Lab Notebook - Week 6 (17/7/17 - 23/7/17)

Project: NU iGEM 2017 Shared Project
Authors: Katerina Cheronis
Dates: 2017-07-17 to 2017-07-23

MONDAY, 7/17

Purpose: OMV purification from JC8031 (no antibiotic) and DeLisa ClyA-GFP (Cm+) fusion
OMVs were purified as described in the SBI protocol - 2 columns (one for GFP OMVs and ones for JC8031 no GFP)

1. Prepare clarified supernatant from bacterial culture
    a. Culture the bacteria in its growth medium overnight at 37 deg.
    b. Spin down bacteria at 5000xg for 15 minutes at 4 deg C
        I. 4000xg for 17 minutes - centrifuge would not go any higher
    c. Transfer the supernatant to a new flask and filter through a 0.45um filter
    d. Spin down the supernatant at 5000xg for 15 minutes at 4 deg C
    e. Transfer the supernatant to a new flask and filter through a 0.22um filter. The filtered supernatant is now ready for OMV isolation
2. Pack the column/bind OMVs
    a. Pipette 200 mL of the resin onto the column
    b. Equillibrate by adding 1mL of the Binding Buffer and allow the solution to flow through. Discard the flow through
    c. Place the yellow cap onto the bottom of the column
    d. Add 10mL of the clarified bacterial supernatant (prepared in step 1) to the resin and incubate on a rotating rack at 4 deg C for 3-4 hours to allow for OMV binding
        I. Used centrifuge at a very low speed.
3. OMV Elution
    a. Place the column onto a rack and allow the resin/supernatant to flow through (collect the flow through for analysis if desired)
    b. Wash the resin with 10mL Binding Buffer 2 times. Discard the flow through.
    c. Add 500uL Elusion Buffer and collect in in 1.5ml Eppendorf tube
        I. Repeat the elusion step for a total of 5 times in separate tubes
4. Analysis
    a. Perform downstream analysis of the five separate elutions (or pool if desired)

GEMSTONE Dye Protocol
Total time for completion: ~ 2 ++ hours
Useful for flow cytometry

- Resuspend 200 - 500ug protein equivalent of OMVs in 500uL PBS
- Add 1uL of the 500X or 2.5uL of the 250X labeling dye to the OMV preparation and incubate at 37degC with shaking for 20 minutes
- Add 167uL ExoQuick-TC to the solution and incubate at 4deg C for 2h-overnight
- Spin the eppendorf tube at 10000rpm for 10 minutes
- Carefully aspirate the supernatant from the corner of the tube
    - No pellet formed.
- Resuspend the OMV pellet in 500 uL PBS and proceed with downstream applications - not done

Imaging: magnification not high enough, did not observe fluorescence or OMV aggregates

TUESDAY, 7/18

Preparation for Western blotting attempt #2

Day 1: 7/18/17
Retransform Competent JC8031:
Pos. Control for retransformation (10pg/μL DNA concentration): mRFP from iGEM distribution - Should appear **red** on cam plates
Neg. Control for retransformation: H_2O transformation - Should **not appear** on cam plates

Exp. Run for retransformation (10pg/µL DNA concentration): Purified plasmid DNA from Cas9-Dsba Miniprep

gRNA Design
Streak glycerol stocks of gRNA-mRFP and His6-Cas9

General:

- Made LB with Cm antibiotic
- Created Cm plates (approximately 40 plates)

Plate results:

mRFP-gRNA

mRFP-gRNA

WEDNESDAY, 7/19

General:

- Made LB : tetR & camR

First Retrasnformation Failed
Retry Retrasnformation
Experimental Setup:
Positive Control: Dh5a Competent Cells from NEB with Cas9-Dsba concentration 100 pg/uL
Experimental 1: JC8031 with with Cas9-Dsba concentration 100 pg/uL
Experimental 1: JC8031 with with Cas9-Dsba concentration 100 ng/uL
Experimental 1: JC8031 with with Cas9-Dsba concentration 100 pg/uL

ClyA-GFP Construct
Determined primers for sequencing ClyA-GFP - JBLF16 and CJK59

gRNA Design
Plates did not grow well, so no colonies were picked
Instead, directly made an 5mL overnight culture from glycerol stock of gRNA-mRFP and His6-Cas9

Prepared overnight cultures of:
mRFP - gRNA
His-Cas9 (Lulu)
His-Cas9 (Jack):
ClyA-GFP
DsbA - Cas9

OMV Experiment:
Retry Gemstone dyes to observe fluorescence:
Red dye used (Ruby)

OMVs taken from Tube 1: 2.953 mg/mL (Nanodrop 2000)
260/280 = 1.40
Tube 1 had the best protein concentration. 2nd 3rd and fouth elusion concentrations were not as high.
Tube label: 29
Incubation time: approximately 4 hours
Stored in +4 for analysis

- In some papers OMVs are stored in -80
    - Where should they be stored?

