Lab Notebook - Week 9 (8/7/2017-8/13/2017)

Project: NU iGEM 2017 Shared Project
Authors: Lulu
Dates: 2017-08-07 to 2017-08-12

MONDAY, 8/7/17

PCR to add His6

Purpose: Add a His tag by PCR and religate the linearized plasmid
Primers: P4 and P5 (non-phosphorylated)
Plasmids: pC49, pC50, pC51, pC52, pC53 (all at conc. 1 ng/uL)

Primers - His6 additi…

	A	B	C
1	**Primer Name**	**Sequence**	**Melting Temperature**
2	P4 (FW)	TCATCATCATgtactagtagcggccgct	56.6, 62.71°C
3	P5 (REV)	TGGTGGTGCATtcagccctttttaataatctgcg	53.5, 64.31°C

Phosphorylation:
Procedure:

1. Prepare the following reaction mixture.
2. Mix thoroughly, spin down and incubate at 37°C for 1 hour.

Table1

	A	B
1	ddH2O	15 uL
2	100 µM primer	2 uL
3	10x T4 ligase buffer with ATP	2 uL
4	10 U/µL T4 polynucleotide kinase	1 uL
5	**Total:**	20 uL

PCR reaction

PCR Table

	A	B
1	Label	Plasmid
2	A	pC49
3	B	pC50
4	C	pC51
5	D	pC52
6	E	pC53

Materials for each PCR:

- ddH20: adjust volume to 50 uL (17.5 uL)
- 2.5 uL 10 uM P4 (Phosphorylated)
- 2.5 uL 10 uM P5 (Phosphorylated)
- 1.5 uL DMSO
- add 1 ng miniprepped DNA (1uL)
- 25 uL of Phusion HS Flex 2x MM
- PCR Tube

Procedure:

1. Add NF Water first
2. Add P4, P5, DMSO, pC34 in any order * Vortex briefly after all added
3. Add MM (Vortex briefly and spin down for a second or two to get material out of lid)
4. Vortex final reaction briefly and spin quickly to collect in bottom of tube
5. Place in Thermocycler at the following conditions:
   - 98 C for 30s
   - 98 C for 15s (Repeat Red 10x)
   - 57 C for 30s
   - 72 C for 2.5 minutes
   - 98 for 15s (Repeat Green 20x)
   - 65 for 30s
   - 72 for 30s
   - 72 C for 5 min
   - 4 C for inf. Time

PCR wash (Do while gel runs)

- Add 250uL of PB and mix well with each PCR reaction
  - Used the one in the Qiagen kit
- Pipette everything into a column from the miniprep kit and spin for 1 minute
- Wash with CWC (500 uL) and spin for 1 minute
- Elute into a steril tube using 30uL of ddH2O

Digestion with DpnI (Prepare while gel runs)

- Add DpnI (1uL(/50uL)) to the purified PCR product and incubate for 1-4 hours (37 degC)
  - Incubated for 60 minutes

Nanodrop results (nanodrop after PCR wash and DpnI treatment):

Nanodrop results - His addit…

	A	B
1	**Plasmid**	**Concentration (ng/uL)**
2	**pC49 A**	5.2*
3	**pC50 B**	3.0
4	**pC51 C**	7.6
5	**pC52 D**	4.0*
6	**pC53 E**	10.4

Gel electrophoresis (for identification of the correct linearized fragment)

- Load 3uL of PCR product and 1uL of dye onto each well (parafilm method)
- Expected band at **5kb**
- **Just before gel was run, sequencing results came in confirming plasmids pC50 (IC55), pC51 (IC56), and pC53 (IC58). These will be used for the remainder of the process. pC49 and pC52 will not be used.**
- Run for ~45 mins **PCR Failed. Set to try again on 8/8 with gradient/touchdown PCR. Will still transform "ligated product"**

