LAB Notebook - Week 7 (24/7/17 - 30/7/17)

Project: NU iGEM 2017 Shared Project
Authors: Lulu
Dates: 2017-07-24 to 2017-07-29

MONDAY, 7/24

Periplasmic GFP experiment:

Purpose: Induction of JC8031 ClyA-GFP with arabinose for observation of fluorescent protein in periplasm
Protocol adapted from: http://2013.igem.org/Team:DTU-Denmark/Methods/Visualizing_GFP_in_the_periplasm

- OD_{600} of overnight culture: 0.84 (blank using LB)
- 4.5 mL of the overnight culture was added to 40.5 mL of 2xyT and allowed to grow with shaking at 37 deg C
- When OD_{600} = 0.5 inducer was added (0.2% arabinose)
- The culture was incubated overnight in a conical flask at 37 deg in the shaker

Nanosight (NTA) information for Devin:
Nanosight measurements on Friday
Expected size distribution of vesicles: 50 nm - 250 nm
Expected yield (OMVs/cell): Unknown
Dilution: PBS

General:
- Meeting with Kibria at Feinberg - Meeting notes on drive

Sequencing results:
- saCas9-His sequenced well
- gRNA-mRFP sequenced well

TUESDAY, 7/25

Periplasmic GFP experiment:
Protocol from Denmark Team for visualization of GFP in the periplasm:
- The culture was transfered to a 50mL falcon tube and span down for 15 minutes at 4000g
- The cells were washed twice in LB and then 25mL clean LB was added. The pellet was then resuspended.
- The falcon tube was incubated (with shaking) at 37deg C for ~3h
- 0.5 mL of culture was added in a microcentrifuge tube (x5) with low melting point agarose and incubated at 50 deg C.
- 20uL of culture were pipetted in 3 slides. They were coversliped.
- The slides were stored in +4 degrees.

General:
- Created primer for the removal of DsbA (for replacement with TorA)
- Started 2x overnight cultures of JC8031 (no antibiotic) for OMV purification the following day

gRNA Experiment - Co-Transformation

1 microliter of each:

mRFP-gRNA with JC8031
mRFP-gRNA with JC8031 and His6-cas9
mRFP-gRNA with JC8031 and DsbA-cas9
mRFP-gRNA with Top10
mRFP-gRNA with Top10 and His6-cas9
mRFP-gRNA with Top10 and DsbA-cas9

Followed iGEM protocol

WEDNESDAY, 7/26

Slides (Danish experiment) were observed under EVOS microscope. Results were inconclusive.

GFP to periplasm microscopy experiment (Repeat with better controls):
Purpose: Determine if the DeLisa plasmid expresses GFP and compare top 10 vs JC8031 expression and localization

Controls

	A	B	C
1	**Positive control**	JC8031 with ClyA-GFP (induced but not chased to periplasm)	(+) Φ
2	**Negative control**	Top 10 with no transformed plasmid	(-) Φ1
3		JC8031 with no transformed plasmid	(-) Φ2

Cells of interest:

- Top10 with ClyA-GFP construct induced and chased to the periplasm (A)
- JC8031 with ClyA-GFP construct induced and chased to the periplasm (B)

ClyA-GFP was minipreped from +4 deg culture prepared the previous day

Nanodrop results:
Number assigned: 30
Concentration: 154.6 ng/uL
260/280: 1.87
260/230: 2.35

The iGEM transformation protocol was followed and transformed cells were allowed to grow overnight at 37 deg C
- JC8031 (2017)
- iGEM 2017 comp cells

OMV purification using materials from SBI:
Purpose: OMV purification from JC8031 for Nanosight

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)

Supernatant Nanodrop:
Protein concentration: 2.740 mg/mL
260/280: 0.99
OMVs labeled O11 - O15 (011 first elusion etc) and stored in +4 for analysis (expected to be analyzed with Devin on Friday)
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gRNA-mRFP Experiment

- Created overnight cultures of the template mRFP-gRNA

THURSDAY, 7/27

Sequencing:

- DsbA-Cas9 (VR and VF primers): sent (ACTG)
- ClyA-GFP (P8 and P9 primers): sent (ACTG)

