A. Culturing:

1. M17 Broth, Agar Preparation

1. Suspend the powder in 975 mL of purified water.

M17 Agar – 55 g;

M17 Broth – 42.5 g.

Mix thoroughly.

2. Heat with frequent agitation and completely dissolve the powder. The agar will inevitably sink to the bottom, but dont worry; it will mix well after autoclaving.

3. Autoclave at 121°C for 15 minutes. Cool to 50°C.

4. Add 25 mL sterile 20% lactose solution and mix well.

5. FOR PLATES:

a.Pour 20-25mL of broth with agar per plate

b. Let the agar solidify, then invert the plate

Prepared plates can be stored in the refrigerator for up to 10 days

2. LB Broth, Agar Preparation

1. Suspend the powder in 1 L of purified water

LB Broth - 25g per liter

LB Agar - 15g per liter

3. Heat with frequent agitation and completely dissolve the powder.

4. Autoclave at 121*C for 15 minutes. Cool to 50*C

5. FOR PLATES:

a.Pour 20-25mL of broth with agar per plate

b. Let the agar solidify, then invert the plate

Prepared plates can be stored in the refrigerator for up to 10 days

2. Hogg-Jago Glucose Broth Preparation

1. Mix into 1800ml of purified water: 60 g tryptone, 20 g yeast extract, 10 g KH_2PO_4, 4 g beef extract, 10 g glucose.

2. After mixing, add the remaining water for a final volume of 2 L

3. Autoclave at 121°C for 30 minutes.

3. Media Preparation for Bacillus subtilis

10X Medium A base:

- Yeast extract 10g
- Casamino acids 2g
- Distilled water to 900mL
- Autoclave, then add :
- 50% glucose, filter sterilized 100mL

10X Bacillus salts:

- $(NH_4)_2SO_4$ 20g
- Anhydrous K_2HPO_4 139.7g
- KH_2PO_4 60g
- Tri-sodium citrate 10g
- $MgSO_4 \cdot 7H_2O$ 2g
- SDW to 1000mL

Medium A

- Sterile water 81mL
- 10X Medium A base 10mL
- 10X Bacillus salts 9mL
- L-Tryptophan (11mg/mL) 0.1mL

Medium B

- Medium A 10mL
- 50mM $CaCl_2 \cdot 2H_2O$ 0.1mL
- 250nM $MgCl_2 \cdot 6H_2O$ 0.1mL

Important:

- Autoclave Medium A base before adding glucose, and autoclave Bacillus salts
- Store aliquots of 10X Medium A base 10mL and 10X Bacillus salts 9mL and keep them in the fridge, never use them twice to avoid contamination

4. Inoculation and Incubation

1. From glycerol stock, Streak M17 agar plate, and incubate at 37 °C overnight.
2. Pick colony from from M17 agar, inoculate M17 Broth and Incubate at 37 °C overnight.
3. For 10 ml volumes and up liquid to liquid inoculations, use 100 microliters of innoculum. Incubate at 37 °C overnight.

5. Checking for contamination of media

1. Take 25mL aliquot of media from stock solution into seperate Falcon tube
2. Use 1mL of the sample as a blank for the photospectrometer.
3. Place the remainder of the cells in the 37*C incubator
4. Check optical density every 2 hours to see if there is any growth for the next 6 hours or so. After that check every 12 hours.

Anything larger than +- 2% should lead to conclusion that contamination has occured

6. Making Glycerol Stock of Bacillus Subtilis

1. To freeze competent Bacillus cells, spin down the fresh competent cells to obtain a pellet.
2. Remove all supernatant.
3. Re-suspend cells in 500µL 60% glycerol.
4. Freeze tubes at -80ºC.

