Table of Contents

September 2023

Tuesday, September 12, 2023

To Do:

1. ~~Patched out LVS to make glycerol stocks~~
2. ~~LB Agar media prep~~
3. PCR

Patching LVS

1. Sterilize workspace with ethanol and Bunsen burner.
2. Aseptically remove one stick from the container then aseptically grab sample of LVS.
3. Draw a line down the middle of the CHA plate. Streak tightly back and forth down the whole plate. Rotate and repeat twice.
4. Close the plate and incubate at 37ºC overnight.

Preparing LB-agar

Protocol written by KMR

1. For 500 mL of LB-agar, weigh out the following components and add to a 1 L (non-baffled) flask:
    a. 6 g agar
    b. 5 g NaCl
    c. 5 g Tryptone
    d. 2.5 g Yeast extract
2. Add stirbar to flask
3. Add 500 mL ddiH20
4. Mix on stirplate until components are dissolved
5. Cover top of flask with foil and add a small piece of autoclave tape
6. Autoclave on 30 minute liquid cycle

Wednesday, September 13, 2023

To Do:

1. ~~Media prep~~
2. ~~Reconstitute Primers for PCR~~
3. PCR

Receiving Primers Protocol

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so dessicated primers go to the bottom of the tube.

2. Add 0.1x EB to a final concentration of 100 uM. Calculate this by multiplying the reported nm by 10 and adding that volume in ul (i.e. 12.7 nmoles = add 127 uL of 0.1xEB).
3. Put on 42°C heat block for 5 minutes to help primers dissolve
4. Vortex and brief spin.
5. Label tubes with KROL numbers on the top and put in the appropriate 100 uM stock box in the -20°C freezer.
6. Optional: Make dilution for intended purpose of primer.
    a. If a PCR primer, make a 10 uM stock by diluting 20 uL of the 100 uM stock into 180 uL of 0.1xEB. Label with purple sticker and put in appropriate freezer box.
    b. If a sequencing primer, make a 2.5 uM stock by diluting 5 uL of the 100 uM stock into 195 uL of 0.1x EB. Label with blue sticker and put in appropriate freezer box.
7. Put Certificate of Analysis sheet in "Oligos Spec Sheet" binder and shipping sheet in Invoices box.

Primer	nm	Volume to add (uL)
KROL660	18.6	186
KROL661	18.6	186
KROL662	17	170
KROL663	20.3	203

PCR Protocol

1. Acquire and label PCR tubes. Be sure to include at least 1 positive and 1 negative control for each PCR experiment.
2. Get a container of ice to keep the components on
3. Acquire the following components and put them on ice, labeling tubes if necessary:
    - Molecular grade H2O in 1.5 mL microfuge tube
    - KOD/primestar buffer
    - dNTPs
    - oligo F (10uM)
    - oligo R (10uM)
    - template (eg. LVS gDNA, plasmid, colony, etc.)
    - Note: KOD/primestar enzyme should be kept in the freezer until it is used as it is expensive and should be added last
4. Centrifuge the microfuge tubes to get any solution out of the microfuge tube cover
5. If any of the solutions are frozen, be sure to vortex the microfuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
    - DO NOT vortex the enzyme itself or any solution with enzyme because vortexing will expose it to oxygen and degrade it
6. Use PCR_worksheet.xlsx to make establish the specifics of what will be added
    - The file is located in the Protocols folder
    - Also setup table below to specify which primers and source DNA will be used
7. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
8. Add ddi H2O to negative control tube
    - Template volume for 1 reaction

9. Prepare a master-mix in a 1.5 mL microfuge tube by adding the following according to the worksheet and using micropipettes:
    - Add ddi H2O
    - Add dNTPs
    - Add KOD buffer
    - Add KOD enzyme
10. Mix the master-mix solution by pipetting up and down
    - Do not vortex to mix
11. Add appropriate volume of master-mix to negative control PCR tube
12. Add template to Master Mix
    - Factor template volume minus 1 template reaction volume
13. Add appropriate volume of master mix to each PCR tube (except negative control) and pipette up and down to mix (conserves tips)
14. Close PCR Tubes until the caps are tight
15. Place the PCR Tubes in the thermocycler on STN 1 – the following settings should be in place:
    - Heat at 94 degrees for 2 minutes,
    - 94 degrees C for 20 seconds
    - 50 degrees C for 30 seconds
    - 68 degrees C for 1 minute/kb (adjust based on expected size of product)
    - Go back to step 2
    - Repeat 32x
    - 68 degrees C for 5 minutes
    - 12 degrees C for infinity

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	FTL0508_F1	LVS gDNA	KROL660, KROL661	531
2	FTL0508_F2	LVS gDNA	KROL662, KROL663	660
3	- controlF1	-	KROL660, KROL661	-
4	- controlF2	-	KROL662, KROL663	-

Total reaction volume	100			
Total number of reactions	4			
				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	5
ddiH2O			62.0	310
PrimeSTAR GXL Buffer	5x	1x	20.0	100
dNTPs	2.5 mM	0.2 mM	8.0	40
oligo F	10 uM	0.3 uM	3.0	15

oligo R	10 uM	0.3 uM	3.0	15
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	10
		Total volume	100	490

Observations

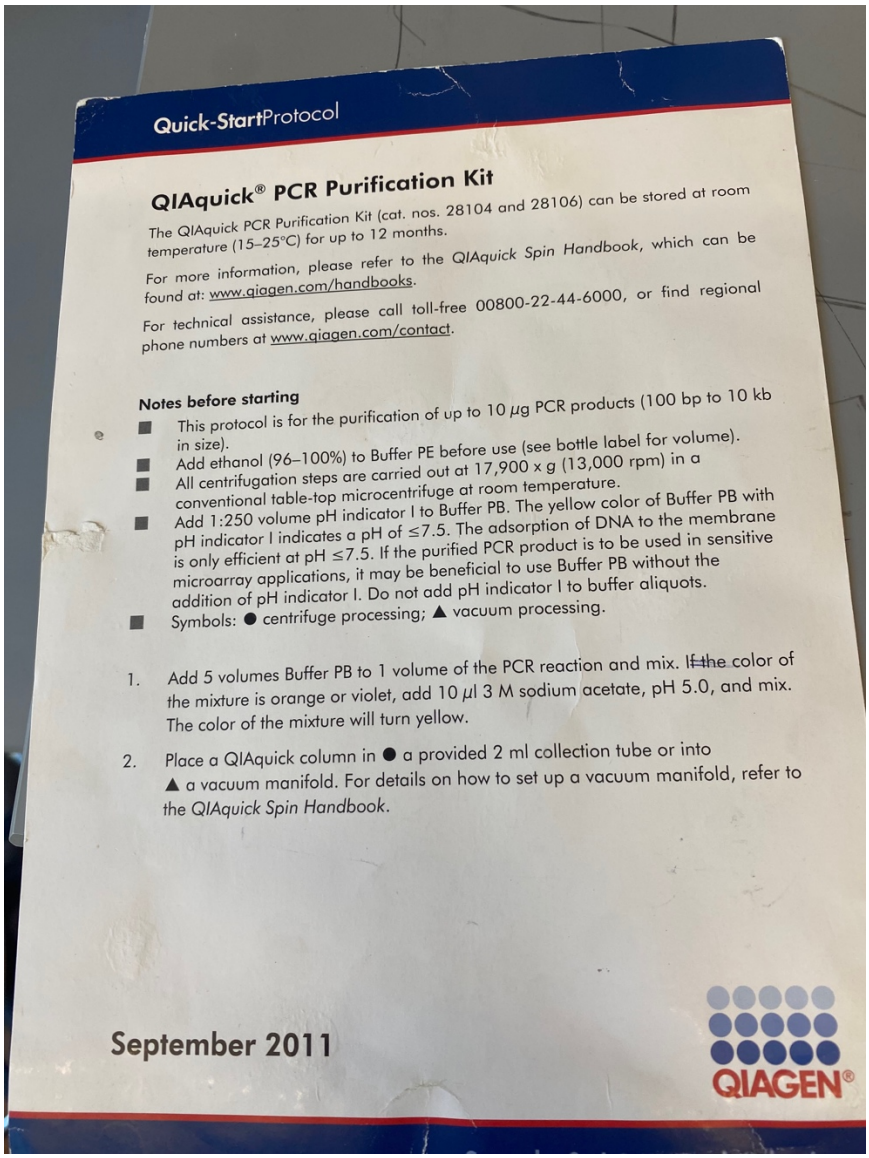
*Johanyx: prepared master mix, added primer and master mix to negative controls
*Christina: prepared templates, added primer and master mix to experimental tubes

Thursday, September 14, 2023

To Do:

1. ~~Media prep~~
2. ~~Reconstitute Primers for PCR~~
3. ~~PCR~~
4. Purify PCR and run on gel.
5. Digest.

QIAquick PCR Purification Kit



Quick-StartProtocol

QIAquick® PCR Purification Kit

The QIAquick PCR Purification Kit (cat. nos. 28104 and 28106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the *QIAquick Spin Handbook*, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- This protocol is for the purification of up to 10 µg PCR products (100 bp to 10 kb in size).
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. The adsorption of DNA to the membrane is only efficient at pH ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.
- Symbols: ● centrifuge processing; ▲ vacuum processing.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. ~~If the~~ color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ● a provided 2 ml collection tube or into ▲ a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.

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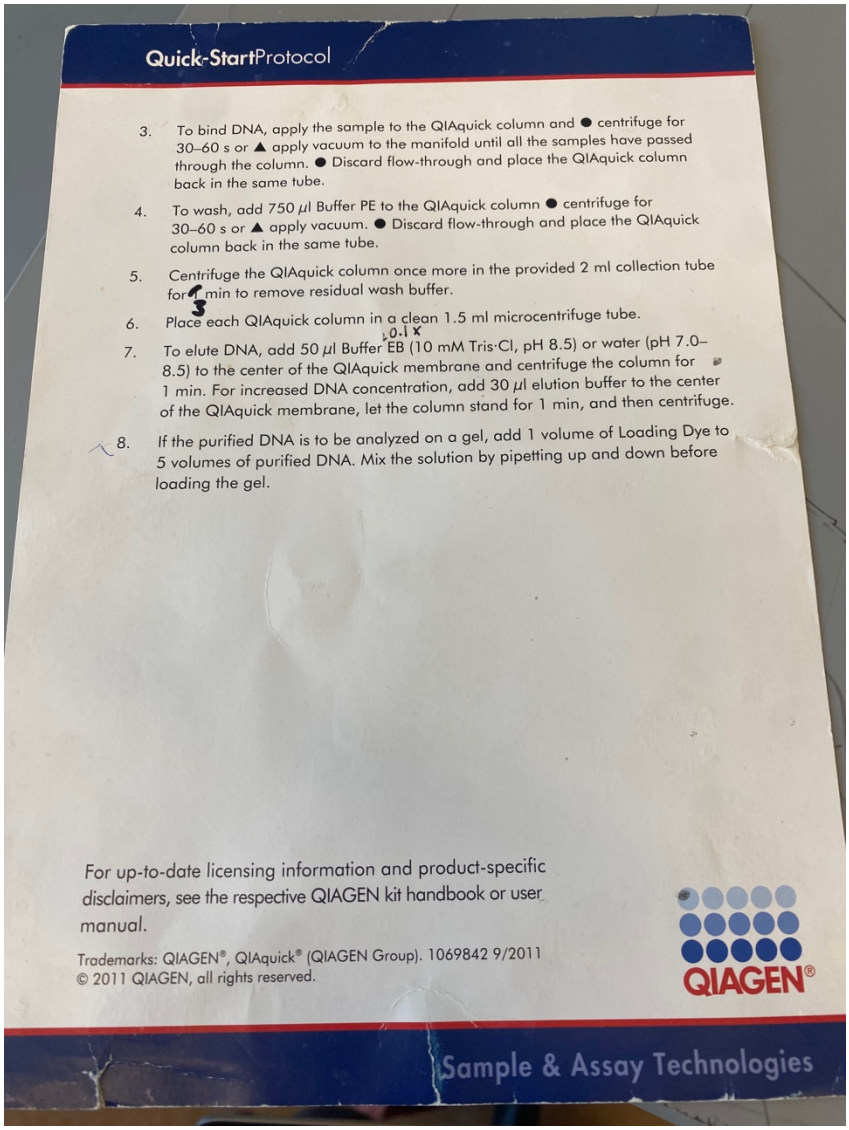

Quick-StartProtocol

3. To bind DNA, apply the sample to the QIAquick column and ● centrifuge for 30–60 s or ▲ apply vacuum to the manifold until all the samples have passed through the column. ● Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 µl Buffer PE to the QIAquick column ● centrifuge for 30–60 s or ▲ apply vacuum. ● Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

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Sample & Assay Technologies

Observations:

There was an error in the PCR where F1 and F2 were flipped, as well as slight contamination of the first negative control (~100bp). The protocol will be repeated on Monday.

Results and Data:

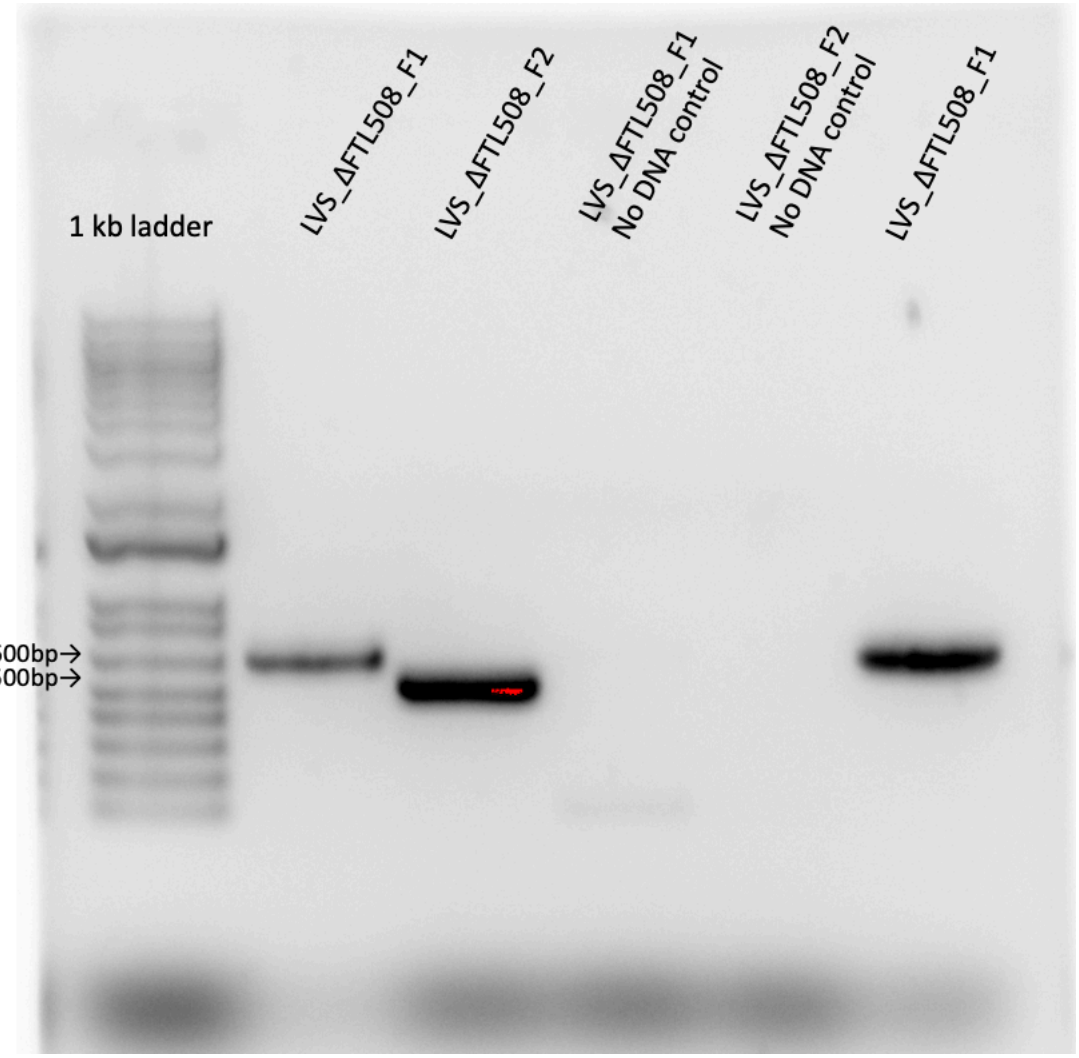


Figure 1. Johanyx and Christina's PCR results. There is an error in F1 and F2. F1 should be 531bp and F2 should be 660bp. The samples seem to be flipped. There is also a slight primer dimer on reaction 3 which is the F1 negative control.

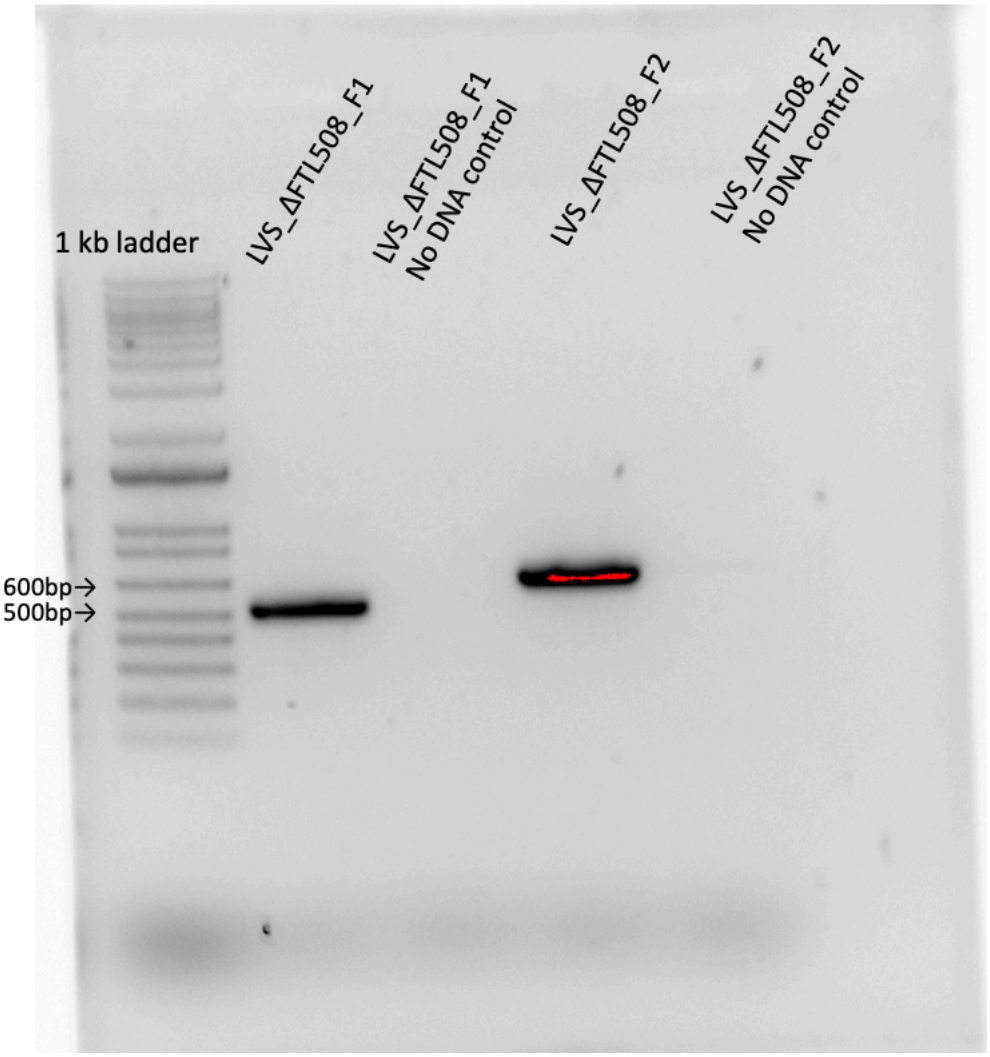


Figure 2. Kira's PCR repeat of F1 and F2 due to the errors in the first run.

Monday, September 18, 2023

To Do:

1. ~~Repeat PCR~~
2. Purify PCR
3. Run PCR products on gel.

PCR Protocol

*See PCR protocol in Protocols/PCR/PCRprotocol_new

Total reaction volume	100			
Total number of reactions	3			
				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	4
ddiH2O			62.0	248
PrimeSTAR GXL Buffer	5x	1x	20.0	80
dNTPs	2.5 mM	0.2 mM	8.0	32
oligo F	10 uM	0.3 uM	3.0	12
oligo R	10 uM	0.3 uM	3.0	12
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	8
		Total volume	100	392

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
3	FTL0508_F2	LVS gDNA	KROL662, KROL663	660
4	+ control	LVS gDNA	KROL3, KROL4	731
5	- controlF2	-	KROL662, KROL663	-

*Johanyx completed reactions 1 and 2.

Tuesday, September 19, 2023

To Do:

1. ~~Repeat PCR~~
2. ~~Purify PCR~~
3. ~~Run PCR products on gel.~~
4. Digest

QIAquick PCR Purification Kit

*See protocol in QIAquick PCR Purification Kit

Observations:

When mixing the PCR products with loading dye, I noticed a stringy thing in the reaction 5 (F2 negative control) tube. However this did not result in contamination.

Results and Data:

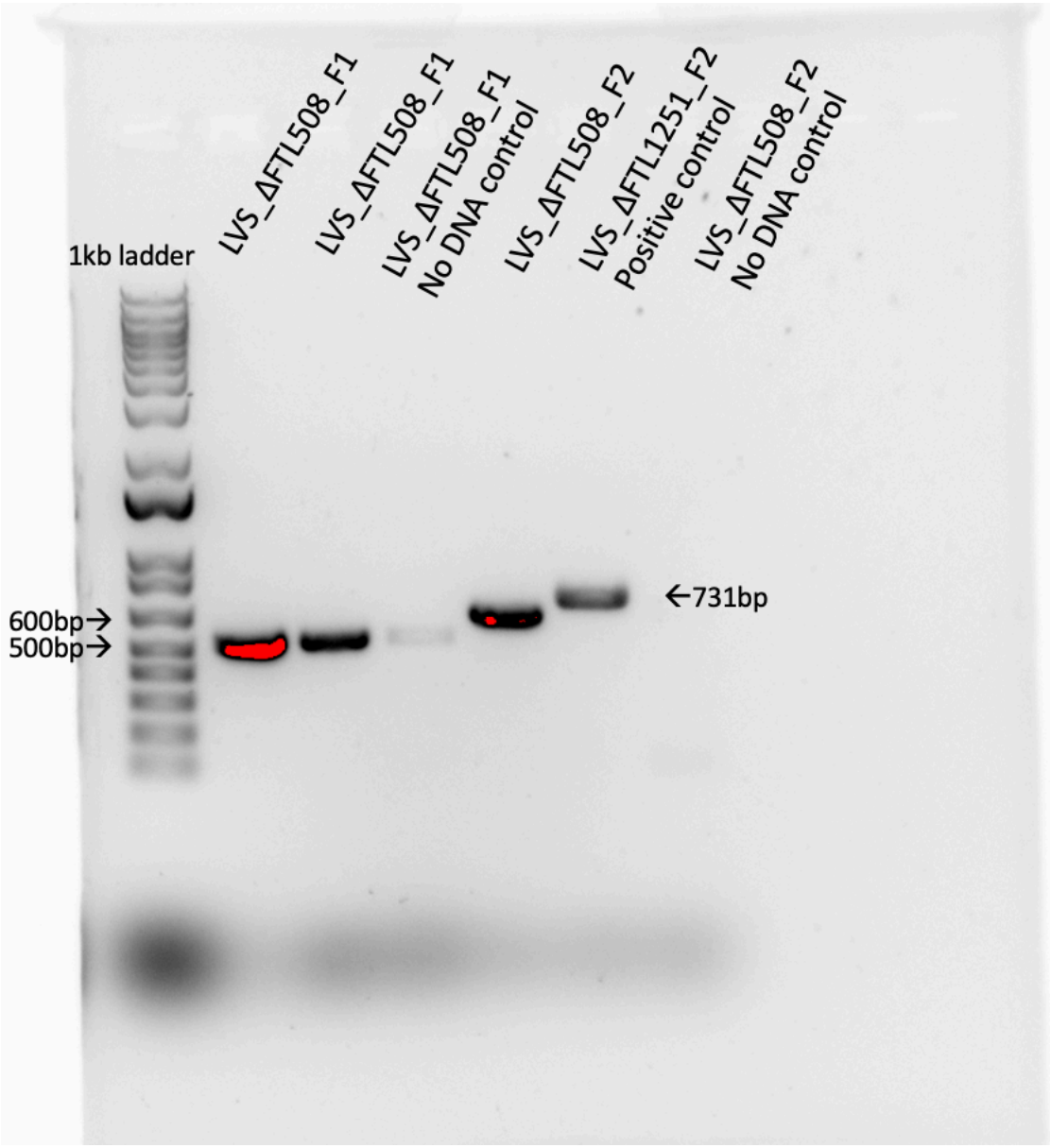


Figure 1. PCR results from 9/19/23. My reactions (3,4, and 5) worked well and had good results with no contamination. There is a slight primer dimer on my negative control. Johanyx's negative control had slight contamination. This could be due to trace contaminants in a pipette tip or technique issues. This can be fixed by more cautious pipetting and aseptic technique.

