Table of Contents

Introduction

Thursday, September 20, 2018

To Do:

~~1. Explain lab notebook formatting~~
2. Media prep

Results and Data:

For each day, copy the previous entry headers and update the date. Save the same ongoing copy of the lab notebook to the lab google drive ONLY. Do not save separate physical copies. At the 1st of each new month, a read-only PDF file of the lab notebook must be saved and given to Dr. Ramsey electronically.

File contents converted to PDF MUST NOT BE EDITED after PDF conversion. Continue to keep using the same word file until the end of the calendar year. New lab notebook files run in 6 month intervals.
For the To do list, update this each day with new tasks, as tasks are done, use the ~~strikethrough~~ font on the day they are completed and leave them out of the list on the next day.

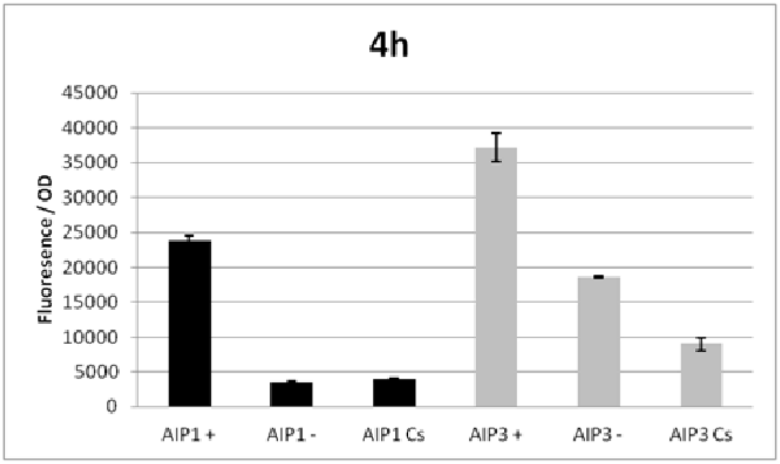

Figure 1

Figures are inserted as inline .png files when possible, .jpg, .gif, .tif otherwise are acceptable. Figure legends are always inserted in Word (right click – 'insert caption') and use "**Heading 2**" text to properly format in the TOC. Table legends are handled the same way. Figure images must be saved in a separate folder where the source data is saved.

Thoughts or questions.

When you have a significant observation, question, confusing point or contradiction that you have identified in your data or protocol, use the "**Heading 3**" text heading on a descriptive brief title or single word heading so you can refer to it in your TOC later.

Protocol 1

1. This is the format for a protocol in your notebook.
2. The protocol title must be formatted in "**Heading 2.**"

3. Reagents which must be made for a specific protocol (buffers, solutions) should be listed in a "Reagents" Section, formatted in "**Heading 3**."
4. The protocol must be in numerical steps.
5. Use standard notation and carefully describe units for your protocol.
6. Use ½" indent for protocol text.

Reagents

Specific buffers

For initial lab notebooks, write as much detail as possible. As time goes by you will be able to refer to written protocols by their heading and/or page number within the document. If you make any modifications to a protocol you must state how and why.

File Formatting Protocol

1. Filenames begin with your initials, an underscore, and the date, formatted as the last 2 digits of the year, the month, then the day, ex: "KMR_180920_Sample_file1v1.xls"
2. This ensures all files will be sorted by their creator and by their date. You must use this file formatting system for all data files (including photos) that will be shared with the lab.

Bibliography data will be saved as (author/date) and using Mendeley at this time with the TOC inserted by order cited at the end of the lab notebook in the Bibliography section.

Example is a recent publication (Ramsey and Dove, 2016).

January 2020

Tuesday, January 28, 2020

To Do:

1. ~~Sucrose selection on single primary integrant from Jamie (LVS pKR58)~~

Results and Data:

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

To select for cross-outs using sucrose selection
-Scrape up small loop of cells and resuspend in 1X PBS
-Dilute culture 1:10 in sterile PBS to 1x10^{-7}
Perform first dilution 1:100 so first dilution tube is 1x10^{-2}, adding 10 uL of resuspended cells to 990 ul 1x PBS
Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS
-Plate 100 uL of last dilution (1x10^{-7}) onto CHAH plates.
-Plate 100 µL of each dilution 10^{-2} – 10^{-7} on CHAH + 10% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.
-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the 1x10^{-7} dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Make freezer stocks of 1° integrants
-Add 800 uL MHB to cryovial per 1° integrant
-Scrape up 1° integrant cells using loop into cryovial
-Completely resuspend cells in cryovial
-Add 200 uL 75% glycerol, close tube, mix by vortexing, and freeze at -80°C.

