		Highest Concentration (x ng/µl)	83.4	
4	1	60	17.98561151	7.014388489
5	2	25	10.41666667	14.58333333
6	3	10	10	15
7	4	5	12.5	12.5
8	5	1	5	20
9	6	0.1	2.5	22.5
10	7	0.01	2.5	22.5
11	8	0.001	2.5	22.5

Result:

HbcAg post dilution

RPA:

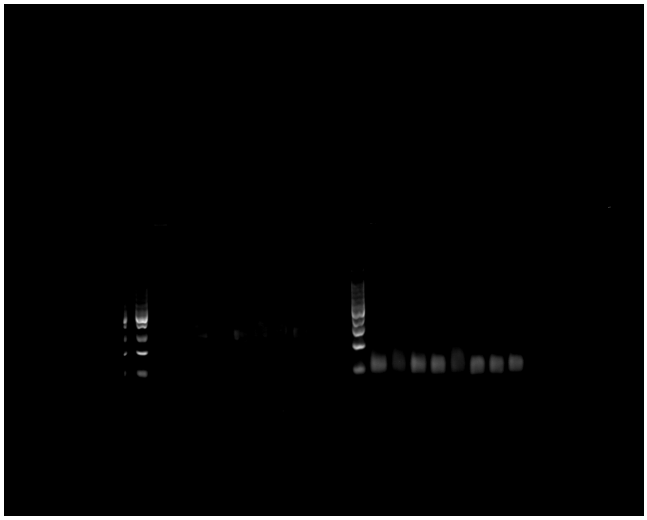
HbcAg_serial_dilutions_RPA_1_primerSet1_2_SEPT24.tif

Left: 1st primer set: Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

Right: 2nd primer set: Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

IS481 post dilution RPA:

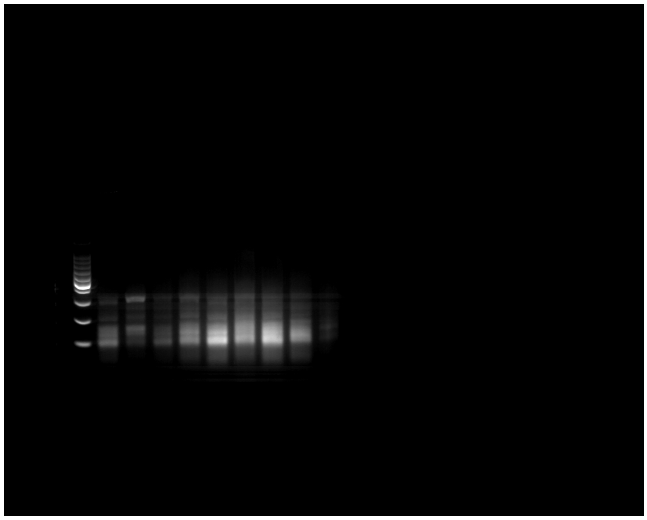
IS481STAR_serial_dilutions_RPA_3_primerSet1_2_SEPT24.tif



Left: 1st primer set: Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control
Right: 2nd primer set: Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

PcaA post dilution RPA:

pcaA RPA set 2 (2).tif



1st primer set: Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

WEDNESDAY, 9/25/2019

Gel Electrophoresis of PCR samples of diluted is481* from Sept 24:

Agarose Gel Preparation:

a. Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
b. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

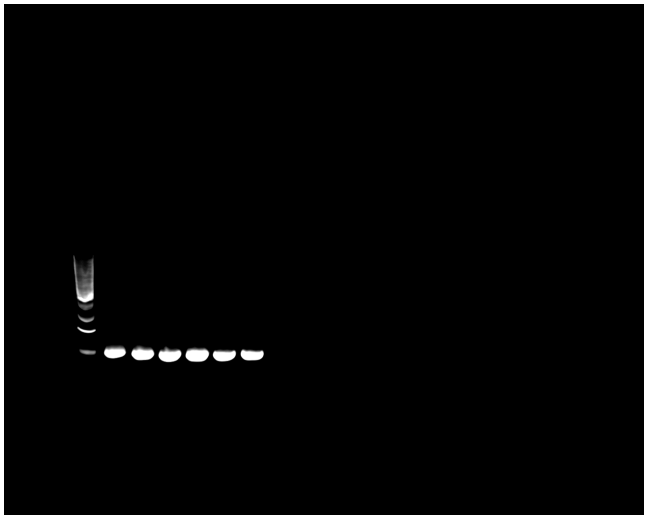
Sample Loading:

1. 5 µl of samples from each PCR diluted sample was added onto a piece of parafilm.
2. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
3. 1 µl of 100bp ladder (molecular ruler) was loaded first (1 µl of ladder was mixed with 5 µl of purple dye), followed by 6 µl of samples in the order:
    a. IS481: ladder, rpa with primer set 1 sample 1 - 9 corresponding to the dilutions from high to low concentrations, empty space, ladder, rpa with primer set 2 sample 1-9 correspondinG to dilutiosn from high to low conc.
4. The gels were left to run for 20 minutes

Result:

IS481 serial dilution PCR:

is481STAR_serial_dilutions_PCR_1_SEPT25_2019.tif



Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

PCR of HbcAg Serial Dilutions and 2 cra samples (extracted from IS481* agar plate)

(following Invitrogen Taq DNA polymerase protocal)

In 1 eppendorf tube added:

- 2.5 µl 10X PCR Buffer
- 0.75 µl 50mM MgCl2
- 2 µl 2mM dNTP Mix
- 1.25 µl 10 µM forward primer
- 1.25 µl 10 µM reverse primer
- 0.1 µl Taq DNA Polymerase
- for Cra:
    - added 18.15µl distilled water
    - scraped colony off of IS481* agar plate to extract Cra gene (Cra is endogenous to E.coli)
- for HbcAg:
    - added 16.15µl distilled wtaer

PCR was left overnight.

THURSDAY, 9/26/2019

50µl final volume RPA for is481 (2nd set of primers)

Protocal followed when doing HbcAg and IS481* RPAs:

I. Reaction mix in 1.5 mL tube:
    1. Primer A (10μM) - 21.6μL
    2. Primer B (10μM) - 21.6μL
    3. Rehydration Buffer - 265.5 μL
    4. dH2O - 118.8 μL
II. Pipetted up and down after addition of each component in step 1
III. Splitted the reaction mix in nine (47.5μL) and added each to 1 freeze dried reaction. Pipetted up and down to mix.
IV. Added 5ul of DNA (with buffer) into each tube
V. Added 2.5μL of 280mM magnesium acetate and mixed well to start the reaction.
VI. Incubated at 38°C for 30 min using thermocycler

Gel Electrophoresis of is481 RPA (2nd primer set)

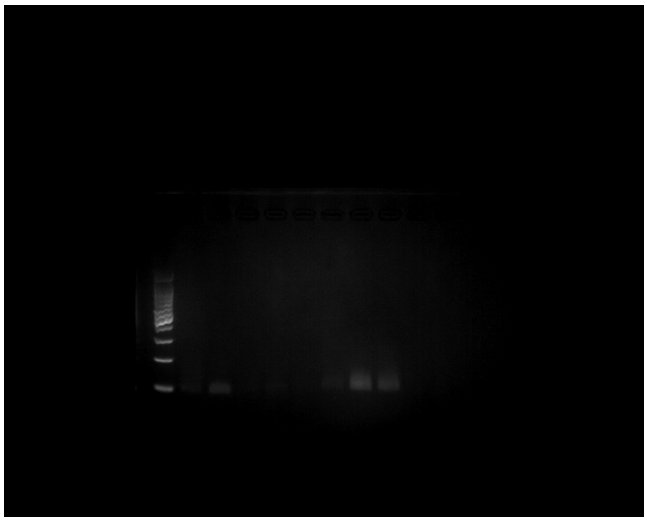
Agarose Gel Preparation:

a. RPA gel: Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
b. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

a. 5 μl of samples from each RPA diluted sample was added onto a piece of parafilm.
b. 1 μl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
c. 5 μl of a) 100bp ladder (molecular ruler) for RPA gel b) 500bp ladder for PCR gel were loaded first (5 μl of ladder was mixed with 1 μl of purple dye), followed by 6 μl of samples in the order from high to low conc.
d. The gels were left to run for 20 minutes

IS481STAR_serial_dilutions_RPA_50ul_primerSet2_SEPT26.tif



Ladder-60 ng/μl dilution- 25ng/μl dilution- 10 ng/μl dilution- 5 ng/μl dilution- 1 ng/μl dilution- 0.1 ng/μl dilution- 0.01 ng/μl dilution- 0.01 ng/μl dilution- negative control

CRISPR of RPA samples of cra after RPA:

CRISPR reaction following NEB protocol:

a. Assemble the reaction at room temperature in the following order*:
    I. 20μl Nuclease-free water
    II. 3μl NEBuffer 2.1 Reaction Buffer (10x)

III. 3µl 300nM gRNA
IV. 1µl 1 µM EnGen Lba Cas12a (Cpf1)

b. Pre-incubate for 10 minutes at 25^{o}C.
c. Add 3 µl of substrate DNA (30 µl final volume).
d. Vortex and pulse-spin in a microfuge.
e. Incubate at 37°C for 10 minutes.
f. Add 1.5 µl of 1uM FQ quencher
g. Incubate for 60 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

Result: No fluorescence was shown.

HbcAg RPA (2nd primer set)

a. Reaction mix in 1.5 mL tube:
    1. Primer A (10µM) - 4.8µL
    2. Primer B (10µM) - 4.8µL
    3. Rehydration Buffer - 59µL
    4. dH2O - 16.4µL
b. Pipetted up and down after addition of each component in step 1
    II. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
    III. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
    IV. Added 1µL of template from each serial dilution in corresponding tube.
    V. Added 0.5µL of 280mM magnesium acetate and mixed well to start the reaction.
    VI. Incubated at 38℃ for 30 min using thermocycler

Gel Electrophoresis of HbcAg RPA (2nd primer set) and PCR from previous day

Agarose Gel Preparation:

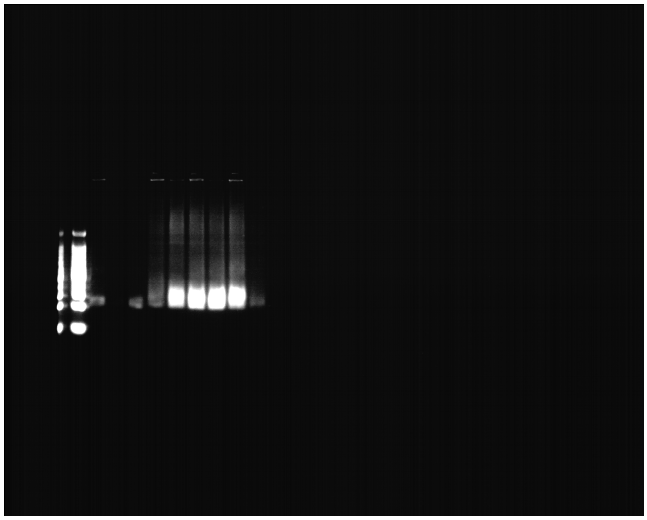
1. a. RPA gel: Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
    b. PCR gel: Prepared a 1x agrose gel by adding 0.5 grams of agrose to 50 ml of TAE buffer.
2. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

a. 5 µl of samples from each RPA diluted sample was added onto a piece of parafilm.
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
c. 5 µl of a) 100bp ladder (molecular ruler) for RPA gel b) 500bp ladder for PCR gel were loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 6 µl of samples in the order from high to low conc.
d. The gels were left to run for 20 minutes

