Lab Book1 (iGEM2020)

Project: iGEM 2020
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Created at: 2020-09-15T17:07:55.323590+00:00

TUESDAY, 9/29/2020

WEDNESDAY, 9/30/2020

LB+AMP Agar Plate Preparation (Sigma-Aldrich protocol for L2987)

1. Suspend 17.5 g in 500mL of distilled water.
2. Heat to boiling while stirring to dissolve all ingredients completel///y.
3. Autoclave for 15 minutes at 121°C.
4. Add 500uL of amphicilin (100mg/ml)

Agar Broth Preparation (Sigma-Aldrich protocol for L3522)

1. Suspend 25 g in 1 L of distilled water.
2. Autoclave for 15 minutes at 121 °C.

To prepare the medium of Luria, Adams and Ting (also known as LC broth: Aseptically add 25 ml of sterile 0.1 M calcium chloride after autoclaving.

Transformation of E.coli via heat shock (SARS-CoV-2 positive control plasmid)

1. Aliquoted 25µL of electrocompetent E.coli into an eppendorf tube
2. Added 10uL SARS-CoV-2 positive control plasmid into an eppendorf tube
3. Iced for 20 minutes,
4. 60 second heat shock in eppendorf heat block at 42°C
5. Returned tubes to ice for 2 minutes
6. Added 800µL of SOC
7. Incubated on shaker at 220 rpm and 37°C for one hour
8. Centrifuged for 1 minute at 13000rpm and discarded 700µL of supernatant
9. Carefully resuspended the pellet and plated on LB+AMP agar
10. Spreaded gently using plastic spreader
11. Incubated overnight (16 hours) at 37 °C

SUNDAY, 10/4/2020

bd primer sets 1 & 2, bsal primer sets 3 & 4

Preparation of Bd gblock (IDT DNA protocol for Bd gBlock 2)

1. Prior to opening, centrifuged the tube at a minimum of 13.3 rpm for 1 minute to ensure that the material was at the bottom of the tube.
2. Added 50µl of TE buffer to the 500ng sample of bd gBlock
3. Vortexed the sample for 5-10 seconds
4. Incubated in the eppendorf heat block at 50 °C for 20 minutes
5. Vortexed the sample for 5-10 seconds
6. Centrifuged the sample for 1 minute

Preparation of Bsal gblock (IDT DNA protocol for Bsal gBlock 2)

1. Prior to opening, centrifuged the tube at a minimum of 3000 x g for 1 minute to ensure that the material was at the bottom of the tube.
2. Added 100µl of TE buffer to the 1000ng sample of bsal gBlock to make 10ng/µl

3. Vortexed the sample
4. Incubated in the eppendorf heat block at 50 °C for 20 minutes
5. Vortexed the sample briefly
6. Centrifuged the sample

Preparation of Bd PCR primers 1 and 2 (fwd and rev)

1. Prior to opening, centrifuged the tube at 13.3 rpm for 1 minute to ensure that the material was at the bottom of the tube.
2. Added X TE buffer to reach a concentration of 100µM for each primer according to manufacturer specification
3. After mixing, put the tubes on ice

Preparation of bsal gblock (IDT DNA protocol for bsal gblock)

1. Prior to opening, centrifuged the tube at a minimum of 3000 x g for 1 minute to ensure that the material was at the bottom of the tube.
2. Added 100µl of TE buffer to the 1000ng sample of bsal gBlock
3. Vortexed the sample
4. Incubated in the eppendorf heat block at 50 °C for 20 minutes
5. Vortexed the sample
6. Centrifuged the sample for 1 minute

Primer dilution - working stock

1. Use C1V1=C2V2 to create a working solution of 10µM, 50µL primer from the stock solution (5µM stock, 45µM TE)
2. Bd 1 FWD, Bd 1 REV, Bd 2 FWD, Bd 2 REV, Bsal 3 FWD, Bsal 3 REV, Bsal 4 FWD, Bsal 4 REV

PCR Protocol

Volume of template DNA used = 5µl
Annealing temperature set for Primer set 4 = 52 °C
Annealing temperature set for Primer set 1, 2 and 3 = 53 °C

1. Four PCR tubes (Bd1, Bd2, Bsal3, Bsal4) were prepared in ice with the following reaction mixture:
    a. 1.25 µL 10 µM Fwd Primer
    b. 1.25 µL 10 µM Rev Primer
    c. 5 µL 10 µM DNA (Bd or Bsal)
    d. 12.5 µL Q5 High-Fidelity 2X Master Mix
    e. 5 µL Nuclease-Free water
2. The PCR tubes was gently mixed and provided a quick spin.
3. The PCR tubes were transfered to a preheated thermocycler (98 °C)
4. The following thermocycling conditions were set:
    a. Initial denaturation: 98 °C for 30 seconds
    b. 35 Cycles:
        I. 98 °C for 10 seconds
        II. 52 °C for Primer set 4 and 53 °C for Primer sets, 1, 2, and 3 for 30 seconds
        III. 72 °C for 10 seconds
    c. Final Extension: 72 °C for 2 minutes
    d. Hold 4 °C
5. The PCR tubes were taken when allowed to do so.

MONDAY, 10/5/2020

PCR products:
Bd primers labeled 1 and 2
Bsal primers labeled 3 and 4

Ligation (Thermofisher Blunt-end Cloning Protocol)

1. The ligation reaction was prepared on ice
2. Added 10µl of 2X reaction buffer to the PCR tube
3. Added 1 µl of non-purified PCR product (Bd primer for tubes 1 and 2, Bsal primers for tubes 3 and 4)
4. Added 1µl of pJET1.2/blunt cloning vector
5. Added 7µl of nuclease free water
6. Added 1 µl of T4 DNA ligase
7. Vortexed for 5 seconds
8. Centrifuged for 5 seconds
9. Ligation mixture was incubated in the PCR machine for 5 minutes at 22˚C.
10. Used mixture directly after for transformation.

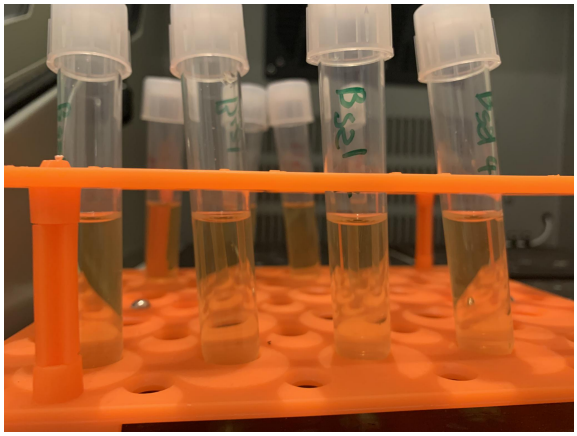
Transformation

1. 4 tubes of NEB 5-alpha Competent *E.coli* cells were thawed on ice for 10 minutes.
2. 4x5 µL of ligated DNA was added to a tube of *E.coli* cells. The tubes were carefully flicked 4-5 times to mix the cells and DNA.
3. The mixtures was placed on ice for 30 minutes
4. The mixtures were heat shocked at 42 °C at exactly 30 seconds.
5. The mixtures were placed on ice for 5 minutes.
6. 950 µL of SOC was pipetted into each mixture
7. The mixtures were placed at 37 °C for 60 minutes. They were allowed to be shaken at 250 rpm.
8. The cells were centrifuged and concentrated
9. Each sample was spread across separate agar plates and left incubated overnight for 37˚C.

