

Table of Contents

May 2022	5
Sunday, May 1, 2022	5
DNA Digest of pKR116 PCR Fragment w/KpnI and BamHI	6
DNA Digest of iLOV-V and LanYFP-V w/EcoRI and BamHI	6
Gels of Digested pKR116 PCR Fragment + Backbone and iLOV-V/LanYFP-V + Backbone	7
Visualizing and Cutting Gel	7
Gel Extraction with QIAquick Gel Extraction Kit	7
Ligation of iLOV-V and LanYFP-V with pF backbone	8
Monday, May 2, 2022	8
Transformation of iLOV-V/LanYFP-V Ligation into <i>E. coli</i>	8
Tuesday, May 3, 2022	9
PCR of pKR113 for fragment to be used in pKR135, EV	10
DNA Digest of pKR113 PCR Fragment w/KpnI and BamHI	11
Gel of Digested pKR113 PCR Fragment and PCR Positive and Negative Control	12
Visualizing and Cutting Gel	12
Gel Extraction with QIAquick Gel Extraction Kit	12
Thursday, May 5, 2022	13
PCR of pKR113 to Troubleshoot Band Size Discrepancy	13
Gel of PCR Fragment of Diluted pKR113	15
Receiving and Dissolving Primers	15
Diluting Primers	15
PCR of pFTR3-rpsu1-V gBlock and KRLVS156 Triple Mutant	15
Friday, May 6, 2022	17
Gel of PCR Fragment from pFTR3-rpsu1-V gBlock and KRLVS156	17
Monday, May 9, 2022	18
Reagents	18
Friday, May 20, 2022	19
Electroporate plasmid into EC cells*	19
Diluting Primers	20
PCR of Diluted pKR113 w/Re-Diluted PCR Primers	20
Gel of PCR Fragment from Diluted pKR113 Plasmid and Re-Diluted Primers	21
Monday, May 23, 2022	22

PCR of pKR113 w/Re-Diluted PCR Primers	22
PCR Purification	24
DNA Digest of pKR113 PCR Fragment w/KpnI and BamHI	24
Gel of Digested pKR113 PCR Fragment and PCR Positive and Negative Control	24
Tuesday, May 24, 2022	25
PCR of Inducible Strains	25
Freezing and Storing Permanent Strains	27
Reagents	27
Wednesday, May 25, 2022	28
PCR of iLOV-V and LanYFP-V gBlock	28
PCR Purification of iLOV-V and LanYFP-V	30
DNA Digest of pKR113 PCR Fragment w/KpnI and BamHI	30
Visualizing and Cutting Gel	30
Gel Extraction with QIAquick Gel Extraction Kit	31
Reagents	31
Thursday, May 26, 2022	32
DNA Digest of iLOV-V, LanYFP-V, and pF w/EcoRI and BamHI	33
Gel of Digested pKR113 PCR Fragment and PCR Positive and Negative Control	33
Visualizing and Cutting Gel	34
Gel Extraction with QIAquick Gel Extraction Kit	34
PCR of Inducible Strains	34
Making 2% Agarose Gel	36
Running 2% Gel of Inducible Strains	36
PCR Purification of Inducible Strains	36
Friday, May 27, 2022	37
PCR of Inducible Strains	37
Making 2% Agarose Gel	39
Running 2% Gel of Inducible Strains	39
June 2022	40
Monday, June 6, 2022	40
Ligation of EV and pKR113 backbone	40
Transformation of EV Ligation into <i>E. coli</i>	40
Tuesday, June 7, 2022	41
Re-Diluting KROL15 and KROL16	41

Reagents	41
Wednesday, June 8, 2022	42
PCR of Inducible Strains	42
Making 2% Agarose Gel	44
Running 2% Gel of Inducible Strains	44
PCR Purification of Inducible Strains	44
Ligation of EV and pKR113 backbone	44
Reagents	45
Thursday, June 9, 2022	45
Transformation of EV Ligation into <i>E. coli</i>	45
Ligation of iLOV-V and LanYFP-V with pF backbone	46
Transformation of iLOV-V/LanYFP-V Ligation into <i>E. coli</i>	46
Friday, June 10, 2022	47
Saturday, June 11, 2022	47
Miniprep of pKR142 and pKR143 from <i>E. coli</i>	48
Thursday, June 16, 2022	49
Transformation of EV Ligation into <i>E. coli</i>	49
Friday, June 17, 2022	50
PCR of Inducible Strains	50
Making 2% Agarose Gel	52
Running 2% Gel of Inducible Strains	52
PCR Purification of Inducible Strains	52
Monday, June 20, 2022	53
Ligation of Empty Vector Control into pKR113 Backbone	53
Reagents	53
Tuesday, June 21, 2022	54
Transformation of EV Ligation into <i>E. coli</i>	54
Reagents	54
Wednesday, June 22, 2022	55
Thursday, June 23, 2022	56
Miniprep of pKR135 from <i>E. coli</i>	56
Reagents	56
Friday, June 24, 2022	57
Making Glycerol Stocks Protocol	57

Preparing Electrocompetent LVS Cells	57
Saturday, June 25, 2022	57
Electroporate pKR142 iLOV-V into EC cells*	58
Monday, June 27, 2022	59
Ligation of LanYFP-V with pF backbone	59
Transformation of iLOV-V/LanYFP-V Ligation into <i>E. coli</i>	60
Diagnostic Digest of Candidate pKR135 Miniprep w/KpnI and BamHI	60
Tuesday, June 28, 2022	62
Wednesday, June 29, 2022	63
Setting Up LVS and $\Delta rpsu$ Cultures for RNA Purification	63
RNA Purification	63
Transformation of Candidate pKR135-2, -3, and -4 into <i>E. coli</i>	64
Miniprep of pKR143 Candidates from <i>E. coli</i>	65
Thursday, June 30, 2022	65
Freezing and Storing Permanent Strain of KRLVS177	65
Bibliography	66

May 2022

Sunday, May 1, 2022

To Do:

1. ~~DNA digest of EV~~
2. ~~DNA digest of iLOV-V and LanYFP-V~~
3. ~~Gel and extraction of EV~~
4. ~~Gel and extraction of iLOV-V and LanYFP-V~~
5. ~~Ligation of iLOV-V and LanYFP-V~~

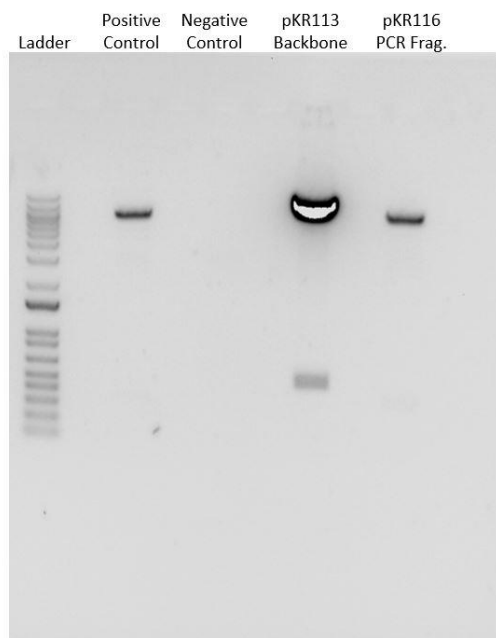
Results and Data:

Figure 1. Unsure why the positive control came out at the banding that it did, since it should be 441bp. Negative control is good. Backbone has the characteristic two bands. PCR fragment looks similar to a plasmid size as it was the wrong plasmid to be amplifying from, so, obviously, the primers did not work on it.

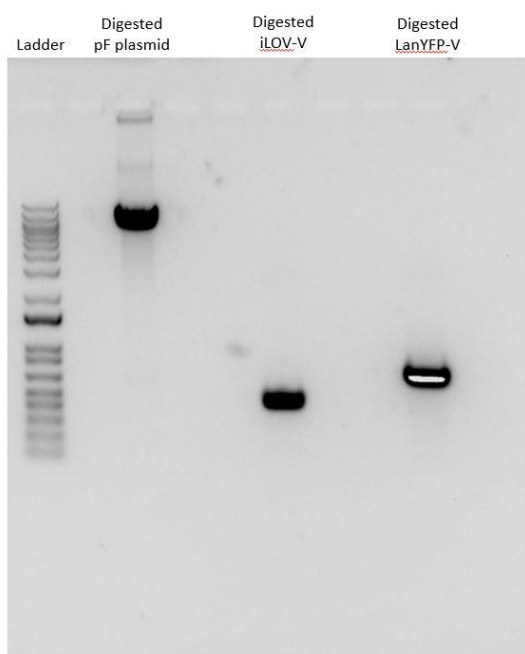


Figure 1. No positive or negative control as this restriction digest was done off of a PCR that CR did previously, in April. Bands were as expected, with some undigested plasmid at the top of the pF lane seemingly. Bands were extracted and purified.

DNA Digest of pKR116 PCR Fragment w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	pKR116 PCR	KpnI, BamHI	15	-

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H ₂ O	10.8	54.0
10x Buffer*	3.0	15.0
DNA	(15.0)	-
KpnI	0.6	3.0
BamHI	0.6	3.0
Total	30.0 (15.0 actual b/c of DNA)	

3. Add indicated amounts of H₂O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme (0.6 uL) to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

DNA Digest of iLOV-V and LanYFP-V w/EcoRI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	iLOV-V	EcoRI, BamHI	15	-
2	LanYFP-V	EcoRI, BamHI	15	-
3	pF	EcoRI, BamHI	5	10

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 3x (uL)
H ₂ O	10.8	54.0
10x Buffer*	3.0	15.0
DNA	(15.0)	-
EcoRI	0.6	3.0
BamHI	0.6	3.0
Total	30.0 (15.0 actual b/c of DNA)	

3. Add indicated amounts of H₂O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme (0.6 uL) to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

Used CR's PCR for the iLOV-V and LanYFP-V digests. Used the purified PCR from March for the empty vector.

Gels of Digested pKR116 PCR Fragment + Backbone and iLOV-V/LanYFP-V + Backbone

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of each sample.
6. Ran for 45 minutes at 113V.

Visualizing and Cutting Gel

1. Using Bio Rad Gel Doc XR+ imager equipped with XcitaBlue Conversion Screen
2. Nucleic Acid Gel > Sybr Safe
3. To cut gel, put on visualizing UV glasses and use a clean razor, cutting straight down. Add to 2mL tube.

pKR116 PCR fragment was not showing up as expected, instead looked to be of a plasmid size. Cut backbone and extracted it along with the pF, LanYFP-V, and iLOV-V fragments from the other gel.

Gel Extraction with QIAquick Gel Extraction Kit

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
3. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
6. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 30uL Buffer EB .01x and let stand for 1-4 minutes. Centrifuge for 1 min at 13,000rpm.

Used wrong tubes for collecting the gel and generally struggled with doing the extraction. May have lost some sample, potentially didn't used the right volumes of QG? Measurements seemed inconsistent. Dunno, was a big mess. Probably best to not do something for the first time when rushing.

Ligation of iLOV-V and LanYFP-V with pF backbone

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

Tube	Insert	Backbone
1	BamHI, EcoRI dig., purified iLOV PCR	BamHI, EcoRI digested, purified pF
2	BamHI, EcoRI dig., purified LanYFP-V PCR	BamHI, EcoRI digested, purified pF
3	BamHI, EcoRI dig., purified pF	BamHI, EcoRI digested, purified pF

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Reaction 3 (uL)	Master Mix (4x)
H ₂ O	11.5	11.5	11.5	46.0
10x ligase buffer	2.0	2.0	2.0	8.0
Insert	4.0	4.0	-	-
Backbone	2.0	2.0	2.0	8.0
Ligase	0.5	0.5	0.5	2.0
TOTAL	20.0	20.0	20.0	64.0

1. 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 in order to avoid degradation. Take 10 uL aliquot of 10X ligase buffer from the buffers box in the -20C.
2. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
3. To the individual tubes, add indicated amounts of H₂O (____uL), 10x buffer (____uL), insert (____uL), and backbone (____uL).
4. Add indicated amount of ligase (____uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
5. After all of the components have been added, mix each tube with a pipette set to 18 uL.
6. Place on bench for 10 minutes.