B. Transformations

1. Preparation of Electrocompetent E. coli

1. Autoclave 1 L SOB and 3 L ddH_2O, and autoclave or filter sterilize 10 mL 10% glycerol.
Chill the water and 10% glycerol.
*2. Dilute a 10 ml overnight culture into 1 L SOB (without Mg or glucose) and
grow until OD550 of 0.7-0.8
3. Spin down (5000 rpm) and re-suspend in 500-1000 ml cold ddH_2O.
Repeat twice, keeping rotors, buckets, and solutions cold by working in the cold room.
4. Resuspend final pellet in 2 ml 10% glycerol. Make 50 ul aliquots and store at -70°C. It is
not necessary to flash freeze in dry ice/ethanol, but you may if you want.

2. Electroporation of E. coli

1. For each ligation, chill a 1 mm electroporation cuvette and an eppendorf.
2. Aliquot 20-25 microliters of frozen electrocompetent cells (thawed on ice) into each eppendorf.
3. Dilute ligation 1:2 with water. Add 0.26 ul (smallest that can be measured with an
eppendorf p10 pipetteman) to cells.
4. Using a normal 200 microliter pipette tip, mix the cells and ligation by pipetting up and down
twice, then transfer to the bottom of the cuvette. It is best to pipette out the cells at one
end of the channel in the cuvette, i.e., with the pipette tip wedged along one of the clear
walls of the cuvette. Do not pipette in any air bubbles! Don't use a gel-loading tip — it

will slow you down and introduce tiny bubbles.
5. Tap the cuvette to bring the cells down into the channel. Look down into the cuvette to make sure that the cells cover the whole floor of the cuvette without any air spaces. Try to keep cuvette on ice.
6. Zap at 1.6 kV, resistance at 2.5 kV and timing at R5 on a .BTX electroporation The time constant should read 4.9 to 5.1.
7. When light goes from constant to blinking, add 200-1000 microliters broth, preferably *SOC, to cuvette. Squirt it right on the cells or mix with the cells.
8. For amp plasmids, you don't need to let the cells grow up before plating, so you can just keep the cells in the cuvette. Otherwise, transfer to an eppie and shake at 37° for 1 hour.
9. To do more electroporations, hit the reset button next to the voltage knob and zap again.
10. Plate at most 1/5 of the cell suspension per plate, or you will get overgrowth. You can save the rest of the bugs in the cuvette at room temp or 4°.

3. Heatshock tranformation of E. coli

1.Thaw cells on ice for ~2min (10ul Nova Blue cells, 20ul BL21 cells)
2. Add DNA to cells – DO NOT pipet up and down, just mix by swirling the pipet tip or flicking the tube. Use ~30ng of strait plasmid DNA. For a ligation add 5ul.
3. Let the cells incubate with the DNA on ice for 5-20min.
4. Heat shock by holding the tube in the 42 water bath for 30sec and then put directly back on ice.
5. Let recover on ice for 2min.
6. Add 250ul SOC or LB (no antibiotic) and shake at 37 for 30-60min.
7. Plate varying amounts onto appropriate antibiotic plates and put in 37 incubator.
8. Check the next morning for colonies.

4. Preparation of Electrocompetent S. thermophilus

1. Innoculate 2-247.5 mL earlmyer flasks full of fresh Hogg-Jago glucose broth (supplemented with 0.4 M of *sorbitol (final conc.)) each with 2.5 ml of overnight M17 culture and incubate at at 37°C without agitation till optical density at 660 nm is .5
2. Add stock powdered glycine to the culture to a 10% final concentration and continue incubation of culture for 1 hour.
3. centrifuge at high speed, and wash pellet twice in ice-cold Electroporation Buffer (EPB) (5 mM potassium phosphate [pH 4.5], 0.4 M sorbitol, 10% glycerol).
4. re-suspend pellet in 2 ml EPB, freeze in an ethanol-dry ice bath, and store at -80°C until further use.

