

# Standard Protocols

Rice University 2017 iGEM Team

<b>Protocol 1: Golden Gate Assembly</b>	<b>2</b>
Table 1: Golden Gate reaction materials	2
Table 2: BsaI Golden Gate assembly thermocycling conditions	2
Table 3: Esp3I Golden Gate assembly thermocycling conditions	3
Procedure	3
<b>Protocol 2: Phusion/Q5 PCR</b>	<b>4</b>
Table 1: Phusion/Q5 PCR materials	4
Table 2: Phusion/Q5 thermocycling conditions	4
Procedure	4
<b>Protocol 3: Colony PCR</b>	<b>5</b>
Table 1: Colony PCR materials	5
Table 2: Phusion/Q5 thermocycling conditions	5
Procedure	5
<b>Protocol 4: Electroporation Transformation</b>	<b>6</b>
Table 1: Electroporation materials	6
Procedure	6
<b>Protocol 5: Heat Shock Transformation</b>	<b>7</b>
Table 1: Heat Shock Transformation materials	7
Procedure	7
<b>Protocol 6: Preparing Heat Shock Competent Cells</b>	<b>8</b>
Table 1: Preparing Heat Shock Comp Cells materials	8
Procedure	8
<b>Protocol 7: Gel Electrophoresis</b>	<b>9</b>
Procedure: Preparing agarose gel	9
Procedure: Loading samples and running agarose gel	9
Procedure: Analyzing Your Gel	10
<b>Protocol 8: LB agar plates preparation</b>	<b>11</b>
Materials:	11
Procedure:	11
Table 1: Antibiotic Concentrations	11
<b>Protocol 9: Miniprep of Plasmid DNA (“Miniprep” for short)</b>	<b>12</b>
Procedure*	12

# Protocol 1: Golden Gate Assembly

Table 1: Golden Gate reaction materials

Component	Volume	Notes
Bsal/Esp3I/BbsI	0.5 $\mu$ L	0.5–0.75 $\mu$ L range.
T <sub>4</sub> DNA Ligase	0.5 $\mu$ L	0.5–1 $\mu$ L range
10 $\times$ T <sub>4</sub> Ligase Buffer	1.5 $\mu$ L	Titurate/vortex to dissolve solids.
10 $\times$ BSA	1.5 $\mu$ L	Enables full Bsal activity at 37°C
DNAs	25 fmol each 0.5 $\mu$ L 50 nM	10–40 fmol equimolar. 2–5-fold less vector to reduce background.
Deionized Water	up to 15 $\mu$ L	10–20 $\mu$ L range.

Table 2: Bsal Golden Gate assembly thermocycling conditions

Bsal Golden Gate, Long, $\geq 6$ parts: 142.5 min / 2:23 Short, $\leq 5$ parts: 97.5 min / 1:38				Basic*: 52.5–75 min		
	Step	Temp	Time		Temp	Time
	Initial Digestion ( <i>opt.</i> )	37°C	10 min		37°C	15 min
Repeat 25 $\times$ / 15 $\times$	Digestion	37°C	1.5 min	Repeat 5–10 $\times$	37°C	1.5 min
	Annealing & Ligation	16°C	3 min		16°C	3 min
	Digestion & Ligase Inact.	50°C	10 min		50°C	5 min
	Inactivation	80°C	10 min		80°C	10 min
	Storage	12°C	$\infty$		12°C	$\infty$

Table 3: Esp3I Golden Gate assembly thermocycling conditions

Esp3I Golden Gate, Long, ≥6 parts: 142.5 min / 2:23 Short, ≤5 parts: 97.5 min / 1:38				Basic*: 52.5–75 min		
	Step	Temp	Time		Temp	Time
	Initial Digestion ( <i>opt.</i> )	37°C	10 min		37°C	15 min
Repeat 25× / 15×	Digestion	37°C	1.5 min	Repeat 5–10×	37°C	1.5 min
	Annealing & Ligation	16°C	3 min		16°C	3 min
	Digestion	37°C	5 min		37°C	5 min
	Digestion & Ligase Inact.	50°C	5 min		50°C	5 min
	Inactivation	80°C	10 min		80°C	10 min
	Storage	12°C	∞		12°C	∞

