Lab Notebook 2

FRIDAY, 10/18/2019

Preparation of LB Broth+Amp:
Added 800ul of 100mg/ml AMP to 800 ml of LB broth (previsouly prepared)

Inoculations on each of the 5 genes:

- Added 10ml of LB+Amp broth to each falcon tube
- Selected a colony from each LB+Agar plate for each gene and introduced it into the falcon tubes, twirling vigorously.
- Incubates in the shaker incubator over night (incubated at 4:00 pm)

PCR on broth:

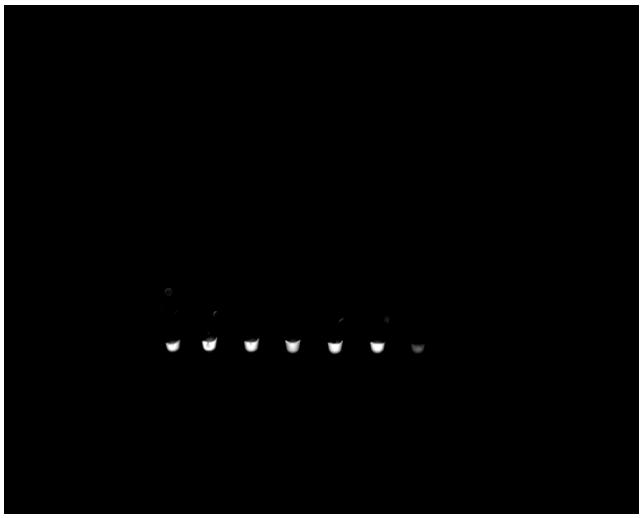
- Took out the stock inculations from the previous day from the fridge (each tube for each gene is expected to contain 10^6 bacterial cells/mL of LB Broth since they were incubated for 18-24 hours in the shaker incubator) (however they were placed in room temperature for a while so we are not sure how that affects the number of cells present)
- Prepared 4 sets of 5 falcon tubes for each gene for the preparation of 5 dilutions of each
- Prepared 10^6, 10^5, 10^4, 10^3, 10^2, 10 cells/ml dilutions for each gene by adding 1ml of the previous solution to 9ml of broth
- Plated 150ul of the 10^3 and 10 cells/ml dilutions of each gene on an LB+Amp agar plate and incubted at 37% overnight inorder to calculate the CFU of each gene the next day.

Running PCR on 3 of the dilutions 10^4, 10^3, 10^2 dilutions for each gene:

- Prepared 10uM PCR primers (both forward and reverse) for each gene by adding 1ul of sample from 100uM stock to 9ul of water.
- Added 20 ul PCR Mastermix to each eppendorph tube
- Added 1 ul of 10 uM forward primers to each appropriate tube. This was done for all genes.
- Added 1 ul of 10 uM reverse primers to each appropriate tube. This was done for all genes.
- Added 2ul LB broth to each tube. None was added to negative control.
    - For HBcAg:
        - 3ul of lysis TAE buffer was added to 1000ul of the 10^3 dilution of the LB Broth
        - PCR was run on both samples: the one with the lysis buffer and the one without the lysis buffer.
- Water was added to reach a total volume ot 40 ul (16 ul for each tube with DNA and 18 ul for negative control
- PCR temperatures were set on machine according to BioRad protocol and 40 cycles were done.
- PCR was for left running for 1 hour 40 minutes.
- The samples were then tested for DNA amplication by adding 0.8μL of 5x sybr green to 8μL of the generated sample

Result:

broth PCR HbcAg 10000 1000 100 ypo 10000 10 00 100 negative.tif



1. HbcAg 10,000 cells/mL, 2. HbcAg 1000 cells/mL, 3. HbcAg 100 cells/mL, 4. ypo2088 10,000celss/mL, 5. ypo2088 1000 cells/mL, 6. ypo2088 100 cells/mL, 7. negative control

Conclusion:

- Verfication of successful PCR amplification on bacteria taken directly from broth.

FQ Optimization - RPA

First FQ optimization:

1.5ul and 1ul of 1X and 0.5X FQ quencher on HbcAg RPA-CRISPR

- RPA*8

1. Reaction mix in 1.5 mL tube:
    a. Primer A (5μM) - 9.6μL
    b. Primer B (5μM) - 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 two (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

    7. Add 1.5ul and 1ul of 0.5X FQ quencher to the RPA mix

    7. Incubate 20 minutes at 37°C.

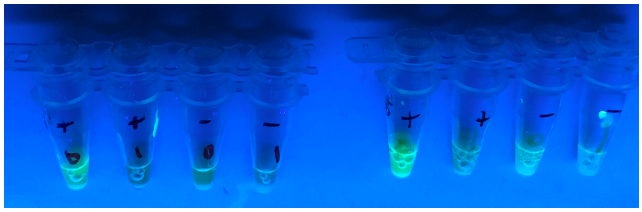
- CRISPR Reagents *8/ for each tube:

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

- Add CRISPR reagents to RPA reaction mix
- Incubate 20 minutes at 37°C.

