Protocol of NJAU_China

College of Life Sciences, Nanjing Agricultural University

NJAU_China iGEMers

Sensor Part Protocol

1. Acquire three sequences ,*copA* promoter（259bp），*maro*（98bp） and *cueO* promoter（103bp） ,from E.coli's genome

1. Extract the whole genome of *E.coli* DH5α as template
2. Utilize PCR to acquire target sequences.(Fig.1)

PCR system

2*Master Mix（Vazyme™）	5μL
DD H_2O	3μL
Template（genome of *E.coli* DH5α）	1μL
Forward primer	0.5μL
Reverse primer	0.5μL
	10μL

PCR procedure

94℃	5min	
94℃	30s	**35cycles**
60℃	30s	
72℃	30s（1kb/min）	
72℃	5min	

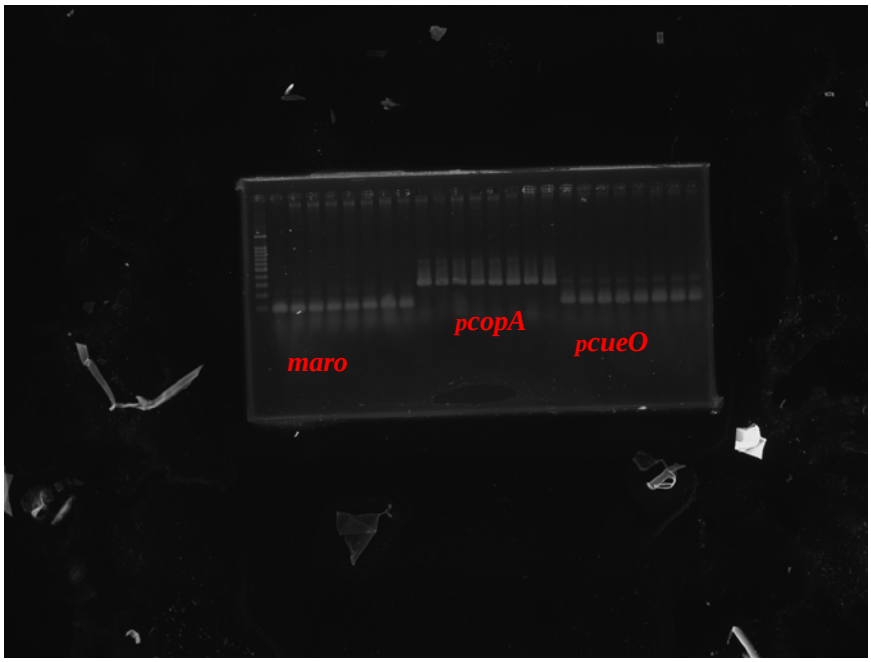


Fig.1 Three sensor candidates was acquired by PCR

2. Gel extraction （purify the PCR product）

For getting three parts and purifying the PCR product,we decided to use method of gel extraction.

1.Cut the DNA stripes from the gel under the UV light（keep the gel thin when you make it.）

2.Use E.Z.N.A Gel Extraction Kit to extract target sequence from the gel just cut off.

3.Test concentration of purified products by Nano Drop

copA promoter 49.5ng/L *maro* 23.2ng/L *cueO* promoter 33.2ng

3. Transformation（acquire plasimid of BBa_E0840 from 2014 distribution）

1.Thaw competent cells on ice. Label one 1.5ml microcentrifuge tube for each concentration and then pre-chill by placing the tubes on ice.
2.Pipet 1μL of DNA into each microcentrifuge tube. For each concentration, use a separate tube.
3.Pipet 50μL of competent cells into each tube. Flick the tube gently with your finger to mix. Incubate on ice for 30 minutes. Pre-heat waterbath now to 42°C.
4.Heat-shock the cells by placing into the waterbath for 1 minute. Be carefμL to keep the lids of the tubes above the water level, and keep the ice close by.
5.Immediately transfer the tubes back to ice, and incubate on ice for 5 minutes.
6.Add 200μL of SOC media per tube, and incubate at 37°C for 1 hours.
7.Prepare the agar plates with proper antibiotic(chl in this transformation).
8. Centrifuge tube 2500rpm/2min and discard supernatant and slightly pipet to resuspend the bacteria
9.Spread the agar plates

4. Miniprep Plasmid（BBa_E0840）

1. Use Plasmid Miniprep Kit
2. Test concentration of plasmid by Nano Drop（74ng/L）

5. Restriction digest and linkage（link three candidates with BBa_E0840）

1. Make single restriction digest to BBa_E0840 in PCR tube

Restriction digest system	
NEB 10*Cutsmart buffer	2 μ L
NEB XbaI	0.5 μ L
DD H_2O	13.5 μ L
BBa_E0840	4 μ L
	20 μ L