### Procedure

1. Prepare a mastermix by adding together enzyme, T<sub>4</sub> DNA Ligase, 10X T<sub>4</sub> Ligase Buffer, 10X BSA, and water
2. Pipette up and down to mix
3. Distribute mastermix into labelled pcr tubes
4. Add DNA components for each reaction to the concentrations specified in the Table 1
5. Flick to mix
6. Place the reaction tubes into the thermocycler and adjust the settings for a given enzyme as shown in Table 2 or Table 3

# Protocol 2: Phusion/Q5 PCR

Source: <https://www.neb.com/protocols/1/01/01/pcr-protocol-m0530>

Table 1: Phusion/Q5 PCR materials

Component	Volume	Final Concentration
H2O	to 50 $\mu$ L	
5X Phusion HF or GC Buffer	10 $\mu$ L	1X
10 mM dNTPs	1 $\mu$ L	200 $\mu$ M
10 $\mu$ M forward primer	2.5 $\mu$ L	0.5 $\mu$ M
10 $\mu$ M reverse primer	2.5 $\mu$ L	0.5 $\mu$ M
template DNA**	variable	10 ng
phusion polymerase	0.5 $\mu$ L	1.0 units per reaction

Table 2: Phusion/Q5 thermocycling conditions

Step	1	2	3	4	5	6	7
Temperature	98C	98C	Tm	72C	GO TO Step 2	72C	4C
Time	0:30	0:10	0:30	30s/kb	34X	5:00	hold

## Procedure

1. Prepare master mix by adding together water, GC/HF buffer, dNTPs, primers and phusion polymerase to the concentrations shown in Table 1
2. Add 10 ng of template DNA
3. Flick to mix
4. Place the reaction tubes into the thermocycler and adjust the settings for a given enzyme as shown in Table 2

# Protocol 3: Colony PCR

Table 1: Colony PCR materials

Component	Volume	Final Concentration
H2O	to 25 $\mu$ L	
5X Phusion HF or GC Buffer	5 $\mu$ L	1X
10 mM dNTPs	0.5 $\mu$ L	200 $\mu$ M
10 $\mu$ M forward primer	1.25 $\mu$ L	0.5 $\mu$ M
10 $\mu$ M reverse primer	1.25 $\mu$ L	0.5 $\mu$ M
template DNA**	1 $\mu$ L	unknown
phusion polymerase	0.25 $\mu$ L	1.0 units per reaction

Table 2: Phusion/Q5 thermocycling conditions

Step	1	2	3	4	5	6	7
Temperature	98C	98C	Tm	72C	GO TO Step 2	72C	4C
Time	3:00	0:10	0:30	30s/kb	34X	5:00	hold

## Procedure

1. Prepare LB with chloramphenicol(17) by adding 0.5uL chloramphenicol to 1mL of LB
2. For 5 PCR reactions per sample:
  - a. Pipette 5 uL of LB solution into each of the 15 PCR tube
  - b. Pick up five colonies per plate and inoculate a colony per PCR tube
3. Prepared a mastermix for the appropriate number of 25uL reactions
4. Add 10 uL of mastermix to each PCR tube
5. Add 1 uL of LB cultures to corresponding tubes
6. Flick the tubes to mix
7. Place the reaction tubes into the thermocycler and adjust the settings as shown in Table 2

# Protocol 4: Electroporation Transformation

Table 1: Electroporation materials

Component	Volume
Electrocompetent cells	50 uL
DNA	1uL
SOB	1 mL
Glucose (1M)	20 uL
Electroporation cuvette	
LB agar plate with appropriate antibiotic	

## Procedure

1. Thaw electrocompetent cells on ice
2. Add 1uL of DNA to the tube containing electrocompetent cells
3. Pipet cells into a chilled cuvette and keep on ice
4. Prepare 1 mL of SOC medium by adding together 1 mL of SOB and 20 uL of 1M glucose
5. Turn on the Microcomputer and set to appropriate program (Ec1 for 0.1 cm cuvettes)
6. Insert cuvette into the slide
7. Pressed the "Pulse" button
8. Immediately remove the cuvette, add 1mL SOC, and gently mix by pipetting up and down
9. Transfer cells to the falcon tubes and place in shaking incubator at 37C for 1h
10. Retrieve the tubes from the incubator and plated 100 uL of cells
11. Place the plates into 37C incubator

