iGEM's Protocols

LB medium preparation

1. Dissolve the following components in MQ water.

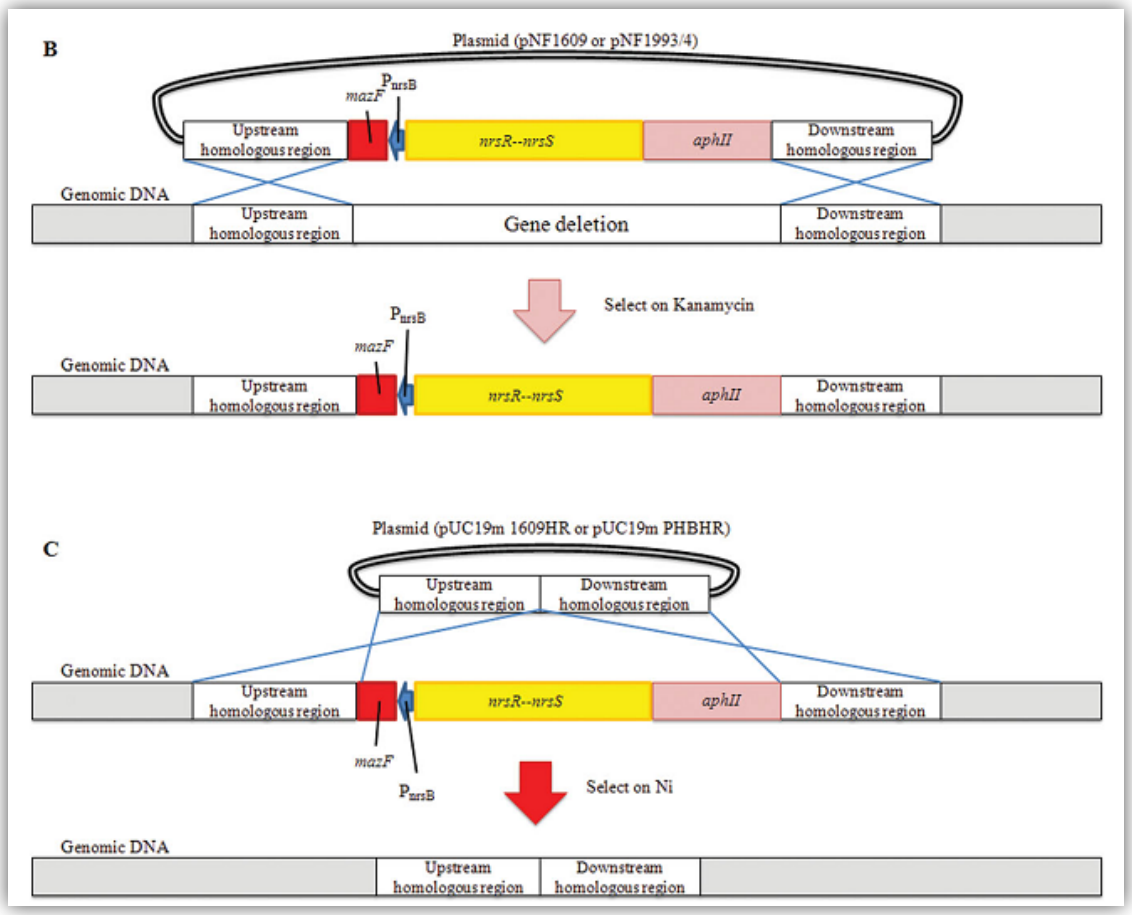
Component	For 1L	For 0.5 L	Labcode
Yeast Extract	5 g	2.5 g	Y4
Tryptone	10 g	5 g	T125
NaCl	10 g	5 g	N59