2 of LVS and 2 of dPmrA already

New LVS stocks were labeled as 3, 4, 5, and 6 and stored in PriM team glycerol stocks at -80.

Thursday, January 30, 2020

To Do:

1. ~~Check sucrose selection plates.~~

Results and Data:

Significant growth on control plate, single colonies on some of the sucrose plates that will be ready to pick tomorrow.

February 2020

Friday, January 31, 2020

To Do:

1. ~~Patch over sucrose selection and leave out for the weekend.~~

Results and Data:

Patch single colonies

Day 9 (maybe 10):

-Pick single colonies (cross-outs) from sucrose plates and patch onto CHAH, 8 – 16 per 1° integrant.

-Optional: cross-patch to CHAH-Kan. Note that it is common to pick up some background cells so some amount of kanamycin-resistant growth is not yet a concern. However, if there is robust kanamycin-resistant growth from all patches from a single 1° integrant on the next day, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Monday, February 3, 2020

To Do:

1. ~~PCR and purification for sequencing tomorrow.~~

Results and Data:

Day 10:

-Take small amounts of each cross-out patch and resuspend in sterile water.

-Heat samples at 95°C for 10' to lyse and kill cells

-Dilute lysates 1:10

-Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation. As of August 2019, the KRamsey lab is primarily using PrimeStar GXL polymerase (Takara Bio). For controls, use ~~LVS cells~~, 17LVS gDNA, the 18pEX-based allelic exchange vector, and 19water only.

2 into 18

Example colony PCR using KOD (use a master mix):

-If necessary, PCR purify and digest only LVS gDNA control and your potential mutants. Diagnostic digest should be 20 uL per reaction with 0.5 uL of enzyme.

-For cross-outs which appear to have the desired mutation (maximum 4), streak to single colony on CHAH plates, incubate at 37° for 3 days (or until single colonies appear).

Samples 1-16 correspond to plate, 17 is LVS gDNA, 18 is pEX plasmid, and 19 is water.

KROL266 and KROL269

4 controls: ~~LVS~~, gDNA, plasmid PCR, negative

16 samples + 4 controls = 20 total samples

Use 50uL sample of reaction volume.

Total reaction volume	50
Total number of reactions	20

				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	21
ddiH2O			31	651
PrimeSTAR GXL Buffer	5x	1x	10	210
dNTPs	2.5 mM	0.2 mM	4	84
oligo F	10 uM	0.3 uM	1.5	31.5
oligo R	10 uM	0.3 uM	1.5	31.5
template	100 ng/ul	2 ng/ul	1	21
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	~~1~~	~~21~~
		Total volume	50	1050

Note: The template does not go into the master mix, but everything else does. Use 2uL of template.

Label 16 tubes (nonsterile) and fill with 50uL of molecular grade H2O. Another 16 will be needed for the dilution.