# Protocol 5: Heat Shock Transformation

Modified from: [http://parts.igem.org/Help:2017\\_Compentent\\_Cell\\_Test\\_Kit](http://parts.igem.org/Help:2017_Compentent_Cell_Test_Kit)

Table 1: Heat Shock Transformation materials

Component	Volume
Electrocompetent cells	50 uL
DNA	1uL
SOB	1 mL
Glucose (1M)	20 uL
LB agar plate with appropriate antibiotic	

## Procedure

1. Thaw competent cells on ice.
2. Pipet 1  $\mu$ L of DNA into each tube with cells.
3. Incubate on ice for 30 minutes.
4. Pre-heat waterbath now to 42°C. Otherwise, hot water and an accurate thermometer works, too!
5. 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.
6. Immediately transfer the tubes back to ice, and incubate on ice for 5 minutes.
7. Add 950  $\mu$ L of SOC media per tube, and incubate at 37°C for 1 hour shaking at 200-300rpm.
8. Pipet 100  $\mu$ L 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.

# Protocol 6: Preparing Heat Shock Competent Cells

Table 1: Preparing Heat Shock Comp Cells materials

Component	Volume
Cells glycerol stock	
LB agar plate (no antibiotic)	
LB	11 mL
TSS buffer	1.6 mL

## Procedure

1. Streak cells from glycerol stock on LB agar plate and incubate in 37C incubator overnight
2. Pick up a colony and prepare liquid culture in 4 mL LB. Incubate overnight in 37 shaking incubator.
3. Retrieve liquid culture from the incubator.
4. Add 100uL of seed culture to 900uL LB media (previously prepared) in a cuvette
5. Blank the spectrophotometer with 1mL LB media
6. Measured the OD600 of the diluted culture
7. Start a new culture by adding 50ul of seed culture to 4.95mL of LB media for a total volume of 5mL to get 100 fold dilution
8. From the OD of the diluted culture and doubling time of the cells, calculate the time needed to reach the OD of about 0.2 and place the culture into 37C incubator.
9. Retrieve the culture from the incubator and place on ice to stop growth.
10. Centrifuge at 4 degrees C for 10 min at 2500 rpm
11. Discard supernatant
12. Resuspend cell pellet in 1.6mL pre-chilled TSS buffer (pre-prepared)
13. Distributed competent cell solution across (32) 1.5mL tubes to make 50uL aliquots
14. (optional) flash freeze the (32) aliquots in liquid nitrogen
15. Store the aliquots in the -80C freezer



# Protocol 7: Gel Electrophoresis

## Procedure: Preparing agarose gel

1. Measure 1 g of agarose (for 100 mL of 1% gel:)
2. Mix agarose powder with 100 mL 1xTAE in a microwavable flask.
3. Microwave for 1-3 min until the agarose is completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.)

Note: Caution HOT! Be careful stirring, eruptive boiling can occur.

Note: It is a good idea to microwave for 30-45 sec, stop and swirl, and then continue towards a boil. Keep an eye on it as the initial boil has a tendency to boil over.

4. Let agarose solution cool down to about 50°C (about when you can comfortably keep your hand on the flask), about 5 mins.
5. Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µl of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.

Note: Caution EtBr is a known mutagen. Wear a lab coat, eye protection and gloves when working with this chemical.

Note: If you add EtBr to your gel, you will also want to add it to the running buffer when you run the gel. If you do not add EtBr to the gel and running buffer, you will need to soak the gel in EtBr solution and then rinse it in water before you can image the gel.

6. Pour the agarose into a gel tray with the well comb in place.

Note: Pour slowly to avoid bubbles which will disrupt the gel. Any bubbles can be pushed away from the well comb or towards the sides/edges of the gel with a pipette tip.