2. Autoclave for 20 min at 121°C.
3. To make solid LB plates, 1.5% (m/v, e.g. 1.5 g in 100 ml liquid LB media) agar (labcode A21) should be added before autoclaving. After the autoclaving, take the bottle to 55°C incubator to make sure the agar does not solidified. Add antibiotic whenever necessary when the media is not hot (antibiotic will be degraded quickly in the media at high temperature).
4. The concentrations for antibiotics commonly used are listed as follows:

Antibiotics	Stock concentration	Add ratio	Final concentration
Ampicillin	100 mg. mL^{-1}	1:1000	100 μg. mL^{-1}
Spectinomycin	50 mg. mL^{-1}	1:1000	50 μg. mL^{-1}
Kanamycin	50 mg. mL^{-1}	1:1000	50 μg. mL^{-1}
Chloramphenicol	34 mg. mL^{-1}	1:1000	34 μg. mL^{-1}

Normal Molecular Cloning (e.g. knock out plasmids construction)

The principle for markerless knock out one gene in the genome of *Synechocystis* is as follows:



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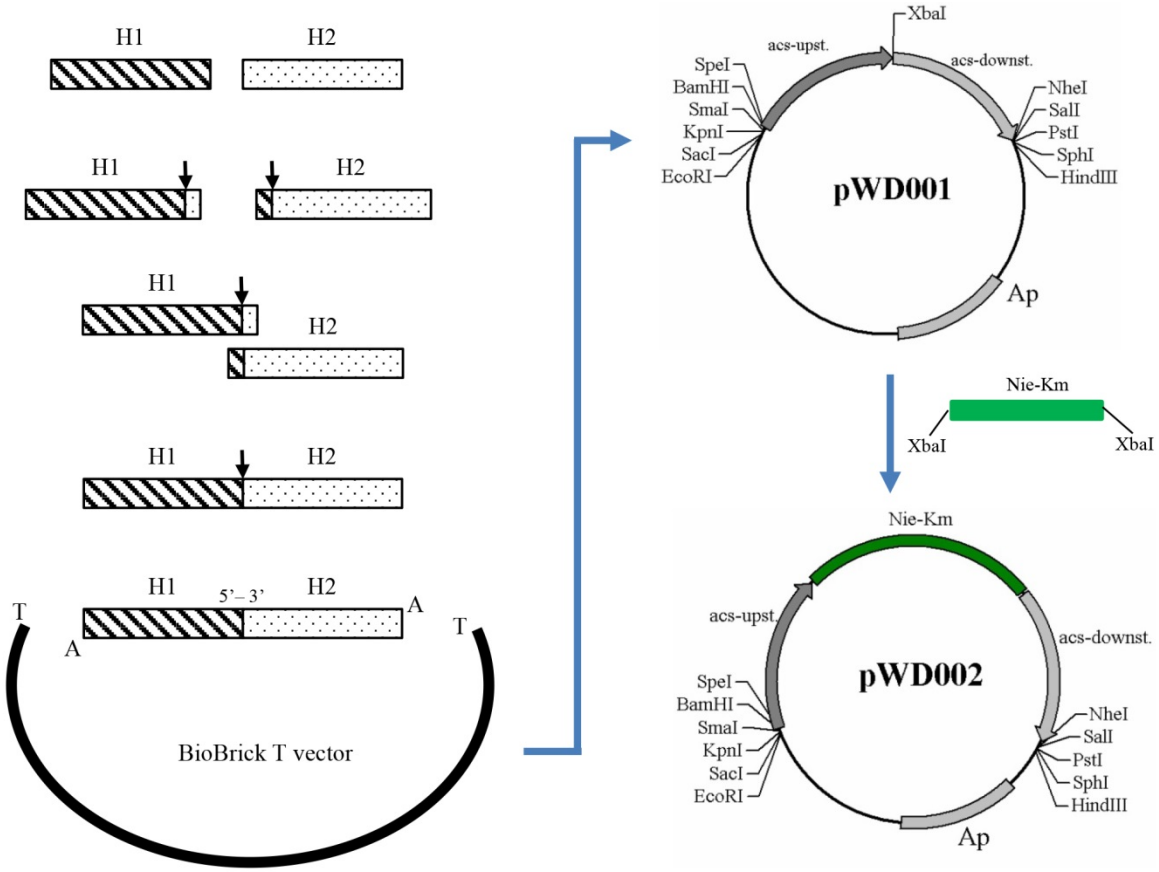
Basically, two plasmids are needed. One contains only the upstream and downstream homologous region, while another one contains an extra selection cassette in the middle of the upstream and downstream homologous region. To construct these two plasmids as convenient as possible, we adopted a fusion-PCR based strategy. The schematic is as follows.