Table2

	A	B	C	D	E	F
1	Lane 1	Lane 3	Lane 3	Lane 4	Lane 6	Lane 6
2	Ladder	B (pC50/IC55)	C (pC51/IC56)	E (pC53/IC58)	C (pC51/IC56) Reload	E (pC53/IC58) Reload
3	All Bands	PCR Fail	PCR Fail	PCR Fail	PCR Fail	PCR Fail

Ligation

Procedure:

1. Calculate the volume of the DpnI-treated PCR product containing 50-150 ng and fill in the table below.
2. Calculate the volume of water to make up to 10 µL.
3. Mix the components below:
4. Incubate for 120 min at room temperature (~22°C).

step 4: or pcr machine, 16C, overnight

Reaction Volumes for Ligat…

	A	B	C	D
1	**Reagent**	**Reaction B**	**Reaction C**	**Reaction E**
2	ddH2O	0 uL	0 uL	0 uL
3	Purified PCR product (50 ng)	8 uL	8 uL	8 uL
4	10x reaction buffer	1 uL	1 uL	1 uL
5	T4 DNA ligase, 5U/uL	1 uL	1 uL	1 uL
6	**Total:**	10 uL	10 uL	10 uL

Perform Transformations:

Volumes for iPCR Transformations

	A	B	C
1	30 mL C-cells	5 uL iPCR ligated product	35 uL rescue media

Multiple plates created to identify ideal efficiencies

Plates

	A	B
1	**PCR B**	**PCR C**
2	with KCM (30 uL plated)	with KCM (30 uL plated)
3	without KCM (20 uL plated)	without KCM (20 uL plated)
4	without KCM (30 uL plated)	without KCM (30 uL plated)

Cas9 activity validation project:

Used newer plates of Trial 1 (mRFP-gRNA + His-Cas9) for overnight culture
Inoculation for + and - controls, trial 2 started in small culture tubes: 2 mL of LB + 4 uL overnight culture

TUESDAY, 8/8/17

PCR to add His6

Purpose: Add a His tag by PCR and religate the linearized plasmid
Primers: P4 and P5 (phosphorylated)
Plasmids: pC50, pC51, pC53 (all at conc. 1 ng/uL)

Primers - His Addition (2)

	A	B	C
1	**Primer Name**	**Sequence**	**Melting Temperature**
2	P4 (FW)	TCATCATCATgtactagtagcggccgct	56.6, 62.71°C
3	P5 (REV)	TGGTGGTGCATtcagccctttttaataatctgcg	53.5, 64.31°C

PCR reaction

Table5

	A	B
1	Label	Plasmid
2	A	pC50
3	B	pC51
4	C	pC53

Materials for each PCR:

- ddH20: adjust volume to 50 uL (15.0 uL)
- 2.5 uL 10 uM P4 (Phosphorylated)
- 2.5 uL 10 uM P5 (Phosphorylated)
- 1.5 uL DMSO
- add 1 ng miniprepped plasmid DNA (3.5 uL)
- 25 uL of Phusion HS Flex 2x MM
- PCR Tube

Procedure:

1. Add NF Water first
2. Add P4, P5, DMSO, plasmid in any order * Vortex briefly after all added
3. Add MM (Vortex briefly and spin down for a second or two to get material out of lid)
4. Vortex final reaction briefly and spin quickly to collect in bottom of tube
5. Place in Thermocycler at the following conditions:
    - 98 C for 30s
    - 98 C for 15s (Repeat Red 10x)
    - 57 C for 30s
    - 72 C for 2.5 minutes
    - 98 for 15s (Repeat Green 20x)
    - 65 for 30s
    - 72 for 2.5 mins ***edited from yesterday***
    - 72 C for 5 min
    - 4 C for inf. Time

PCR wash (Can do while gel runs)

- Add 250uL of PB and mix well with each PCR reaction
    - Used the one in the Qiagen kit
- Pipette everything into a column from the miniprep kit and spin for 1 minute (Max speed)
- Wash with CWC (400 uL) and spin for 1 minute
- Add 30 uL NF water and incubate at RT for 1 minute

