CRISPR/CAS9 Lab Notebook

Dan X + Danny
Danny + Kirsten + Jack
Kirsten + Tina
Kirsten + Jack
Kosuke
Brown

05.27.15

- Performed PCR of gDNA for CRISPR/Cas9 demo (Brown)

05.28.15

- Performed PCR cleanup of gDNA for CRISPR/Cas9 demo (Brown)
- Performed RNA transcription of gDNA into gRNA for CRISPR/Cas9 demo (Brown)

05.29.15

- Purified gRNA (RFP, Term, Chlor) with DNA digestion followed by RNA clean-up (Brown)
- Assessed purity of gRNA samples using Nanodrop (Brown)
- Attempted to prepare gels for RNA electrophoresis, but realized more agarose was needed (Brown)

06.01.15

- Added Cas9 to 3 types of gRNA (Brown)
- Added Cas9/gRNA complexes to corresponding plasmids (RFP or Term) (Brown)
- Ran DNA samples on gel after Cas9/gRNA addition (Term supported our hypothesis; RFP had strange results) (Brown)

06.11.15

- Isolated GFP and Term gRNA from transcription on 6/10 (Dan X + Danny)
- Added Cas9 to gRNAs (Dan X + Danny)
- Digested RFP and GFP plasmids with Cas9 (Dan X + Danny)
- Ran digested plasmids on gel; GFP gRNA/Cas9 selectively cut GFP plasmid; n.d. for RFP plasmid (Dan X + Danny)

06.12.15

- Isolated RFP plasmid from E. coli (Dan X + Danny)
- Added Cas9 to RFP, GFP, and Term gRNAs (Dan X + Danny)
- Digested RFP plasmid with RFP, GFP, and Term gRNAs (Dan X + Danny)
- Ran RFP plasmid digests on gel; RFP and Term gRNAs cut the RFP plasmid, while GFP gRNA did not (Dan X + Danny)

06.15.15

- Drafted protocol for CRISPR/Cas9 transformation (Dan X + Danny + Kirsten)
- Prepared dilutions of gRNA (GFP+RFP) and plasmid stock (GFP+RFP+FDC) (Dan X + Danny + Kirsten)

06.16.15

- Prepared four Cas9 plasmid digests (Dan X + Danny)
  - RFP/GFP/FDC plasmids with no gRNA (negative control)
  - RFP/GFP/FDC plasmids with RFP gRNA
  - RFP/GFP/FDC plasmids with GFP gRNA
  - RFP/GFP/FDC plasmids with RFP and GFP gRNA
- Transformed bacteria with Cas9 plasmid digests and plated on 1.5% LB agar plates (Dan X + Danny)
  - Four plates at 1X concentration
    - (-) gRNA & RFP gRNA
    - RFP/GFP gRNA & GFP gRNA
  - Four plates at 100X concentration

06.17.15

- Visualized plates; RFP was overgrown, even on plate where RFP gRNA was added (Danny + Kirsten + Jack)
- Performed 3 *E. coli* transformations (Danny + Kirsten + Jack):
  - New GFP
    - 2µL of 300 pg/µL GFP plasmid added to competent cells
  - Old GFP/RFP/FDC (100:1:1 concentration)
    - 3µL of 55000:550:550 pg/µL GFP/RFP/FDC plasmid mix
  - New GFP/RFP/FDC (100:1:1 concentration)
    - 5µL of 300:3:3 pg/µL GFP/RFP/FDC plasmid mix

06.18.15

- Visualized plates for the three *E. coli* transformations (Danny + Kirsten + Jack)
  - New GFP
  - Old GFP/RFP/FDC (100:1:1 concentration)
  - New GFP/RFP/FDC (100:1:1 concentration)
- Incubated GFP-expressing colony in LB Chlor broth (Danny + Kirsten + Jack)

06.19.15

- Performed mini-prep of new GFP (tGFP) plasmid (Danny + Kirsten + Jack)
  - tGFP plasmid concentration: 19.3 ng/μL
- Performed Cas9 digestion of tGFP plasmid with GFP gRNa (Danny + Kirsten + Jack)
- Ran gel of tGFP plasmid digest; forgot to add loading dye to tGFP plasmid control, so no useful results (Danny + Kirsten + Jack)
- Re-suspended tGFP-containing bacterial culture in new LB Chlor broth (incubate at room temperature over weekend) (Danny + Kirsten + Jack)

06.22.15

- Performed mini-prep of tGFP plasmid (Danny + Kirsten + Jack)
  - tGFP plasmid concentration: 19.1 ng/μL
- Ran gel of tGFP plasmid digest (Danny + Kirsten + Jack)
- Made tGFP/RFP/FDC dilutions for new transformation experiment (Danny + Kirsten + Jack)
- Performed 2 *E. coli* transformations (Danny + Kirsten + Jack):
  - tGFP/RFP/FDC at 100:10:1 ratio (concentrated)
    - 5 μL of 10:1:0.1 nM tGFP/RFP/FDC plasmid mix
    - Stored at 37ºC and 30ºC
  - tGFP/RFP/FDC at 100:10:1 ratio (dilute)
    - 5 μL of 1:0.1:0.01 nM tGFP/RFP/FDC plasmid mix
    - Stored at 37ºC and 30ºC

06.23.15

- Visualized transformation plates from previous day (Danny + Kirsten + Jack)
  - Under visible light (dilute, 37ºC)
  - Under UV light (dilute, 37ºC)
- Performed four double digestions of the RFP plasmid (Danny + Kirsten + Jack):
  - EcoRI-HF/PstI-HF (E/P)
  - EcoRI-HF/SpeI-HF (E/S)

    - XbaI/PstI-HF (X/P)
    - XbaI/SpeI-HF (X/S)
    - Each reaction tube was 40µL:
        - 29 µL DI MilliQ water
        - 5 µL RFP plasmid (47.2 ng/µL) (~250ng)
        - 4 µL CutSmart buffer
        - 1 µL of each of two restriction enzymes
- Ran double digest products on a 1% TAE gel with a 1kb ladder (Danny + Kirsten + Jack)
- Performed re-ligation of the RFP plasmid double digests (Danny + Kirsten + Jack)
    - 3 µL plasmid digest
    - 3 µL Instant Sticky End buffer
- Performed transformation of 25 µL competent cells with each re-ligated RFP digest sample (2 µL digest per tube) (Danny + Kirsten + Jack)
    - Plated 100 µL of each sample
    - Let dry for 5 minutes in fume hood
    - Parafilmed and wrapped in aluminum foil
    - Incubated plates overnight at 37ºC
- Resuspended 4 samples in 5 mL LB Chlor broth overnight (Danny + Kirsten + Jack)
    - GFP colony (from today's plate)
    - RFP colony (from today's plate)
    - FDC colony (from today's plate)
    - Dilute plasmid mix (from yesterday's transformation) (15 µL)
    - Concentrated plasmid mix (from yesterday's transformation) (15 µL)
- Prepared FDC gDNA (Danny + Kirsten + Jack)
    - Performed PCR of reverse template primer and FDC forward primer
    - Performed PCR cleanup of sample

06.24.15

- Visualized transformation plates from previous day (Danny + Kirsten + Jack)
    - E/S
    - E/P
    - X/S
    - X/P
- Transcribed FDC gRNA from gDNA (Danny + Kirsten + Jack):
    - 20 µL reaction:
        - 3.5 µL DI MilliQ water
        - 10 µL NTP buffer
        - 4.5 µL FDC gDNA (~1 µg)
        - 2 µL T7 polymerase
    - Incubated at 37ºC for 2 hours
    - Purified gRNA