1. PCR amplify both H1 and H2 homologous regions. All the sequence information about Synechocystis can be found on the website of Cyanobase (http://genome.microbedb.jp/cyanobase/Synechocystis). For gene knockout plasmid construction, 1 kb fragment of **each** H1 and H2 are needed. For primers design, I often use this website (http://eu.idtdna.com/calc/analyzer) to check the tendency of primers to form self-dimer and hetero-dimer. The restriction site which introduced depends on the sequence of both H1 H2 homologous regions and the T vector, could be either AvrII, NheI, SpeI, or XbaI, which all have the same compatible ends after enzyme digestion.
2. For PCR amplification, *pfu* DNA polymerase is used. Other polymerase which has a high fidelity can also be used. After gel purification of H1 and H2, a fusion step is carried out which only add H1 and H2, DNA polymerase, Buffer and dNTP for about 13 cycles. The program for the fusion-step is as follows.

Step 1	95°C	30 s
Step 2	55°C	30 s
Step 3	72°C	4 min

3. After fusion step, normally, 5 µl fusion product will be taken as template for whole PCR amplification of the fusion fragment.
4. The fusion fragment will be further purified through gel purification. Then one single "A" will be added to the 3 overhang of the fragment, according to the protocol below.

Add "A" to the blunt end of PCR fragment

For 50 µl Mixture

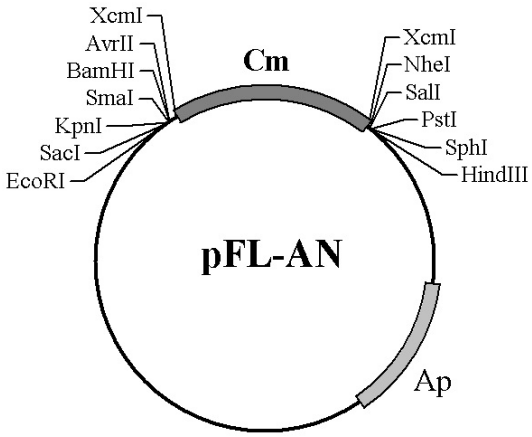
DNA	?
10×Buffer +$(NH_4)_2SO_4$-$MgCl_2$	5µl
$MgCl_2$	4µl
dATP(10mM)	1µl
Taq DNA Pol.	0.5µl
ddH_2O	fill up to 50µl