5. Electroporation of S. thermophilus

1. Thaw cells on ice. Add 1 µg plasmid DNA to 100 µl of electrocompetent cells. Homogenize by gently mixing with pipette several times. Transfer mixture into a prechilled cuvette.
2. perform electroporation with the standard settings

Mode	Prokaryotes “O“
Voltage (V)	1,600 V
Time constant (T)	5 ms

3. After the electric pulse, dilute cells in 1 ml of ice-cold M17 broth containing 0.4 M sorbitol and incubate for 3 h without at 37°C.
4. Spread 100 ul onto antibiotic M17 agar plates, and incubate at 37°C for 48 to72 h, store the cuvette for precautionary measures.

6. Preparation of Competent Bacillus Subtilis

1. Grow one blank plate of *Bacillus subtilis* (or several if you want to transform different strains) for 20 hours at 37ºC (plate been kept on the bench for several days would be better)
2. Inoculate about 12mL of medium with several colonies. Mix the contents of the tube. Check with OD650. Start OD should be between 0.1 and 0.2. Be careful to pipette 0.8mL of this mixture into the cuvette to measure and dispose of it after measurement to avoid contamination in the main mixture.
3. Incubate at 37ºC with vigorous shaking. Read the OD650 every 20min (never keep the solution you used for measuring!)
4. Plot log(OD650) in function of time. After a brief lag, you should observe a exponential increase. After awhile, it will leave the exponential growth; the moment at which it leaves the exponential path is denoted as t0 (3 on the graph). It should take about 100min and the OD should be between 0.35 and 0.55.

1. At t0, incubate for 90 minutes at 37ºC with vigorous shaking.
2. Transfer 0.05mL of this culture into 0.45mL of pre-warmed Medium B in an Eppendorf tube. You have to prepare one tube for each transformation, plus an extra tube for a DNA-less control.
3. Incubate the diluted cultures at 37ºC with shaking for 90min. At this moment, the cells are HIGHLY COMPETENT.
4. To check for competency, you can look at cells under the microscope; competent cells are very motile.

7. Transforming Competent Cells

1. Spin Eppendorf tubes containing cells. Remove 400µL of liquid to keep only 100µL of the culture (to concentrate cells). Re-suspend the cell pellet in the remaining culture.
2. To transform from competent glycerol stocks, spin the tube at about 1600rpm for 20min, remove the supernatant (glycerol), and add 100µL of pre-warmed medium B.
3. Mix the cells thoroughly.
4. Add 0.6µg of DNA to the competent cells.
5. Incubate for 30min at 37ºC with shaking.
6. Plate 100µL of transformed cells onto selective agar.

C. Plasmid Stability Analysis

1. Transform *S. thermophilus* LMG 18311, or *S. thermophilus* DGCC7710 with a kanamycin resistant shuttle vector

2. Plate transformants on a large LM17 plate containing X µg /ml of kanamycin.

3. Inoculate 10 mL of LM17 broth without kanamycin and let grow for 12 hours.

4. Add 1 ml of innoculumn on a large (250 ml) LM17 agar screening plate, add sterile glass beads and let them roll across the entire surface for a consistent lawn. incubate the agar at 37 degrees Celsius overnight.

5. Prepare 1L of X µg /ml kanamycin LM17 broth

6. Add 200ul of prepared kan LM17 broth to each well of 10 96 well plates, and load the plates, and the agar tray into the Genetix Qpix2xt colony picker. set the colony picker to take down coordinates of each picked colony and map each plate-well inoculation accordingly.

7. Take the agar plate out, seal with parafilm, and store in the refrigerator. remember to not the orientation of the plate when you take it out.

8. Take out the 96 well plates put on the lids, and incubate at 37 degrees Celsius overnight.

9. load the plates into the plate reader, CHECK ORIENTATION and read optical density at 660 nm of the entire plate. export the file into excel.