- Performed flow cytometry of 4 samples (Danny + Kirsten + Jack):
  - GFP (from yesterday's mixed plasmid plate) (statistics)
  - RFP (from yesterday's mixed plasmid plate) (statistics)
  - FDC (from yesterday's mixed plasmid plate) (statistics)
  - GFP/RFP/FDC more conc. plasmid mix (from transformation on 6/22) (statistics)
- Digested FDC plasmid with Cas9 and FDC gRNA (Danny + Kirsten + Jack)
  - Prepared 300nM solution of FDC gRNA
  - Performed *in vitro* digestion
- Ran FDC, GFP, and RFP plasmid digests on a 0.8 % gel (0.4 g agarose, 50 mL TAE) (Danny + Kirsten + Jack)

06.25.15

- Ran gel of GFP, RFP, and FDC plasmids to ensure purity (Danny + Kirsten + Jack)

06.26.15

- Ran gel of other GFP and other two FDC plasmids to see if other samples contained purer/more viable plasmid; FDC5 seemed to be the right size (~3.5kb), though GFP plasmid had a lower travel distance than expected (Danny + Kirsten + Jack)
- Volume of FDC5, RFP, and GFP plasmids was low, so performed transformation with RFP and FDC5 plasmids (for eventual mini-prep) (already had GFP stock plate) (Danny + Kirsten + Jack)

06.27.15 (Saturday with Kosuke)

- Inoculated GFP (3 tubes at 3 mL), RFP, FDC5, and X/S (2 white and 6 red) colonies in LB Chlor broth (Danny)

06.28.15 (Sunday with Kosuke)

- Placed overnight LB Chlor liquid cultures in the fridge (Danny)

06.29.15 (Danny)

- Mini-prepped GFP, RFP, FDC5, and X/S cultures
  - Note: From the overnight culture, inoculated another tube of GFP, RFP, and FDC5 to grow overnight and use as control for flow cytometer
- Sent out X/S colonies for sequencing

- Performed Cas9 digestion of GFP/RFP/FDC plasmid mix (0.5:0.1:0.05 and 0.5:0.1:0.1 nM ratios) with the 3 different gRNAs (GFP, RFP, and FDC), along with RFP, GFP, and FDC plasmids with their corresponding gRNAs

06.30.15 (Danny + Kirsten + Jack)

- Ran gel of digestion (GFP with GFP gRNA, RFP with RFP gRNA, FDC with FDC gRNA)
    - Plasmids appeared uncut
    - Determined length of plasmid backbone and insert by running EcoRI and E/S-cut GFP, RFP, and FDC plasmids on a 0.7% TAE gel (100 V, 70 min); RFP and FDC Cas9 digests appeared as expected, but GFP Cas9 digest did not seem to work

07.01.15 (Danny + Kirsten + Jack)

- Performed Cas9 digestion and EcoRI digestion of GFP plasmid (21.1 ng/μL stock)
- Ran GFP plasmid, Cas9 digest, and EcoRI digest on a 0.7% TAE gel
- Made X/S gDNA from forward and reverse primers (PCR followed by PCR cleanup)

07.02.15 (Kirsten + Jack)

- Finished making X/S religation and RFP reverse gRNA

07.03.15

- FEDERAL HOLIDAY

07.06.15 (Kirsten)

- Nanodropped X/S gRNA's made on Thursday
- Redid Cas9 digestions with all tubes containing GFP (used new GFP)
- Transformed *E. coli* with GFP/RFP/FDC plasmid mix (0.5:0.1:0.05 and 0.5:0.1:0.1 nM ratios) with the 3 different gRNAs (GFP, RFP, and FDC)
    - Plated and grew culture overnight to use for flow cytometer
- Picked colony from RFP plate and grew in LB chlor overnight to miniprep tomorrow (note that amount of plasmid is low as styrene group used some)

07.07.15 (Kirsten)

- Made LB broth (Dan X)

- Analyzed GFP/RFP/FDC + gRNA plates → very little growth
- Analyzed transformations (and GFP, RFP, and FDC controls) using flow cytometer
- Analyzed GFP + RFP plasmid mix on flow cytometer; grew up overnight to analyze +/+ cells on flow cytometer tomorrow

07.08.15 (Kirsten)

- Analyzed GFP + RFP plasmid mix on flow cytometer
- Finished analyzing and gating Phase II transformations on flow cytometer
- Picked colonies (3 each) from RFP and new GFP plate and grew in LB chlor (wrapped tubes in foil) overnight to miniprep tomorrow

07.09.15 (Kirsten + Tina)

- Miniprepped GFP and RFP
  - Nanodropped GFP and RFP plasmids
  - 6 tubes are now living in the -20 C fridge (small pink CRISPR box)
- Use X-S RFP digested plasmid in large pink box (labelled X-S digested RFP plasmid) for ligation assay:
  - mix:
    - X-S cut RFP plasmid (from freezer): 9 µL
    - 10X ligase buffer: 3 µL
    - DW: 17 µL + **10 µL**
    - T4 ligase: 1 µL
    - (total: **40** µL)
  - incubate ligated product for 30 minutes at room temperature (while this is incubating, start the Cas9 digest below)
  - incubate ligated product for 10 minutes at 65 degree Celsius to inactivate T4 ligase (keep on ice until you need after 25 degree incubation in Cas 9 digest)
  - Cas9 digest: complete with three tubes (X/S religation gRNA, reverse RFP gRNA, GFP gRNA)
    -

	GFP gRNA tube (Neg. Control)	X/S tube	X/S and RFP reverse tube
X/S religated gRNA	-	3 µL	1.5 µL
reverse RFP gRNA	-	-	1.5 µL
GFP gRNA	3 µL	-	-

10 X cas 9 buffer	3 µL	3 µL	3 µL
Deionized milliQ water	13 µL	13 µL	13 µL
Cas 9	1 µL	1 µL	1 µL

- incubate 10 minutes at 25 degree Celsius
- add **10** µL of **40** µL ligated product and incubate at 37 degrees for 1.5 hours

- Four tubes living in the -20 freezer (small pink CRISPR box)

07.10.15 (Kirsten + Tina)

- Transform the four tubes using NEB 5 Alpha Competent Cells
- Plate 100 µL on LB chlor plates
- Grow overnight at 37 degrees covered in foil (there are some LB chlor plates already made in the bottom right of the clear doored cooler that are wrapped in plastic and labeled “CRISPR team” on green tape)
- Add 50 µL of the remaining transformant mix to 3 ml of LB chlor, cover in foil, and grow in the 37 degree shaker overnight (keep any remaining mix of comp cells labeled in a rack in the clear doored cooler)
- Plates - in 37ºC incubator, covered in foil, should be 4
- Cultures - in 37ºC shaker, also foil, 4 tubes
- Put plates and overnight cultures in the clear doored cooler (covered in foil)

07.11.15

- Moved the X/S transformation plates from the incubator to the fridge (Kosuke)

07.13.15

- All members absent

07.14.15

- All members absent

07.15.15 (Kirsten)

- ASL Presentation and Lab Meeting (Kirsten)
- Analyzed X/S transformation plates (Kirsten)
  - no gRNA: white (253 colonies), red (26); transformation efficiency: 9.31%
  - GFP gRNA (control): white (251), red (28), transformation efficiency: 10.04%

