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Competent Cell Check

Present: Rika, Marina, An-Chi, Erika, Tara, Isla, Hikaru

Cells used: NEB 5-alpha competent E.coli (high-efficiency)

Procedure:

1. Clean your working area by wiping down with 70% ethanol.
2. Thaw competent cells on ice. Label one 1.5 mL microcentrifuge tubes for each transformation and then pre-chill by placing the tubes on ice.
    - Do triplicates (3 each) of each concentration if possible, so you can calculate an average colony yield.
3. Spin down the DNA tubes from the Competent Cell Test Kit/Transformation Efficiency Kit to collect all of the DNA into the bottom of each tube prior to use. A quick spin of 20-30 seconds at 8,000-10,000 rpm will be sufficient. *Note:* There should be 50 µL of DNA in each tube sent in the Kit.
4. Pipet 1 µL of DNA into each microcentrifuge tube.
5. Pipet 50 µL of competent cells into each tube. Flick the tube gently with your finger to mix.
6. Incubate on ice for 30 minutes. Start: 11:44 (group 1), 11:50 (group 2)
    - Preheat water bath now to 42°C. Otherwise, hot water and an accurate thermometer works, too!
7. Heat-shock the cells by placing into the waterbath for 45 seconds (no longer than 1 min). Be careful to keep the lids of the tubes above the water level, and keep the ice close by.
8. Immediately transfer the tubes back to ice, and incubate on ice for 5 minutes.
9. Add 950 µL of SOC media per tube, and incubate at 37°C for 1 hour shaking at 200-300rpm.
    - Prepare the agar plates during this time: label them, and add sterile glass beads if using beads to spread the mixture.
10. Pipet 100 µL from each tube onto the appropriate plate, and spread the mixture evenly across the plate. Incubate at 37°C overnight or approximately 16 hours. Position the plates with the agar side at the top, and the lid at the bottom.
11. Count the number of colonies on a light field or a dark background, such as a lab bench. Use the following equation to calculate your competent cell efficiency. If you've done triplicates of each sample, use the average cell colony count in the calculation.
    - Efficiency (in cfu/µg) = [colonies on plate (cfu) / Amount of DNA plated (ng)] x 1000 (ng/µg)
    - *Note: The measurement "Amount of DNA plated" refers to how much DNA was plated onto each agar plate, not the total amount of DNA used per transformation. You can calculate this number using the following equation:*

Amount of DNA plated (ng) = Volume DNA added (1 µL) x concentration of DNA (refer to vial, convert to ng/µL) x [volume plated (100 µL) / total reaction volume (1000 µL)]

Group 1 (Isla, Marina, An-Chi)	Group 2 (Hikaru, Tara, Erika, Rika)
Step 6 (incubation in ice): Concentrations: 20, 50 pg/ul ● Start: 11:44am ● End: 12:19pm ○ The temperature wasn't high enough so we had to wait for the temp. to rise. **Step 7**: Heat shock the cells for 45 seconds in a 42°C water bath **Step 8:** Put the microcentrifuge tube in the ice bath for 5 minutes. **Step 9:** Add 950µL of of SOC media per tube, and incubate for 1 hour and a half, shaking manually ● Start: 12:29pm ● End: 2:00pm **Step 10**: Pipette 100 µL from each tube (20, 50 pg/ul) onto the appropriate plate. Spread the mixture across the plate by swirling gently. ● Incubate at 37°C ○ Start: 2:05pm ○ End:	Step 6 (incubation in ice): Concentrations: 5,10, 0.5 pg/ul ● Start 11:50 ● End: 12:29 Step 9: ● Start: 12:29 ● End: 14:00 Step 10: ● Start: 14:05 ● End:

Changes to be made:

1. Use an inoculating loop to spread the mixture evenly across the plate.
2. Don't write all over the top of the plates→ makes it hard to see the colonies
3. Put the plates in the incubator upside down

Competent Cell Check Results

Present: Athena and An-Chi

The table below documents the number of colonies seen on the plates at 6/6/17 at 12:05 after being in the incubator since the afternoon of 6/5/17. The NEB 5-alpha competent e.coli (high-efficiency) were transformed with varying concentrations of BBa_J04450 received in the Competent Cell Check test kit from iGEM.

DNA Concentration	Plate A	Plate B	Plate C
0.5 pg/ul	9	17	50
5 pg/ul	4	40	0
10 pg/ul	110	38	13
20 pg/ul	0	30	46
50 pg/ul	24	2	1

Analysis: Only distinct colonies were counted and bacterial coverage of plate was ignored as it would not be useful when all plates have samples spread evenly on them. After counting the individual colonies on all 15 plates, the concentration of DNA that yielded the most consistent high number of colonies was at 10 pg/ul. Based on these results, we are going to be conducting the rest of our experiments that involves the transformation of NEB 5-alpha competent e.coli (high-efficiency) in with 10 pg/ul DNA.