Result:

fq_1_1ul_1.5ul_1x_0.5x_oct_18_2019.png



HbcAg RPA-CRISPR with FQ reporter: 1ul 1X positive - 1ul 0.5X positive - 1ul 1X negative - 1ul 0.5X negative - 1.5ul 1X positive - 1.5ul 0.5X positive - 1.,5ul 1X negative - 1.5ul 0.5X negative -

Conclusion & Discussion:

- 1ul and 1.5ul of 1X FQ have high background fluorescence, so preferable to use 0.5X.
- Since 1,5ul of 0.5X has the lowest background fluorescence with positive control fluorescence, 1.5ul of 0.5X will be used for further FQ optimization.
- High background fluorescence might due to extended period of incubation. Incubation time of the quencher should be rechecked.

Second FQ optimization:

Time of adding FQ quencher: 20minutes incubation or 40 minutes incubation. (20minutes incubation means adding quencher when pre-incubated CRISPR is added to the RPA reaction mix/ 40minutes incubation means adding quencher to the RPA mix before RPA incubation/reaction)

- Following the standard protocol, FQ reporter should be added after pre-incubation of the CRISPR. However, prior experiment shows that they are no significant difference in fluorescence between the one with pre-incubated quencher and another one after incubation of quencher.

- 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 5µl of DNA added to tube 1 and tube 3.
7. Tube 1 and 2: 1ul of 0.5X FQ quencher was added
8. Incubate 20 minutes at 37°C.

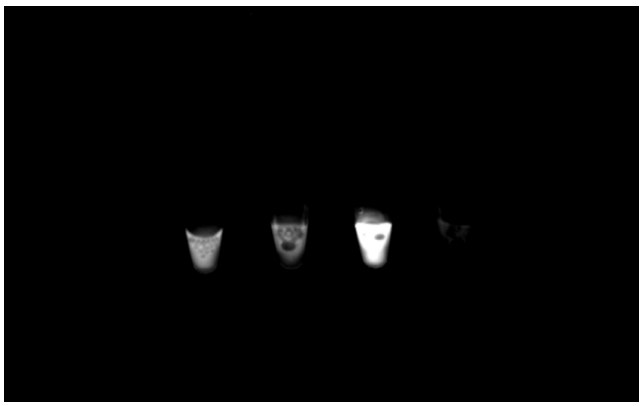
- CRISPR Reagents *4/ for each tube:

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

- Add CRISPR reagents to RPA reaction mix
- Tube 3 and 4: 1ul of 0.5X FQ quencher was added
- Incubate 20 minutes
- Checked under the blue light based E-imager.

Result:

fq_hbcag_rpa_crispr_1ul_0.5x_fq_40min_20min_heating_+-+-_3_18_oct_2019.png



RPA-CRISPR with 1ul of 0.5X FQ quencher: 40minutes FQ incubation positive- 40minutes FQ incubation negative- 20minutes FQ incubation positive-20minutes FQ incubation negative

Conclusion & Discussion:

- High intensity fluorescence of the postivie samples of 20 minutes incubation is observed. No negative fluorscence for the 20 minutes incubation.
- Therefore, higher contrast between 20minutes incubation of FQ quencher is observed.
- When FQ was added to the RPA mix prior to the initial incubation, there is higher nagative fluorescence
- Revision in the chip structure, so that FQ quencher is bnot heated up until CRISPR reaction, is necessary.

Third FQ optimization:

Reconfirm the volume of quencher and 20 minutes incubation of the quencher

- 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

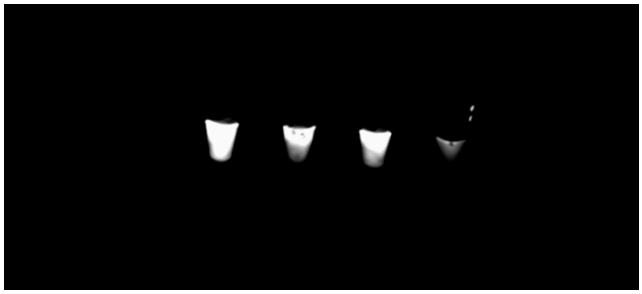
2. Pipetted up and down after addition of each component in step 1
3. Splitted the reaction mix in two (42.5μL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into 4 volumes - 15μL to 4 separate PCR tubes.
5. Added 1μL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA
7. Incubate 20 minutes at 37°C.

- CRISPR Reagents *4/ for each tube:

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

- Add CRISPR reagents to RPA reaction mix
- Add 1.5ul and 1ul of 0.5X FQ quencher to the RPA+ preincubated CRISPR mix
- Incubate 20 minutes at 37°C.
- Checked under the blue light based E-imager.

fq_hbcag_rpa_crispr_1.5ul_1ul_0.5x_fq_20min_heating_+-+-_2_18_oct_2019.png



RPA-CRISPR: 1.5ul of 0.5X FQ quencher positive-1.5ul of 0.5X FQ quencher negative- 1ul of 0.5X FQ quencher positive- 1ul of 0.5X FQ quencher negative

- High intensity fluorescence of the negative control when 1.5ul of quencher isi added compared to 1ul of quencher was added to the reaction.
- Therefore, higher contrast between 20minutes incubation of FQ quencher is observed.
- 1ul of FQ quencher should be used with 20minutes incubation time.

Miniprep HbcAg

Nano-drop of HbcAg Miniprep:

Table 1

	A	B	C	D
1		Nucleic Acid Conc. (ng/ul)	260/280	260/230
2	hbcag mp1	8.5	1.97	1.77
3	hbcag mp2	13.1	1.82	1.00

Discussion:

- Since 5ml of broth was used, not 10ml, maybe it wasn't engouh nutrients for bacteria to grow.