- X/S re-ligated gRNA: white (336), red (24), transformation efficiency: 6.67%
- X/S re-ligated gRNA and RFP reverse gRNA: white (178), red (5), transformation efficiency: 2.73%

07.16.15 (Danny + Kirsten)

- Redid Phase II with only GFP and RFP; Cas 9 digest of:
  - GFP/RFP plasmid + GFP gRNA
  - GFP/RFP plasmid + RFP gRNA (note used RFP tube of unknown concentration and another with known concentration)
  - GFP/RFP plasmid + GFP gRNA + RFP gRNA (for curiosity sake)
  - GFP/RFP plasmid + no gRNA (control)
- Grew on LB chlor plates and in broth overnight (covered both in foil)
- Feeling peachy as Danny is back
- Discussed best paper format for publication, currently thinking Nature Communications--read relevant papers and finish introduction by the end of the weekend (and draft by Wednesday)

07.17.15 (Danny + Kirsten)

- Analyze sequencing from red colonies (RFP X/S digestion) to determine percent of RFP forward and reverse plasmids
  - R1 is reverse
  - R2 is reverse
  - R3 is reverse
  - R4 is forward
  - R5 is forward
  - R6 is forward
  - Conclusion: 50-50 mix of forward and reverse RFP plasmids
- Performed Cas9 digestion of R5, R1, and W1 plasmids with X/S re-ligation gRNA, reverse-RFP gRNA, and GFP gRNA
- Performed EcoRI restriction enzyme digestions of W1 and R5
- Ran Cas9 and restriction enzyme digests on a gel to determine efficacy of gRNAs
- Analyzed Phase II transformations by counting colonies and using flow cytometry

07.20.15 (Danny + Kirsten + Jack)

- Worked on paper draft
- Re-digested RFP plasmid with XbaI and SpeI and re-ligate plasmid using T4 ligase
- Re-ran Phase I Cas9 digestion of GFP and RFP with respective gRNAs

- Re-ran Phase I gel (GFP plasmid, GFP + G gRNA, GFP + R gRNA, RFP plasmid, RFP + G gRNA, RFP + R gRNA, GFP EcoRI digest, RFP EcoRI digest)
- Re-ran Phase III Cas9 digestion of RFP plasmid with X/S re-ligation and RFP-reverse gRNAs
- Redid Phase II negative control transformation
- Redid Phase III transformations

07.21.15 (Danny + Kirsten + Jack)

- Worked on paper draft
- Analyzed Phase III transformations (plates and liquid cultures on flow cytometer)
    - X/S rel gRNA + RFP rev: majority white colonies (1 red)
    - X/S rel gRNA: mostly white colonies (little growth)
    - GFP gRNA: no growth
    - As these results were not expected, ran Cas9 digests, X/S re-ligated plasmid mix, X/S re-ligated plasmid (W2), X/S re-ligated plasmid EcoRI digest, RFP plasmid, and RFP EcoRI digests on a gel
- Noted that there was no growth on the Phase II control (no gRNA) → Redid the Cas9 digestion, transformed, and plated
- Re-transformed GFP and RFP (30nM tubes prepared on 7/20/15) and plated
- Analyzed GFP monoculture on flow cytometer (and RFP gRNA or other culture with GFP and RFP expression if we still have in -20℃ cooler)

07.22.15 (Danny + Kirsten + Jack)

- Took pictures of Phase II plates
- Visualized GFP and RFP control plates; they both had ~100% transformation efficiency
    - Flow cytometry:
    - Microscopy: Green colonies fluoresced very brightly
- Analyze Phase II negative control (no gRNA)
    - Green, red, and white colonies on plate
    - Flow cytometry:
    - Microscopy: White colonies showed very dim GFP fluorescence

07.23.15 (Danny + Kirsten + Jack)

- Ordered/Located chromogenic BioBricks:
    - Part K1073022 (red-pink): Ordered through iGEM HQ (Braunschweig 2013)
        - 2813 bp
    - Part BBa_K1033931 (yellow): Ordered through iGEM HQ (Uppsala 2013)

        - 2842 bp
        - No promoter/RBS
    - Part BBa_K1033916 (yellow-green): Plate 4, Well 6M (Uppsala 2013)
        - 2818 bp
        - No promoter/RBS
    - Part BBa_K592011 (dark green): Plate 4, Well 2I (Uppsala 2011)
        - 2827 bp
    - Part BBa_K1033902 (blue): Ordered through iGEM HQ (Uppsala 2013)
        - 2794 bp
        - No promoter/RBS
        - 24-48 hours to appear
    - Part BBa_K1033929 (deep blue): Ordered through iGEM HQ (Uppsala 2013)
        - 2842 bp
        - No promoter/RBS
    - Part BBa_K592009 (indigo): Plate 1, Well 19E (Uppsala 2011)
        - 2794 bp
        - No promoter/RBS
    - Part BBa_K1033906 (purple): Ordered through iGEM HQ (Uppsala 2013)
        - 2815 bp
        - No promoter/RBS

07.23.15 (Danny + Jack)

- First we performed restriction enzyme digestion of RFP2 (100.6 ng/μL) with XbaI and SpeI according to the following protocol:
    - 32 μL DI MilliQ water
    - 10 μL RFP plasmid (100.6 ng/μL) (~1000ng)
    - 5 μL CutSmart buffer
    - 1.5 μL of each of two restriction enzymes XbaI/SpeI-HF (X/S)
    - Final volume: 50 μL
    - Incubated at 37ºC for 30 minutes
- Then we performed enzymatic cleanup using the MinElute Reaction Cleanup Kit from Qiagen per the manufacturer's instructions. 20 μL of elution buffer were used.
- Then we used the Nanodrop to determine the new DNA concentration: 31.0 ng/μL
- Next we performed ligation using using T4 DNA Ligase from New England BioLabs per the manufacturer's instructions:
    - 7 μL DI MilliQ water
    - 10 μL digested RFP plasmid
    - 2 μL T4 ligation buffer
    - 1 μL T4 ligase
    - Final volume: 20 μL
    - Incubated at 22-23ºC (room temperature) for 10 minutes

    - Incubated at 65ºC for 10 minutes to heat inactivate the ligase

07.24.15 (Danny + Jack)

- Designed and ordered primers for all chromogenic gDNAs
    - Pink
    - Yellow
    - Lime (yellow-green)
    - Dark green
    - Blue
    - Deep blue
    - Indigo
    - Purple
- Resuspended DNA from distribution kit for indigo (Part BBa_K592009), dark green (BBa_K592011), and yellow/green (BBa_K1033916) (a.k.a. lime) chromogenic proteins, as well as backbone with promoter + RBS (BBa_K608002). Transformed and plated *E. coli* with these 4 plasmids in order to miniprep them.

07.25.15 (Kosuke)

- Moved plates with chromogenic protein/backbone plasmids from 37ºC incubator to fridge. Colonies are all white, because chromogenic protein plasmids have no promoter/RBS

07.27.15 (Danny + Jack)

- Performed Cas9 digestion of RFP re-ligation mix
    - (-) gRNA
    - X/S re-ligation gRNA
    - X/S re-ligation gRNA + reverse-RFP gRNA
    - GFP gRNA (control)

	No gRNA	**X/S gRNA**	**X/S gRNA + rRFP gRNA**	**GFP gRNA (control)**
X/S-religated gRNA	---	3 µL	1.5 µL	---
Reverse-RFP gRNA	---	---	1.5 µL	---
GFP gRNA	---	---	---	3 µL
10X Cas9 Buffer	3 µL	3 µL	3 µL	3 µL
Deionized MilliQ Water	23 µL	20 µL	20 µL	20 µL
Cas9	1 µL	1 µL	1 µL	1 µL
Total	27µL	27µL	27µL	27µL

After mixing the above solutions and incubating at 37ºC for 10 min to let the gRNA and Cas9 associate, 3µL of the RFP re-ligation mix was added for a final volume of 30µL. Then we incubated at 37ºC for 1 hour for the digestion.