2. Put tube at 37℃ and wait for 3-4h
3. Add 1 μ L NEB Alkaline phosphatase for preventing BBa_E0840 from linking itself,and wait for 30min-1h（Needn't add any buffer）
4. Make double restriction digest to three candidates

Restriction digest system（*copA*）	
NEB 10*Cutsmart buffer	2 μ L
NEB XbaI	0.5 μ L
NEB SpeI	0.5 μ L
DD H_2O	12 μ L
copA	5 μ L
	20 μ L

Restriction digest system（*maro*）	
NEB 10*Cutsmart buffer	2 μ L

NEB XbaI	0.5 μL
NEB SpeI	0.5 μL
DD H_2O	6 μL
maro	11 μL
	20 μL

Restriction digest system（*cueO*）

NEB 10*Cutsmart buffer	2 μL
NEB XbaI	0.5 μL
NEB SpeI	0.5 μL
DD H_2O	12 μL
cueO	5 μL
	20 μL

6.Use E.Z.N.A Cycle-pure kit to purify restriction digest of *copA* promoter and BBa_E0840，
And put the *maro* and *cueO* promoter in 65℃ waterbath for 25min for inactiving enzyme

7.Use NEB T4 DNA Ligase and 10* Reaction Buffer

Linkage system（*maro*）

10* Reaction Buffer	2 μL
T4 DNA ligase	0.5 μL
BBa_E0840（purified）	5 μL
maro	8 μL
DD H_2O	4.5 μL
	20 μL

Linkage system（*copA*）

10* Reaction Buffer	1 μL
T4 DNA ligase	0.5 μL
BBa_E0840（purified）	2 μL
copA	1 μL
DD H_2O	5.5 μL
	10 μL

Linkage system（*cueO*）

10* Reaction Buffer	2 μL
T4 DNA ligase	0.5 μL
BBa_E0840（purified）	4 μL
cueO	6 μL
DD H_2O	7.5 μL
	20 μL

8.Use reactive product to make transformation

9.Individually spread bacteria on three the agar plates,and put them in 37℃ overnight

6.colony PCR（For examing whether the candidates link with BBa_E0840 in forward direction）

1.Choose a forward primier, prefix,from pSB1C3 and a reverse primier in those three candidates individually.
2.Prepare mix solution（above）and pipet 9 μL in each PCR tube

2*Master Mix（Vazyme^{TM}）	5 μL*n
DD H_2O	3 μL*n
Forward primer	0.5 μL*n
Reverse primer	0.5 μL*n
	9n

（n stands for the number of colony which you want to test, usually it need to be more than what you want ）

3.Prepare a empty agar plates containing chl.
4.Use sterile tip mildly dip on a single colony on plates（Change a new tip before dip a new colony）
5.Use tip with bacteria to dip empty plates
6.And drop the tip in one tube with 9 μL mix solution
7．Discard the tip and send those tubes to PCR machine
8. The construction is accurate if DNA strips appear(positive), in reverse the construction is fa μLt (negative)(Fig.2).