Promoters Gibson Assembly

Protocol (https://www.neb.com/protocols/2012/12/11/gibson-assembly-protocol-e5510)

0422-32-1100

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly	4-6 Fragment Assembly	Positive Control**
Total Amount of Fragments	0.02–0.5 pmols* X µl	0.2–1 pmols* X µl	10 µl
Gibson Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

Used Anderson promoters, seen in iGEM registry

Promoters Transformation

1. Thaw competent cells on ice.
2. Chill approximately 5 ng (2 µl) of the ligation mixture in a 1.5 ml microcentrifuge tube.
3. Add 50 µl of competent cells to the DNA. Mix gently by pipetting up and down or flicking the tube 4–5 times to mix the cells and DNA. Do not vortex.
4. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds*. Do not mix.
6. Add 950 µl of room temperature media* to the tube.
7. Place tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 50–100 µl of the cells and ligation mixture onto the plates.
10. Incubate overnight at 37°C.
11. * Please note: For the duration and temperature of the heat shock step as well as for the media to be used during the recovery period, please follow the recommendations provided by the competent cells' manufacturer.

***Gibson Assembly Code (for thermocycler) = 001**

Group 1 (Ria, Isla, Katelyn, Erika, Hikaru) COX-2	Group 2 (Rika, An-chi, Tara, Yukina) c-Myc
Testing on COX-2 ● Diluted COX-2= Green top **Step 1:** 1ul of promoter , 10 ul of COX-2 , 10 ul of distilled water, 10 ul of master mix Changes: Modified the ratio → different from protocol ● += longest 0= medium - = shortest Blank=control **Step 2:** Control: add 11 ul of control DNA (to maintain ratio), 10 ul of master mix, and 10 ul of COX-2 Total Volume= 31 ul Incubate samples in thermocycler at 50ºC for 15 minutes ● Start: 11:16am	Testing on c-Myc Row 1A start ● Diluted c-Myc: red top Colored - → short 110 0 → 106 + → 101 Colored blank → control **Step 1:** (start 11:03) master mix → dH2O → dna **Step 2:** (start 11:16, end 11:31) Orange Container: Row A → 6/6 Gibson Constructs

- End: 11:31am

Yellow Container:
Column 1→ 6/6 Gibson Constructs

TRANSFORMATION:

1) Transfer 50 ul of competent cells to 1.5 ml centrifuge tube
2) Add 2ul of assembled product into the appropriate tube containing NEB competent cells
3) Mix gently (tapping) and place the mixtures on ice for 30 minutes.
    a) Start: 11:59 am
    b) End: 12:29 pm
4) Heat shock at 42°C for 30 seconds
5) Transfer to ice for 2 minutes
6) Add 950 ul of room temp. SOC media to tubes

- Change: we put it back into ice for about 1 minute while we wait for the other group

7) Place the tubes in a 37°C water bath for 60+ minutes
    a) Start: 12:34pm
    b) End: 1:59pm
8) Spread 200ul of the cells onto the plates with the inoculating loop

- Positive controls in LB plate and CAM plate

9) Let the mixture absorb into the agar plate for 2 minutes
10) Turn the plates over and incubate them at 37°C

Promoter: 01 (Isla, Ria, Katelyn)

Promoter: 06 (Isla, Ria, Katelyn)

Promoter: 10 (Hikaru, Erika)

TRANSFORMATION:

1) Transfer 50 ul of competent cells to 1.5 ml centrifuge tube
2) Add 2ul of assembled product into the appropriate tube containing NEB competent cells
3) Mix gently (tapping) and place the mixtures on ice for 30 minutes.
    a) Start: 12:00 am
    b) End: 12:29
11) Heat shock 42C for 30 seconds
12) Ice for 2 minutes (recover from heat)
13) Add 950 ul of SOC broth
14) Place tubes in warm water bath for 60 minutes (start: 12:34, end 13:34)

Promoter Strength Results (Day 1)

Present: Athena and An-Chi

The table below shows the number of distinct colonies see on plates on 6/12/17 8:50 after being in the incubator since 6/6/7. The NEB 5-alpha competent e.coli (high-efficiency) were transformed with a plasmid created with two DNA fragments through Gibson Assembly.

Promoter	LB/camr COX-2	LB/camr c-Myc
Weak A	0	0
Weak B	0	0
Weak C	0	0
Medium A	0	0
Medium B	0	0
Medium C	0	0
Strong A	0	0
Strong B	0	0
Strong C	0	2
Positive Control	78 & lawn	5

LB: Lawn

	LB COX-2	LB c-Myc
Weak	210	lawn
Medium	110	27
Strong	lawn	182+lawn
Positive Control	11	100+lawn

Promoter Strength Results (Day 2)

*All have a light lawn

Promoter	LB/camr COX-2	LB/camr c-Myc
Old Weak A (-)	9	9
Old Weak B (-)	80	50
Old Weak C (-)	500	50
Old Medium A (0)	250	44
Old Medium B (0)	300	140
Old Medium C (0)	63	66
Old Strong A (+)	200	150
Old Strong B (+)	57	1
Old Strong C (+)	450	110
New Weak A (-)	30	120
New Weak B (-)	40	250
New Weak C (-)	82	150
New Medium A (0)	0	500
New Medium B (0)	2	500
New Medium C (0)	3	160
New Strong A (+)	100	200
New Strong B (+)	270	50
New Strong C (+)	350	4
New Positive Control	47	

Construct Repeats + Modified Gibson Assembly & Transformation

Present: Ria, Hikaru, Isla, Erika, Sarah
Modified:
(New) Modifications to the ratio: 2 ul of promoters, 20 ul of dna, 20 ul of master mix, 10 ul of deionized H20. Total volume: 42 ul

(Old) Modifications to the ratio: 1 ul of promoters, 10 ul of dna, 10 ul of master mix, 10 ul of deionized H20. Total volume: 31 ul