QIAquick PCR Purification Kit Protocol

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. (Have approximately 100 uL of PCR, so 500 uL Buffer PB will be used.)
2. Place a QIAquick column in the provided 2 mL tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 uL Buffer PE to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 min to remove the residual wash buffer.
6. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, add ~~50~~ 30 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 uL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1	Default	2/3/2020	3:59 PM	145.42	2.908	1.582	1.84	2.05	50.00	230	1.417	0.091
2	Default	2/3/2020	4:00 PM	149.43	2.989	1.631	1.83	2.24	50.00	230	1.333	0.026
3	Default	2/3/2020	4:01 PM	184.89	3.698	1.988	1.86	2.28	50.00	230	1.619	0.048
5	Default	2/3/2020	4:02 PM	131.88	2.638	1.417	1.86	2.28	50.00	230	1.158	0.035
6	Default	2/3/2020	4:03 PM	109.24	2.185	1.237	1.77	2.46	50.00	230	0.889	12.975
7	Default	2/3/2020	4:04 PM	122.72	2.454	1.482	1.66	2.14	50.00	230	1.149	1.136
8	Default	2/3/2020	4:05 PM	177.66	3.553	2.229	1.59	1.98	50.00	230	1.797	2.884
9	Default	2/3/2020	4:06 PM	180.51	3.610	1.953	1.85	2.29	50.00	230	1.574	0.014
10	Default	2/3/2020	4:07 PM	167.62	3.352	2.310	1.45	1.46	50.00	230	2.294	6.418
11	Default	2/3/2020	4:08 PM	148.42	2.968	1.769	1.68	2.19	50.00	230	1.357	0.781
12	Default	2/3/2020	4:09 PM	132.23	2.645	1.696	1.56	1.85	50.00	230	1.430	2.521
13	Default	2/3/2020	4:10 PM	228.36	4.567	3.560	1.28	1.27	50.00	230	3.606	16.427
13	Default	2/3/2020	4:11 PM	155.01	3.100	1.976	1.57	1.86	50.00	230	1.668	3.093
14	Default	2/3/2020	4:12 PM	198.71	3.974	2.297	1.73	2.33	50.00	230	1.707	0.499
15	Default	2/3/2020	4:13 PM	137.06	2.741	1.581	1.73	2.26	50.00	230	1.214	0.307
16	Default	2/3/2020	4:14 PM	186.16	3.723	2.029	1.83	2.32	50.00	230	1.607	0.030
17	Default	2/3/2020	4:15 PM	126.55	2.531	1.391	1.82	2.18	50.00	230	1.160	0.006
18	Default	2/3/2020	4:16 PM	185.26	3.705	2.021	1.83	2.30	50.00	230	1.609	0.034
19	Default	2/3/2020	4:17 PM	7.62	0.152	0.081	1.89	0.74	50.00	230	0.206	0.033

Tuesday, February 4, 2020

To Do:

1. ~~Sequencing for colony PCR.~~

Results and Data:

PCR Tube 1	Sample 1
PCR Tube 2	Sample 2
PCR Tube 3	Sample 3
PCR Tube 4	Sample 5
PCR Tube 5	Sample 6
PCR Tube 6	Sample 7
PCR Tube 7	Sample 8
PCR Tube 8	Sample 9
PCR Tube 9	Sample 10
PCR Tube 10	Sample 11
PCR Tube 11	Sample 12
PCR Tube 12	Sample 13
PCR Tube 13	Sample 14
PCR Tube 14	Sample 15
PCR Tube 15	Sample 16

Wednesday, February 5, 2020

To Do:

1. ~~Check sequencing.~~
2. ~~Streak for single colonies.~~

Results and Data:

Sequencing looks good.

Streaked for single colonies by using a different stick for each quarter of the plate.

Thursday, February 6, 2020

To Do:

1. ~~Make EC cells.~~
2. ~~Electroporate pKR58 into dPmrA.~~

Results and Data:

Prepare electrocompetent (EC) cells

-Scrape up entire plate of cells into 400 uL of sterile 10% sucrose and resuspend (alternately, cells can be grown to mid-log in MHB)

-Add 1.1 mL of 10% sucrose to a final volume of 1.5 mL

-Spin for 3 minutes at 10,000 rpm

-remove supernatant, throw out, and resuspend in fresh 1 mL 10% sucrose

-Repeat 3x-5x in 10% sucrose

-After final spin, remove all supernatant.

-Resuspend cells in 10% sucrose at high density (corresponding to ~1x10^{11} cells /mL); these are EC cells by slowly adding 110 uL at a time. It should be about equal amounts of cells as sucrose.