TUESDAY, 10/6/2020

1. Labeled 2 tubes Bd 1, 2x Bd 2, 2x Bsal 3, 2x Bsal 4 (transformed product)
2. Pipetted 6ml of LB broth into each tube
3. Took respective bacteria colonies from agar plates and mixed with the broth
4. Left inoculated for 18-24 hours at 37˚C.

Transformed Product with Bacteria Colonies before Inculation



WEDNESDAY, 10/7/2020

Miniprep

1. Miniprep of the 8 innoculations was performed (2x[Bd1, Bd2, Bsal3, Bsal4])
2. Resulted in 50 µL of each (8 total tubes)
   Notes:
   - did not add optional LyseBlue reagant
   - step (6) (7), and (8) were performed with centrifuge
   - step (11) was not performed at this time

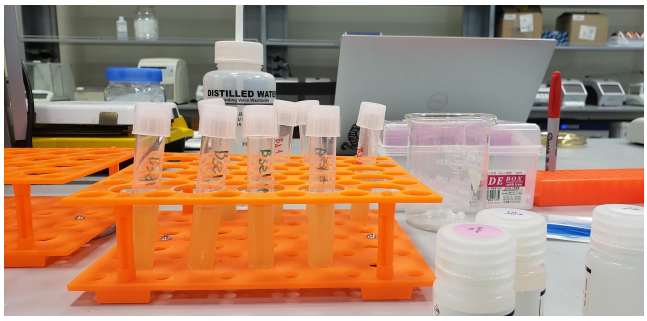
Miniprep:

Following QIAprep Spin Miniprep Kit protocol

All centrifugation steps are carried out at 13000rpm in a table-top microcentrifuge

1. Centrifuged bacterial overnight culture at 7380 rpm for 3 minutes at room temp.
2. Liquid was removed so that only pellet was left in the tube.
3. Pellet was resuspended in 250 µl Buffer P2 and was transferred into a microcentrifuge tube
4. 250 µl of Buffer P2 was added and the tube was inverted 6 times in order to mix the solution.
5. Added 350µl of Buffer N3 and mixed immediately by inverting the tube 6 times.
6. Centrifuged for 10 minutes at 13000 rpm.
7. Applied 800µl of supernatant to the QIAprep 2.0 spin column, centrifuged for 30 seconds and discarded the flow-through.
8. Washed the QIAprep 2.0 spn column by adding 0.5 ml of Buffer PB, centrifuged for 30 seconds and discarded the flow-through.
9. Washed the QIAprep 2.0 spin column by adding 0.75 ml of Buffer PE, centrifuged for 20 seconds, and discarded the flow through.
10. Transferred the QIAprep spin column to the collection tube
11. Centrifuged for 1 minute to remove residual wash buffer
12. Placed the column in a clean 1.5 ml microcentrifuge tube.
13. Eluted DNA with 50 µl of Buffer EB to the center of the spin column and let it stand for 1 minute then centrifuged for 1 minute.

07.10.20 miniprep innoculation tubes.jpg



07.10.20 miniprep solutions.jpg

Nanodrop

1. Nanodrop performed with 2 µL droplets and concentrations obtained
    a. the machine was blanked using the elution buffer from the last miniprep step
    b. results were all desirable

PCR Protocol (Taq 2X Master Mix)

Volume of template DNA used = 5µl
Annealing temperature set for Primer set 4 = 52 °C
Annealing temperature set for Primer set 1, 2 and 3 = 53 °C

1. Four PCR tubes (Bd1, Bd2, Bsal3, Bsal4) were prepared in ice with the following reaction mixture:
    a. 1.25 µL 10 µM Fwd Primer
    b. 1.25 µL 10 µM Rev Primer
    c. 5 µL 10 µM DNA (Bd or Bsal)
    d. 12.5 µL Q5 High-Fidelity 2X Master Mix
    e. 5 µL Nuclease-Free water
2. The PCR tubes was gently mixed and provided a quick spin.
3. The PCR tubes were transfered to a preheated thermocycler (98 °C)
4. The following thermocycling conditions were set:
    a. Initial denaturation: 98 °C for 30 seconds
    b. 35 Cycles:
        I. 98 °C for 10 seconds
        II. 52 °C for Primer set 4 and 53 °C for Primer sets, 1, 2, and 3 for 30 seconds
        III. 72 °C for 10 seconds
    c. Final Extension: 72 °C for 2 minutes
    d. Hold 4 °C
5. The PCR tubes were taken when allowed to do so.

THURSDAY, 10/8/2020

Quality control

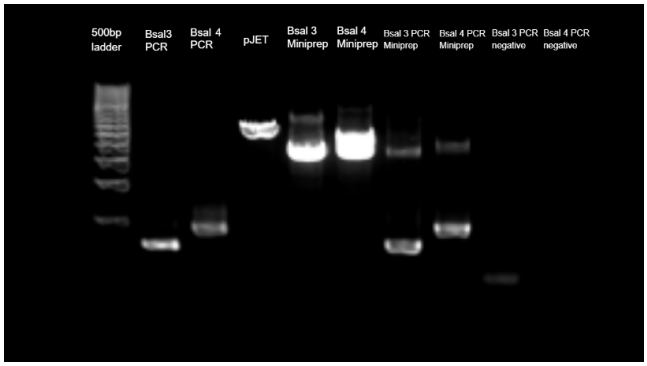
Making gels

1. Mixed 40 ml of 50X TAE buffer with 1960ml of MilliQ water to make 2L of 1X TAE buffer
2. Measured out 2x 0.5g of Agarose
3. Mesaured out 2x 50ml of 1X TAE buffer and poured into two conical flasks
4. Mixed 0.5g of Agarose into each of the conical flasks with 1X TAE and gently swirled
5. Microwaved mixtures in increments until boiling to dissolve the Agarose
6. Poured solutions into two separate casting trays and placed the well combs into them, allowed them to set.

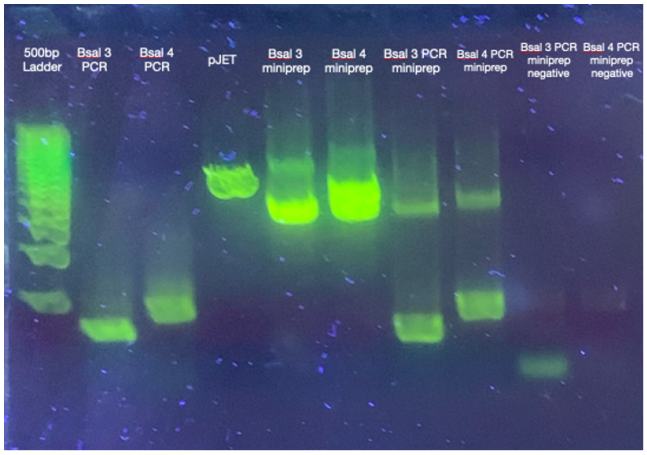
Loading Gels

1. Collected all the samples necessary (listed in table below) and placed them on ice
2. Drop 1 µl of nucleic acid sample loading buffer for each sample labeled on parafilm (except on ladder)
3. Drop 5 µl of each sample and mixed with loading buffer on parafilm.
4. Placed set gel into electrophoresis chamber.
5. Put samples into the wells of the chamber in order shown in table (Bd Gel/Bsal Gel)
6. Poured 1X TAE buffer carefully into the chamber to cover the gel.
7. Connected the electrophoresis chamber to MyRun electrophoresis.
8. Ran gel on 135 V for 20 minutes.