- Elute by spinning for 30 seconds

Gel electrophoresis (for identification of the correct linearized fragment)

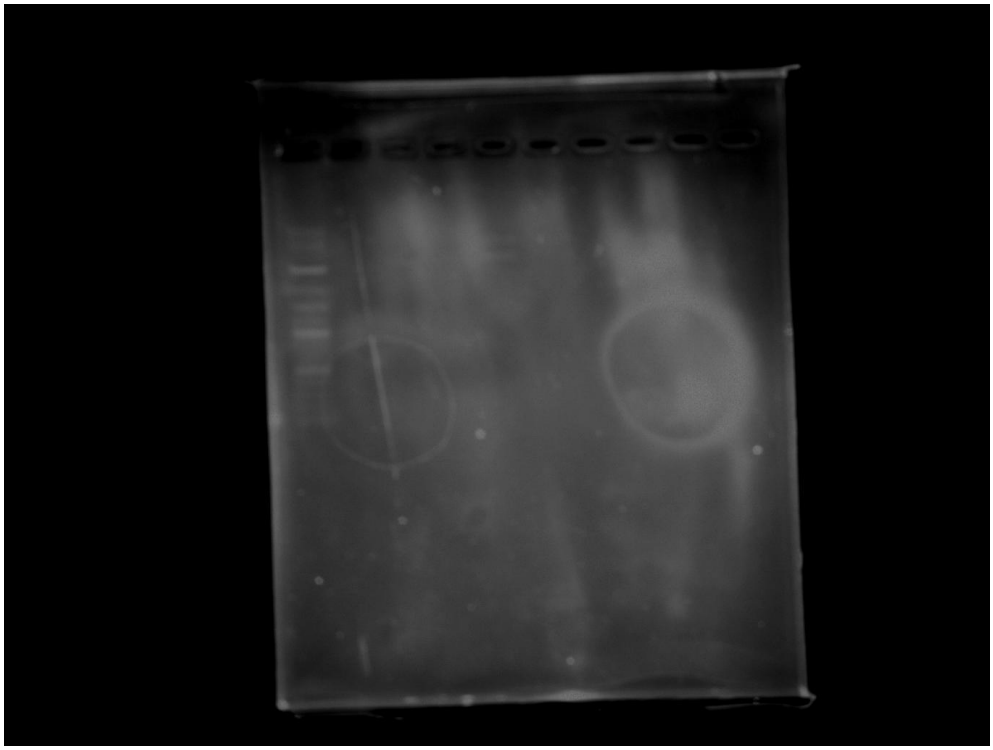
- Load 3uL of PCR product and 1uL of dye onto each well (parafilm method)
- Expected band at **5kb**
- Run for ~45 mins

Table7

	A	B	C	D
1	Lane 1	Lane 3	Lane 4	Lane 5
2	Ladder	A (IC54)	B (IC55)	C (IC56)
3	All Bands	None	5 kB	5 kB and >5 kB

Gel Result:

Camera 0.jpg



Digestion with DpnI (Prepare when gel finished)

- Add DpnI (1uL) to the purified PCR product and incubate for 1-4 hours (37 degC)
    - Incubated for 60 minutes

Nanodrop results (nanodrop after PCR wash and DpnI treatment):

Table6

	A	B
1	**Plasmid**	**Concentration (ng/uL)**
2	**pC50 A**	N/A
3	**pC51 B**	10.1
4	**pC53 C**	25.1

Ligation

Procedure:

1. Calculate the volume of the DpnI-treated PCR product containing 50-150 ng and fill in the table below.
2. Calculate the volume of water to make up to 10 µL.
3. Mix the components below:
4. Incubate for 120 min at room temperature (~22°C). (or PCR at 16C overnight)