Result:

HbcAg_serial_dilutions_RPA_primerSet2_SEPT26.tif



Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

HbcAg Serial Dilution (MP3)

Table 15

	A	B	C	D	E
1	**Serial Dilutions**	**Concentration (ng/µl)**	**From Previous Stock**	**dH2O**	**Remaining**
2		Highest Concentration (x ng/µl)	62.1		30
3	1	60	48.3	1.7	30
4	2	25	20	28	28
5	3	10	20	30	37.5
6	4	5	12.5	12.5	20
7	5	1	5	20	22.5
8	6	0.1	2.5	22.5	22.5
9	7	0.01	2.5	22.5	22.5
10	8	0.001	2.5	22.5	25
11	9	Negative Control (dH2O)	0	25	

2nd HbcAg RPA (2nd primer set)

I. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 4.8µL
   b. Primer B (10µM) - 4.8µL
   c. Rehydration Buffer - 59µL

d. dH2O - 16.4µL

1. Pipetted up and down after addition of each component in step 1
2. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
3. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
4. Added 1µL of template from each serial dilution in corresponding tube.
5. Added 0.5µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Incubated at 38℃ for 30 min using thermocycler

2nd HbcAg and Cra (1st primer set) PCR using 2x mastermix

I. Labelled each eppendorf tube with name of gene and the concentration
II. Added 20 µl PCR Mastermix to each tube
III. Added 1 µl of 10 µM forward primers to each tube.
IV. Added 1 µl of 10 µM reverse primers to each tube.
V. Added 2ul of each dilution to each tube. None was added to negative control.
    1. For cra samples, colonies are scraped from IS481* plate and put directly into the master mix
VI. Water was added to reach a total volume ot 40 µl (16 µl for each tube with DNA and 18 µl for negative control and Cra sample tubes)
VII. PCR temperatures were set on machine according to BioRad protocol and 35 cycles were done.
VIII. PCR was left overnight.

Gel Electrophoresis of 2nd HbcAg RPA (2nd primer set)

Agarose Gel Preparation:

a. RPA gel: Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
b. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

a. 5 µl of samples from each RPA diluted sample was added onto a piece of parafilm.
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
c. 5 µl of a) 100bp ladder (molecular ruler) for RPA gel b) 500bp ladder for PCR gel were loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 6 µl of samples in the order from high to low conc.
d. The gels were left to run for 20 minutes

Result:

HbcAg serial dilution

RPA

HbcAg_serial_dilutions_RPA_primerSet2_2_SEPT26.tif

Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

FRIDAY, 9/27/2019

HbcAg RPA (2nd primer set)

I. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 4.8µL
   b. Primer B (10µM) - 4.8µL
   c. Rehydration Buffer - 59µL
   d. dH2O - 16.4µL

II. Pipetted up and down after addition of each component in step 1

2. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
3. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
4. Added 1µL of template from each serial dilution in corresponding tube.
5. Added 0.5µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Incubated at 39°C for 30 min using thermocycler

Gel Electrophoresis of HbcAg RPA (2nd primer set) from today and PCR from previous day

Agarose Gel Preparation:

1. a. RPA gel: Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.

   b. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough
2. PCR gel: Prepared a 1x agarose gel by adding 0.5 grams of agarose to 50 ml of TAE buffer.

Sample Loading:

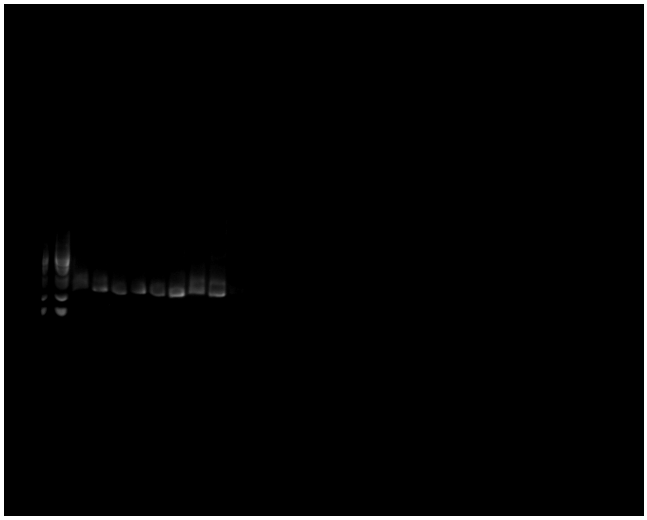
a. 5 µl of samples from each RPA diluted sample was added onto a piece of parafilm.
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
c. 5 µl of a) 100bp ladder (molecular ruler) for RPA gel b) 500bp ladder for PCR gel were loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 6 µl of samples in the order from high to low conc.
d. The gels were left to run for 20 minutes

Result:

HbcAg serial dilution

RPA:

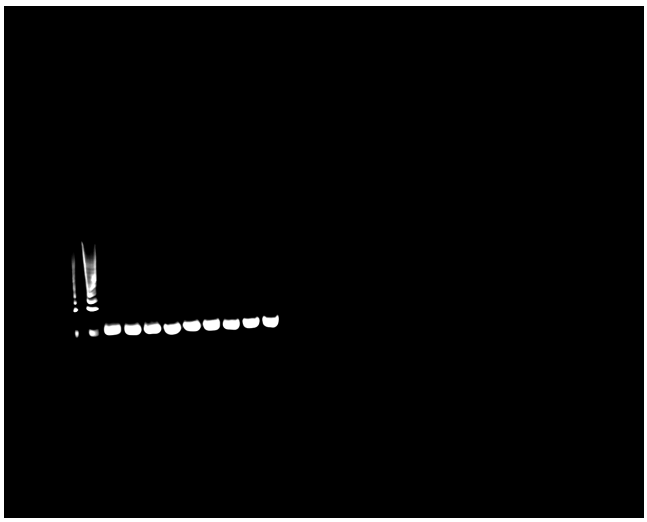
HbcAg_serial_dilutions_RPA_primerSet2_2_SEPT27.tif



Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

HbcAg serial dilution PCR:

HbcAg_serial_dilutions_PCR_2_SEPT27.tif



Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

SATURDAY, 9/28/2019

NEB Protocol for RPA on pcaA (for the engineers- total volume is 50 µl) done at 10 min and 20 min

1. Prepare reaction mix in a 1.5 ml tube:
   a. Primer A (10 µl): 2.4 µl
   b. Primer B (10 µl): 2.4 µl
   c. Rehydration buffer: 29.5 µl

d. Nuclease free water: 11.2 ul

e. Template: 2 µl

2. Vortex
3. Add mixture to a TwistAmp Basic Reaction
4. Add 2.5 µl of 280mM of Magnesium Acetate
5. Incubate at 39 C for 10/20 minutes

Gel Electrophoresis of pcaA

Agarose Gel Preparation:

1.
    a. RPA gel: Prepared a 1x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
2. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

a. 5 µl of samples from each RPA diluted sample was added onto a piece of parafilm.

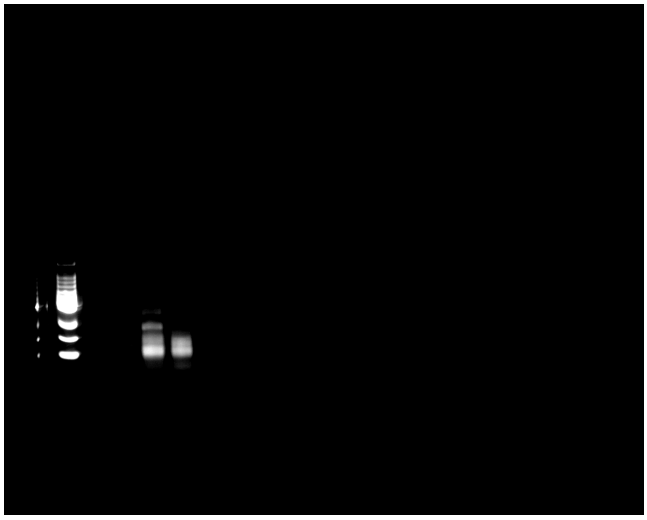
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.

c. 5 µl of 500bp ladder for PRA gel were loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 6 µl of samples in the order:

d. The gel was left to run for 20 minutes

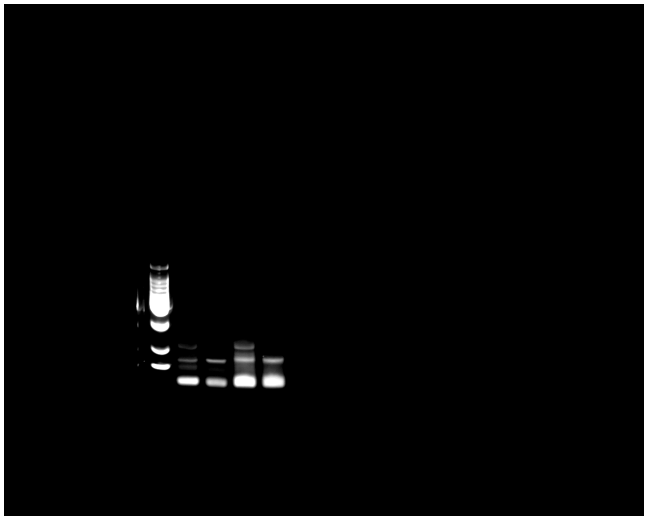
Result:

RPA pcaA 10 min 20 min.tif



Ladder- 10minutes RPA (positive). - 10minutes RPA (negative) - 20minutes RPA (positive) - 20minutes RPA (negative)

RPA pcaA 10 min 20 min part 2!.tif



Ladder- 10minutes RPA (positive). - 10minutes RPA (negative) - 20minutes RPA (positive) - 20minutes RPA (negative)

NEB CRISPR + FQ reporter on PCR-amplified Cra (modified)

CRISPR reaction following NEB protocol:

1. Assemble the reaction at room temperature in the following order:
    a. 5µl Nuclease-free water
    b. 3µl NEBuffer 2.1 Reaction Buffer (10x)
    c. 3µl 300nM gRNA
    d. 1µl 1 µM EnGen Lba Cas12a (Cpf1)
    e. 16.5µl 1µM FQ reporter
2. a. for tube 1, Pre-incubated for 10 minutes at 25^{o}C.
    b. for tube 2, pre-incubated for 10 minutes at 37^{o}C
3. Add 3 µl of substrate DNA (30 µl final volume).
4. Incubate at 37°C for 10 minutes.
5. Incubate for 60 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

Gel Electrophoresis of CRISPR'ed PCR-amplified Cra

Agarose Gel Preparation:

1. a. PCR gel: Prepared a 1x agarose gel by adding 0.5 grams of agarose to 50 ml of TAE buffer.
2. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

a. 5 µl of samples from each PCR diluted sample was added onto a piece of parafilm.
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
c. 5 µl of 500bp ladder for PCR gel were loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 6 µl of samples in the order:

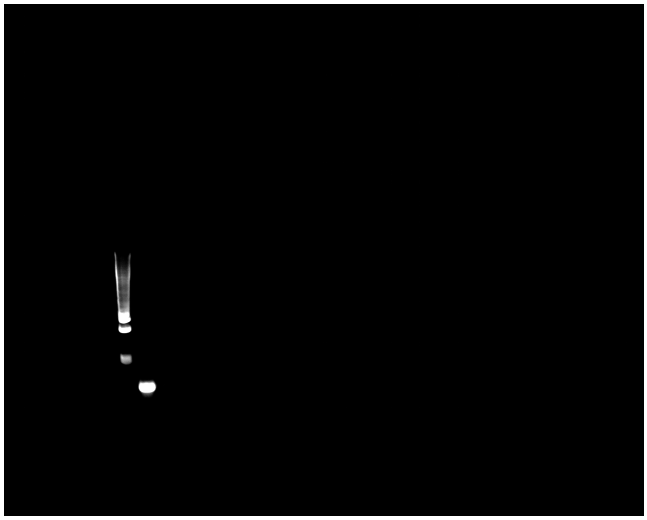
PCR'ed sample, CRISPR'ed sample with binding incubated at 37^{o}C, CRISPR'ed sample with binding incubated at 25^{o}C, negative control

a. The gel was left to run for 20 minutes

Result:

CRISPR'ed PCR- amplified Cra

Cra_NEB_CRISPR_FQ_PCR_CRISPRED_CRISPRED_Control_Sept28.tif



Ladder-PCR'ed sample- CRISPR'ed sample with binding incubated at 37^{0}C- CRISPR'ed sample with binding incubated at 25^{0}C- negative control

- **LAMP on ypo 2088**

LAMP primer mix for ypo2088 (previous one is contaminated)

Preparation of LAMP Primer Mix ypo2088

1. Dilute 100µM FIP, BIP, F3, B3 primers to be 10µM following lastyear's LAMP optimization.
2. Mix them in one ependorff tube.
3. Pipetted up and down to mix

Preparation of LAMP Primer Mix stock (total volume 100µl) for ypo2088

1. Add 4µl of FIP (100µM), 4µl of BIP (100µM), 1µl of F3 (100µM),, 1µl of B3 (100µM), and 45µl of dH2O in the ependorff tube.
2. Pipetted up and down to mix.
3. Vortex the primer mix.

Agarose Gel Preparation:

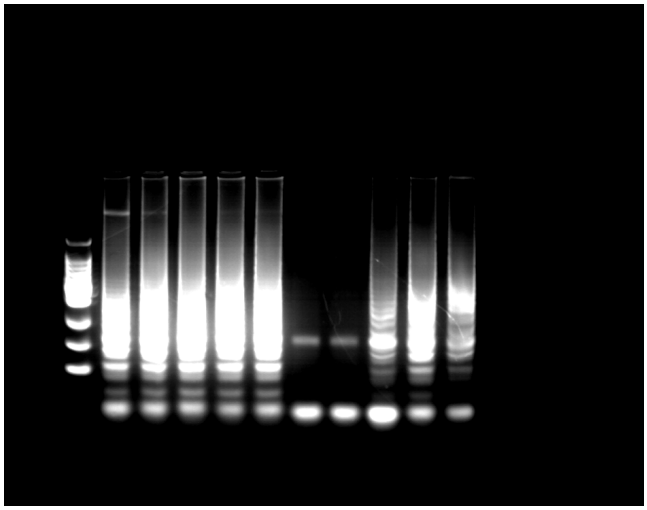
1. a. LAMP GEL: Prepared a 1x agarose gel by adding 0.5 grams of agarose to 50 ml of TAE buffer.
2. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

a. 5 µl of samples from each PCR diluted sample was added onto a piece of parafilm.
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
c. 5 µl of 500bp ladder for gel was loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 8 µl of samples in the order: 60, 25, 10, 5, 1, 0.1, 0.01, 0.001 and negative control.
d. Whilst pipeting lamp reaction on 0.1 dilution, twice the gel was punctured and had to be repeated.

Results :

lamp 2 ypo, 2x 0.1.tif



From the left, 500bp ladder - 60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution (punctured) , 0.1 ng/µl dilution (punctured)- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

SUNDAY, 9/29/2019

15µlRPA with a) 4µb) 10µl sample DNA (done with HbcAg and IS481)

I. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 9.6µL
   b. Primer B (10µM) - 9.6µL
   c. Rehydration Buffer - 118µL
   d. dH2O - 32.8µL

II. Pipetted up and down after addition of each component in step 1
   2. Splitted the reaction mix in four (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
   3. Splitted the reaction into 9 volumes - 15µL to 9 separate PCR tubes.
   4. Added:
      a. 4µL of template from each serial dilution in corresponding tube.
      b. 10µL of template from each serial dilution in corresponding tube
   5. Added:
      a. 1µL of 280mM magnesium acetate and mixed well to start the reaction.
      b. 1.25µL of 280mM magnesium acetate and mixed well to start the reaction.
   6. Incubated at 39°C for 30 min using thermocycler

Gel Electrophoresis of HbcAg and IS481 RPA from today and PCR-amplified Cra

Agarose Gel Preparation:

1.
   a. RPA gel: Prepared a 3x agarose gel by adding 1.5 grams of agarose to 50 ml of TAE buffer.
   b. PCR gel: Prepared a 1x agarose gel by adding 0.5 grams of agarose to 50 ml of TAE buffer.
2. Heating in the solution up in 20 second interval (in the microwave) and stop after making sure that the solution is clear enough

Sample Loading:

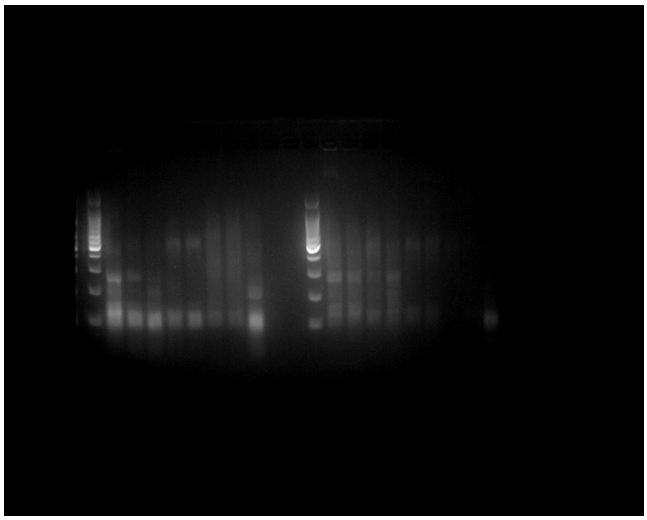
   a. 5 µl of samples from each RPA diluted sample was added onto a piece of parafilm.
   b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.

c. 5 µl of 100bp ladder (molecular ruler) for RPA gel were loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 6 µl of samples in the order from high to low conc (first samples with 4µl DNA added, then samples with 10 µl added)
d. The gels were left to run for 20 minutes

Result:

IS481 RPA optimization: 15µl RPA + Left) 15ul DNA/ Right)
15µl

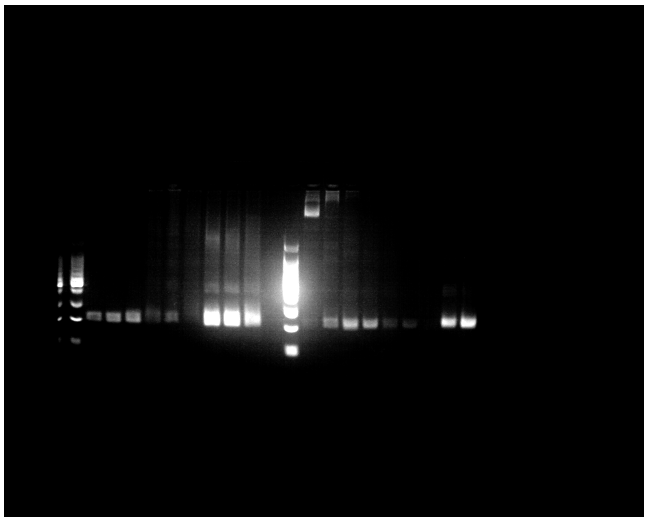
IS481_serial_dilutions_15ulRPA_4ulDNA_10ulDNA_SEPT29.tif



Left: Ladder- 10µl of 60 ng/µl DNA dilution - 10µl of 25ng/µl dilution-10µl of 10 ng/µl dilution-10µl of 5 ng/µl dilution-10µl of 1 ng/µl dilution-10µl of 0.1 ng/µl dilution-10ul of 0.01 ng/µl dilution-10ul of 0.01 ng/µl dilution- negative control
Right: Ladder- 4ul of 60 ng/µl dilution - 4ul of 25ng/µl dilution-15ul of 10 ng/µl dilution- 4ul of 5 ng/µl dilution- 4ul of 1 ng/µl dilution- 4ul of 0.1 ng/µl dilution- 4ul of 0.01 ng/µl dilution-4ul of 0.01 ng/µl dilution- negative control

HbcAg RPA optimization: 15ul RPA + Left) 15ul DNA/ Right) 15ul

HbcAg_serial_dilutions_15ulRPA_4ulDNA_10ulDNA_SEPT29.tif

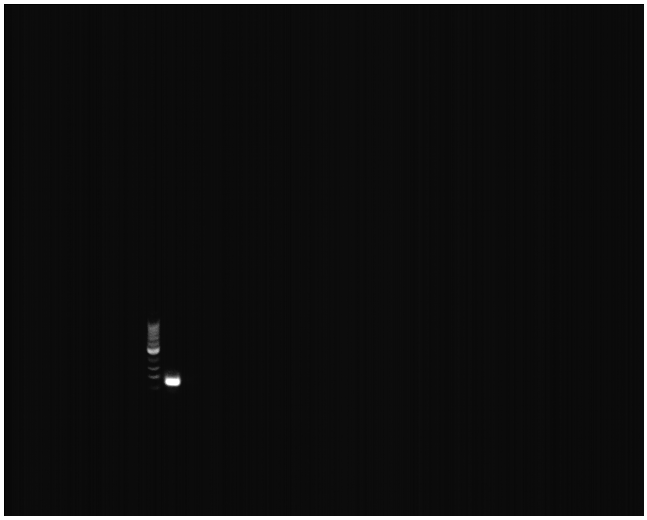


Left: Ladder- 10ul of 60 ng/µl DNA dilution - 10ul of 25ng/µl dilution-10ul of 10 ng/µl dilution-10ul of 5 ng/µl dilution-10ul of 1 ng/µl dilution-10ul of 0.1 ng/µl dilution-10ul of 0.01 ng/µl dilution-10ul of 0.01 ng/µl dilution- negative control

Right: Ladder- 4ul of 60 ng/µl dilution - 4ul of 25ng/µl dilution-15ul of 10 ng/µl dilution- 4ul of 5 ng/µl dilution- 4ul of 1 ng/µl dilution- 4ul of 0.1 ng/µl dilution- 4ul of 0.01 ng/µl dilution-4ul of 0.01 ng/µl dilution- negative control

Amplified PCR Cra:

Cra_NEB_CRISPR_FQ_PCR_CRISPRED_CRISPRED_Control_Sept29.tif



Ladder-PCR'ed sample- CRISPR'ed sample with binding incubated at 37^{o}C- CRISPR'ed sample with binding incubated at 25^{o}C- negative control

Conclusion & Discussion:

Yesterday and Today's Cra PCR- CRISPR:

- the purpose of Cra PCR- CRISPR to show whether FQ quencher is working.
- Cra is used instead of other genes, becuase for the four genes, only As scaffold gRNA are currently avaiable, not Lb scaffold gRNA.
- This three base pair difference might affect CRISPR and FQ reporter reaction.
- In this Cra test, Lb scaffold gRNA is used.
- Gel shows that CRIPSR was successful as band disappears, but no fluorescence was observed under the fluorescence microscope.
- FQ quencher is not working
- DNase Alert (another fluorescence reporter) should be ordered and used.