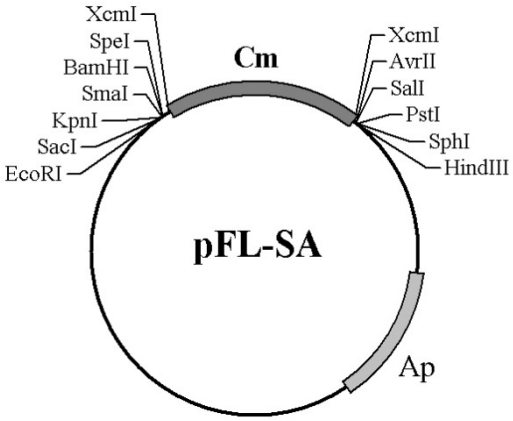
72℃ 10~30 min should be enough

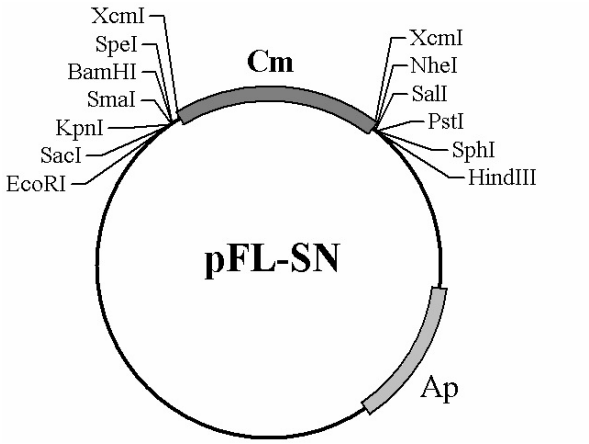
PS：

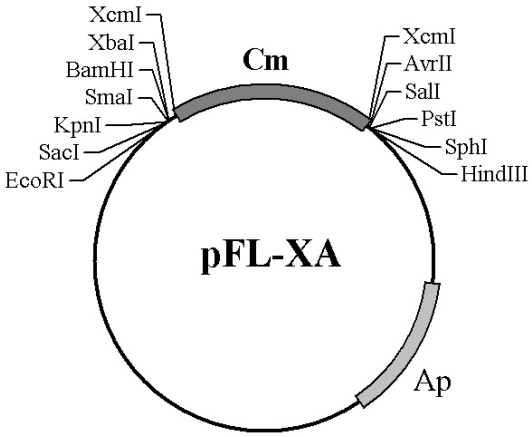
- Do not add water. During the last step of gel extraction, use 45 µl ddH_2O to elute, and so you can get at least 39.5 µl of DNA product, which can be used directly for add "A" reaction;
- PCR machine at 72℃ for 20 min;
- After add "A" reaction, use the PCR Cycle Pure Kit to purify the DNA, then the fragments can be directly used for TA cloning;
- Taq DNA pol. has a specific substrate requirement for only dATP (probably 99%). So if you add dNTP by accident, theoretically, it should be fine.

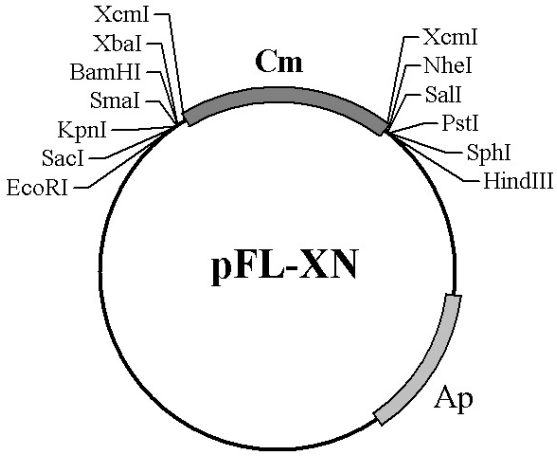
5. Product will be further purified through PCR purification kit. And the product with an extra single "A" after purification can be readily used for ligation to the BioBrick T vector.
6. For a detail construction of BioBrick T vectors, please check this paper-- *Enhancing photosynthetic production of ethylene in genetically engineered Synechocystis sp. PCC 6803*. There are 6 different T vectors in stock, and the plasmid map are as follows: (A-AvrII, N-NdeI, S-SpeI, X-XbaI)