Monday, May 2, 2022

To Do:

1. Transform iLOV-V/LanYFP-V into *E. coli*

Results and Data:

N/A.

Transformation of iLOV-V/LanYFP-V Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 10 uL and 200 uL.
 - a. Positive control: pF; Backbone ligation: BamHI-EcoRI digested pF

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pF	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Backbone Ligation	pF (digested)	8 uL	10 uL, 200 uL	2
4	Ligation 1	pKR142	8 uL	10 uL, 200 uL	2
5	Ligation 2	pKR143	8 uL	10 uL, 200 uL	2
Total number of plates					10

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 heatblock 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.

Tuesday, May 3, 2022

To Do:

1. ~~PCR of pKR113 for EV~~
2. ~~PCR Purification~~
3. ~~Restriction Digest~~
4. ~~Gel of RD~~
5. ~~Gel extraction + purification~~
6. ~~Count transformation plates~~

Results and Data:

No transformants on any plates.

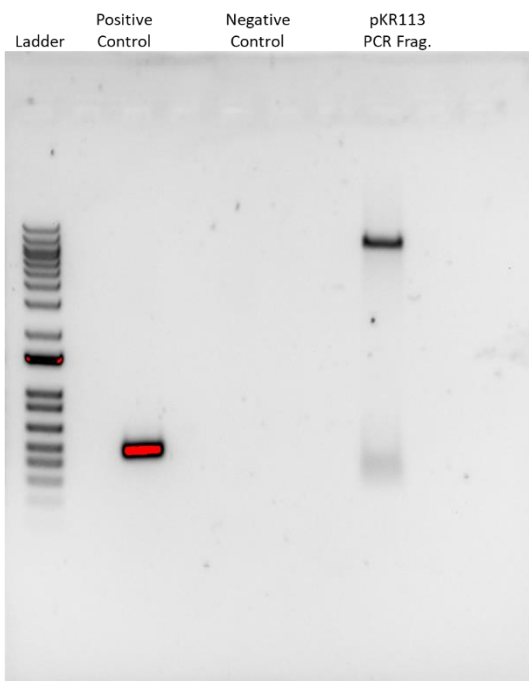


Figure 2. Positive control looks good. Negative control is good. PCR fragment. The smeary lighter band may be the 241bp fragment expected from the primers with this plasmid, the heavier band may be the plasmid itself as the plasmid DNA was not diluted prior to the PCR.

PCR of pKR113 for fragment to be used in pKR135, EV

1. Acquired and labelled PCR tubes: pKR113, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL495, KROL16, KROL496 (10uM)
 - pKR113 and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
4. If any of the solutions are frozen, be sure to vortex the microcentrifuge 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
5. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
6. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
7. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
8. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
9. Add appropriate volume of master-mix to negative control PCR tube
10. Add template to Master Mix
 - Factor template volume minus 1 template reaction volume
11. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
12. Close PCR Tubes until the caps are tight
13. 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 15 seconds
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	100
Total number of reactions	3

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			62.0	248.0
PrimeSTAR GXL Buffer	5x	1x	20.0	80.0
dNTPs	2.5 mM	0.2 mM	8.0	32.0
oligo F	10 uM	0.3 uM	3.0	
oligo R	10 uM	0.3 uM	3.0	
template	100 ng/ul	2 ng/ul	2.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2.0	4.8
Total volume			100.0	400.0

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	EV	pKR113	KROL495, KROL496	245
3	- control	-	KROL495, KROL496	-

Forgot to PCR purify because I am DUMB and didn't double check my to-do list ☹. Might [reduce the power of the ligation](#), but I'll go forward and try it.

DNA Digest of pKR113 PCR Fragment w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	pKR113 PCR	KpnI, BamHI	15	-

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)
H ₂ O	10.8
10x Buffer (Cutsmart)	3.0
DNA	(15.0)
KpnI	0.6
BamHI	0.6
Total	30.0 (15.0 actual b/c of DNA)

3. Add indicated amounts of H₂O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme (0.6 uL) to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

Gel of Digested pKR113 PCR Fragment and PCR Positive and Negative Control

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sybr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of positive, negative, and digested pKR113.
6. Ran for 45 minutes at 113V.

Visualizing and Cutting Gel

1. Using Bio Rad Gel Doc XR+ imager equipped with XcitaBlue Conversion Screen
2. Nucleic Acid Gel > Sybr Safe
3. To cut gel, put on visualizing UV glasses and use a clean razor, cutting straight down. Add to 2mL tube.

Gel Extraction with QIAquick Gel Extraction Kit

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
3. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
6. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 30uL Buffer EB .01x and let stand for 1-4 minutes. Centrifuge for 1 min at 13,000rpm.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
pKR113 PCR Frag	90mg	270uL	90uL

Thursday, May 5, 2022

To Do:

1. ~~20 μ L PCR of pKR113 to troubleshoot for EV~~

Results and Data:

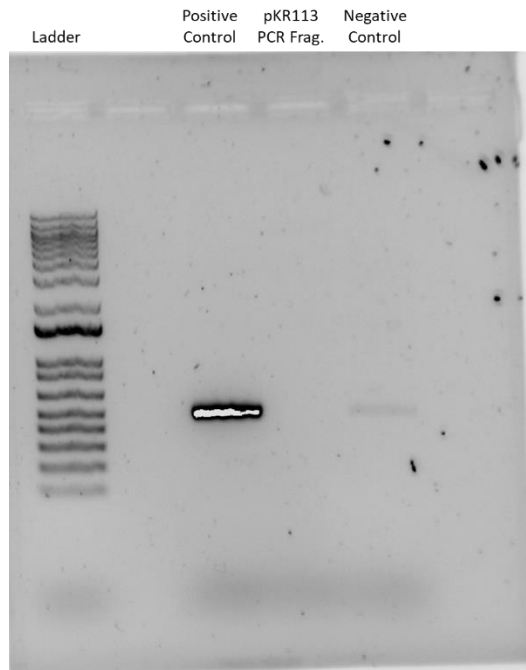


Figure 3. Positive control looks good. No PCR fragment. Negative control had contamination, so changed the molecular grade water to be fresher.

Made 1:100 dilution of pKR113 stock to use for PCR- large band could just be plasmid DNA since there is so much of it in the stock.

PCR of pKR113 to Troubleshoot Band Size Discrepancy

1. Acquired and labelled PCR tubes: pKR113, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL495, KROL16, KROL496 (10 μ M)
 - pKR113 and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
4. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - NOT vortexing the enzyme as it will expose it to oxygen and degrade it
5. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
6. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction

7. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
8. Mix the master-mix solution by pipetting up and down
9. Add appropriate volume of master-mix to negative control PCR tube
10. Add template to Master Mix
 - Factor template volume minus 1 template reaction volume
11. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
12. Close PCR Tubes until the caps are tight
13. 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 30 seconds
 - i. Note: Changed extension time to 30 seconds since 245 bp is SO close to 250
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	20
Total number of reactions	3

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			12.4	49.6
PrimeSTAR GXL Buffer	5x	1x	4.0	16
dNTPs	2.5 mM	0.2 mM	1.6	6.4
oligo F	10 uM	0.3 uM	0.6	2.4
oligo R	10 uM	0.3 uM	0.6	2.4
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	1.6
Total volume			20	78.4

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	EV	pKR113	KROL495, KROL496	245
3	- control	-	KROL15, KROL16	-

pKR113 promoter region not amplified. Based on the previous gels, it was probably just ample amount of plasmid DNA (since it wasn't dilute) which makes sense as to why it had a backbone-like banding pattern when restriction digested. It might simply be an issue with trying to amplify off of the plasmid, so instead going to try to amplify the promoter region from the gBlock pFTR3-rpsU1-V, which appears to work based on SnapGene with KROL495 and KROL496. There is just a little bit of hanging primer at the start of the gBlock, but I don't see why it shouldn't still anneal so I will go forward with that.

Gel of PCR Fragment of Diluted pKR113

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of positive, negative, and amplified diluted pKR113.
6. Ran for 45 minutes at 113V.

Receiving and Dissolving Primers

1. Spin primers at maximum speed in tabletop centrifuge for 3 minutes so desiccated 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. Put Certificate of Analysis sheet in "Oligos Spec Sheet" binder and shipping sheet in Invoices box.

Diluting Primers

1. 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.

PCR of pFTR3-rpsu1-V gBlock and KRLVS156 Triple Mutant

1. Acquired and labelled PCR tubes: EV, 141, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL495, KROL16, KROL496, KROL514, KROL515 (10uM)
 - pFTR3-rpsu1-V gBlock, KRLVS156 gDNA, and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
4. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)

- NOT vortexing the enzyme as it will expose it to oxygen and degrade it
5. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) to PCR tubes
 6. Add ddi H₂O to negative control tube
 7. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
 8. Mix the master-mix solution by pipetting up and down
 9. Add appropriate volume of master-mix to negative control PCR tube
 10. Add template to Master Mix
 - Factor template volume minus 1 template reaction volume
 11. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
 12. Close PCR Tubes until the caps are tight
 13. 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 30 seconds
 - i. Note: Changed extension time to 30 seconds since 245 bp is SO close to 250
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	20
Total number of reactions	4

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	5
ddiH ₂ O			12.4	62
PrimeSTAR GXL Buffer	5x	1x	4.0	20
dNTPs	2.5 mM	0.2 mM	1.6	8
oligo F	10 uM	0.3 uM	0.6	
oligo R	10 uM	0.3 uM	0.6	
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	2
			Total volume	20
				92

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	- control	-	KROL15, KROL16	-
3	EV	gBlock	KROL495, KROL496	245
4	KRLVS156	KRLVS156	KROL514, KROL515	277

Friday, May 6, 2022

To Do:

1. 20 uL PCR of pKR113 to troubleshoot for EV

Results and Data:

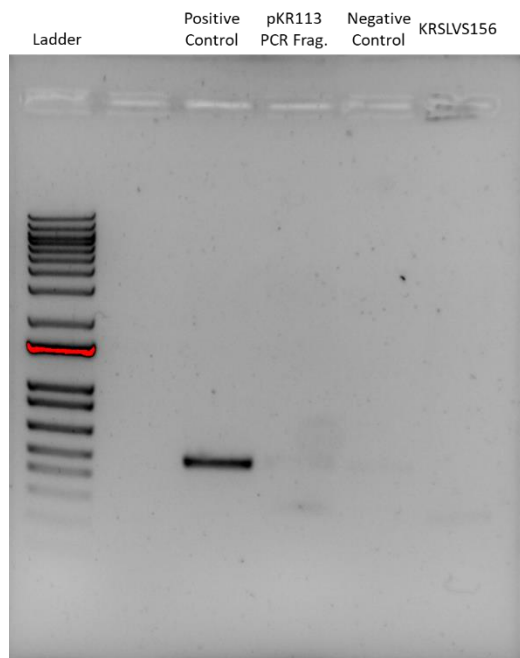


Figure 4. Positive control looks good. No PCR fragment. Negative control good. Checked KRLVS156 for sequencing results validation but no fragment showed up, did not dilute gDNA (end me) so will retry with diluted gDNA. Might need to redo gDNA prep as there is not very much left.

Gel of PCR Fragment from pFTR3-rpsu1-V gBlock and KRLVS156

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 24 uL of positive, negative, pFTR3-rpsu1-V gBlock fragment, and KRLVS156 gDNA KROL514 and 515 amplified.
6. Ran for 45 minutes at 113V.