GFP to periplasm microscopy experiment:
- ClyA-GFP plates (Top10 and JC8031) were sprayed with arabinose and incubated at 37 deg
- Started overnight cultures at the end of the day. No GFP expression was observed when the plates were placed under UV light.

gRNA experiment
Miniprepped the mRFP template as a negative control for repeating the transformation experiment - Jack

FRIDAY, 7/28

Experiments:
mRFP-gRNA and Cas9 cytoplasmic functionality experiment

Controlled Variables:
All transformations are to be performed in **JC8031**
The volume of comp cells is to be **20 uL** per trial and will be rescued in **20 uL** of rescue media
The amount of DNA per trial will be **1 uL total of 1 pg/uL - 10 ng/uL plasmid DNA**
.5uL for dual transformation of each plasmid
The protocol we will use is "**Protocol for Transformation - Chelsea (detailed)**"

Experimental Setup

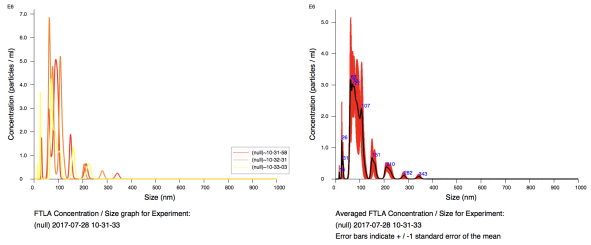
	A	B	C	D
1	Sample	Trial	Description	Plate
2	A	+ Control 1	mRFP-gRNA plasmid to examine if mRFP from this plasmid can be expressed in the comp cells (RED)	tet
3	B	+ Control 2	mRFP-template gRNA plasmid to examine if mRFP from this plasmid can be expressed in the comp cells (RED)	tet
4	C	- Control 1	His6-Cas9 plasmid to get a baseline flourescene reading for quantitative analysis (WHITE)	cam
5	D	- Control 2	DsbA-Cas9 plasmid to get a baseline flourescene reading for quantitative analysis (WHITE)	cam
6	E	Exp. Trial 1	mRFP-gRNA and His6-Cas9 to observe Cas9 functionality (WHITE)	tet+cam
7	F	Exp. Trial 2	mRFP-template gRNA and His6-Cas9 to observe Cas9 Specificity (RED)	tet+cam
8	G	Exp. Trial 3	mRFP-gRNA and DsbA-Cas9 to observe Cas9 functionality (WHITE)	tet+cam
9	H	Exp. Trial 4	mRFP-template gRNA and DsbA-Cas9 to observe Cas9 Specificity (RED)	tet+cam

OMV Visualization

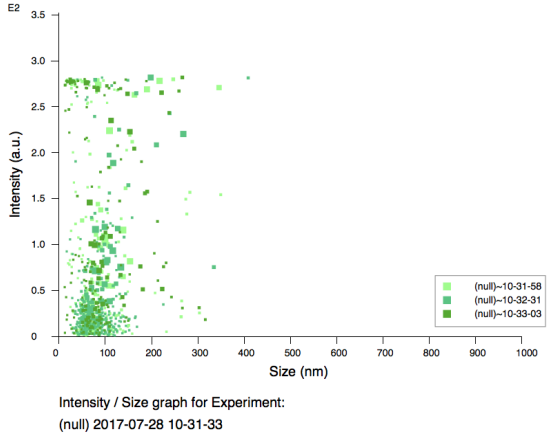
OMVs were visualized using the Nanosight for Flourescent NTA

Results:

NanoSight distribution: 2017-07-31



Intensity vs Size of nanoparticles



NanoSight Results

	A	B	C
1	Mean	99.7 nm	
2	Mode	62.0 nm	
3	SD	50.6 nm	
4	D10	59.9 nm	
5	D50	85.6 nm	
6	D90	159.5 nm	
7	Concentration	1.84e+008 +/- 2.35e+007 particles/ml	9.3 +/- 1.2 particles/frame 13.1 +/- 0.2 centres/frame

Notes/Observations:
Tried 1:100 and 1:50 dilutions, with 1:50 working best.
Tube used: O11 (First elution)
Linear aggregates (~6) of OMVs were observed during video. Might be a result of the purification method used.