Table8

	A	B	C
1	**Reagent**	**Reaction B**	**Reaction C**
2	ddH2O	3 uL	6 uL
3	Purified PCR product (50 ng)	5 uL	2 uL
4	10x reaction buffer	1 uL	1 uL
5	T4 DNA ligase, 5U/uL	1 uL	1 uL
6	**Total:**	10 uL	10 uL

Perform Transformations:

Table9

	A	B	C
1	35 mL C-cells (Dh5a from NEB)	5 uL iPCR ligated product	35 uL rescue media

Multiple plates created to identify ideal efficiencies

Table10

	A	B
1	**PCR B (pC57)**	**PCR C (pC58)**
2	without KCM (30 uL plated) A	without KCM (30 uL plated) C
3	with KCM (30 uL plated) B	with KCM (30 uL plated) D

Cas9 activity validation project:

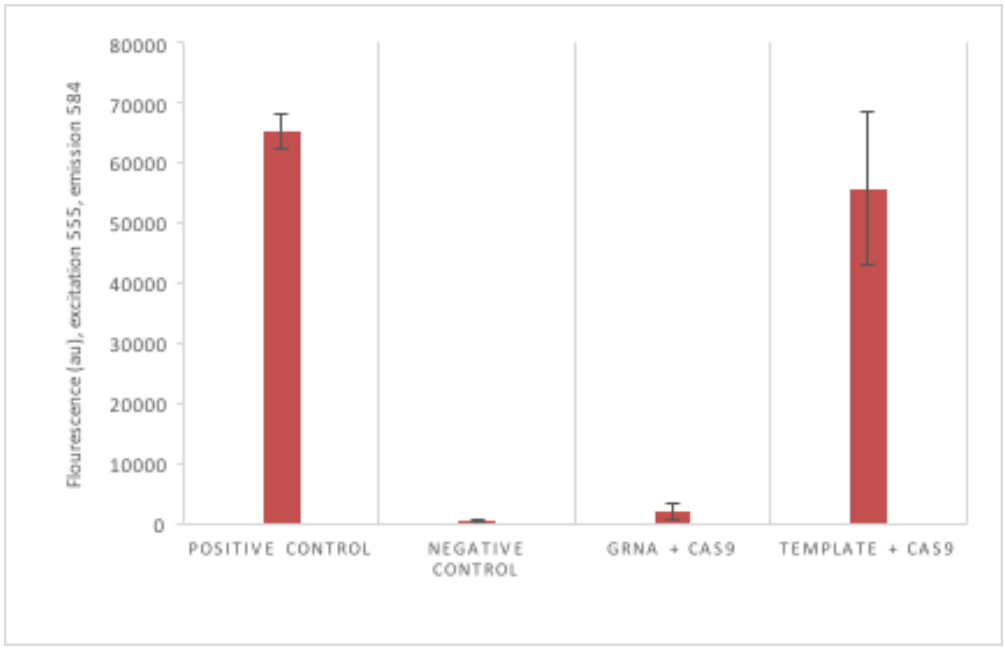
Created M9 media, stored in +4°C (see protocol)
Inoculate all 12 overnights (including Trial 1) in 2mL of M9 media at 1:25 dilution (80 uL of overnight in 1.92 mL of M9)
Cells were left in shaker at 37°C for 5.5 hours.

Plate reading:
96-well black-bottom plate used
100 uL of cells pipetted into each well, 1x PBS as blank
OD 600, fluoresence 555 (emission), 584 (excitation)

Table4

	A	B	C	D	E
1		Positive control (AU/OD)	Negative control (AU/OD)	gRNA + Cas9 (AU/OD)	Template + Cas9 (AU/OD)
2	1	62375	239.13	3333.33	65603.45
3	2	67869.565	272.73	2150.94	60000
4	3	65333.33	448.98	407.41	41202.53
5	average	65192.63167	320.28	1963.893333	55601.99333
6	std dev	2749.9833	112.7164917	1471.900745	12781.16079

image.png



WEDNESDAY, 8/9/17

Growth results:
Pictures of Plates*

THURSDAY, 8/10/17

Cloning Day 3:

Promega miniprep:
Miniprep DNA from His6 addition. This will be sent to sequencing and used for cloning in the meantime.