D. DNA Extraction (Mini-Prep)

1. Plasmid DNA Extraction

1. Pellet 1–5 ml 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 the supernatant from step 5 to the QIAprep spin column by decanting or pipetting. Centrifuge for 30–60 s and discard the flow-through, or apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.
7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB. Centrifuge for 30–60 s and discard the flow-through, or apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source. Note: This step is only required when using endA+ strains or other bacteria strains with high nuclease activity or carbohydrate content.
8. Wash the QIAprep spin column by adding 0.75 ml Buffer PE. Centrifuge for 30–60 s and discard the flow-through, or apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source. Transfer the QIAprep spin column to the collection tube.
9. Centrifuge for 1 min to remove residual wash buffer.
10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.
11. Determine concentration using Nanodrop 2000 spectrophotometer.

2. Genomic DNA Extraction

1. Pellet cells by centrifuging 10ml of overnight culture for 10 minutes at 4750 RPM.
Discard the supernatant.
2. Suspend cells in 480µl 50mM EDTA. incubate at 37 degrees C for 10 minutes
3. Add lytic enzyme(s) (120µl) [lysozyme and/or lysostaphin].
4. Incubate at 37°C for 60 minutes.
5. Centrifuge for 2 minutes at 13,000–16,000 × g and remove supernatant.
6. Add 600µl Nuclei Lysis Solution. Pipet gently to mix.
7. Incubate for 5 minutes at 80°C, then cool to room temperature.
8. Add 3µl of RNase Solution. Mix, incubate at 37°C for 60 minutes, then
cool to room temperature.
9. Add 200µl of Protein Precipitation Solution. Vortex thoroughly.
10. Incubate on ice for 5 minutes.
11. Centrifuge at 13,000–16,000 × g for 3 minutes.
12. Transfer the supernatant into two clean tubes containing 600µl of room temperature
isopropanol. Mix.
13. Centrifuge as above, and decant the supernatant.
14. Add 600µl of room temperature 70% ethanol to one tube. Mix and transfer the suspension into the other tube, then mix again.
15. Centrifuge for 2 minutes at 13,000–16,000 × g
16. Aspirate the ethanol and air-dry the pellet for 10–15 minutes.
17. Rehydrate the DNA pellet in 100µl of Rehydration Solution for 1 hour at 65°C
or overnight at 4°C.

E. Isolation of the CRISPR System

Protocol for Annealing Oligonucleotides		

Annealing Buffer: 10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA

1. Resuspending the Oligonucleotides: Resuspend both complementary oligonucleotides at the same molar concentration, using Annealing Buffer (see note below). For convenience, keep Annealing Buffer volume below 500 µl for each oligo. Annealing should perform well over a wide range of oligo concentrations. For larger scale oligo syntheses, it may be necessary to use larger volumes that can be aliquoted after resuspension.
2.
3. Annealing the Oligonucleotides:
    a. Mix equal volumes of both complementary oligos (at equimolar concentration) in a 1.5 ml microfuge tube.
    b. Place tube in a standard heatblock at 90–95 °C for 3–5 minutes.
    c. Remove the heat block from the apparatus and allow to cool to room temperature (or at least below 30 °C) on the workbench. Slow cooling to room temperature should take 45–60 minutes.
    d. Store on ice or at 4 °C until ready to use.
    e. An alternative procedure for annealing involves the use of a thermal cycler. Dispense 100 µl aliquots of the mixed oligos into PCR tubes (500 µl size). Do not overlay the samples with oil. Place the tubes in a thermal cycler and set up a program to perform the following profile:

4.		5.	i6. 7. Heat to 95 °C and remain at 95 °C for 2 minutes; .
8.		9.	i10.11. Ramp cool to 25 °C over a period of 45 minutes; i .
12.		13.	i14.15. Proceed to a storage temperature of 4 °C. Briefly spin the tubes in a microfuge to i draw all moisture from the lid. Pool samples into a larger tube, store on ice or at 4 °C i until ready to use. .

16.
17. Long Term Storage: It may be necessary to aliquot and lyophilize the annealed sample. After drying, the sample may be stored at –20 °C in a desiccated container. Resuspend the annealed oligos at the desired concentration with sterile distilled water. The annealed pair of oligonucleotides is ready for use.