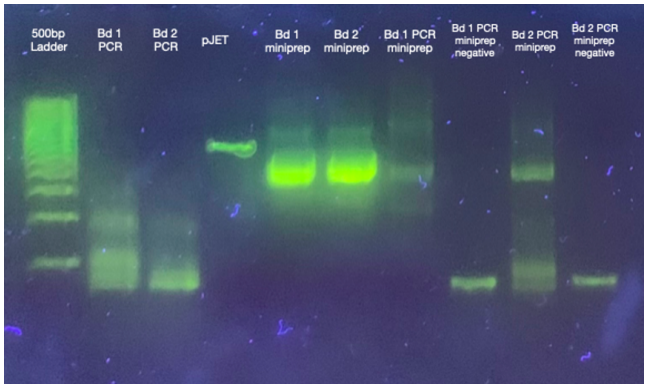
Bsal Gel 8_10_2020.png



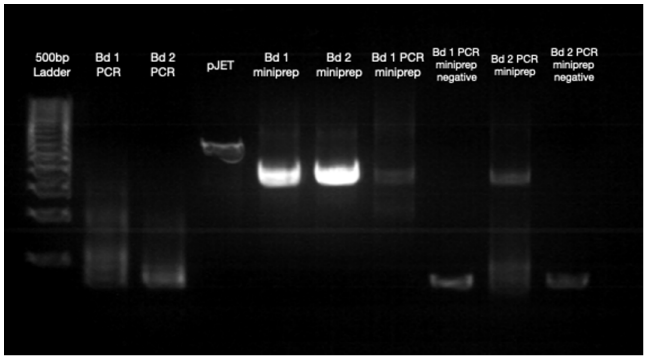
UVBsal Gel 8_10_2020.png



UVBd Gel 8_10_2020.png



Bd Gel 8_10_2020.png



Results: Bsal Successful. Bsal 4 will be used for the rest of the downstream experiments. Bd requires another PCR run on the minipreps.

Bd Primer Set 1 Amplicon length: 364 base pairs
Bd Primer Set 2 Amplicon length: 373 base pairs
Bsal Primer Set 3 Amplicon length: 476 base pairs
Bsal Primer Set 4 Amplicon length: 486 base pairs

SUNDAY, 10/11/2020

Bd PCR and Innoculate

Bd PCR:

4 PCR tubes were prepared: Bd1 and its negative control, and Bd2 and its negative control.

1. The thermocycler was preheated to 95 ˚C.
2. The reagents were allowed to thaw in ice
3. 4 PCR tubes were prepared with the following mixture compositions as shown in the table below. The order of reagents added to each PCR tube is in order as you go down each row in the table

PCR Tubes

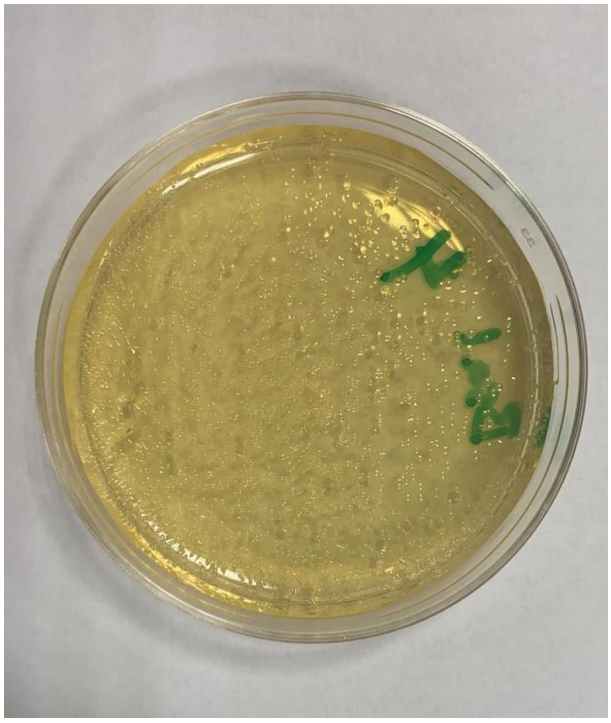
	A	B	C	D	E
1		Bd Primer Set 1	Negative (Primer set 1)	Bd Primer Set 2	Negative (Primer set 2)
2	Taq 2x MasterMix	12.5 µl	12.5 µl	12.5 µl	12.5 µl
3	Forward Primer	0.5 µl	0.5 µl	0.5 µl	0.5 µl
4	Reverse Primer	0.5 µl	0.5 µl	0.5 µl	0.5 µl
5	DNA	3 µl	none	3 µl	none
6	DNase-Free Water	8.5 µl	11.5 µl	8.5 µl	11.5µl

4. The tubes were spun for a few seconds
5. The tubes were transferred to the thermocycler and the following temperatures and settings were set:
    a. Initial Denaturation: 95 ^{o}C (30 seconds)
    b. 30 Cycles
        I. 95 ^{o}C (30 seconds)
        II. 53 ^{o}C (60 seconds)
        III. 68 ^{o}C (30 seconds)
    c. Final Extension: 68 ^{o}C (5 minutes)
    d. Hold: 4 ^{o}C
6. The tubes were taken the next day

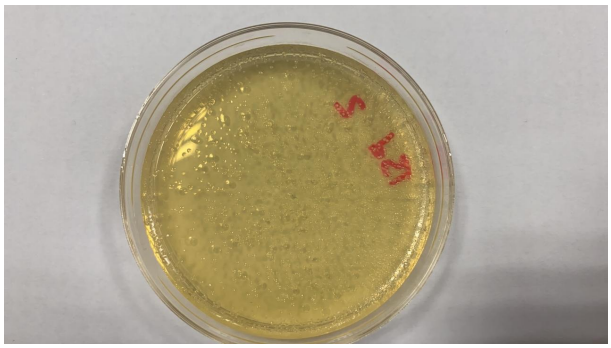
Innoculate:

1. The agar plate lableled as Bsal 4 was taken for innoculation.
2. Four innoculation tubes were prepared, all labeled "4".
3. 6 mL of LB Broth was pipetted into each tube.
4. A innoculation loop was used to take a few colonies from the agar plate and transferred to the tubes containing the LB Broth
5. The tubes were incubated at 37 ^{o}C at 225 RPM and was left overnight.

Bsal4 Agar Plate.jpg



Bd2 Agar Plate.jpg



Transformation was successful. *E.coli* Colonies have clearly grown.

MONDAY, 10/12/2020

Miniprep for Bsal 4 (QIAprep Spin Miniprep Kit Protocol)

1. Centrifuged bacterial overnight culture at 7380 rpm for 3 minutes at room temp.
2. Liquid was removed so that only pellet was left in the tube.
3. Pellet was resuspended in 250 µl Buffer P2 and was transfered into a microcentrifuge tube
4. 250 µl of Buffer P2 was added and the tube was inverted 6 times in order to mix the solution.
5. Added 350µl of Buffer N3 and mixed immediately by inverting the tube 6 times.
6. Centrifuged for 10 minuteds at 13000 rpm.
7. Applied 800µl of supernatant to the QIAprep 2.0 spin column, centrifuged for 30 seconds and discarded the flow-through.
8. Washed the QIAprep 2.0 spn column by adding 0.5 ml of Buffer PB, centrifuged for 30 seconds and discarded the flow-through.
9. Washed the QIAprep 2.0 spin column by adding 0.75 ml of Buffer PE, centrifuged for 20 seconds, and discarded the flow through.