-For any extra EC cells, aliquot ~110 µL / sterile tube (enough for 2 electroporations) and freeze at -80°C

*Electroporate plasmid into EC cells**

-For each electroporation, aliquot 4 mL MHB into glass test tubes for recovery, warm in shaker at 37°C

-For each electroporation, in a 2 mm sterile electroporation cuvette, combine:

5 µL of pEX-based allelic exchange construct (mini-prep concentration, at least 100 ng/µL)

50 µL electrocompetent cells

-Have recovery media ready

-Electroporate using the following settings: 2.5 kV, 25 µF, and 600 Ω **(EC2)**

-Immediately after individual electroporations, use 1 mL warm recovery media from test tube to wash cells out of cuvette and transfer cells to recovery test tube

-Recover cells for 4-8 hours, shaking at 37°C

-Pellet cells by centrifugation in sterile 2 mL tubes, 1' at 10,000 x g.

-Plate on CHAH-Kan plates, using 1 plate for each 2 mL tube (2 plates per electroporation)

-Incubate plates at 37°C for 3 days (or until single colonies appear)

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

Friday, February 7, 2020

To Do:

1. ~~Patch out single colonies from LVS 3.1 and 3.2 plates.~~

Results and Data:

Patched out 5 single colonies each of LVS 3.1 and 3.2 onto Kan and CHA.

Monday, February 10, 2020

To Do:

1. ~~Redo colony PCR.~~
2. ~~Restreak 5 single colonies from EP plates.~~

Results and Data:

Day 10:

-Take small amounts of each cross-out patch and resuspend in 50ul sterile water.

-Heat samples at 95°C for 10’ to lyse and kill cells

-Dilute lysates 1:10

-Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation. As of August 2019, the KRamsey lab is primarily using PrimeStar GXL polymerase (Takara Bio). For controls, use ~~LVS cells~~, 17LVS gDNA, the 18pEX-based allelic exchange vector, and 19water only.

2 into 18

Example colony PCR using KOD (use a master mix):

-If necessary, PCR purify and digest only LVS gDNA control and your potential mutants. Diagnostic digest should be 20 uL per reaction with 0.5 uL of enzyme.

-For cross-outs which appear to have the desired mutation (maximum 4), streak to single colony on CHAH plates, incubate at 37° for 3 days (or until single colonies appear).

Samples 1-16 correspond to plate, 17 is LVS gDNA, 18 is pEX plasmid, and 19 is water.

KROL266 and KROL269

4 controls: ~~LVS~~, gDNA, plasmid PCR, negative

16 samples + 4 controls = 20 total samples

Use 50uL sample of reaction volume.

Total reaction volume	50
Total number of reactions	10

				Factor
Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	11
ddiH2O			31	341
PrimeSTAR GXL Buffer	5x	1x	10	110
dNTPs	2.5 mM	0.2 mM	4	44
oligo F	10 uM	0.3 uM	1.5	16.5
oligo R	10 uM	0.3 uM	1.5	16.5
template	100 ng/ul	2 ng/ul	1	11
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	1	11
		Total volume	50	550

Note: The template does not go into the master mix, but everything else does. Use 2uL of template.

Label 16 tubes (nonsterile) and fill with 50uL of molecular grade H2O. Another 16 will be needed for the dilution.

QIAquick PCR Purification Kit Protocol

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. (Have approximately 100 uL of PCR, so 500 uL Buffer PB will be used.)
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3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 uL Buffer PE to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 min to remove the residual wash buffer.
6. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, add ~~50~~ 30 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 uL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.		Cursor abs.
	340 raw											
Sample 1	Default	2/10/2020	2:07 PM	124.75	2.495	1.348	1.85	2.45	50.00	230	1.019	-0.002
Sample 2	Default	2/10/2020	2:08 PM	139.76	2.795	1.511	1.85	2.45	50.00	230	1.139	0.007
Sample 3	Default	2/10/2020	2:10 PM	99.80	1.996	1.100	1.81	2.29	50.00	230	0.870	-0.007

Sample 4	Default	2/10/2020 2:10 PM	139.36	2.787	1.530	1.82	2.43	50.00	230	1.145	0.013
Sample 5	Default	2/10/2020 2:11 PM	132.97	2.659	1.451	1.83	2.43	50.00	230	1.096	0.017
Sample 6	Default	2/10/2020 2:13 PM	131.34	2.627	1.428	1.84	2.43	50.00	230	1.081	0.022
Sample 7	Default	2/10/2020 2:14 PM	111.13	2.223	1.214	1.83	2.44	50.00	230	0.911	0.011
Sample 8	Default	2/10/2020 2:14 PM	141.52	2.830	1.536	1.84	2.46	50.00	230	1.149	0.020
Sample 9	Default	2/10/2020 2:15 PM	107.81	2.156	1.164	1.85	2.40	50.00	230	0.898	0.007
Sample 10	Default	2/10/2020 2:16 PM	93.26	1.865	1.012	1.84	2.39	50.00	230	0.780	0.018