1. Protocol for PCR

Add sequentually to a pcr tube, keep the last three on ice.

1. MilliQ water 33 uL
2. Template DNA 3.5 uL
3. Forward and Reverse Primers .8 uL each
4. 10 uM DNTP 1.5 uL

5. Buffer 10 uL
6 Longtaq polymerase 2 uL

PCR Cleanup

2. Protocol for Creating a Gel

1. Prepare 50 mL of 0.7% agarose gel. Add 0.35 g of agarose to 50 mL of TAE buffer and boil in microwave until all agarose is melted. Stop microwave every 20 to 30 seconds and swirl the solution to help the agarose dissolve. Required about 2 minutes for all of the agarose to dissolve.
a. 50mL 1X TAE buffer
OR 10 mL of 50X TAE
* use 490 mL of DI water to prepare 1X concentrated
2. Let agarose gel solution cool to about 55C (hot but not burning to the touch). This will take only a few minutes. add 10 µL of sybrgold (10 mg/mL). Swirl solution to mix for at least 30 seconds.

3. Prepare the Dye

1. Dilute the stock SYBR® Gold stain 10,000-fold to make a 1X staining solution.
2. Dilute into TBE buffer. Staining with SYBR® Gold stain is somewhat pH sensitive. For optimal sensitivity, verify that the pH of the staining solution at the temperature used for staining is between 7.0 and 8.5.
3. Incubate the gel in 1X staining solution for 10–40 minutes.
4. Place the gel in the staining container, such as a petri dish, the lid of a pipet-tip box-, or a polypropylene container.

Band Extraction

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg ~ 100 µl). For >2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold.
7. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin/apply vacuum again.
8. If the DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 0.5 ml Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
9. To wash, add 0.75 ml Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube. Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2–5 min after addition of Buffer PE.
10. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min at 17,900 x g (13,000 rpm) to remove residual wash buffer.
11. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
12. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center

of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

13. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

F. Biobrick Assembly

Digestion

Digest Upstream Part with EcoRI-HF™ and SpeI.

Upstream Part Plasmid:	500 ng
EcoRI-HF:	1 µl
SpeI:	1 µl
10X NEBuffer 2:	5 µl
100X BSA:	0.5 µl
H2O:	to 50 µl

Digest Downstream Part with XbaI and PstI.

Upstream Part Plasmid:	500 ng
Xbal:	1 µl
Pstl:	1 µl
10X NEBuffer 2:	5 µl
100X BSA:	0.5 µl
H2O:	to 50 µl

Digest the Destination Plasmid with EcoRI-HF and PstI: The Destination Plasmid DNA should either be prepared with PCR or contain a toxic gene (e.g. ccdB, sacB) in the cloning site to avoid the need for gel purification. The Destination Plasmid should also have a different antibiotic resistance marker from both the plasmid containing the Upstream Part and the plasmid containing the Downstream Part to avoid the need to purify the Upstream and Downstream Parts.

Destination Plasmid DNA:	500 ng
EcoRI-HF:	1 µl
Pstl:	1 µl
10X NEBuffer 2:	5 µl
100X BSA:	0.5 µl
H2O:	to 50 µl

Incubate all three restriction digest reactions at 37°C for 10 minutes and then heat inactivate at 80°C for 20 minutes.

Ligation

Ligate the Upstream and Downstream Parts into the digested Destination Plasmid.

Upstream Part digestion:	2 µl
Downstream Part digestion:	2 µl
Destination Plasmid digestion:	2 µl
10X T4 DNA Ligase Buffer:	2 µl
T4 DNA Ligase:	1 µl
H2O:	11 µl

Incubate at room temperature for 10 minutes and then heat inactivate at 80°C for 20 minutes.

Transform 2 µl of the ligation product into 50 µl of competent *E. coli* cells (or other suitable host strain). Select using the antibiotic corresponding to the Destination Plasmid.