***Gibson Assembly Code (for thermocycler) = 001**

Old: blank, New: color
+:110
o:106
- :101

COX-2 (Hikaru, Ria)	c-Myc (Isla, Erika)
Old: (Hikaru) Step 1: 1ul of promoter, 10 ul of COX-2, 10 ul of distilled water, 10 ul of master mix Total volume: 31 ul Step 2: Incubate samples in thermocycler at 50°C for 15 minutes ● Start: 11:34am ● End: 11:49am Step 3: 25ul of competent cells, 2ul of ligation mixture	Old: (Isla) Step 1: 1ul of promoter, 10 ul of c-Myc, 10 ul of distilled water, 10 ul of master mix Total volume: 31 ul Step 2: Incubate samples in thermocycler at 50°C for 15 minutes ● Start: 11:34am ● End: 11:49am Step 3: 25ul of competent cells, 2ul of ligation mixture
New: (Ria) +positive control (22 ul of control) Step 1: 2ul of promoter, 20 ul of COX-2, 10 ul of distilled water, 20 ul of master mix Total Volume= 42 ul Step 2: Incubate samples in thermocycler at 50°C for 15 minutes	New: (Erika) +positive control (22 ul of control) Step 1: 2ul of promoter, 20 ul of c-Myc, 10 ul of distilled water, 20 ul of master mix Total Volume= 42 ul Step 2: Incubate samples in thermocycler at 50°C for 15 minutes

● Start: 11:34am ● End: 11:49am Step 3: 25ul of competent cells, 2ul of ligation mixture **TRANSFORMATION:** 15) Transfer 25 ul of competent cells to 1.5 ml centrifuge tube 16) Add 2ul of assembled product into the appropriate tube containing NEB competent cells 17) Mix gently (tapping) and place the mixtures on ice for 30 minutes. a) Start: 12:11 am b) End: 12:41 pm 18) Heat shock at 42°C for 30 seconds 19) Transfer to ice for 2 minutes 20) Add 950 ul of room temp. SOC media to tubes - Change: we put it back into ice for about 2 minute while we wait for the other beaker 21) Place the tubes in a 37°C water bath for 60+ minutes a) Start: 12:34pm b) End: 1:59pm c) Total volume: 977ul each 22) Spread 300ul of the cells onto the plates with the inoculating loop - Positive controls in LB plate and CAM plate - Add 77ul in LB broth tube 23) Let the mixture absorb into the agar plate for 2 minutes 24) Turn the plates over and incubate them at 37°C	● Start: 11:34am ● End: 11:49am Step 3: 25ul of competent cells, 2ul of ligation mixture **TRANSFORMATION:** 25) Transfer 25 ul of competent cells to 1.5 ml centrifuge tube 26) Add 2ul of assembled product into the appropriate tube containing NEB competent cells 27) Mix gently (tapping) and place the mixtures on ice for 30 minutes. a) New: i) Start: 12:11 pmm ii) End: 12:41 pm b) Old: i) End: 12:42pm 28) Heat shock at 42°C for 30 seconds 29) Transfer to ice for 2 minutes 30) Add 950 ul of room temp. SOC media to tubes - Change: we put it back into ice for about 2 minute while we wait for the other beaker 31) Place the tubes in a 37°C water bath for 60+ minutes a) Start: 12:50pm b) End: 1:50pm c) Total volume: 977ul each 32) Spread 300ul of the cells onto the plates with the inoculating loop - Positive controls in LB plate and CAM plate - Add 77ul in LB broth tube 33) Let the mixture absorb into the agar plate for 2 minutes 34) Turn the plates over and incubate them at 37°C

Construct Repeats Optical Density Results

Wavelength 600 nm	New COX-2 +	New COX-2 o	New COX-2 -	Old COX-2 +	Old COX-2 o	Old COX-2 -
Absorbance Trial 1	0.311	0.374	0.352	0.215	0.312	0.342
Absorbance Trial 2	0.323	0.399	0.364	0.209	0.313	0.343
Absorbance Trial 3	0.325	0.411	0.354	0.215	0.310	0.342
Average	0.320	0.395	0.357	0.213	0.312	0.342

Wavelength 600 nm	New c-Myc +	New c-Myc o	New c-Myc -	Old c-Myc +	Old c-Myc o	Old c-Myc -
Absorbance Trial 1	0.294	0.302	0.349	0.274	0.301	0.09
Absorbance Trial 2	0.295	0.30	0.348	0.268	0.297	0.093
Absorbance Trial 3	0.296	0.30	0.352	0.267	0.298	0.091
Average	0.295	0.301	0.350	0.270	0.300	0.091

06/14/17

Wavelength 600 nm	New COX-2 +	New COX-2 o	New COX-2 -	Old COX-2 +	Old COX-2 o	Old COX-2 -
Absorbance	0.424	0.796	0.640	0.370	0.490	0.400

Trial 1						
Absorbance Trial 2	0.446	0.803	0.643	0.369	0.485	0.404
Absorbance Trial 3	0.430	0.796	0.653	0.387	0.488	0.407
Average	0.433	0.798	0.645	0.375	0.488	0.404

Wavelength 600 nm	New c-Myc +	New c-Myc o	New c-Myc -	Old c-Myc +	Old c-Myc o	Old c-Myc -
Absorbance Trial 1	0.433	0.417	0.437	0.624	0.565	0.314
Absorbance Trial 2	0.438	0.422	0.436	0.623	0.529	0.311
Absorbance Trial 3	0.437	0.423	0.438	0.623	0.533	0.319
Average	0.436	0.421	0.437	0.623	0.542	0.315

06/15/17

Wavelength 600 nm	New c-Myc +	New c-Myc o	New c-Myc -	Old c-Myc +	Old c-Myc o	Old c-Myc -
Absorbance Trial 1	0.791	0.709	0.700	0.905	0.655	(????)
Absorbance Trial 2	0.793	0.755	0.700	0.899	0.633	(????)
Absorbance Trial 3	0.807	0.760	0.721	0.936	0.671	(????)