RPA Optimization for engineers:

- Due to negative control contaminatior HbcAg, only IS481 should taken account.
- Adding 4ul of DNA shows higher specificity in RPA reaction with 15ul RPA mix compared to 10ul of DNA, because the band iwth .
- On the microfluidic chip (our final product), 4ul of DNA sample will be loaded and reacted with 15ul of RPA mix.

MONDAY, 9/30/2019

Test SYBR Green

Dilute SYBR Green 10,000X to 1,000X using TE buffer

Add SYBR Greeen to yesterday's IS481 RPA samples (both 4µL and 10µL DNA) : SYBR green volume was adjusted to last year's protocol.

1. Adjust the last year's SYBR Green protocol (1µL of SYBR Green to 25µL sample) for the total sample volume of 14µL and 20µL.
    a. Add 0.56µL of SYBR Green to RPA sample with 4µL of DNA
    b. Add 0.80µL of SYBR Green to RPA sample with 10µL of DNA

2. Test the fluorescence under UV light

Dilute SYBR Green to 50X and 100X:

1. 50X SYBR Green: Add 5µL of 1000X SYBR Green to 95µL of TE buffer
2. 100X SYBR Green: Add 10µL of 1000X SYBR Green to 90µL of TE buffer

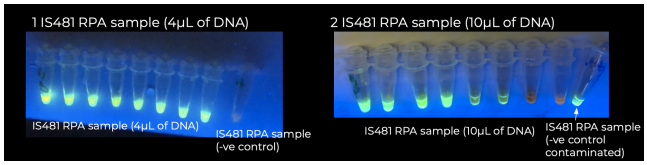
Test 50X, 100X, and 32X on yesterday's three HbcAg RPA samples (both 4µL and 10µL DNA) that show band in the gel electrophoresis.

1. Total volume of 14µL:
    a. 1000X (32X): 0.56ul
    b. 50X (1X): 0.28ul
    c. 100X (1X): 0.14ul
2. Total volume of 20µL:
    a. 1000X (32X): 0.8ul
    b. 50X (1X): 0.4ul
    c. 100X (1X): 0.2ul

Result:

IS481 SYBR Green testing use different RPA dilution under UV light:

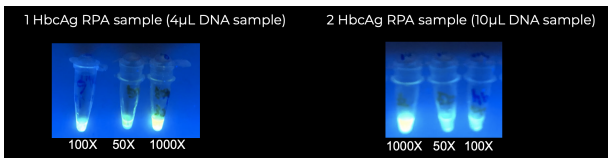
Screen Shot 2019-10-18 at 3.57.59 AM.png



Left: 1000X SYBR green added to IS481 RPA (+4ul DNA sample of 60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control)
Right: 1000X SYBR green added to IS481 RPA (+10ul DNA sample of 60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control)

HbcAg SYBR Green testing - use different SYBR green concentration under UV light:

Screen Shot 2019-10-18 at 3.59.48 AM.png



Left: 0.28ul of 100X added to RPA sample (+4ul DNA) - 0.14ul of 50X added to RPA sample (+4ul DNA) - 0.56ul of 1000X added to RPA sample (+4ul DNA)
Right: 0.8ul of 1000X added to RPA sample (+10ul DNA) - 0.4ul of 50X added to RPA sample (+10ul DNA) - 0.2ul of 100X added to RPA sample (+10ul DNA)

Conclusion & Discussion:

- Tested and compared with yesterday's RPA optimizaiton gels for IS481 and HbcAg.
- If gel shows band, SYBR green shows fluorescence
- If gel doesn't show band, SYBR green doesn't fluoresence

- This also applied to fluorescing negative control (that shows band).and non-fluorescing sample containing DNA (that doesn't show band)

HbcAg Innoculations:

1. 10mL LB broth was added to 15mL culture tubes
2. A plastic inoculation loop was used to select 4 colonies from HbcAg plate and was swirled in the corresponding broth to dislodge the colony
3. The tubes were loosely capped and incubated on a shaker at 220rpm and 37°C (48 hours)

TUESDAY, 10/1/2019

15µl RPA with 10µl sample DNA (done with pcaA)

I. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 9.6µL
   b. Primer B (10µM) - 9.6µL
   c. Rehydration Buffer - 118µL
   d. dH2O - 32.8µL

II. Pipetted up and down after addition of each component in step 1

2. Splitted the reaction mix in four (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
3. Splitted the reaction into 9 volumes - 15µL to 9 separate PCR tubes.
4. Added:
   a. 4µL of template from each serial dilution in corresponding tube.
   b. 10µL of template from each serial dilution in corresponding tube
5. Added:
   a. 1µL of 280mM magnesium acetate and mixed well to start the reaction.
   b. 1.25µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Incubated at 39℃ for 20 min using thermocycler

WEDNESDAY, 10/2/2019

Re-preparation of FQ quencher:

1. Added 380 µl of TE buffer to lyophilized quencher to get it to a working stock of 100 µM
2. Took 10 µl of 100 µM and added 10 µl of TEbuffer to get a stock of 50 µM in 20 µl
3. Took 2 µl of 50 µM solution and added 98 µl of TE buffer to get a concentration of 1uM in 100 µl

Dicussion:

- One of the reason that FQ reporter didn't work was that FQ reporter was diluted using water, not TE buffer.
- Even though both TE buffer and water is okay for the FQ reporter dilution, diluting using TE buffer is a better option as the water is slightly acidic, which degrades DNA following the sigma aldrich explanation. Thus, diluting in the TE buffer might destabilize DNA and hinder from giving fluorescence (https://www.sigmaaldrich.com/technical-documents/articles/biology/handling-guidelines-for-dna-and-rna-oligonucleotides.html)

NEB CRISPR + re-diluted FQ reporter on PCR-amplified Cra (modified)

CRISPR reaction following NEB protocol:

1. Assemble the reaction at room temperature in the following order*:
   a. 5µl Nuclease-free water
   b. 3µl NEBuffer 2.1 Reaction Buffer (10x)
   c. 3µl 300nM gRNA

d. 1µl 1 µM EnGen Lba Cas12a (Cpf1)
e. 16.5µl 1µM FQ reporter

2. Pre-incubation of CRISPR:
   a. for tube 1, Pre-incubated for 10 minutes at 25^{0}C.
   b. for tube 2, pre-incubated for 10 minutes at 37^{0}C
3. Add 3 µl of substrate DNA (30 µl final volume).
4. Incubate at 37°C for 10 minutes.
5. Incubate for 60 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

Miniprep 4 HbcAg Innoculations:

Miniprep procedures for each innoculation:

1. Pellet 5ml bacterial overnight culture by centrifugation at 7830 rpm (6800 x g) for 3 min at room temperature (15–25°C).
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
6. Apply the supernatant from step 5 to the QIAprep spin column by decanting or pipetting. Centrifuge for 60 s and discard the flow-through,
7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB. Centrifuge for 60 s and discard the flow-through.
8. Wash the QIAprep spin column by adding 0.75 ml Buffer PE. Centrifuge for 60 s and discard the flow-through.
9. Centrifuge for 1 min to remove residual wash buffer.
10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

THURSDAY, 10/3/2019

Nano-drop of HbcAg Minipreps from yesterday:

Table 16

	A	B	C	D
1		Nucleic Acid Conc. (ng/µl)	260/280	260/230
2	HbcAg mp1	81.7	1.89	2.30
3	HbcAg mp2	39.2	1.99	2.42
4	HbcAg mp3	76.3	1.89	2.08
5	HbcAg mp4	82.4	1.91	2.21

SUNDAY, 10/6/2019

HbcAg Miniprep 4 Serial Dilutions

Table 18

	A	B	C	D	E
1	**Serial Dilutions**	**Concentration (ng/µl)**	**From Previous Stock**	**dH2O**	**Remaining**
2	Highest Concentration (x ng/µl)	82.4			30
3	1	60	39.32	14.68	34
4	2	25	20	28	28
5	3	10	20	30	25
6	4	5	25	25	40
7	5	1	5	20	45
8	6	0.1	5	45	45
9	7	0.01	5	45	45
10	8	0.001	5	45	25
11	9	Negative Control (dH2O)	0	50	

PCR of minipreped plasmid pcaA

Resuspension of Lyophilized Primers

a. Added volume of water indicated on order sheet (differed for each primer) to make 100 µM of each primer
b. Pipetted up and down to mix

Preparation of PCR mix

a. Prepared 10 µM working stock of each primer by extracting 5 µl of 100 µM stock and adding 45 µl of distilled water to a total of 50 µl.
b. Labelled each eppendorf tube with name of gene and sample number (1,2,3, postive control, negative control)
c. Added 20 µl PCR Mastermix to each tube
d. Added 1 µl of 10 µM forward primers to each appropriate tube. This was done for all genes.
e. Added 1 µl of 10 µM reverse primers to each appropriate tube. This was done for all genes.
f. Added 2ul DNA minipreps (1, 2, 3, and gblock) to each tube. None was added to negative control.
g. Water was added to reach a total volume ot 40 µl (16 µl for each tube with DNA and 18 µl for negative control)
h. PCR temperatures were set on machine according to BioRad protocol and 40 cycles were done.
i. PCR was left overnight.

Preparation of 16µl RPA+SYBR Green Mastermix + 10.75µl HbcAg 60ng/µl sample to be run on microfluidics chip

a. in one RPA reaction tube:
    a. Primer A (10µM) - 2.4µL
    b. Primer B (10µM) - 2.4µL
    c. Rehydration Buffer - 29.5µL
    d. dH2O - 8.2µL
b. added 15µL each from tube to 2 PCR tubes
c. added 1µL dilluted SYBR Green to the 2 PCR tubes
d. In 10µL 60ng/µL DNA sample and 10µL negative control, 0.75 µL Magnesium Acetate was added to each.
e. The tubes were given to the engineering team.