10. Transfered the QIAprep spin column to the collection tube
11. Centrifuged for 1 minute to remove residual wash buffer
12. Placed the column in a clean 1.5 ml microcentrifuge tube.
13. Eluted DNA with 50 µl of Buffer EB to the center of the spin column and let it stand for 1 minute then centrifuged for 1 minute.

Nanodrop for Bsal 4

1. With the sampling arm open, 2µl of sample (purified plasmid) pipetted onto lower measurment pedestal
2. The sampling arm closed and a spectral measurment intitiated using the operative system ("Nanodrop2000")
3. When the measurment completed, the sampling arm opened and the sample wiped from both the upper and lower pedestal using labaratory wipe
4. Based on the absorbition spectrum concentration and puritity of nucleic acids deduced

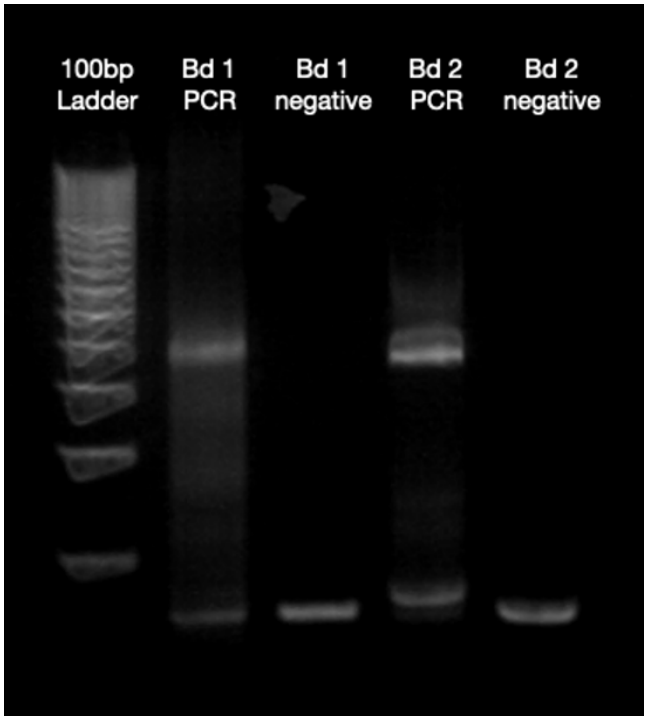
Gel Preparation

1. Measured 0.5g of agarose and 50mL of TAE Buffer
2. Dissolved the mixture by heating in a microwave
3. Added 3 µL of GelGreen and swirled gently
4. Poured in agarose mixture into the casting tray and applied the comb and was allowed to sit until solidified (~30 minutes)

Electrophoresis

1. Took the four PCR samples done on Bd (Sunday 11/10/2020)
2. The gel was set in place onto the chamber
3. Pipetted 6 µL of the DNA ladder onto the first well
4. Pipetted 1 µL of nucleic acid sample loading dye on parafilm for each PCR sample
5. Pipetted 5 µL of each PCR sample onto each 1 µL dyes on the same parafilm
6. The droplet was homogenized with a pipette and transferred onto each well.
7. Ran gel on 135 V for 20 minutes.
8. Ran gel on 135 V for another 5 minutes.
9. Gel was observed under UV.

Bd PCR Gel 12_10_2020.png



Results: Inconclusive. Need to re-run the test.

TUESDAY, 10/13/2020

RPA

1. Bsal RPA FWD1 and REV1 were resuspended to 100μM
2. A 5 μM dilution was made for each primer for RPA reactions (19μL nuclease free water, 1 μL primer)

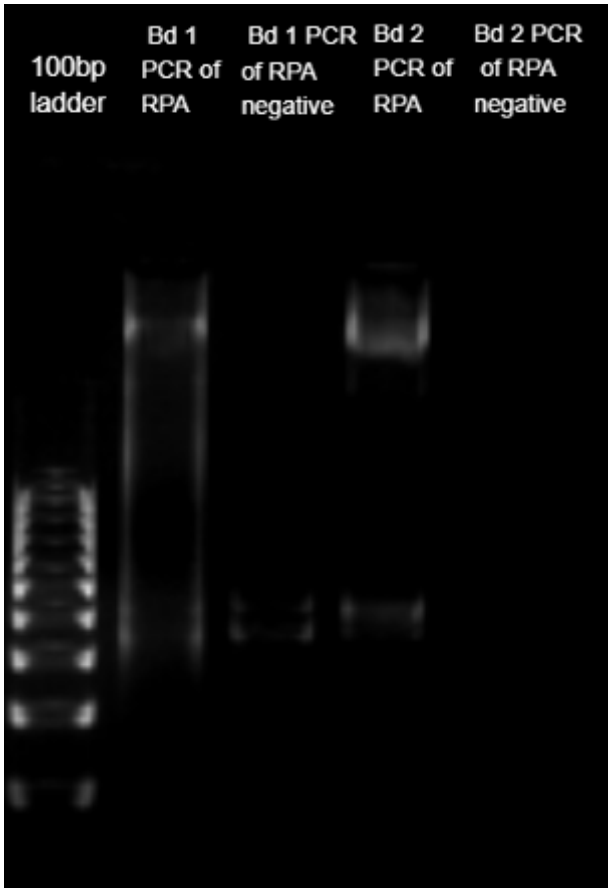
3. Reaction mix in 1.5 mL tube:
    a. Primer Forward (5μM) - 2.4μL
    b. Primer Reverse (5μM) - 2.4μL
    c. Rehydration Buffer - 29.5μL
    d. dH2O - 8.2μL
2. Pipetted up and down after addition of each component in step 1
3. Add the reaction mix (42.5uL) to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into2 volumes - 15μL to 2 separate PCR tubes.
5. Added 1μL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA (Bsal4 243, first batch made) to one tube and 5ul of nuclease-free water to one tube
7. Incubate 20 minutes at 37°C.

Electrophoresis

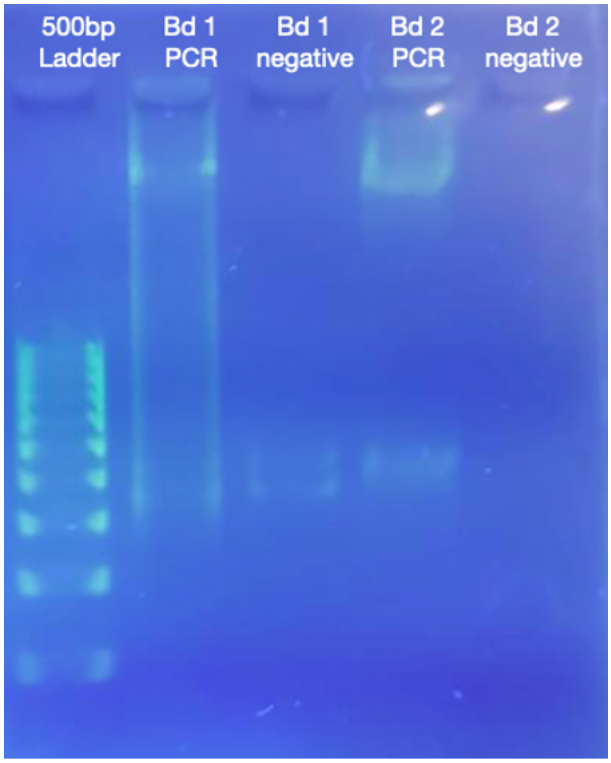
1. Took the four PCR samples done on Bd (Tuesday 13/10/2020) and two RPA samples done on Bsal 4 (Tuesday 13/10/2020)
2. The gel was set in place onto the chamber
3. Pipetted 6 μL of the DNA ladder onto the first well
4. Pipetted 1 μL of nucleic acid sample loading dye on parafilm for each PCR sample
5. Pipetted 5 μL of each PCR sample onto each 1 μL dyes on the same parafilm
6. The droplet was homogenized with a pipette and transferred onto each well.
7. Ran gel on 135 V for 20 minutes.