GFP to periplasm microscopy experiment:
Team Denmark's protocol was again completed and the team attempted to observe periplasmic localized GFP in our JC8031 and our Top10 cells. The cells did not fluoresce under the EVOS microscope suggesting that the DeLisa plasmid does not contain GFP.

PCR to relocate His6
This PCR will relocate the His6 from the DsbA::His6::Cas9 to DsbA::Cas9::His6
See Protocol

- A gel was run but 30 minutes were inadequate for band detection. The PCR samples were stored at +4 overnight to repeat the experiment the following day.

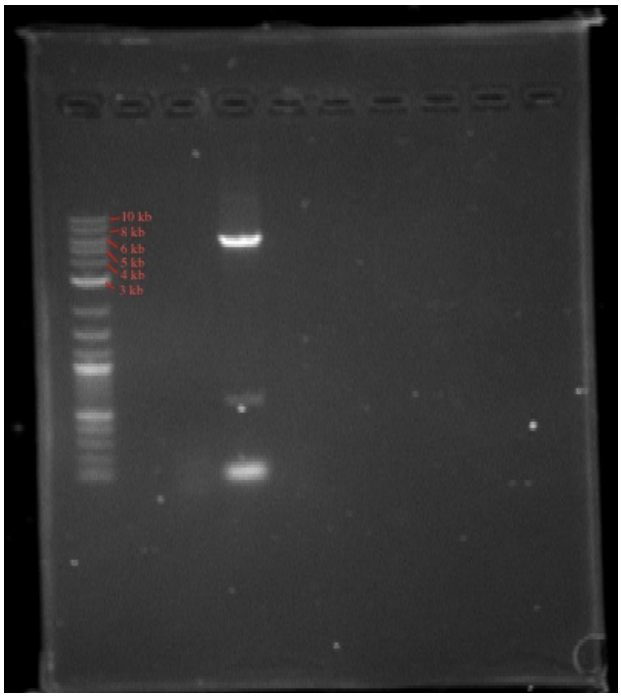
Sequencing:

- DsbA-Cas9 (VR and VF primers): sequenced right
- ClyA-GFP (P8 and P9 primers): did not sequence well

SATURDAY, 7/29

PCR to relocate His6
Results: A bright band was observed at 5kb as expected.

PCR 20170729.png



Gel stored at +4 deg C overnight in TAE buffer for extraction the following day

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

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- 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

PCR for Removal of His6 Tag from DsbA::His6::saCas9

Introduction

This protocol outlines PCR for the removal of His6 Tag from DsbA::His6::saCas9 using Phusion HS Flex 2x Master Mix (MM). This will amplify the Region of the plasmid that is NOT the His6 and create a linear DNA fragment which can then be used in iPCR to insert a new His6

Materials

Materials (Per RXN)

- 2.5 uL 10 uM P6
- 2.5 uL 10 uM P7
- 1.5 uL DMSO
- 18.5 uL of 100 pg/uL pC34 (add 1 ng)
- 25 uL of Phusion HS Flex 2x MM
- PCR Tube

Procedure

Procedure

1. Add NF Water first
2. Add P6, P7, 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 35x)
    61 C for 30s
    72 C for 2.5 minutes
    72 C for 10 min
    4 C for inf. Time

6. Gel Electrophoresis

    - Heat 1% agarose in TAE and pour into mold
    - Add 3 uL Sybr Green and mix with pipette tip
    - Let harden and orient in the chamber
    - Pour 1 x TAE over the gel to fully submerge
    - Mix 50 uL sample with 10 uL loading dye
    - Add 5 uL sample to each lane
    - Add ladder to 1 lane
    - Apply 100 V for 30 min

- Image gel to see if obtained **Correct Band 5 kb**
- PCR Purify

iGEM Registry: Transformation Protocol

Estimated bench time: 1 hour
Estimated total time: 3 hours (plus 14-18 hour incubation)

Transformations are essential to using the DNA Distribution Kits. However, they can also be one of the more fickle laboratory techniques.