We then calculated that the final solution after digestion had ~5.5 ng/µL dsDNA. We transformed using 25 µL NEB 5-alpha competent cells and 1 µL (~5.5 ng) DNA per tube, and resuspended in 425 µL SOC after heat shocking.

- Grew up broths of *E. coli* containing indigo, dark green, lime, and promoter+RBS backbone plasmids for mini-prep
- iGEM HQ mailed out chromogenic BioBricks we ordered
- Prepared/Poured more LB Chlor plates (about 30)

07.28.15 (Danny + Jack)

- Visualized Phase III transformation plates: the labeled “RFP2” plasmid was actually a GFP plasmid. The GFP gRNA, which was supposed to be a negative control, made the colonies all white. The X/S gRNA did not produce all green colonies, which is unexpected. So today we re-performed the experiment from July 23.

- First we performed restriction enzyme digestion of the RFP plasmid “R4” (see July 17) (40 ng/µL) with XbaI and SpeI according to the following protocol:
    - 17 µL DI MilliQ water
    - 25 µL RFP4 (see July 17) plasmid (40 ng/µL) (~1000ng)
    - 5 µL CutSmart buffer
    - 1.5 µL of each of two restriction enzymes XbaI/SpeI-HF (X/S)
    - Final volume: 50 µL
    - Incubated at 37ºC for 30 minutes
- Then we performed enzymatic cleanup using the MinElute Reaction Cleanup Kit from Qiagen per the manufacturer’s instructions. 20 µL of elution buffer were used.
- Then we used the Nanodrop to determine the new DNA concentration: 24.5 ng/µL
- Next we performed ligation using using T4 DNA Ligase from New England BioLabs per the manufacturer’s instructions:
    - 7 µL DI MilliQ water
    - 10 µL digested RFP plasmid
    - 2 µL T4 ligation buffer
    - 1 µL T4 ligase
    - Final volume: 20 µL
    - Incubated at 22-23ºC (room temperature) for 10 minutes
    - Incubated at 65ºC for 10 minutes to heat inactivate the ligase

- Performed Cas9 digestion of RFP re-ligation mix
    - (-) gRNA
    - X/S re-ligation gRNA
    - X/S re-ligation gRNA + reverse-RFP gRNA
    - GFP gRNA (control)

	No gRNA	X/S gRNA	X/S gRNA + rRFP gRNA	GFP gRNA (control)
X/S-re-ligated gRNA	---	3 µL	1.5 µL	---
Reverse-RFP gRNA	---	---	1.5 µL	---
GFP gRNA	---	---	---	3 µL
10X Cas9 Buffer	3 µL	3 µL	3 µL	3 µL
MilliQ Water	23 µL	20 µL	20 µL	20 µL
Cas9	1 µL	1 µL	1 µL	1 µL
Total	27µL	27µL	27µL	27µL

After mixing the above solutions and incubating at room temp for 10 min to let the gRNA and Cas9 associate, 3µL of the RFP re-ligation mix was added for a final volume of 30µL. Then we incubated at 37ºC for 1 hour for the digestion.

We then calculated that the final solution after digestion had ~5.5 ng/μL dsDNA. We transformed using 25 μL NEB 5-alpha competent cells and 1 μL (~5.5 ng) DNA per tube, and resuspended in 425 μL SOC after heat shocking. Last, we plated on LB chloramphenicol and prepared LB chloramphenicol liquid cultures for flow cytometry the following day.

- Mini-prepped chromogenic and promoter+RBS backbone broths from previous day; 30 μL of elution buffer were used to elute the plasmid DNA
- Performed restriction enzyme digestion of promoter/RBS backbone and chromogenic plasmids
    - S/P digestion: Both promoter+RBS backbone liquid cultures
    - X/P digestion: Indigo, Dark Green, and Lime liquid cultures

	DI MilliQ Water	**CutSmart Buffer**	**DNA**	**PstI**	**SpeI**	**XbaI**
Promoter+RBS Backbone-1	30.4 μL	4 μL	3.6 μL	1 μL	1 μL	---
Promoter + RBS Backbone-2	31.6 μL	4 μL	2.4 μL	1 μL	1 μL	---
Indigo	31.0 μL	4 μL	3.0 μL	1 μL	---	1 μL
Dark Green	31.6 μL	4 μL	2.4 μL	1 μL	---	1 μL
Lime	32.0 μL	4 μL	2.0 μL	1 μL	---	1 μL

    - Cleaned up digests with Qiagen cleanup kit
- Ligated chromogenic genes into promoter/RBS backbone using Instant-Sticky Ligation Mix
    - 1.5 μL backbone DNA (Backbone 1 or Backbone 2)
    - 1.5 μL chromogenic DNA (Indigo, Dark Green, or Lime)
    - 3 μL Instant-Sticky Ligation Mix
    - 6 tubes total
- Transformed/Plated NEB 5-alpha competent cells with 2 μL of ligation product

07.29.15 (Danny + Jack)

- Visualized transformation plates from previous day
    - Phase III
        - (-) control: 38.8 ± 5.8% efficiency

        - X/S gRNA: 35.4 ± 4.9% efficiency
        - X/S + reverse-RFP gRNA: 2.3 ± 0.3% efficiency
        - GFP gRNA: 12.5 ± 1.5% efficiency
        - Possible explanations:
            - Incompatible buffers
            - Inefficient X/S re-ligation gRNA → Ordered alternate gRNA primer
    - Phase II-chromo
        - After 16 hours:
            - All plates (indigo, dark green, and lime with one of the two backbones) expressed no color
            - Possible explanations:
                - Backbone colonies picked did not contain the backbone plasmid (there may have been point mutations)
                - Chromogenic colonies picked did not contain the chromogenic plasmid (there may have been point mutations)
                - Dark green and lime require 24-48 hours of incubation for color expression
        - After 24 hours:
            - All plates expressed no color
- Amplified chromogenic gDNAs (followed by PCR cleanup)
    - Forward primer: 2 µL
    - Reverse primer: 2 µL
    - DI MilliQ water: 21 µL
    - Q5 Master Mix: 25 µL
    - Total: 50 µL
    - Run thermal cycler on “Q5-gRNA” settings
- Performed PCR cleanup of chromogenic gDNAs
    - Pink: 387.5 ng/µL
    - Yellow: 225.0 ng/µL
    - Lime: 530.9 ng/µL
    - Dark Green: 170.4 ng/µL
    - Blue: 515.5 ng/µL
    - Deep Blue: 671.0 ng/µL
    - Indigo: 141.0 ng/µL
    - Purple: 535.2 ng/µL

07.30.15 (Danny + Jack)

- Visualized indigo, lime, and dark green plates with two different promoter+RBS backbones
    - After 40 hours:
        - 10-20 colonies expressed indigo chromogen