Table 1. This table shows the nanodrop results for our PCR products that we took before loading the gel. "F1" refers to LVS_FTL508_F1, "F2" refers to LVS_FTL508_F2, and "Positive" refers to LVS_FTL508_F2 positive control.

#	Sample ID	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	F1	9/19/2023 2:44:27 PM	219.5	ng/µl	4.39	2.666	1.65	1.21	DNA	50
2	F2	9/19/2023 2:46:45 PM	161.6	ng/µl	3.231	1.731	1.87	2.44	DNA	50
3	Positive	9/19/2023 2:48:04 PM	380.5	ng/µl	7.611	4.359	1.75	1.65	DNA	50

Wednesday, September 20, 2023

To Do:

1. ~~Repeat fragment 1~~
2. ~~Dilute plasmid~~
3. Amplify pKR201 by PCR

PCR Protocol

*See PCR protocol in Protocols/PCR/PCRprotocol_new

Total reaction volume	100			
Total number of reactions	3			
				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	4
ddiH2O			62.0	248
PrimeSTAR GXL Buffer	5x	1x	20.0	80
dNTPs	2.5 mM	0.2 mM	8.0	32
oligo F	10 uM	0.3 uM	3.0	12
oligo R	10 uM	0.3 uM	3.0	12
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	8
		Total volume	100	392

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
4	pKR184	LVS gDNA	KROL524B, KROL667	739
5	- control	-	KROL524B, KROL667	-

*Numbered 4 and 5 since they are being run on the same gel as Johanyx's experiment

Observations:

After I left the lab, I realized that I made the incorrect volume for the master mix. I did a three-reaction volume instead of two. This is because I was initially prepared to include a positive control along with the experimental and negative. We ended up not using a positive control. I don't think this will affect my results, I just had extra unused master mix.

Thursday, September 21, 2023

To Do:

1. ~~Dilute plasmid~~
2. Amplify pKR184 by PCR
3. Purify PCR product

4. Run PCR products on gel

QIAquick PCR Purification Kit

*see protocol QIAquick PCR Purification Kit

Results and Data:

Table 1. This table shows our nanodrop data for the PCR purified products. "F1" refers to LVS_FTL508_F1.

1	pKR184	Science	9/21/2023 3:05:23 PM	156.5	ng/µl	3.13	1.725	1.81	2.65	DNA
2	F1	Science	9/21/2023 3:08:08 PM	143.9	ng/µl	2.879	1.599	1.8	2.3	DNA
3	F1 positive control	Science	9/21/2023 3:09:27 PM	519.4	ng/µl	10.388	5.916	1.76	1.8	DNA

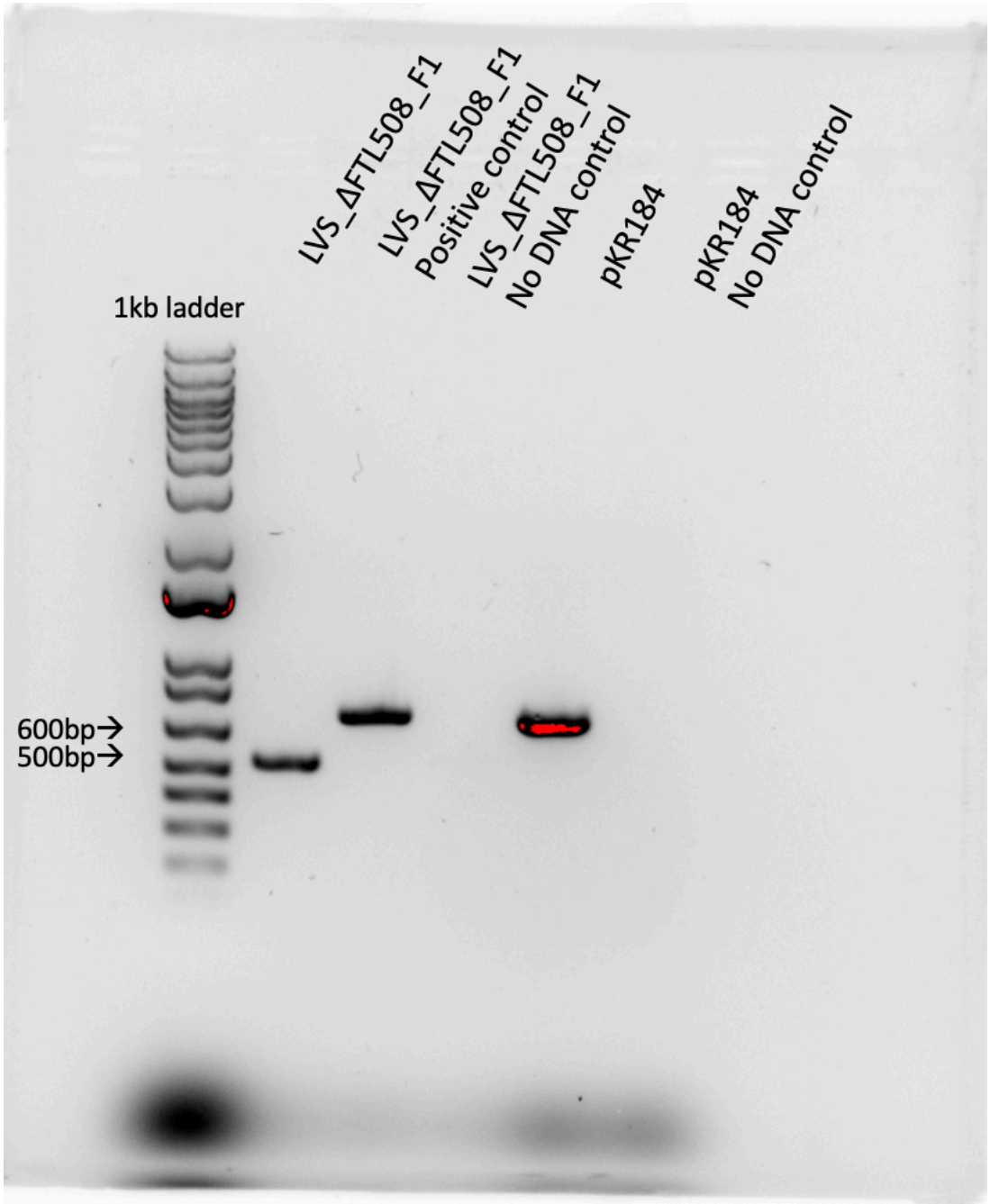


Figure 1. Our PCR results from today. Johanyx completed the LVS samples, and I completed the pKR184 samples. The results were great and had no contamination.

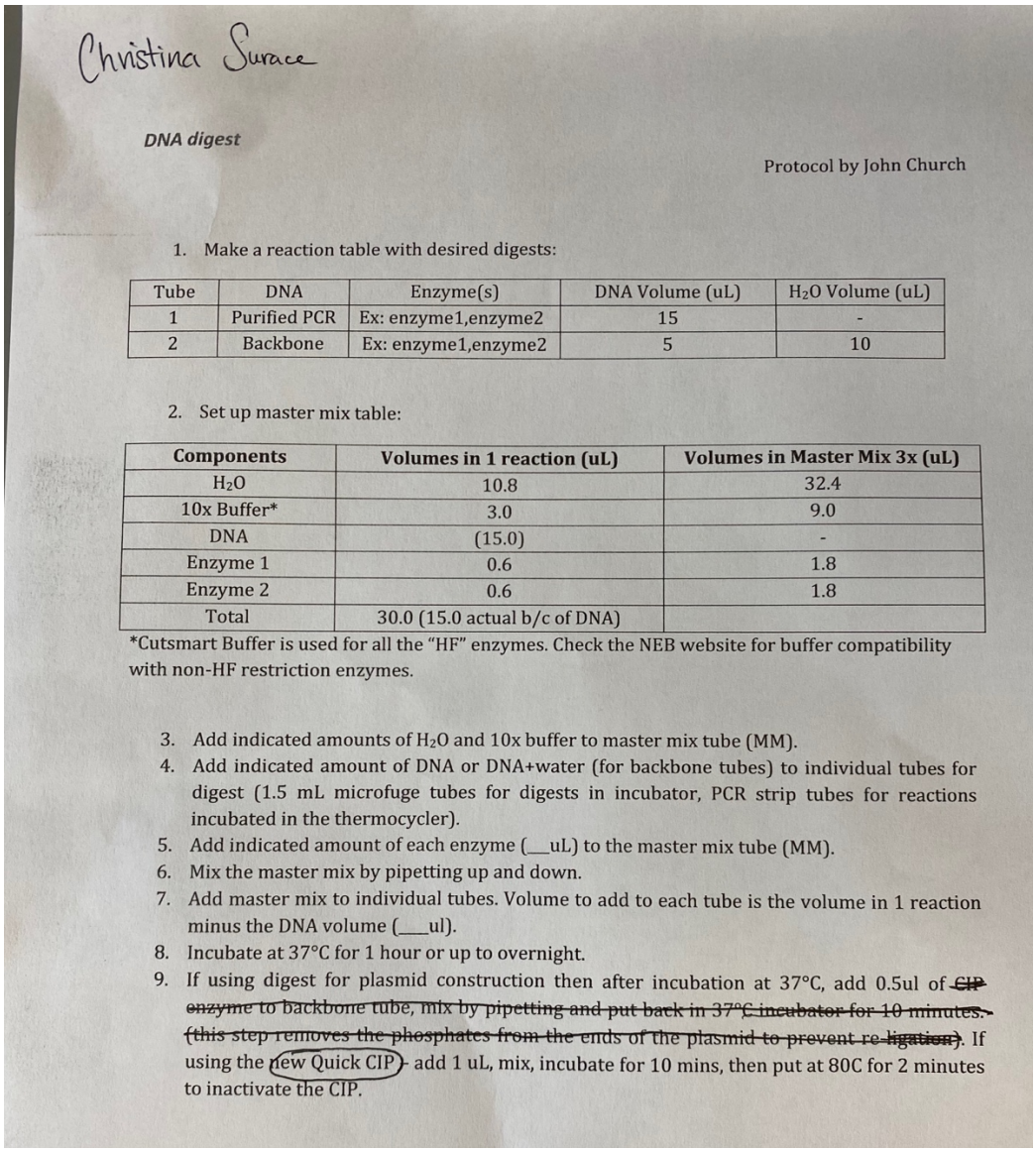
Friday, September 22, 2023

To Do:

1. ~~Dilute plasmid~~
2. ~~Amplify pKR184 by PCR~~
3. ~~Purify PCR product~~

~~4. Run PCR products on gel~~
5. Digest PCR products and plasmid backbone

DNA Digest Protocol



Christina Surace

DNA digest

Protocol by John Church

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	Purified PCR	Ex: enzyme1,enzyme2	15	-
2	Backbone	Ex: enzyme1,enzyme2	5	10

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H_2O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
Enzyme 1	0.6	1.8
Enzyme 2	0.6	1.8
Total	30.0 (15.0 actual b/c of DNA)	

*Cutsmart Buffer is used for all the "HF" enzymes. Check the NEB website for buffer compatibility with non-HF restriction enzymes.

3. Add indicated amounts of H_2O and 10x buffer to master mix tube (MM).
4. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
5. Add indicated amount of each enzyme (___uL) to the master mix tube (MM).
6. Mix the master mix by pipetting up and down.
7. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (___ul).
8. Incubate at 37°C for 1 hour or up to overnight.
9. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of ~~CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation)~~. If using the new Quick CIP - add 1 uL, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.

This protocol will be used with these volumes and reactions:

tube	DNA	Enzyme(s)	DNA Volume (uL)	H2O volume (uL)
1	pKR184 PCR Pure	NotI-HF	15	n/a
2	pKR11 backbone	NotI-HF	5	10

Master mix:

Components	Volumes in 1 rxn (uL)	Volumes in Master mix 3x (uL)
H2O	10.8	32.4
10x Buffer*	3	9
DNA	15	n/a
Enzyme 1	1.2	3.6
Total	**30**(-15 DNA)	**45**

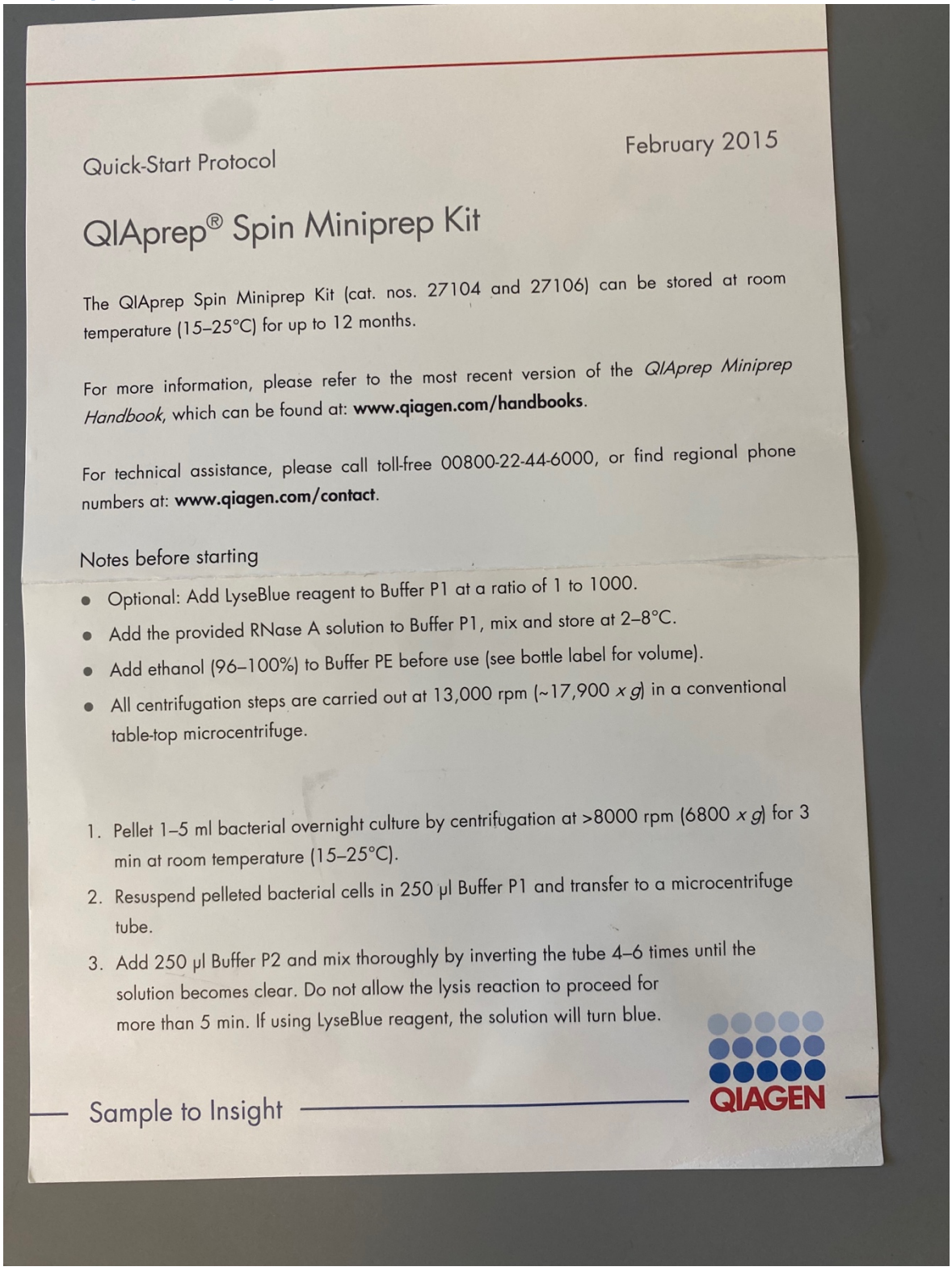
Wednesday, September 27, 2023

To Do:

1. Digest PCR products and plasmid backbone
2. ~~Assist Ben and Jake with Mini Prep~~

*note: Jake completed the mini prep protocol for colonies 1-3. I completed the protocol for 4-6.

QIAprep Spin Miniprep Kit



Quick-Start Protocol

February 2015

QIAprep® Spin Miniprep Kit

The QIAprep Spin Miniprep Kit (cat. nos. 27104 and 27106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the most recent version of the *QIAprep Miniprep Handbook*, which can be found at: **www.qiagen.com/handbooks**.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at: **www.qiagen.com/contact**.

Notes before starting

- Optional: Add LyseBlue reagent to Buffer P1 at a ratio of 1 to 1000.
- Add the provided RNase A solution to Buffer P1, mix and store at 2–8°C.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 13,000 rpm (~17,900 *x g*) in a conventional table-top microcentrifuge.

1. Pellet 1–5 ml bacterial overnight culture by centrifugation at >8000 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.

Sample to Insight

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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 800 µl supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. For centrifuge processing, follow the instructions marked with a triangle (▲). For vacuum manifold processing, follow the instructions marked with a circle (●). ▲ Centrifuge for 30–60 s and discard the flow-through, or ● apply vacuum to the manifold to draw the solution through the QIAprep 2.0 spin column and switch off the vacuum source.
7. Recommended: Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. ▲ Centrifuge for 30–60 s and discard the flow-through, or ● apply vacuum to the manifold to draw the solution through the QIAprep 2.0 spin column and switch off the vacuum source.

   Note: This step is only required when using endA+ strains or other bacteria strains with high nuclease activity or carbohydrate content.
8. Wash the QIAprep 2.0 spin column by adding 0.75 ml Buffer PE. x3 ▲ Centrifuge for 30–60 s and discard the flow-through, or ● apply vacuum to the manifold to draw the solution through the QIAprep 2.0 spin column and switch off the vacuum source. Transfer the QIAprep 2.0 spin column to the collection tube.
9. Centrifuge for 3 min to remove residual wash buffer.
10. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB 0.1X (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.
11. If the extracted DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Results and Data:

Table 1. Nanodrop data for Miniprepped DNA. pKR187 is the plasmid that was cloned by Ben and Jake. The numbers after the underscore represent the transformed colony number that the DNA came from.

1	pKR187_1	Science	9/27/2023 1:25:07 PM	285	ng/µl	5.7	3.049	1.87	2.4
2	pKR187_2	Science	9/27/2023 1:26:40 PM	318.9	ng/µl	6.379	3.41	1.87	2.35
3	pKR187_3	Science	9/27/2023 1:27:48 PM	333.8	ng/µl	6.677	3.583	1.86	2.34
4	pKR187_4	Science	9/27/2023 1:29:21 PM	256.2	ng/µl	5.125	2.754	1.86	2.39
5	pKR187_5	Science	9/27/2023 1:30:31 PM	301.9	ng/µl	6.038	3.223	1.87	2.4
6	pKR187_6	Science	9/27/2023 1:31:33 PM	519.2	ng/µl	10.383	5.558	1.87	2.3

Observations:

The next step in this process is to prepare the samples for sequencing, then send them to the INBRE facility in Avedisian Hall to be sequenced. The sequencing will confirm that the cloning process was done correctly. The results should be in by Friday.

Table 2. The sequencing reactions were set up using the following volumes from the table. In addition to the plasmid and the water, 2.56uL of the stock solution of 2.5uM KROL525 primer was added to the reaction tubes. The template stock concentration is from the nanodrop results.

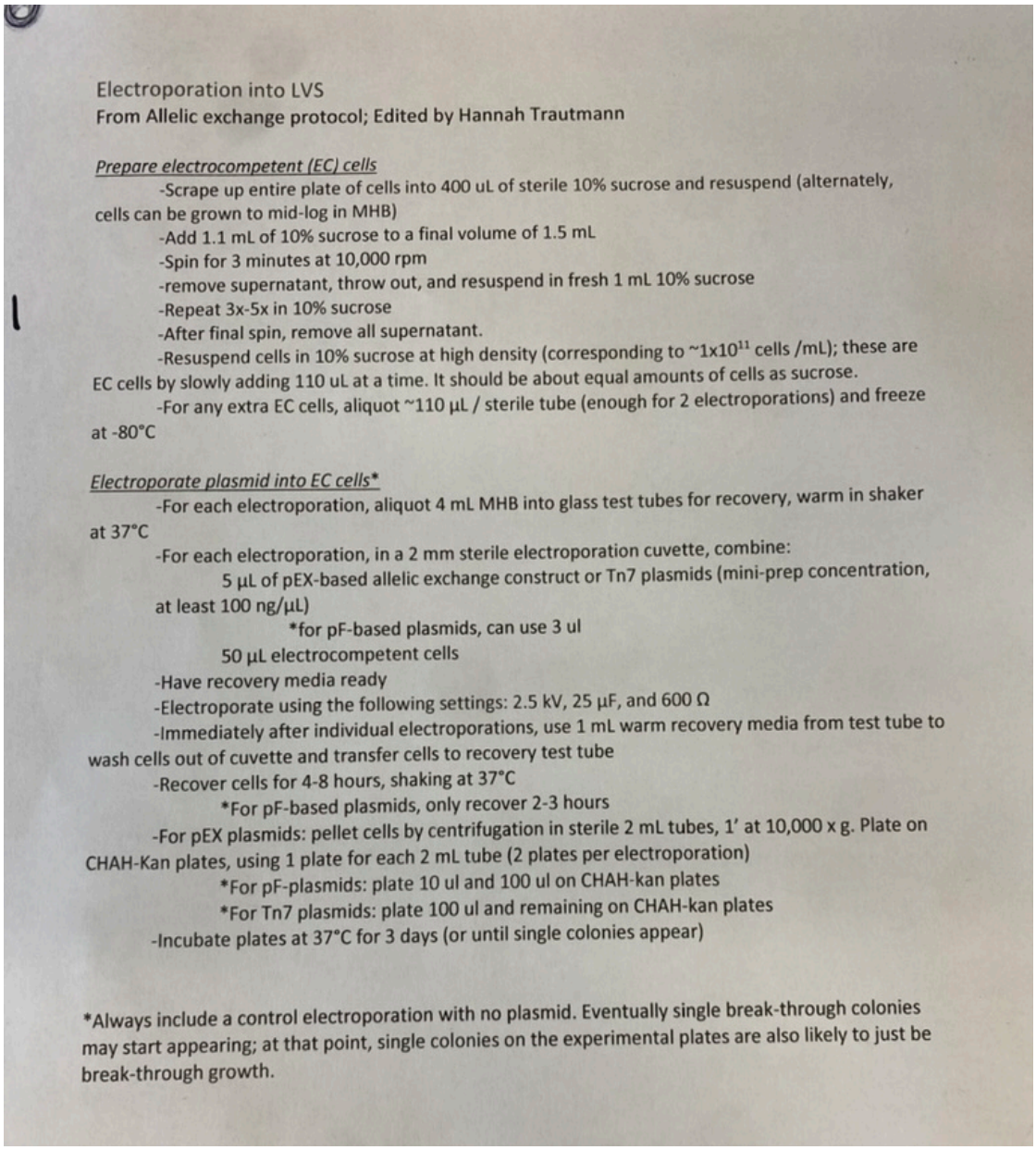
Sample number	**Well**	**Template Type**	**Template Name**	**Primer Name[a]**	**A.**	**B.**	**E.**	**F.**
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/µl)	**PLASMID** template:	Volume H_2O **needed**
							2x(~200 ÷ B)µl	**(12 less D or E - 2.56)µl**
BM1		PLASMID	pKR187	KROL525	7861	285	1.40	8.04
BM2		PLASMID	pKR187	KROL525	7861	318.9	1.25	8.19
BM3		PLASMID	pKR187	KROL525	7861	333.8	1.20	8.24
BM4		PLASMID	pKR187	KROL525	7861	256.2	1.56	7.88
BM5		PLASMID	pKR187	KROL525	7861	301.9	1.32	8.12
BM6		PLASMID	pKR187	KROL525	7861	519.2	0.77	8.67

Thursday, September 28, 2023

To Do:

1. Digest PCR products and plasmid backbone
2. ~~Assist Ben with electroporation prep.~~

Preparation of electrocompetent (EC) cells



Electroporation into LVS
From Allelic exchange protocol; Edited by Hannah Trautmann

Prepare electrocompetent (EC) cells

-Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)
-Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL
-Spin for 3 minutes at 10,000 rpm
-remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose
-Repeat 3x-5x in 10% sucrose
-After final spin, remove all supernatant.
-Resuspend cells in 10% sucrose at high density (corresponding to ~$1x10^{11}$ cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.
-For any extra EC cells, aliquot ~110 µL / sterile tube (enough for 2 electroporations) and freeze at -80°C