Innoculate HbcAg

1. 10mL LB broth was added to 15mL culture tubes
2. A plastic inoculation loop was used to select a colony from each plate and was swirled in the corresponding broth to dislodge the colony
3. Step 2 was repeated for 3 colonies on each of the 5 plates
4. The tubes were loosely capped and incubated on a shaker at 220rpm an ernight (19 hours)

SATURDAY, 10/19/2019

- Miniprep on ypo2088

ypo2088 MP 2′ serial dilutions:

Table 2

	A	B	C
1	Concetration (ng/ul)	Volume from previous stock (ul)	Voume of water (ul)
2	60	19.81833196	20.18166804
3	5	3.333333333	36.66666667
4	0.01	0.08	39.92

Nano-drop of ypo2088 Miniprep:

Table 3

	A	B	C	D
1		Nucleic Acid Conc. (ng/ul)	260/280	260/230
2	ypo2088 MP2'	121.1		

1. Dilute ypo2088 PCR primers (both forward and reverse) by adding 1ul from the stock to 9ul of water.

Nano-drop of HbcAg Miniprep:

Table 4

	A	B	C	D
1		Nucleic Acid Conc. (ng/ul)	260/280	2
2	hbcag mp1	124.8	1.88	2.60
3	hbcag mp2	100.0	1.72	1.30
4	hbcag mp3	98.0	1.88	2.14
5	ypo2088	22.0	2.42	1.65

IS481 serial dilution:

Table 5

	A	B	C	D	E
1	IS481				
2	**Serial Dilutions**	**Concentartion (ng/µl)**	**From Previous Stock**	**dH2O**	**Remaining**
3		Highest Concentration (x ng/µl)	71.8		
4	1	60	20.8913649	4.108635097	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	Nagative Control (dH2O)	0	25	

HbcAg serial dilution:

Table 6

	A	B	C	D	E
1	HbcAg				
2	**Serial Dilutions**	**Concentartion (ng/µl)**	**From Previous Stock**	**dH2O**	**ıing**
3		Highest Concentration (x ng/µl)	71.8		
4	1	60	20.8913649	4.108635097	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	Nagative Control (dH2O)	0	25	

Ypo2088 serial dilution:

Table 7

	A	B	C	D	E
1	YPO				
2	**Serial Dilutions**	**Concentartion (ng/µl)**	**From Previous Stock**	**dH2O**	**ıing**
3		Highest Concentration (x ng/µl)	121		
4	1	60	12.39669421	12.60330579	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	Nagative Control (dH2O)	0	25	

IS481 and HbcAg PCR (total volume: 16ul) following Taq 2X Master Mix NEB Protocol

1. Add the following components in each pcr tube
    a. Taq 2X Master Mix: 8ul
    b. Forward Primer (10uM): 0.32ul
    c. Reverse Primer (10uM): 0.32ul
    d. Template DNA: 5ul
    e. Nuclease-free water: 2.36ul (up to 16ul)
2. Thermocycling following BioLab protocol
    a. Initial Denaturation: 95°C 30 seconds
    b. 30 cycles
        I. 95°C 20 seconds (15-30 seconds)
        II. *60°C 30 seconds (15-60 seconds)
        *Annealing temperature of the gene
        III. 68°C 30 seconds
    c. Final extension: 68°C 5 minutes
    d. Hold: 4°C

CRISPR on IS481 RPA (post serial diluation and postive, negative control), IS481 PCR (serial dilution)

- CRISPR Reagents *10

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer

4. Incubate 20 minutes at 37°C.

- Add 1ul of 0.5X FQ quencher to the CRIPSR mix
- Add CRISPR reagents mix with quencher to RPA reaction mix
- Incubate 20 minutes at 37°C.
- Check under the blue light based E-gel imager.

Results

IS481 RPA-CRISPR positive (undiluted IS481 MP was used: 71.8ng/ul) and negative control

is481_rpa_crispr_+-_19_oct_2019.png

IS481 RPA-CRISPR: positive control - negative control

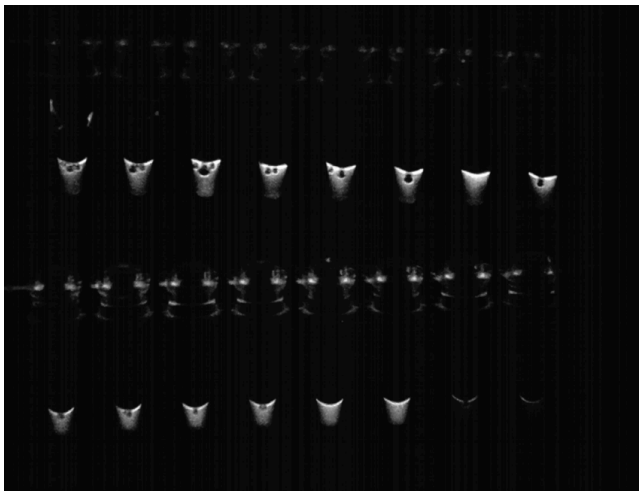
Conclusion & Discussion

- CRISPR using the new Lb Scaffold gRNA for IS481 is working.
- Compare specificity of CRISPR on PCR and RPA'ed IS481 serial dilution.