Monday, May 9, 2022

To Do:

- ~~1. Make 2.5% iron pyrophosphate~~
- ~~2. Supplement MHB~~
- ~~3. Make LB + Kan plates~~
- ~~4. Start dishwasher~~

Results and Data:

N/A.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH₂O (type 1), dissolved overnight, fresh solution every 2 weeks

LB Agar + Kanamycin

Melt LB agar and then cool at 56°C

0.5 mL 50 ug/mL Kanamycin

Pour plates (24 mL)

Friday, May 20, 2022

To Do:

1. ~~Electroporate LanYFP and iLOV into LVS~~
2. ~~Re dilute KROL495 and KROL496~~
3. ~~PCR of EV stuff~~

Results and Data:

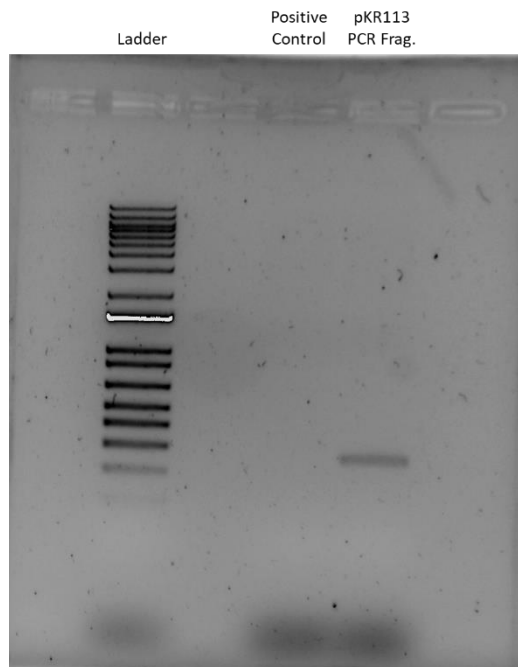


Figure 5. Positive control didn't work, was a little unsure I pipetted it when doing it. But PCR fragment from pKR113 looks right! Negative looks good.

Electroporate plasmid into EC cells*

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
 - 3 μ L of iLOV and LanYFP plasmid DNA
 - 50 μ L electrocompetent cells
3. Have recovery media ready
4. Electroporate using the EC2 program
5. 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
6. Recover cells for 2-3 hours, shaking at 37°C
7. Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
 - a. Plated 10 μ L and 200 μ L of each, straight from recovery tube (because pF plasmid)
8. Incubate plates at 37°C for 3 days (or until single colonies appear)

Both arced, Hannah thinks they will be okay since they're pF plasmids, which are super efficient. Did not include a negative control, as there were not enough cells, maybe. Took iLOV cells from recovery at ~2.5 hours and LanYFP at ~2.75 hours. Plated 10 μ L and 200 μ L rather than remaining due to pF efficiency.

Should have single colonies by Monday, will patch four onto a single CHAH+Kan plate and freeze down for permanent strains. Should also check on our Gel Doc as we might be able to see the fluorescence. Additionally, will want to check on the plate reader in MRamsey lab, need to reach out and ask for permission/training.

Diluting Primers

1. 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.

Re-diluted PCR primer set of KROL495 and KROL496 from the master stock, as when I did it the first time I had no diluted on the same day I received the primers. Due to the odd tube, it's possible that the master had not fully thawed by the time I made the dilution, and I also don't think I vortexed so probably a bad time.

PCR of Diluted pKR113 w/Re-Diluted PCR Primers

1. Acquired and labelled PCR tubes: EV, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL495, KROL16, KROL496, (10uM)
 - Diluted pKR113 and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
4. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - NOT vortexing the enzyme as it will expose it to oxygen and degrade it
5. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
6. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
7. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
8. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
9. Add appropriate volume of master-mix to negative control PCR tube
10. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
11. Close PCR Tubes until the caps are tight
12. 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 30 seconds

- Go back to step 2
- Repeat 32x
- 68 degrees C for 5 minutes
- 12 degrees C for infinity

Total reaction volume	20
Total number of reactions	3

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			12.4	49.6
PrimeSTAR GXL Buffer	5x	1x	4.0	16
dNTPs	2.5 mM	0.2 mM	1.6	6.4
oligo F	10 uM	0.3 uM	0.6	
oligo R	10 uM	0.3 uM	0.6	
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	1.6
			Total volume	20
				78.4

Reaction Number	Plasmid/Region	Source DNA	Primers	Length (bps)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	- control	-	KROL15, KROL16	-
3	EV	pKR113	KROL495, KROL496	245

Gel of PCR Fragment from Diluted pKR113 Plasmid and Re-Diluted Primers

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 24 uL of positive, negative, and dil. pKR113 fragment
6. Ran for 45 minutes at 113V.

Monday, May 23, 2022

To Do:

1. ~~Put away dishes~~
2. ~~PCR of EV stuff :D~~
3. ~~PCR PURIFICATION OH MY GOD~~
4. ~~Restriction Digest~~
5. ~~Patch out transformants (hopefully)~~

Results and Data:

Ladder Positive Control Negative Control pKR113 Backbone pKR113 PCR Frag.

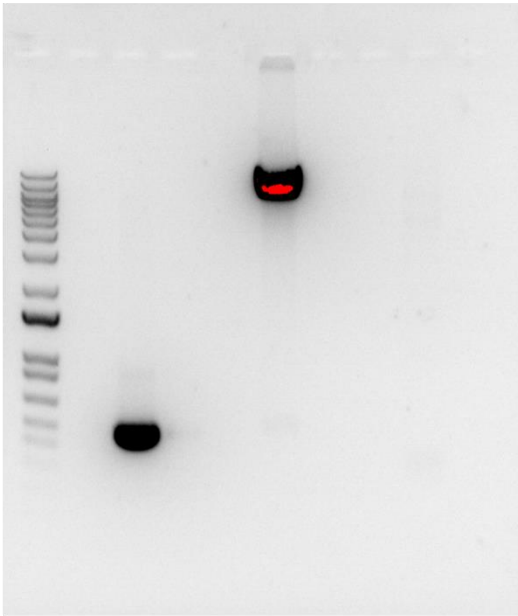


Figure 6. Positive and negative control look good, from PCR. Backbone from restriction digest looks like there's maybe a very faint second band, unsure. No EV fragment.

Kathryn looked at gel and we determined that the stain had run too far on the gel, and so we were unable to see the EV fragment that we were expecting based on last weeks gel. Will plan to re-do the digest and run it again, not allowing it to run as long.

PCR of pKR113 w/Re-Diluted PCR Primers

1. Acquired and labelled PCR tubes: EV, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL495, KROL16, KROL496, (10uM)
 - Diluted pKR113 and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
4. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - NOT vortexing the enzyme as it will expose it to oxygen and degrade it

5. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
6. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
7. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
8. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
9. Add appropriate volume of master-mix to negative control PCR tube
10. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
11. Close PCR Tubes until the caps are tight
12. 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 30 seconds
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	100
Total number of reactions	3

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	4
ddiH ₂ O			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	
oligo R	10 uM	0.3 uM	3.0	
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#	Plasmid/Region	Source DNA	Primers	Length (bp)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	- control	-	KROL15, KROL16	-
3	EV	pKR113	KROL495, KROL496	245

PCR Purification

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 30uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

DNA Digest of pKR113 PCR Fragment w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	pKR113 PCR	KpnI, BamHI	15	-
2	pKR113 plasmid	KpnI, BamHI	5	10

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)
H ₂ O	10.8
10x Buffer (Cutsmart)	3.0
DNA	(15.0)
KpnI	0.6
BamHI	0.6
Total	30.0 (15.0 actual b/c of DNA)

3. Add indicated amounts of H₂O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme (0.6 uL) to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.
7. If using digest for plasmid construction then after incubation at 37°C, add 1 uL of QuickCIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes, mix, incubate for 10 mins, then put at 80°C for 2 minutes to inactivate the enzyme.

Gel of Digested pKR113 PCR Fragment and PCR Positive and Negative Control

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.

2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of positive, negative, and digested pKR113.
6. Ran for 45 minutes at 113V.

Science has not rained its praise on me today. No PCR fragment on gel.

Tuesday, May 24, 2022

To Do:

1. ~~2.5% iron pyrophosphate~~
2. ~~PCR of inducible strains~~
3. ~~Freezing down permanent stocks of fluorescent patches~~

Results and Data:

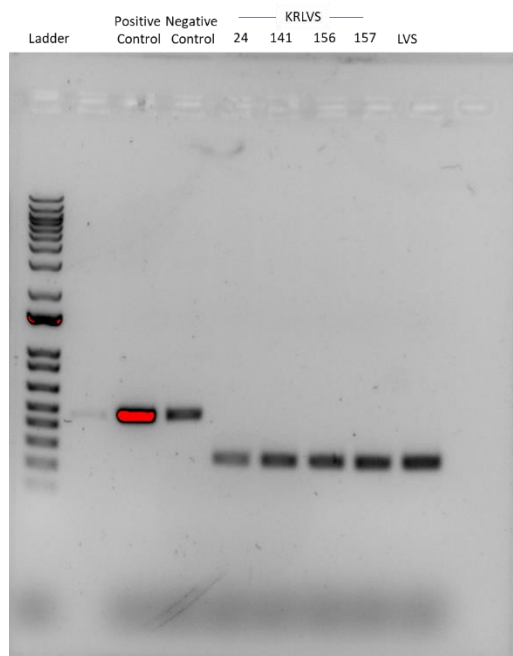


Figure 1. Positive control good. Negative control obviously got LVS gDNA in it. KRLVS24 (the background of the triple mutants) and LVS both had a suspected band size of 235 bp. The three triple mutant strains had an expected band size of 275bp.

Edited plasmids worksheet and created new maps to reflect the error in the current fluorescent strains not containing a VSVG tag. Also confirmed the KROL514 and KROL515 primers on the sequence maps to confirm the size of fragment expected from the PCR. Additionally, checked that there would be a fragment from LVS gDNA in order to set-up a positive control for these primers specifically.

Had Kathryn look at gel of EV from yesterday, stain travels up on a gel, and based on the faintness of the lower ladder bands it's quite possible the band was there but simply unstained. Will try again with the remaining 15 uL of the purified PCR product tomorrow.

PCR of Inducible Strains

1. Acquired and labelled PCR tubes: LVS, 24, 141, 156, 157, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL16, KROL514, KROL515 (10uM)
 - Diluted KRLVS24/141/156/157 gDNA and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
2. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - NOT vortexing the enzyme as it will expose it to oxygen and degrade it
3. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
4. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
5. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
6. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
7. Add appropriate volume of master-mix to negative control PCR tube
8. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
9. Close PCR Tubes until the caps are tight
10. 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 30 seconds
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	20
Total number of reactions	7

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			12.4	99.2
PrimeSTAR GXL Buffer	5x	1x	4.0	32
dNTPs	2.5 mM	0.2 mM	1.6	12.8
oligo F	10 uM	0.3 uM	0.6	
oligo R	10 uM	0.3 uM	0.6	
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	3.2
Total volume			20	156.8

Reaction #	Plasmid/Region	Source DNA	Primers	Length (bp)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	- control	-	KROL15, KROL16	-
3	KRLVS24 gDNA	KRLVS24 gDNA	KROL514, KROL515	235
4	pKR113 Plasmid Recom	KRLVS141 gDNA	KROL514, KROL515	277
5	pKR113 Plasmid Recom	KRLVS156 gDNA	KROL514, KROL515	277
6	pKR113 Plasmid Recom	KRLVS157 gDNA	KROL514, KROL515	277
7	LVS gDNA	LVS gDNA	KROL514, KROL515	235

Freezing and Storing Permanent Strains

1. Per strain, label 2 cryotubes with strain number. Include LVS, strain number, genotype, date.
2. Add 200 uL sterile 75% glycerol to each tube (2 per strain).
3. In a sterile 2 mL tube (1 per strain), add 400 uL of MHB
4. Resuspend patch (all of what you have) in MHB to homogeneity
5. Correct volume to 1700 uL (add 2x 650 uL MHB)
6. Transfer 800 uL to each cryotube (final volume should be 1 mL)
7. Vortex cryotube
8. Quickly spin (mini-fuge) to get liquid to the bottom of the tube
9. Freeze at -80°C in appropriate strain box

Reagents

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH₂O (type 1), dissolved overnight, fresh solution every 2 weeks

Wednesday, May 25, 2022

To Do:

1. ~~Supplement MHB~~
2. ~~PCR of iLOV-V and LanYFP gBlocks~~
3. ~~PCR PURIFICATION OF iLOV-V and LanYFP-V gBlocks~~
4. ~~Restriction digest of EV, BB, iLOV-V, and LanYFP-V PCR~~

Results and Data:

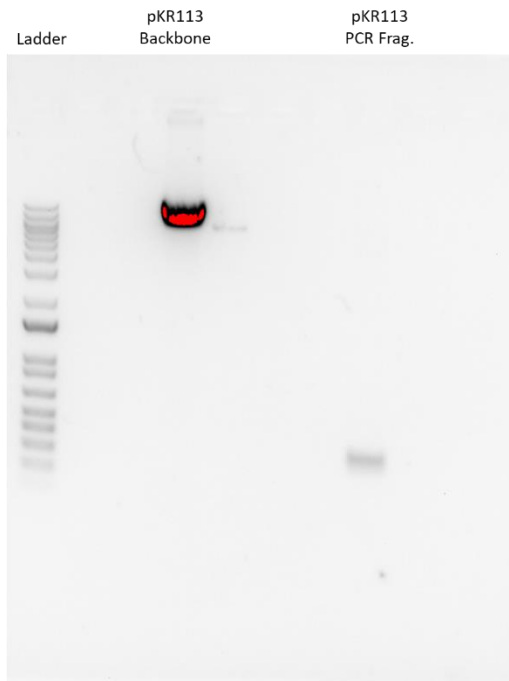


Figure 7. Ran positive and negative PCR on last gel, and did not include in new digest. Both the backbone and the Ev fragment were visible and cut + purified.