Tuesday, February 11, 2020

To Do:

1. ~~Sequencing round 2 for pKR58 in LVS to confirm strain.~~
2. ~~Sucrose selection for pKR58 in dPmrA tube 3.~~
3. ~~Pick colonies from transformation of pKR58 for overnight cultures.~~

Results and Data:

Sucrose selection on dPmrA 3
To select for cross-outs using sucrose selection

-Scrape up small loop of cells and resuspend in 1X PBS
-Dilute culture 1:10 in sterile PBS to 1x10^{-7}
Perform first dilution 1:100 so first dilution tube is 1x10^{-2}, adding 10 uL of resuspended cells to 990 ul 1x PBS
Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS
-Plate 100 uL of last dilution (1x10^{-7}) onto CHAH plates.
-Plate 100 µL of each dilution 10^{-2} – 10^{-7} on CHAH + 10% sucrose plates. If you have extra sucrose plates, can plate some or all dilutions twice.
-Incubate plates at 37°C for 3-4 days, or until single colonies appear. It is generally a good idea to test both large and smaller single colonies. Note that there should be many more colonies on the CHAH plate with the 1x10^{-7} dilution than on the sucrose plate with the corresponding dilution. If there seem to be similar numbers of colonies, the 1° integrant may have an inactivating mutation in *sacB* and is not worth keeping.

Make freezer stocks of 1° integrants
-Add 800 uL MHB to cryovial per 1° integrant
-Scrape up 1° integrant cells using loop into cryovial
-Completely resuspend cells in cryovial
-Add 200 uL 75% glycerol, close tube, mix by vortexing, and freeze at -80°C.

dPmrA 3, 4, 5, 6, and 7 were placed in the PriM team glycerol stocks box at -80.

Overnight Cultures Protocol

By John Church

1. Add 45 mL of LB broth to a sterile 50 mL conical (makes enough for 8 tubes: 9*5=45)
2. Add 45 uL of kanamycin (Kan50) to the conical.

3. Label 4 glass sterile test tubes for each new plasmid and 2 for each existing plasmid (controls are not needed).
4. Pipette out 5 mL of LB + Kan media into each of the tubes.
5. Pick 4 colonies from each reaction and inoculate the tube.
    - Do this by using a stick and picking an isolated colony from plate
6. Place in the 37° shaking incubator overnight. ~16-22 hours

Wednesday, February 12, 2020

To Do:

1. ~~Check sequencing for strain.~~
2. ~~Freeze down strain according to protocol.~~
3. ~~Miniprep pKR58.~~
4. ~~Nanodrop pKR58.~~

Results and Data:

Freezing permanent bacterial strain stocks

Add strain to strain list

If you are freezing down a strain generated by allelic exchange, remember to add the 1° integrant that generated the final strain. Include it on the strain list first.
For each strain you add, include:

- Genotype
- Notes (for example, FTL number corresponding to a gene name or vice versa)
- Location (Strain boxes are stored at -80°C)
- The genotype of the original strain you started with (Background)
- Any integrated plasmid (no plasmids in clean deletions, but plasmids are in 1° integrants)
- The descriptive name of any integrated plasmid
- Strain verification data: should include the name of the person who verified and the date for each type of verification
- Any additional notes (notable phenotypes, etc)

Freeze down strain

Use aseptic technique

Per strain, label 2 cryotubes with strain number. Include LVS, strain number, genotype, date.

Add 200 uL sterile 75% glycerol to each tube (2 per strain).