8. Ran gel on 135 V for another 5 minutes.
9. Gel was observed under UV.

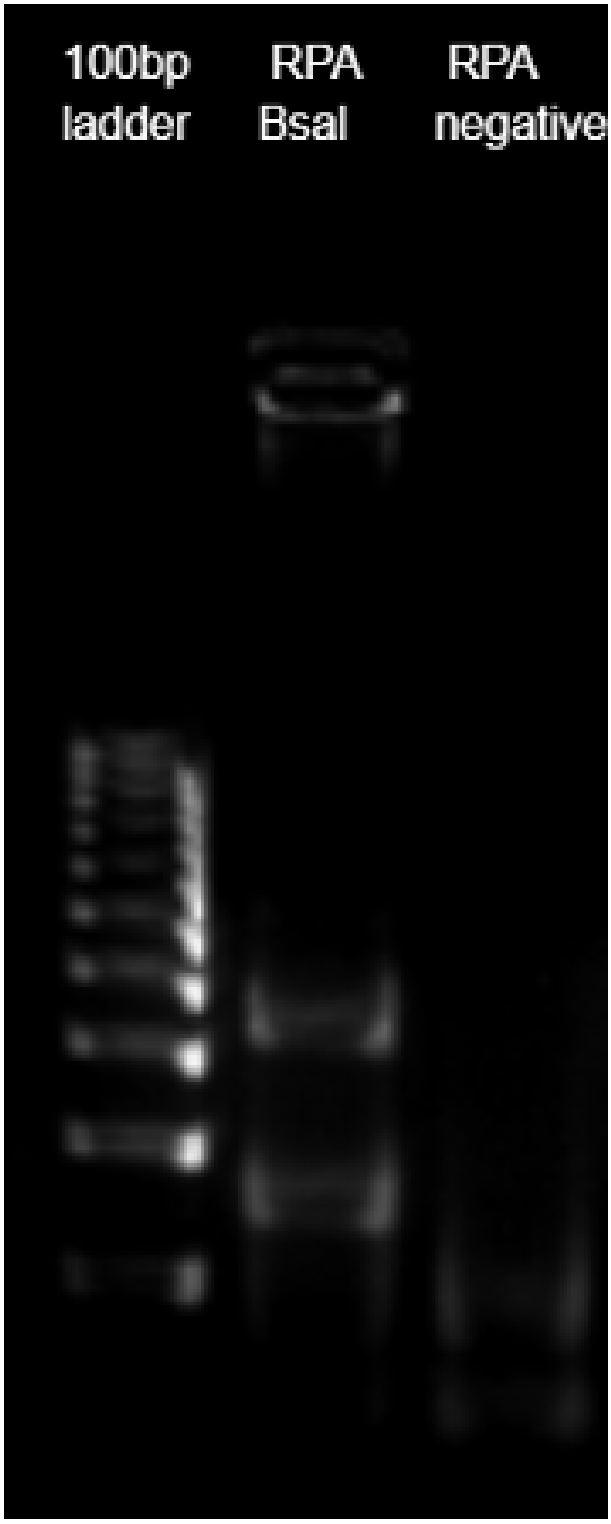
Bd PCR Gel 13_10_2020.png



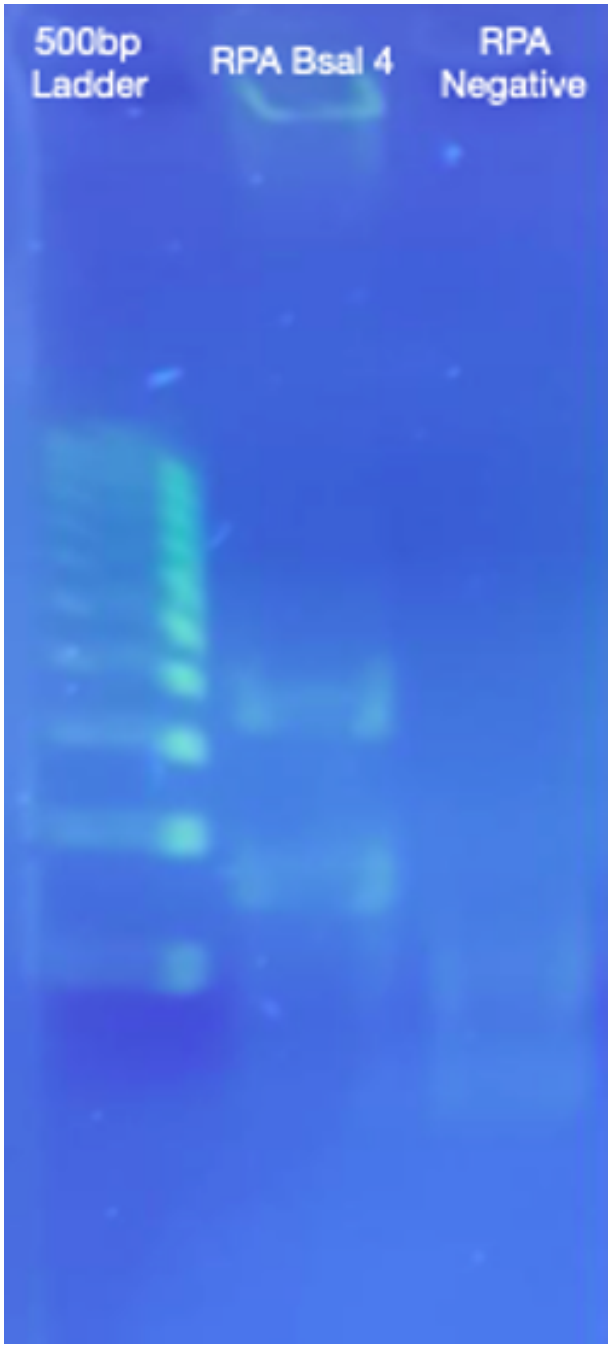
UVBd PCR Gel 13_10_2020.png



Bsal RPA 13_10_2020.png



UVBsal RPA 13_10_2020.png



Result: RPA worked on the Bsal gene with Bsal RPA primers. Bd PCR finally worked. We can proceed on working with Bd amplified by primer set 2

WEDNESDAY, 10/14/2020

1. Bd RPA FWD2 and REV2 were resuspended to 100µM (centrifuged first)
2. A 5 µM dilution was made for each primer for RPA reactions (48 µL nuclease free water, 2 µL primer)

8 RPA reactions - 3 with Bsal DNA + Bsal primers, 3 with Bsal DNA + Bd primers, 1 with Bsal primers + no DNA, 1 with Bd primers + no DNA

1. Reaction mix in 1.5 mL tube:
    a. Primer Forward (5µM) - 2.4µL
    b. Primer Reverse (5µM) - 2.4µL

c. Rehydration Buffer - 29.5µL
d. dH2O - 8.2µL

2. Pipetted up and down after addition of each component in step 1
3. Add the reaction mix (42.5uL) to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into2 volumes - 15µL to 2 separate PCR tubes.
5. Added 1µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA (Bsal4 243, first batch made) to one tube and 5ul of nuclease-free water to one tube
7. Incubate 20 minutes at 37°C.