Results

IS481 PCR - CRISPR and RPA-CRISPR:

is481_top_rpa_bot_pcr_crispr_2_19_oct_2019.png



Top- RPA:60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution

Bottom- PCR:60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution

Conclusion & Discussion

- CRISPR worked with both PCR and RPA at high DNA concentration
- CRISPR on RPA worked with lower concentration of DNA sample compa -CRISPR

CRISPR on HbcAg RPA (postive, negative control)

- CRISPR Reagents *10

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

- Add 1ul of 0.5X FQ quencher to the CRIPSR mix
- Add CRISPR reagents mix with quencher to RPA reaction mix
- Incubate 20 minutes at 37°C.
- Check under the blue light based E-gel imager.

Results

HbcAg RPA-CRISPR positive (undiluted HbcAg MP was used: 100ng/ul) and negative control

hbcag_rpa_crispr_newgrna_+-_2_19_oct_2019.png

HbcAg RPA-CRISPR: positive control - negative control

Conclusion & Discussion

- CRISPR using the new Lb Scaffold gRNA for HbcAg is working.
- Compare specificity of CRISPR on PCR and RPA'ed HbcAg serial dilution.

Preparation of HbcAg RPA sample for engineers (total volume of RPA mix: 16ul)

- RPA Reagents *8

Reaction mix in 1.5 mL tube:

a. Primer A (5μM) - 7.2μL
b. Primer B (5μM) - 7.2μL
c. Rehydration Buffer - 88.5μL
d. dH2O - 24.6μL

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

3. Splitted the reaction mix in three (42.5μL) and added each half to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into 6 volumes - 15μL to 6 separate PCR tubes.
5. Added 1μL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of eight diluted DNA, undiluted DNA, and water
7. Incubate 20 minutes at 37°C.

- CRISPR Reagents *10/ for each tube:

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

- Add 1ul of 0.5X FQ quencher to the CRIPSR mix
- Add CRISPR reagents mix with quencher to RPA reaction mix
- Incubate 20 minutes at 37°C.

SUNDAY, 10/20/2019

Miniprep HBcAg

CRIPSR on RPA'ed and PCR'ed HbcAg

- CRISPR Reagents/ for each tube:

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

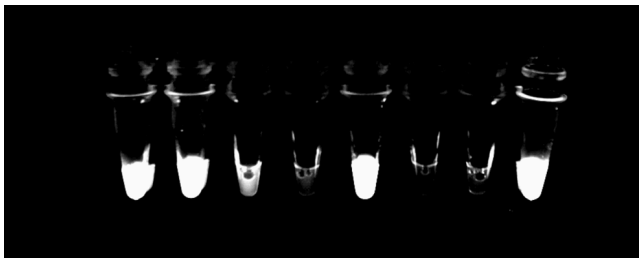
- Add 1ul of 0.5X FQ quencher to the CRIPSR mix
- Add CRISPR reagents mix with quencher to RPA reaction mix
- Incubate 20 minutes at 37°C.

Result

HbcAg_serial_dilutions_PCR_CRISPR_SEPT20_2019.png

PCR-CRISPR:60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution- negative control

HbcAg_serial_dilutions_RPA_CRISPR_SEPT20_2019.png



PCR:60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution- negative control

Specificity Test on HbcAg and IS481

Table 9

	A	B	C	D	E	F	G	H	I
1	**Tube**	1	2	3	4	5	6	7	
2	**DNA**	HbcAg	X (water)	HbcAg	X (water)	IS481	X (water)	IS481	X (water)
3	**Primer**	HbcAg and IS481 Forward and Reverse Primers (5µM)							
4	**gRNA**	HbcAg		IS481		HbcAg		IS481	

- RPA*8

1. Reaction mix in 1.5 mL tube:
    a. IS481 Primer Forward (5μM) - 9.6μL
    b. IS481 Primer Reverse (5μM) - 9.6μL
    c. HbcAg Primer Forward (5μM) - 9.6μL
    d. HbcAg Primer Reverse (5μM) - 9.6μL
    e. Rehydration Buffer - 118μL
    f. 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 following the chart
    7. Incubate 20 minutes at 37°C.

- CRISPR Reagents *8/ for each tube:

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA following the chart
3. 2ul NEBuffer

4. Incubate 20 minutes at 37°C.

- Add CRISPR reagents to RPA reaction mix
- Add 1ul of 0.5X FQ quencher
- Incubate 20 minutes at 37°C.