In a sterile 2 mL tube (1 per strain), add 400 uL of MHB

Resuspend patch (all of what you have) in MHB to homogeneity

Correct volume to 1700 uL (add 2x 650 uL MHB)

Transfer 800 uL to each cryotube (final volume should be 1 mL)

Vortex cryotube

Quickly spin (mini-fuge) to get liquid to the bottom of the tube

Freeze at -80°C in appropriate strain box

Plate 50ul of one of the strains for using later

Thursday, February 13, 2020

To Do:

1. ~~Nanodrop pKR58.~~

Results and Data:

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
pKR58 1	Default	2/13/2020	8:35 AM	223.65	4.473	2.374	1.88	2.61	50.00	230	1.711	0.012
pKR58 2	Default	2/13/2020	8:36 AM	300.63	6.013	3.245	1.85	2.47	50.00	230	2.432	0.132
pKR58 3	Default	2/13/2020	8:37 AM	265.81	5.316	2.838	1.87	2.56	50.00	230	2.078	0.057
pKR58 4	Default	2/13/2020	8:38 AM	161.18	3.224	1.706	1.89	2.61	50.00	230	1.234	0.031

Combined and placed in the plasmids box

Friday, February 14, 2020

To Do:

1. ~~Patch out single colonies from sucrose selection.~~
2. ~~Make stocks of LVS strain.~~

Results and Data:

Monday, February 17, 2020

To Do:

1. ~~Colony PCR on dPmrA samples.~~
2. ~~Purification of PCR.~~
3. ~~Observe macrophage assay with Jamie.~~

Results and Data:

Day 10:

-Take small amounts of each cross-out patch and resuspend in sterile water.

-Heat samples at 95°C for 10' to lyse and kill cells

-Dilute lysates 1:10

-Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation. As of August 2019, the KRamsey lab is primarily using PrimeStar GXL polymerase (Takara Bio). For controls, use ~~LVS cells~~, 17LVS gDNA, the 18pEX-based allelic exchange vector, and 19water only.

2 into 18

Example colony PCR using KOD (use a master mix):

-If necessary, PCR purify and digest only LVS gDNA control and your potential mutants. Diagnostic digest should be 20 uL per reaction with 0.5 uL of enzyme.

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KROL266 and KROL269

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dNTPs	2.5 mM	0.2 mM	4	84
oligo F	10 uM	0.3 uM	1.5	31.5
oligo R	10 uM	0.3 uM	1.5	31.5
template	100 ng/ul	2 ng/ul	1	21
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	~~1~~	~~21~~

Total volume	50	1050

Note: The template does not go into the master mix, but everything else does. Use 2uL of template.

Label 16 tubes (nonsterile) and fill with 50uL of molecular grade H2O. Another 16 will be needed for the dilution.

QIAquick PCR Purification Kit Protocol

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. (Have approximately 100 uL of PCR, so 500 uL Buffer PB will be used.)
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6. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, add ~~50~~ 30 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 uL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Sample 1	Default	2/17/2020	3:00 PM	122.64	2.453	1.356	1.81	1.39	50.00	230	1.762	0.409
Sample 2	Default	2/17/2020	3:01 PM	128.23	2.565	1.390	1.85	2.37	50.00	230	1.084	0.014
Sample 3	Default	2/17/2020	3:02 PM	92.17	1.843	0.989	1.86	1.04	50.00	230	1.780	0.039
Sample 3	Default	2/17/2020	3:03 PM	138.81	2.776	2.265	1.23	0.93	50.00	230	2.986	21.212
Sample 4	Default	2/17/2020	3:04 PM	155.95	3.119	1.718	1.82	2.36	50.00	230	1.323	0.002
Sample 5	Default	2/17/2020	3:04 PM	109.38	2.188	1.190	1.84	2.32	50.00	230	0.942	0.051
Sample 6	Default	2/17/2020	3:05 PM	103.25	2.065	1.122	1.84	2.41	50.00	230	0.856	0.013
Sample 7	Default	2/17/2020	3:06 PM	12.56	0.251	0.160	1.57	1.51	50.00	230	0.167	0.076
Sample 7	Default	2/17/2020	3:07 PM	11.43	0.229	0.127	1.80	1.40	50.00	230	0.164	0.086
Sample 8	Default	2/17/2020	3:08 PM	127.51	2.550	1.571	1.62	2.24	50.00	230	1.137	1.378
Sample 9	Default	2/17/2020	3:09 PM	125.97	2.519	1.664	1.51	1.84	50.00	230	1.366	3.537
Sample 10	Default	2/17/2020	3:09 PM	83.06	1.661	0.949	1.75	2.31	50.00	230	0.720	0.086
Sample 11	Default	2/17/2020	3:10 PM	64.13	1.283	0.703	1.82	2.37	50.00	230	0.542	0.034
Sample 12	Default	2/17/2020	3:11 PM	113.29	2.266	1.223	1.85	2.49	50.00	230	0.911	0.700
Sample 13	Default	2/17/2020	3:13 PM	148.08	2.962	1.870	1.58	2.09	50.00	230	1.415	2.721
Sample 14	Default	2/17/2020	3:13 PM	72.47	1.449	0.853	1.70	2.30	50.00	230	0.631	0.085
Sample 15	Default	2/17/2020	3:14 PM	131.11	2.622	1.450	1.81	2.36	50.00	230	1.111	-0.210
Sample 16	Default	2/17/2020	3:15 PM	64.92	1.298	0.783	1.66	2.27	50.00	230	0.571	0.524