*Electroporate plasmid into EC cells**

-For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
-For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
5 µL of pEX-based allelic exchange construct or Tn7 plasmids (mini-prep concentration, at least 100 ng/µL)
*for pF-based plasmids, can use 3 ul
50 µL electrocompetent cells
-Have recovery media ready
-Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω
-Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube
-Recover cells for 4-8 hours, shaking at 37°C
*For pF-based plasmids, only recover 2-3 hours
-For pEX plasmids: pellet cells by centrifugation in sterile 2 mL tubes, 1' at 10,000 x g. Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
*For pF-plasmids: plate 10 ul and 100 ul on CHAH-kan plates
*For Tn7 plasmids: plate 100 ul and remaining on CHAH-kan plates
-Incubate plates at 37°C for 3 days (or until single colonies appear)

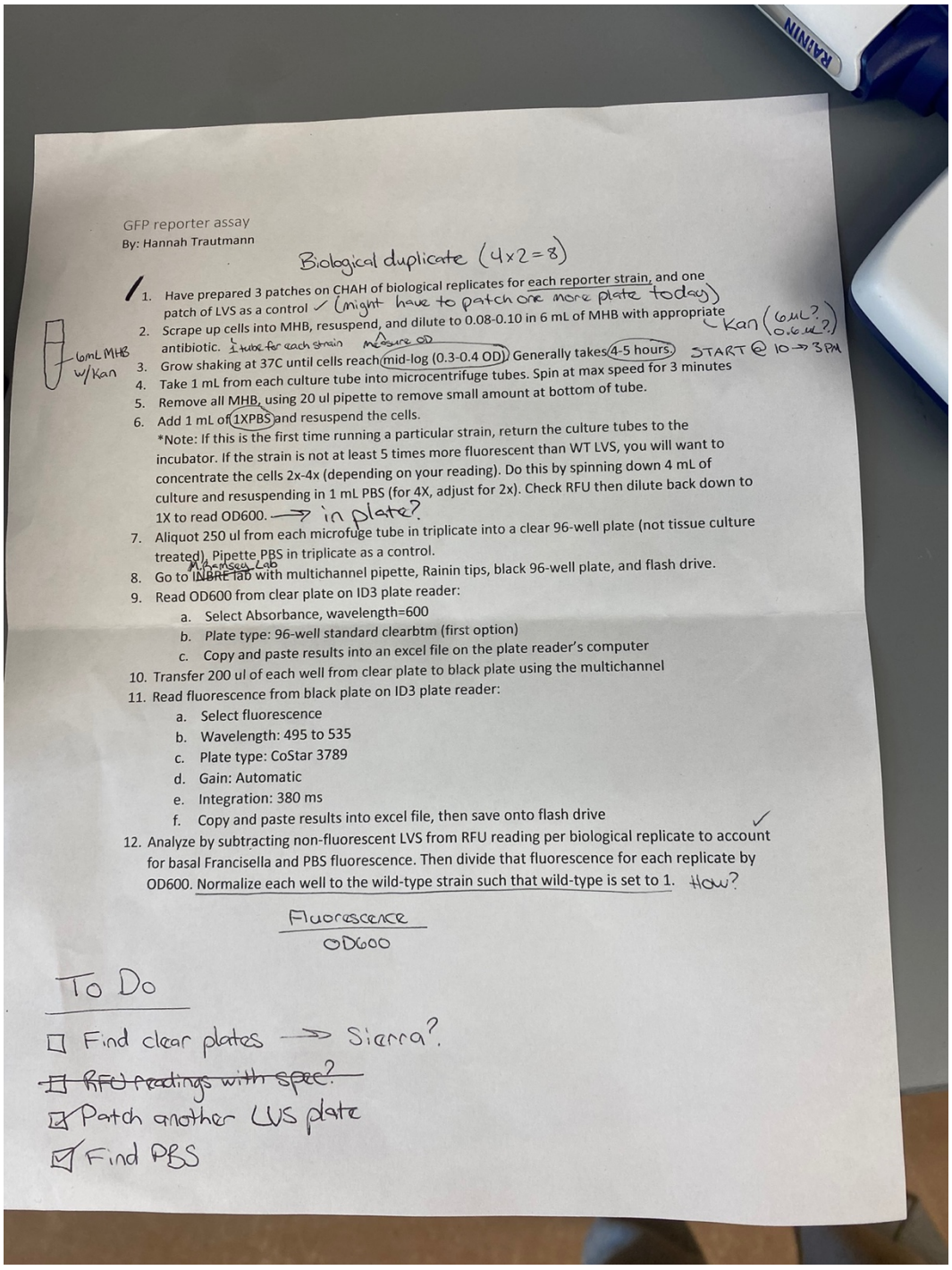
*Always include a control electroporation with no plasmid. Eventually single break-through colonies may start appearing; at that point, single colonies on the experimental plates are also likely to just be break-through growth.

Friday, September 29, 2023

To Do:

1. Digest PCR products and plasmid backbone
2. Assist Ben and Jake with GFP Reporter Assay preparation.

GFP Reporter Assay



GFP reporter assay
By: Hannah Trautmann

Biological duplicate (4x2=8)

1. Have prepared 3 patches on CHAH of biological replicates for each reporter strain, and one patch of LVS as a control ✓ (might have to patch one more plate today)
2. Scrape up cells into MHB, resuspend, and dilute to 0.08-0.10 in 6 mL of MHB with appropriate antibiotic. 1 tube for each strain measure OD ← Kan (6uL? 0.6uL?)
3. Grow shaking at 37C until cells reach mid-log (0.3-0.4 OD). Generally takes 4-5 hours. START @ 10 → 3PM
4. Take 1 mL from each culture tube into microcentrifuge tubes. Spin at max speed for 3 minutes
5. Remove all MHB, using 20 ul pipette to remove small amount at bottom of tube.
6. Add 1 mL of 1XPBS and resuspend the cells.
   *Note: If this is the first time running a particular strain, return the culture tubes to the incubator. If the strain is not at least 5 times more fluorescent than WT LVS, you will want to concentrate the cells 2x-4x (depending on your reading). Do this by spinning down 4 mL of culture and resuspending in 1 mL PBS (for 4X, adjust for 2x). Check RFU then dilute back down to 1X to read OD600. → in plate?
7. Aliquot 250 ul from each microfuge tube in triplicate into a clear 96-well plate (not tissue culture treated). Pipette PBS in triplicate as a control.
8. Go to ~~INBRE~~ Ramsey Lab with multichannel pipette, Rainin tips, black 96-well plate, and flash drive.
9. Read OD600 from clear plate on ID3 plate reader:
   a. Select Absorbance, wavelength=600
   b. Plate type: 96-well standard clearbtm (first option)
   c. Copy and paste results into an excel file on the plate reader's computer
10. Transfer 200 ul of each well from clear plate to black plate using the multichannel
11. Read fluorescence from black plate on ID3 plate reader:
    a. Select fluorescence
    b. Wavelength: 495 to 535
    c. Plate type: CoStar 3789
    d. Gain: Automatic
    e. Integration: 380 ms
    f. Copy and paste results into excel file, then save onto flash drive ✓
12. Analyze by subtracting non-fluorescent LVS from RFU reading per biological replicate to account for basal Francisella and PBS fluorescence. Then divide that fluorescence for each replicate by OD600. Normalize each well to the wild-type strain such that wild-type is set to 1. How?

$$\frac{\text{Fluorescence}}{\text{OD600}}$$

To Do

- ☐ Find clear plates → Sierra?
- ~~☐ RFU readings with spec?~~
- ☑ Patch another LVS plate
- ☑ Find PBS

January 2024

Wednesday, January 24, 2024

To Do:

1. ~~Review project items with Ben and Dr. Ramsey~~

2. ~~Make aliquots of NF water, PBS, EB (0.1x), PE.~~
3. Digest and ligation.

Thursday, January 25, 2024

To Do:

1. ~~Make two half liters of MHB.~~
2. ~~Repeat digest.~~
3. Gel purify.
4. Gel extract.
5. Ligation.

Mueller-Hinton broth protocol- by Jamie Wandzilak

For 500 mL of MHB

1. Weigh out 10.5g of Mueller-Hinton broth into 1L square-bottle
2. Add 500mL of ddiH_2O (type I)
3. Autoclave. Cool down to 37 ℃ or cooler.
4. Can keep this sterile media indefinitely without supplements.

DNA Digest Protocol

Protocol by John Church

1. Make a reaction table with desired digests:

tube	DNA	Enzyme(s)	DNA Volume (uL)	H2O volume (uL)
1	pKR184 PCR Pure	NotI-HF	15	n/a
2	pKR11 backbone	NotI-HF	5	10

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H_2O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
Enzyme 1	1.2	3.6
Total	**30.0 (15.0 actual b/c of DNA)**	**45**

*Cutsmart Buffer is used for all the "HF" enzymes. Check the NEB website for buffer compatibility with non-HF restriction enzymes.

3. Add indicated amounts of H_2O and 10x buffer to master mix tube (MM).
4. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).

5. Add indicated amount of each enzyme (___uL) to the master mix tube (MM).
6. Mix the master mix by pipetting up and down.
7. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (____ul).
8. Incubate at 37°C for 1 hour or up to overnight.
9. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). **If using the new Quick CIP – add 1 uL, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.**

Thoughts & Observations:

Gel purify and extract before ligation.
When performing the QuickCIP step, I misread the instructions and put the backbone with QuickCIP directly onto the heat block at 80C. I did not realize my mistake until 2 minutes later. I quickly removed the reaction tube from the heat block and placed into the incubator for 10 minutes, then back to the heat block for 2 minutes. Hopefully this has no effect on the ligation, but I will continue with the gel purification and extract for now. If necessary, I will repeat the digest step for a successful ligation.

Gel Purification (Digest)

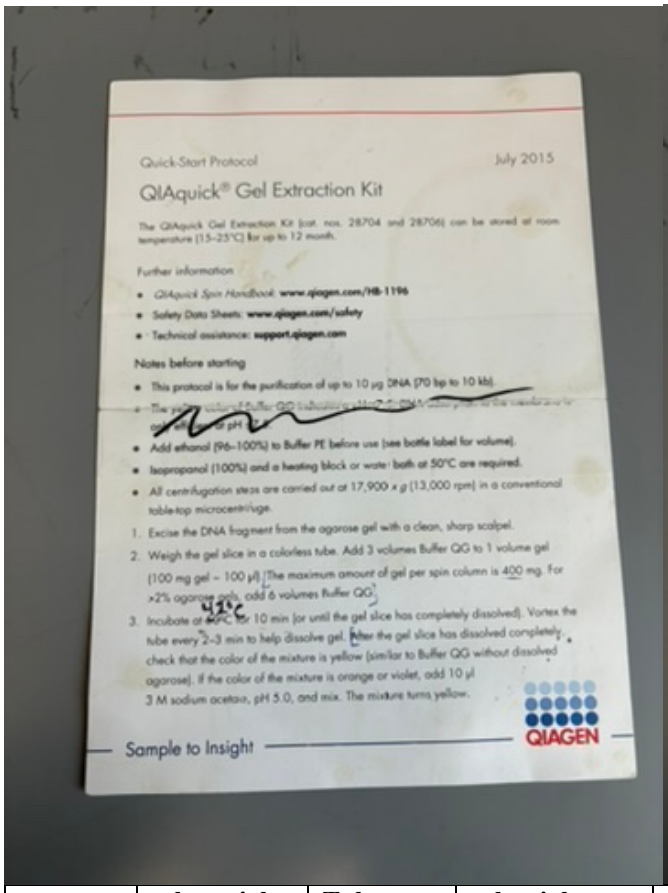
1. Pour about 100 mL of agarose gel into the gel rig set up.
2. Add 12 uL of SybrSafe.
3. Let the gel solidify, then place the gel in the correct position.
4. Pour TAE buffer until it completely covers the gel.
5. Add 6 uL of loading dye to the 30 uL digest reaction tubes.
6. Load 10 uL of the DNA ladder in the first well.
7. Leave a space between wells and load the full volume of the reaction tubes.
8. Run at 113V for an hour.

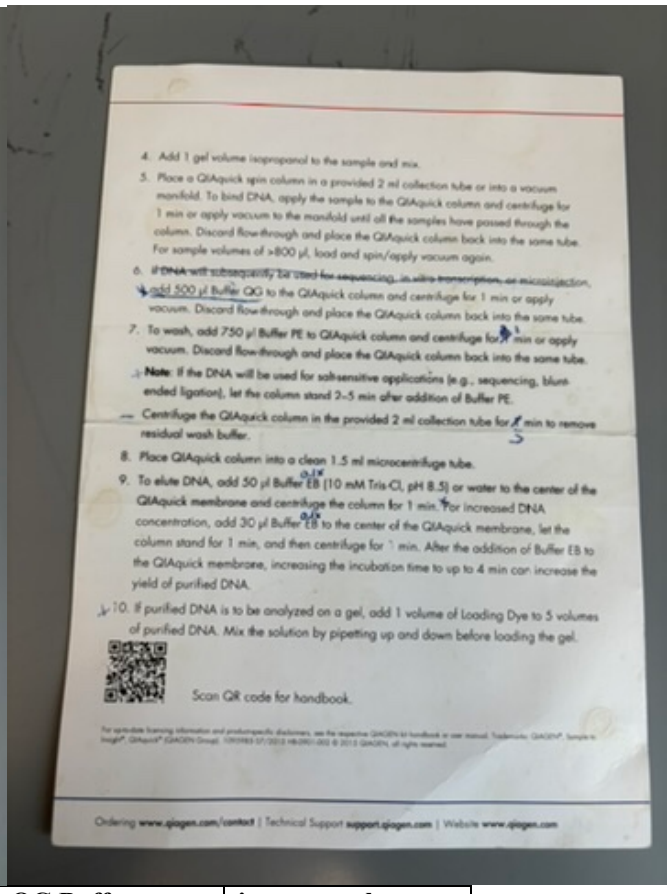
Friday, January 26, 2024

To Do:

1. ~~Gel extract/purify.~~
2. Ligation set up.
3. Transform into E. coli.

QIAquick Gel Extraction Protocol





	tube weight (g)	Tube w gel(g)	gel weight (mg)	QG Buffer Volume (uL)	isopropanol volume (uL)
tube 1	0.97	1.34	370	1110	370
tube 2	0.97	1.39	420	1260	420

Thoughts & Observations

I finished the purification protocol and left my samples in the freezer over the weekend.
Edit: 1/29/2024. I realized I did not check the concentrations of my gel purified extracts on the nanodrop.

Monday, January 29, 2024

To Do:

1. ~~Gel extract/purify.~~
2. Ligation set up.
3. Transform into E. coli.

Ligation Protocol

The purpose of this is to ligate the cut plasmid backbone to the strand of PCR DNA.

1. Make a reaction table with desired ligations. Always include a backbone only control for each plasmid backbone used.

Tube	Insert	Backbone
1	NotI-HF digested pKR184 PCR pure	NotI-HF digested, purified pKR11

2	-	NotI-HF digested, purified pKR11

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (3x)
H_2O	11.5	11.5	34.5
10x ligase buffer	2.0	2.0	6
Insert	4.0	-	-
Backbone	2.0	2.0	6
Ligase	0.5	0.5	1.5
TOTAL	20.0	20.0	48

3. Obtain ice to assemble and keep the reactions on. This is important, as the reaction happens at 16°C and the ligase buffer (which contains ATP) needs to be kept cold to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
4. Obtain and label PCR tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H_2O (___uL), 10x buffer (_____uL), insert (____uL), and backbone (____uL).
6. Add indicated amount of ligase (___uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Place in the thermocycler or water bath overnight at 16°C. **Can do at room temp for 10 minutes if necessary. (Place in -20 after incubation)**

Transformation

1. Set up reaction table. **Always include a positive and negative control for each antibiotic**. If transforming plasmids (from previous plasmid prep), use 0.5 – 1 uL of plasmid. If transforming ligations, use 8 uL per ligation. If transforming plasmid, plate 20 uL and 100 uL. If transforming a ligation, plate 100 uL and remaining culture.
    - **Note**: The plasmid used for the positive control plates should be the regular circular plasmid, not the digest/purified one.
    - There should always be a backbone control if testing a ligation. This is the digested and ligated backbone with NO INSERT.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	(+) control	pKR11	1 uL	100 ul	1
2	(-) control	None	0	100 ul	1
3	Backbone Ligation	pKR11 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1	pKR201	8 uL	100 uL, remaining	2
				Total number of plates	6

2. Check to be sure you have enough LB-Kan plates with appropriate antibiotic. If plates were stored at 4°C, warm at 37°C until needed.
3. Obtain DNA and thaw on ice if necessary.
4. Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
5. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
6. When competent cells are thawed (check by probing for frozen cells using a sterile pipette tip), gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.

7. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
8. Place tubes with cells and DNA onto 42°C heat block for 30 seconds (heat shock step).
9. After heat shock, place tubes back on ice until next step (don't keep them here too long).
10. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
11. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
12. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
13. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry. For "remaining" volume, spin tubes at max speed in benchtop centrifuge for 30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.

Making Hemoglobin 2% Solution

1. Add 6g freeze-dried hemoglobin to a 500 mL flask along with a large stir bar.
2. Add 300mL of ddiH_2O (type I).
3. Place the flask on a stir plate and stir for a few minutes to dissolve the hemoglobin.
4. Add aluminum foil and autoclave tape to the top of the flask.
5. Autoclave on a 20-minute liquid cycle with water in the bin.
6. Cool down (ideally to ~55C – can be in water bath set to 56C) and store in 4C fridge.

Tuesday, January 30, 2024

To Do:

1. ~~Ligation set up.~~
2. ~~Spread plate.~~
3. Transform into E. coli.

Thoughts & Observations:

Today I stopped in to check on the spread plates Ben did from my ligation experiment last night. The results were not good, so I decided to trash the plates and repeat the experiment starting from the digest. Tomorrow I will digest and gel purify. I will weigh and save the gel chunks for purification and ligation on Thursday.

Edit: 1/31/24- Plate count estimates:

Ligation 1: ~10 colonies

Backbone: ~10 colonies

I did not get an exact count, but these are estimates of what was on the REM plates. These are most likely background colonies since there are equal amounts on both plates. *Think of another source of error- religation of the backbone is not likely since the growth efficiency was low.

Wednesday, January 31, 2024

To Do:

1. Digest.
2. Run digested products on gel.
3. Cut out gel bands, weigh, and put in the fridge.
4. Ligation.

Digest Protocol

10. Make a reaction table with desired digests:

tube	DNA	Enzyme(s)	DNA Volume (uL)	H2O volume (uL)
1	pKR184 PCR Pure	NotI-HF	15	n/a
2	pKR11 backbone	NotI-HF	5	10

11. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H_2O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
Enzyme 1	1.2	3.6
Total	**30.0 (15.0 actual b/c of DNA)**	**45**

*Cutsmart Buffer is used for all the "HF" enzymes. Check the NEB website for buffer compatibility with non-HF restriction enzymes.

12. Add indicated amounts of H_2O and 10x buffer to master mix tube (MM).
13. Add indicated amount of DNA or DNA+water (for backbone tubes) to individual tubes for digest (1.5 mL microfuge tubes for digests in incubator, PCR strip tubes for reactions incubated in the thermocycler).
14. Add indicated amount of each enzyme (___uL) to the master mix tube (MM).
15. Mix the master mix by pipetting up and down.
16. Add master mix to individual tubes. Volume to add to each tube is the volume in 1 reaction minus the DNA volume (____ul).
17. Incubate at 37°C for 1 hour or up to overnight.
18. If using digest for plasmid construction then after incubation at 37°C, add 0.5ul of CIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes. (this step removes the phosphates from the ends of the plasmid to prevent re-ligation). **If using the new Quick CIP – add 1 uL, mix, incubate for 10 mins, then put at 80C for 2 minutes to inactivate the CIP.**

Thoughts & Observations:

Gel purify and extract before ligation.
Gel results:

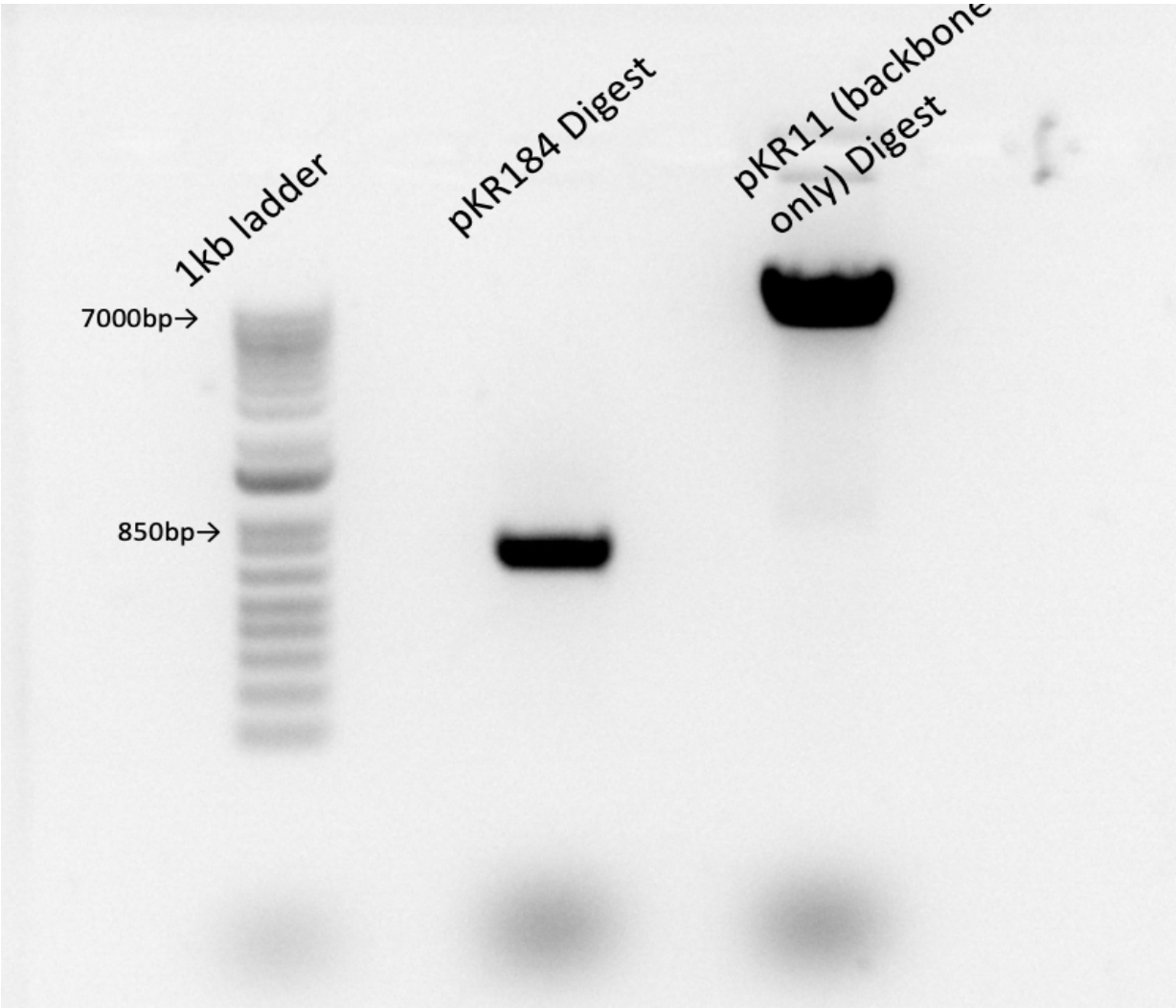


Figure 1. Digested products run on gel (before extraction).