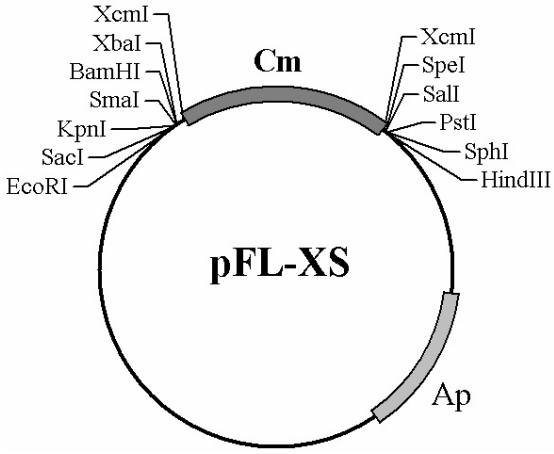












7. These T-vectors can be prepared simply by enzyme digestion using XcmI, and further gel purification. The only differences between each T-vector is the combination of two restriction site from a total of AvrII, NheI, SpeI, and XbaI.

8. Before the ligation, run a gel to check the quality and concentration of both fragment and T vector. A mole ratio of from 3 to 8 fragment to 1 T vector is often adopted. (For ligation time, if there is no time for further transformation, put the ligation mixture at room temperature for 30 min then take it to the fridge overnight, and do the transformation the next day. Otherwise, do the ligation at 16°C for 4 hours, then transform to *E.coli* DH5α).
9. Here is a protocol to prepare chemically competent cells.

1. Inoculate a single colony or a glycerol stock in LB plus the appropriate marker, and grow this overnight.

2. Add 200 µL to 50 mL pre-warmed LB and grow until it reaches an OD_{600} of 0.3-0.6 at 37°C, 250 rpm.
   Note: You can also pick one single colony and directly inoculate into the liquid medium.
   Note: For optimal results, grow to an OD_{600} of 0.35-0.4.
   Note: The procedure can be up- or downscaled by multiplying all amounts with the same factor.

3. Centrifuge for 8 min at 800 ×g, 4°C in a Sorvall RC-5B with an SS-34 rotor. Keep the cells on ice for all subsequent steps.

4. Remove the supernatant and gently resuspend the cells in 1/10 volume (5 mL) ice-cold *0.1 M $CaCl_2$* (solution A) using a pipet.

5. Centrifuge for 8 min at 800 ×g, 4°C.
   Note: The cell pellet should now have an abnormal shape.

6. Remove the supernatant and gently resuspend the cells in 1/10 volume (5 mL) ice-cold *0.1 M $CaCl_2$+16%glycerol* (solution B).

7. Allocate 100 µL each to sterilized pre-cooled EP tubes, and store them at -80°C.

   Note: The cells can be stored for months without much loss of transformation efficiency if repeated freeze/thaw cycles are prevented.

10. For *E.coli* DH5α transformation, please check the following protocol

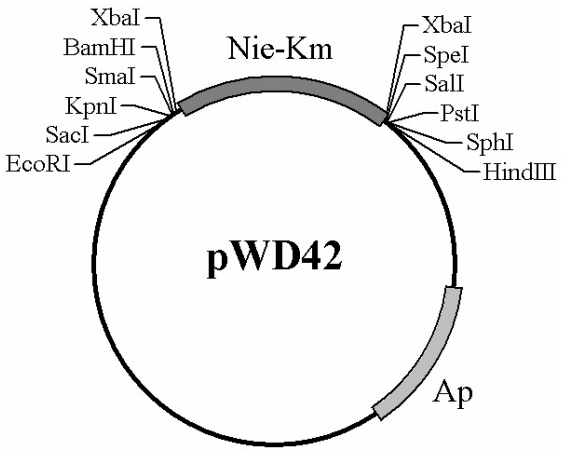
1. Add an appropriate amount(5 µl out of total 10 µl ligation mixture) of DNA to 100 µL thawed cells, and incubate for 30 min on ice. Include a negative control without any DNA.
   Note: The transformation efficiency should be so high that it is suitable to dilute plasmid purifications to less than a 100 ng. Alternatively, fewer cells can be used.

2. Heat-shock the cells for 90 s in a water bath at 42°C. Immediately transfer the cells to ice and incubate for 1-2 min.