Bacterial Stocks Prepped

	A	B	C	D	E	F
1	Label	Stock	Purified Label	Concentration (ng/uL)	260/280	260/230
2	A	pC58 with KCM	pC59	206.3	1.90	2.48
3	B	pC58 with KCM	pC60	190.8	1.88	2.11
4	C	pC57 no KCM	pC61	385.2	1.88	2.37

PelB:
Purpose: Add a PelB SS by PCR and religate the linearized plasmid
Primers: P59and P60 (phosphorylated)
Plasmids: pC59, pC60, pC61 (all at conc. 5 ng/uL)

Primers - PelB addition

	A	B	C
1	**Primer Name**	**Sequence**	**Melting Temperature**
2	P59 (FW)	TGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCaagagaaattacattctggga	49.0, 74.2°C
3	P60 (REV)	GACCAGCAGCAGCGGTCGGCAGCAGGTATTTCATttgtcccctctttcgtg	52.9, 72.0°C

PCR reaction
3 simultaneous reactions ran due to not having sequenced the plasmid yet.

Name assignment

	A	B	C
1	Label	Plasmid	Sequecning Code
2	A	pC59	iPC1
3	B	pC60	iPC2
4	C	pC61	iPC3

Materials for each PCR:

- ddH20: adjust volume to 50 uL (16.5 uL)
- 2.5 uL 10 uM P59 (Phosphorylated)
- 2.5 uL 10 uM P60 (Phosphorylated)
- 1.5 uL DMSO ****REMOVE! replace with water*****
- add 10 ng miniprepped plasmid DNA (2 uL)
- 25 uL of Phusion HS Flex 2x MM
- PCR Tube

Procedure:

1. Add NF Water first
2. Add P59, P60, DMSO, plasmid in any order * Vortex briefly after all added
3. Add MM
4. spin quickly to collect in bottom of tube
5. Place in Thermocycler at the following conditions:
    - 98 C for 30s
    - 98 C for 15s (Repeat Red 10x)
    - 53 C for 30s
    - 72 C for 2.5 minutes
    - 98 for 15s (Repeat Green 20x)
    - 71 for 30s
    - 72 for 2.5 mins ***edited from yesterday***
    - 72 C for 5 min
    - 4 C for inf. Time

PCR wash (Can do while gel runs) ***WASH AFTER DPN1 DIGEST AFTER GEL**

- Add 250uL of PB and mix well with each PCR reaction
    - Used the one in the Qiagen kit
- Pipette everything into a column from the miniprep kit and spin for 1 minute (Max speed)
- Wash with CWC (400 uL) and spin for 1 minute
- Add 30 uL NF water and incubate at RT for 1 minute
- Elute by spinning for 30 seconds

Gel electrophoresis (for identification of the correct linearized fragment)

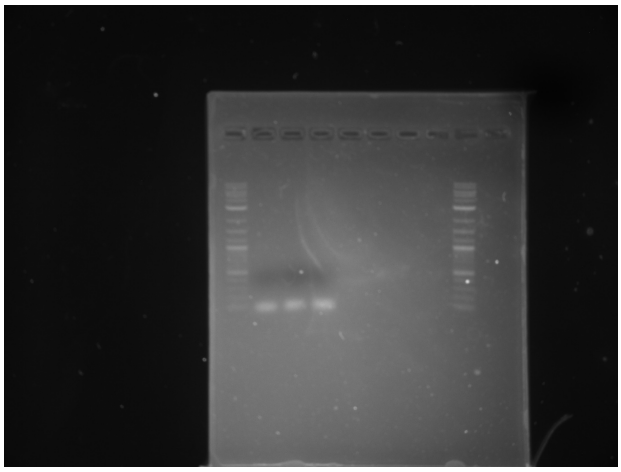
- Load 3uL of PCR product and 1uL of dye onto each well (parafilm method)
- Expected band at **5.5kb**
- Run for ~45 mins