** we should do a positive control also. I didn't have time today. Instructions: twistdx.co.uk/en/products/product/twistamp-basic

Innoculate:

1. The agar plate lableled as Bd 2 was taken for innoculation.
2. Four innoculation tubes were prepared, all labeled "Bd 2".
3. 6 mL of LB Broth/AMP was pipetted into each tube.
4. A innoculation loop was used to take a few colonies from the agar plate and transferred to the tubes containing the LB Broth
5. The tubes were incubated at 37 ^{o}C at 225 RPM and was left overnight.

THURSDAY, 10/15/2020

Miniprep for Bd 2 (QIAprep Spin Miniprep Kit Protocol)

1. Centrifuged 4 Bd 2 bacterial cultures overnight at 7380 rpm for 3 minutes at room temp.
2. Supernatant was removed so that only pellet was left in the tube.
3. Pellet was resuspended in 250 µl Buffer P1 and was transfered into a microcentrifuge tube
4. 250 µl of Buffer P2 was added and the tube was inverted 6 times in order to mix the solution.
5. Added 350µl of Buffer N3 and mixed immediately by inverting the tube 6 times.
6. Centrifuged for 10 minuteds at 13000 rpm.
7. Applied 800µl of supernatant to the QIAprep 2.0 spin column, centrifuged for 30 seconds and discarded the flow-through.
8. Washed the QIAprep 2.0 spn column by adding 0.5 ml of Buffer PB, centrifuged for 30 seconds and discarded the flow-through.
9. Washed the QIAprep 2.0 spin column by adding 0.75 ml of Buffer PE, centrifuged for 20 seconds, and discarded the flow through.
10. Transfered the QIAprep spin column to the collection tube
11. Centrifuged for 1 minute to remove residual wash buffer
12. Placed the column in a clean 1.5 ml microcentrifuge tube.
13. Eluted DNA with 50 µl of Buffer EB to the center of the spin column and let it stand for 1 minute then centrifuged for 1 minute.

Nanodrop

1. Nanodrop performed with 2 µL droplets and concentrations obtained
    a. the machine was blanked using the elution buffer from the last miniprep step
    b. results were all desirable

MONDAY, 10/19/2020

Used RPA primers prepared from Wednesday (14/10/2020)

RPA Specificity Test

9 RPA Reactions in total:

- 3 Reactions with Bd Primers and Bd DNA
- 1 Negative Control with Bd Primers and no Bd DNA
- 3 Reactions with Bsal Primers and Bd DNA
- 1 Negative Control with Bsal Primers and no Bd DNA

- 1 Positive control with TwistAMP primers and TwistAMP DNA

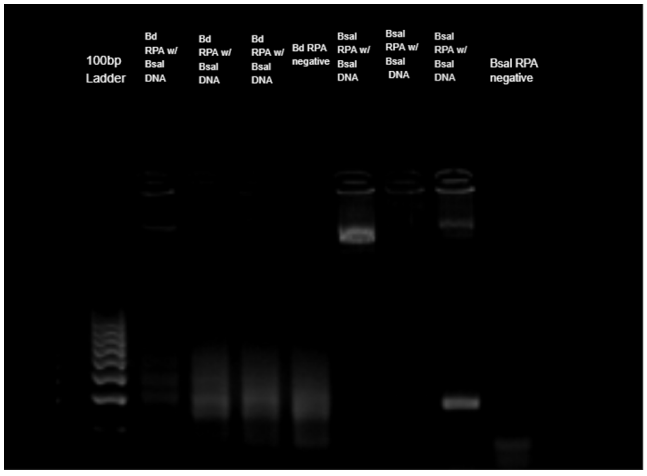
First 8 RPA Reactions:

1. For each reaction, the following reaction mix was prepared in PCR tubes:
    a. Primer Forward (5μM) - 2.4μL
    b. Primer Reverse (5μM) - 2.4μL
    c. Rehydration Buffer - 29.5μL
    d. dH2O - 8.2μL
2. Pipetted up and down after the addition of each component in step 1
3. Each reaction mix prepared in step 1 was added to powdered TwistAMP freeze-dired reaction. Pipetted up and down to mix.
4. Splitted the reaction into2 volumes - 15μL to 2 separate PCR tubes.
5. Added 1μL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA (Bd2 243, first batch made) to one tube and 5ul of nuclease-free water to one tube
7. Incubate 20 minutes at 37°C.

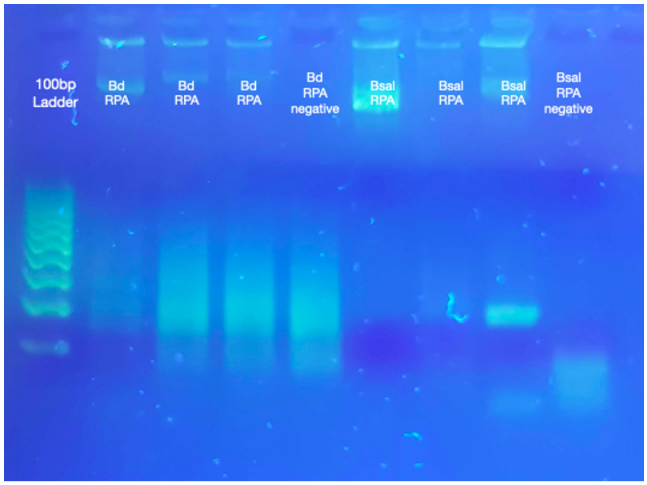
Positive Control RPA Reaction:
twistdx.co.uk/en/products/product/twistamp-basic

1. Prepare reaction mix in 1.5ml tube:
    - Positive control primer mix 8 µl
        - Primer Free Rehydration buffer 29.5 µl
        - Positive control DNA template 1 µl
        - Water 9 µl
        - (Total volume 47.5 µl)
2. Add reaction mix to a TwistAmp® Basic reaction. Pipette to mix
3. Took 15 µL from the reaction mix to a new separate PCR tube
4. Added 1µL of 280 mM magnesium acetate
5. Joined this PCR tube with the 8 PCR tube reactions prepared above.
6. Incubated for 20 minutes at 37 °C.