Serial dilution of is481 mp2

Table 17

	A	B	C	D	E	F
1	IS481					
2	**Serial Dilutions**	**Concentration (ng/µl)**	**From Previous Stock**	**dH2O**	**Remaining**	
3		Highest Concentration (x ng/µl)	90.7			
4	1	60	16.53803749	8.461962514	14.58333333	
5	2	25	10.41666667	14.58333333	15	
6	3	10	10	15	12.5	
7	4	5	12.5	12.5	20	
8	5	1	5	20	22.5	
9	6	0.1	2.5	22.5	22.5	
10	7	0.01	2.5	22.5	22.5	
11	8	0.001	2.5	22.5		
12	9	Negative Control (dH2O)	0	25		
13						

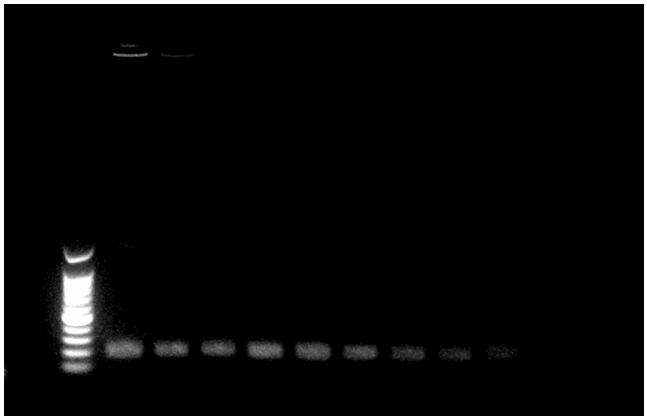
HbcAg RPA with 15µl RPA with 10µl sample DNA

I. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 9.6µL
   b. Primer B (10µM) - 9.6µL
   c. Rehydration Buffer - 118µL
   d. dH2O - 32.8µL

II. Pipetted up and down after addition of each component in step 1
   2. Splitted the reaction mix in four (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
   3. Splitted the reaction into 9 volumes - 15µL to 9 separate PCR tubes.
   4. Added 10µL of template from each serial dilution in corresponding tube
   5. Added 1.25µL of 280mM magnesium acetate and mixed well to start the reaction.
   6. Incubated at 39°C for 20 min using thermocycler

Result:
HbcAg RPA optimization:

HbcAg_serial_dilutions_15ulRPA_10ulDNA_OCT7_2.png



Ladder- 10ul of 60 ng/µl DNA dilution - 10ul of 25ng/µl dilution-10ul of 10 ng/µl dilution-10ul of 5 ng/µl dilution-10ul of 1 ng/µl dilution-10ul of 0.1 ng/µl dilution-10ul of 0.01 ng/µl dilution-10ul of 0.01 ng/µl dilution- negative control

HbcAg PCR with optimized annealing temperature (61℃)

1. Added 20 µl PCR Mastermix to each PCR tube
2. Added 2 µl of 10 µM forward primers to each tube.
3. Added 2 µl of 10 µM reverse primers to each tube.
4. Added 2ul of each dilution to each tube. None was added to negative control.
5. Water was added to reach a total volume ot 40 µl (16 µl for each tube with DNA and 18 µl for negative control)
6. Annealing temperature was set to slightly below Tm of the primers. (61 degrees)
7. PCR temperatures were set on machine according to BioRad protocol and 35 cycles were done.

IS481 with BioLab protocol and optimized annealing temperature (60℃)

1. Added 20 µl PCR Mastermix to each PCR tube
2. Added 1 µl of 10 µM forward primers to each tube.
3. Added 1 µl of 10 µM reverse primers to each tube.
4. Added 2ul of each dilution to each tube. None was added to negative control.
5. 16 µl of water was added for each tube with DNA and 18 µl of water was added for negative control
6. Annealing temperature was set to slightly below Tm of the primers. (61 degrees)
7. PCR temperatures were set on machine according to BioRad protocol and 35 cycles were done.

Gel Electrophoresis of Optimized HbcAg RPA

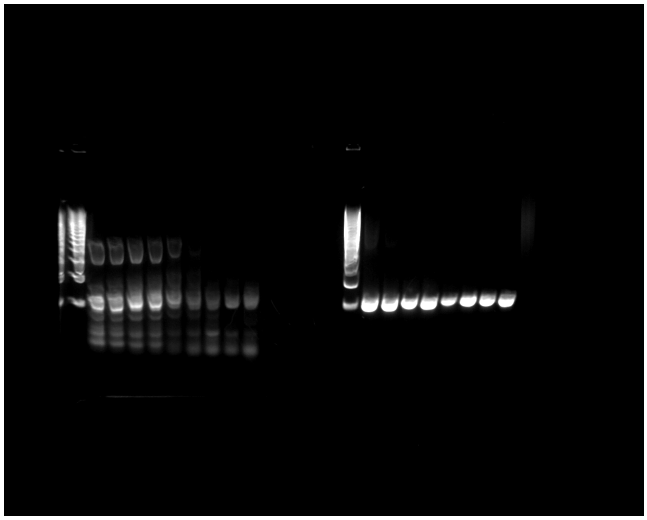
Sample Loading:

a. 5 µl of samples from each RPA diluted sample was added onto a piece of parafilm.
b. 1 µl of purple loading dye was added to each sample drop and mixed by pipetting up and down.
c. 5 µl of 100bp ladder (molecular ruler) for RPA gel were loaded first (5 µl of ladder was mixed with 1 µl of purple dye), followed by 6 µl of samples in the order from high to low conc (first samples with 4µl DNA added, then samples with 10 µl added)
d. The gels were left to run for 20 minutes

Result:

IS481 and HbcAg PCR:

IS481_newPCR_HbcAg_PCR_OCT7.tif



Left IS481 PCR : Ladder- 15ul of 60 ng/µl dilution - 15ul of 25ng/µl dilution-15ul of 10 ng/µl dilution-15ul of 5 ng/µl dilution-15ul of 1 ng/µl dilution-15ul of 0.1 ng/µl dilution-15ul of 0.01 ng/µl dilution-15ul of 0.01 ng/µl dilution- negative control
Right HbcAg PCR: Ladder- 4ul of 60 ng/µl dilution - 4ul of 25ng/µl dilution-15ul of 10 ng/µl dilution- 4ul of 5 ng/µl dilution- 4ul of 1 ng/µl dilution- 4ul of 0.1 ng/µl dilution- 4ul of 0.01 ng/µl dilution-4ul of 0.01 ng/µl dilution- negative control

Discussion

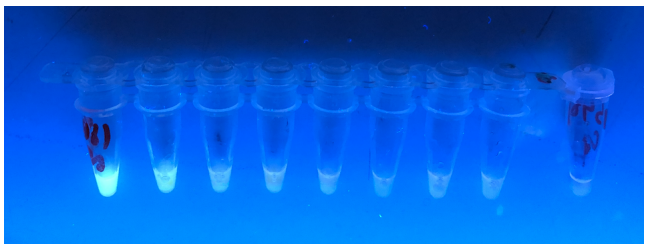
- New annealing temperature was chosen with consideration of the PCR primers' denaturing temperature; it was chosen to be 1-2C lower than the lower primer of each gene.
- New protocol of PCR is used (with different volume of master mix and primer) following the BioLab (the manufacturer of our new PCR Master mix)
- For IS481, the new protocol shows negative control contamination and primer dimers, so

SYBR Green Verification of Optimized HbcAg RPA

1. 0.8µl of diluted SYBR Green (25X) was added to each tube.
2. The reactions were placed under UV Lamp at 384 nm to observe fluorescence.

Result

IS481_RPA_SYBR. green_7_OCT_2019.png



60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

Conclusion & Discussion

- SYBR Green 25X shows fading of RPA reaction along its serial dilution.

Serial Dilution of ypo2088 minprep 1

Table 19

	A	B	C	D	E
1	**Serial Dilutions**	**Concentration (ng/µl)**	**From Previous Stock**	**dH2O**	**Remaining**
2		Highest Concentration (x ng/µl)	75		
3	1	60	20	5	14.58333333
4	2	25	10.41666667	14.58333333	15
5	3	10	10	15	12.5
6	4	5	12.5	12.5	20
7	5	1	5	20	22.5
8	6	0.1	2.5	22.5	22.5
9	7	0.01	2.5	22.5	22.5
10	8	0.001	2.5	22.5	
11	9	Negative Control (dH2O)	0	25	

15µl RPA with 10µl sample DNA (done with ypo2088)

I. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 9.6µL
   b. Primer B (10µM) - 9.6µL
   c. Rehydration Buffer - 118µL
   d. dH2O - 32.8µL

II. Pipetted up and down after addition of each component in step 1

2. Splitted the reaction mix in four (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
3. Splitted the reaction into 9 volumes - 15µL to 9 separate PCR tubes.
4. Added:
   a. 4µL of template from each serial dilution in corresponding tube.
   b. 10µL of template from each serial dilution in corresponding tube
5. Added:
   a. 1µL of 280mM magnesium acetate and mixed well to start the reaction.
   b. 1.25µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Incubated at 39℃ for 20 min using thermocycler

image.png

- From the left , Ladder - 0.1 ng/µl dilution- 1 ng/µl dilution- 5 ng/µl dilution- 10ng/µl dilution- 25 ng/µl dilution- 60 ng/µl dilution - negative control

THURSDAY, 10/10/2019

Cra PCR

I. Reaction mix in 1. PCR tube:
   a. Forward Primer (10µM) - 1.25µL
   b. Reverse Primer (10µM) - 1.25µL
   c. 2x Mastermix - 12.5µL
   d. dH2O - 10µL
II. In Tube 1: Dislodged E.coli colony from IS481 plate using pipette tip
III. Tube 2: -ve control
IV. Followed New England BioLabs protocal in setting run parameters
V. PCR left to run overnight

FRIDAY, 10/11/2019

Cra CRISPR + Quencher Trial 1

CRISPR reaction following NEB protocol:

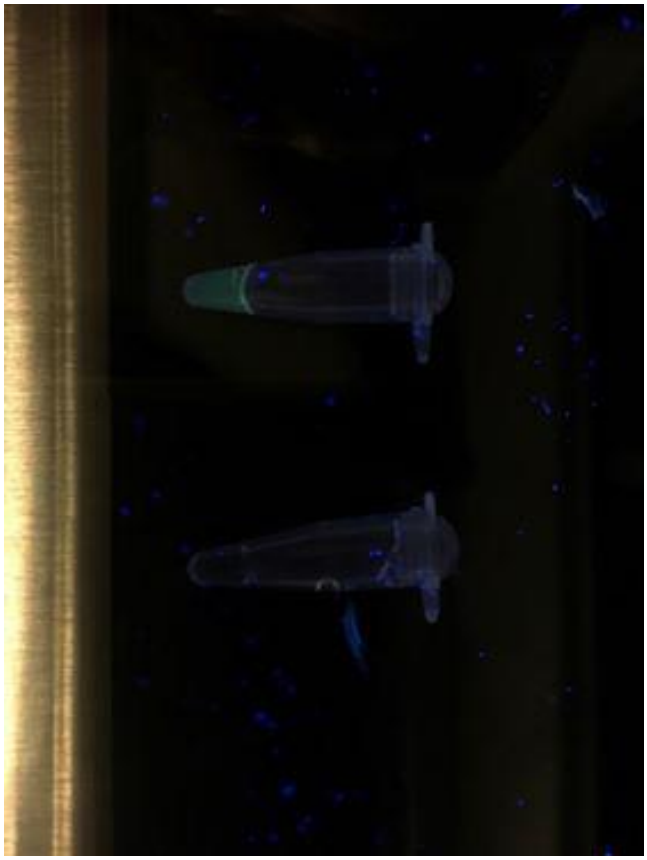
1. Assemble the reaction at room temperature in the following order*:
   a. 17.5µl Nuclease-free water
   b. 3µl NEBuffer 2.1 Reaction Buffer (10x)
   c. 3µl 300nM gRNA
   d. 1µl 1 µM EnGen Lba Cas12a (Cpf1)
   e. 2.5µl 100µM FQ reporter
2. pre-incubated for 10 minutes at 37^{o}C
3. Add 3 µl of substrate DNA (30 µl final volume).
4. Incubate for 60 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