PCR of iLOV-V and LanYFP-V gBlock

1. Acquired and labelled PCR tubes: iLov, Lan, positive, and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL16, KROL500, KROL501, KROL502, KROL503 (10uM)
 - Diluted iLOV-V gBlock, LanYFP gBlock, and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
4. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - NOT vortexing the enzyme as it will expose it to oxygen and degrade it
5. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
6. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction

7. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
8. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
9. Add appropriate volume of master-mix to negative control PCR tube
10. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
11. Close PCR Tubes until the caps are tight
12. 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 45 seconds
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	100
Total number of reactions	4

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	5
ddiH ₂ O			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	
oligo R	10 uM	0.3 uM	3.0	
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

Reaction #	Plasmid/Region	Source DNA	Primers	Length (bps)
1	iLOV-V	iLOV-V gBlock	KROL500, KROL501	450
2	+control	LVS gDNA	KROL15, KROL16	441
3	- control	-	KROL500, KROL501	-
4	LanYFP-V	LanYFP-V gBlock	KROL502, KROL503	733

PCR Purification of iLOV-V and LanYFP-V

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 30uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Stored in cloning box.

DNA Digest of pKR113 PCR Fragment w/KpnI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	pKR113 PCR	KpnI, BamHI	15	-
2	pKR113 plasmid	KpnI, BamHI	5	10

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)
H ₂ O	10.8
10x Buffer (Cutsmart)	3.0
DNA	(15.0)
KpnI	0.6
BamHI	0.6
Total	30.0 (15.0 actual b/c of DNA)

3. Add indicated amounts of H₂O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme (0.6 uL) to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.
7. If using digest for plasmid construction then after incubation at 37°C, add 1 uL of QuickCIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes, mix, incubate for 10 mins, then put at 80°C for 2 minutes to inactivate the enzyme.

Visualizing and Cutting Gel

1. Using Bio Rad Gel Doc XR+ imager equipped with XcitaBlue Conversion Screen
2. Nucleic Acid Gel > Sybr Safe
3. To cut gel, put on visualizing UV glasses and use a clean razor, cutting straight down. Add to 2mL tube.

UuuuUUUUUGH. Had issues doing the extraction, didn't seem to get all of the backbone but its always in such a high concentration think it will be fine. Got all of the EV fragment which appeared to be less based on the lightness, but the ladder was also somewhat faint, though these appeared darker after the backbone was cut. Who knows, if the ligation and transformation works then it doesn't matter anyways.

Gel Extraction with QIAquick Gel Extraction Kit

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
3. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
6. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 30uL Buffer 0.01X EB and let stand for a minute. Centrifuge for 1 min at 13,000rpm.

Component	Weight	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
pKR113 PCR Frag	380mg	1140uL	380uL
pKR113 Backbone	390mg	1170uL	390uL

Re-eluted the backbone because I wanted to let the EB sit for 1-4 minutes for high concentration, but then forgot which tube I added the EB to, so I spun them both then took the EB from the backbone and reapplied it to the column, and fresh EB to the EV column and let them sit for 2 minutes, then spun again and saved the flow-through.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

Thursday, May 26, 2022

To Do:

- 1. Restriction digest of iLOV-V and LanYFP
- 2. Gel purification of iLOV-V and LanYFP-V
- 3. Ligation of EV:pKR113BB and iLOV-V:pFBB and LanYFP-V:pFBB
- 4. PCR of sequencing confirmation
- 5. Gel of sequencing confirmation
- 6. PCR Purification of seq. PCR

Results and Data:

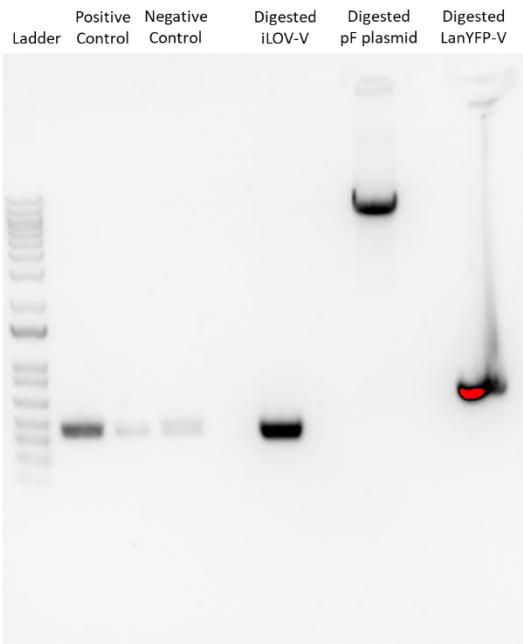


Figure 1. Positive control looked good, but there was obvious contamination in the negative control. However, the iLOV-V and LanYFP-V bands looked good, as well as the digested pF backbone, so these were cut and purified.

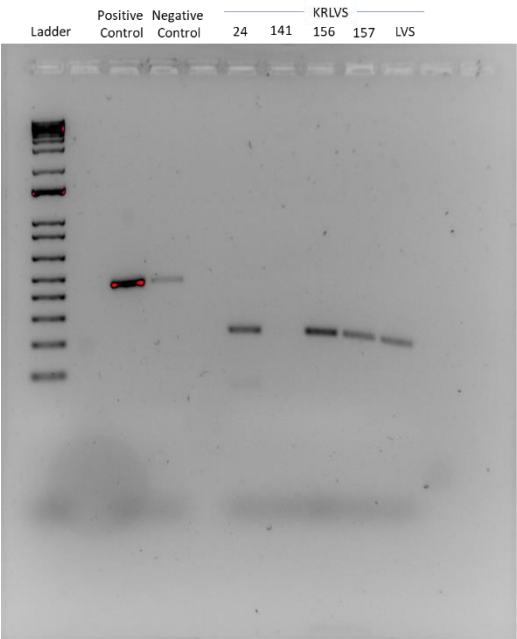


Figure 2. Positive control good. Negative control has obvious contamination. Additionally, it seems that the ion gradient was a little funky, so the lanes ran weirdly and cannot be easily interpreted.

Re-did PCR, digest, and gel purification of the iLOV-V and LanYFP-V samples, as I was not very confident in my gel extraction on May 1st. There was contamination in the negative control and clearly the LanYFP-V was rather smeary, but I went ahead and cut these out the best that I could.

DNA Digest of iLOV-V, LanYFP-V, and pF w/EcoRI and BamHI

1. Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	iLOV-V PCR	EcoRI, BamHI	15	-
2	LanYFP-V PCR	EcoRI, BamHI	15	-
3	pF plasmid	EcoRI, BamHI	5	10

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 4x (uL)
H ₂ O	10.8	43.2
10x Buffer*	3.0	12.0
DNA	(15.0)	-
EcoRI	0.6	2.4
BamHI	0.6	2.4
Total	30.0	60.0

3. Add indicated amounts of H₂O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme (0.6 uL) to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.
7. If using digest for plasmid construction then after incubation at 37°C, add 1 uL of QuickCIP enzyme to backbone tube, mix by pipetting and put back in 37°C incubator for 10 minutes, mix, incubate for 10 mins, then put at 80°C for 2 minutes to inactivate the enzyme.

Gel of Digested pKR113 PCR Fragment and PCR Positive and Negative Control

1. Melt agarose gel until completely dissolved, then place in 50°C water bath until cool enough to touch.
2. Set up gel rig to cast gel, with ladder.
3. Add 6uL of Sbyr Safe dye to gel rig, pour ~60uL of agarose gel, use ladder to mix, then replace ladder and allow to set.
4. Turn gel, add used TAE, remove ladder.
5. Loaded 15 uL ladder, and 36 uL of positive (PCR), negative (PCR), and digested iLOV-V, LanYFP-V, and pF.
6. Ran for 45 minutes at 113V.

Lanes									
1	2	3	4	5	6	7	8	9	10
Ladder	Positive?		Negative		iLOV-V		pF		LanYFP

Visualizing and Cutting Gel

1. Using Bio Rad Gel Doc XR+ imager equipped with XcitaBlue Conversion Screen
2. Nucleic Acid Gel > Sybr Safe
3. To cut gel, put on visualizing UV glasses and use a clean razor, cutting straight down. Add to 2mL tube.

Gel Extraction with QIAquick Gel Extraction Kit

1. Excise the DNA fragment from the agarose gel with a clean, sharp razor
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel. (100mg~100uL)
3. Incubate at 42C for 10 minutes or until slice is dissolved. Vortex every 2-3min to help slice dissolve.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a 2mL collection tube. Centrifuge for 1 min at 13,000rpm. Discard flow through. For samples >800uL, load and spin the rest of the volume.
6. Add 500uL Buffer QG to the QIAquick column. Centrifuge for 1 min at 13,000rpm. Discard flow through. Place back into the same tube.
7. Wash: Add 750uL Buffer PE to the QIAquick column. Centrifuge at 1 min at 13,000rpm. Discard flow through. Place back into the same tube. Centrifuge again for 3 minutes. Discard flow through.
8. Place columns in a fresh 1.5mL microcentrifuge tube.
9. Elute: Add 30uL Buffer 0.01X EB and let stand for a minute. Centrifuge for 1 min at 13,000rpm.

Component	Weight (mg)	Buffer QG 3 vol (uL)	Isopropanol 1 vol (uL)
iLOV-V	180	540	180
LanYFP-V	200	600	200
pF Backbone	260	780	260

PCR of Inducible Strains

1. Acquired and labelled PCR tubes: LVS, 24, 141, 156, 157, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL16, KROL514, KROL515 (10uM)
 - Diluted KRLVS24/141/156/157 gDNA and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
2. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - NOT vortexing the enzyme as it will expose it to oxygen and degrade it
3. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes

4. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
5. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
6. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
7. Add appropriate volume of master-mix to negative control PCR tube
8. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
9. Close PCR Tubes until the caps are tight
10. 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 30 seconds
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	50
Total number of reactions	7

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			31.0	248
PrimeSTAR GXL Buffer	5x	1x	10.0	80
dNTPs	2.5 mM	0.2 mM	4.0	32
oligo F	10 uM	0.3 uM	1.5	12
oligo R	10 uM	0.3 uM	1.5	12
template	100 ng/ul	2 ng/ul	1.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	1.0	8
Total volume			50	392

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bps)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	- control	-	KROL15, KROL16	-
3	KRLVS24 gDNA	KRLVS24 gDNA	KROL514, KROL515	235
4	pKR113 Plasmid Recom	KRLVS141 gDNA	KROL514, KROL515	277
5	pKR113 Plasmid Recom	KRLVS156 gDNA	KROL514, KROL515	277
6	pKR113 Plasmid Recom	KRLVS157 gDNA	KROL514, KROL515	277
7	LVS gDNA	LVS gDNA	KROL514, KROL515	235

Making 2% Agarose Gel

1. Add 2.4 g agarose powder to 120 mL 1x TAE buffer in 250 mL.
2. Add stirbar to container.
3. Heat to dissolve the agarose while stirring (don't let it overboil, should look like clear liquid, no solids).
4. Let the 2% agarose solution cool to approximately 50-55°C.