3. Add 900 µL pre-warmed LB and incubate for 60 min at 37°C, 250 rpm.
   Note: Instead of LB, other rich media such as TSB also work.

4. Gently spin down (5000 rpm for 3 min) to concentrate all the cells and plate all the cells on an LB plate. Incubate the plates overnight at 37°C.
   Note: incubate less than 14 hours when using only ampicillin as marker.

11. Normally, 10 clones on the plate will be picked for PCR confirmation. Then choose two correct clones and inoculate in the 5 ml liquid culture overnight. Extract the plasmids for sequencing, and for further plasmid construction.
12. Till now, plasmid with only H1 H2 homologous region is constructed. Next step, selection cassette (3.7 kb) prepared from pWD42 through enzyme digestion by XbaI, will be ligated to the vector cut by the restriction enzyme which recognize the restriction site introduced through primers.



13. So far, the plasmids for a markerless gene knockout are constructed. The transformation protocol for *Synechocystis* is as follows.

Counter-selected marker-less gene knock-out method

1. **First round transformation**: selecting fully segregated mutant through kanamycin
    1) Plasmid construction: the whole cassette containing nickel induced expression of *mazF* flanked by both upstream and downstream fragments of the target gene. For each upstream and downstream fragment, 1 kb is recommended.
    2) Strains preparation: cells for transformation can be picked directly from the plate, or collected from liquid culture (normally 1 ml cultures OD=1 is enough for one transformation). Better cell quality is recommended.
    3) Washing the cells twice using fresh BG11 media (without antibiotic) by centrifuging at 5000 rpm for 5 min, then concentrated the total liquid volume to about 200 μl.
    4) Add plasmid into the concentrated cultures to a finally plasmid concentration of 10 μg mL^{-1}, mix gently and put it into the light incubator for about 4 to 6 hours.
    5) Spread the mixture on the commercial membrane resting on the BG11 plate (without antibiotic), and put the plate into the light incubator for about 16~24 hours;
    6) Then transfer the membrane with cultures to another BG11 plate containing the corresponding antibiotic for selection. Normally, single colony appears around one week;
    7) Pick the colony and streak sequentially on the BG11 plate containing kanamycin and BG11 plate with nickel, respectively. Colonies which grow on the BG11 plate containing kanamycin but not on BG11 plate with nickel are candidates for PCR confirmation. Further segregated in the liquid culture with kanamycin (50 μg mL^{-1}) if necessary.

2. **Second round transformation**: remove the cassette to get marker-less mutant by nickel
    1) Plasmid construction: only upstream and downstream fragments of the target gene;
    2) the protocols for second round transformation are almost the same as the first round transformation, except for transferring the membrane with cultures to the BG11 plate with nickel (20 μM) and streaking sequentially on the BG11 plate with nickel and BG11 plate containing kanamycin, respectively;
    3) Then pick the colonies which grow on the BG11 plate with nickel but not on BG11 plate containing kanamycin for further PCR confirmation.

PS: normally it takes 5 weeks to marker-less knock out one gene: 3 weeks for the first round and 2 weeks for the second round transformation. For PCR confirmation, at least 35 cycles are recommended. Besides, please make sure you get fully segregated mutant in the first round transformation.