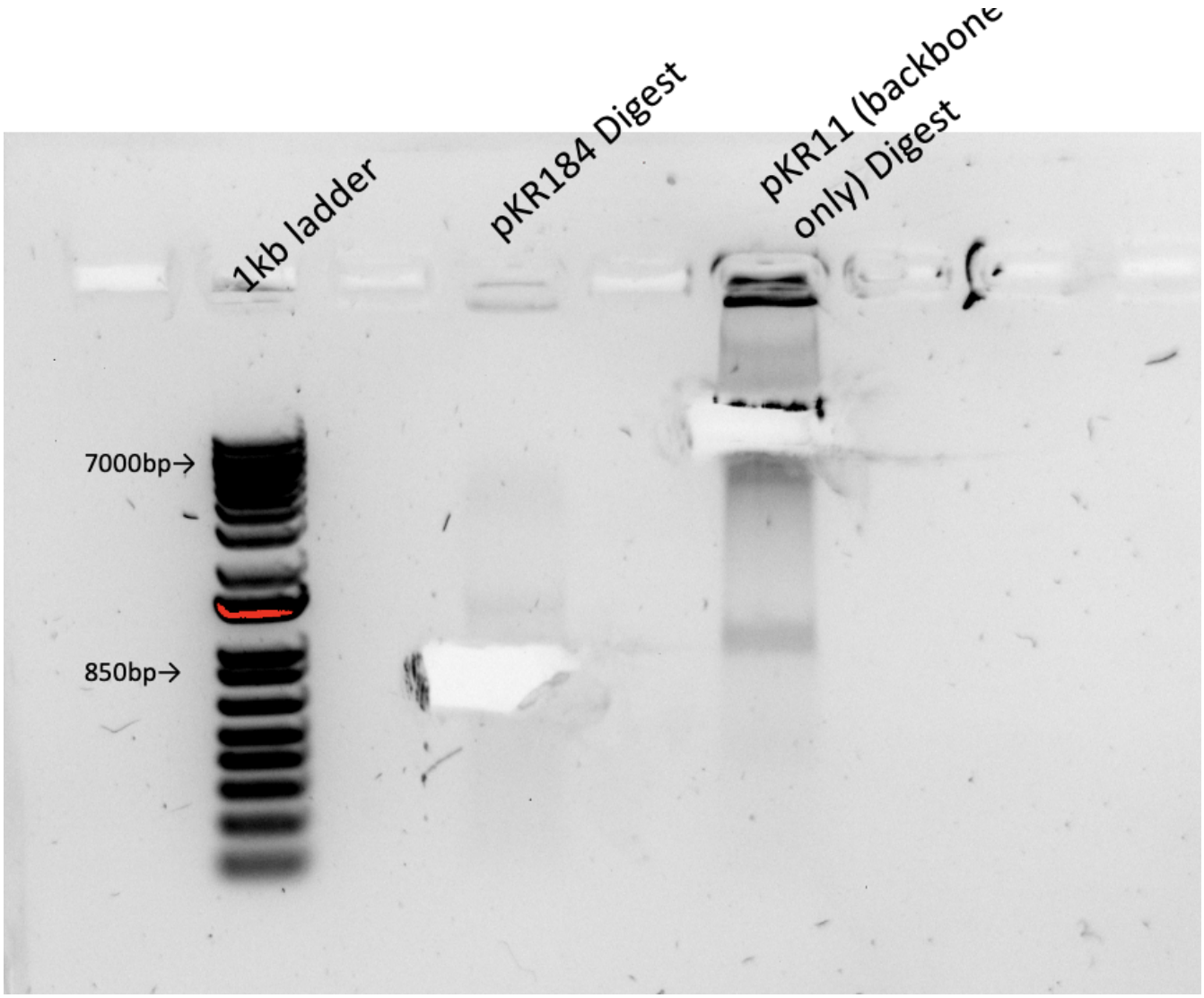


Figure 2. Gel image showing extraction points.

Gel Purification (Digest)

9. Pour about 100 mL of agarose gel into the gel rig set up.
10. Add 12 uL of SybrSafe.
11. Let the gel solidify, then place the gel in the correct position.
12. Pour TAE buffer until it completely covers the gel.
13. Add 6 uL of loading dye to the 30 uL digest reaction tubes.
14. Load 10 uL of the DNA ladder in the first well.
15. Leave a space between wells and load the full volume of the reaction tubes.
16. Run at 113V for an hour.

Thursday, February 1, 2024

To Do:

1. ~~Digest.~~
2. ~~Gel extract.~~
3. Gel purify.

4. Ligation.
5. Spread plate.
6. Repeat PCR for more PCR pure pKR184.

QIAquick Gel Extraction

*See QIAquick Gel Extraction kit

	tube weight (g)	Tube with gel(g)	gel weight (mg)	QG Buffer Volume (uL)	isopropanol volume (uL)
tube 1	0.95	1.21	260	780	260
tube 2	0.99	1.17	180	540	180

Monday, February 5, 2024

To Do:

1. ~~Ligation.~~
2. Spread plate.
3. Repeat PCR for more PCR pure pKR184.

Ligation Protocol

The purpose of this is to ligate the cut plasmid backbone to the strand of PCR DNA.

1. Make a reaction table with desired ligations. Always include a backbone only control for each plasmid backbone used.

Tube	Insert	Backbone
1	NotI-HF digested pKR184 PCR pure	NotI-HF digested, purified pKR11
2	-	NotI-HF digested, purified pKR11

2. Set up master mix table:

	3X	5X	BB only
Water	10.31	4.81	12.43
Ligation Buffer	2	2	2
Backbone	5.07	5.07	5.07
Insert	2.12	7.62	-
Ligase	0.5	0.5	0.5
	20.00	20.00	20.00

See page 27 of my notebook for full protocol.

Nanodrop concentrations used in ligation calculation:

Sample ID	User name	Date and Time	Nucleic Acid	Unit
pKR184	Science	2/5/2024 2:28:34 PM	32.8	ng/µl
pKR11	Science	2/5/2024 2:30:01 PM	49.3	ng/µl

Ligation calculator below:

Ligation Calculator

ng vector	sample name	ratio of insert/bb bps	sample concentration	molar ratio	ng of insert	size of fragment	backbone size
50	pKR11	0.092822724		1	4.641136191	732	7886
50	ΔrpsU2 -pEX-GFP	0.092822724		3	13.92340857	732	7886
50	ΔrpsU2 -pEX-GFP	0.092822724		5	23.20568095	732	7886
		concentration	Concentration 1:5	ng needed	uL needed		
3X		32.8	6.56	13.92340857	2.12		
5X		32.8	6.56	23.20568095	7.62		
backbone		49.3	9.86		5.07		
		3X		5X	BB only		
Water		7.76		10.31	15.38		
Ligation Buffer		2		2	2		
Backbone		2.12		2.12	2.12		
Insert		7.62		5.07	-		
Ligase		0.5		0.5	0.5		
		20.00		20.00	20.00		

Ligation Calculator							
ng vector	sample name	ratio of insert/bb bps	sample concentration	molar ratio	ng of insert	size of fragment	backb size
50	pKR11	0.092822724		1	4.641136191	732	7886
50	ΔrpsU2 - pEX-GFP	0.092822724		3	13.92340857	732	7886
50	ΔrpsU2 - pEX-GFP	0.092822724		5	23.20568095	732	7886
		concentration	Concentration 1:5	ng needed	uL needed		
3X		32.8	6.56	13.92340857	2.12		
5X		32.8	6.56	23.20568095	7.62		
backbone		49.3	9.86		5.07		

		3X		5X	BB only		
Water		7.76		10.31	15.38		
Ligation Buffer		2		2	2		
Backbone		2.12		2.12	2.12		
Insert		7.62		5.07	-		
Ligase		0.5		0.5	0.5		
		20.00		20.00	20.00		

Protocol Modifications

To perform this protocol, I used three microfuge tubes, one for the insert dilution, one for the backbone dilution, and one for a master mix containing the (diluted) backbone, ligase buffer, and the ligase. I added the indicated amounts of water to labeled PCR tubes. In the microfuge tubes I did a 1:5 dilution of the insert DNA, using 4 uL of DNA to 16 uL of 0.1x EB. I did the same dilution at a higher volume for the backbone. After diluting, I added the indicated amounts of DNA to the PCR tubes and mixed with water, then added the master mix (ligase buffer and ligase were added last to the master mix to preserve integrity).
After incubating at room temp for 10 minutes I placed the PCR tubes in the -20 in my cloning box.

Wednesday, February 7, 2024

To Do:

1. ~~Ligation.~~
2. ~~Transform.~~
3. Spread plate.
4. Repeat PCR for more PCR pure pKR184.

Transformation

14. Set up reaction table. **Always include a positive and negative control for each antibiotic**. If transforming plasmids (from previous plasmid prep), use 0.5 – 1 uL of plasmid. If transforming ligations, use 8 uL per ligation. If transforming plasmid, plate 20 uL and 100 uL. If transforming a ligation, plate 100 uL and remaining culture.
    - **Note**: The plasmid used for the positive control plates should be the regular circular plasmid, not the digest/purified one.
    - There should always be a backbone control if testing a ligation. This is the digested and ligated backbone with NO INSERT.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	(+) control	pKR11	1 uL	100 ul	1
2	(-) control	None	0	100 ul	1
3	Backbone Ligation	pKR11 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1 (3x)	pKR201	8 uL	100 uL, remaining	2
5	Ligation 2 (5x)	pKR201			2
				Total number of plates	8

15. Check to be sure you have enough LB-Kan plates with appropriate antibiotic. If plates were stored at 4°C, warm at 37°C until needed.
16. Obtain DNA and thaw on ice if necessary.
17. Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
18. Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
19. When competent cells are thawed (check by probing for frozen cells using a sterile pipette tip), gently pipette 100 uL of cells into each reaction tube directly onto DNA using aseptic technique.
20. Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
21. Place tubes with cells and DNA onto 42°C heat block for 30 seconds (heat shock step).
22. After heat shock, place tubes back on ice until next step (don't keep them here too long).
23. Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
24. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
25. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
26. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry. For "remaining" volume, spin tubes at max speed in benchtop centrifuge for 30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.

Thoughts & Observations:

This transformation will have an extra two plates since we had to change the concentrations for the ligation. The ligation has not been working, so we decided to do a 3x ligation and a 5x ligation in hopes for better results on the transformation.
After spread plating with the sterile beads, I dried the plates by the Bunsen burner and placed them into the 37 degree incubator overnight.

Thursday, February 8, 2024

To Do:

1. ~~Ligation.~~
2. Transform.
3. Spread plate.
4. Overnight culture for pKR11 (working stock).

Transformation

See page 31 of my lab notebook for steps.
Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	(+) control	pKR11 (original stock)	1 uL	20 uL, 100 ul	2
2	(-) control	None	0	100 ul, remaining	2
				Total number of plates	4

Protocol Modifications

This transformation is a test to confirm the identity of pKR11. As seen in my digest gel results, there is a faint band in the lane where pKR11 was run. This band should not be there since there is only one NotI cut site on the plasmid, meaning there would not be a fragment produced. To test this, I am using a special aliquot of the original pKR11 stock from the -80 freezer as my positive control. Dr. Ramsey diluted the plasmid 1:10 with 0.1x EB. After doing the spread plate I place my plates in the 37 degree incubator.
*I accidentally added only 800uL of LB media instead of 1mL for recovery.

After I did the spread plating, I did overnight stock cultures of the pKR11 plate from my last transformation on Wednesday (2/7). First, I used two culture tubes, and added 5 mL of LB media to each, then 5 uL of kanamycin to each. I inoculated each tube with one colony from my plate. I then set them in the shaker at 37 degrees overnight.

Friday, February 9, 2024

To Do:

1. ~~Ligation.~~
2. ~~Transform.~~
3. Spread plate.
4. Overnight culture for pKR11 (original stock).

Pelleting Cells from overnight culture

My overnight cultures from Thursday were ready to be pelleted. I used two 2mL sterile tubes. Aseptically I added 2mL of my overnight culture to their respective tubes, then spun them in the centrifuge at 13,000 rpm for three minutes. I decanted the supernatant, then repeated the process by adding 2mL more stock to each tube and spinning again, then decanting. I was left with large cell pellets in my microfuge tubes which I placed in the -20 freezer in my cloning box.

After journal club, I did overnight stock cultures of the pKR11 (original stock) plate from my last transformation on Thursday (2/8). First, I used two culture tubes, and added 5 mL of LB media to each, then 5 uL of kanamycin to each. I inoculated each tube with one colony from my plate. I then set them in the shaker at 37 degrees overnight. Ben will be in on Saturday to pellet my cells for me, then put them in the -20 freezer until Monday.

Monday, February 12, 2024

To Do:

1. ~~Pellet cells~~
2. Mini prep
3. Prep for sequencing.

Miniprep

*See pg. 18-19 for QIA miniprep kit instructions

Table 1. Nanodrop results for the miniprep on pKR11 working stock (201 and 202) and pKR11 original stock (OG1 and OG2). My DNA concentrations were really high so I had to dilute them before putting together the sequencing reaction.

1	pKR11_201	534.7	ng/µl	10.694	5.726	1.87	2.3	DNA
2	pKR11_202	607.9	ng/µl	12.158	6.341	1.92	2.41	DNA
3	pKR11_OG1	690.5	ng/µl	13.809	7.405	1.86	2.33	DNA
4	pKR11_OG2	745.6	ng/µl	14.913	7.832	1.9	2.35	DNA

Table 2. Nanodrop results for the 1:10 dilution of plasmid DNA to be used in the sequencing reaction. For the dilution I used 2uL of DNA and 18 uL of 0.1x EB in microfuge tubes to then assemble the sequencing reaction in the PCR tubes.

1	pKR11_201	54	ng/µl	1.08	0.604	1.79	2.35	DNA
2	pKR11_202	60.1	ng/µl	1.202	0.665	1.81	2.38	DNA
3	pKR11_OG1	71.6	ng/µl	1.432	0.787	1.82	2.3	DNA
4	pKR11_OG2	76.6	ng/µl	1.532	0.853	1.8	2.29	DNA

Send for sequencing

Table 3. Sequencing reaction table.

Sample number	**Well**	**Template Type**	**Template Name**	**Primer Name**	**A.**	**B.**	**E.**	**F.**
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/µl)	**PLASMID** template:	Volume H_2O **needed**
CS1		plasmid	pKR11	KROL7	7886	54	7.41	2.03
CS2		plasmid	pKR11	KROL7	7886	60.1	6.66	2.78
CS3		plasmid	pKR11	KROL7	7886	71.6	5.59	3.85
CS4		plasmid	pKR11	KROL7	7886	76.6	5.22	4.22
a.Add 2.56 µl of 2.5 µM stock to each reaction								

Indications:
CS1= pKR11 working stock, number 1
CS2= pKR11 working stock, number 2
CS3= pKR11 original stock, number 1
CS4= pKR11 original stock, number 2

Wednesday, February 14, 2024

To Do:

1. ~~Mini prep~~
2. ~~Prep for sequencing.~~
3. ~~Make hemoglobin 2% solution.~~
4. Set up diagnostic digest.

Making Hemoglobin 2%

*See pg 27 of my notebook.
I made four flasks of hemoglobin 2%.

Diagnostic Digest

*See pg 28-29 for DNA digest protocol.

tube	**DNA**	**Enzyme(s)**	**DNA Volume (uL)**	**H2O volume (uL)**
1	pKR11 (-80) backbone	NotI-HF	5	10

2	pKR11 (-80) backbone	NotI-HF	5	10

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H_2O	10.8	32.4
10x Buffer*	3.0	9.0
DNA	(15.0)	-
Enzyme 1	1.2	3.6
Total	**30.0 (15.0 actual b/c of DNA)**	**45**

*I may have the hour incubation time slightly off for these samples. I set the timer, but it didn't start immediately, but the difference in time should be minimal.

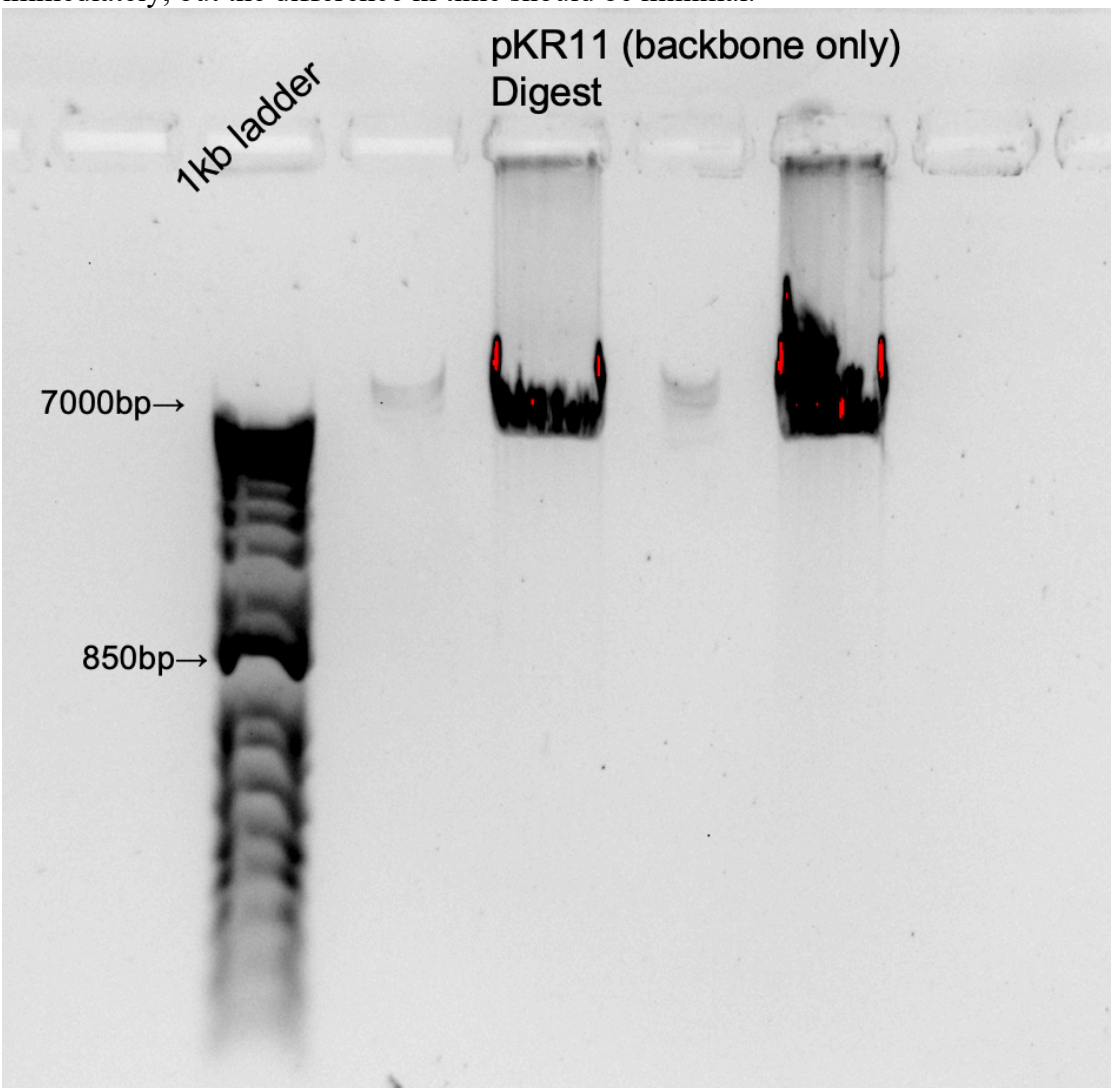


Figure 1. Gel imaging from digest. There is no second band, but I seem to have had a "loading accident" which is why the bands look so distorted and smudgy and carried over to the next-door lanes.

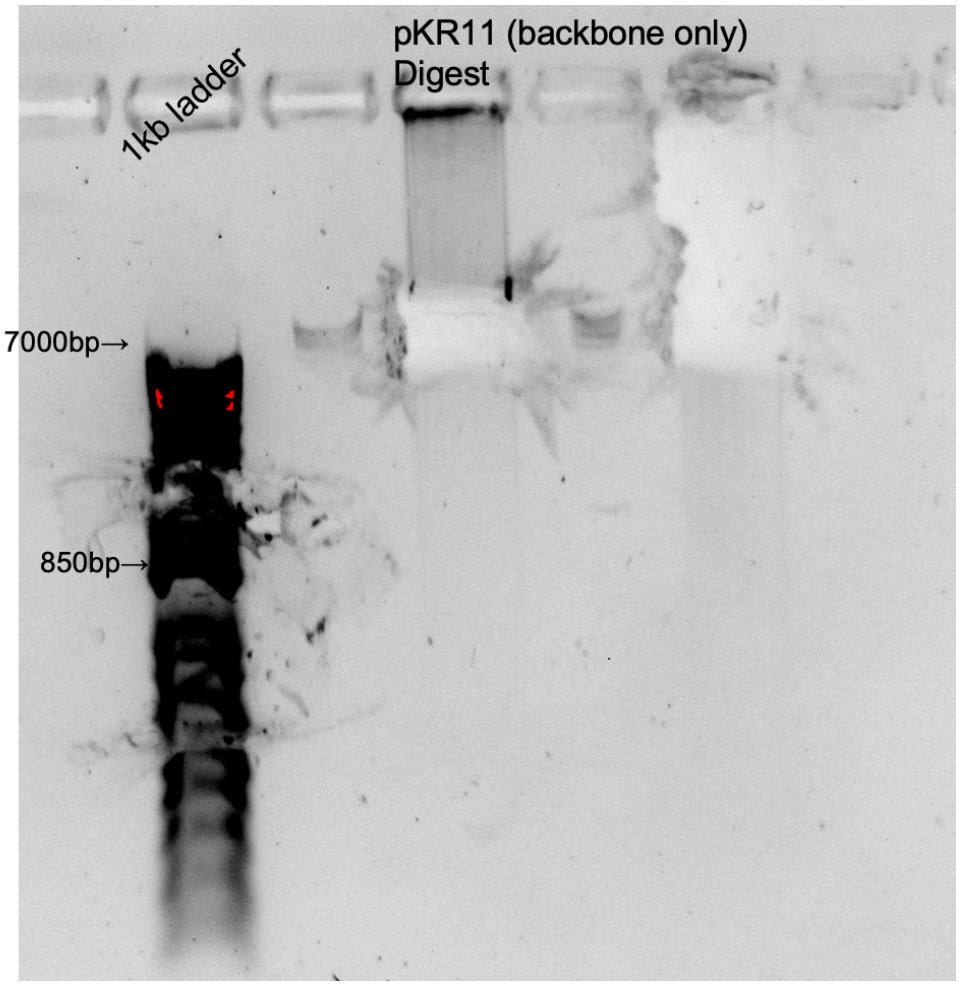


Figure 2. Cutout imaging of gel digest.

Thursday, February 15, 2024

To Do:

1. ~~Set up diagnostic digest.~~
2. Gel purify.
3. Ligation.
4. Transformation.

Gel Purification

*See pg. 25 for gel extraction/purification protocol

	tube weight (g)	Tube w gel(g)	gel weight (mg)	QG Buffer Volume (uL)	isopropanol volume (uL)
tube 1	0.98	1.12	140	420	140
tube 2	0.99	1.17	180	540	180
Tube 3	0.97	1.32	350	1050	350

Table 1. Nanodrop results for gel purified pKR11 (original stock).

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	pKR11OG1_pure	4.4	ng/µl	0.087	0.027	3.24	0.02
2	pKR11OG2_pure	14.3	ng/µl	0.287	0.135	2.12	0.05

Thoughts & Observations:

The DNA concentration is super low, likely due to the loading accident I had when running the gel. I don't think this will be acceptable to use for a ligation so I will have to repeat the gel and purification.

Ligation

*See pg. 27

Protocol Modifications:

Kira gave me pKR7 (backbone) and V5 (insert) to use as a control. I actually messed up on the control reactions because I accidentally added extra backbone to the insert reaction tube. I also realized I didn't have enough LB-kan plates to do a transformation today, so I'm going to do the overnight ligation protocol. I left the reaction tubes in the thermal cycler at 16 degrees C for tomorrow. I'm going to make the media I need in the meantime and I will pick up with the transformation tomorrow night.

Making LB-Kan Plates

1. Keep antibiotic stocks on ice. For kanamycin, add 500 uL to 500 mL of LB-agar media. Stir on stirplate to mix media.
2. Open a new sleeve of sterile plates. Don't open plates unless next to flame.
3. Next to flame, open foil top of flask. Pass flask opening through flame.
4. Open a plate next to flame and pour ~20-25 mL LB-agar into plate; the bottom of the plate should be covered in media.
5. Continue to pour media into plates until media is gone.
6. Immediately rinse out flask
7. Stack plates and mark the side to indicate which antibiotic is used. (blue = kanamycin, black = carbenicillin / ampicillin).
8. Let plates sit at room temperature for one night, then store at 4°C with tape indicating date poured. Don't use antibiotic plates older than 3 months.

Friday, February 16, 2024

To Do:

1. ~~Set up diagnostic digest.~~
2. ~~Gel purify.~~
3. ~~Ligation~~.
4. Transformation.
5. Make overnight cultures.

Transformation

See page 31 of my lab notebook for transformation protocol.