Running 2% Gel of Inducible Strains

1. Loaded 10uL of ladder and 5uL of each sample
2. Ran at 113V for ~2-2.5 hours

PCR Purification of Inducible Strains

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 30uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Friday, May 27, 2022

To Do:

1. ~~20 μ L PCR of sequencing confirmation, just to be sure.~~
2. ~~Gel of sequencing confirmation~~

Results and Data:

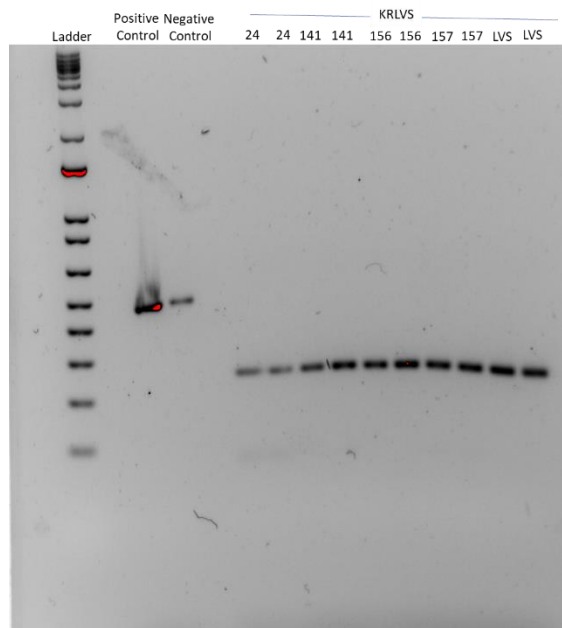


Figure 3. Positive control is okay, though it is smeary. However, clear contamination in the negative control, and once again an uneven ion gradient makes it difficult to interpret.

PCR of Inducible Strains

1. Acquired and labelled PCR tubes: LVS, 24, 141, 156, 157, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL16, KROL514, KROL515 (10 μ M)
 - Diluted KRLVS24/141/156/157 gDNA and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
4. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
 - NOT vortexing the enzyme as it will expose it to oxygen and degrade it
5. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
6. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
7. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O

- Add dNTPs
 - Add PrimeStar buffer
 - Add PrimeStar enzyme
8. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
 9. Add appropriate volume of master-mix to negative control PCR tube
 10. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
 11. Close PCR Tubes until the caps are tight
 12. 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 30 seconds
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	20
Total number of reactions	7

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			12.4	99.2
PrimeSTAR GXL Buffer	5x	1x	4.0	32
dNTPs	2.5 mM	0.2 mM	1.6	12.8
oligo F	10 uM	0.3 uM	0.6	4.8
oligo R	10 uM	0.3 uM	0.6	4.8
template	100 ng/ul	2 ng/ul	0.4	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	0.4	3.2
Total volume			20	156.8

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bps)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	- control	-	KROL15, KROL16	-
3	KRLVS24 gDNA	KRLVS24 gDNA	KROL514, KROL515	235
4	pKR113 Plasmid Recom	KRLVS141 gDNA	KROL514, KROL515	277
5	pKR113 Plasmid Recom	KRLVS156 gDNA	KROL514, KROL515	277
6	pKR113 Plasmid Recom	KRLVS157 gDNA	KROL514, KROL515	277
7	LVS gDNA	LVS gDNA	KROL514, KROL515	235

Making 2% Agarose Gel

1. Add 2.4 g agarose powder to 120 mL 1x TAE buffer in 250 mL.
2. Add stirbar to container.
3. Heat to dissolve the agarose while stirring (don't let it overboil, should look like clear liquid, no solids).
4. Let the 2% agarose solution cool to approximately 50-55°C.

Running 2% Gel of Inducible Strains

1. Loaded 10uL of ladder and 5uL of each sample
2. Ran at 113V for ~2-2.5 hours

Lane																			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
-	La	-	(+)	(-)	-	24	24	141	141	156	156	157	157	LVS	LVS	-	-	-	-

June 2022

Monday, June 6, 2022

To Do:

1. ~~Ligation of EV pKR113 backbone~~
2. ~~Transform *E. coli* with ligation + plate~~
3. ~~Make 2.5% pyrophosphate~~

Results and Data:

Transformation: No growth. 😞

Ligation of EV and pKR113 backbone

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

Tube	Insert	Backbone
1	BamHI, KpnI dig., purified PCR	BamHI, KpnI digested, purified pKR113
2	-	BamHI, KpnI digested, purified pKR113

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (3x)
H ₂ O	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 in order to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer.
4. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H₂O (____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 on bench for 10 minutes.

Transformation of EV Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 100 uL and remaining culture.
 - Positive control: pKR113; Backbone ligation: BamHI-KpnI digested pKR113

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR113	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Backbone Ligation	pR113 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1	pKR135	8 uL	100 uL, remaining	2
Total number of plates					8

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 heatblock 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.

Tuesday, June 7, 2022

To Do:

- ~~1. Re dilute KROL15 and KROL16~~
- ~~2. Supplement MHB~~
- ~~3. Run dishwasher~~

Results and Data:

N/A

Re-Diluting KROL15 and KROL16

1. 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.

Potential LVS gDNA contamination in the KROL15 and KROL16 primers based on the negative controls that Marissa and I have had contamination in. After

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

Wednesday, June 8, 2022

To Do:

- ~~1. Set up inducible strain PCR with new aliquots~~
- ~~2. PCR purify products and run 5 uL~~
- ~~3. Make and run 2% agarose gel~~
- ~~4. Ligate EV~~

Results and Data:

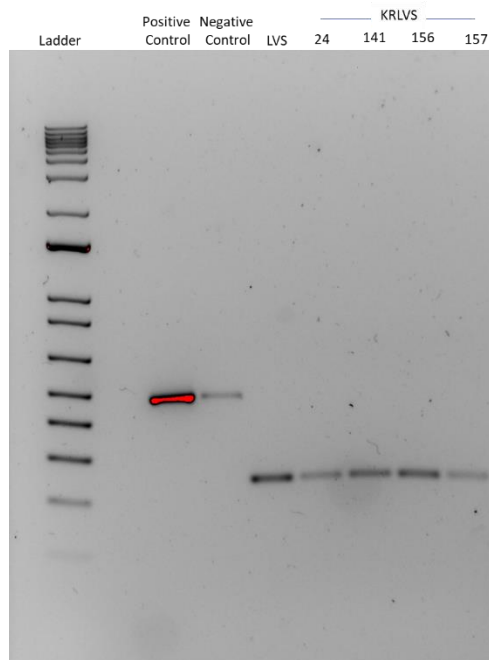


Figure 4. Positive control good. Negative control has obvious contamination. Additionally, it seems that the ion gradient was still odd and the curve makes it difficult to interpret.

PCR of Inducible Strains

1. Acquired and labelled PCR tubes: LVS, 24, 141, 156, 157, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL16, KROL514, KROL515 (10uM)
 - Diluted KRLVS24/141/156/157 gDNA and LVS gDNA
3. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)
4. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
5. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
6. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O

- Add dNTPs
 - Add PrimeStar buffer
 - Add PrimeStar enzyme
7. Mix the master-mix solution by pipetting up and down
 8. Add appropriate volume of master-mix to negative control PCR tube
 9. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
 10. Close PCR Tubes until the caps are tight
 11. 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 30 seconds
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	50
Total number of reactions	7

Component	Stock concentration	Final concentration	1 rxn volume	Factor
ddiH ₂ O			31.0	248
PrimeSTAR GXL Buffer	5x	1x	10.0	80
dNTPs	2.5 mM	0.2 mM	4.0	32
oligo F	10 uM	0.3 uM	1.5	12
oligo R	10 uM	0.3 uM	1.5	12
template	100 ng/ul	2 ng/ul	1.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	1.0	8
Total volume			50	392

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bps)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	- control	-	KROL15, KROL16	-
3	KRLVS24 gDNA	KRLVS24 gDNA	KROL514, KROL515	235
4	pKR113 Plasmid Recom	KRLVS141 gDNA	KROL514, KROL515	277
5	pKR113 Plasmid Recom	KRLVS156 gDNA	KROL514, KROL515	277
6	pKR113 Plasmid Recom	KRLVS157 gDNA	KROL514, KROL515	277
7	LVS gDNA	LVS gDNA	KROL514, KROL515	235

Got new aliquots of dNTPs, Buffer, and MG H₂O, in addition to the re-diluted KROL15 and KROL16.

Making 2% Agarose Gel

1. Add 2.4 g agarose powder to 120 mL 1x TAE buffer in 250 mL.
2. Add stirbar to container.
3. Heat to dissolve the agarose while stirring (don't let it overboil, should look like clear liquid, no solids).
4. Let the 2% agarose solution cool to approximately 50-55°C.

Running 2% Gel of Inducible Strains

1. Loaded 10uL of ladder and 5uL of each sample
2. Run at 113V for ~2-2.5 hours

PCR Purification of Inducible Strains

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 30uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Ligation of EV and pKR113 backbone

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

Tube	Insert	Backbone
1	BamHI, KpnI dig., purified PCR	BamHI, KpnI digested, purified pKR113
2	-	BamHI, KpnI digested, purified pKR113

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (3x)
H ₂ O	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 in order to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
4. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H₂O (___uL), 10x buffer (___uL), insert (___uL), and backbone (___uL).
6. Add indicated amount of ligase (___uL) to the individual tubes.

7. After all of the components have been added, mix each tube with a pipette set to 18 uL.
8. Let incubate at 16°C overnight in the thermocycler.

Reagents

LB Agar + Kanamycin

Melt LB agar and then cool at 56°C

0.5 mL 50 ug/mL Kanamycin

Pour plates

Thursday, June 9, 2022

To Do:

- ~~1. Transformation of pKR135~~
- ~~2. Ligation of LanYFP-V and iLOV-V~~
- ~~1. Transformation of LanYFP-V and iLOV-V~~

Results and Data:

N/A.

Transformation of EV Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 100 uL and remaining culture.
 - a. Positive control: pKR113; Backbone ligation: BamHI-KpnI digested pKR113

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR113	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Backbone Ligation	pR113 (digested)	8 uL	100 uL, remaining	2
4	Ligation 1	pKR135	8 uL	100 uL, remaining	2
Total number of plates					8

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 heatblock 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.

Ligation of iLOV-V and LanYFP-V with pF backbone

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

Tube	Insert	Backbone
1	BamHI, EcoRI dig., purified iLOV PCR	BamHI, EcoRI digested, purified pF
2	BamHI, EcoRI dig., purified LanYFP-V PCR	BamHI, EcoRI digested, purified pF
3	-	BamHI, EcoRI digested, purified pF

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Reaction 3 (uL)	Master Mix (4x)
H ₂ O	11.5	11.5	11.5	46.0
10x ligase buffer	2.0	2.0	2.0	8.0
Insert	4.0	4.0	-	-
Backbone	2.0	2.0	2.0	8.0
Ligase	0.5	0.5	0.5	2.0
TOTAL	20.0	20.0	20.0	64.0

1. 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 in order to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
2. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
3. To the individual tubes, add indicated amounts of H₂O (____uL), 10x buffer (____uL), insert (____uL), and backbone (____uL).
4. Add indicated amount of ligase (____uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
5. After all of the components have been added, mix each tube with a pipette set to 18 uL.
6. Place on bench for 10 minutes.