        - All white colonies on lime and dark green plates
    - After 48 hours:
        - 1 lime colony expressed protein on BB1 plate
        - Dark green still not expressing → Picked a different colony from plate on 7/24
- Transcribed chromogenic gRNAs using the T7 High Yield RNA Synthesis Kit from New England Biolabs per the manufacturer's instructions, using yesterday's PCR products as template. Performed RNA cleanup using the Life Technologies Ambion MEGAclear kit, purification of transcription reactions, per the manufacturer's instructions. NOTE: solution was kept on ice for 30-60 minutes before first centrifugation step, because I couldn't find enough tubes. Purified RNA was then quantified using the Nanodrop:
    - Blue: 492.4 ng/μL
    - Purple: 374.9 ng/μL
    - Pink: 645.7 ng/μL
    - Dark green: 1956.6 ng/μL
    - Indigo: 1616.1 ng/μL
    - Yellow: 1027.1 ng/μL
    - Lime: 237.2 ng/μL
    - Dark blue: 492.8 ng/μL
- Grew up liquid cultures of:
    - Indigo plasmid (pick colored colony)
    - Lime plasmid (pick colored colony)
    - Dark green plasmid
    - New chromogenic plasmids (5)
        - Pink
        - Yellow
        - Blue
        - Deep blue
        - Purple

07.31.15 (Danny + Jack)

- Visualized liquid cultures from previous day
    - After 16 hours:
        - Pink culture expressed pink; this plasmid already has a promoter and RBS
        - Remaining cultures appeared white; let indigo and lime cultures incubate for longer to see if color expresses
- Mini-prepped the following liquid cultures:
    - Pink
    - Lime
    - Indigo
    - Yellow (non-expressing)

    - Dark green (non-expressing)
    - Blue (non-expressing)
    - Deep blue (non-expressing)
    - Purple (non-expressing)
- Ligated 5 non-expressing chromogenic plasmids into promoter+RBS backbone
    - Restriction enzyme digestion followed by Qiagen cleanup
    - Instant-Sticky End ligation
    - Transformed NEB 5-alpha cells with 2 µL of DNA, and plated on LB chloramphenicol agar plates; stored at room temperature over the weekend

08.03.15 (Danny + Kirsten + Jack)

- Visualized chromogenic plates
    - After 64 hours at room temperature:
        - All colonies appeared white
- Amplified X/S-1 and X/S-2 re-ligation gDNAs; performed PCR cleanup of gDNAs
- Performed T7 transcription of X/S-1 and X/S-2 re-ligation gDNAs; purify the two gRNAs using MEGAclear (***ran out of binding buffer concentrate for new x/s tube, used binding buffer from mRNA kit as substitute for 250 of the 350 µL)
    - Old X/S: 290 ng/µL
    - New X/S: 231 ng/µL
- Met with Lynn to discuss paper at 2PM
- Picked remaining chromogenic colonies and grew up liquid cultures for mini-prep
    - Also re-plated purple chromogenic colonies as they were very small (colonies were very dense and normal [white] colonies were outcompeting the chromogenic colonies)

08.04.15 (Danny + Kirsten + Jack)

- Performed X/S digestion of R6 RFP plasmid, followed by Qiagen Enzymatic Reaction Cleanup
- Performed T4 ligation of R6 RFP plasmid
- Mini-prepped blue and yellow chromogenic protein plasmids. Both clearly showed color, especially when pelleted.
    - Yellow: 49.8 ng/µL
    - Blue: 9.0 ng/µL
- Inoculated another broth culture of the purple E. coli using the triple re-plate.

08.05.15 (Danny + Kirsten + Jack)

- Performed Phase III Cas9 digestion with old and new X/S re-ligation gRNAs
    - Used the R6 RFP ligation product

- Realized did not add enough EtOH to wash buffers in PCR cleanup of old and new X/S gRNAs on 8/3 → Ran Phase III Cas9 digests on a 0.8% TAE gel test viability of gRNAs
    - Gel was very messy; decided to just re-make X/S gRNAs
- Re-PCR amplified old and new X/S gDNAs; performed PCR cleanup with EtOH-containing wash buffer
    - Old X/S: 516.7 ng/μL
    - New X/S: 215.1 ng/μL

08.06.15 (Danny + Kirsten + Jack)

- Mini-prepped dark green, blue, deep blue, and purple chromogenic liquid cultures
    - Dark Green: 95.1 ng/μL
    - Blue: 133.1 ng/μL
    - Deep Blue: 26.0 ng/μL
    - Purple: 64.8 ng/μL
- Performed T7 transcription of old and new X/S gDNAs
    - ~1 μg gDNA needed
        - Old X/S: 1.9 μL used
        - New X/S: 4.6 μL used
    - Performed RNA purification using the Macherey-Nagel NucleoSpin miRNA Kit
        - Old X/S: 529.3 ng/μL
        - New X/S: 1311.2 ng/μL
    - Made 300 nM dilutions of X/S gRNAs
        - Old X/S: 16 μL + 984 μL DI MilliQ (1000 μL total)
        - New X/S: 7 μL + 993 μL DI MilliQ (1000 μL total)
- Discussed similarity of chromogens and decided which colors to use in Cas9 digestions/transformations
    - Yellow and lime were essentially identical under blue UV light, so we chose yellow over lime
    - Blue and deep blue appeared almost identical, and since deep blue had a lower plasmid stock concentration, we chose blue over deep blue
    - *Dark green looked very similar to blue as well, so we are waiting to see whether our liquid culture appears more green when it begins to express the chromogen
    - We will also add RFP to the mix of colors
    - New list of colors:
        - Pink
        - RFP (red)
        - Yellow
        - Blue
        - Indigo
        - Purple
- Made dilutions of chromogenic gRNAs and plasmids

	ng/μL	μmol	#μL per mL for 300 nmol
Pink	645.7	22.306	13.4495
Yellow	1027.1	35.481	8.4552
Lime	237.2	8.194	36.6118
Dark green	1956.6	67.591	4.4385
Blue	492.4	17.01	17.6367
Deep blue	492.8	17.024	17.6224
Indigo	1616.1	55.828	5.3736
Purple	374.9	12.951	23.1644

	ng/μL	nmol	#μL per 30 μL for 30 nmol
Pink	21.8	12.54	24
Yellow	49.8	28.36	11
Lime	65.2	37.44	8
Dark green	95.1	54.44	6
Blue	133.1	77.09	4
Deep blue	26	14.8	20
Indigo	42.1	24.38	12
Purple	64.8	37.25	8

- Performed Cas9 digestion of R6 RFP plasmid with old and new X/S gRNAs, reverse-RFP gRNA, and GFP gRNA, along with a negative control (Phase III)

	No gRNA	X/S gRNA (1 old, 1 new)	X/S gRNA + rRFP gRNA (1 old, 1 new)	GFP gRNA (control)
X/S-re-ligated gRNA	---	3 μL	1.5 μL	---

Reverse-RFP gRNA	---	---	1.5 µL	---
GFP gRNA	---	---	---	3 µL
10X Cas9 Buffer	3 µL	3 µL	3 µL	3 µL
MilliQ Water	23 µL	20 µL	20 µL	20 µL
Cas9	1 µL	1 µL	1 µL	1 µL
Total	27µL	27µL	27µL	27µL

After mixing the above solutions and incubating at room temp for 10 min to let the gRNA and Cas9 associate, 3µL of the RFP re-ligation mix was added for a final volume of 30µL. Then we incubated at 37ºC for 1 hour for the digestion.

We transformed using 25 µL NEB 5-alpha competent cells and 1 µL DNA per tube, and resuspended in 425 µL SOC after heat shocking. Last, we plated on LB chloramphenicol and prepared LB chloramphenicol liquid cultures for flow cytometry the following day.