THURSDAY, 7/20

Minipreped cultures prepared the previous day.
Miniprep Nanodrop results:
Using old Promega protocol

Attempt 1

	A	B	C	D
1		**Conc (ng/uL)**	**260/280**	**260/230**
2	**mRFP - gRNA**	5.0	1.10	1.06
3	**His-Cas9 Lulu**	6.9	1.63	1.06
4	**His-Cas9 Jack**	40.0	1.64	0.71
5	**ClyA-GFP**	11.7	1.71	0.76
6	**DsbA-Cas9**	24.4	1.86	1.38

Using new protocol, Chelsea's revisions (new protocol available in miniprep kit):

Attempt 2

	A	B	C	D
1		**Conc (ng/uL)**	**260/280**	**260/230**
2	**mRFP - gRNA**	9.4	1.71	0.66
3	**His-Cas9 Lulu**	44.8	1.91	2.21
4	**His-Cas9 Jack**	107.3	1.93	2.26
5	**ClyA-GFP**	27.8	1.94	2.60
6	**DsbA-Cas9**	168.4	1.90	2.22

Second miniprep attempt of ClyA - GFP was sent for sequencing (after 2:00 pm). Should receive early next week.

- Primers used:
  - CJGk59 (REV)// 5uL
  - JBLF16 (FW)//5uL
- Plasmid volume: 20uL (no dilution following nanodrop)

Prepared rich medium for Western blot with Jazzy (1/2 Dose)

Recipe for 1L 2xYT:

- ☒ Measure ~900ml of distilled H_2O.
- ☒ Add 16g Bacto Tryptone.
- ☒ Add 10g Bacto Yeast Extract.
- ☒ Add 5g NaCl.
- ☒ Adjust pH to 7.0 with 5N NaOH.
- ☒ Adjust to 1L with distilled H_2O.
- ☒ Sterilize by autoclaving.

Created new Tetracyclin 1000x stocks (10 stocks of 1 mL each) - based on protocol book concentrations

- In antibiotics cryo box (-20 deg). Aluminum foil becuase light sensitive.

Nanosight information from Devin:

Laser goes through the particles and uses Stokes Einstein equation for quantification

Size and concentration information

Sensitive to concentration - need to find best ratio (1*10^8 particles/mL)

- But we don't know OMV concentration following purification so this will require trial and error
- Western blot with flag tag would be the best for GFP detection
- Nanosight is able to detect fluoreschence at certain excitations - worth a try
- Probably will try out using Nanosight early next week

gRNA Design:

Streatek plates of gRNA-mRFP from glycerol stock - Lulu

Prepared an overnight culture of mRFP-gRNA for miniprep the following day - Lulu

FRIDAY, 7/21

Plate results - Many aggregated colonies but there are some isolated ones to pick

mRFP - gRNA

Results from Retrasnformation 7/19/2017

Overall: Since colonies were left for more than 18 hours, the results are not to be used/trusted, will retry transformation again today. However, we will use the results as a reference to see what conditions to use for our retransformation

Positive Control: Dh5a Competent Cells from NEB with Cas9-Dsba concentration 100 pg/uL - **Contaminated with Ice, thrown out**

Experiment 1: JC8031 with with Cas9-Dsba concentration 100 pg/uL - **Few colonies**

Experiment 1: JC8031 with with Cas9-Dsba concentration 100 ng/uL - **Many colonies, grew extremely well**

Experiment 1: JC8031 with with Cas9-Dsba concentration 100 pg/uL - **Few colonies**

gRNA Experiment:

This experiment is to check the viability of the constructs **BBa_K2019002** and **BBa_K2019000**.