Transformation of iLOV-V/LanYFP-V Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 10 uL and 200 uL.

a. Positive control: pF; Backbone ligation: BamHI-EcoRI digested pF

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pF	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Backbone Ligation	pF (digested)	8 uL	10 uL, 200 uL	2
4	Ligation 1	pKR142	8 uL	10 uL, 200 uL	2
5	Ligation 2	pKR143	8 uL	10 uL, 200 uL	2
Total number of plates					10

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 heatblock 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.

Friday, June 10, 2022

To Do:

- ~~1. Start overnight cultures of any transformants~~
- ~~2. Count plates.~~

Results and Data:

Transformation Plate Counts				
	Ligation		Backbone Only	
Plasmid	100 uL	Remaining	100 uL	Remaining
pKR135	☹	☹	☹	☹

Plasmid	10 uL	100 uL	10 uL	100 uL
pKR142	10	>100	3	71
pKR143	10	>100	3	71

Did not see colonies on pKR135 plates. Will test the DNA concentrations, but may have flubbed the gel extraction. I was nervous and having a harder time seeing that day and did struggle to excise the gel. Will have to start from PCR again if the concentrations are bad. Will also leave the plates out overnight juuust in case.

Made four 5mL LB + 50 ug/mL Kan overnight cultures each for pKR142 and pKR143.

Saturday, June 11, 2022

To Do:

- ~~1. Check Aisling's plates~~
- ~~2. Check EV plates for potential growth~~
- ~~3. Miniprep pKR142 and pKR143~~
- ~~4. Nanodrop miniprep~~
- ~~5. Run dishwasher~~

Results and Data:

Definitely no growth on the pKR113 plates, the backbone, or the pKR135 plate. Maybe should try a control with a better plasmid to confirm the transformation itself is working? Other transformation done that day worked, so not super sure why this one didn't. I believe Hannah said she had a difficult time making the strains, but I'm not sure that was a pKR113 problem. But maybe?

Sample	Conc. (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
iLOV-V 1	238.4	4.769	2.519	1.89	2.32
iLOV-V 2	216.5	4.329	2.287	1.89	2.44
iLOV-V 3	289.9	5.798	3.068	1.89	2.37
iLOV-V 4	219.0	4.379	2.313	1.89	2.04
LanYFP-V 1	236.8	4.737	2.506	1.89	2.40
LanYFP-V 2	259.7	5.194	2.742	1.89	2.38
LanYFP-V 3	245.5	4.909	2.596	1.89	2.39
LanYFP-V 4	210.1	4.201	2.226	1.89	2.38

Miniprep of pKR142 and pKR143 from *E. coli*

1. Pellet overnight cultures by centrifugation at 15,000rpm for 3 minutes.
2. Resuspend pellets in a 250uL Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow lysis reaction to proceed for more than 5 minutes.
4. Add 350uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 minutes at 13,000rpm.
6. Transfer 800uL of supernatant to a QIAprep 2.0 spin column through pipetting.
7. Wash the QIAprep column by adding 500uL Buffer PB. Centrifuge for 30-60s and discard flow through.
8. Wash the QIAprep column by adding 750uL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Centrifuge for 3 minutes at 13,000rpm to remove residual buffer.
10. Place QIAprep column in a clean 1.5mL microcentrifuge tube. Add 50uL of Buffer EB. Let stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Thursday, June 16, 2022

To Do:

1. Transformation of pKR135 using previous ligation

Results and Data:

Oh my god it's NAT RESISTANT NOT KAN RESISTANT. End me. ☹️ Repeating transformation with LB+Nat plates.

Transformation of EV Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 100 uL and remaining culture.
 - a. Positive control: pKR113; Backbone ligation: BamHI-KpnI digested pKR113

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control - 1	pKR113	1 uL	20 uL, 100 uL	2
3	(-) control	None	0	20 uL, 100 uL	2
4	Backbone Ligation	pR113 (digested)	8 uL	100 uL, remaining	2
5	Ligation 1	pKR135	8 uL	100 uL, remaining	2
Total number of plates					8

2. Check to be sure you have enough LB 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.

Friday, June 17, 2022

To Do:

1. ~~Inducible strain PCR~~
2. ~~PCR purification of inducible strains~~
3. ~~Inducible strain 2% gel~~
4. ~~Count transformation plates~~

Results and Data:

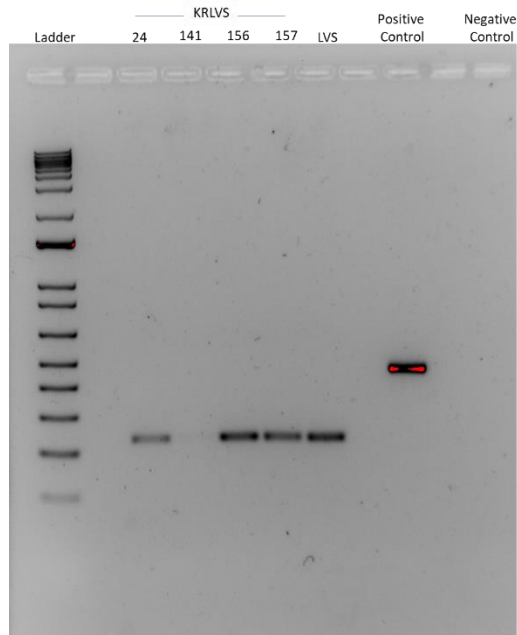


Figure 5. Positive control good. Negative control good. KRLVS141 is very low, as there was little DNA left. That said it does not appear as though there is any difference in the band sizes. Will test through sequencing.

Transformation Plate Counts				
	Ligation		Backbone Only	
Plasmid	100 uL	Remaining	100 uL	Remaining
pKR135	13	88	14	96

Predictably the proper antibiotic plates worked. However, the 1:1 ratio of the ligation:backbone is not promising. Will probably needed to do the 3X and 5X ligation. Additionally, will need to make more LB+Nat plates after I do the transformation next week.

PCR of Inducible Strains

1. Acquired and labelled PCR tubes: LVS, 24, 141, 156, 157, positive and negative control.
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
 - Primestar buffer
 - dNTPs
 - KROL15, KROL16, KROL514, KROL515 (10uM)
 - Diluted KRLVS24/141/156/157 gDNA and LVS gDNA
 - Note: Primestar enzyme kept in the freezer until it is used-- added last
4. If any of the solutions are frozen, be sure to vortex the microcentrifuge tube in order to dissolve it (tubes with frozen components may not be homogenized)

5. Add appropriate volume (based on PCR worksheet) of each experiment specific primer (forward and reverse) and respective template to PCR tubes
6. Add ddi H₂O to negative control tube
 - Template volume for 1 reaction
7. Prepare a master-mix in a 1.5 mL microcentrifuge tube by adding the following according to the worksheet and using micropipettes:
 - Add ddi H₂O
 - Add dNTPs
 - Add Primestar buffer
 - Add Primestar enzyme
8. Mix the master-mix solution by pipetting up and down
 - Do not vortex to mix
9. Add appropriate volume of master-mix to negative control PCR tube
10. Add appropriate volume of master mix to each PCR tube (except negative control) and pipet up and down to mix
11. Close PCR Tubes until the caps are tight
12. 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 30 seconds
 - Go back to step 2
 - Repeat 32x
 - 68 degrees C for 5 minutes
 - 12 degrees C for infinity

Total reaction volume	50
Total number of reactions	7

				Factor
Component	Stock concentration	Final concentration	1 rxn volume	8
ddiH ₂ O			31.0	248
PrimeSTAR GXL Buffer	5x	1x	10.0	80
dNTPs	2.5 mM	0.2 mM	4.0	32
oligo F	10 uM	0.3 uM	1.5	12
oligo R	10 uM	0.3 uM	1.5	12
template	100 ng/ul	2 ng/ul	1.0	
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	1.0	8
			Total volume	50
				392

Reaction#	Plasmid/Region	Source DNA	Primers	Length (bps)
1	+ control	LVS gDNA	KROL 15, KROL16	441
2	- control	-	KROL15, KROL16	-
3	KRLVS24 gDNA	KRLVS24 gDNA	KROL514, KROL515	235
4	pKR113 Plasmid Recom	KRLVS141 gDNA	KROL514, KROL515	277
5	pKR113 Plasmid Recom	KRLVS156 gDNA	KROL514, KROL515	277
6	pKR113 Plasmid Recom	KRLVS157 gDNA	KROL514, KROL515	277
7	LVS gDNA	LVS gDNA	KROL514, KROL515	235

Got new aliquot of enzyme and ethanol'd literally everything multiple times.

Making 2% Agarose Gel

1. Add 2.4 g agarose powder to 120 mL 1x TAE buffer in 250 mL.
2. Add stirbar to container.
3. Heat to dissolve the agarose while stirring (don't let it overboil, should look like clear liquid, no solids).
4. Let the 2% agarose solution cool to approximately 50-55°C.

Running 2% Gel of Inducible Strains

1. Loaded 10uL of ladder and 5uL of each sample
2. Run at 113V for ~2-2.5 hours

Lanes											
1	2	3	4	5	6	7	8	9	10	11	12
Ladder	-	24	141	156	157	LVS	-	(+)	-	(-)	-

Using wider comb and skipping lane between positive and negative control.

NO NEGATIVE CONTAMINATION :D

PCR Purification of Inducible Strains

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a 2mL collection tube.
3. Centrifuge tube for 30-60s at 13,000rpm. Discard flow through.
4. Wash: add 750uL of Buffer PE to the QIAquick column. Centrifuge for 30-60s at 13,000rpm. Discard flow through.
5. Centrifuge again for 3 minutes at 13,000rpm to remove any residual wash buffer.
6. Place the QIAquick column in a fresh 1.5mL centrifuge tube.
7. Elute: add 30uL of Buffer 0.1x EB. Let column stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Monday, June 20, 2022

To Do:

1. ~~3x and 5x ligation of pKR135~~
2. ~~Make and filter sterilize 2.5% iron pyrophosphate~~
3. ~~Run dishwasher~~

Results and Data:

Sample	Nucleic Acid (ng/ul)	A260 (Abs)	A280 (Abs)	260/280	260/230
Empty Vector Insert	10.5	0.209	0.124	1.69	0.06
pKR113 BamHI/KpnI dig.	27.8	0.556	0.271	2.05	0.04

Ligation of Empty Vector Control into pKR113 Backbone

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

Tube	Insert	Backbone
1	BamHI, KpnI dig., purified pKR113 PCR	BamHI, KpnI digested, purified pKR113
2	-	BamHI, KpnI digested, purified pKR113

2. Set up master mix table:

	3X	5X	BB only
Water	15.21	14.89	15.70
Ligation Buffer	2	2	2
Backbone	1.80	1.80	1.80
Insert	0.49	0.82	-
Ligase	0.5	0.5	0.5
	20.00	20.00	20.00

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 in order to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
4. Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
5. To the individual tubes, add indicated amounts of H₂O (___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 on bench for 10 minutes.

Reagents

2.5% Iron pyrophosphate

0.75g iron pyrophosphate (cabinet) in 30mL of ddiH₂O (type 1), dissolved overnight, fresh solution every 2 weeks

Tuesday, June 21, 2022

To Do:

1. ~~Supplement MHB~~
2. ~~Put away dishes~~
3. ~~Transform pKR135 into *E. coli*~~

Results and Data:

N/A.