Gel Lanes

	A	B	C	D
1	Lane 1	Lane 3	Lane 4	Lane 5
2	Ladder	A (IC62)	B (IC63)	C (IC64)
3	All Bands	Primer DImer	Primer Dimer	Primer Dimer

Gel Result:

08102017_PCR_1.tif



SATURDAY, 8/12/17

PelB:

Purpose: Add a PelB SS by PCR and religate the linearized plasmid

Primers: P59and P60 (phosphorylated)

Plasmids: pC59, pC60, pC61 (all at conc. 5 ng/uL)

Primers - PelB Additi…

	A	B	C
1	**Primer Name**	**Sequence**	**Melting Temperature**
2	P59 (FW)	TGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCaagagaaattacattctggga	49.0, 74.2°C
3	P60 (REV)	GACCAGCAGCAGCGGTCGGCAGCAGGTATTTCATttgtcccctctttcgtg	52.9, 72.0°C

PCR reaction

(3 simultaneous reactions ran due to not having sequenced the plasmid yet.)

Table15

	A	B	C
1	Label	Plasmid	Sequecning Code
2	A	pC59	iPC1
3	B	pC60	iPC2
4	C	pC61	iPC3

Materials for each PCR:

- ddH20: adjust volume to 50 uL (18 uL)
- 2.5 uL 10 uM P59 (Phosphorylated)
- 2.5 uL 10 uM P60 (Phosphorylated)
- add 10 ng miniprepped plasmid DNA (2 uL each)
- 25 uL of Phusion HS Flex 2x MM

- PCR Tube

Procedure:

1. Add NF Water first
2. Add P59, P60, plasmid in any order * Vortex briefly after all added
3. Add MM
4. spin quickly to collect in bottom of tube
5. Place in Thermocycler at the following conditions:
    - 98 C for 30s
    - 98 C for 15s (Repeat Red 10x)
    - 53 C for 30s
    - 72 C for 2.5 minutes
    - 98 for 15s (Repeat Green 20x)
    - 71 for 30s
    - 72 for 2.5 mins
    - 72 C for 5 min
    - 4 C for inf. Time

Gel electrophoresis (for identification of the correct linearized fragment)

- Load 4uL of Ladder into first well
- Load 3uL of PCR product and 1uL of dye onto each well (parafilm method)
- Expected band at **5.5kb**
- Run for ~60 mins

Gel Lanes (2)

	A	B	C	D
1	Well 1	Well 3	Well 4	Well 6
2	Ladder	A (IC62)	B (IC63)	C (IC64)
3	All Bands	Primer DImer	Primer Dimer	Primer Dimer

Gel Result: (on computer desktop under name "PelB 2")

Protocol for Transformation - Chelsea (detailed)

Introduction

Protocol for Experiment 1 - Functional Analysis of Cas9 and DsbA-Cas9 to Cut mRFP sequence using specific gRNA

Materials

›

- Competent cells (10 uL/trial for Top10, 20 uL/trial for JC8031)
- Plasmid DNA (conc. 1 pg/uL- 10 ng/uL)
- 2 mL microcentrifuge tubes (chilled in -20 freezer)
- Agar plates (with right antibiotic resistance if needed)
- Ice (in bucket)
- Spreader beads/wand
- SOC Media for rescuing cells
- Water Bath (set to 42 C)
- Incubator/Shaker @ 37 C
- Floating test tube rack for water bath