Result

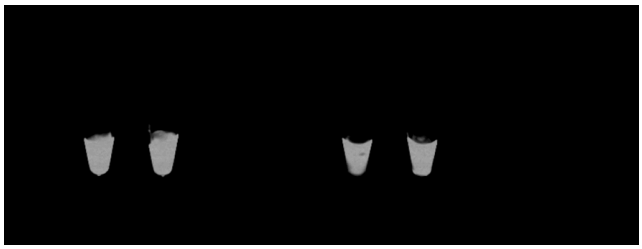
Trial1: Specificity test for RPA- CRISPR

specificity_hbcag_is481_1_20_oct_2019.png

HbcAg DNA with HbcAg gRNA - water with HbcAg gRNA - HbcAg DNA with IS481 gRNA - water with IS481 gRNA - IS481 DNA with HbcAg gRNA - water with HbcAg gRNA - IS481 DNA with IS481 gRNA - water with IS481 gRNA

Trial2: Specificity test for RPA- CRISPR

specificity_hbcag_is481_2_20_oct_2019.png



HbcAg DNA with HbcAg gRNA - water with HbcAg gRNA - HbcAg DNA with IS481 gRNA - water with IS481 gRNA - IS481 DNA with HbcAg gRNA - water with HbcAg gRNA - IS481 DNA with IS481 gRNA - water with IS481 gRNA

HbcAg serial dilution:

Table 10

	A	B	C	D	E
1	HbcAg				
2	**Serial Dilutions**	**Concentartion (ng/µl)**	**From Previous Stock**	**dH2O**	**ıing**
3		Highest Concentration (x ng/µl)	100		
4	1	60	15	10	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	Nagative Control (dH2O)	0	25	
13					

HbcAg PCR (total volume: 16ul) following Taq 2X Master Mix NEB Protocol

1. Add the following components in each pcr tube
    a. Taq 2X Master Mix: 8ul
    b. Forward Primer (10uM): 0.32ul
    c. Reverse Primer (10uM): 0.32ul
    d. Template DNA: 5ul
    e. Nuclease-free water: 2.36ul (up to 16ul)
2. Thermocycling following BioLab protocol
    a. Initial Denaturation: 95°C 30 seconds
    b. 30 cycles
        I. 95°C 20 seconds (15-30 seconds)
        II. *60°C 30 seconds (15-60 seconds)
        *Annealing temperature of the gene
        III. 68°C 30 seconds
    c. Final extension: 68°C 5 minutes
    d. Hold: 4°C

CRISPR on HbcAg PCR (post serial diluation + negative control)

- CRISPR Reagents *10

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA

3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

MONDAY, 10/21/2019

CRISPR on yesterday HbcAg PCR (post serial diluation + negative control)

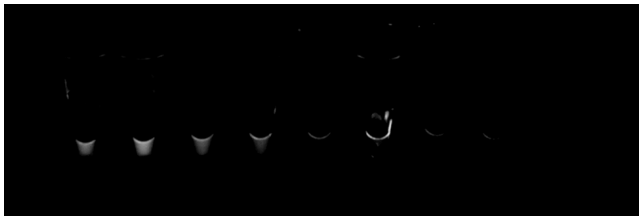
- CRISPR Reagents *10/ in each tube:

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

Result

HbcAg PCR- CRISPR - FQ

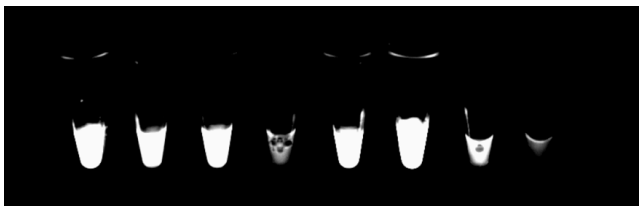
HbcAg_serial_dilutions_PCR2_CRISPR_SEPT21_2019.png



PCR-CRISPR:60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution- negative control

HbcAg RPA- CRISPR -FQ

HbcAg_serial_dilutions_RPA2_CRISPR_SEPT21_2019.png



RPA-CRISPR:60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution- negative control

Conclusion & Discussion

- CRISPR worked with both PCR and RPA at high DNA concentration
- CRISPR on RPA worked with lower concentration of DNA sample compared to PCR-CRISPR

Specificity Test on HbcAg and PcaA

Table11

	A	B	C	D	E	F	G	H	I
1	**Tube**	1	2	3	4		6	7	
2	**DNA**	HbcAg	X (water)	HbcAg	X (water)	Pca	X (water)	PcaA	X (water)
3	**Primer**	HbcAg and IS481 Forward and Reverse Primers (5µM)							
4	**gRNA**	HbcAg		PcaA		HbcAg		PcaA	

- RPA*8

1. Reaction mix in 1.5 mL tube:
    a. PcaA Primer Forward (5μM) - 9.6μL
    b. PcaA Primer Reverse (5μM) - 9.6μL
    c. HbcAg Primer Forward (5μM) - 9.6μL
    d. HbcAg Primer Reverse (5μM) - 9.6μL
    e. Rehydration Buffer - 118μL
    f. 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 following the chart
    7. Incubate 20 minutes at 37°C.

- CRISPR Reagents *8/ for each test tube

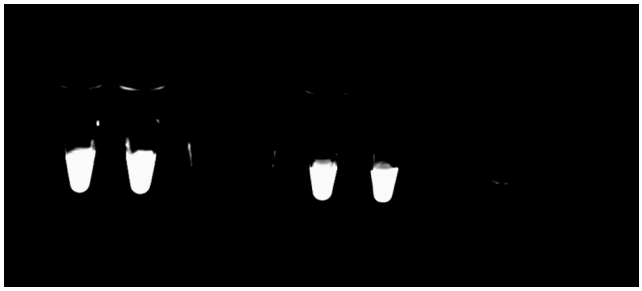
1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA following the chart
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

- Add CRISPR reagents to RPA reaction mix
- Add 1ul of 0.5X FQ quencher
- Incubate 20 minutes at 37°C.