Transformation of EV Ligation into *E. coli*

1. Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 100 uL and remaining culture.
 - a. Positive control: pKR113; Backbone ligation: BamHI-KpnI digested pKR113

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR113	1 uL	20 uL, 100 uL	2
3	(-) control	None	0	20 uL, 100 uL	2
4	Backbone Ligation	pR113 (digested)	8 uL	100 uL, remaining	2
5	3x Ligation	pKR135	8 uL	100 uL, remaining	2
6	5x Ligation	pKR135	8 uL	100 uL, remaining	2
Total number of plates					10

2. Check to be sure you have enough LB 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.

Reagents

Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalax (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

Wednesday, June 22, 2022

To Do:

1. ~~Count transformant plates~~
2. ~~Make overnights of pKR135 transformants~~
3. ~~Set up sequencing for pKR142 and pKR143~~
4. ~~Set up sequencing for inducible strains~~

Results and Data:

Transformation Plate Counts						
	3x Ligation		5x Ligation		Backbone Only	
Plasmid	100 uL	Remaining	100 uL	Remaining	100 uL	Remaining
pKR135	3	25	10	100	4	48

Made overnights of six colonies in LB+50 ug/mL Nat. The ratios are pretty terrible, but making six overnights allegedly makes it a 1.5% chance that none of the colonies would be what we're looking for.

Sample#	Template	Template Name	Primer Name	A.	B.	E.	F.
	(plasmid or PCR)			Template Size (bp)	Template Conc. (ng/μl)	<u>PLASMID</u> <u>template:</u>	<u>Volume</u> <u>H₂O</u>
SS1	Plasmid	pKR142-1	KROL44	7644	238.40	1.68	7.76
SS2	Plasmid	pKR142-2	KROL44	7644	216.50	1.85	7.59
SS3	Plasmid	pKR142-3	KROL44	7644	289.90	1.38	8.06
SS4	Plasmid	pKR142-4	KROL44	7644	219.00	1.83	7.61
SS5	Plasmid	pKR143-1	KROL44	7927	236.80	1.69	7.75
SS6	Plasmid	pKR143-2	KROL44	7927	259.70	1.54	7.90
SS7	Plasmid	pKR143-3	KROL44	7927	245.50	1.63	7.81
SS8	Plasmid	pKR143-4	KROL44	7927	210.10	1.90	7.54

Sample	Nucleic Acid (ng/ul)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS24 PCR	2.5	0.051	0.026	1.98	-2.73
KRLVS141 PCR	6.0	0.120	0.059	2.02	7.33
KRLVS156 PCR	6.7	0.134	0.075	1.80	4.24
KRLVS157 PCR	6.2	0.124	0.085	1.45	5.13
LVS PCR	6.8	0.135	0.085	1.59	14.42

Sample	Template	Template Name	Primer Name	A.	B.	C.	D.	F.
	(plasmid or PCR)			Template Size (bp)	Template Conc. (ng/μl)	<u>PCR</u> <u>template</u>	<u>PCR</u> <u>template</u>	<u>Volume</u> <u>H₂O</u>
SS9	PCR	KRLVS24_Induc_PCR	KROL514	235	2.5	5.88	2.35	7.09
SS10	PCR	KRLVS141_Induc_PCR	KROL514	277	6	6.93	1.15	8.29
SS11	PCR	KRLVS156_Induc_PCR	KROL514	277	6.7	6.93	1.03	8.41
SS12	PCR	KRLVS157_Induc_PCR	KROL514	277	6.2	6.93	1.12	8.32
SS13	PCR	LVS_Induc_PCR	KROL514	235	6.8	5.88	0.86	8.58

Had to dilute purified PCR products in order to make a pipetable amount. Using KROL514 diluted from 10 uM to 2.5 uM, arbitrarily. Could have used KROL515 too.

Thursday, June 23, 2022

To Do:

1. ~~Miniprep pKR135 transformants~~
2. ~~Nanodrop miniprep~~
3. ~~Make CHAH+Kan plates~~
4. ~~Patch out LVS for EC~~
5. ~~Patch out LVS for more SUA~~
6. ~~Streak *Arpsu2* cells to single colony~~

Results and Data:

Sample	Nucleic Acid (ng/ul)	A260 (Abs)	A280 (Abs)	260/280	260/230
pKR135-1	581.4	11.629	6.042	1.92	2.40
pKR135-2	555.6	11.113	5.850	1.90	2.40
pKR135-3	590.8	11.815	6.265	1.89	2.38
pKR135-4	564.0	11.280	5.915	1.91	2.35
pKR135-5	238.2	4.764	2.524	1.89	2.32
pKR135-6	595.4	11.907	6.275	1.90	2.34

Looked at plasmid map, will use KROL44 for sequencing. Covers the whole insert in >400bp and has a 169 lead before the insert.

Miniprep of pKR135 from *E. coli*

1. Pellet overnight cultures by centrifugation at 15,000rpm for 3 minutes.
2. Resuspend pellets in a 250uL Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow lysis reaction to proceed for more than 5 minutes.
4. Add 350uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 minutes at 13,000rpm.
6. Transfer 800uL of supernatant to a QIAprep 2.0 spin column through pipetting.
7. Wash the QIAprep column by adding 500uL Buffer PB. Centrifuge for 30-60s and discard flow through.
8. Wash the QIAprep column by adding 750uL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Centrifuge for 3 minutes at 13,000rpm to remove residual buffer.
10. Place QIAprep column in a clean 1.5mL microcentrifuge tube. Add 50uL of Buffer EB. Let stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Reagents

CHAH+Kan

To a 1L flask add:

- 30.6g of cystine heart agar
- 300mL of ddiH2O (type I)
- stirbar

Heat on 60°C, stirring, for 10 minutes (media should be totally dissolved)

Autoclave on 30' liquid cycle, filling the water bin up to the height of the media

Add 2% hemoglobin to water bath, after autoclave add CHA as well
Using sterile technique, pour hemoglobin into CHA
For 5 ug/ml, add 60 ul of 50 mg/mL kanamycin to 600 mL of media

Friday, June 24, 2022

To Do:

- ~~1. Prepare electrocompetent LVS~~
- ~~2. Prepare new single use aliquots of LVS (16)~~

Results and Data:

Sequencing results received today for inducible strains PCR and pKR142/pKR143.

Making Glycerol Stocks Protocol

1. Prepare 0.8 mL of MHB in a 1.5mL tube
2. Take at least half of a thickly spread plate and add cells to the MHB tube
3. Resuspend until there are no clumps in the MHB
4. Add 200ul of 75% glycerol to the 0.8 mL resuspension, mix by pipetting
5. Aliquot 50ul of solution to tubes for single use

Preparing Electrocompetent LVS Cells

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

Saturday, June 25, 2022

To Do:

- ~~1. Streak LVS to single colony~~
- ~~2. Electroporate pKR142 and pKR143 into LVS~~

Results and Data:

N/A

Looking at the sequence files from Janet, it appears that only one of the four miniprep colonies of pF_iLOV-V was a successful ligation, and the other three were backbone self-ligations. There were no mutations in the iLOV gene nor the VSVG tag, though there was one in the promoter. Went ahead and decided to electroporate it into LVS. Alternatively, and unfortunately, the four miniprep colonies from pF_LanYFP-V were all self-ligations, and so I was unable to go forward with the electroporation of this plasmid. I double checked all sequence files from that day, ensuring that there was no way that I had mixed up the iLOV and LanYFP plasmids for sequencing, on both plasmid maps and what was said previously remained accurate. Will plan to do a tabletop ligation and transformation of the gel purified

backbone and LanYFP-V insert on Monday. Previously, doing the tabletop ligation seemed to work just fine, so I see no issue in going forward with this.

Electroporate pKR142 iLOV-V into EC cells*

1. For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C
2. For each electroporation, in a 2 mm sterile electroporation cuvette, combine:
 - 3 µL of iLOV-V plasmid DNA
 - 50 µL electrocompetent cells
3. Have recovery media ready
4. Electroporate using the EC2 program
5. 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
6. Recover cells for 2-3 hours, shaking at 37°C
7. Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)
 - a. Plated 10 µL and 200 µL of each, straight from recovery tube (because pF plasmid)
8. Incubate plates at 37°C for 3 days (or until single colonies appear)

The electroporation arced for the positive control, but otherwise went perfectly for pKR142. Accidentally used pKR113 as the positive control instead of pF, but it was formed on a pF plasmid so I think that will be okay. Have a three reaction tube of electrocompetent LVS left, for pKR143 in the future.

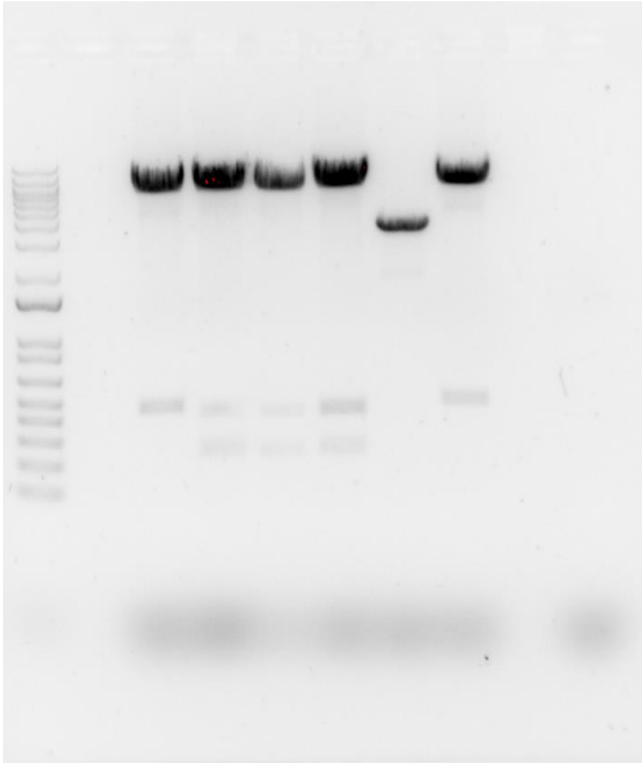
Monday, June 27, 2022

To Do:

1. Patch out Arspu2 from single colony (two)
2. Ligation of pKR143
3. Transformation of pKR143
4. Diagnostic digest of pKR135 minipreps
5. Run gel of diagnostic digest
6. Set up sequencing of pKR135 miniprep

Results and Data:

Ladder MP 1 MP 2 MP 3 MP 4 MP 5 MP 6 Neg



Ligation of LanYFP-V with pF backbone

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

Tube	Insert	Backbone
1	BamHI, EcoRI dig., purified LanYFP-V PCR	BamHI, EcoRI digested, purified pF
2	-	BamHI, EcoRI digested, purified pF

2. Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Master Mix (4x)
H ₂ O	11.5	11.5	34.5
10x ligase buffer	2.0	2.0	6.0
Insert	4.0	-	-
Backbone	2.0	2.0	6.0
Ligase	0.5	0.5	1.5
TOTAL	20.0	20.0	48.0

- 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 in order to avoid degradation. Take a 10 uL aliquot of 10X ligase buffer from the miscellaneous buffers box in the -20C.
- Obtain and label 1.5mL tubes for the reactions. Be sure to include the date and your initials.
- To the individual tubes, add indicated amounts of H₂O (___uL), 10x buffer (___uL), insert (___uL), and backbone (___uL).
- Add indicated amount of ligase (___uL) to the individual tubes. Remember to keep the ligase in a mini cooler.
- After all of the components have been added, mix each tube with a pipette set to 18 uL.
- Place on bench for 10 minutes.

Transformation of iLOV-V/LanYFP-V Ligation into *E. coli*

- Set up reaction table. **Always include a positive and negative control for each antibiotic.** Use 8 uL per ligation and plate 10 uL and 200 uL.
 - Positive control: pF; Backbone ligation: BamHI-EcoRI digested pF

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pF	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
3	Backbone Ligation	pF (digested)	8 uL	10 uL, 200 uL	2
4	Ligation 1	pKR143	8 uL	10 uL, 200 uL	2
Total number of plates					8

- 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.
- Obtain DNA and thaw on ice if necessary.
- Thaw appropriate number of competent cell tubes on ice (5 reactions per tube of competent cells)
- Label sterile tubes as indicated in reaction table. Add indicated volume of indicated DNA on ice.
- 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.
- Incubate cells on ice for 20 minutes. During incubation, find or set heat block to 42°C.
- Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step).
- After heat shock, place tubes back on ice until next step (don't keep them here too long).
- Using aseptic technique, add 1 mL LB (no antibiotic) to each microfuge tube.
- Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
- Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
- Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Kan), spreading until plates look dry.

