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September 2023

Saturday, September 16, 2023

To Do:

1. Start cloning

Results and Data:

Would really like to get the P_{rpsU2} -5'UTR_{tu14}-lacZ construct cloned! Try to make pKR122- this has a low copy (R6Kgamma) origin. Try growing at 30°C and selecting using carbenicillin. Also try Ben's minimal media approach.

But first, start at the beginning. Need to PCR from a gBlock to amplify the promoter-UTR region, using KROL472 and KROL350.

Reaction	Locus	Primers	DNA	Expected size
1	P_{rpsU2} -5'UTR _{tu14}	KROL472, KROL350	gBlock	244
2	P_{rpsU2} -5'UTR _{tu14}	KROL472, KROL350	-DNA	-

Total reaction volume	100
Total number of reactions	2
Number of reactions plus error	2.3

Component	Stock concentration	Final concentration	1 rxn volume	Master Mix
ddiH ₂ O			63	144.9
PrimeSTAR GXL Buffer	5x	1x	20	46
dNTPs	2.5 mM	0.2 mM	8	18.4
PrimeSTAR GXL DNA Polymerase	1.25 U/ul	0.025 U/ul	2	4.6
KROL472	10 uM	0.3 uM	3	6.9
KROL350	10 uM	0.3 uM	3	6.9
template	100 ng/ul	2 ng/ul	1	indiv
		Total volume	100	227.7

Use STN1 program for PCR:

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

Run 5 uL on gel:

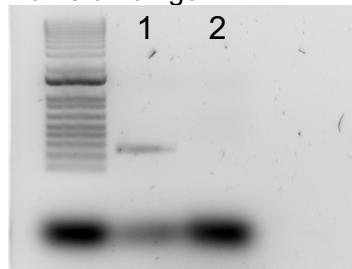


Figure 1. PCR of P_{rpsU2} -5'UTR_{tu14} for Tn7-lacZ reporter plasmids.

5 ul of PCR products, not run too far but appears to be ~ 244 bp! Looks good, no product in negative control! Move forward.

Monday, September 18, 2023**To Do:**

1. Purify PCR

Results and Data:

Purify PCR product, elute in 35 uL 0.1x EB

Tuesday, September 19, 2023**To Do:**

1. Set up digest
2. Run gel of digest
3. Gel-purify digests
4. Set up ligations

Results and Data:

Set up digest:

	1 PCR 1 P_{rpsU1}	pKR89 backbone
H ₂ O	10.8	22.8
10x CSB	3.0	3.0
DNA	15.0	3.0
KpnI-HF	0.6	0.6
NotI-HF	0.6	0.6
Total	30.0	30.0

Incubate O/N at 37°C.

CIP backbone with new Quick CIP – add 1 uL, mix, incubate for 10 mins at 37°C, then put at 80°C for 2 minutes to inactivate the CIP.

Run digests (entire volume) on 1% agarose gel.

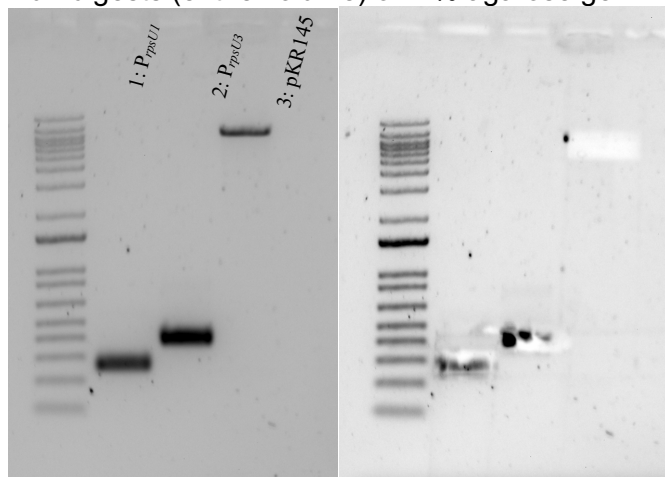


Figure 2. Digest and gel purification of *rpsU* promoters for GFP fusion plasmids.

Cutting was tough, it's not dark in the lab! Move forward with what I've got. Gel-purify slices, elute in 35 uL 0.1x EB.

Plan ligations:

Tube	Insert	Backbone
1	NotI, KpnI digested, purified PCR1	NotI, KpnI digested, purified pKR145
2	NotI, KpnI digested, purified PCR2	NotI, KpnI digested, purified pKR145
3	-	NotI, KpnI digested, purified pKR145

Set up master mix table:

Component	Reaction 1 (uL)	Reaction 2 (uL)	Reaction 3 (uL)	Master Mix (3.5x)
H ₂ O	11.5	11.5	15.5	40.25
10x ligase buffer	2	2	2	7
Insert	4	4	-	-
Backbone	2	2	2	7
Ligase	0.5	0.5	0.5	1.75
TOTAL	20	20	20	-

Add 4 uL of either DNA (insert, reactions 1&2) or water (reaction 3) to 16 uL of master mix in 0.2 mL PCR strip tubes. Incubate at 16°C O/N in thermocycler.