At iGEM HQ, we run test transformations of the DNA Distribution Kit with the following protocol. We have found that it is the best protocol to use with the DNA Distribution Kit and ensures high efficiency transformations.

- At iGEM HQ, we make our own stocks of NEB 10b competent cells. Competent cells purchased from vendors will have better efficiency.
- Make sure to test the competency of your cells with the provided Competent Cell Test Kit.
- **Read through the entire protocol before starting!**

Materials

Resuspended DNA	Resuspend DNA Distribution Kit well(s) with 10µl dH20. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye.
10pg/ul Control DNA	**1µl for control transformation.** pSB1C3 w/ BBa_J04450, RFP on high-copy chloramphenicol resistant plasmid. Located in the Competent Cell Test Kit.
Competent Cells	**50µl per transformation.** iGEM HQ stores competent cells in aliquots of 260µl (5rxns total) at -80°C.
2ml Microtubes	**One tube per transformation.** Label tubes with part name or well location before starting.
Floating Foam Tube Rack	Place 2ml tubes in floating tube rack for better support when working on ice and for the heat shock in the water bath.
Ice & ice bucket	Fill bucket with ice, and pre-chill 2ml tubes (5min). Thaw competent cell stock on ice (10-15min).
Lab Timer	
42°C water bath	Set water bath to 42°C before starting.
SOC Media	**200µl per transformation.** SOC Media is better than LB Media for higher transformation efficiency. SOC Media should not contain antibiotics, and can be easily contaminated.
37°C incubator	Preferably with a rotor/shaker for 2ml tubes. Incubate petri plates overnight (non-agitated).
Petri plates w/ LB agar and antibiotic	**2 plates per transformation:** for 20µl and 200µl platings. Make sure to use appropriate antibiotic. Label with part name or well location before starting.
Sterile spreader or glass beads	Used to spread transformation across petri plates. Be sure to use sterile technique in between platings.
Pipettes and Tips	10µl, 20µl, 200µl tips and pipettes recommended

Setup:

When transforming competent cells, both timing and temperature are very important. Use a lab timer, follow the incubation temperatures closely, and keep materials on ice when required.

Resuspend DNA in selected wells in the Distribution Kit. Label 2ml tubes with part name or well location. Fill lab ice bucket with ice, and pre-chill 2ml tubes (one tube for each transformation, including your control) in a floating foam tube rack.

1. Thaw competent cells on ice
   This may take 10-15min for a 260µl stock. Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.
2. Pipette 50µl of competent cells into 2ml tube
   50µl in a 2ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. **Don't forget a 2ml tube for your control.**
3. Pipette 1µl of resuspended DNA into 2ml tube
   Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
4. Pipette 1µl of control DNA into 2ml tube
   Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
5. Close 2ml tubes, incubate on ice for 30min
   Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
6. Heat shock tubes at 42°C for 1 min
   2ml tubes should be in a floating foam tube rack. Place in water bath to ensure the bottoms of the tubes are submerged. Timing is critical.
7. Incubate on ice for 5min
   Return transformation tubes to ice bucket.
8. Pipette 200µl SOC media to each transformation
   SOC should be stored at 4°C, but can be warmed to room temperature before use. Check for contamination.
9. Incubate at 37°C for 2 hours, shaker or rotor recommended
10. Pipette each transformation on two petri plates for a 20µl and 200µl plating
    Pipette 20µl and 200µl of the transformation onto appropriately labeled plates. Spread with sterilized spreader or glass beads immediately. This helps ensure that you will be able to pick out a single colony.
11. Incubate transformations overnight (14-18hr) at 37°C
    Incubate the plates upside down (agar side facing up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; un-transformed cells will begin to grow.
12. Pick single colonies
    Pick single colonies from transformations: do a colony PCR to verify part size, make glycerol stocks, grow up cell cultures and miniprep.
13. Count colonies for control transformation
    Count colonies on the 20µl control plate and calculate your competent cell efficiency. Competent cells should have an efficiency of $1.5x10^8$ to $6x10^8$ cfu/µg DNA.