- Performed Cas9 digestion of the 5 chromogenic plasmids and RFP with corresponding gRNAs
    - Ran plasmids and Cas9 digests on a 0.8% TAE gel with a 1 kb ladder to test efficacy of gRNAs

08.07.15 (Danny + Kirsten + Jack)

- Visualized Phase III plates from previous day
    - Almost no red colonies
    - Ran Cas9 digests on a 0.8% TAE gel with RFP and X/S-religated plasmids, along with EcoRI digests of RFP and X/S-religated plasmids
- Performed EcoRI digest of chromogenic plasmids and RFP
    - Each reaction tube was 30µL:
        - 21.5 µL DI MilliQ water
        - 3 µL of 10 nM plasmid DNA
        - 5 µL CutSmart buffer
        - 0.5 µL of EcoRI restriction enzyme
- Ran a 0.8% TAE gel of the 5 chromogenic plasmids and RFP, plus their respective Cas9 and EcoRI digests:
- Ran a 0.8% TAE gel of the 6 digests from yesterday, plus the RFP religation without any Cas9, plus RFP EcoRI digested, plus RFP religation EcoRI digested

- Mini-prepped additional blue and deep blue plasmid
  - Blue: 80.3 ng/μL
  - Deep blue: 30.8 ng/μL
- Ran gRNAs on a 20% PAGE gel (sybrGold post-staining) with a micro-RNA ladder (up to 25 bp)
  - Darkest bands for each sample appeared to be ~100 bp, as expected

08.09.15 (Danny + Kirsten + Jack)

- Mini-prepped additional purple plasmid
- Performed E/S double digest of pink, yellow, blue, and indigo plasmids to discern lengths of inserts and backbones
  - Ran digests + plasmids on a gel, but gel quality was poor, likely because of buffer and gel TAE incompatibility
  - Remade another 0.8% TAE gel and will run digests again tomorrow morning

08.10.15 (Danny + Kirsten + Jack)

- Reran 0.8% TAE gel with E/S chromogenic double digests and plasmids, along with new purple plasmid to ensure purity
- Performed Cas9 digestion of 22 nM "W1" X/S-re-ligated backbone with old and new X/S re-ligation gRNAs
  - Ran digests on a gel with the 22 nM "W1" plasmid
- Prepared/Poured LB chloramphenicol plates
- Ran pink, blue, indigo, old X/S, and new X/S gDNAs on a 3% TAE gel to ensure purity (Kosuke)
  - Indigo and old X/S gDNAs showed some larger fragments, suggesting primer dimerization during PCR amplification
- Reinoculated blue and yellow chromogenic bacteria on plates and in liquid cultures

08.11.15 (Danny + Kirsten + Jack)

- Mini-prepped blue and yellow plasmids
  - "Blue plasmid 3": 169.3 ng/μL
  - "Yellow plasmid 2": 219.2 ng/μL
- Considered possible ways to increase efficiency of X/S re-ligation gRNAs:
  - **Increase Cas9 concentration**
  - **Increase reaction time**
  - Use Life Technologies' Cas9 digestion kit
    - Ordered the kit and plan to use it when it arrives
  - Use Cas9 nickase in conjunction with two gRNAs for increased specificity (i.e. reduced chance of gRNA cleaving intact RFP plasmids)

        - Not an option for X/S re-ligated plasmid (only NGG sequence option leads to primer that has hairpin Tm of ~51°C)
    - Use other Cas9 orthologs, which have alternate PAMs and may allow for other gRNA design options
        - Not an option for X/S re-ligated plasmid (no PAM sequences present in cleavage region of interest)
- Performed Cas9 digestion of RFP (with RFP gRNA) and W2 (re-ligated) plasmid (with new X/S re-ligation gRNA)
    - Tested various reaction times and Cas9 concentrations
        - Times tested: 1 hour and 2 hours
        - Concentrations tested: 1 µL and 2 µL Cas9 nuclease in 30 µL reaction
    - Ran the 8 Cas9 digests on a 0.8% TAE gel with the RFP and W2 plasmids, along with their E digests
        - Cas9 concentration seemed to make RFP cutting more efficient
        - Increased reaction time seemed to increase cleavage efficiency of both plasmids
        - Most significantly: New X/S re-ligation gRNA appeared to cut the W2 plasmid; however, we the X/S E digest showed up at a different length, so we should perform E and P digests of the W2 plasmid to ensure linear W2 plasmid is the same length as the Cas9-digested W2 plasmid
- Transformed NEB 5-alpha competent cells with RFP plasmid ("30 nM RFP") and the chromo-mix
    - The chromo-mix contained 5 µL each of:
        - 10 nM pink plasmid
        - 10 nM "yellow plasmid 2"
        - 10 nM "blue plasmid 3"
        - 10 nM indigo plasmid
        - 10 nM purple plasmid
        - 10 nM RFP plasmid (diluted "30 nM RFP")
    - Transformed with 1 µL of "30 nM RFP"
    - Transformed with 2 µL of the chromo-mix
    - Plated 50 µL of transformants on LB chloramphenicol plates; let plates grow at 37°C for ~16 hours

08.12.15 (Danny + Kirsten + Jack)

- Performed E/S double digest of blue and yellow plasmids to ensure purity
- Performed two separate single restriction enzyme digests of W2 plasmid with E and P to determine length of linearized plasmid
- Ran chromogenic double digests and W2 single digests on a 0.8% TAE gel, along with a 1 kb ladder, the intact plasmids, and the W2 Cas9 digest from the previous day (2 µL Cas9 nuclease, 1 hour)

- Picked one red colony on transformation plate from previous day, along with one white colony on transformation plate from 6/23, and inoculated each in 10 mL LB chloramphenicol broth for mini-prep tomorrow
- Visualized chromo-mix transformation plate (may need to wait until tomorrow for all colors to appear)
    - After 17 hours: Red colonies were visible under visible light, and red and yellow colonies were visible under UV light

08.13.15 (Danny + Kirsten + Jack)

- Mini-prepped RFP and X/S re-ligated plasmids
    - Separated each 10 mL stock into four eppendorf tubes with ~2.5 mL each (8 tubes total)
    - Nanodropped plasmids to determine DNA concentration

08.14.15 (Danny + Kirsten + Jack)

- Digested RFP plasmid ("RFP1") with XbaI and SpeI
    - 29 µL DI MilliQ water
    - 4 µL CutSmart buffer
    - 5 µL (~250 ng) "RFP1" plasmid
    - 1 µL XbaI
    - 1 µL SpeI
    - Incubated at 37ºC for 30 minutes
- Performed cleanup of X/S double digest using the Qiagen Enzymatic Reaction Cleanup Kit
- Performed T4 ligation of "RFP1" X/S double digest
    - 7 µL DI MilliQ water
    - 10 µL digested RFP plasmid
    - 2 µL T4 ligation buffer
    - 1 µL T4 ligase
    - Final volume: 20 µL
    - Incubated at 22-23ºC (room temperature) for 10 minutes
    - Incubated at 65ºC for 10 minutes to heat inactivate the ligase
- Drafted CRATER survey to send out to all iGEM teams
- Remade 300 nM stock of Phase II gRNAs
- Remade Phase II chromogenic plasmid stock
    - 2 µL red
    - 2 µL yellow
    - 4 µL pink
    - 11 µL blue
    - 11 µL indigo
    - Total: 20 µL