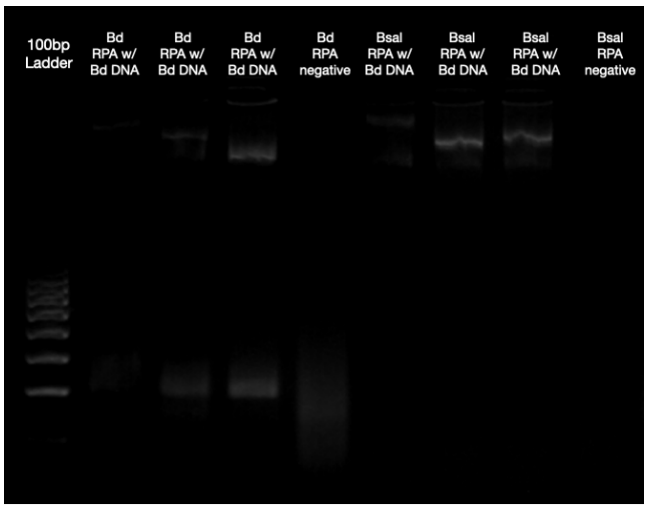
Bsal RPA specificity 19_10_2020.png



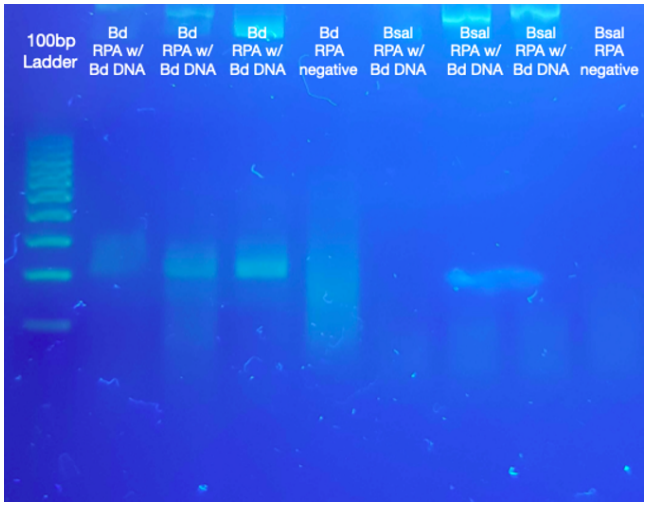
UVBsal RPA specificity 19_10_2020.png



Bd RPA specificity 19_10_2020.png



UVBd RPA specificity 19_10_2020.png



TUESDAY, 10/20/2020

RPA of BsaI and Bd DNA using RPA BsaI1 and Bd1 primers

a. A 5 µM dilution was made for each primer for RPA reactions (47.5 µL TE buffer, 2.5 µL primer)

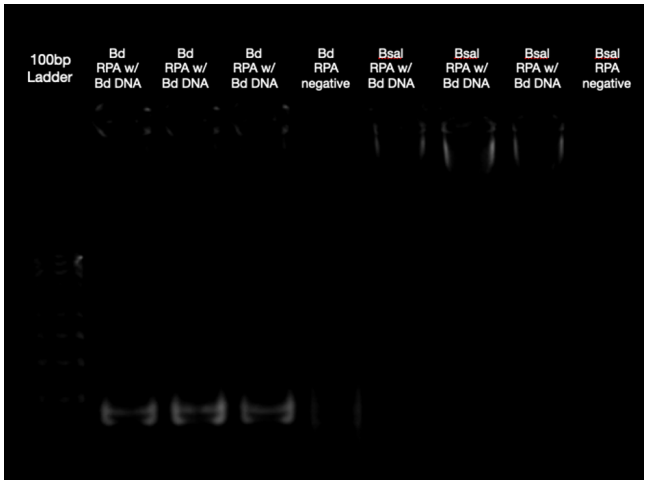
b. Reaction mix in 1.5 mL tube:
    a. Primer Forward (5µM) - 2.4µL
    b. Primer Reverse (5µM) - 2.4µL
    c. Rehydration Buffer - 29.5µL
    d. dH2O - 8.2µL

2. Pipetted up and down after addition of each component in step 1
3. Add the reaction mix (42.5uL) to 1 freeze dried reaction. Pipetted up and down to mix.
4. Splitted the reaction into2 volumes - 15µL to 2 separate PCR tubes.
5. Added 1µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA (BsaI4 243, first batch made) to one tube and 5ul of nuclease-free water to one tube
7. Incubate 20 minutes at 37°C.

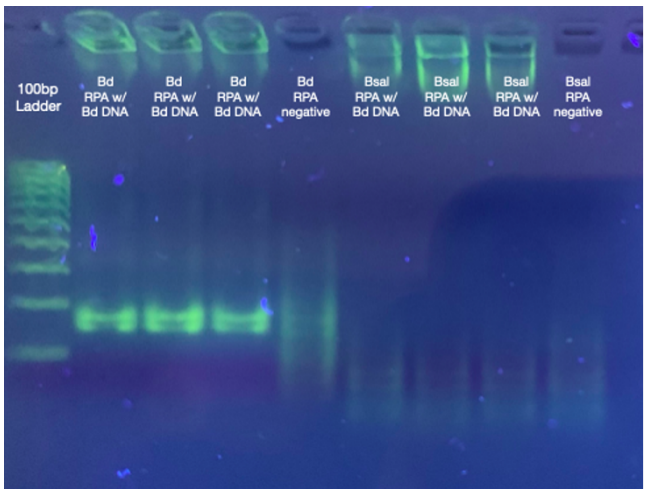
Gel Electrophoresis

1. Took the eight RPA samples done on BsaI (Tuesday 20/10/2020) and eight RPA samples done on Bd (Tuesday 20/10/2020)
2. The gel was set in place onto the chamber
3. Pipetted 6 µL of the DNA ladder onto the first well
4. Pipetted 1 µL of nucleic acid sample loading dye on parafilm for each PCR sample
5. Pipetted 5 µL of each PCR sample onto each 1 µL dyes on the same parafilm
6. The droplet was homogenized with a pipette and transferred onto each well.
7. Ran gel on 135 V for 20 minutes.
8. Ran gel on 135 V for another 5 minutes.
9. Gel was observed under UV.

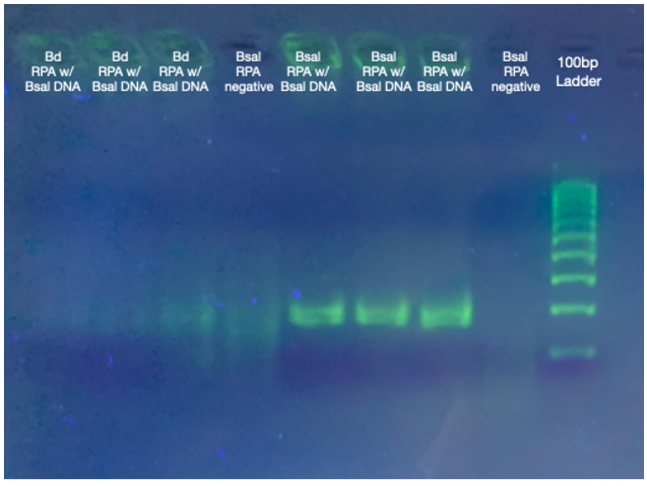
Bd RPA Specificity gel 20_10_2020.png



UVBd RPA Specificity gel 20_10_2020.png



UVBsal RPA Specificity gel 20_10_2020.png



WEDNESDAY, 10/21/2020

CRISPR Cas12a

Resuspend the guides - sgRNA (Bd_sgRNA_1, Bsal_sgRNA_1)

- Lypholized product centrifuged for 60 seconds
- 10nm to 100uM --> added 100uL IDTE buffer pH 7.5

Serial Dilutions of gRNA (Bd_sgRNA_1 & Bsal_sgRNA_1), Cpf1, FQ reporter

1. 100 uM stock solution of each was diluted to a 10 uM, 50uL solution with 5uL of stock solution and:
    a. 45uL IDTE pH 7.5 for gRNA
    b. 45uL NEBubber 2.1 for Cpf1
    c. 45uL TE buffer for FQ reporter
2. The 10 uM solutions were each diluted to 1uM, 50uL solutions using 5 uL of the 10uM solution and:
    a. 45uL IDTE pH 7.5 for gRNA
    b. 45uL NEBubber 2.1 for Cpf1
    c. 45uL TE buffer for FQ reporter

Literature DETECTR RPA CRISPR cas12a protocol

https://science.sciencemag.org/content/sci/suppl/2018/02/14/science.aar6245.DC1/aar6245_Chen_SM.pdf