Procedure

Procedue

1. Set water bath to 42 C
2. Thaw comp cell aliquots on ice for 15 minutes
3. Remove agar plates (containing the appropriate antibiotic) from storage at 4°C and let warm up to room temperature
4. Pipette 10 uL of competent cells in each 2 mL microcentrifuge tube (20 uL for JC8031). Keep tubes on ice at all times.
5. Pipette 1 uL of 1 pg/uL-10 ng/uL of plasmid DNA into each microcentrifuge tube, do not disturb in anyway (not even flick). For dual transformation, use 0.5 uL of each plasmid DNA to get to 1 uL total DNA per rxn
    a. e.g. If stocks of DNA are at 50 ng/ul, dilute 50 ng/uL to 10 ng/uL
6. Incubate tubes on ice for 20 mins
7. Heat shock tubes in water bath at 42 C for 60 secs. --- Timing must be exact or cells will die ---
8. Immediately move tubes to ice bucket, cover with ice, and incubate on ice for 5 mins
9. Add 10 uL (20 uL if using JC8031) of SOC (no antibiotics) and rescue for an hour and 15 minutes at 37 C in shaker

- [x] 10. Plate on appropriate agar + antibiotic plates
- [x] 11. Incubate overnight at 37

M9 Supplemented Media

Introduction

Prof. Lucks lab's protocol for preparing supplemented M9 media

Storage:

* Media should be stored at **4°C**, wrapped in foil and stored away from light
* Media has short shelf life of ~1 week. Check the media for precipitation before using. **For best results, mix the media the day it is to be used**
* Individual media components can be stored long term at **4°C**, except for **thiamine hydrochloride**, **which should be made fresh and stored away from light**

Materials

- 1x M9 salts
- 1 mM thiamine hydrochloride
- 0.4% glycerol
- 0.2% casamino acids
- 2 mM MgSO4
- 0.1 mM CaCl2

Procedure

1. 5x M9 Minimal Salts

   11.3 g M9 minimal salts
   300 mL ddH2O
   Autoclave to sterilize

2. 10 mg/mL thiamine hydrochloride

   450 mg thiamine hydrochloride
   45 mL ddH2O
   0.22 uM filter sterilize

   Storage:
   Aliquot into 5mL tubes
   Freeze with aluminum foil at 20°C
   For fridge-stored (+4°C), good for 2 weeks

3. 40% glycerol

   80 mL glycerol
   120 mL ddH2O
   Autoclave to sterilize

4. 10% casamino acids

   10 g Bacto casamino acids
   100 mL ddH2O

0.22 uM filter sterilize

Storage:
Aliquot into 10mL tubes
Freeze at 20°C

5. 1M MgSO4

6.02 g MgSO4
50 mL ddH2O
Autoclave to sterilize

6. 1M CaCl2

5.55 g CaCl2
50 mL ddH2O
Autoclave to sterilize

7. ddH2O

Autoclave to sterilize

Recipe for mixing:

MIX UNDER FLAME
Add CaCl2 LAST; goes back into solution once mixed

200 mL M9

	A	B
1	**Volume**	**Component**
2	40 mL	5x M9 minimal salts
3	6.8 mL	10 mg/mL thiamine hydrochloride
4	2 mL	40% glycerol
5	4 mL	10% casamino acids
6	400 uL	1M MgSO4
7	20 uL	1M CaCl2
8	Fill to 200mL (~147 mL)	Sterilized ddH2O

50 mL

	A	B
1	**Volume**	**Component**
2	10 mL	5x M9 minimal salts
3	1.7 mL	10 mg/mL thiamine hydrochloride
4	500 uL	40% glycerol
5	1 mL	10% casamino acids
6	100 uL	1M MgSO4
7	5 uL	1M CaCl2
8	Fill to 50 mL	Sterilized ddH2O

65 mL

	A	B	C	D
1	**Volume**	**Component**		
2	13 mL	5x M9 minimal salts		
3	2.21 mL	10 mg/mL thiamine hydrochloride		
4	650 uL	40% glycerol		
5	1.3 mL	10% casamino acids		
6	130 uL	1M MgSO4		
7	6.5 uL	1M CaCl2		
8	Fill to 65	Sterilized ddH2O	~47.7 mL	