Will use pKR89 as backbone. Note that I should have started to prep to re-clone pKR123 as positive control!

Looked at disc diffusion assay plates from Kira and Adja's experiment on 7/20. Kira had already found some potentially laurenobiolide-resistant colonies. I found a new one on the plate with just the wild-type *S. aureus*, KRSA1.

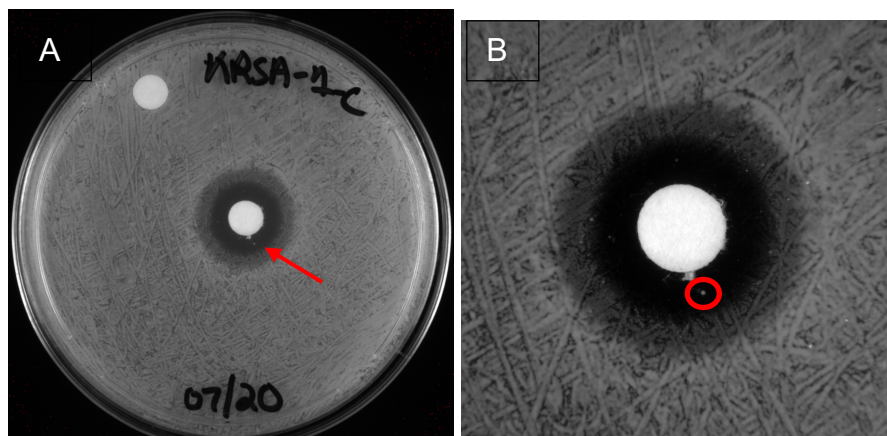


Figure 3. Detection of potential laurenobiolide-resistant mutant.

A. Plate from Adja and Kira's disc diffusion assay from 7/20/23, with lawn of KRSA1 (SA113) and disc with laurenobiolide (8 mg/mL). Arrow points to colony in zone of inhibition. **B.** Close-up view of colony (circled).

Picked out indicated colony and struck to single colony on LB, incubated O/N at 37°C.

Friday, July 28, 2023

To Do:

1. Check on *S. aureus* plate, discuss with Kira

Results and Data:

Kira and I discussed a plan:

Kira will start 6 O/N cultures from the single colonies of potentially laurenobiolide(LB)-resistant cells.
I will freeze them down on Saturday
Since there is ~100 uL of LB left at 8mg/mL, I could screen a few of colonies for resistance to LB.
Asked Kira to also streak out KRSA1 (wild-type) and KRSA4 (LB-resistant) for me for controls.

Saturday, July 29, 2023

To Do:

1. Freeze down potentially laurenobiolide resistant strains
2. Pellet some of each stock, in case they are LB-resistant and we want to isolate gDNA
3. Pour plates for disc diffusion assay tomorrow
4. Patch out cells for disc diffusion assay tomorrow

Results and Data:

Froze down potential LB-resistant cells that Kira started O/N. Make new box (S. aureus Potential laurenobiolide^R?) and store in section 8C of the -80°C freezer.

KRSA1-PLBRA

KRSA1-PLBRB

KRSA1-PLBRC

KRSA1-PLBRD

KRSA1-PLBRE

KRSA1-PLBRF

Also pellet and freeze 1 mL of cells for gDNA isolation later, if necessary.

Pulled out LB plates from 4°C to patch out cells for tomorrow. While doing so, check that we have enough LB.

!!! Not even 40 uL of laurenobiolide left!

Either scrap experiment or resuspend more laurenobiolide. Check how much we have in the freezer. There are three vials, two of which have legible labels.



Figure 4. Vials containing laurenobiolide.

Difficult to see labels of red vials in this photo, but one is 1.2 mg and the other is 2.3 mg. Unclear what the number of the blue-capped vial represents- if it is in ng, then it should be 12.7083 mg? I don't think we've ever received that much!

Resuspend and combine the drug from the two red-capped vials, combining with drug that Kira and Adja resuspended last week.

	Laurenobiolide (mg)	Desired conc (mg/mL)	volume methanol (uL)
Vial 1	1.2	8	150
Vial 2	2.3	8	287.5
	total		437.5

disc diffusion assays (20 ul/plate) 21.875

At this point, I do have enough to screen all six colonies. But if the first three are laurenobiolide-resistant, it seems wasteful to continue using up drug. Test three colonies and compare to WT and LB^R controls.