06/16/17

Wavelength 600 nm	Old c-Myc +	Old COX-2 o

Absorbance Trial 1	0.423	0.402
Absorbance Trial 2	0.422	0.400
Absorbance Trial 3	0.430	0.410

06/19/17

*made new solution

Wavelength 600 nm	Old c-Myc +	Old COX-2 o
Absorbance Trial 1	0.319	0.437
Absorbance Trial 2	0.345	0.396
Absorbance Trial 3	0.357	0.396

COX-2 +	COX-2 o	COX-2 -	c-Myc +	c-Myc o	c-Myc -
0.213	0.312	0.342	0.270	0.300	0.091
0.375	0.488	0.404	0.623	0.542	0.315

Promoter/Reporter System Gibson Assembly

Protocol (https://www.neb.com/protocols/2012/12/11/gibson-assembly-protocol-e5510

Present: Athena, Rika, An-Chi, Maya, Yukina

Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly	4-6 Fragment Assembly	Positive Control**
Total Amount of Fragments	0.02–0.5 pmols* X µl	0.2–1 pmols* X µl	10 µl
Gibson Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

Modifications to the ratio: 1 ul of promoters, 10ul of dna, 10 ul of master mix, 10 ul of deionized H20.
Total volume: 31 ul

11ul of positive control to maintain ratio

* Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in Gibson Assembly reaction should not exceed 20%.
** Control reagents are provided for 5 experiments.
*** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.

Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section).

Transform NEB 5-alpha Competent E. coli cells (provided with the kit) with 2 µl of the assembly reaction, following the transformation protocol.

PCR Tube Label Legend:

1 - 2 ul of COX-2 promoter + 4ul of FRB + 2ul of nLuc **** (no plasmid)

2- 2ul of COX-2 promoter + 4ul of FRB + 2ul of nLuc + 4ul of plasmid (pSB1C3)

3 - 2ul of c-Myc promoter + 4ul of FRB + 2ul of nLuc (no plasmid)****

4 - 2ul of c-Myc promoter + 4ul of FRB + 2ul of nLuc + 4ul of plasmid

5 - 2ul of COX-2 promoter + 4ul of FKBP + 2ul of cLuc *** (no plasmid)

6 - 2ul of COX-2 promoter + 4ul of FKBP + 2ul of cLuc + 4 ul of plasmid

7 - 2ul of c-Myc promoter + 4ul of FKPB + 2ul of cLuc (no plasmid)****

8 - 2ul of c-Myc promoter + 4ul of FKBP + 2ul of cLuc + 4ul of plasmid (pSB1C3)

Method

1. 10:50 Iced nLuc, cLuc, COX-2, c-Myc, FRB (Right), FKBP (right), Master Mix
2. 11:05 started mixing each cell
3. 11: 30 Mixed water + master mix in each cell

Thermocycler in at 12:00 pm

Promoter/ Reporter System Transformation

1. Thaw competent cells on ice.
2. Chill approximately 5 ng (2 µl) of the ligation mixture in a 1.5 ml microcentrifuge tube.
3. Add 25 µl of competent cells to the DNA. Mix gently by pipetting up and down or flicking the tube 4–5 times to mix the cells and DNA. Do not vortex.
4. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds*. Do not mix.
6. Add 950 µl of room temperature media* to the tube.
7. Place tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 50–100 µl of the cells and ligation mixture onto the plates.
10. Incubate overnight at 37°C.
11. * Please note: For the duration and temperature of the heat shock step as well as for the media to be used during the recovery period, please follow the recommendations provided by the competent cells' manufacturer.

35) Transfer 25 ul of competent cells to 1.5 ml centrifuge tube
36) Add 2ul of assembled product into the appropriate tube containing NEB competent cells
37) Mix gently (tapping) and place the mixtures on ice for 30 minutes.
    a) Start: 10:38 am
    b) End: 11:08 am
38) Heat shock at 42ºC for 30 seconds
39) Transfer to ice for 2 minutes
40) Add 950 ul of room temp. SOC media to tubes
41) Change: placed in ice for 3 minutes while we waited for the water bath to heat up a bit
42) Place the tubes in a 37ºC water bath for 60 minutes
    a) Start: 11:19pm
    b) End: 1:59pm
    c) Total volume: 977ul each
43) Spread 300ul of the cells onto the plates with the inoculating loop

- Positive controls in LB plate and CAM plate
- Add 77ul in LB broth tube

44) Let the mixture absorb into the agar plate for 2 minutes
45) Turn the plates over and incubate them at 37ºC

Promoter/ Reporter System Results (Day 1)

Strong Promoter	2	4	6	8
Absorbance Trial 1	0.370	0.405	0.435	0.234
Absorbance Trial 2	0.376	0.430	0.443	0.341
Absorbance Trial 3	0.373	0.392	0.443	0.337