Results:

Top: positive Cra Crispr'ed sample

Bottom: -ve control

75224777_825678924515296_5055833229366394880_n.jpg



Cra CRISPR + Quencher Trial 2

CRISPR reaction following NEB protocol:

1. Assemble the reaction at room temperature in the following order*:
    a. 17.5µl TAE Buffer
    b. 3µl NEBuffer 2.1 Reaction Buffer (10x)
    c. 3µl 300nM gRNA
    d. 1µl 1 µM EnGen Lba Cas12a (Cpf1)
    e. 2.5µl 100µM FQ reporter
2. pre-incubated for 10 minutes at 37^{o}C
3. Add 3 µl of substrate DNA (30 µl final volume).
4. Incubate for 60 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

Results: Fluorescence similar to that of Trial 1

Testing IS481 RPA with adding magnesium acetate

I. Reaction mix in 1.5 mL tube:
    a. Primer A (10µM) - 4.8µL
    b. Primer B (10µM) - 4.8µL
    c. Rehydration Buffer - 59µL
    d. dH2O - 16.4µL
1. Pipetted up and down after addition of each component in step 1
2. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.

3. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
4. Added 1µL of template from each serial dilution in corresponding tube.
5. Added 0.5µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Incubated at 39°C for 30 min using thermocycler

2. SYBR green was added

Result: Fluorescence was observed under UV light.

Testing IS481 RPA without pipetting

I. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 4.8µL
   b. Primer B (10µM) - 4.8µL
   c. Rehydration Buffer - 59µL
   d. dH2O - 16.4µL

II. Pipetted up and down after addition of each component in step 1
   2. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
   3. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
   4. Added 1µL of template from each serial dilution in corresponding tube.
   5. Added 0.5µL of 280mM magnesium acetate.
   6. Incubated at 39°C for 30 min using thermocycler

2. SYBR green was added (no pipetting to mix SYBR green to RPA reaction mix) and fluorescence was checked under UV light.

Result: Fluorescence was observed, but only in the top half of the pcr tube. No fluorescence was observed in the bottom.

Sample preparation for engineers: 4 Sample sets

- 5ul DNA sample *4
  : 5ul of IS481 mp1 (without dilution)
- (16ul RPA mix. + 1ul SYBR Green) *4

1. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 4.8µL
   b. Primer B (10µM) - 4.8µL
   c. Rehydration Buffer - 59µL
   d. dH2O - 16.4µL
2. Pipetted up and down after addition of each component in step 1
3. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into 4 volumes - 16µL to 4 separate PCR tubes.
5. Added 1µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Added 1ul of SYBR Green (25X).

SATURDAY, 10/12/2019

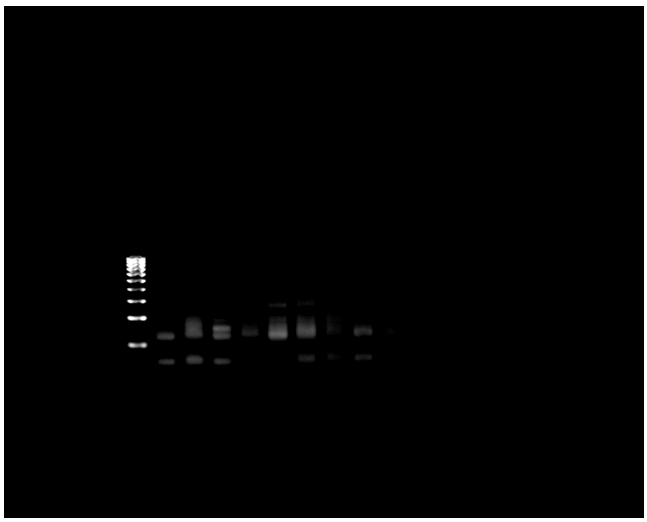
pcaA RPA (1st primer set)

a. Reaction mix in 1.5 mL tube:
   1. Primer A (10µM) - 2.55µL
   2. Primer B (10µM) - 2.55µL
   3. Rehydration Buffer - 59µL
   4. dH2O - 20.3µL
b. Pipetted up and down after addition of each component in step 1
   II. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.

III. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
IV. Added 1µL of template from each serial dilution in corresponding tube.
V. Added 0.5µL of 280mM magnesium acetate and vortex to start the reaction.
VI. Incubated at 39°C for 30 min using thermocycler

Result:

pcaA RPA set 1 Oct 12.tif



Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

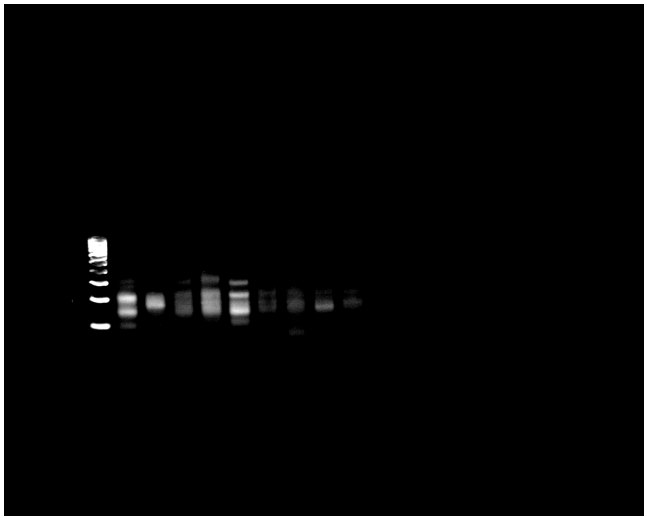
Discussion: Primer dimers were evident in this gel, so the primer concentration was lowered.

pcaA RPA (1st primer set)

a. Reaction mix in 1.5 mL tube:
    1. Primer A (10µM) - 1.8 µL
    2. Primer B (10µM) - 1.8 µL
    3. Rehydration Buffer - 59µL
    4. dH2O - 22.4µL
b. Pipetted up and down after addition of each component in step 1
    II. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
    III. Splitted the reaction into 9 volumes - 8.5µL to 9 separate PCR tubes.
    IV. Added 1µL of template from each serial dilution in corresponding tube.
    V. Added 0.5µL of 280mM magnesium acetate and vortex to start the reaction.
    VI. Incubated at 39°C for 30 min using thermocycler

Result:

RPA pcaA Oct 12.tif



Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

Discussion: When the concentration of primers is reduced significantly, the primer dimers disappear. however, in these results, it seems like there was negative control contamination.

Dilution of Minipreps (ypo MP1)

Table 20

	A	B	C	D	E
1	ypo2088				
2	**Serial Dilutions**	**Concentration (ng/µl)**	**From Previous Stock**	**dH2O**	**Remaining**
3		Highest Concentration (x ng/µl)	77.3		
4	1	60	19.35483871	5.64516129	14.58333333
5	2	25	10.41666667	14.58333333	15
6	3	10	10	15	12.5
7	4	5	12.5	12.5	20
8	5	1	5	20	22.5
9	6	0.1	2.5	22.5	22.5
10	7	0.01	2.5	22.5	22.5
11	8	0.001	2.5	22.5	
12	9	Negative Control (dH2O)	0	25	

PCR of pcaA (NEB protocol)

image.png

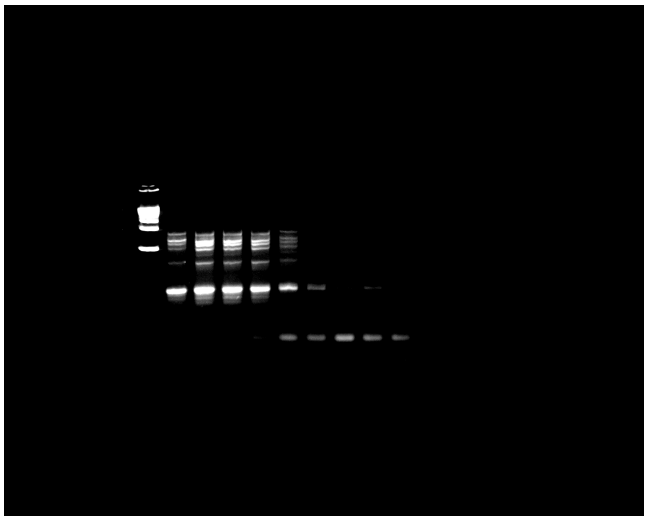
Component	25 µl Reaction
Q5 High-Fidelity 2X Master Mix	12.5 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl
Template DNA	variable
Nuclease-Free Water	to 25 µl

PCR protocol (adapted from: https://international.neb.com/protocols/2012/12/07/protocol-for-q5-high-fidelity-2x-master-mix-m0492?fbclid=IwAR0fNdT9A0uVPSL6DXTOkcES7Dl4z_bPRgfEwZw3rNH42IhBgk_tQRJyWCg)

Denaturation: 98 C for 30 sec
35 cycles: 98 C for 10 sec, 67 C for 30 sec (based on Tm calculator), 72 C for 15 sec
Final Extension: 72 C for 2 min
Hold: 4 C

Result:

pcaA PCR 12 Oct.tif



Ladder-60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control

SUNDAY, 10/13/2019

Nanodrop of IS481

Table 21

	A	B	C	D
1	Sample	Nucleic acid conc	260/280	260/230
2	IS481 mp1 (A)	17.8	2.09	2.20

Nanodrop of ypo2088

Table 22

	A	B	C	D
1	Sample	Nucleic acid conc	260/280	260/230
2	ypo2088	78.3	2.12	2.34

SYBR Green of ypo2088

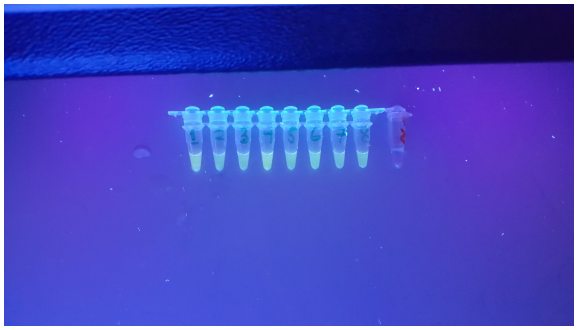
TUESDAY, 10/15/2019

15µl RPA with 4µl sample DNA (done with ypo2088 25ng/µl)

I. Reaction mix in 1.5 mL tube:
   a. Primer A (10µM) - 2.4µL
   b. Primer B (10µM) - 2.4µL
   c. Rehydration Buffer - 29.5µL
   d. dH2O - 8.2µL
II. Pipetted up and down after addition of each component in step 1
III. added reaction mix to 1 freeze dried reaction. Pipetted up and down to mix.
IV. Splitted the reaction into 2 volumes - 15µL to 2 separate PCR tubes.
V. Added:
   a. 4µL of template from each serial dilution in corresponding tube.
VI. Added:
   a. 1µL of 280mM magnesium acetate and mixed well to start the reaction.
VII. Incubated at 37°C for 20 min using thermocycler
7. Added 1µL diluted SYBR Green to the samples together with the negative control and observed under UV Lamp

Results:

20191015_165328.jpg



- **SYBR Green + 60 ng/µl dilution- 25ng/µl dilution- 10 ng/µl dilution- 5 ng/µl dilution- 1 ng/µl dilution- 0.1 ng/µl dilution- 0.01 ng/µl dilution- 0.01 ng/µl dilution- negative control**

HbcAg CRISPR + Quencher

CRISPR reaction following NEB protocol:

1. Assemble the reaction (2 reactions one +ve one -ve control) at room temperature in the following order*:
    a. 3µl NEBuffer 2.1 Reaction Buffer (10x)
    b. 3µl 300nM gRNA
    c. 1.5µl 1 µM EnGen Lba Cas12a (Cpf1)
    d. 2.5µl 100µM FQ reporter
2. pre-incubated for 10 minutes at 37^{o}C
3. Added to RPA reactions
4. Incubate for 30 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

Result: Fluroscence was observed.