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	(+) control	pKR11 (original stock)	1 uL	100 ul	1
2	(-) control	None	0	100 ul	1
3	Backbone only (pKR11)	pKR11 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1 (pKR201)	pKR201 ligated	8 uL	100 uL, remaining	2

5	Backbone only (pKR7)	pKR7 (digested)	8 uL	100 uL, remaining	2
6	Ligation 2 (V5)	V5 ligated	8 uL	100 uL, remaining	2
				Total number of plates	10

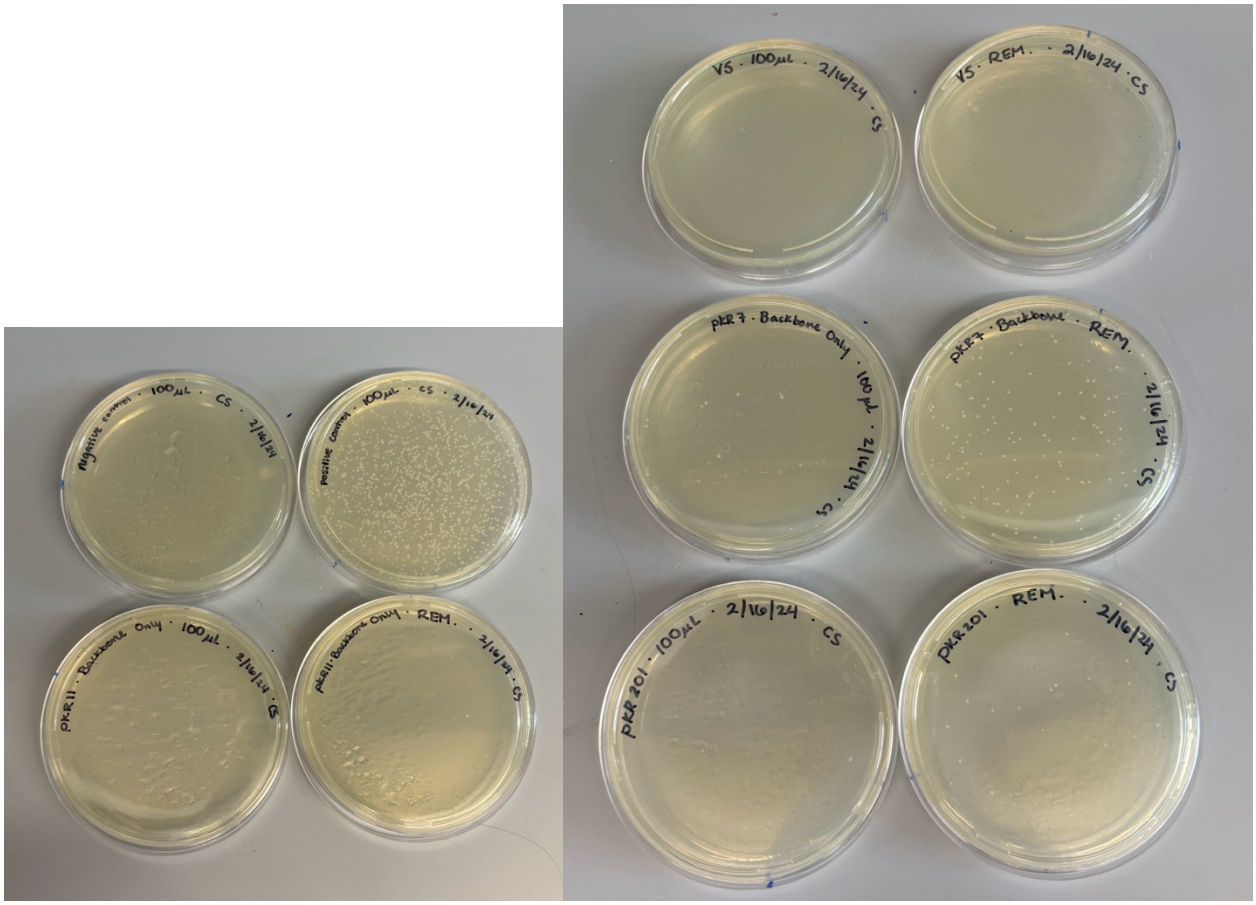
The transformations were plated and placed in the 37-degree incubator overnight.

Saturday, February 17, 2024

To Do:

1. ~~Transformation.~~
2. Make overnight cultures.

Plate results:



There is a small amount of colonies on pKR201, but it worked! So I decided to make three overnight cultures from the pKR201 plate.

Table 1. Plate counts for transformation.

(-)	(+)	pKR7 (100uL)	pKR7 REM	V5 (100uL)	V5 REM	pKR11 (100uL)	pKR11 REM	pKR201 (100uL)	pKR201 REM
0	TMTC	13	140	0	2	0	3	3	21

Making overnight stocks

*see pg. 35 for O/N culture protocol

I chose three colonies to make overnight cultures from. I left them in the shaking incubator at 37 degrees in the M. Ramsey lab overnight.

Sunday, February 18, 2024

To Do:

1. ~~Make overnight cultures.~~
2. Pellet cells and miniprep.

Pelleting cells

*See pg. 35

Miniprep

*See pg. 18-19 for QIA miniprep kit instructions

Table 1. Nanodrop results from miniprep.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
1	1) pKR201	264.9	ng/µl	5.297	2.805	1.89	2.35	DNA
2	2) pKR201	273.7	ng/µl	5.475	2.922	1.87	2.3	DNA
3	3) pKR201	237.8	ng/µl	4.756	2.529	1.88	2.36	DNA

Tuesday, February 20, 2024

To Do:

1. ~~Make overnight cultures.~~
2. ~~Pellet cells and miniprep.~~
3. Send minipreps for sequencing.
4. Miniprep 3 more cultures.
5. Run diagnostic digest.

To sequence pKR201 – KROL121
Do I have insert and is it in the right direction?
*20 uL DNA reaction digest
NcoI & BamHI – 705 bp for correct product
Incorrect product: 1802bp
Use pKR11 for control – only 1 fragment
BACKUP- EcoRV
Correct orientation: Fragments at 4742 and 3867; Wrong: 5015 and 3594

Overnight cultures

*See pg. 35

Diagnostic Digest

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR201	NcoI-HF, BamHI-HF	3	7
2	pKR201	NcoI-HF, BamHI-HF	3	7
3	pKR201	NcoI-HF, BamHI-HF	3	7

4	pKR11	NcoI-HF, BamHI-HF	3	7

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 5x (uL)
H_2O	7.2	36
10x Buffer* (CutSmart)	2	10
DNA	(10)	-
Enzyme 1	0.4	2
Enzyme 2	0.4	2
Total	20.0 (10.0 actual b/c of DNA)	

*do not perform quickCIP step for diagnostic digests

Tuesday, February 20, 2024

To Do:

1. ~~Make overnight cultures.~~
2. ~~Pellet cells and miniprep.~~
3. Send minipreps for sequencing.
4. Miniprep 3 more cultures.
5. Run diagnostic digest.

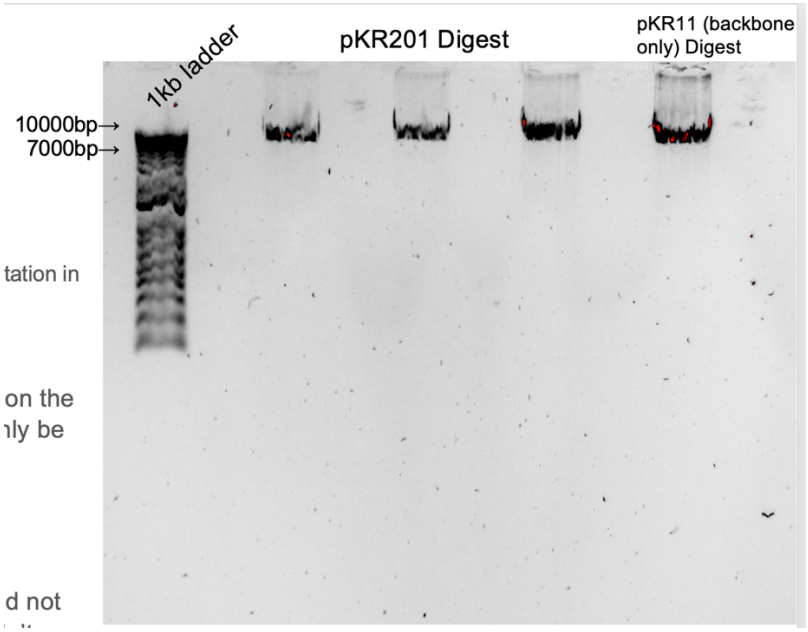


Figure 1. Diagnostic digest gel results. The agarose I used was chunky and weird so the resolution is not good. The three pKR201 lanes should have two bands in each lane to show that the ligation and digest worked correctly but there is only one band. The pKR11 lane is a control, but all the bands seem to match the backbone only, suggesting that the digest or ligation were unsuccessful (at least in the colonies that I chose).

Wednesday, February 21, 2024

To Do:

1. Send minipreps for sequencing.
2. ~~Miniprep 3 more cultures.~~
3. ~~Run diagnostic digest.~~

Miniprep

*See pg. 35

Table 1. Nanodrop results from 3 more colonies of pKR201.

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
4	627.4	ng/µl	12.548	6.755	1.86	2.31	DNA
5	528.3	ng/µl	10.567	5.649	1.87	2.31	DNA
6	891.9	ng/µl	17.838	9.599	1.86	2.31	DNA

Sequencing prep

Table 2. Diluted samples of the miniprep I did earlier today. I did a 1:10 dilution with 2 uL DNA and 18 uL 0.1x EB.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
4	4diluted	80.2	ng/µl	1.603	0.872	1.84	2.39	DNA
5	5diluted	52.8	ng/µl	1.057	0.558	1.89	2.45	DNA
6	6diluted	146.8	ng/µl	2.936	1.586	1.85	2.34	DNA

Table 3. Sequencing reaction setup and calculations.

Sample number	Well	Template Type	Template Name	Primer Name[a]	A.	B.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/µl)	**PLASMID** template:	Volume **H_2O needed**
							Volume =	
							2x(~200 ÷ B)µl	**(12 less D or E - 2.56)µl**
CS4		plasmid	pKR201	KROL121	8609	80.2	4.99	4.45
CS5		plasmid	pKR201	KROL121	8609	52.8	7.58	1.86
CS6		plasmid	pKR201	KROL121	8609	146.8	2.72	6.72

*Note: I will wait for the results of my diagnostic digest today before sending for sequencing.

Diagnostic Digest

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR201	NcoI-HF, BamHI-HF	3	7
2	pKR201	NcoI-HF, BamHI-HF	3	7

3	pKR201	NcoI-HF, BamHI-HF	3	7
4	pKR11	NcoI-HF, BamHI-HF	3	7

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 5x (uL)
H_2O	7.2	36
10x Buffer* (CutSmart)	2	10
DNA	(10)	-
Enzyme 1	0.4	2
Enzyme 2	0.4	2
Total	20.0 (10.0 actual b/c of DNA)	

*See pg. 43-44 for reaction table. Same volumes and number of reactions used. The only difference for today is that I used 3 different colonies that I cultured Tuesday and miniprepped today. Hopefully the results for yesterday's digest were due to a bad selection of colonies.

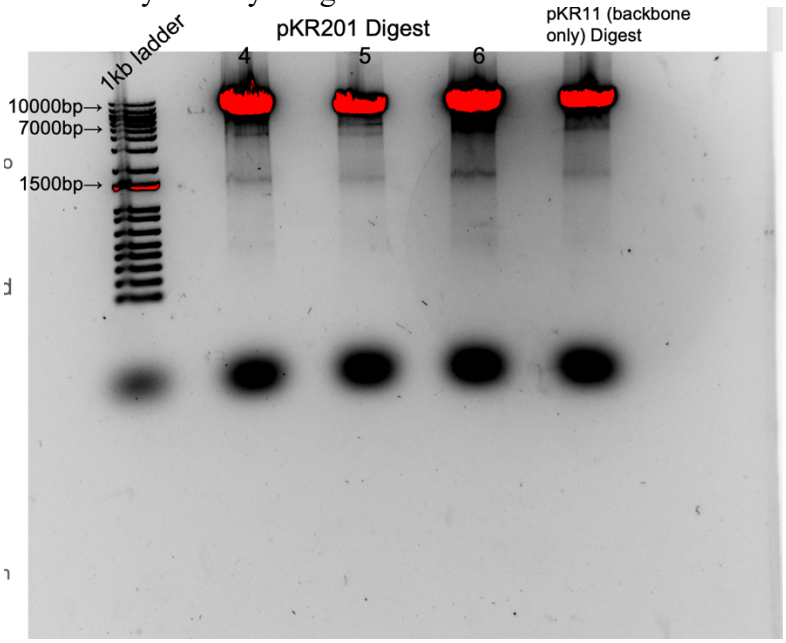


Figure 1. Gel results for second diagnostic digest of pKR201 colonies 4, 5, and 6. Results are confusing. I didn't end up sending any samples for sequencing.

Thursday, February 22, 2024

To Do:

1. Send minipreps for sequencing.
2. ~~Miniprep 3 more cultures.~~
3. Run diagnostic digest.

Diagnostic Digest

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)

1	pKR201	NotI-HF	3	7
2	pKR201	NotI-HF	3	7
3	pKR201	NotI-HF	3	7
4	pKR201	NotI-HF	3	7
5	pKR201	NotI-HF	3	7
6	pKR201	NotI-HF	3	7
7	pKR11	NotI-HF	3	7
8	pKR11	n/a	3	7

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 8x (uL)
H_2O	7.2	57.6
10x Buffer* (CutSmart)	2	16
DNA	(10)	-
Enzyme 1	0.8	6.4
Total	20.0 (10.0 actual b/c of DNA)	80

*Using tube 8 as a control- undigested pKR11

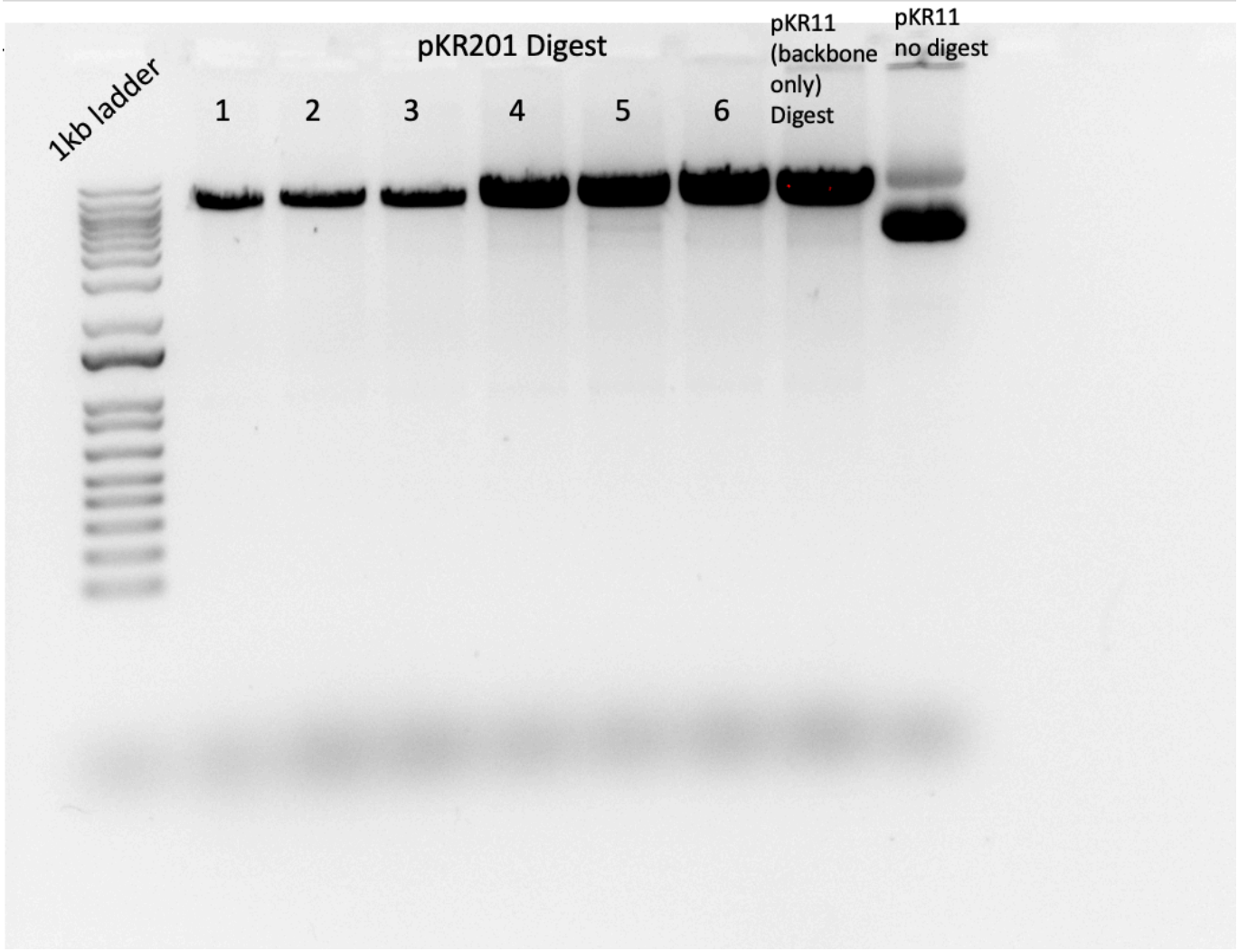


Figure 1. Gel results of diagnostic digest. Results do not show the expected band of insert at ~700 bp.

Friday, February 23, 2024

To Do:

1. ~~Run diagnostic digest.~~
2. Check nanodrops of gel purified product (ethanol contamination?)
3. PCR for new pKR184 insert product. (Monday)

Monday, February 26, 2024

To Do:

1. PCR for new pKR184 insert product.

PCR Protocol

*See PCR protocol in Protocols/PCR/PCRprotocol_new

Total reaction volume	100			
Total number of reactions	3			
				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	4
ddiH2O			62.0	248

PrimeSTAR GXL Buffer	5x	1x	20.0	80
dNTPs	2.5 mM	0.2 mM	8.0	32
oligo F	10 uM	0.3 uM	3.0	12
oligo R	10 uM	0.3 uM	3.0	12
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	8
		Total volume	100	392

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	pKR184	LVS gDNA	KROL524B, KROL667	739
2	- control	-	KROL524B, KROL667	-
3	+ control	LVS gDNA	KROL3, KROL4	731

*I placed the PCR reactions on the thermocycler using STN1 settings. I changed the extension time to 50 seconds instead of 3 minutes since my fragments are only ~730 bp.

Tuesday, February 27, 2024

To Do:

1. ~~PCR purification.~~
2. ~~Run PCR on gel.~~
3. Digest.

PCR purification

*see pg. 8-9 of my lab notebook

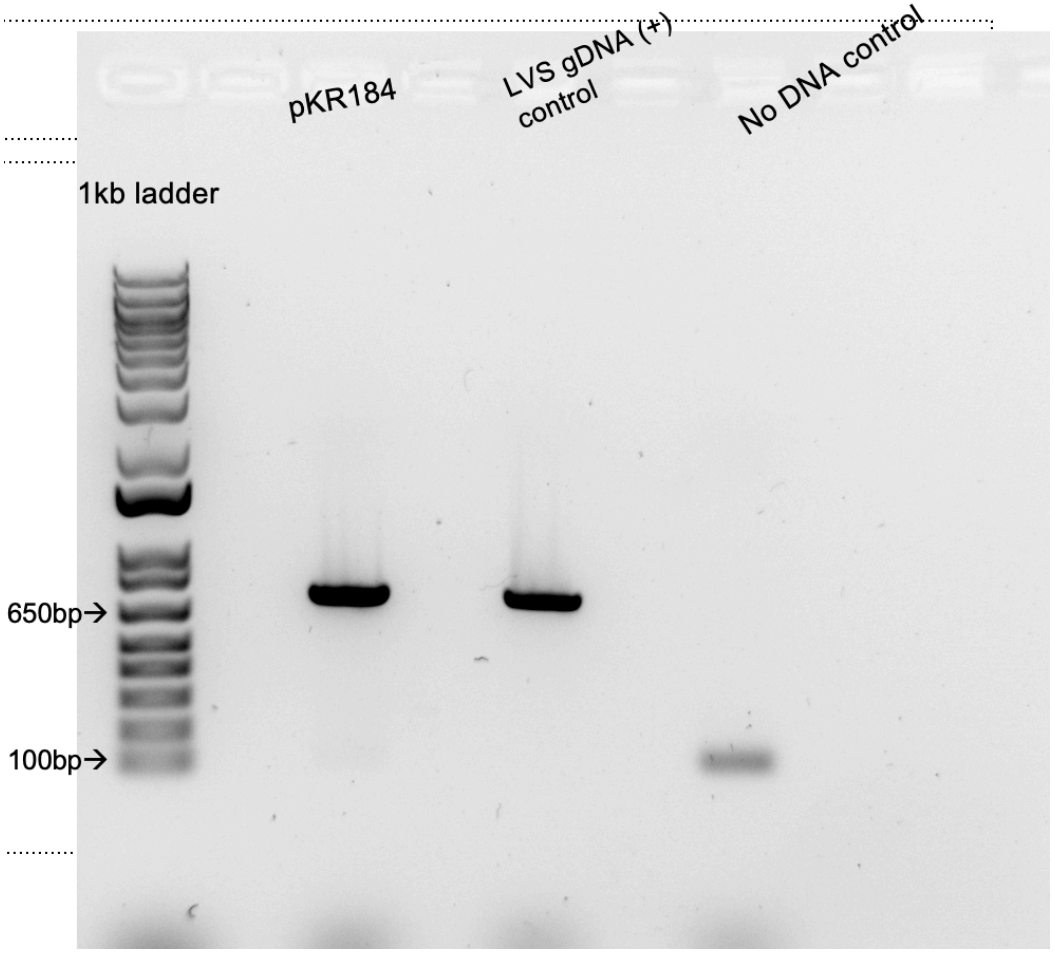


Figure 1. PCR gel results. The expected band size for pKR184 is 731bp which is accurate on the gel. The control is 739bp which also looks accurate. The negative control had only water and should not have any visible bands. Luckily the band is small enough that it is likely leftover primers and not contamination, so I will be moving onto the digest.

Wednesday, February 28, 2024

To Do:

1. ~~Digest.~~
2. ~~Gel extraction~~.
3. Gel purification.
4. Ligation.

Digest

*See pg 28-29 for DNA digest protocol.

tube	DNA	Enzyme(s)	DNA Volume (uL)	H2O volume (uL)
1	pKR184	NotI-HF	15	-
2	pKR11 backbone	NotI-HF	5	10

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H_2O	10.8	32.4
10x Buffer* (cutsmart)	3.0	9.0
DNA	(15.0)	-
Enzyme 1	1.2	3.6
Total	**30.0 (15.0 actual b/c of DNA)**	**45**

*perform quick CIP step for plasmid construction
*After CIP, add 6 uL loading dye to each sample and run the full 36 uL samples on a 100 mL agarose gel for about an hour at 113V.

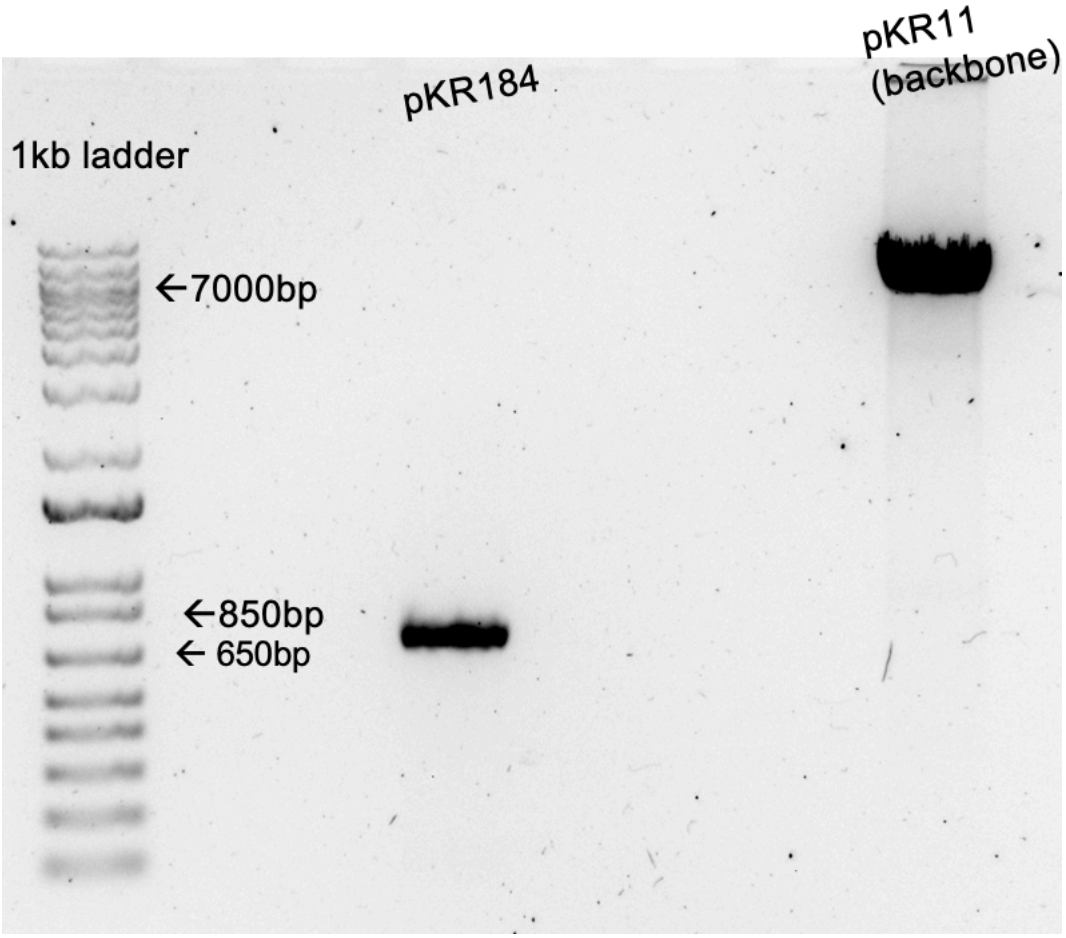


Figure 1. Digest gel results.