Result

Trial3: Specificity test for RPA- CRISPR

specificity_hbcag_pcaa_20_oct_2019.png



RPA-CRISPR using:HbcAg DNA with HbcAg gRNA - water with HbcAg gRNA - HbcAg DNA with PcaA gRNA - water with PcaA gRNA - PcaA DNA with HbcAg gRNA - water with HbcAg gRNA - PcaA DNA with PcaA gRNA - water with PcaA gRNA/ All pcr tube contains IS481 and PcaA RPA primers

Conclusion & Discussion

- Contamination of used water is expected with HbcAg DNA that undergo CRISPR when HbcAg gRNA is present.

Specificity Test on IS481 and PcaA

Table12

	A	B	C	D	E	F	G	H	I
1	**Tube**	1	2	3	4	5	6	7	
2	**DNA**	PcaA	X (water)	PcaA	X (water)	IS481	X (water)	IS481	X (water)
3	**Primer**	HbcAg and IS481 Forward and Reverse Primers (5µM)							
4	**gRNA**	PcaA		IS481		PcaA		IS481	

- RPA*8

1. Reaction mix in 1.5 mL tube:
    a. IS481 Primer Forward (5μM) - 9.6μL
    b. IS481 Primer Reverse (5μM) - 9.6μL
    c. PcaA Primer Forward (5μM) - 9.6μL
    d. PcaA Primer Reverse (5μM) - 9.6μL
    e. Rehydration Buffer - 118μL
    f. 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 following the chart
7. Incubate 20 minutes at 37°C.

- CRISPR Reagents *8/ for each test tube:

1. 1.5ul 1uM Lb Cas 12a

2. 0.5 5uM gRNA following the chart
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

- Add CRISPR reagents to RPA reaction mix
- Add 1ul of 0.5X FQ quencher
- Incubate 20 minutes 37°C.

Result

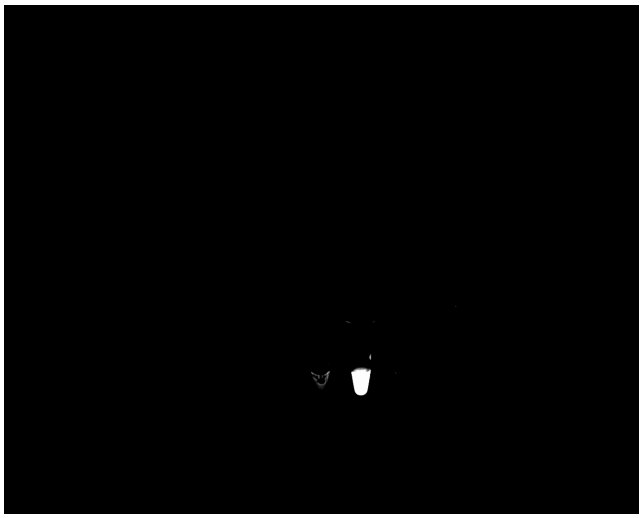
Trial 4: Specificity test for RPA-CRISPR (Test tube 1-4)

specificity_pcaa_with_is481primer_1_20_oct_2019.tif

RPA-CRISPR using: PcaA DNA with PcaA gRNA - water with PcaA gRNA - PcaA DNA with IS481 gRNA - water with IS481 gRNA/ All pcr tube contains IS481 and PcaA RPA primers

Trial 4: Specificity test for RPA- CRISPR (Test tube 5-8)

specificity_is481_with_pcaaprimer_2_20_oct_2019.tif



RPA-CRISPR using: IS481 DNA with PcaA gRNA - water with PcaA gRNA - IS481 DNA with IS481 gRNA (test tube that shows high fluorescence) - water with IS481 gRNA/ All pcr tube contains IS481 and PcaA RPA primers

Repeated Specificity Test on IS481 and PcaA

Table13

	A	B	C	D	E
1	**Tube**	1	2	3	4
2	**DNA**	IS481	X (water)	IS481	X (water)
3	**Primer**	IS481 and PcaA primers			
4	**gRNA**	IS481		PcaA	

- RPA*8

1. Reaction mix in 1.5 mL tube:
    a. IS481 Primer Forward (5μM) - 9.6μL
    b. IS481 Primer Reverse (5μM) - 9.6μL
    c. PcaA Primer Forward (5μM) - 9.6μL
    d. PcaA Primer Reverse (5μM) - 9.6μL
    e. Rehydration Buffer - 118μL
    f. 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 following the chart
    7. Incubate 20 minutes at 37°C.

- CRISPR Reagents *8

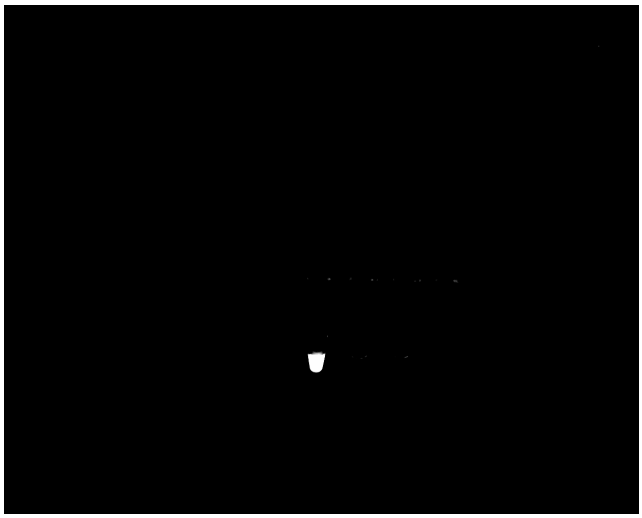
1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA following the chart
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

- Add CRISPR reagents to RPA reaction mix
- Add 1ul of 0.5X FQ quencher
- Incubate 20 minutes 37°C.