*Testing whether nLuc or cLuc works better

2- 2ul of COX-2 promoter + 4ul of FRB + 2ul of nLuc + 4ul of plasmid

4 - 2ul of c-Myc promoter + 4ul of FRB + 2ul of nLuc + 4ul of plasmid

6 - 2ul of COX-2 promoter + 4ul of FKBP +2ul of cLuc + 4ul of plasmid

8 - 2ul of c-Myc promoter + 4ul of FKBP + 2ul of cLuc + 4ul of plasmid

* cLuc works better with c-Myc promoter
nLuc works better with COX-2 promoter

Promoter/ Reporter System Results (Day 2)

Counted:10:25

	A	B	C
2 - 2ul of COX-2 promoter + 4ul of FRB + 2ul of nLuc	24	12	25
4 - 2ul of c-Myc promoter + 4ul of FRB . + 2ul of nLuc	120	120	200
6 - 2ul of COX-2 promoter + 4ul of FKBP + 2ul of cLuc	8	150	20
8 - 2ul of c-Myc promoter + 4ul of FKBP + 2ul of cLuc	200	200	100

Gibson Assembly w/ Confirmed Promoters & Reporters

Promoter for COX-2: Medium
Promoter for c-Myc: Strong

Protocol (https://www.neb.com/protocols/2012/12/11/gibson-assembly-protocol-e5510

Present: Isla, Ria, Hikaru, An-Chi
Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly	4-6 Fragment Assembly	Positive Control**
Total Amount of Fragments	0.02–0.5 pmols* X µl	0.2–1 pmols* X µl	10 µl
Gibson Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

Modifications to the ratio: 20 ul of master mix, 20 ul of deionized H20. Total volume: 31 ul

11ul of positive control to maintain ratio

* Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in Gibson Assembly reaction should not exceed 20%.
** Control reagents are provided for 5 experiments.
*** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.

Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section).

Transform NEB 5-alpha Competent E. coli cells (provided with the kit) with 2 µl of the assembly reaction, following the transformation protocol.

Group 1 (Ria, An-chi, Isla)	Group 2 (Hikaru, Rika, Athena)
1) Put 2ul of c-Myc promoter in PCR tubes with pipet 2) Put 4ul of FRB in the PCR tubes 1, 2, 3, and 4 3) Add 2ul of nLuc to PCR tubes 3 and 4 4) Add 2ul of cLuc to PCR tubes 7 and 8 5) Add 4ul of plasmid to PCR tubes 4 and 8 6) Add 20ul of deionized water to the PCR tubes 7) Add 20ul of master mix to the PCR tubes 8) Place tubes in thermocycler and let it run for an hour a) Start: 12:56pm b) End: 1:56pm	1. Put 2ul of COX-2 promoter in PCR tubes with pipet 2. Put 4ul of FKPB in the PCR tubes 5, 6, 7, and 8 3. Add 2ul of nLuc to PCR tubes 1 and 2 4. Add 2ul of cLuc to PCR tubes 5 and 6 5. Add 4ul of plasmid to PCR tubes 2 and 6 6. Add 20ul of deionized water to the PCR tubes 7. Add 20ul of master mix to the PCR tubes 8. Place tubes in thermocycler and let it run for an hour a. Start: 11:41am b. End: 12:41pm

PCR Tube Label Legend:

1 - 2 ul of COX-2 promoter + 4ul of FRB + 2ul of nLuc **** (no plasmid)

2 - 2ul of COX-2 promoter + 4ul of FRB + 2ul of nLuc + 4ul of plasmid

3 - 2ul of c-Myc promoter + 4ul of FRB + 2ul of nLuc (no plasmid)****

4 - 2ul of c-Myc promoter + 4ul of FRB + 2ul of nLuc + 4ul of plasmid

5 - 2ul of COX-2 promoter + 4ul of FKBP + 2ul of cLuc *** (no plasmid)

6 - 2ul of COX-2 promoter + 4ul of FKBP + 2ul of cLuc + 4 ul of plasmid

7 - 2ul of c-Myc promoter + 4ul of FKPB + 2ul of cLuc (no plasmid)****

8 - 2ul of c-Myc promoter + 4ul of FKBP + 2ul of cLuc + 4ul of plasmid

Transformation w/ Confirmed Promoters & Reporters

Present: Ria, Maya, Sarah, Hikaru and An-Chi

Chemically Competent Cell Transformation (after Gibson)
Procedure:

1. Thaw competent cells on ice.
2. Chill approximately 5 ng (2 µl) of the ligation mixture in a 1.5 ml microcentrifuge tube.
3. Add 25 µl of competent cells to the DNA. Mix gently by pipetting up and down or flicking the tube 4–5 times to mix the cells and DNA. Do not vortex.
4. Place the mixture on ice for 30 minutes. Do not mix.
    a. Start: 10:31am
    b. End: 11:09am
5. Heat shock at 42°C for 30 seconds*. Do not mix.
6. Add 950 µl of room temperature media* to the tube.
7. Place tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
    a. Start: 11:13am
    b. End: 12:13pm
8. Warm selection plates to 37°C.
9. Spread 50–100 µl of the cells and ligation mixture onto the plates.
10. Incubate overnight at 37°C.

* Please note: For the duration and temperature of the heat shock step as well as for the media to be used during the recovery period, please follow the recommendations provided by the competent cells' manufacturer.