WEDNESDAY, 10/16/2019

HbcAg RPA + SYBR Green and CRISPR + FQ Very Diluted Samples

Dilutions made from 0.001ng/µl HbcAg sample

Table 23

	A	B	C	D
1	Concentration (ng/µL)	dH2O (µL)	Sample from previous dilution (µL)	Volume remaining (µL)
2	1e-3	22.5µL	2.5µL	22.5µL
3	1e-4	22.5µL	2.5µL	22.5µL
4	1e-5	22.5µL	2.5µL	22.5µL
5	1e-6	22.5µL	2.5µL	22.5µL
6	1e-7	22.5µL	2.5µL	22.5µL
7	1e-8	22.5µL	2.5µL	22.5µL
8	1e-9	22.5µL	2.5µL	22.5µL
9	1e-10	22.5µL	2.5µL	22.5µL
10	1e-11	22.5µL	2.5µL	22.5µL
11	1e-12	22.5µL	2.5µL	22.5µL
12	1e-13	22.5µL	2.5µL	22.5µL
13	1e-14	22.5µL	2.5µL	22.5µL
14	1e-15	22.5µL	2.5µL	22.5µL
15	1e-16	22.5µL	2.5µL	22.5µL
16	1e-17	22.5µL	2.5µL	22.5µL
17	1e-18	22.5µL	2.5µL	22.5µL
18	1e-19	22.5µL	2.5µL	22.5µL
19	1e-20	22.5µL	2.5µL	22.5µL

*Samples with concentrations 1e-3, 1e-4, 1e-5, 1e-6, 1e-7, 1e-8, 1e-9, and 1e-10 were used

15µl RPA with 4µl sample DNA (done with HbcAg further serial dilutions *2sets and 25ng/µl)

I. Reaction mix in 1.5 mL tube:
    a. Primer A (10µM) - 19.2µL
    b. Primer B (10µM) - 19.2µL
    c. Rehydration Buffer - 118µL
    d. dH2O - 23.8µL
II. Pipetted up and down after addition of each component in step 1
III. added reaction mix to 8 freeze dried reaction. Pipetted up and down to mix.
IV. Splitted the reaction into 19 volumes - 15µL to 19 separate PCR tubes.
V. Added:
    a. 4µL of template from each serial dilution in corresponding tube (no mixing).
    b. 1µL of 280mM magnesium acetate (added to DNA sample) and mixed well.
    c. To one of the two sets of samples: added 2.5µl 100µM FQ reporter
VI. Incubated at 37°C for 20 min using thermocycler
VII. Added 1µL diluted SYBR Green to 1 of the 2 sets of the samples and observed under UV Lamp
II. Results:

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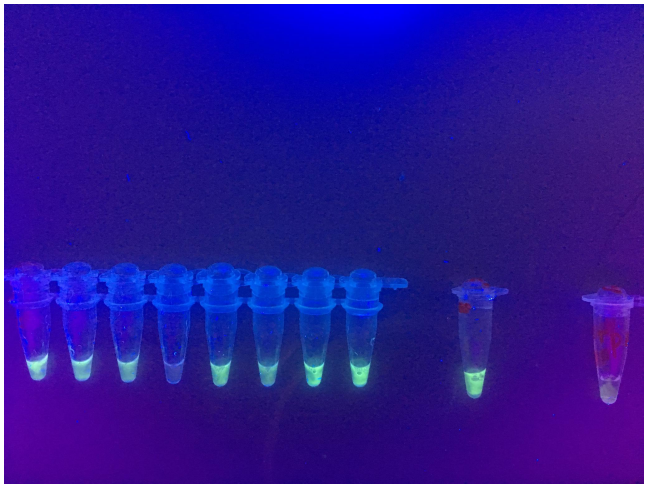


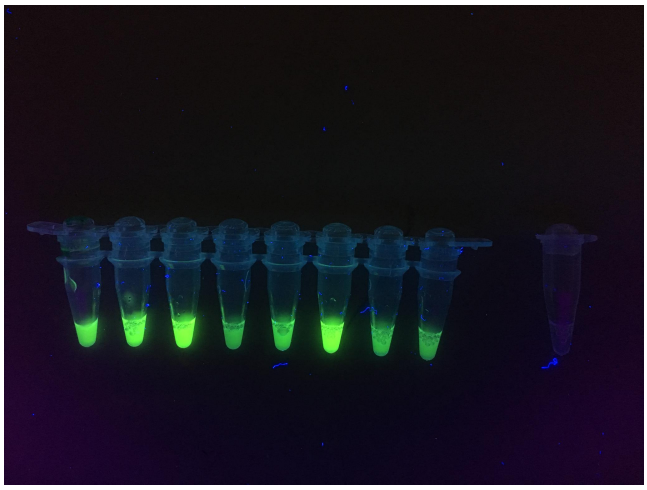
From left -> right: high to low concentration samples, -ve control, SYBR GREEN with 10µl RPA primers

HbcAg CRISPR + Quencher (done on the set of RPA'd samples remaining including the 25ng/µl sample)

CRISPR reaction following NEB protocol:

1. Assemble the reaction at room temperature in the following order*:
    a. 2µl NEBuffer 2.1 Reaction Buffer (10x)
    b. 0.5µl 3µM gRNA
    c. 1.5µl 1 µM EnGen Lba Cas12a (Cpf1)
2. pre-incubated for 10 minutes at 37^{0}C
3. Added to RPA reactions
4. Incubate for 20 minutes at 37°C. Check the fluorescence every 10 minutes using fluorescent microscope.

微信图片_20191018225020.jpg



From left-> right: samples with high->low initial DNA concentrations, -ve control

Running RPA-CRISPR on microfluidic chip

- CRISPR reaction following the optimized volume:

1. Assemble the two reaction at room temperature in the following order*:

a. 2µl NEBuffer 2.1 Reaction Buffer (10x)
b. 0.5µl 5uM gRNA
c. 1.5µl 1 µM EnGen Lba Cas12a (Cpf1)

2. pre-incubated for two for 10 minutes and two for 20 minutes at 37^{o}C in the pcr thermocycler (not on the chip)

*For incubation, the heater that engineers made was used:

- RPA (16ul)
    I. Reaction mix in 1.5 mL tube:
        a. Primer A (10µM) - 4.8µL
        b. Primer B (10µM) - 4.8µL
        c. Rehydration Buffer - 59µL
        d. dH2O - 16.4µL
    II. Pipetted up and down after addition of each component in step 1
    III. added reaction mix to 1 freeze dried reaction. Pipetted up and down to mix.
    IV. Splitted the reaction into 4 volumes - 15µL to 2 separate PCR tubes.
    V. Added:
        a. 4µL of template from each serial dilution in corresponding tube.
    VI. Added:
        a. 1µL of 280mM magnesium acetate and mixed well to start the reaction.
        b. 2.5ul of undiluted FQ reporter
    VII. Incubated at 39°C on the microfluidic chip for 20 minutes

- 10 minutes and 20 minutes pre-incubated CRISPR reagents were added to the two microfluidic chips.
- The reaction was heated for 20 minutes.

Result: No fluorescence was observed on the chip.

chip_16_oct_2019.png

No flurescence on the chip channels under UV light

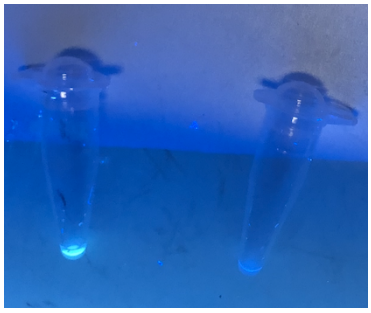
*For incubation, the incubator (Set as 39C) was used.

- RPA (16ul)
    I. Reaction mix in 1.5 mL tube:
        a. Primer A (10μM) - 4.8μL
        b. Primer B (10μM) - 4.8μL
        c. Rehydration Buffer - 59μL
        d. dH2O - 16.4μL
    II. Pipetted up and down after addition of each component in step 1
    III. added reaction mix to 1 freeze dried reaction. Pipetted up and down to mix.
    IV. Splitted the reaction into 4 volumes - 15μL to 2 separate PCR tubes.
    V. Added:
        a. 4μL of template from each serial dilution in corresponding tube.
    VI. Added:
        a. 1μL of 280mM magnesium acetate and mixed well to start the reaction.
        b. 2.5ul of undiluted FQ reporter
    VII. Incubated at 39°C in the incubator for 20 minutes

- 10 minutes and 20 minutes pre-incubated CRISPR reagents were added to the two microfluidic chips.
- The reaction was heated for 20 minutes.

Results:

samplefromchip_incubator_16_oct_2019.png



Left: 20 minutes pre-incubated CRISPR/ Right: 10 minutes pre-incubated CRISPR
After few minutes, 10 minutes pre-incubated CRISPR slowly shows fluorescence

Conclusion & Discussion:

- No fluorescence was observed on the chip. When 10ul of the sample was extractacted from the chip and transfer to the pcr tube, the fluroescence was observed for both pre-incubation time. This shows that RPA and CRISPR reactions work on the chip as well as both 10 minutes and 20 minutes pre-incubation work.
- Also, since the chip itself is giving out fluorscence (including channel), it is hard to visualize the fluorescence on the chip. Modification on the channels of the microfluidic chips will be made.

THURSDAY, 10/17/2019

Performing serial dilutions on LB Broth for different genes to determine the minimum sample needed for the detection of each gene.

Stock solution was cultured for each gene for 16 hours in the shaker incubator. CFU = 10^6 bacterial cells/ml.