Simple gDNA preparation protocol for *Synechocystis*

1. Cell collection: get some cells either from plate or liquid culture then centrifuge (12,000 rpm for 1 min) in a 1.5 EP tube (For OD=1~1.5, I normally take 1 ml);
2. Remove the supernatant. Resuspend cells in 200 μl TE buffer, and add 200 μl phenol/chloroform/isoamylalcohol(25:24:1). Then add some glass beads. Vortex for 5 min (I suggest all the steps involving chloroform should be carried out in fume cupboard);
3. Centrifuge for 5 min at 12,000 rpm, and transfer the up transparent phase to a new 1.5 EP tube. Add equal volume (about 200 μl) of chloroform/isoamylalcohol(24:1), then invert the tube several times;
4. Centrifuge for 5 min at 12,000 rpm, and transfer the up transparent phase to a new 1.5 EP tube. Add 1/5 volume (about 40 μl) 5 M NaCl and 2～3 volumes (about 400 μl) absolute ethanol, invert the tube several times for mixing, and put the tube in the -20℃ freezer for at least 30 min to make DNA precipitation.(Normally you cannot see the DNA by naked eyes);
5. Centrifuge for 10 min at 12,000 rpm, remove the liquid. Add 500 μl 70% (V/V) ethanol to wash the DNA;
6. Centrifuge for 5 min at 12,000 rpm and remove the liquid carefully. (Normally I use the pipette with 10 μl tips to remove the remaining liquid);
7. Dry the tube in room temperature for about 10 min. (I normally turn on the flow hood for drying the samples);
8. Add 20~30 ddH_2O or TE buffer to dissolve the gDNA, and measure the concentration by Nanodrop if you want.

BG11 media preparation

To make BG11 media, first is to make all the Stock solutions (1000 x concentrated)

Composition

#	Lab code	Formula	Molecular Mass [g / mol]	Molarity [mol/L]	Mass [g/L]	Remarks
1		K_2HPO_4 . 3 H_2O	228.22	0.1754	40	I, II
2		$MgSO_4$. $7H_2O$	246.47	0.3043	75	
3		$CaCl_2$. $2H_2O$	147.01	0.2449	36	
4		Citric Acid	189.01 (?)	0.0317	6	
5		Ferric ammonium citrate	unknown		6	I, II, III
6		EDTA . 2Na	unknown		1	
7		Na_2CO_3	105.99	0.1887	20	
A5		H_3BO_3	61.83	0.046256	2.86	
		$MnCl_2$. $4H_2O$	197.90	0.009146	1.81	
		$ZnSO_4$. $7H_2O$	287.56	0.000765	0.222	
		Na_2MoO_4 . $2H_2O$	241.95	0.001611	0.39	
		$CuSO_4$. $2H_2O$	195.64	0.000409	0.08	
		$CoCl_2$. $6H_2O$	237.93	0.000042	0.01	

Remarks

I: Autoclave separately for 20 minutes at 121 C

II: Store at 4˚C

III: xFe . $C_6H_5O_7$. yNH_4 / ***RES20400-A7 SIGMA***

Procedure

1. Find appropriate bottles for all the stocks (usually 100ml or 1L bottles).

Per stock, for 1 litre:

2. Measure 1 litre of MQ water and pour into bottle
3. Mark water level
4. Remove part of water and keep aside
5. Dissolve Chemical
6. Add water until mark is reached

PS: For stock solution A5 preparation, add chemical one by one. Make sure the chemical is completely dissolved, then add the next one.

BG11 (1X) media preparation:

For 1 litre of BG-11, using 1000x concentrated stock solutions.

1. Clean flasks and bottle (rinse with tap and rinse with distilled)
2. Add 992 mL of MQ water to flask
3. Add 1mL of stock solution 2, 3, 4, 6, 7 and A5 to flask
4. Add 1.5 gr/L of Sodium Nitrate to flask
5. Stir solution (magnetic stirrer)
6. Pour solution from flask into bottle
7. Autoclave bottle for 20 min at 121 ºC. Loose cap secured with 2 tapes
8. Let bottle cool down to room temperature
9. Add 1mL of stock solution 1 and 5 to bottle under flow-hood keep sterile
   If necessary (long cultivations):
10. Add 10 mL TES-NaOH(1 M, pH 8.0) Buffer

BG11 (1X) media plate preparation:

For 1 litre of BG-11, using 1000x concentrated stock solutions.