METHOD:

1. Make the **RPA reactions** on target gene [refer to RPA protocol](total volume 21µl)
2. Incubate the RPA reaction for 20 minutes at 37°C.
3. Take 5uL of RPA amplified product onto gel electrophoresis
4. Add the following reagents in this order to the RPA [16uL]:
    - 1.25uL 1 uM gRNA (final [] in reaction mixture: 62.5nM)
    - 1uL 1uM EnGen Lba Cas12a (Cpf1) (final [] in reaction mixture: 50nM)
    - 1uL 1uM FQ reporter (final [] in reaction mixture: 50nM)
5. Incubate 40 minutes at 37°C.
6. Check under the UV light and blue light every 10 minutes

** after 40 minutes with no sign of florescence, the reagants were tripled (extra 2.5uL gRNA, 2uL Cpf1, 2uL FQ) in the tubes and again incubated at 37°C and checked under UV light after 10 minutes for florescence (#7 Bsal DNA may have 4uL Cpf1)

SYBR green dilution

- A 1000x, 50mL solution of SYBR green was prepared by using 5uL stock 10000x SYBR green and 45uL DMSO
- A 100X 100mL solution of SYBR green was prepared by using 10uL stock 1000x SYBR green and 90uL DMSO

RPA Bsal SYBR Green detection

- RPA taken from first specificity experiment 14.10.2020

1. 1uL SYBR green (1000x) was added to each RPA tube (16uL)

results: too much background noise - SYBR green concentration too high

- RPA with 5x SYBR worked well

Going Forward with SYBR green:

- Test out SYBR green concentrations of 10X, 5X, 1X

THURSDAY, 10/22/2020

Miniprep for 4xBsal 4 and 4xBd 2 (QIAprep Spin Miniprep Kit Protocol)

1. Centrifuged bacterial overnight culture at 7380 rpm for 3 minutes at room temp.
2. Liquid was removed so that only pellet was left in the tube.

3. Pellet was resuspended in 250 µl Buffer P2 and was transfered into a microcentrifuge tube
4. 250 µl of Buffer P2 was added and the tube was inverted 6 times in order to mix the solution.
5. Added 350µl of Buffer N3 and mixed immediately by inverting the tube 6 times.
6. Centrifuged for 10 minuteds at 13000 rpm.
7. Applied 800µl of supernatant to the QIAprep 2.0 spin column, centrifuged for 30 seconds and discarded the flow-through.
8. Washed the QIAprep 2.0 spn column by adding 0.5 ml of Buffer PB, centrifuged for 30 seconds and discarded the flow-through.
9. Washed the QIAprep 2.0 spin column by adding 0.75 ml of Buffer PE, centrifuged for 20 seconds, and discarded the flow through.
10. Transfered the QIAprep spin column to the collection tube
11. Centrifuged for 1 minute to remove residual wash buffer
12. Placed the column in a clean 1.5 ml microcentrifuge tube.
13. Eluted DNA with 50 µl of Buffer EB to the center of the spin column and let it stand for 1 minute then centrifuged for 1 minute.

Nanodrop for 4xBsal 4 and 4xBd 2

1. With the sampling arm open, 2µl of sample (purified plasmid) pipetted onto lower measurment pedestal
2. The sampling arm closed and a spectral measurment intitiated using the operative system ("Nanodrop2000")
3. When the measurment completed, the sampling arm opened and the sample wiped from both the upper and lower pedestal using labaratory wipe
4. Based on the absorbition spectrum concentration and puritity of nucleic acids deduced

RPA (2 sample, 2 negative for both Bd and Bsal):

1. For each reaction, the following reaction mix was prepared in PCR tubes:
    a. Primer Forward (5µM) - 4.8 µL
    b. Primer Reverse (5µM) - 4.8 µL
    c. Rehydration Buffer - 59 µL
    d. dH2O - 16.4µL
2. Pipetted up and down after the addition of each component in step 1
3. Aproximately 42.5uL reaction mix prepared in step 1 was added to powdered TwistAMP freeze-dired reaction. Pipetted up and down to mix.
4. Splitted the reaction into2 volumes - 15µL to 2 separate PCR tubes.
5. Added 1µL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of DNA (Bsal 177, Bd 254) to sample tubes and 5ul of nuclease-free water to negative control tubes
7. Incubate 20 minutes at 37°C.

1uM solutions of Bd gRNA, Bsal gRNA, CPF1 were prepared

Optimized NEB CRISPR-LbCas12a Protocol (from NYUAD iGEM 2019)

Reagents/ materials needed: NEBuffer 2.1 Reaction Buffer (10x), gRNA, EnGen Lba Cas12a (Cpf1), custom Fluorescence-Quencher (FQ) reporter (50µM)

Method:

1. **CRISPR complex:** Add the following reagents in this order at the room temperature:
    - 2µl NEBuffer 2.1 Reaction Buffer (10x)
    - 0.5µl 5uM gRNA
    - 1.5µl 1 µM EnGen Lba Cas12a (Cpf1)
2. Make the **RPA reactions** on target gene [refer to RPA protocol] (total volume 21µl)
3. Incubate both CRISPR complex for 10 minutes at 37°C.
4. Add 1µl of 50µM FQ quencher to the CRISPR complex
5. Add CRISPR complex mix (5uL) with quencher to RPA reaction mix
6. Incubate 30 minutes at 37°C.
7. Check under the blue light based E-gel imager.

Results:

Bd samples worked well, BsaI did not. Possible problem with BsaI gRNA.

20 minutes incubation of step 6 was enough to begin to optimize, 30 minutes optimal visualization

SYBR green at 5X was run and showed favorable results for both Bd and BsaI (254nm)

Next up: run a gel with the CRISPR and SYBR to see if BsaI gRNA is working

TUESDAY, 10/27/2020

DETECTR Proof of Concept

RPA:

1. The following reaction mix was prepared in an eppendorf tube:
    a. Primer Forward (5μM) - 4.8 μL
    b. Primer Reverse (5μM) - 4.8 μL
    c. Rehydration Buffer - 59 μL
    d. dH2O - 16.4μL
2. Pipetted up and down after the addition of each component in step 1
3. Aproximately 42.5uL reaction mix prepared in step 1 was added to powdered TwistAMP freeze-dired reaction. Pipetted up and down to mix.
4. Splitted the reaction into2 volumes - 15μL to 2 separate PCR tubes.
5. Added 1μL of 280mM magnesium acetate and mixed well to start the reaction.
6. Add 5ul of Bd DNA ([Bd] = 254.4 ng/μL) to a reaction mixture, repeat until triplicated. Add 5ul of nuclease-free water to the last negative control tube.
7. Incubate 20 minutes at 37°C.

CRISPR/Cas12a:

1. **CRISPR complex:** Add the following reagents in this order at the room temperature to a single eppendorf tube:
    - 8 μl NEBuffer 2.1 Reaction Buffer (10x)
    - 2 μl 5uM gRNA
    - 6 μl 1 μM EnGen Lba Cas12a (Cpf1)
2. Incubate the CRISPR complex for 10 minutes at 37°C.
3. Add 4μl of 50μM FQ quencher to the CRISPR complex
4. 5 μL of the CRISPR mixture was added to each RPA mixture (When the RPA mixture has finished amplifying) and incubated for 20 minutes at 37 °C.
5. The reaction tubes were observed under UV light.

Proof Of Concept DETECTR Bd.jpg