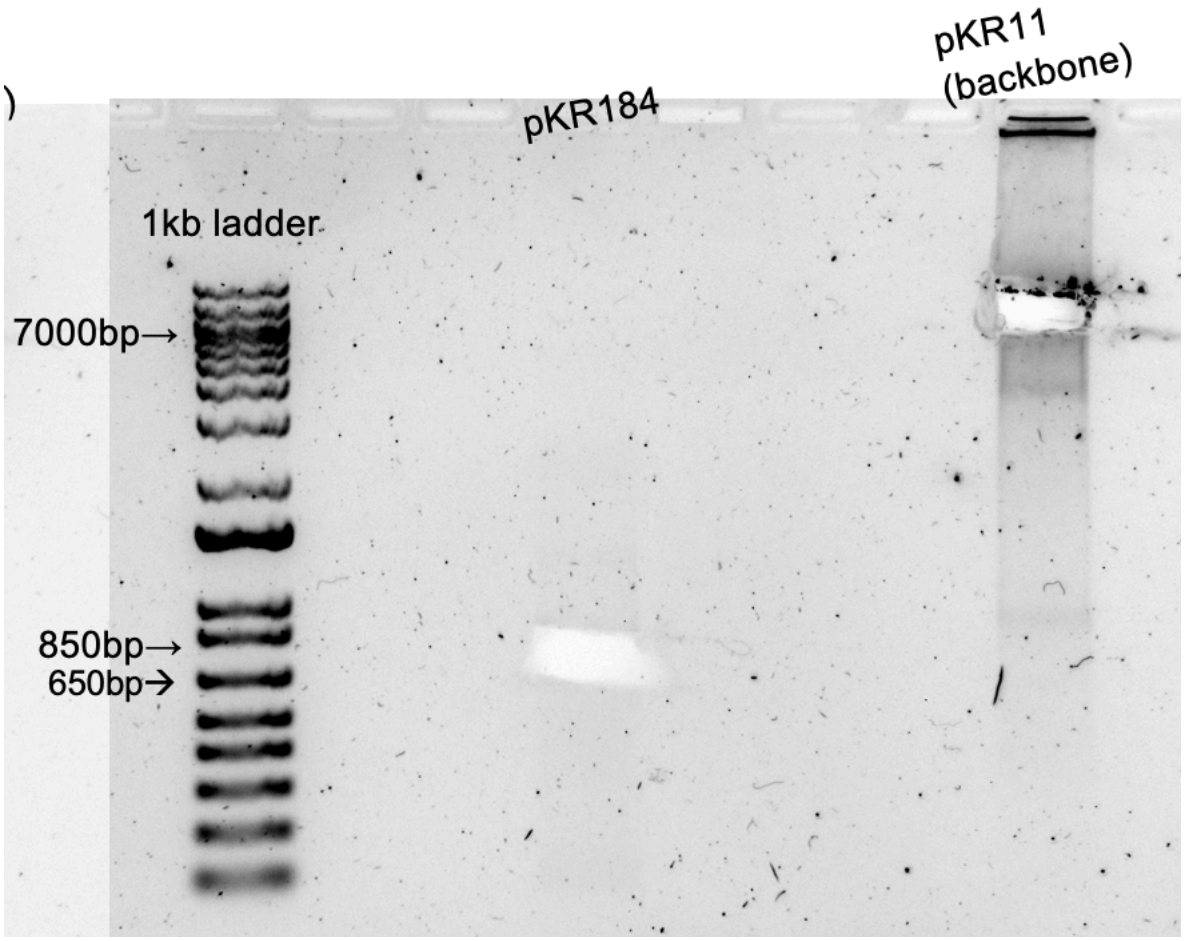


Figure 2. Gel extraction image.

Thursday, February 29, 2024

To Do:

1. ~~Digest.~~
2. ~~Gel extraction.~~
3. Gel purification.
4. Ligation.

Gel purification

*See pg. 26 for QIAquick gel purification protocol

Performed using these volumes:

	tube weight (g)	Tube w gel(g)	gel weight (mg)	QG Buffer Volume (uL)	isopropanol volume (uL)
tube 1	0.99	1.26	270	810	270
tube 2	0.98	1.19	210	630	210

Table 1. Nanodrop results for gel purified digest products.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	pKR184	15.3	ng/µl	0.307	0.154	2	0.62
2	pKR11	22.8	ng/µl	0.456	0.225	2.02	0.3

Ligation

*See pg. 27 of my notebook for full protocol.

Tube	Insert	Backbone
1	NotI-HF digested pKR184 PCR pure	NotI-HF digested, purified pKR11
2	-	NotI-HF digested, purified pKR11
3	V5 insert (from Kira)	pKR7 digested
4	-	pKR7 digested

1. Set up master mix table:

Component	Reaction 1 & 3 (uL)	Reaction 2 & 4 (uL)	Master Mix (5x)
H_2O	11.5	11.5	57.5
10x ligase buffer	2.0	2.0	10
Insert	4.0	-	-
Backbone	2.0	2.0	-
Ligase	0.5	0.5	2.5
TOTAL	20.0	20.0	70

Transformation

*See pg. 31 for Transformation protocol

Reaction table

Tube number	**Purpose**	**DNA**	**Volume of DNA**	**Final volume to plate**	**Number of kanamycin-containing plates**
1	(+) control	pKR11 (original stock)	1 uL	100 ul	1
2	(-) control	None	0	100 ul	1
3	Backbone only (pKR11)	pKR11 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1 (pKR201)	pKR201 ligated	8 uL	100 uL, remaining	2
5	Backbone only (pKR7)	pKR7 (digested)	8 uL	100 uL, remaining	2
6	Ligation 2 (V5)	V5 ligated	8 uL	100 uL, remaining	2
				Total number of plates	10

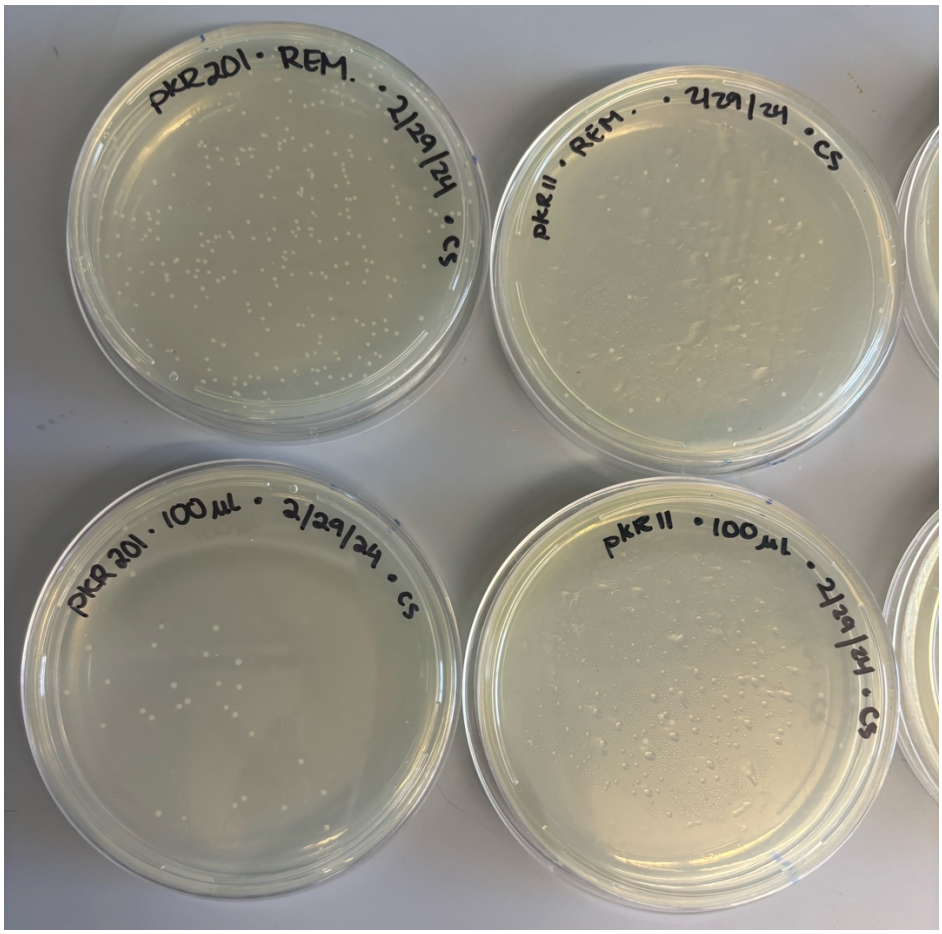
The transformations were spread plated using sterile beads and placed in the 37-degree incubator overnight.

Friday, March 1, 2024

To Do:

1. Ligation.
2. Transformation
3. Plate counts
4. Make cultures

Plate count results:


pKR201 · REM. · 2/29/24 · CS
pKR11 · REM. · 2/29/24 · CS
pKR201 · 100 µL · 2/29/24 · CS
pKR11 · 100 µL · 2/29/24 · CS

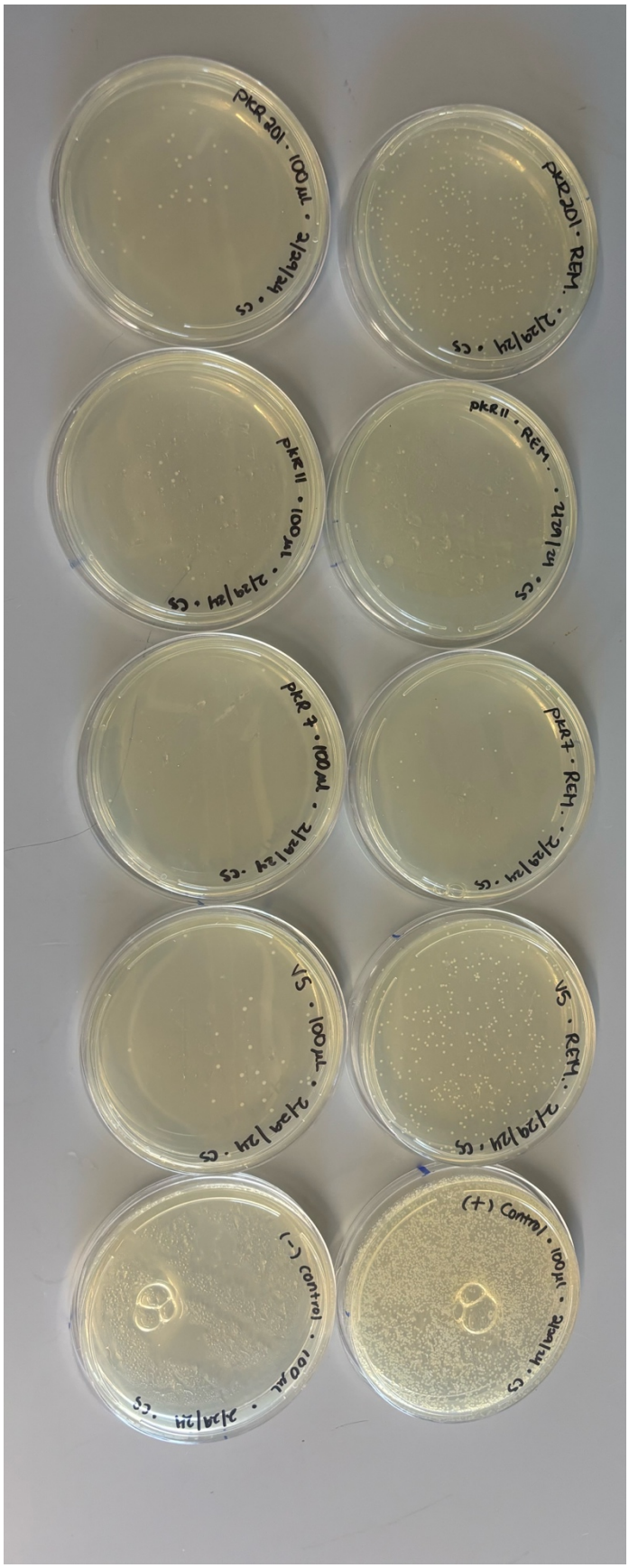

pkR201 · 100 µl · 2/29/24 · CS
pkR201 · REM · 2/29/24 · CS
pkR11 · 100 µl · 2/29/24 · CS
pkR11 · REM · 2/29/24 · CS
pkR7 · 100 µl · 2/29/24 · CS
pkR7 · REM · 2/29/24 · CS
VS · 100 µl · 2/29/24 · CS
VS · REM · 2/29/24 · CS
(-) control · 100 µl · 2/29/24 · CS
(+) Control · 100 µl · 2/29/24 · CS

Table 1. Plate count results.

(-)	(+)	pKR7 (100uL)	pKR7 REM	V5 (100uL)	V5 REM	pKR11 (100uL)	pKR11 REM	pKR201 (100uL)	pKR201 REM
0	TMTC	3	20	28	292	5	35	32	320

Ratios:
pKR201/pKR11: 320/35= 9.14
V5/pKR7: 292/20= 14.6
I made 6 overnight cultures and left them in the shaking incubator at 37 degrees.

Saturday, March 2, 2024

To Do:

1. ~~Make cultures~~
2. Miniprep

Miniprep

*See QIAquick miniprep protocol.
After miniprepping my 6 samples I left them in the -20 degree freezer in my cloning box. I will pick up on Tuesday or Wednesday with a diagnostic digest.

Table 1. Miniprep nanodrop results.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	1	712.1	ng/µl	14.242	7.569	1.88	2.35
2	2	430.4	ng/µl	8.609	4.548	1.89	2.3
3	3	544.4	ng/µl	10.887	5.984	1.82	2.4
4	4	832.4	ng/µl	16.648	8.972	1.86	2.32
5	5	637.3	ng/µl	12.745	6.968	1.83	2.35
6	6	802.7	ng/µl	16.055	8.715	1.84	2.31

Tuesday, March 5, 2024

To Do:

1. ~~Miniprep~~
2. ~~Diagnostic Digest~~
3. Sequencing prep

Diagnostic Digest

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR201	NotI-HF	3	7
2	pKR201	NotI-HF	3	7
3	pKR201	NotI-HF	3	7
4	pKR201	NotI-HF	3	7
5	pKR201	NotI-HF	3	7
6	pKR201	NotI-HF	3	7
7	pKR11	NotI-HF	3	7
8	pKR11	n/a	2	5

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 8x (uL)
H_2O	7.2	57.6
10x Buffer* (CutSmart)	2	16
DNA	(10)	-
Enzyme 1	0.8	6.4
Total	20.0 (10.0 actual b/c of DNA)	80

*Using tube 8 as a control- undigested pKR11. Added 2 uL loading dye to visualize.
I ran the agarose gel at 118V for 35 min.
When loading the gel, I noticed that the wells were not fully formed. They had slight slanting shapes to them, so when I loaded the reactions, they kind of spilled over above the wells, so I predict the image will not be pretty, but the separation should still work.

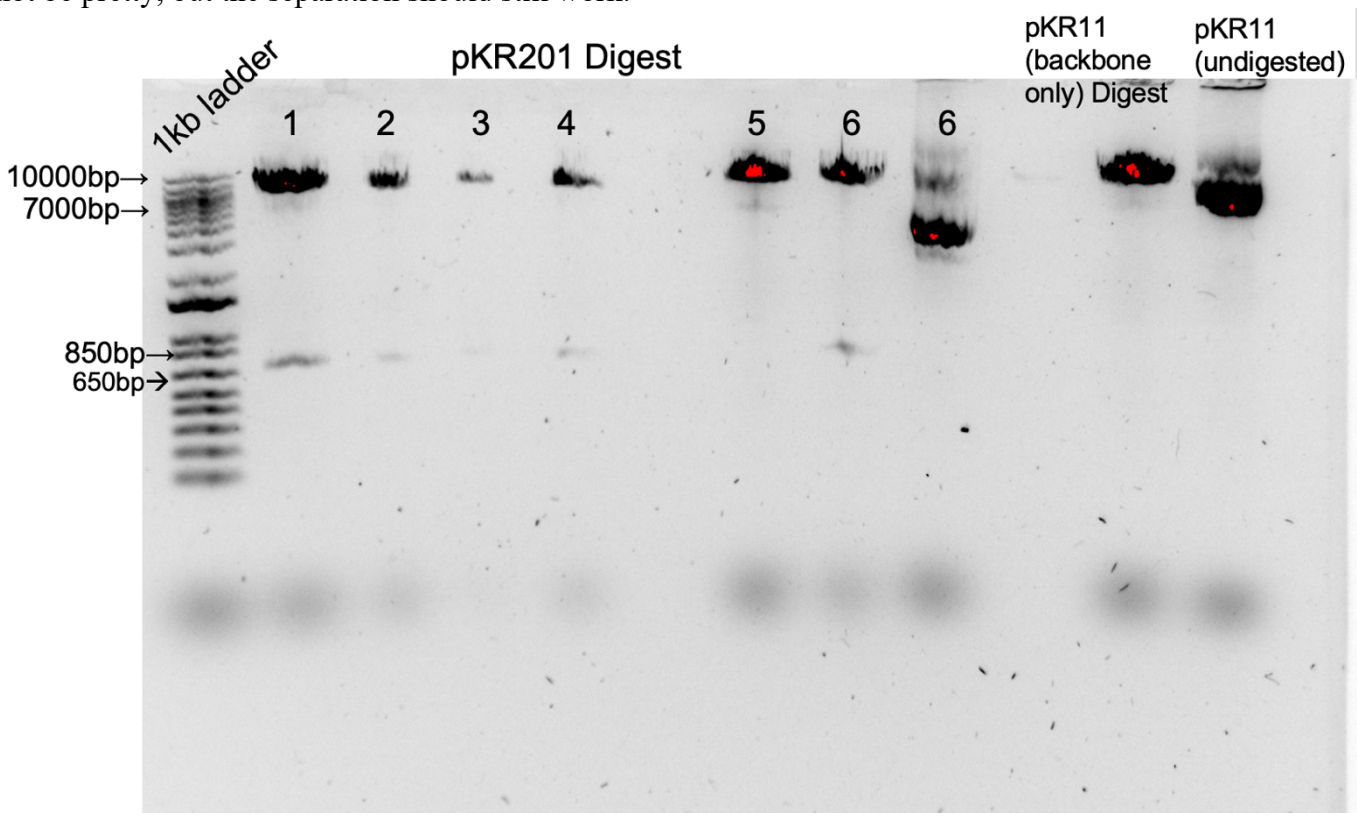


Figure 1. Diagnostic digest gel results. There are faint bands at the expected 731 bp which means that the ligation was successful. Sample 5 doesn't really have a band so I may exclude that sample when making the pKR201 plasmid stock.

Wednesday, March 6, 2024

To Do:

1. ~~Diagnostic Digest~~
2. Sequencing prep

Table 1. Dilutions of the miniprep samples for sequencing.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	1 dil	60.6	ng/µl	1.211	0.819	1.48	3.76
2	2 dil	65.4	ng/µl	1.309	0.858	1.53	3.54
3	3 dil	94.4	ng/µl	1.889	1.178	1.6	3.02
4	4 dil	49.6	ng/µl	0.992	0.667	1.49	4.34
5	5 dil	45.5	ng/µl	0.911	0.656	1.39	4.65
6	6 dil	62.6	ng/µl	1.253	0.818	1.53	3.56

Table 2. Sequencing order.

Sample number	**Template Type**	**Template Name**	**Primer Name[a]**	**A.**	**B.**	**E.**	**F.**
	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/µl)	**PLASMID** template:	Volume **H_2O needed**
						Volume =	
						2x(~200 ÷ B)µl	**(12 less D or E - 2.56)µl**
CS1	plasmid	pKR201	KROL121	8609	60.6	6.60	2.84
CS2	plasmid	pKR201	KROL121	8609	65.4	6.12	3.32
CS3	plasmid	pKR201	KROL121	8609	94.4	4.24	5.20
CS4	plasmid	pKR201	KROL121	8609	49.6	8.06	1.38
CS5	plasmid	pKR201	KROL121	8609	45.5	8.79	0.65
CS6	plasmid	pKR201	KROL121	8609	62.6	6.39	3.05

+ 2.56 uL 2.5uM stock to each.

After making up the sequencing reactions, I brought the samples over to the INBRE facility in Avedisian and left them in the sequencing freezer.

Monday, March 18, 2024

To Do:

1. ~~Sequencing prep~~
2. Sequencing results

The sequencing results showed that only one of my six samples had the insert in the correct direction, but the GFP fragment was not present. The GFP may be toxic to *E. coli,* so I will have to go back and do a low temperature transformation. After spread plating, I will leave the plates to grow at room temperature so that not as much DNA is produced. I can also prepare a transformation with a media that allows for less growth.

Wednesday, March 20, 2024

To Do:

1. ~~Make LB-Kan plates.~~
2. Make M9 minimal media.
3. Ligation.
4. Transformation.

LB-Kan

*See protocols/media/LB agar

M9 Minimal Media

Combine M9 salts and water as indicated below and autoclave (liquid cycle, 30 minutes). When cool, aseptically add glucose, $MgSO_4$, and $CaCl_2$.

Reagent	Amount to add (for 100 mL)
M9 salts (5X)	20 mL
Glucose (20%)[a]	2 mL
$MgSO_4$ (1 M)[b]	200 µL
$CaCl_2$ (1 M)[b]	10 µL
ddiH_2O	78 mL

[a]Filter-sterilized and stored at 4°C.

[b]Autoclaved and stored at room temperature.

5X M9 Salts

Chemical	Mass
Na_2HPO_4•$7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5.0 g
ddiH_2O	to 1 L

Divide the salt solution into 200-ml aliquots
Sterilize by autoclaving for 15-20 minutes

Thursday, March 21, 2024

To Do:

1. ~~Ligation.~~
2. Transformation.
3.

Ligation

*See pg. 27 of my notebook for full protocol.

Tube	Insert	Backbone
1	NotI-HF digested pKR184 PCR pure	NotI-HF digested, purified pKR11
2	-	NotI-HF digested, purified pKR11

1. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (3x)
H_2O	11.5	11.5	34.5
10x ligase buffer	2.0	2.0	6
Insert	4.0	-	-
Backbone	2.0	-	6

Ligase	0.5	0.5	1.5
TOTAL	20.0	20.0	48

*Instead of adding 4uL insert to the BB only tube (reaction 2) I added 4 uL of NF water. The backbone was added to the master mix, and 16 uL of master mix was added to each reaction tube. I let the reactions sit at room temperature for 10 minutes, then started the transformation.

Transformation

*See pg. 31 for Transformation protocol

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	(+) control	pKR11 (original stock)	1 uL	100 ul	1
2	(-) control	None	0	100 ul	1
3	Backbone only (pKR11)	pKR11 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1 (pKR201)	pKR201 ligated	8 uL	100 uL, remaining	2
Total					**6**

*I spread plated the transformations using sterile beads and left them on my benchtop to grow at room temperature over the weekend. I will come in on Sunday to check for colonies.