1. Clean flasks and bottle (rinse with tap and rinse with distilled)
2. Add 992 mL of MQ water to flask
3. Add 1mL of stock solution 2, 3, 4, 6, 7 and A5 to flask
4. Add 1.5 gr/L of Sodium Nitrate to flask
5. Stir solution (magnetic stirrer)
6. Pour solution from flask into bottle
7. Add agar (for bacterial) at a final concentration of 1.5% (m/v)
8. Autoclave bottle for 20 min at 121 ºC. Loose cap secured with 2 tapes
9. Take the bottle to the 55 ºC incubator
10. Before making the plates, add 1 mL of stock solution 1 and 5 to bottle, filter sterilized 30% $Na_2S_2O_3$ (1 ml to 100 ml total volume), filter sterilized 1 M TES-NaOH Buffer (pH 8.0, 1 ml

to 100 ml total volume) under flow-hood to keep sterile, also add antibiotics if necessary at a final concentration: Kanamycin (50 μg. mL^{-1}); Spectinomycin (20 μg. mL^{-1}); Chloramphenicol (10 μg. mL^{-1}).

Glycerol stock preparation and revive the cells from glycerol stock

Glycerol stock for *E.coli*:

1. Pick a single clone and inoculate into the liquid LB media, with antibiotics if necessary. 37°C, 250 rpm, overnight.
2. For 1 ml glycerol stock, add 800 μl cultures and mixed with 200 μl sterilized 80% glycerol. Gently mixed, and put into the -80 °C freezer.

Glycerol stock for *Synechocystis*:

1. Prepare relatively large amount of liquid culture at a OD around 1 to 2.5 (better if OD less than 3 in the flask). Concentrated the cultures 10 times by spin down the cells (5000 rpm, 5 min);
2. Wash the cell pellets twice with fresh BG11 media (without antibiotics) through 5000 rpm, 5 min;
3. Remove the supernatant, add 800 μl fresh BG11 media, re-suspend the cells. Add 800 μl cultures and mixed with 200 μl sterilized 80% glycerol. Gently mixed, and put into the -80 °C freezer.

Revive the *E.coli* from glycerol stock:

1. Get the glycerol stock from -80 °C freezer with a cool container. With the protection of the flame to keep everything sterilize, pick some ice cultures using a inoculation needle and inoculate to the liquid LB media (with antibiotic if necessary).
2. 37°C, 250 rpm, overnight.

Revive the *Synechocystis* from glycerol stock:

1. Get the glycerol stock from -80 °C freezer with a cool container. With the protection of the flame to keep everything sterilize, pick some ice cultures using a inoculation needle and inoculate to the liquid BG11 media in the flask without antibiotic. Mix them by gently shaking the flask, add more ice cultures until you can see the green color of the culture. (I normally add 10 ml BG11 media, and pick more ice cultures from the glycerol stock)
2. Put the flask into the light incubator (not high light), and cells will be ready for further experiment about 5 days later.

Colony PCR confirmation

For *E.coli*:

1. Prepare the PCR reaction mixture, and 20 μl mixture is allocated to each PCR reaction tubes. 2 times MyTaqmix is recommended.
2. Use the sterilized toothpicks or tips to pick the clones, first to streak to another new plate, then put into the PCR reaction mixture. Stir as hard as possible.
3. Start the PCR reaction, and gel electrophoresis.

For *Synechocystis*:

1. Prepare PCR tubes, and fill in 4 μl MQ water to each tube;
2. Use the tips to pick some *Synechocystis* cells on the plate, then re-suspend in the MQ water inside the tube till you can see the green color (more cells are recommended);
3. Put all the tubes on a float foam, and throw it to the container with liquid N_2 to freeze the samples;
4. Around 1 min later, put this float foam into a container with hot water (above 60 °C), wait for 2 min then throw back to the liquid N_2 container;
5. After 5 cycles, spin down the cell debris, and take 1 μl of the supernatant as template for colony PCR confirmation (2 times MyTaqmix is recommended).