- Perform Cas9 digestion of chromo-mix with different combinations of 5 gRNAs
    - Negative control: No gRNA
    - Experimental controls (0.6 µL of each gRNA):
        - Expected pink: RFP, yellow, blue, and indigo gRNAs
        - Expected yellow: RFP, pink, blue, and indigo gRNAs
        - Expected blue: RFP, pink, yellow, and indigo gRNAs
        - Expected indigo: RFP, pink, yellow, and blue gRNAs
        - Expected red: pink, yellow, blue, and indigo gRNAs
    - 2 µL Cas9 were used for each reaction
    - Incubated at room temperature for 10 minutes, and then added the new chromogenic mix (3 µL) and incubated at 37ºC for one hour
- Transformed NEB 5-alpha competent cells with chromogenic Cas9 digests

08.15.15 (Danny + Kirsten + Jack)

- Visualized chromogenic transformation plates
    - After 16 hours:
        - Negative controls plates expressed yellow, pink, red, and white colonies
        - “Pink” plates had mostly pink colonies, with ~10 yellow
        - “Red” plates had mostly red colonies, with ~10 yellow and some pink
        - “Yellow” plates had mostly yellow colonies, with a few red
        - “Blue” plates had mostly white colonies, with some red and yellow
        - “Indigo” plates had little growth, with some red, yellow, and white colonies

08.17.15 (Danny + Kirsten + Jack)

- Visualized chromogenic transformation plates
    - After 16 hours in 37ºC incubator, and 48 hours in 30ºC incubator:
        - Negative control plates
            - 10 µL
            - 50 µL
                - yellow: 153
                - indigo: 28
                - blue: 92
                - pink: 276
                - red: 420
            - 250 µL
        - Pink plates
            - 10 µL
            - 50 µL
                - pink: 282

            - indigo: 489
            - yellow: 1
            - blue: 27
            - red: 2
        - 250 µL
    - Red plates
        - 10 µL
        - 50 µL
            - red: 237
            - yellow: 2
            - blue: 15
            - indigo: 162
            - pink: 0
        - 250 µL
    - Yellow plates
        - 10 µL
        - 50 µL
            - yellow: 60
            - indigo: 112
            - blue: 19
            - red: 0
            - pink: 0
        - 250 µL
    - Blue plates
        - 10 µL
        - 50 µL
            - blue: 774
            - indigo: 213
            - red: 3
            - yellow: 0
            - pink: 0
        - 250 µL
    - Indigo plates
        - 10 µL
        - 50 µL
            - indigo: 113
            - yellow: 2
            - red: 1
            - blue: 48
            - pink: 0
        - 250 µL
- Performed Phase III Cas9 digestions with new X/S gRNA and “RFP1” re-ligated plasmid

- Ran Cas9 digests on a 0.8% TAE gel with the RFP ligation mix, RFP plasmid, RFP plasmid cut with P, W1 plasmid, and W1 plasmid cut with P
- Transformed NEB 5-alpha competent cells with Cas9 digests
  - Plated on LB chloramphenicol plates
  - Let plates grow overnight in 37ºC incubator

08.18.15 (Danny + Kirsten + Jack)

- Visualized Phase III transformation plates
  - After 16 hours in 37ºC incubator
    - Negative control plates
    - X/S gRNA plates
    - X/S + rRFP gRNA plates
    - GFP gRNA plates
- Performed Cas9 digestions of chromogenic plasmids and RFP, except indigo
  - 16μL blue, 3μL red, 3μL yellow, 8μL pink (30 total, all stocks at 10nM)
  - Transformed NEB 5-alpha competent cells with Cas9 digests
  - Plated on LB chloramphenicol plates, and let grow overnight in 37ºC incubator
- Performed Cas9 digestion of Thai and Erica's ligation product to select against RFP (used RFP gRNA)
  - Transformed NEB 5-alpha competent cells with this Cas9 digest
  - Plated on LB chloramphenicol plates, and let grow overnight in 37ºC incubator
- Performed Cas9 digestion of W1 plasmid with X/S gRNA, using varying concentrations and brands of Cas9 nuclease
  - Performed 4 digests:
    - 1 μL NEB Cas9
    - 2 μL NEB Cas9
    - 0.5 μL Life Technologies Cas9
    - 1 μL Life Technologies Cas9
  - Ran Cas9 digests on a 0.8% TAE gel, along with the W1 plasmid, "W2" cut with PstI, and a 1kb ladder

08.19.15 (Danny + Kirsten + Jack)

- Visualized Phase II transformation plates
  - After 20 hours in 37ºC incubator
    - Negative control plates
      - 50 μL
      - 150 μL
    - Pink plates
      - 50 μL
      - 150 μL

        - Red plates
            - 50 µL
            - 150 µL
        - Yellow plates
            - 50 µL
            - 150 µL
        - Blue plates
            - 50 µL
            - 150 µL
        - Thai's *BirA* plates
            - 50 µL
                - One white colony
            - 150 µL
                - ~ 5 red colonies and 10 white colonies
- PCR amplified anti-X/S crRNA forward primer with trcrRNA reverse template primer using the Q5-gRNA settings
    - Performed PCR cleanup of the amplification product

08.20.15 (Danny + Kirsten + Jack)

- Performed T7 transcription of anti-X/S re-ligation gDNA
- Performed RNA purification of anti-X/S re-ligation gRNAs
- Transformed NEB 5-alpha competent cells with *BirA* re-ligation product
    - Negative control (no Cas9 digestion with RFP gRNA)
    - Experimental plate (Cas9 digestion with RFP gRNA)
    - Plated ___ µL on LB chloramphenicol plates, and let grow overnight at 37°C

08.21.15 (Danny + Kirsten + Jack)

- Analyzed *BirA* and CRATERed *BirA* plates (Kirsten)
- Analyzed *BirA* transformants on flow cytometer (Kirsten)
- Re-ran RNA gel with gRNA's: new X/S, anti-X/S, RFP, Blue, Yellow, Indigo (Danny)
- Made LB chloramphenicol plates (Kirsten)
    - 7.5 g agar
    - 500 mL DI water
    - 10 tablets of LB agar
- Cas9 digested X/S re-ligated plasmid mix with sense+anti-sense X/S gRNAs, anti-sense X/S gRNA, sense+antisense X/S gRNA + rRFP gRNA, anti-sense gRNA + rRFP gRNA, GFP gRNA, control with Cas9 and no gRNAs, control with no Cas-9 and no gRNAs
- Cas9 digested W1/RFP1 mix with X/S sense ("new X/S") gRNA
- Cas9 digested spore coat cellulose binding gene for Charles with RFP gRNA

- Cas9 digested FDC gene ligated in RFP gene for Dan X. with RFP gRNA
- Cas9 digested *BirA* gene for Thai with RFP gRNA
- Took pictures of chromogenic plates in 30ºC incubator (transformations from yesterday) (Jack)