Monday, March 25, 2024

To Do:

1. Count plates.
2. Make overnight cultures.

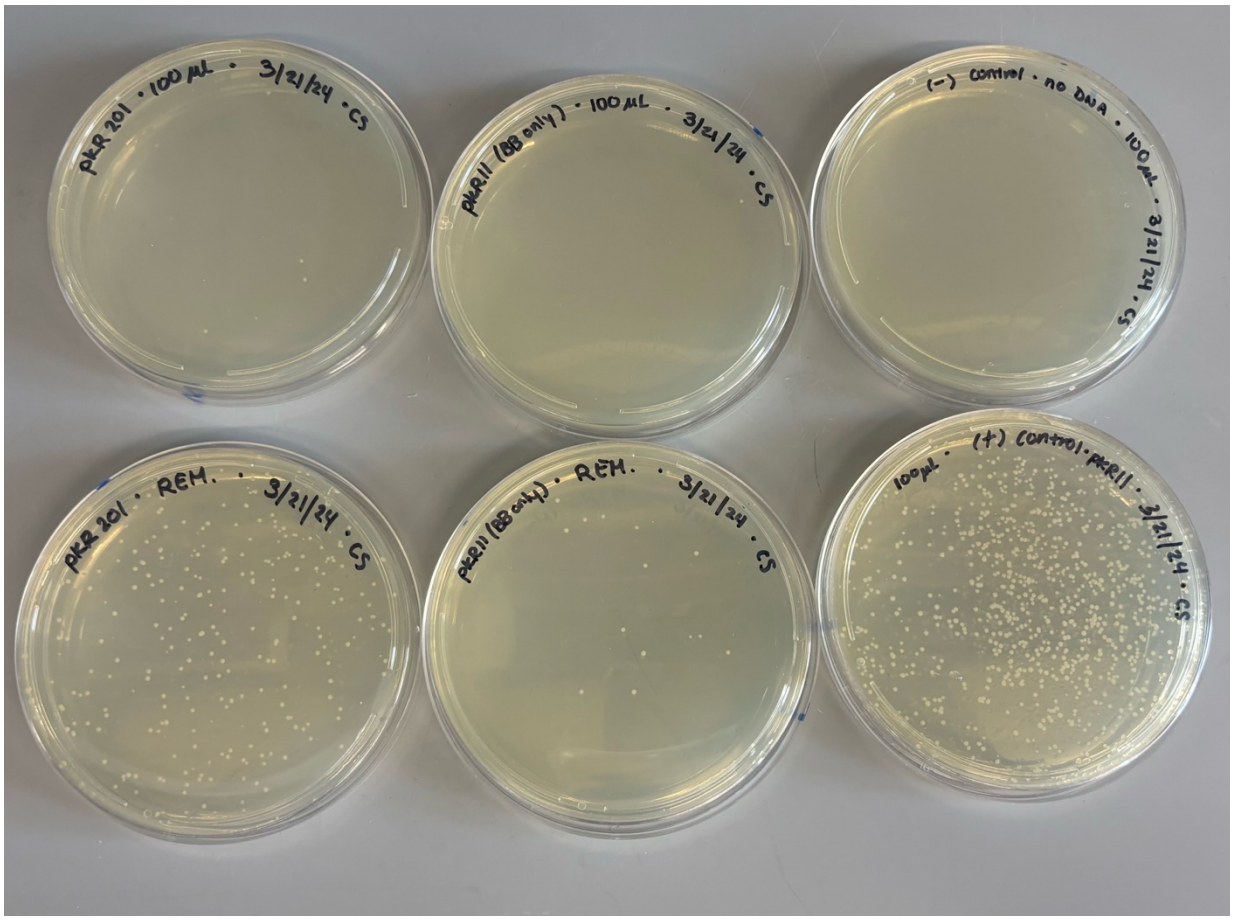


Table 1. Plate count results.

(-)	(+)	pKR11 (100uL)	pKR11 REM	pKR201 (100uL)	pKR201 REM
0	TMTC	1	18	5	287

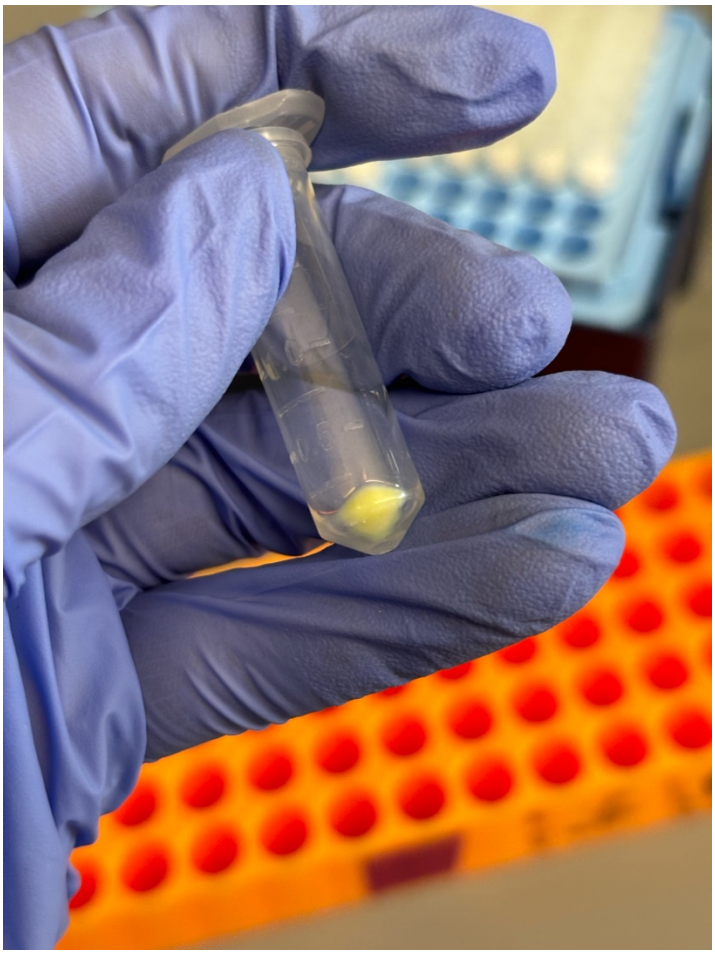
*Made 6 overnight cultures from different colonies.

Tuesday, March 26, 2024

To Do:

1. Make overnight cultures.
2. Miniprep

*When pelleting my cells for the miniprep I noticed that colony #6 had a green pellet!



Miniprep

*See QIAquick Miniprep on page 18-19
* I did overnight cultures of 12 more colonies and grew them in the shaking incubator at 26 degrees.

Wednesday, March 27, 2024

To Do:

1. Miniprep
2. Sequencing reactions

*I did not have any green pellets from the room temp overnight cultures, so I discarded them. I cultured 12 more colonies at the same temperature and will check again tomorrow. The first three cultures are from my 100 uL transformation in hopes that these colonies will have GFP.

Thursday, March 28, 2024

To Do:

1. ~~Miniprep~~
2. Sequencing reactions
3. ~~Diagnostic digest~~

*20 uL DNA reaction digest
NcoI & BamHI – 705 bp for correct product
Incorrect product: 1802bp

*When pelleting my cells in preparation for the miniprep, I still noticed lysis in most of the cultures. 0/12 pellets were green, so I decided to choose colonies 7-12 to miniprep and do a diagnostic digest.

Miniprep

*See protocol pg. 18-19

Table 1. Miniprep concentration results. The first four samples were errors due to an old blank sample. The concentrations for these samples were expected to be low since they were grown at room temperature.

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	8	-146.1	ng/µl	-2.921	-0.171	17.11	1.11
2	7	-99.7	ng/µl	-1.995	0.313	-6.37	0.9
3	7	-102	ng/µl	-2.04	0.283	-7.21	0.92
4	7	-104.4	ng/µl	-2.088	0.261	-8.01	0.9
5	7(Reblank)	78.7	ng/µl	1.574	0.746	2.11	2.33
6	8	36	ng/µl	0.72	0.339	2.13	1.7
7	8	64.9	ng/µl	1.298	0.596	2.18	2.2
8	10	65	ng/µl	1.301	0.633	2.05	1.52
9	11	105.1	ng/µl	2.102	1.029	2.04	2.29
10	12	35.1	ng/µl	0.702	0.28	2.51	2.03

Diagnostic Digest

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR201	NotI-HF	3	7
2	pKR201	NotI-HF	3	7
3	pKR201	NotI-HF	3	7
4	pKR201	NotI-HF	3	7
5	pKR201	NotI-HF	3	7
6	pKR201	NotI-HF	3	7
7	pKR11	NotI-HF	3	7
8	pKR11	n/a	2	5

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 8x (uL)
H_2O	7.2	57.6
10x Buffer* (CutSmart)	2	16
DNA	(10)	-
Enzyme 1	0.8	6.4
Total	20.0 (10.0 actual b/c of DNA)	80

*Using tube 8 as a control- undigested pKR11. Added 2 uL loading dye to visualize.
I ran the agarose gel at 120V for ~35 min.

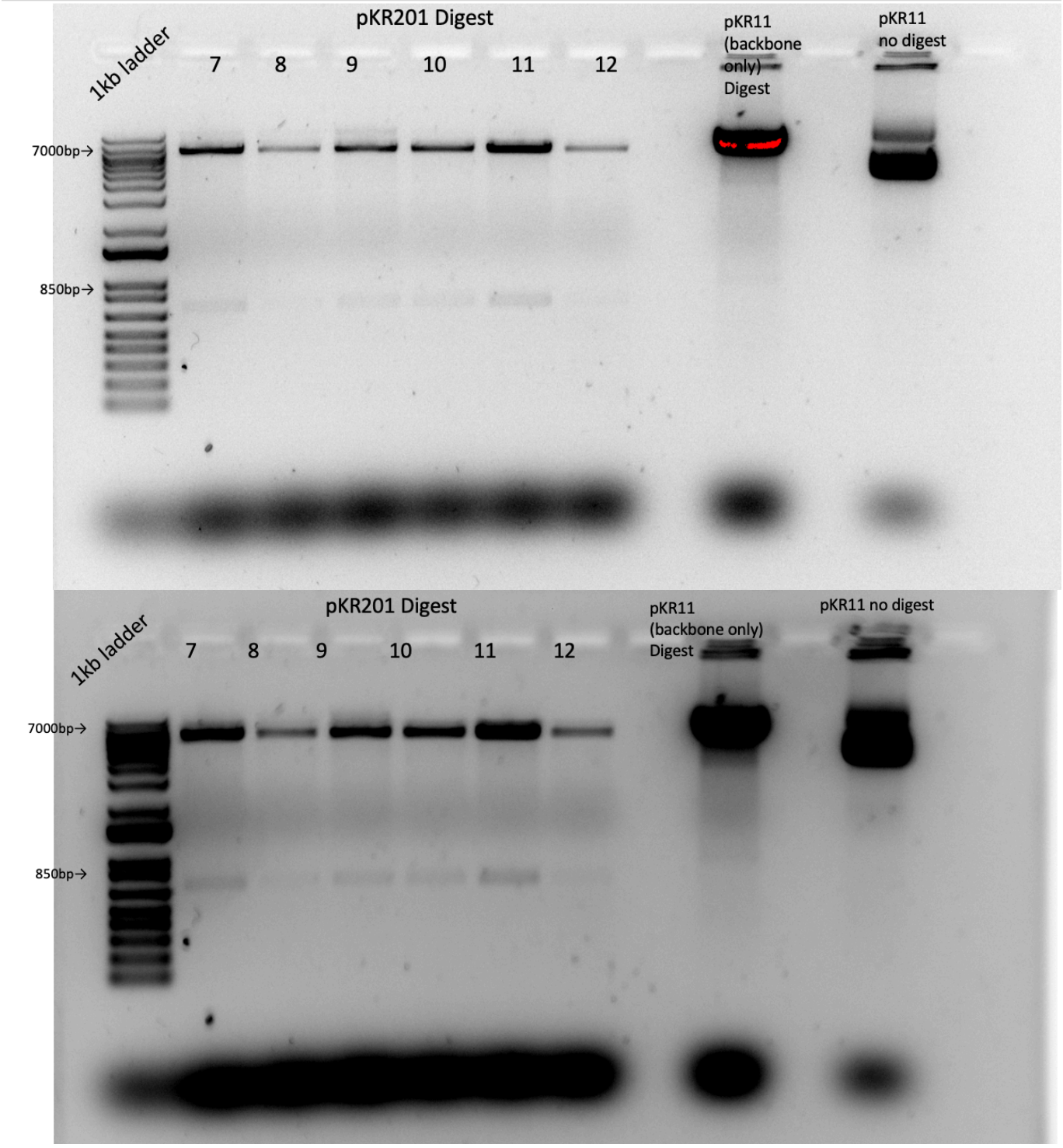


Figure 1&2. Gel results for digest. The second image has higher exposure to show the second bands at ~700bp. These results do not tell us about the orientation of the fragment because I used NotI-HF. I will repeat with NcoI-HF and BamHI-HF to check for orientation.

Diagnostic Digest

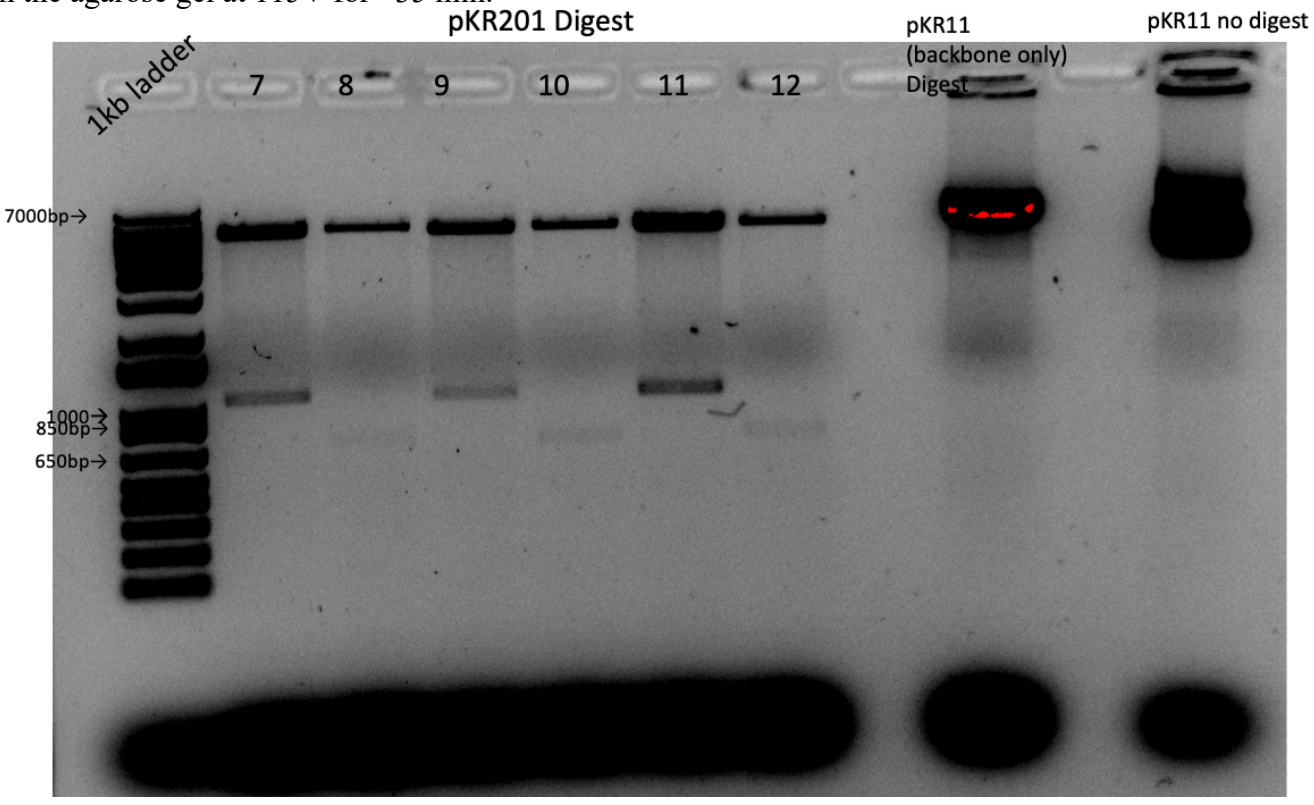
1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H_2O Volume (uL)
1	pKR201	NcoI-HF, BamHI-HF	3	7
2	pKR201	NcoI-HF, BamHI-HF	3	7
3	pKR201	NcoI-HF, BamHI-HF	3	7
4	pKR201	NcoI-HF, BamHI-HF	3	7
5	pKR201	NcoI-HF, BamHI-HF	3	7
6	pKR201	NcoI-HF, BamHI-HF	3	7
7	pKR11	NcoI-HF, BamHI-HF	3	7
8	pKR11	n/a	2	5

2. Set up master mix table:

Components	**Volumes in 1 reaction (uL)**	**Volumes in Master Mix 8x (uL)**
H_2O	7.2	57.6
10x Buffer* (CutSmart)	2	16
DNA	(10)	-
Enzyme 1	0.8	6.4
Total	20.0 (10.0 actual b/c of DNA)	80

*Using tube 8 as a control- undigested pKR11. Added 2 uL loading dye to visualize.
I ran the agarose gel at 115V for ~35 min.



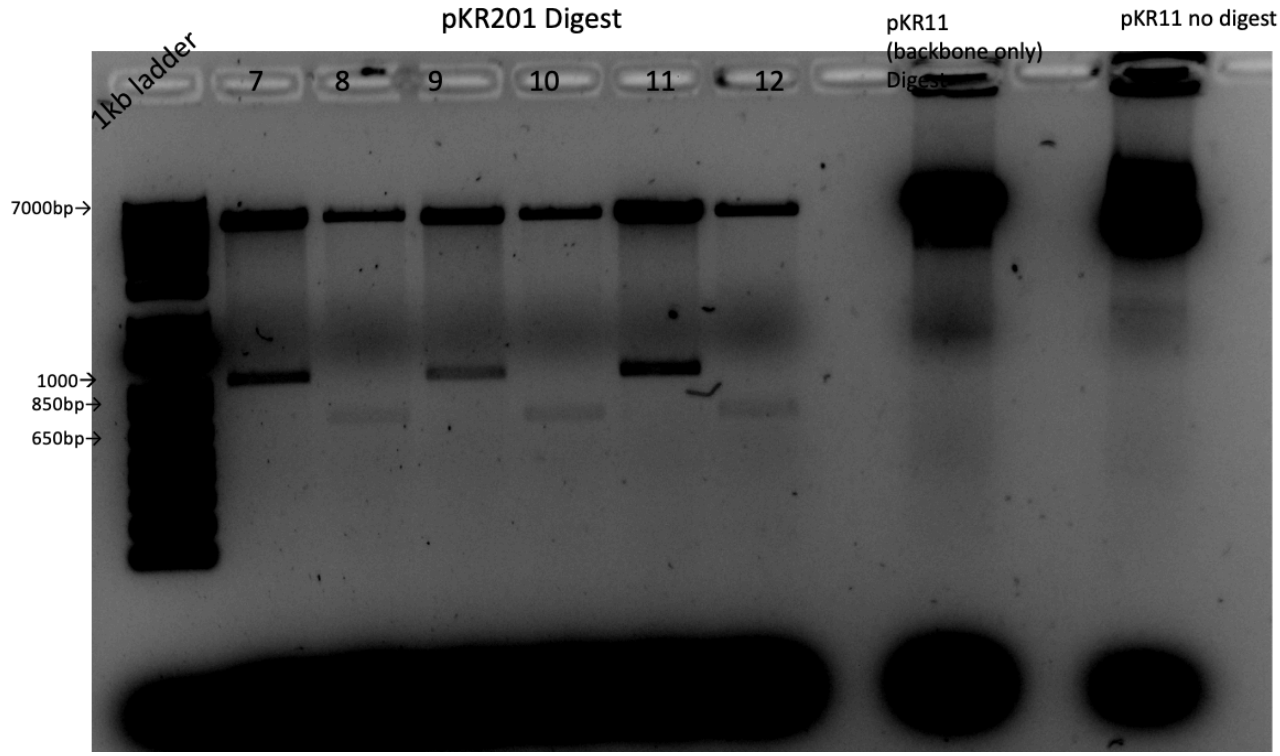


Figure1&2. Gel results for colonies 7-12. Colonies 8, 10, and 12 seem to have the correct orientation of the insert, indicated by the band around 700bp. Colonies 7, 9, and 11 have the incorrect orientation. The band size for incorrect orientation should be about 1800bp, but this band looks a little smaller.
*checked snap gene fille, my estimate for incorrect insertion is 1200bp fragment between cut sites

Monday, April 1, 2024

To Do:

1. Sequencing reactions

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
6	98.2	ng/µl	1.964	1.091	1.8	2.46
8	20	ng/µl	0.401	0.221	1.81	2.88
10	30.5	ng/µl	0.609	0.376	1.62	2.64
12	17.5	ng/µl	0.351	0.225	1.56	2.76

*As I started my sequencing reactions, I realized that the DNA concentration is too low for a 12 uL reaction. Ben suggested that next time I miniprep, I elute in a smaller volume of EB to concentrate the samples more.
I diluted the KROL 560 10 uM primer with 0.1x EB. I made a total of 25 uL using 6.25 uL primer stock and 18.75 uL EB.

Sample number	Well	Template Type	Template Name	Primer Name[a]	A.	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/µl)	**PCR** template:	**PCR** template:	**PLASMID** template:	Volume **H_2O needed**
							ng needed =	Volume =	Volume =	
							(A ÷ 100) × 2.5	**(C ÷ B)µl**	**2x(~200 ÷ B)µl**	**(12 less D or E - 2.56)µl**

CS1		plasmid	pKR201	KROL 121	8609	98.2			4.07	5.37
CS2		plasmid	pKR201	KROL 560	8609	98.2			4.07	5.37
a.Add 2.56 µl of 2.5 µM stock to each reaction										
3130xl Plate Record		Date	4/1/24	Name	Christina Surace					
PI	Kathryn Ramsey	Dept	CMB	Email	csurace@uri.edu	PO No.				

Thursday, April 4, 2024

To Do:

1. ~~Make LB Agar~~
2. Retransform plasmids (6, 8, 10, 12) and pKR11 and pKR200 – to make more
3. Sequencing reactions (#6 w KROL 460)
4. Culture & Miniprep

LB AGAR

Preparing LB-agar

1. For 500 mL of LB-agar, weigh out the following components and add to a 1 L (non-baffled) flask:
    a. 6 g agar
    b. 5 g NaCl
    c. 5 g Tryptone
    d. 2.5 g Yeast extract
2. Add stirbar to flask
3. Add 500 mL ddiH20
4. Mix on stirplate until components are dissolved
5. Cover top of flask with foil and add a small piece of autoclave tape
6. Autoclave on 30 minute liquid cycle

Transformation

*See pg. 31 for Transformation protocol

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of kanamycin-containing plates
1	(+) control	pKR11	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL	1
3	pKR200	pKR200	1 uL	20 uL, 100 uL	2
4	pKR 201 (6)	pKR 201 colony 6	1 uL	20 uL, 100 uL	2
5	pKR 201 (8)	pKR 201 colony 8	1 uL	20 uL, 100 uL	2
6	pKR 201 (10)	pKR 201 colony 10	1 uL	20 uL, 100 uL	2
7	pKR 201 (12)	pKR 201 colony 12	1 uL	20 uL, 100 uL	2
Total					**13**

*I spread plated the transformations using sterile beads and left them on my benchtop to grow at room temperature over the weekend. I will come in on Monday to check for colonies.

Monday, April 8, 2024

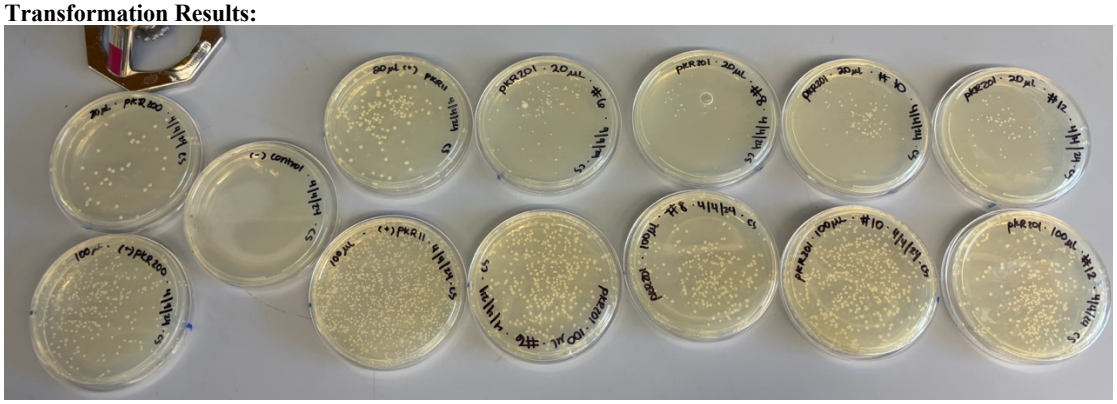
To Do:

1. ~~Retransform plasmids (6, 8, 10, 12) and pKR11 and pKR200 – to make more~~
2. ~~Sequencing reactions (#6 w KROL- 460)~~
3. Culture & Miniprep

Sequencing Reaction

Sample number	Well	Template Type	Template Name	Primer Name[a]	A.	B.	C.	D.	E.	F.
	(GSC use only)	(plasmid or PCR)			Template Size (bases)	Template Stock Conc. (ng/µl)	**PCR** template:	**PCR** template:	**PLASMID** template:	Volume H_2O **needed**
							ng needed =	Volume =	Volume =	
							(A ÷ 100) × 2.5	**(C ÷ B)µl**	**2x(~200 ÷ B)µl**	**(12 less D or E - 2.56)µl**
CS1		plasmid	pKR201	KROL 460	8609	98.2			4.07	5.37

Transformation Results:



Wednesday, April 10, 2024

To Do:

1. ~~Culture~~ & Miniprep

Overnight Cultures

The sequencing results for the green pellet (#6) looked good so I did 6 cultures from the plate and grew at 37 degrees in the shaking incubator overnight. I also did two cultures each for pKR11 and pKR200. All cultures were 5mL LB with 5uL kanamycin.