Promoter/ Reporter System Colony Count

*light lawn in all except 4C

	A	B	C
2 - 2ul of COX-2 medium promoter + 4ul of FRB + 2ul of nLuc	407 + full lawn	2	150
4 - 2ul of c-Myc strong promoter + 4ul of FRB + 2ul of nLuc	28	536	200
6 - 2ul of COX-2 medium promoter + 4ul of FKBP +2ul of cLuc	2	65	20
8 - 2ul of c-Myc strong promoter + 4ul of FKBP. + 2ul of cLuc	60 + full lawn	25	11

Promoter/Reporter System Optical Density

*2 and 6= medium promoter
4 and 8= strong promoter

Promoter Strength:	Medium	Strong	Medium	Strong
	2	4	6	8
Absorbance Trial 1	0.992	0.312	0.532	0.942
Absorbance Trial 2	0.995	0.324	0.536	0.930
Absorbance Trial 3	1.028	0.322	0.537	0.955
Average	1.005	0.319	0.535	0.423

*Testing whether nLuc or cLuc works better

2 - 2ul of COX-2 promoter + 4ul of FRB + 2ul of nLuc + 4ul of plasmid

4 - 2ul of c-Myc promoter + 4ul of FRB . + 2ul of nLuc + 4ul of plasmid

6 - 2ul of COX-2 promoter + 4ul of FKBP +2ul of cLuc + 4ul of plasmid

8 - 2ul of c-Myc promoter + 4ul of FKBP. + 2ul of cLuc + 4ul of plasmid

COX-2 and nLuc
c-Myc and cLuc

Transformation of Promoter/Reporter Constructs Plated with Rapamycin

12. Thaw competent cells on ice.
13. Chill approximately 5 ng (2 µl) of the ligation mixture in a 1.5 ml microcentrifuge tube.
14. Add 25 µl of competent cells to the DNA. Mix gently by pipetting up and down or flicking the tube 4–5 times to mix the cells and DNA. Do not vortex.
15. Place the mixture on ice for 30 minutes. Do not mix.
16. Heat shock at 42°C for 30 seconds*. Do not mix.
17. Add 950 µl of room temperature media* to the tube.
18. Place tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
19. Warm selection plates to 37°C.
20. Spread 50–100 µl of the cells and ligation mixture onto the plates.
21. Incubate overnight at 37°C.
22. * Please note: For the duration and temperature of the heat shock step as well as for the media to be used during the recovery period, please follow the recommendations provided by the competent cells' manufacturer.

Present: An-Chi, Ria, and Maya

1. 4 tubes made with: COX-2 + nLuc; c-Myc + cLuc; c-Myc/cLuc + COX-2/nLuc; COX-2/cLuc + c-Myc/nLuc
2. 25 ul of competent cells in all tubes
3. 2 ul DNA
4. Incubate on ice for 30 minutes
    a. Start: 12:14
    b. End: 12:44
5. Heat shock all tubes at 12:53 for 30 sec. At 42 degrees
6. (??)

Results of Plating in Presence of Rapamycin

Name	**Colony Count**	**Glowing Colony Count**
COX-2 nLuc (rap)	None	None
c-Myc cLuc (rap)	12	12
c-Myc nLuc + COX-2 cLuc	4	None
c-Myc nLuc + COX-2 cLuc (rap)	40	11
c-Myc cLuc + COX-2 nLuc	40	None
c-Myc cLuc + COX-2 nLuc (rap)	8	8

Electrophoresis (Test)

Procedure:

1. Defrost markers, backbone plasmid, p-c-Myc, p-COX-2, COX-2, c-Myc
2. Warm up agarose in microwave until no more matter in floating/nothing is sparkling *avoid bubbling/boiling
3. Cool until beaker is room temperature or able to pick up by hand
4. Pour agarose ⅓ of the depth into electrophoresis device, wait until it solidifies
5. Pull out the black walls
6. Add buffer on one side until it evens out on both sides up to the maximum point
7. Pipet in 10 ul of plasmid/backbones/c-Myc/COX-2 into holes *COX-2 and c-Myc must be placed next to each respective plasmid
    a. Markers in lanes 1 and 8
    b. COX-2, c-Myc in lanes 2 and 7
    c. P-COX-2, p-myc in lanes 3 and 6
    d. Backbones in lanes 4 and 5
    e. **change: only put in one backbone and one marker because there weren't enough → lane numbers viewed by red side closest to you on the right side
        i. 1. Empty (supposed to be marker)
        ii. 2. COX-2
        iii. 3. P-COX-2
        iv. 4. Empty (supposed to be backbone)
        v. 5. Backbone
        vi. 6. P-myc
        vii. 7. c-Myc
        viii. 8. Marker
8. Plug in electrophoresis device to power bank and place lid on device
9. Turn on voltage to 150 volts **keep eye on device to make sure the markers etc… doesn't overflow from agar
10. Stain agar using a stain card

(Re-Do) Gibson Assembly w/ Confirmed Promoters & Reporters

Re-do because we spilled them
Promoter for COX-2: Medium
Promoter for c-Myc: Strong

Protocol (https://www.neb.com/protocols/2012/12/11/gibson-assembly-protocol-e5510

Present: Isla, An-Chi, Erika
Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly	4-6 Fragment Assembly	Positive Control**
Total Amount of Fragments	0.02–0.5 pmols* X µl	0.2–1 pmols* X µl	10 µl
Gibson Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

Modifications to the ratio: 20 ul of master mix, 20 ul of deionized H20. Total volume: 31 ul

11ul of positive control to maintain ratio

* Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in Gibson Assembly reaction should not exceed 20%.
** Control reagents are provided for 5 experiments.
*** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.

Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section).

Transform NEB 5-alpha Competent E. coli cells (provided with the kit) with 2 µl of the assembly reaction, following the transformation protocol.

Group 1 (Isla, An-chi, Erika)

9) Put 2ul of c-Myc promoter in PCR tubes (3,4,7,8)
10) Put 2ul of COX-2 promoter in PCR tubes (1,2,5,6)
11) Put 4ul of FRB in the PCR tubes 1, 2, 3, and 4
12) Put 4ul of FKPB in the PCR tubes 5, 6, 7, and 8
13) Add 2ul of nLuc to PCR tubes 1,2, 3 and 4
14) Add 2ul of cLuc to PCR tubes 5, 6, 7 and 8
15) Add 4ul of plasmid to PCR tubes 2, 4, 6 and 8
16) Add 20ul of deionized water to the PCR tubes
17) Add 20ul of master mix to the PCR tubes
18) Place tubes in thermocycler and let it run for an hour (100 for an hour)
    a) Start: 12:56pm
    b) End: 1:56pm

PCR Tube Label Legend: (RSG+number)

1 - 2 ul of COX-2 promoter +4ul of FRB + 2ul of nLuc **** (no plasmid)

2- 2ul of COX-2 promoter + 4ul of FRB + 2ul of nLuc + 4ul of plasmid

3 - 2ul of c-Myc promoter+ 4ul of FRB + 2ul of nLuc (no plasmid)****

4 - 2ul of c-Myc promoter + 4ul of FRB . + 2ul of nLuc + 4ul of plasmid

5 - 2ul of COX-2 promoter + 4ul of FKBP + 2ul of cLuc *** (no plasmid)

6 - 2ul of COX-2 promoter + 4ul of FKBP +2ul of cLuc + 4 ul of plasmid

7 - 2ul of c-Myc promoter+ 4ul of FKPB + 2ul of cLuc (no plasmid)****

8 - 2ul of c-Myc promoter + 4ul of FKBP + 2ul of cLuc + 4ul of plasmid

(Re-Do) Transformation w/ Confirmed Promoters & Reporters

Present: Erika, Ria,

23. Thaw competent cells on ice.
24. Add 100ul of rapamycin on plates. Let it absorb into plates.
25. Chill approximately 5 ng (2 µl) of the ligation mixture in a 1.5 ml microcentrifuge tube.
26. Add 25 µl of competent cells to the DNA. Mix gently by pipetting up and down or flicking the tube 4–5 times to mix the cells and DNA. Do not vortex.
27. Place the mixture on ice for 30 minutes. Do not mix.
    a. Start: 10:50
    b. End: 11:20
28. Heat shock at 42°C for 30 seconds*. Do not mix.
29. Add 950 µl of room temperature media* to the tube.
30. Place tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
    a. Start: 11:27
    b. End: 12:27
31. Warm selection plates to 37°C.
32. Spread 50–100 µl of the cells and ligation mixture onto the plates.
33. Incubate overnight at 37°C.
34. * Please note: For the duration and temperature of the heat shock step as well as for the media to be used during the recovery period, please follow the recommendations provided by the competent cells' manufacturer.

(Redo) Promoter/Reporter System Results

*No luminescence was observed on any plates.
Plated With Rapamycin

	2	4	6	8	4+6	2+8
A	0	4	1	2	4	(?????)
B	230	7	515	3	13	29 *large lawn covering
C	5	36 *large lawn covering	4 * large covering	*heavy lawn	78	623

(Redo) Promoter Reporter Rapamycin Results

	2 + rap	4 + rap	6 + rap	8 + rap	2+8	2+8 +rap	4+6	4+6 +rap
A	lawn	Light lawn	70 +lawn	lawn	376	4+lawn	3	4
A (glowing)	0	0	0	0	6	4	0	0
B	70	118	120	302	41	41	lawn	13+law n
B (glowing)	0	0	0	0	32	10	0	0
C	23	31 +lawn	4	lawn	10	250	2	101
C (glowing)	0	0	0	0	10	19	0	0

NOTE: 2+8 w/o rap was not supposed to glow, but Ms. Crissy added rapamycin to all the plates on sunday.

Restriction Digest

1. Use permanent marker to label four 1.5-ml tubes, in which restriction reactions will be performed

P = PstI
E = EcoRI
S = SpeI
X = XbaI
- = no enzyme

2. Use table below as a checklist by adding reagents to each reaction.

Tube	DNA	Buffer	PstI	EcoRI	SpeI	XbaI	H2O
1	4µl	5µl	-	-	-	1µl	6µl
2	4µl	5µl	-	1µl	-	-	6µl
3	4µl	5µl	-	-	1µl	-	6µl
4	4µl	5µl	1µl	-	-	-	6µl
5	4µl	10µl	-	-	1µl	1µl	-
6	4µl	10µl	1µl	-	-	1µl	-
7	4µl	10µl	-	1µl	1µl	-	-
8	4µl	10µl	1µl	1µl	-	-	-

3. Collect reagents, and place in test tube rack on lab bench.

4. Add 4 µl of DNA to each reaction tube. Touch pipet tip to side of reaction tube, as near to the bottom as possible, to create capillary action to pull solution out of tip.