After Aisling's potential mix-up I felt extra cautious in plating of the transformation points so I made sure to keep hold of the tube I was working with, and to do only the plates for that specific tube, as per usual.

Diagnostic Digest of Candidate pKR135 Miniprep w/KpnI and BamHI

- Make a reaction table with desired digests:

Tube	DNA	Enzyme(s)	DNA Volume (uL)	H ₂ O Volume (uL)
1	pKR135-1	KpnI, BamHI	4.0	-
2	pKR135-2	KpnI, BamHI	4.0	-
3	pKR135-3	KpnI, BamHI	4.0	-
4	pKR135-4	KpnI, BamHI	4.0	-
5	pKR135-5	KpnI, BamHI	4.0	-
6	pKR135-6	KpnI, BamHI	4.0	-
7	Water	KpnI, BamHI	-	4.0

2. Set up master mix table:

Components	Volumes in 1 reaction (uL)	Volumes in Master Mix 8x (uL)
H ₂ O	13.0	104.0
10x Buffer (Cutsmart)	2.0	16.0
DNA	(4.0)	-
KpnI	0.5	4.0
BamHI	0.5	4.0
Total	20.0 (16.0 actual b/c of DNA)	128.0

3. Add indicated amounts of H₂O, 10x buffer, and DNA to individual tube for digest (1.5 mL microfuge tubes for digest in incubator).
4. Add indicated amount of each enzyme (0.6 uL) to the tube.
5. Mix by pipetting up and down.
6. Incubate at 37°C for 1 hour.

Ran the digest for one hour and then froze the digests for about an hour while I met with Kathryn. I looked it up online first and the consensus seemed to suggest that this was an okay storage method, and that at -20 it should reduce any star activity and should stop the reaction. After this, I thawed (though they had not frozen completely) and added loading dye, loaded 20uL of each digest and ran the 1% agarose gel as per a normal set-up, 113V for 1 hour. Saw the band sizes I was expecting, as well as an additional weird extra band in 2, 3, and 4; decided to send these out for sequencing anyways.

Sample#	Template	Template Name	Primer Name	A.	B.	E.	F.
	(plasmid or PCR)			Template Size (bp)	Template Conc. (ng/μl)	PLASMID template:	Volume H ₂ O needed
SS1	Plasmid	pKR135-2	KROL44	7399	555.6	0.72	8.72
SS2	Plasmid	pKR135-3	KROL44	7399	590.8	0.68	8.76
SS3	Plasmid	pKR135-4	KROL44	7399	564.0	0.71	8.73

During meeting with Kathryn, discussed also looking at our GFP construct under the microscope. The strain is KRLVS113, so will need to patch that out on Thursday along with the others. Colby seems to be making the gel pad for us, says that the minimal media in the gel pad will help keep the cells alive for a little while, but not forever. Our cells don't necessarily need to be alive or replicating, just looking for fluorescence.

Tuesday, June 28, 2022

To Do:

1. ~~Patch out LVS from single colony (two)~~
2. ~~Patch out pKR142 single colonies onto CHAH+Kan~~
3. ~~Count pKR143 ligation and backbone plates~~
4. ~~Make overnights of pKR143 candidate colonies in LB+50 ug/mL Kan~~
5. ~~Make LB+Nat plates~~

Results and Data:

Discussed weird banding in the candidate pKR135 miniprep diagnostic digests in lab meeting, Kathryn agreed that sequencing was a good idea. Discussed the possibility that I have a mixed population plasmid, since the slightly heavier band could be explained since the pKR113 digest would have a bigger insert with the *rpsu* gene. Checked on SnapGene, and the insert from pKR113 is 469bp which aligns perfectly with that extra band. As such, I will be transforming these plasmids back into *E. coli* and picking single colonies in order to (hopefully) isolate the plasmid I want. I figure I will transform all three and pick ~2-4 colonies from each, I'll make overnights on Thursday, miniprep on Friday and run a diagnostic digest on Friday, as well. I may ask if I can set-up a sequencing reaction early and have it sequenced while I am out for vacation.

Additionally, looked at the transformation plates and there was no growth on the positive control for the pKR142 transformation into LVS. However the positive control arced so I believe that could be a big reason why. There were a bunch of colonies on the 10uL plate, so patched four onto CHAH+Kan. I'm pretty confident I kept track of the electroporation tubes, but a tiny bit of anxiety has seeped in that I didn't.

Hannah reminded me that we only re-use electroporation cuvettes once. I used two new ones so when I electroporate pKR143 into LVS I can reuse the two that I already used. Additionally, the Nat is kept in its own box in the -20, in an orange box. So, radical.

Need to clarify where the empty vector is going once I get the plasmid. I assume into a triple deletion mutant, but I'm not sure what that entails, there doesn't seem to just be a triple deletion with no plasmid, so I might have to make it lose the plasmid? Dunno.

Transformation Plate Counts				
	Ligation		Backbone Only	
Plasmid	100 uL	Remaining	100 uL	Remaining
pKR143	18	326	1	255

Awesome, ratio is somewhere between 1:18 and 1:1.2. Sweet, perfect. Will make overnights of six cultures in LB + 50ug/mL Kan and miniprep tomorrow. Hopefully, will also have time to do a diagnostic digest and set up sequencing for Thursday.

Wednesday, June 29, 2022

To Do:

1. ~~Set up cultures for RNA purification~~
2. ~~RNA Purification~~
3. ~~Miniprep pKR143~~
4. ~~Re-transform pKR135-2, pKR135-3, and pKR135-4 into *E. coli*~~

Results and Data:

N/A.

Setting Up LVS and $\Delta rpsu$ Cultures for RNA Purification

1. Scrape up each triplicate patch of LVS and $\Delta rpsu$ into individual tubes of 400uL MHB
2. Check OD's in a 1:20 dilution (50uL of sample in 950uL of MHB)
3. Calculate volume to add to tubes of 8 mL MHB for an OD of 0.08 for LVS or 0.10 for $\Delta rpsu$
4. Check OD's with 600uL
5. Shake @37°C and grow to mid-log, checking OD's at ~2-2.5 hours initially

RNA Cultures OD's				
	0 hours	2 hours	4 hours	4.6 hours
LVS-1	0.079	0.182	0.327	0.384
LVS-2	0.076	0.173	0.324	0.376
LVS-3	0.075	0.177	0.315	0.375
$\Delta rpsu$ -1	0.090	0.160	0.258	0.287
$\Delta rpsu$ -2	0.083	0.144	0.219	0.255
$\Delta rpsu$ -3	0.102	0.167	0.257	0.291

When setting up the cultures, I added a couple extra microliters to try to account for the volume I was not taking out, however that clearly was not enough so both sets of cultures started low, but particularly $\Delta rpsu2$ which obviously is not great. Hannah reminded me I could aim for a little high, ~30%, to get a more accurate OD. Kathryn decided we would go ahead after 4 hours and 40 minutes despite the OD's, as it would be better for some to be undergrown than for LVS to be overgrown. Next time REMEMBER TO CALCULATE A HIGHER OD.

RNA Purification

1. Pellet 1.8mLx2 of culture at max speed @room temperature
2. Resuspend cell pellet in 1 mL TRI-Reagent
3. Incubate at 60°C for 10 min
4. Spin at 4°C for 10 min at max speed
5. Transfer supernatant to new 2mL tube (~950 uL).
6. Add equal volume 100% ethanol
7. Pass sample over Directzol column, 600 uL per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
8. Place spin column in new collection tube
9. Wash twice with 400 uL RNA PreWash buffer, discarding flow-through in phenol and methanol waste
10. Add 700 ul of Wash buffer, let sit on column for 3 min
11. Spin max speed for 2 min
12. Wash again with 700 ul Wash buffer

13. Spin max speed for 2 min
14. Place column in new collection tube
15. Spin max speed for 3 min
16. Place column in clean 1.5 mL tube
17. Add 90 uL RNase-free water, let sit on column 2 min
18. Spin max speed 1 min
19. Place flow-through on column again, spin 1 min
20. Store nucleic acids at -80°C if not moving directly to the next step

Stored total nucleic acids in -80C freezer in new RNA box. DNase treatment has not been completed yet and will need to be before the next step. Labelled according to the following chart:

Labelled	Contents
1 RNA	LVS BR-1
2 RNA	LVS BR-2
3 RNA	LVS BR-3
4 RNA	Δ rpsu2 BR-1
5 RNA	Δ rpsu2 BR-2
6 RNA	Δ rpsu2 BR-3

Transformation of Candidate pKR135-2, -3, and -4 into *E. coli*

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. Plate 20 uL and 100 uL.
 - a. Positive control: pKR113

Reaction table

Tube#	Purpose	DNA	Vol. of DNA	Final vol. to plate	# of LB-kan plates
1	(+) control	pKR113	1 uL	20 uL, 100 uL	2
3	(-) control	None	0	20 uL, 100 uL	2
4	Mixed Pop MP 1	pKR135-2	1 uL	20 uL, 100 uL	2
5	Mixed Pop MP 2	pKR135-3	1 uL	20 uL, 100 uL	2
6	Mixed Pop MP 3	pKR135-4	1 uL	20 uL, 100 uL	2
Total number of plates					10

2. 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
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.

Miniprep of pKR143 Candidates from *E. coli*

1. Pellet overnight cultures by centrifugation at 15,000rpm for 3 minutes.
2. Resuspend pellets in a 250uL Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250uL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow lysis reaction to proceed for more than 5 minutes.
4. Add 350uL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 minutes at 13,000rpm.
6. Transfer 800uL of supernatant to a QIAprep 2.0 spin column through pipetting.
7. Wash the QIAprep column by adding 500uL Buffer PB. Centrifuge for 30-60s and discard flow through.
8. Wash the QIAprep column by adding 750uL Buffer PE. Centrifuge for 30-60s and discard flow through.
9. Centrifuge for 3 minutes at 13,000rpm to remove residual buffer.
10. Place QIAprep column in a clean 1.5mL microcentrifuge tube. Add 50uL of Buffer EB. Let stand for 1 minute. Centrifuge for 1 minute at 13,000rpm.

Thursday, June 30, 2022

To Do:

- ~~1. Take new transformant colonies of pKR135-2, pKR135-3, and pKR135-4 and make overnights~~
- ~~2. Make permanent stocks of KRLVS177~~
- ~~3. Patch out KRLVS174, KRLVS175, KRLVS177, and KRLVS113 for fluorescent microscopy~~

Results and Data:

N/A.

KRLVS174, KRLVS175, KRLVS177, and KRLVS113 are all Kan resistant, so will patch out onto CHAH+Kan since the regular CHAH plates that Kathryn made seem to be growing slow. Plus, it's probably better to encourage them to not lose their plasmids.

Freezing and Storing Permanent Strain of KRLVS177

1. Per strain, label 2 cryotubes with strain number. Include LVS, strain number, genotype, date.
2. Add 200 uL sterile 75% glycerol to each tube (2 per strain).
3. In a sterile 2 mL tube (1 per strain), add 400 uL of MHB
4. Resuspend patch (all of what you have) in MHB to homogeneity
5. Correct volume to 1700 uL (add 2x 650 uL MHB)
6. Transfer 800 uL to each cryotube (final volume should be 1 mL)
7. Vortex cryotube
8. Quickly spin (mini-fuge) to get liquid to the bottom of the tube
9. Freeze at -80°C in appropriate strain box

Made three overnights in 5mL LB + 50 ug/mL Nat from each candidate plasmid that was retransformed to miniprep tomorrow. Additionally saved the relevant plates in the fridge, wrapped in parafilm, in the event that none of them are the plasmid. That said, if none of these are the plasmid, I may have to redo the transformation anyways as there is a whole bunch of colonies, not with super great isolation.

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.