Thursday, April 11, 2024

To Do:

1. Miniprep

Miniprep

Refer to QIA Miniprep kit instructions.

Table 1. Nanodrop results for my plasmid samples (labeled as 6).

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
6.1	502.5	ng/µl	10.051	5.217	1.93	2.28
6.2	300.4	ng/µl	6.009	3.059	1.96	2.28
6.3	306.1	ng/µl	6.122	3.138	1.95	2.25
6.4	313	ng/µl	6.259	3.195	1.96	2.25
6.5	275	ng/µl	5.5	2.801	1.96	2.22
6.6	317.2	ng/µl	6.345	3.233	1.96	2.27
11.1	691.8	ng/µl	13.837	7.394	1.87	2.21
11.2	920	ng/µl	18.4	9.827	1.87	2.32
200.1	742	ng/µl	14.839	8.109	1.83	2.35
200.1	557.9	ng/µl	11.159	5.981	1.87	2.29

Friday, April 12, 2024

To Do:

1. Miniprep
2. Sucrose selection

Day 6:

-Kanamycin-resistant cells should be 1° integrants. Make stocks of up to 4 potential 1° integrants, but only move forward with identifying cross-outs from two 1° integrants per strain

To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 1X PBS (400 uL)

-Dilute culture 1:10 in sterile PBS to $1x10^{-7}$

Perform first dilution 1:100 so first dilution tube is $1x10^{-2}$, adding 10 uL of resuspended cells to 990 ul 1x PBS

Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS

-Plate 100 uL of last dilution ($1x10^{-7}$) onto CHAH plates.

-Plate 100 µL of each dilution 10^{-2} – 10^{-7} on CHAH + 10% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice. *Spread plate using sterile beads

-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the $1x10^{-7}$ dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Monday, April 15, 2024

To Do:

1. ~~Miniprep~~
2. ~~Sucrose selection~~

Today I looked at my plates. The growth on the sucrose plates was not obvious enough, so I'm going to incubate the plates for another day or two. There were about 40 colonies on the no sucrose plate.

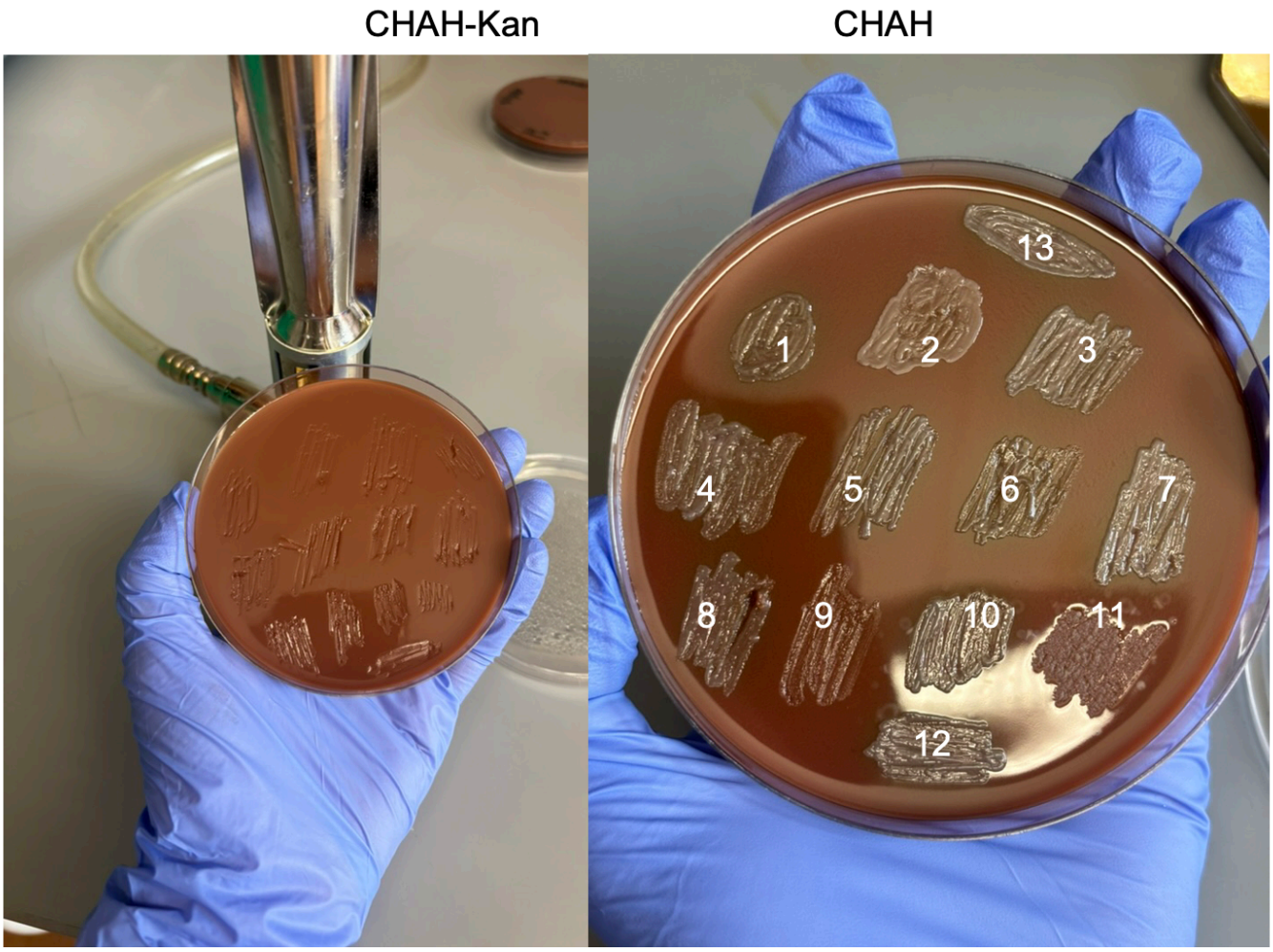
Tuesday, April 16, 2024

To Do:

1. ~~Cross patch on CHAH and CHAH Kan~~

Cross Patching

Aseptically take up part of an LVS colony from 10^-4 to 10^-7 dilution plates. Patch onto CHAH, then cross patch with the same sterile toothpick onto CHAH-Kan. Repeat for 12 more colonies. Incubate at 37 degrees for one day.



Wednesday, April 17, 2024

To Do:

1. ~~Cross patch on CHAH and CHAH Kan~~
2. Colony PCR

Colony PCR

Resuspend patches in PCR tubes with 50uL NF water aseptically. Heat kill by incubating on thermocycler at 95 degrees C for 10 min. Dilute lysates 1:10 with NF water.

Sample Number	DNA	Expected size
1	pKR201-1 patch 1	1275 or 1773
2	pKR201-1 patch 2	1275 or 1773
3	pKR201-1 patch 3	1275 or 1773
4	pKR201-1 patch 4	1275 or 1773
5	pKR201-1 patch 5	1275 or 1773
6	pKR201-1 patch 6	1275 or 1773
7	pKR201-1 patch 7	1275 or 1773
8	pKR201-1 patch 8	1275 or 1773
9	pKR201-1 patch 9	1275 or 1773
10	pKR201-1 patch 10	1275 or 1773
11	pKR201-1 patch 11	1275 or 1773
12	pKR201-1 patch 12	1275 or 1773
13	pKR201-1 patch 13	1275 or 1773
14	pKR201-1	
15	pKR201 (1:100)	8609
16	LVS gDNA	1275
17	- DNA	-

Total reaction volume	15
Total number of reactions	17
Number of reactions plus error	19.55

Assemble Master mix:

Component	Stock concentration	Final concentration	1 rxn volume	Master Mix
ddiH2O			8.6	168.13
PrimeSTAR GXL Buffer	5x	1x	3	58.65
dNTPs	2.5 mM	0.2 mM	1.2	23.46
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.3	5.865
KROL147	10 uM	0.3 uM	0.45	8.7975

KROL148	10 uM	0.3 uM	0.45	8.7975
template	100 ng/ul	2 ng/ul	1	indiv
		Total volume	15	273.7

-Use PCR program:

1. 94°C 2'
2. 94°C 20"
3. 50°C 30"
4. 68°C (1' per kb)
5. Go to step 2, rep 32x
6. 68°C 5'
7. Hold 12°C

Thursday, April 18, 2024

To Do:

1. Colony PCR
2. Run colony PCR on gel

Gel settings

Mix 3uL loading dye with 15uL reaction. Load full volume on the gel. Run at 120V for ~1 hour.

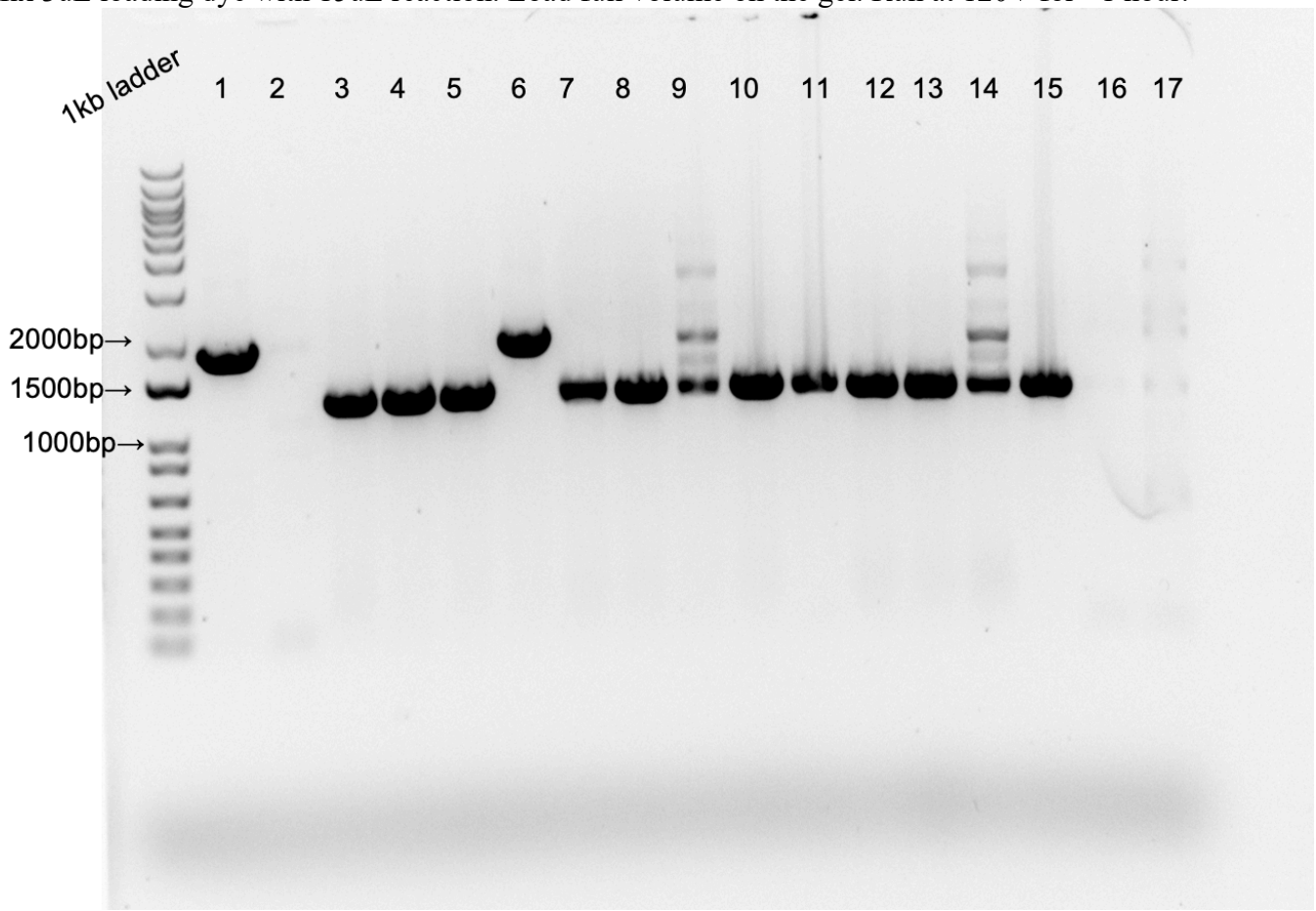


Figure 1. Colony PCR results. Mutants at patch 1 and patch 6!!
1-13 = patches 1-13

14= pKR201-1
15= LVS gDNA (WT)
16= no DNA control
17= pKR201 diluted 1:100
The next step would be to make glycerol stocks for my mutants. My patches were quite small, so I patched them each onto new CHA plates following the protocol on page 4 of my notebook. I will make the glycerol stocks tomorrow.

Friday, April 19, 2024

To Do:

1. ~~Colony PCR~~
2. ~~Run colony PCR on gel~~
3. Streak for isolation
4. Cross patch single colonies
5. Glycerol stocks

Streaking for isolation

Aseptically, take a small loop of potential mutant and streak onto CHAH plate using the 4 quadrant method.
I did this twice since I had two potential mutants, then left the plates to grow at 37 degrees over the weekend.

Monday, April 22, 2024

To Do:

1. ~~Streak for isolation~~
2. Cross patch single colonies
3. Glycerol stocks

Patching single colonies

Patch single colonies of potential mutants on CHAH and cross-patch to CHAH-Kan plates (3 colonies are usually sufficient).

Tuesday, April 23, 2024

To Do:

1. ~~Streak for isolation~~
2. ~~Cross patch single colonies~~
3. Colony PCR
4. Glycerol stocks

Colony PCR

Resuspend patches in PCR tubes with 50uL NF water aseptically. Heat kill by incubating on thermocycler at 95 degrees C for 10 min. Dilute lysates 1:10 with NF water. (I did 2 uL lysate to 18 uL NF water).

Sample Number	DNA	Expected size
1	pKR201-1-1 patch 1	1275 or1773
2	pKR201-1-1 patch 2	1275 or1773
3	pKR201-1-1 patch 3	1275 or1773
4	pKR201-1-6 patch 1	1275 or1773
5	pKR201-1-6 patch 2	1275 or1773

6	pKR201-1-6 patch 3	1275 or1773
7	pKR201-1	
8	pKR201 (1:100)	8609
9	LVS gDNA	1275
10	- DNA	-
11	pKR11 (1:100)	7886

Total reaction volume	15
Total number of reactions	11
Number of reactions plus error	12.65

Component	Stock concentration	Final concentration	1 rxn volume	Master Mix
ddiH2O			8.6	108.79
PrimeSTAR GXL Buffer	5x	1x	3	37.95
dNTPs	2.5 mM	0.2 mM	1.2	15.18
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.3	3.795
KROL147	10 uM	0.3 uM	0.45	5.6925
KROL148	10 uM	0.3 uM	0.45	5.6925
template	100 ng/ul	2 ng/ul	1	indiv
		Total volume	15	177.1

-Use PCR program:

1. 94°C 2'
2. 94°C 20"
3. 50°C 30"
4. 68°C (1' per kb)
5. Go to step 2, rep 32x
6. 68°C 5'
7. Hold 12°C

Wednesday, April 24, 2024

To Do:

1. ~~Colony PCR~~
2. Run PCR on gel
3. Glycerol stocks

I loaded my PCR samples with 3 uL loading dye and ran on 1% agarose gel at 120 V for about an hour.

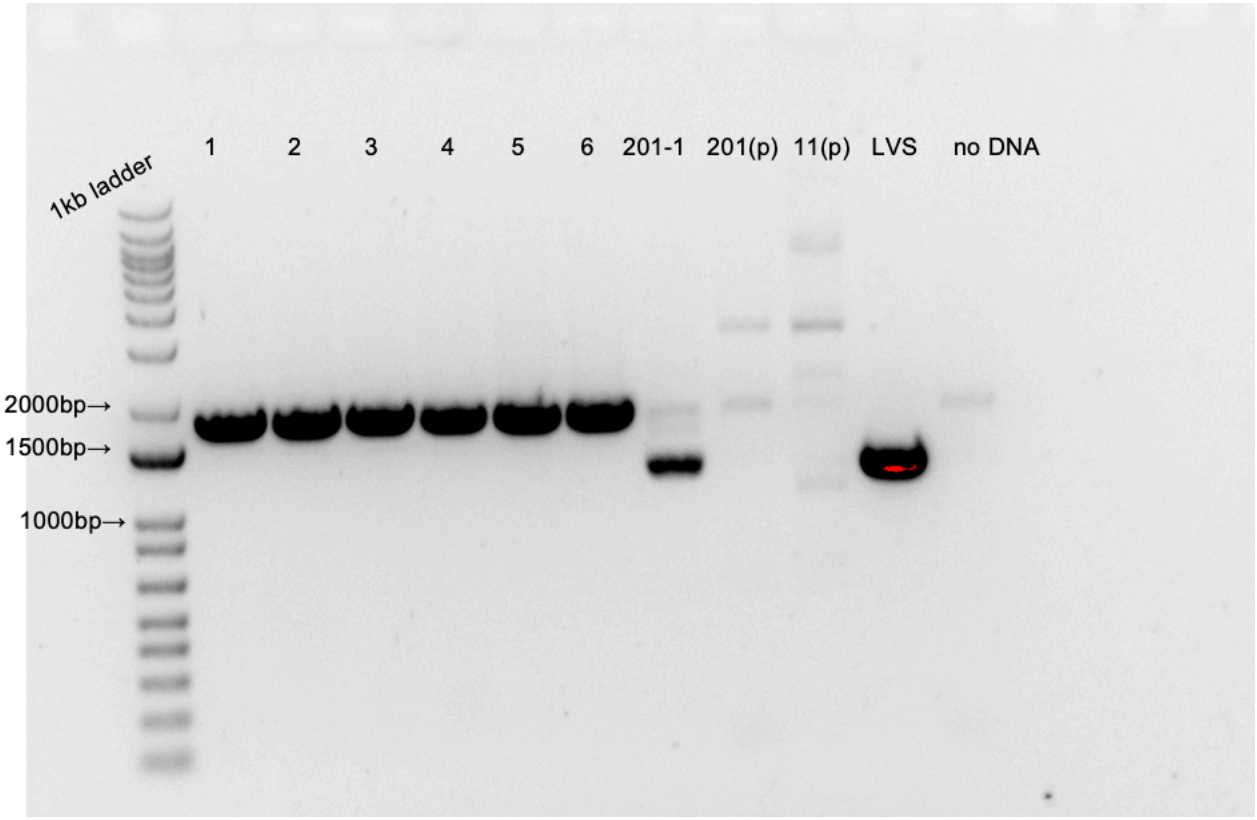


Figure 1. Colony PCR results. All samples are mutants.

Making Freezer Stocks

1. Add 800uL supplemented MHB to sterile 2mL tube. Resuspend cells using loop. Add 800uL more MHB and mix completely. Aliquot 800uL into 2 labeled cryovials. Add 200uL 75% glycerol. Vortex to mix.

Thursday, April 25, 2024

To Do:

1. ~~Streak LVS mutant strains onto CHA.~~
2. Make single use aliquots.
3. ~~Combine pKR201 minipreps for working stock,~~ save 5-10uL for permanent stock.
4. Extract gDNA and run PCR.

Streaking LVS

I streaked out my LVS mutants onto CHAH plates (one plate for each mutant). Aseptically take some liquid from cryovial onto a sterile stick. Streak the entire plate. Rotate and repeat twice.

Saturday, April 27, 2024

To Do:

1. Make single use aliquots.
2. ~~Combine pKR201 minipreps for working stock,~~ save 5-10uL for permanent stock.
3. Extract gDNA
4. run PCR on gDNA.

I pelleted some of my KRLVS 279.1 and 279.2 cells for the gDNA extraction. I noticed that 279.1 seems to grow faster than 279.2.

Monday, April 29, 2024

To Do:

1. Make single use aliquots.
2. ~~Combine pKR201 minipreps for working stock,~~ save 5-10uL for permanent stock.
3. Extract gDNA
4. run PCR on gDNA.

***note** about the gDNA extraction kit: I left the kit on my bench over the weekend and the reagents got warm because the bench gets warmed by the incubator.

gDNA Extraction of LVS

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scraped up patches of KRLVS279.1 and KRLVS279.2 and resuspended in supplemented MHB
3. Pellet cells by centrifugation (3 min at 8,000 rpm) and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2ul of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes. (start cooling centrifuge)
9. Place the samples on ice for 3-5 minutes.
10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds.
11. Pellet the debris by centrifugation at 4°C for 10 minutes at max speed in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry.
17. Resuspend the DNA in 35uL of 0.1x EB.

Table 1. Nanodrop results for gDNA extraction.

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
279.1	1635.2	ng/µl	32.703	16.787	1.95	1.81
279.2	1828.2	ng/µl	36.564	18.72	1.95	1.66

Tuesday, April 30, 2024

To Do:

1. Make single use aliquots.
2. ~~Combine pKR201 minipreps for working stock,~~ save 5-10uL for permanent stock.
3. ~~Extract gDNA~~
4. ~~run PCR on gDNA.~~

gDNA PCR

*Follow PCR protocol. Dilute gDNA samples 1:100.

Sample Number	DNA	Expected size
1	KRLVS279.1	1773
2	KRLVS279.2	1773
3	pKR201 (1:100)	8609
4	pKR11	7886
5	LVS gDNA	1275
6	- DNA	-

Component	Stock concentration	Final concentration	1 rxn volume	Master Mix
ddiH2O			8.6	59.34
PrimeSTAR GXL Buffer	5x	1x	3	20.7
dNTPs	2.5 mM	0.2 mM	1.2	8.28
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.3	2.07
KROL147	10 uM	0.3 uM	0.45	3.105
KROL148	10 uM	0.3 uM	0.45	3.105
template	100 ng/ul	2 ng/ul	1	indiv
		Total volume	15	96.6

Before adding my KRLVS templates to the PCR reaction tubes, I diluted them 1:100 in 0.1x EB (2 uL DNA + 198 uL EB).

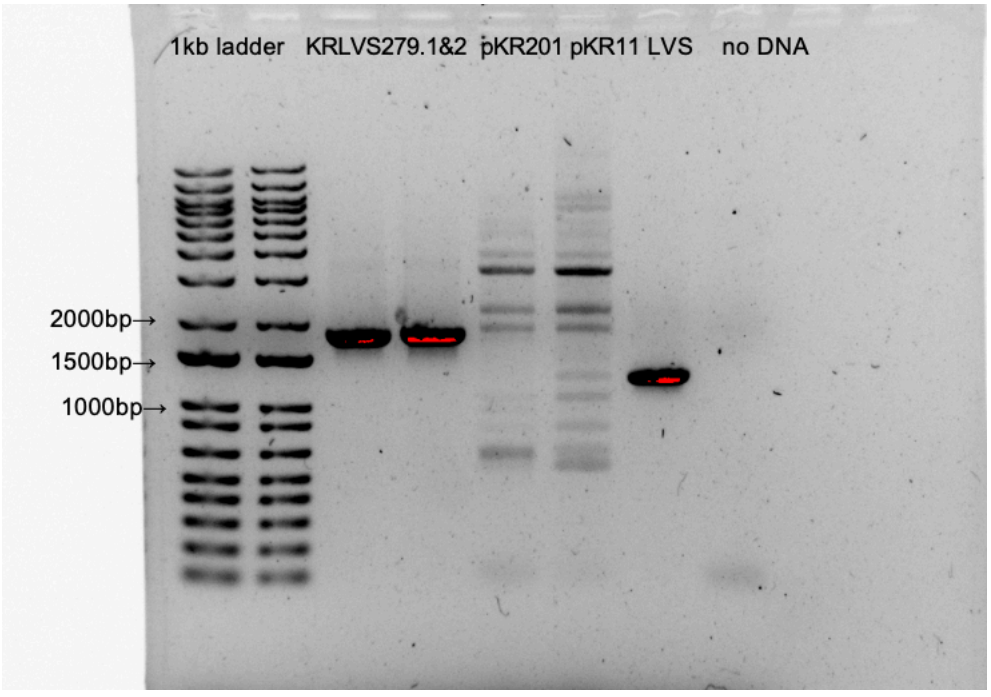


Figure 1. Results for gDNA PCR. The mutant strain DNA have the expected band size for the correct *gfp* mutation sequence.

Bibliography

Ramsey, K. M. and Dove, S. L. (2016) ‘ A response regulator promotes Francisella tularensis intramacrophage growth by repressing an anti-virulence factor ’, *Molecular Microbiology*. doi: 10.1111/mmi.13418.