Result

Trial 5: Specificity test for RPA- CRISPR

specificity_is481_with_pcaaprimer_1_20_oct_2019.tif



RPA-CRISPR using: IS481 DNA with IS481 gRNA - water with IS481 gRNA - IS481 DNA with HbcAg gRNA - water with HbcAg gRNA/ All pcr tube contains IS481 and PcaA RPA primers

Conclusion & Discussion

- When the reaction run with multiple primer sets, fluorescence from FQ reporter was observed only when gRNA matched with DNA.
- When there is no DNA, but only primers and uykiloo
- This was reconfirmed when the experiment was replicated.
- In the device, the channel with gRNA, that matches with the sample's disease, will give the fluorescence.

Ypo2088 PCR (total volume: 16ul) following Taq 2X Master Mix NEB Protocol

1. Add the following components in each pcr tube
    a. Taq 2X Master Mix: 8ul
    b. Forward Primer (10uM): 0.32ul
    c. Reverse Primer (10uM): 0.32ul
    d. Template DNA: 5ul
    e. Nuclease-free water: 2.36ul (up to 16ul)
2. Thermocycling following BioLab protocol
    a. Initial Denaturation: 95°C 30 seconds
    b. 30 cycles
        I. 95°C 20 seconds (15-30 seconds)
        II. *60°C 30 seconds (15-60 seconds)
        *Annealing temperature of the gene
        III. 68°C 30 seconds
    c. Final extension: 68°C 5 minutes
    d. Hold: 4°C

Ypo2088 RPA (total volume: 16ul)

- RPA*8

1. Reaction mix in 1.5 mL tube:
    a. Primer Forward (5μM) - 9.6μL

b. Primer Reverse (5μM) - 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 fre[illegible]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 following the chart
7. Incubate 20 minutes at 37°C.

CRISPR on Ypo2088 RPA (post serial diluation and postive, negative control), Ypo2088 PCR (serial dilution)

- CRISPR Reagents *10/ for each tube:

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

- Add 1ul of 0.5X FQ quencher to the CRIPSR mix
- Add CRISPR reagents mix with quencher to RPA reaction mix
- Incubate 20 minutes at 37°C.
- Check under the blue light based E-gel imager.

CRISPR on ypo2088 PCR (post serial diluation + negative control)

- CRISPR Reagents *10/ for each test tube:

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

Result

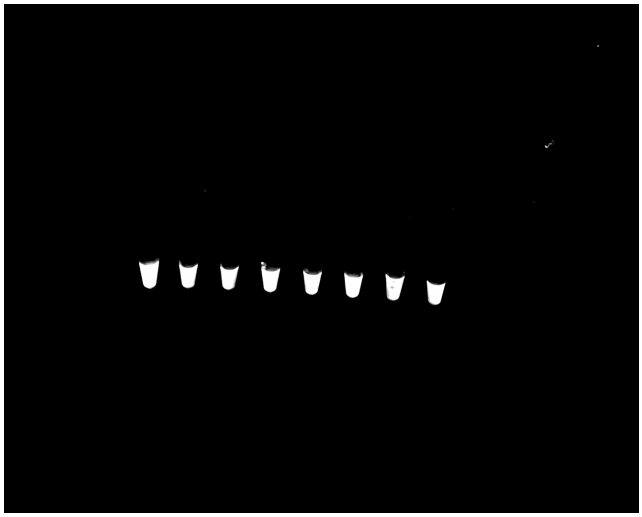
Ypo2088 PCR- CRISPR -FQ

ypo2088_serial_dilutions_PCR2_1_CRISPR_SEPT21_2019.tif

RPA-CRISPR:60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution- negative control

Ypo2088 RPA- CRISPR -FQ

ypo2088 new_rpa_crispr_newgrna serial dilutions_21_oct_2019.tif



RPA-CRISPR:60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution- negative control

Conclusion & Discussion

- CRISPR worked with both PCR and RPA at high DNA concentration
- CRISPR on RPA worked with lower concentration of DNA sample compared to PCR-CRISPR

10µl final volume RPA for ypo2088 (primer set 1)

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 freez ction. 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°C for 40 min using thermocycler