5. Always add buffer to reaction tubes before adding enzymes. Use fresh tip to add 5 µl of restriction buffer to clean spot on each reaction tube.

6. Use fresh tips to add 1 µl of PstI, EcoRI, SpeI, XbaI to appropriate tubes.

7. Use fresh tip to add 1µl of dH20 to tube labeled “-”.

8. Close tube tops. Pool and mix reagents by pulsing in a microfuge or by sharply tapping the tube bottom on lab bench.

9. Place reaction tubes in 37 C water bath, and incubate for a minimum of 20 minutes. Reactions can be incubated for a longer period of time.

Labeling system:

#1-8 on row B COX-2 p/reporter construct (colored in tabs)

#1-8 on row E c-Myc p/reporter construct (not colored tabs)

1 - Xbal + H2O

2 - EcoRI + H2O

3 - SpeI + H2O

4 - PstI + H2O

5 - Xbal + SpeI

6 - Xbal + PstI

7 - EcoRI + SpeI

8 - EcoRI + PstI

Electrophoresis Gel Set-Up:

Backbone	1	2	3	4	5	6	7	8	Gene

*on the chamber: Black is on the top left corner and red is on the bottom right

Bradford Protein Assay

1. The standard protocol can be performed in three different formats, 5 ml and a 1 ml cuvette assay, and a 250 µl microplate assay. The linear range of these assays for BSA is 125–1,000 µg/ml, whereas with gammaglobulin the linear range is 125–1,500 µg/ml.
2. Remove the 1x dye reagent from 4°C storage and let it warm to ambient temperature. Invert the 1x dye reagent a few times before use.
3. If 2 mg/ml BSA or 2 mg/ml gamma-globulin standard is used, refer to the tables in the 11 4110065A.qxp 9/25/2007 2:39 PM Page 17 appendix as a guide for diluting the protein standard. (The dilutions in the tables are enough for performing triplicate measurements of the standards.) For the diluent, use the same buffer as in the samples (refer to Troubleshooting section for more information). Protein solutions are normally assayed in duplicate or triplicate. For convenience, the BSA or gamma-globulin standard sets can be used, but blank samples (0 µg/ml) should be made using water and dye reagent.
4. Pipet each standard and unknown sample solution into separate clean test tubes or microplate wells (the 1 ml assay may be performed in disposable cuvettes). Add the 12 4110065A.qxp 9/25/2007 2:39 PM Page 18 1x dye reagent to each tube (or cuvette) and vortex (or invert). For microplates, mix the samples using a microplate mixer. Alternatively, use a multichannel pipet to dispense the 1x dye reagent. Depress the plunger repeatedly to mix the sample and reagent in the wells. Replace with clean tips and add reagent to the next set of wells. Assay Volume of Volume of Standard and Sample 1x Dye Reagent 5 ml 100 µl 5 ml 1 ml 20 µl 1 ml Microplate 5 µl 250 µl
5. Incubate at room temperature for at least 5 min. Samples should not be incubated longer than 1 hr at room temperature. 13 4110065A.qxp 9/25/2007 2:39 PM Page 19
6. Set the spectrophotometer to 595 nm. Zero the instrument with the blank sample (not required for microplate readers). Measure the absorbance of the standards and unknown samples. Refer to Section 3 for data analysis. Note: If the spectrophotometer has a reference and sample holder, the instrument can be zeroed with two blank samples. If the effect of buffer on absorbance is required, zero the instrument with a cuvette filled with water and dye reagent in the reference holder.

PAGE

1. Make the **separating gel**:

- Set the casting frames (clamp two glass plates in the casting frames) on the casting stands.
- Prepare the gel solution (as described above) in a separate small beaker.
- Swirl the solution gently but thoroughly.
- Pipet appropriate amount of separating gel solution (listed above) into the gap between the glass plates.
- To make the top of the separating gel be horizontal, fill in water (either isopropanol) into the gap until a overflow.
- Wait for 20-30min to let it gelate.
- Make the **stacking gel**:
- Discard the water and you can see separating gel left.
- Pipet in stacking gel until a overflow.
- Insert the well-forming comb without trapping air under the teeth. Wait for 20-30min to let it gelate.

2. Make sure a complete gelation of the stacking gel and take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow until the buffer surface reaches the required level in the outer chamber.

3. Prepare the samples:

- Mix your samples with sample buffer (loading buffer).
- Heat them in boiling water for 5-10 min.

4. Load prepared samples into wells and make sure not to overflow. Don't forget loading protein marker into the first lane. Then cover the top and connect the anodes.

5. Set an appropriate volt and run the electrophoresis when everything's done.

6. As for the total running time, stop SDS-PAGE running when the downmost sign of the protein marker (if no visible sign, inquire the manufacturer) almost reaches the foot line of the glass plate. Generally, about 1 hour for a 120V voltage and a 12% separating gel. For a separating gel possessing higher percentage of acrylamide, the time will be longer.

Note: Various factors affect the properties of the resulting gel.

- Higher concentration of ammonium persulfate and TEMED will lead to a faster gelation, on the other hand, a lower stability and elasticity.
- The optical temperature for gel gelation is 23°C-25°C. Low temperature will lead to turbid, porous and inelastic gels.
- The pH is better to be neutral and the gelation time should be limited in 20-30 min.