Plan for tomorrow:

Plate	Bacteria	Drug	Control	Expectation
1	KRSA1	laurenobiolide (8 mg/mL)	methanol	LB sensitive
2	KRSA4	laurenobiolide (8 mg/mL)	methanol	LB resistant
3	KRSA1-PLBR-A	laurenobiolide (8 mg/mL)	methanol	?
4	KRSA1-PLBR-B	laurenobiolide (8 mg/mL)	methanol	?
5	KRSA1-PLBR-C	laurenobiolide (8 mg/mL)	methanol	?
6	KRSA1	Thiostrepton (50 mg/mL)	DMSO	TBD
7	KRSA4	Thiostrepton (50 mg/mL)	DMSO	TBD
8	KRSA1	fusidic acid (50 mg/mL)	EthOH	TBD
9	KRSA4	fusidic acid (50 mg/mL)	EthOH	TBD
10	KRSA1	Lincomycin (50 mg/mL)	water	TBD
11	KRSA4	Lincomycin (50 mg/mL)	water	TBD
12	KRSA1	Tiamulin (12.5 mg/mL)	EtOH	TBD
13	KRSA4	Tiamulin (12.5 mg/mL)	EtOH	TBD

Poured a flask of LB plates, 24 mL per plate, for the disc diffusion assays tomorrow.

Sunday, July 30, 2023

To Do:

1. Disc diffusion assay

Results and Data:

Plan for today:

Plate	Bacteria	Drug	Control	Expectation
1	KRSA1	laurenobiolide (8 mg/mL)	methanol	LB sensitive
2	KRSA4	laurenobiolide (8 mg/mL)	methanol	LB resistant
3	KRSA1-PLBR-A	laurenobiolide (8 mg/mL)	methanol	?
4	KRSA1-PLBR-B	laurenobiolide (8 mg/mL)	methanol	?
5	KRSA1-PLBR-C	laurenobiolide (8 mg/mL)	methanol	?
6	KRSA1	Thiostrepton (50 mg/mL)	DMSO	TBD
7	KRSA4	Thiostrepton (50 mg/mL)	DMSO	TBD
8	KRSA1	fusidic acid (50 mg/mL)	EthOH	TBD
9	KRSA4	fusidic acid (50 mg/mL)	EthOH	TBD
10	KRSA1	Lincomycin (50 mg/mL)	water	TBD
11	KRSA4	Lincomycin (50 mg/mL)	water	TBD
12	KRSA1	Tiamulin (12.5 mg/mL)	EtOH	TBD
13	KRSA4	Tiamulin (12.5 mg/mL)	EtOH	TBD

Label DDA plates (13) with number of experiment, strain, drug, and date.

Scrape up cells of different strains into LB. Check OD600 (50 uL into 950). Too concentrated, dilute and check again.

Number	Strain	Measured OD600	Actual OD600	Desired OD600	Desired volume	Volume cells to add	Volume LB
1	KRSA1	0.143	2.86	0.05	1500	26.2	1473.8
2	KRSA4	0.148	2.96	0.05	1500	25.3	1474.7
3	KRSA1-PLBR-A	0.128	2.56	0.05	1500	29.3	1470.7
4	KRSA1-PLBR-B	0.181	3.62	0.05	1500	20.7	1479.3
5	KRSA1-PLBR-C	0.139	2.78	0.05	1500	27.0	1473.0

Dilute as in table above.

Plate 100 uL per plate, as per table, spreading with beads

Let plates dry.

While drying, prepare abx discs. Need 20 uL per disc- looks like we don't have >40 uL for the four trial abx. Dilute some of what is left to test.

	tiamulin	fusidic acid	thiostrepton	lincomycin
Stock (mg/mL):	12.5	50	50	50
volume stock (ul)	5	15	15	15
volume dilutant (ul)	45	45	45	45
final conc (mg/ml)	1.25	12.5	12.5	12.5
dilutant	EtOH	EtOH	DMSO	water

Revised DDA sheet:

Plate	Bacteria	Drug	Control	Expectation
1	KRSA1	laurenobiolide (8 mg/mL)	methanol	LB sensitive
2	KRSA4	laurenobiolide (8 mg/mL)	methanol	LB resistant
3	KRSA1-PLBR-A	laurenobiolide (8 mg/mL)	methanol	?
4	KRSA1-PLBR-B	laurenobiolide (8 mg/mL)	methanol	?
5	KRSA1-PLBR-C	laurenobiolide (8 mg/mL)	methanol	?
6	KRSA1	thiostrepton (12.5 mg/mL)	DMSO	TBD
7	KRSA4	thiostrepton (12.5 mg/mL)	DMSO	TBD
8	KRSA1	fusidic acid (12.5 mg/mL)	EthOH	TBD
9	KRSA4	fusidic acid (12.5 mg/mL)	EthOH	TBD
10	KRSA1	lincomycin (12.5 mg/mL)	water	TBD
11	KRSA4	lincomycin (12.5 mg/mL)	water	TBD
12	KRSA1	tiamulin (1.25 mg/mL)	EtOH	TBD
13	KRSA4	tiamulin (1.25 mg/mL)	EtOH	TBD

In sterile petri dishes, add 20 ul of dilutant or drug to sterile discs. Let dry (methanol and ethanol, <10 minutes; DMSO >20 minutes!)

Plate discs on appropriate plates, incubate O/N at 37°C. Put in incubator around 2pm.