Reaction	Plasmid	gRNA 1	gRNA 2	gRNA 3	1 µM cas 9 nuclease	DI H20	10X Cas-9 nuclease buffer
~~**(-) + Cas9**~~	~~3 µL X/S rel. mix 2~~	-	-	-	~~2 µL~~	~~19 µL~~	~~3 µL~~
(-) - Cas9	3 µL X/S rel. mix 2	-	-	-	-	24 µl	3 µL
X/S mix	3 µL X/S rel. mix 2	1.5 µL X/S antisense	1.5 µL X/S sense	-	1 µL Life Tech	19 µL	3 µL
Anti-X/S	3 µL X/S rel. mix 2	3 µL X/S antisense	-	-	1 µL Life Tech	19 µL	3 µL
~~**X/S mix + rRFP**~~	~~3 µL X/S rel. mix 2~~	~~1 µL X/S antisense~~	~~1 µL X/S sense~~	~~1 µL rRFP~~	~~2 µL~~	~~19 µL~~	~~3 µL~~
~~**Anti-X/S + rRFP**~~	~~3 µL X/S rel. mix 2~~	~~1.5 µL X/S antisense~~	~~1.5 µL rRFP~~	~~-~~	~~2 µL~~	~~19 µL~~	~~3 µL~~
~~**GFP**~~	~~3 µL X/S rel. mix 2~~	~~3 µL GFP~~	~~-~~	~~-~~	~~2 µL~~	~~19 µL~~	~~3 µL~~
W1/RFP1 mix	3 µL W2 + RFP1 mix	3 µL X/S antisense	-	-	1 µL Life Tech	19 µL	3 µL
Spores	3 µL Charles' spore coat	3 µL RFP gRNA	-	-	2 µL NEB	19 µL	3 µL
Styrene	3 µL Dan's FDC	3 µL RFP gRNA	-	-	2 µL NEB	19 µL	3 µL
P(3HB)	3 µL Thai's BirA	3 µL RFP gRNA	-	-	2 µL NEB	19 µL	3 µL

08.22.15 (Danny)

- Transformed all Cas9 digests from previous day
  - Plated each sample with two concentrations ("dilute" and "concentrated") on LB chloramphenicol plates, and also grew up samples in LB chloramphenicol broth. (Note: Charles's spore coat transformations were plated on LB ampicillin plates and grown in LB ampicillin broth.)

08.23.15 (Kosuke)

- Moved plates/broths from incubator to 4ºC fridge.

08.24.15 (Danny + Kirsten + Jack)

- Visualized plates/broths from 8/22
  - Ran *BirA* and FDC liquid cultures in flow cytometer
- Inoculated cultures for interlab study

08.25.15 (Danny + Kirsten + Jack)

- Counted colonies from 8/22 plates
  - *Bir*A dil:
    - white: ~73*4 = 292 (21.6%)
    - red: ~ 269*4 = 1076 (78.4%)
  - *Bir*A dil + cas9:
    - white: 114 (99.1%)
    - red: 1 (0.9%)
  - FDC dil:
  - FDC dil +cas9:

08.27.15 (Danny + Kirsten + Jack)

- Performed Cas9 digestion of FDC ligation mix with RFP gRNA
  - Transformed NEB 5-alpha competent cells with 5 µL of digest
  - Plated on LB chloramphenicol plates and let grow overnight at 37ºC

08.31.15 (Danny + Kirsten + Jack)

- Redo 2 sets of chromogenic digestions and transform-plate on LB chlor
  - 8.5µL blue, 1.6µL red, 1.6µL yellow, 4µL pink (16 total, all stocks at 10nM)
  - added above to original chromo mix from 8/18
  - digested mix with cas9:

	No gRNA	blue (example)
blue gRNA	---	---
yellow gRNA	---	1 µL

pink gRNA	---	1 µL
RFP gRNA	---	1 µL
10X Cas9 Buffer	3 µL	3 µL
MilliQ Water	22 µL	19 µL
Cas9	2 µL	2 µL
Total	27µL	27µL

After mixing the above solutions and incubating at room temp for 10 min to let the gRNA and Cas9 associate, 3µL of the chromo mix 3 was added for a final volume of 30µL. Then we incubated at 37ºC for 1 hour for the digestion.

- Digest each chromogenic plasmid with cas9/gRNA and PstI → run on gel
- Count colonies from FDC CRATER transformation
- Get graphs from flow cytometer to add to manuscript (figure out how to compile triplicate information)
- Redo BirA and FDC digestions and transformation
- Picked two pink colonies from early transformation (Phase II-C) plate

09.01.15 (Danny + Kirsten + Jack)

- Ran gel of chromogenic plasmid cas9 digests and PstI digests
  - Pink plasmid appeared nicked → Possibly an endogenous nickase in DH5-alpha strain of *E. coli*
  - Transformed pink plasmid into K-12 *E. coli* in hopes that the plasmid would not appear nicked in this strain
- BirA and FDC plates had no colonies
-

09.02.15 (Danny + Kirsten + Jack)

- Performed restriction enzyme digestion/clean up and T4 ligation of new *FDC*/*BirA* genes into RFP plasmid
  - PCR cleanup kit used for *BirA* cleanup
  - Qiagen Enzymatic Reaction cleanup kit used for *FDC* cleanup
- Performed Cas9 digestion of *BirA* and *FDC* ligation mixes with RFP gRNA
  - Transformed 5 µL of digests into NEB 5-alpha competent cells, along with a double-negative control for *BirA* and *FDC* (0.5 µL ligation mix with nothing else added)

    - Plated 75 µL on LB chloramphenicol plates, and let grow in 37ºC incubator overnight
- Picked two pink colonies from K-12 transformation plate and grew in LB chloramphenicol liquid cultures for mini-prep tomorrow

09.03.15 (Danny + Kirsten + Jack)

- Redo 2 replicates of pink chromogenic digestions
    - Made new chromo mix: 5.3µL blue, 1µL red, 1µL yellow, 2.7µL pink (10 total, all stocks at 10nM)
    - digested mix with cas9:

	pink
blue gRNA	1µL
yellow gRNA	1µL
pink gRNA	---
RFP gRNA	1µL
10X Cas9 Buffer	3 µL
MilliQ Water	22 µL
Cas9	2 µL
Total	27µL

After mixing the above solutions and incubating at room temp for 10 min to let the gRNA and Cas9 associate, 3µL of the chromo mix 3 was added for a final volume of 30µL. Then we incubated at 37ºC for 1 hour for the digestion. Digests were stored in the –20 freezer in the blue CRISPR Phase II box.

09.04.15 (Kirsten + Jack)

- Count colonies on FDC plates
- Run FDC cultures on flow cytometer
    - Ask Griffin about compiling data points
- Redo PstI digestions of RFP and Pink and redo Cas 9 digestion of Pink
- Transform Phase II pink in ThermoFisher Top 10 Chemically Competent Cells (Plate 50 and 150 µl on LB chlor)--grow over the weekend at room temperature
- Work on paper
    - Make table for number of colonies of chromogenic and FDC plates
    - Format flow cytometer images
    - Work on Discussion and Results

09.07.15 (Kirsten)

- Run chromogenic PstI and Cas 9 digests on 0.8% gel
- Make LB chlor plates

09.08.15 (Kirsten + Jack)

- Re-do digestion, ligation, and cas 9 digestion of FDC and transform in Top 10 Comp Cells, plate 200 ul and inoculate 50 ul in LB chlor for FC analysis tomorrow
- Count chromogenic colonies and make table for paper
- Grow cultures of just RFP and FDC for FC controls (2 each)

09.09.15 (Kirsten + Jack)

- Little growth on FDC plates and cultures- will redo tomorrow
- Worked on paper

09.10.15 (Kirsten + Jack)

- Work on paper
- Redo FDC transformations in Top 10 Competent Cells

09.14.15 (Kirsten)

- Digest RFP and reverse RFP gDNA genes with EcoRI and SpeI and ligate into RFP backbone

09.15.15 (Kirsten)

- Transform RFP and reverse RFP gDNA genes into NEB5 Electrocompetent cells and plate on LB chlor

9.16.15 (Kirsten)

- Pick colonies and grow in LB chlor

9.17.15 (Kirsten)

- Miniprep, and send for sequencing
- Dry out samples for Biobricks tomorrow