Tuesday, February 18, 2020

To Do:

1. ~~Submit sequencing.~~
2. ~~Look at mac assay cells under microscope with Jamie.~~

Results and Data:

Sample 7 was not sequenced because it had a low concentration.

Wednesday, February 19, 2020

To Do:

1. ~~Check sequencing of dPmrA.~~
2. ~~Streak colonies 1, 2, and 3 to single colony.~~

Results and Data:

Sequencing is good for samples 1, 2, and 3.

Thursday, February 20, 2020

To Do:

1. ~~Make new 1X TAE.~~

Results and Data:

Friday, February 21, 2020

To Do:

1. ~~Check plates struck for single colonies.~~
2. ~~Patch our single colonies and leave out for weekend.~~

Results and Data:

Monday, February 24, 2020

To Do:

1. ~~Colony PCR Round 2.~~

Results and Data:

1	3.1.3
2	3.1.4
3	3.1.5
4	3.1.6
5	3.2.1
6	3.2.3
7	3.2.4

8	3.3.1
9	3.3.2
10	3.3.3

Day 10:

-Take small amounts of each cross-out patch and resuspend in sterile water.

-Heat samples at 95°C for 10' to lyse and kill cells

-Dilute lysates 1:10

-Use 1-2 µL of heat-killed cells as template in colony PCR to check for desired mutation. As of August 2019, the KRamsey lab is primarily using PrimeStar GXL polymerase (Takara Bio). For controls, use ~~LVS cells~~, 17LVS gDNA, the 18pEX-based allelic exchange vector, and 19water only.

2 into 18

Example colony PCR using KOD (use a master mix):

-If necessary, PCR purify and digest only LVS gDNA control and your potential mutants. Diagnostic digest should be 20 uL per reaction with 0.5 uL of enzyme.

-For cross-outs which appear to have the desired mutation (maximum 4), streak to single colony on CHAH plates, incubate at 37° for 3 days (or until single colonies appear).

Samples 1-16 correspond to plate, 17 is LVS gDNA, 18 is pEX plasmid, and 19 is water.

KROL266 and KROL269

4 controls: ~~LVS~~, gDNA, plasmid PCR, negative

16 samples + 4 controls = 20 total samples

Use 50uL sample of reaction volume.

Total reaction volume	50
Total number of reactions	10

Factor

Component	**Stock concentration**	**Final concentration**	**1 rxn volume**	11
ddiH2O			31	341
PrimeSTAR GXL Buffer	5x	1x	10	110
dNTPs	2.5 mM	0.2 mM	4	44
oligo F	10 uM	0.3 uM	1.5	16.5
oligo R	10 uM	0.3 uM	1.5	16.5
template	100 ng/ul	2 ng/ul	1	11
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	1	11
		Total volume	50	550

Note: The template does not go into the master mix, but everything else does. Use 2uL of template.

Label 16 tubes (nonsterile) and fill with 50uL of molecular grade H2O. Another 16 will be needed for the dilution.