7. Place newly poured gel at 4°C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified.

## Procedure: Loading samples and running agarose gel

1. Add loading buffer to each of your digest samples.

Note: Loading buffer serves two purposes: 1) it provides a visible dye that helps with gel loading and will also allow you to gauge how far the gel has run while you are running your gel; and 2) it contains a high percentage of glycerol, so it increases the density of your DNA sample causing it to settle to the bottom of the gel well, instead of diffusing in the buffer.

2. Once solidified, place the agarose gel into the gel box (electrophoresis unit).
3. Fill gel box with 1x TAE (or TBE) until the gel is covered.
4. Carefully load a molecular weight ladder into the first lane of the gel.

Note: When loading the sample in the well, maintain positive pressure on the sample to prevent bubbles or buffer from entering the tip. Place the very top of the tip of the pipette into the buffer just above the well. Very slowly and steadily, push the sample out and

watch as the sample fills the well. After all of the sample is unloaded, push the pipettor to the second stop and carefully raising the pipette straight out of the buffer.

5. Carefully load your samples into the additional wells of the gel.
6. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel.

Note: Black is negative, red is positive. (The DNA is negatively charged and will run towards the positive electrode.) Always Run to Red.

Note: A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage.

7. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
8. (Optional) If you did not add EtBr to the gel and buffer, place the gel into a container filled with 100 mL of TAE running buffer and 5  $\mu$ L of EtBr, place on a rocker for 20-30 mins, replace EtBr solution with water and destain for 5 mins.

9. Using any device that has UV light, visualize your DNA fragments.

Note: When using UV light, protect your skin by wearing safety goggles or a face shield, gloves and a lab coat.

Note: If you will be purifying the DNA for later use, use long-wavelength UV and expose for as little time as possible to minimize damage to the DNA.

Note: The fragments of DNA are usually referred to as 'bands' due to their appearance on the gel.

#### Procedure: Analyzing Your Gel

Using the DNA ladder in the first lane as a guide (the manufacturer's instruction will tell you the size of each band), you can interpret the bands that you get in your sample lanes to determine if the resulting DNA bands that you see are as expected or not.

# Protocol 8: LB agar plates preparation

## Materials:

- 5 g NaCl
- 2.5 g yeast extract
- 50 uL NaOH
- 5 g tryptone
- agar 7.5 g
- 500 mL H<sub>2</sub>O
- 250 uL of 34 mg/ml chloramphenicol\*

*\*Note: this protocol is for preparing plates with 17 µg/mL of chloramphenicol antibiotics. We also prepared plates throughout the course of our work with the antibiotics and concentrations listed in Table 1 below.*

## Procedure:

1. Add all materials except antibiotic to a 1L flask, and use a stir bar/stir plate to dissolve all the solids.
2. Cover top of flask with loose foil and autoclave. Autoclaved on setting #3 (liquids)
3. Once autoclaved agar is cool enough to handle without gloves, add 250uL of 34mg/ml chloramphenicol for a final concentration of 17ug/ml chloramphenicol
4. Use a serological pipette in the biosafety cabinet to add 20mL of agar+antibiotic to each plate
5. Label plates
6. Allow plates to solidify and then transfer to 4C fridge

Table 1: Antibiotic Concentrations

Antibiotic	Working Concentration	Shorthand
Ampicillin	100 µg/mL	Amp100
Chloramphenicol	17 or 34 µg/mL	Chl17 / Chl34
Kanamycin	50 µg/mL	Kan50

# Protocol 9: Minipreparation of Plasmid DNA

## ("Miniprep" for short)

### Procedure\*

1. Pellet bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15–25°C).
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
6. Apply 800 µl supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30–60 s and discard the flow-through
7. Wash the QIAprep 2.0 spin column by adding 0.75 ml Buffer PE. Centrifuge for 30–60 s and discard the flow-through. Transfer the QIAprep 2.0 spin column to the collection tube.
8. Centrifuge for 2 min to remove residual wash buffer.
9. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl water to the center of the QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.
10. Measure concentration of the purified plasmid DNA using nanodrop

*\*We use the Qiagen Spin Miniprep Kit and reference the Qiagen protocols*