- Performing serial dolutions on each LB broth tube for each gene six times with a dilution gactor of 10
- Hence, for each gene we have end up having 6 tubes of the following concentrations: 10^6, 10^5, 10^4, 10^3, 10^2, 10.

Colony PCR on Serial Dilution of LB Broths (all genes):

a. Prepared 10 µM working stock of each primer by extracting 1 µl of 100 µM stock and adding 9 µl of distilled water to a total of 10ul.
b. Labelled each Eppendorf tube with the name of the gene and sample number (serial dilution, negative control)
c. Added 20 µl PCR Mastermix to each tube
d. Added 1 µl of 10 µM forward primers to each appropriate tube. This was done for all genes.
e. Added 1 µl of 10 µM reverse primers to each appropriate tube. This was done for all genes.
f. Added 2ul DNA minipreps to each tube. None was added to negative control.
g. Water was added to reach a total volume ot 40 µl (16 µl for each tube with DNA and 18 µl for negative control)
h. PCR temperatures were set on machine according to BioRad protocol and 40 cycles were done.
i. PCR was for left running for 1 hour 40 minutes.

Result:

image.png

From Left to right 1-ladder, 2- IS481 10^3 CFU/ml , 3-IS481 10^2 CFU/ml, 4-pcAa 10 CFU/ml, 5-pcAa 1 CFU/ml, 6- Negative Contol, 8-ypo2088 10^3 CFU/ml, 9- ypo2088 10^2 CFU/ml, 10-HbcAg 10^3 CFU/ml, 11- HbcAg 10^2 CFU/ml, 12- Negative Control

Nano-drop of IS481 Minipreps and HbcAg Miniprep (previously made):

Table 24

	A	B	C	D
1		Nucleic Acid Conc. (ng/µl)	260/280	260/230
2	IS481 MP1	34.2	1.95	2.05
3	IS481 MP2	78.2	1.88	2.28
4	IS481 MP3	399.4	1.87	2.22
5	IS481 MP4	71.6	1.77	1.28
6	HBcAG mp1	84.3	1.88	2.32
7	HBcAG mp2	51.7	1.80	1.45
8	HBcAG mp3	96.7	1.71	1.22
9	HbcAg mp1 (red)	309.4	1.90	2.19

FQ Optimization

First FQ Optimization:

Check the fluorescence of different FQ reporter concentrations on 16ul RPA. For each FQ reporter dilution, one positive (with DNA) and negative (without DNA) was tested in order to confirm negative control and check the background noise.

- RPA*8 replicates

1. Reaction mix in 1.5 mL tube:
    a. Primer A (10µM) - 9.6µL
    b. Primer B (10µM) - 9.6µL
    c. Rehydration Buffer - 118µL
    d. dH2O - 32.8µL
2. Pipetted up and down after addition of each component in step 1
3. Splitted the reaction mix in four (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into 8 volumes - 15µL to 8 separate PCR tubes.
5. Added 1µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA to five of them, add 5ul water to three of them
7. Incubate 20 minutes at 37°C.

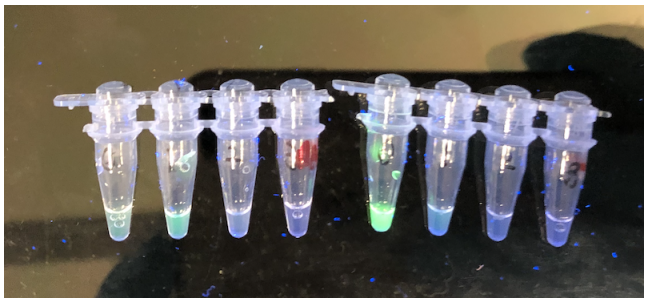
- CRISPR Reagents *8 replicates

1. 1.5ul 1uM Lb Cas 12a

2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. 2.5ul of FQ reporter: 100uM (undiluted, 1X), 10uM, 1uM, 0.1uM + negative control of the four dilution
    a. FQ reporter is diluted using TE buffer
5. Incubate 20 minutes

Results

fq_1_1x_0.1x_0.001x_0.0001x_+-_oct_18_2019.png



FQ reporter on HbcAg RPA-CRISPR: Positive 1X - Positive 0.1X -Positive 0.01X - Positive 0.001X - Negative 1X - Negative 0.1X -Negative 0.01X - Negative 0.001

Conclusion & Discussion:

- Under UV light, fluorescence were observed for 1X and 0.1X FQ reporter concentration. However, this includes fluorescence in negative control.
- Low intenstiy fluorescence was observed for 0.1X FQ reporter dilution.
- No fluorescence was observed for FQ reporter dilution lower than 0.5X.
- More optimization testing is required to confirm the fluorescence of 1X and 0.5X FQ reporter dilution.

Second FQ Optimization:

2.5ul of 1X and 0.1X FQ reporter on HbcAg RPA-CRISPR / repeated twice

- RPA*4

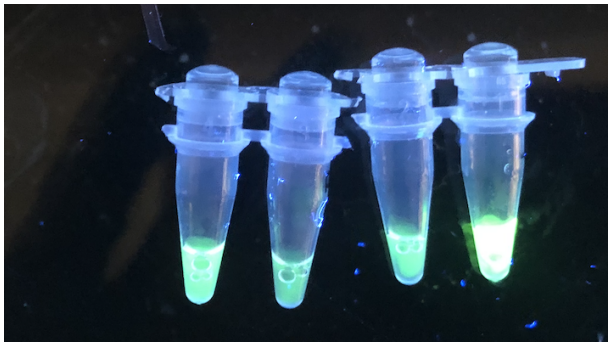
1. Reaction mix in 1.5 mL tube:
    a. Primer A (5μM) - 4.8μL
    b. Primer B (5μM) - 4.8μL
    c. Rehydration Buffer - 59μL
    d. dH2O - 16.4μL
2. Pipetted up and down after addition of each component in step 1
3. Splitted the reaction mix in two (42.5μL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into 4 volumes - 15μL to 4 separate PCR tubes.
5. Added 1μL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA
7. 2.5ul of FQ reporter (1X, 0.5X): two positive, two negative
    I. FQ reporter is diluted using TE buffer
8. Incubate 20 minutes at 37°C.

- CRISPR Reagents *4

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes

Result:

fq_2_1x_0.1x_2.5ul_++--_oct_18_2019.png



FQ reporter on HbcAg RPA-CRISPR: Positive 1X - Positive 0.5X - Negative 1X - Negative 0.5X

Conclusion & Discussion:

- Since neagative control fluoresce better than positive control, contamination of buffer or water is expected.

Determining the source of contamination:

1. 10ul of NEBuffer 2.1 was added to PCR tube.
2. 0.5ul of 1X FQ quencher was added.
3. Check under UV light.

Results: Fluorscence was observed for NEBuffer 2.1.

Conclusion & Discussion:

- NEBuffer 2.1 is contaminated. Aliquote of New NEBuffer 2.1 should be used.
- Use lower volume of FQ reporter than 2.5 µl; detected high background fluorescence may not only because of the contamination of buffer.

Third FQ Optimization:

1X and 0.5x of 1.5ul FQ quencher is used

- RPA*4

1. Reaction mix in 1.5 mL tube:
    a. Primer A (5µM) - 4.8µL
    b. Primer B (5µM) - 4.8µL
    c. Rehydration Buffer - 59µL
    d. dH2O - 16.4µL
2. Pipetted up and down after addition of each component in step 1
3. Splitted the reaction mix in two (42.5µL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into 4 volumes - 15µL to 4 separate PCR tubes.
5. Added 1µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA
7. 1X and 0.5X 1.5ul of FQ reporter: two positive and two negative
    I. FQ reporter is diluted using TE buffer
8. Incubate 20 minutes at 37°C.

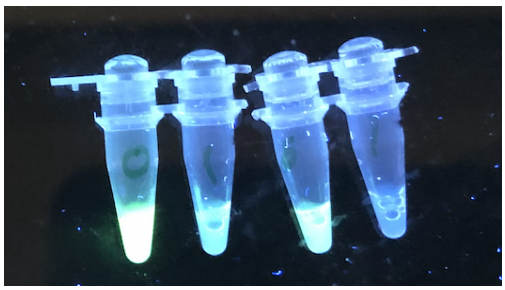
- CRISPR Reagents *4

1.. 1.5ul 1uM Lb Cas 12a

2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes

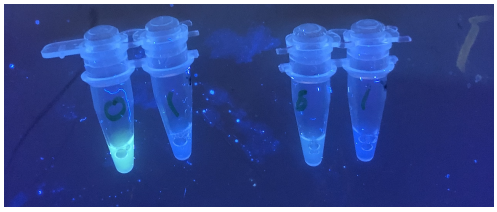
Result:

fq_3_1x_0.5x_uv_oct_18_2019.png



1.5ul FQ reporter on HbcAg RPA-CRISPR: Positive 1X - Positive 0.5X - Negative 1X - Negative 0.5X under UV light (used for gel detection)

fq_3_1x_0.5x_1.5ul_oct_18_2019.png



1.5ul FQ reporter on HbcAg RPA-CRISPR: Positive 1X - Positive 0.5X - Negative 1X - Negative 0.5X under UV light

Conclusion & Discussion:

- 1.5ul of FQ quencher is enough for detection.
- High background fluorescence is detected when 1X FQ reporter is used. Therefore, 0.5X FQ reporter should be used.

HbcAg Innoculations:

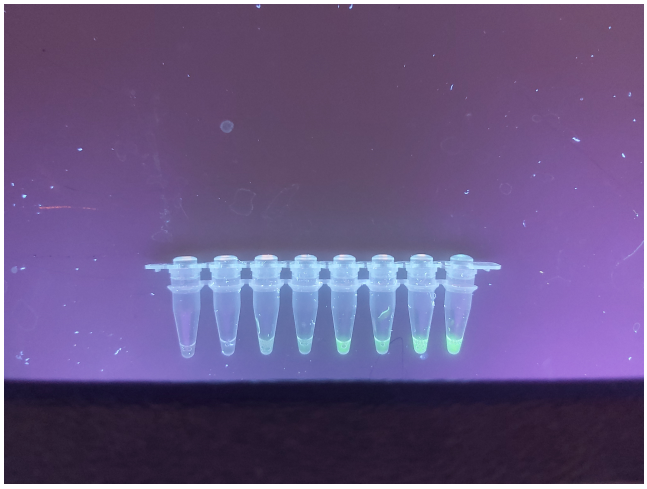
1. 5mL LB broth was added to three 15mL culture tubes. (falcon tubes).
2. A plastic inoculation loop was used to select 3 colonies from HbcAg plate and was swirled in the corresponding broth to dislodge the colony
3. The tubes were loosely capped and incubated on a shaker at 220rpm and 37°C (16 hours).

SYBR Green Optimization

1. SYBR Green was diluted from 25x to 15x 10x 5x and 1x
2. One negative control and one sample (10 µl RPA mix and 4 µl DNA)

Results:

image.png



Right to left: 15x SYBR green RPA positive- 15X SYBR green RPA negative- 10X SYBR green RPA positive- 10X SYBR green RPA negative- 5X SYBR green RPA positive- 5X SYBR green RPA negative- 1X SYBR green RPA positive- 1X SYBR green RPA negative