QIAquick PCR Purification Kit Protocol

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. (Have approximately 100 uL of PCR, so 500 uL Buffer PB will be used.)
2. Place a QIAquick column in the provided 2 mL tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 uL Buffer PE to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 min to remove the residual wash buffer.
6. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, add ~~50~~ 30 uL 0.1x Buffer EB (10mM Tris.Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 uL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Sample 1	Default	2/24/2020	2:35 PM	106.44	2.129	1.192	1.79	2.00	50.00	230	1.063	0.054
Sample 2	Default	2/24/2020	2:36 PM	204.08	4.082	2.245	1.82	2.49	50.00	230	1.638	0.028
Sample 3	Default	2/24/2020	2:37 PM	210.94	4.219	2.333	1.81	2.48	50.00	230	1.702	0.026
Sample 4	Default	2/24/2020	2:38 PM	160.15	3.203	1.782	1.80	2.88	50.00	230	1.112	0.067
Sample 5	Default	2/24/2020	2:39 PM	187.97	3.759	2.082	1.81	2.82	50.00	230	1.335	0.035
Sample 6	Default	2/24/2020	2:40 PM	134.96	2.699	1.495	1.81	2.97	50.00	230	0.910	0.026
Sample 7	Default	2/24/2020	2:40 PM	155.34	3.107	1.721	1.81	2.86	50.00	230	1.088	0.048
Sample 8	Default	2/24/2020	2:41 PM	178.49	3.570	1.979	1.80	2.84	50.00	230	1.258	0.065

Sample 9	Default	2/24/2020	2:42 PM	126.51	2.530	1.404	1.80	3.01	50.00	230	0.842	0.020
Sample 10	Default	2/24/2020	2:44 PM	39.24	0.785	0.494	1.59	4.80	50.00	230	0.164	0.144

Tuesday, February 25, 2020

To Do:

1. ~~Sequencing.~~

Results and Data:

Purified PCR samples should always be stored in the freezer.

Wednesday, February 26, 2020

To Do:

1. ~~Check sequencing.~~
2. ~~Freeze down strains.~~
3. ~~Streak out a 50uL of cells from each strain for working stocks of dPmrA.~~

Results and Data:

Freeze down 1 and 2.

Freezing permanent bacterial strain stocks

Add strain to strain list

If you are freezing down a strain generated by allelic exchange, remember to add the 1° integrant that generated the final strain. Include it on the strain list first.

For each strain you add, include:

- Genotype
- Notes (for example, FTL number corresponding to a gene name or vice versa)
- Location (Strain boxes are stored at -80°C)
- The genotype of the original strain you started with (Background)
- Any integrated plasmid (no plasmids in clean deletions, but plasmids are in 1° integrants)
- The descriptive name of any integrated plasmid
- Strain verification data: should include the name of the person who verified and the date for each type of verification
- Any additional notes (notable phenotypes, etc)

Make freezer stocks of mutants

-Label 2 cryovials per strain

-Add 800 uL MHB to a sterile 2 mL tube per mutant

-Scrape up mutant cells using loop into 2 mL tube

-Completely resuspend cells

-Add 800 uL more MHB, completely mix cells

-Aliquot 800 uL cells to each labeled cryovial

-Add 200 uL 75% glycerol to each labeled cryovial, close tube, mix by vortexing, and freeze at -80°C.

Plate 50ul of one of the strains for using later
Pink for strain, yellow for primary integrant

Thursday, February 27, 2020

To Do:

1. ~~Make glycerol stocks of dPmrA plates from yesterday.~~
2. ~~Streak out 50uL of cells from WT snip (KRLVS69.1 and 69.2).~~
3. ~~Make single use aliquots of dPmrA snip.~~

Results and Data:

Working stocks and single use aliquots were stored in the Undergrads box in the -80.

Friday, February 28, 2020

To Do:

1. ~~Make working glycerol stocks from LVS plates struck out.~~
2. ~~Make single use aliquots from LVS plates.~~

Results and Data:

Working stocks and single use aliquots were stored in the Undergrads box in the -80.

March 2020

Monday, March 2, 2020

To Do:

1. ~~Macrophage assay with Jamie.~~

Results and Data:

Tuesday, March 3, 2020

To Do:

1. ~~Macrophage assay with Jamie.~~

Results and Data:

Ended here due to Coronavirus.

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.