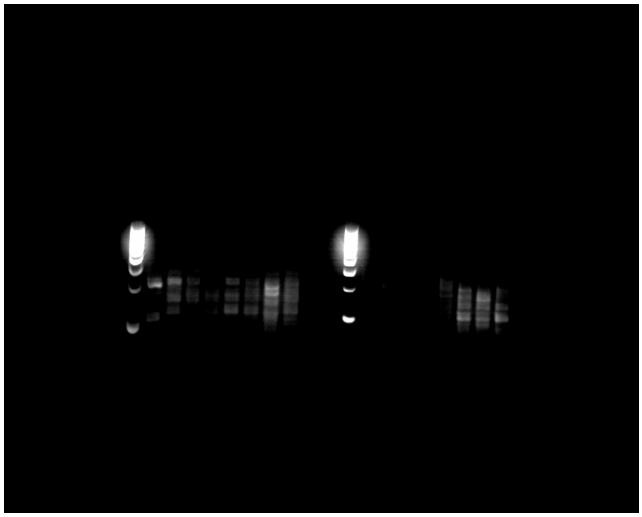
Running Agarose Gel with SYBR Green x 10,000

1. Add 5 μL of the 10,000× SYBR Green solution in DMSO to 50 mL of 1× TE, TBE, or TAE buffer (for mid-sized gels). Mix thoroughly with a spatula, rod, or magnetic stirrer.
2. Add 1.5g of agarose.
3. Pour the diluted dsGreen solution into an appropriate tray or pan and submerge the gel.
4. Soak the gel for 5–10 min.
5. View or document the gel using available light source and an green/yellow filter. Transilluminators with blue light, or with UV low pressure mercury lamp (254 nm) can be used to visualize gels stained with dsGreen. High pressure mercury lamp (365 nm) can be used too, but this light source gives somewhat less efficient excitation.

Result

ypo2088 post serial dilution RPA

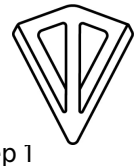
YPO2088_serial_dilutions_RPA_2_primerSet1_OCT21.tif



Left (Replicate 1): Ladder-60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution- negative control
Right (Replicate 2): 2nd primer set: Ladder-60 ng/ul dilution- 25ng/ul dilution- 10 ng/ul dilution- 5 ng/ul dilution- 1 ng/ul dilution- 0.1 ng/ul dilution- 0.01 ng/ul dilution- 0.01 ng/ul dilution- negative control

RPA+ CRISPR and Quencher Verifcation on broth for ypo2088 and Cra:

- Prepared 5 falcon tubes for each gene for the preparation of 5 dilutions of each
- Prepared 10^6, 10^4, 10^2, 10, and 1 cells/mL dilutions of ypo2088 LB+Amp Broth.
- Prepared RPA reaction mix in 1.5 mL tube twice, once for Cra and once for ypo2088:
    a. Primer Forward (5μM) - 9.6μL
    b. Primer Reverse (5μM) - 9.6μLR
    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, only 6 tubes were used for each gene.
5. Added 1μL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA following the chart
7. Incubate 20 minutes at 37°C.

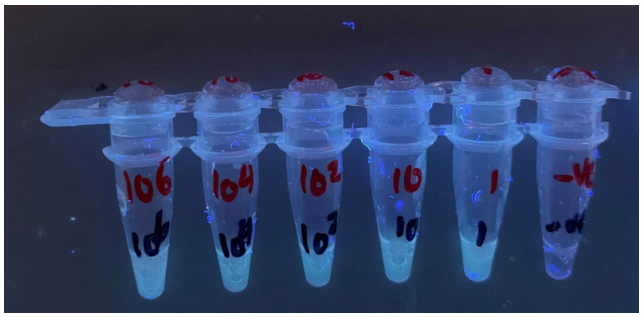
- CRISPR Reagents for each tube (done 12 times, 6 times for each tube of each gene after the RPA incubation):

1. 1.5ul 1uM Lb Cas 12a
2. 0.5 5uM gRNA
3. 2ul NEBuffer
4. Incubate 20 minutes at 37°C.

- Add 1ul of 0.5X FQ quencher to the CRIPSR mix
- Add CRISPR reagents mix with quencher to RPA reaction mix
- Incubate 20 minutes at 37°C.
- Check under the blue light based E-gel imager and UV light:

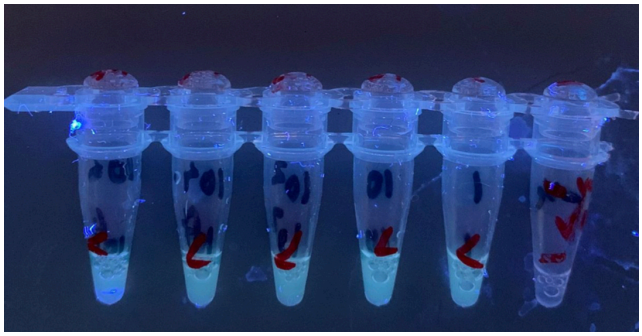
Results:

Broth RPA+CRISPR on ypo2088 gene (ligated into pJET vector that is transformed into E. coli)



1. ypo2088 1,000,000 cells/mL, 2. ypo2088 10,000 cells/mL, 3. ypo2088 100, 4. ypo2088 10, 5. ypo2088 1, 6. Negative control

Broth RPA+CRISPR on Cra gene



1. cra 1,000,000 c . cra 10,000 cells/mL, 3. cra 100, 4. cra 10, 5. cra 1, e control

Conclusion:

- The RPA-Crispr Cas 12a detection in the presence of of an fq quencher sequence works in broth with veru high sensitivity up to 1 bacterial cell/ mL.
- The detection protocl with RPA and CRISPR is quite robust even with a low plasmid copy number with the endogenous cra gene found in E. coli bacteria.