This follows the Biobricks submissions:

http://parts.igem.org/Part:BBa_K2019002

http://parts.igem.org/Part:BBa_K2019000

For mRFP-gRNA, the backbone is pSB1T3: http://beta.labgeni.us/registries/parts_registry/?part=BBa_K2019002

For His-Cas9, the backbone is pSB1C3: http://beta.labgeni.us/registries/parts_registry/?part=BBa_K2019000

Miniprep mRFP-gRNA cells from overnight culture (started on Thursday 7/20)

mRFP - gRNA

	A	B	C	D
1	**Ammount of DNA during miniprep**	**DNA concentration**	**260/280**	**260/230**
2	*High Concentration (#35)	349.1 ng/uL	1.89	2.08
3	**Low concentration (#36)	138.4 ng/uL	1.92	2.17

* Bigger pellet generated becuase of higher volume of bacteria culture used (not enough to use 2x1.5 mL for both preparations)

** 1.5 mL the first time and ~800 uL the second time the culture was centrifuged

Sequencing:

- Cas9-His was sent for sequencing with Forward (VF) and Reverse (VR) primer (10 uL template and 2 uL of each primer in separate tubes)

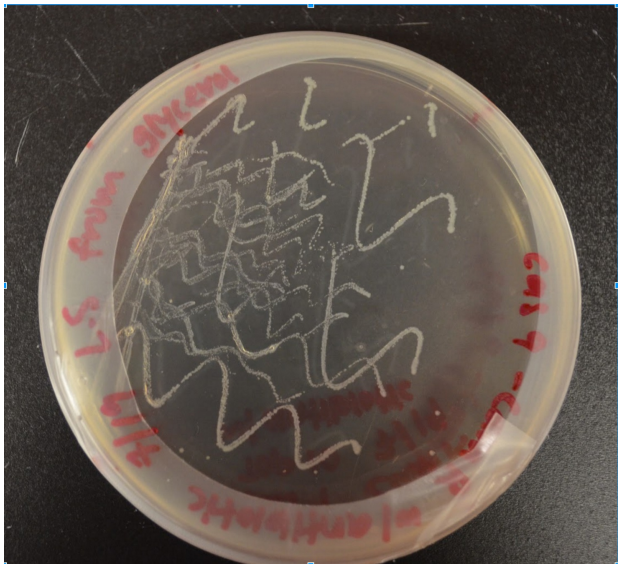
- gRNA - mRFP with the revense primer only (VR)

Results for ClyA-GFP:
ClyA identified in sequence but GFP not there. Potentially downstream.
Next steps: sequence again with different primer.

Transformations of Cas9-DsbA construct from 2016 team

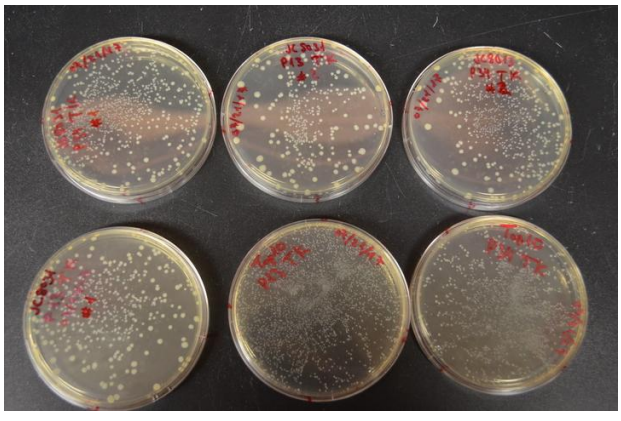
- 2 vials of Top10 comp. cells 2 vials of JC8031 comp cells
    - Thaw in ice for 15 mins
- 50 uL of each vial of comp cells was put in separate 1.7 mL microcentrifuge tubes
- 1 uL of plasmid 13 and plasmid 34 added to each cell strain (see bacterial database for more info)
- Vials were left in ice for 30 minutes
- Heat shock for 53 seconds followed by 5 minute incbation in ice
- Add 200 uL of SOC
- Aspirate and transafer to falcon tubes
- Incubate at 37 deg for 1 h and 15 m

cas9 - CamR w/ antibiotic



SUNDAY, 7/23

Colonies



Transformation results:
Note: the plates were left in the incubator for an additional day and then placed in the +4.

Started overnight cultures of JC8031 (no antibiotic) and JC8031 (ClyA-GFP) for induction with arabinose the following day (2 of each)