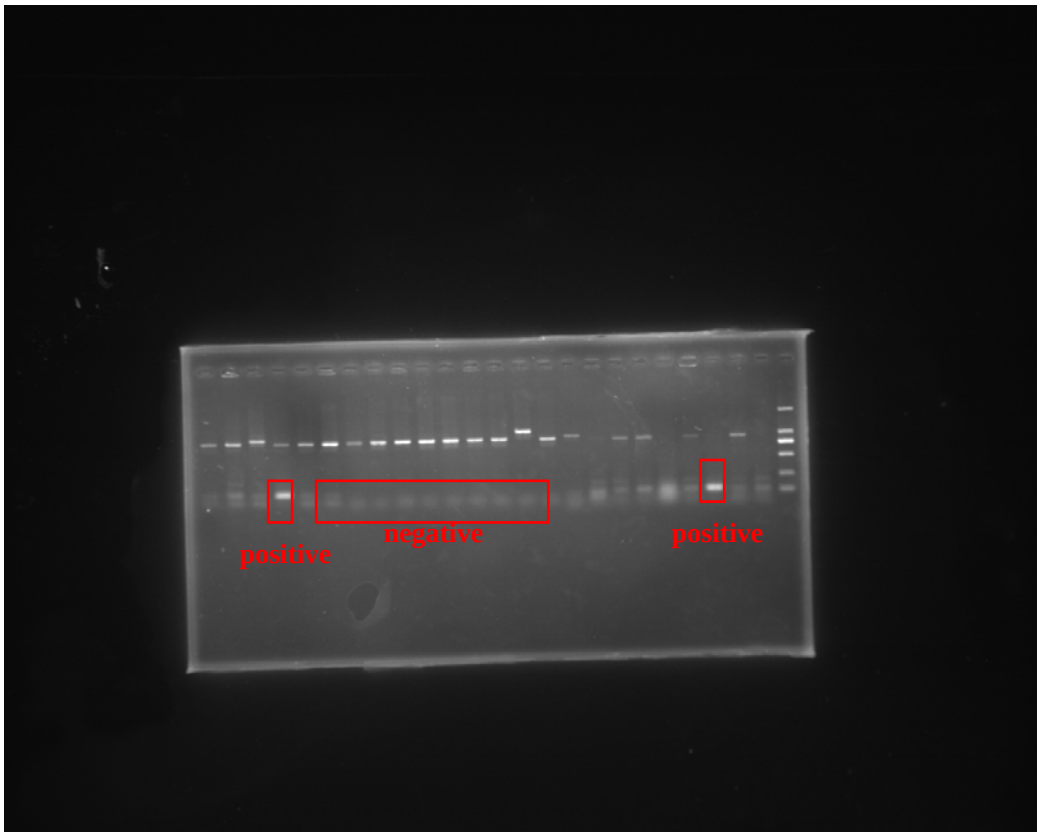


Fig.2 Colony PCR

7.sensitivity experiment

1.Do the transformation and acquire the three DH5α single colonies on the plates

2. InocμLate the single colony individually to LB and cμLture overnight at 37℃

3.Pipet 1% bacteria to 100ml fresh LB and cμLture for 4h

4.Pipet 200μL bacteria to 96-well tissue cμLture plate's each well（Fig.1）

5.Set six different concentration of copper,0.25mg/L ,0.5mg/L,1mg/L,2mg/L,4mg/L and 8mg/L,to treat three types of DH5α containing three constructs individually.

6.Utilize microplate reader to test fluorescence each 20min in 490nm exciting light

8.specificity experiment

1. InocμLate the single colony of three types of DH5α individually to LB and cμLture overnight at 37℃

2.Pipet 1% bacteria to 100ml fresh LB and cμLture for 4h

3.Pipet 200μL bacteria to 96-well tissue cμLture plate's each well（Fig.1）

4.Set six metal,Al^{3+},Li^{+},Mn^{2+},Pb^{2+},Zn^{2+} and Cu^{2+} at the same concentration,4mg/L, to treat three types of DH5α containing three constructs individually.

5.Utilize microplate reader to test fluorescence each 3h in 490nm exciting light

*gsh*F Protocol

1. GSHf extraction, PCR and transformation

Extract *gsh*F:

1. Cultivatie Streptococcus thermophilus in MRS medium, 42 ° c, 72 h.

2. Add1ml liquid into a 2ml centrifuge tubes, 8000rpm,2min.

3. Resuspend the sterile water 3 times, 8000rpm,2min.

4. Add 270ul,1XTE and suspend, shake vigorously and then add 15ul lysozyme, put in 0 ° c ice bath immediately for 30min.

5. Add 15ul10% SDS at room temperature and mix, adding 3~4ul of proteinase k, put in 37 ° c water bath immediately for 30min.

6.Add 200ul, 5mM of Nacl, and violent. Then add 500ul of of Phenol:chloroform:isoamylalcohol (25:24:1)and shake vigorously . Centrifuge at 10000 rpm for 110 min. After centrifugation, take supernatant and repeat the extract again until no a white precipitate.

7. Take the supernatant to a new centrifuge tube, add 0.8 volume of 1XTE,1 volume of isoamylalcohol, and mix gently. Precipitate overnight at -20oC or at -70 ° C for 15~20min.

8. Centrifuge at10000 rpm for 10min and wash the precipitate for 3 with 70% cold ethanol and air dry. Resuspend the precipitate in 30~50dd water soluble.

2.PCR

Primer sequences

Forward Primer: CGGAATTCATGACATTAAACCAACTTCTT

Reversed Primer: GAATTCTTAAGTTTGACCAGCCACTATTTCT

Without knowing the annealing temperature conditions, we set up a series of annealing temperature gradient, 50.9 ° c, 51.8 ° c, 52.7 ° c, 53.6 ° c, 54.5 ° c and 55.5 ° c, 56.4 ° c, 57.3 ° c,

58.2 ° C,59.1 ° c.
Ten sets of experimental data.

PCR solution (20ul)

Reagent	/μL
Buffer	2
DNTP	1.6
Mg ion	1.2
R	1
F	1
Taq enzyme	0.1
DD water	12.1
DNA	1

PCR procedure system

Temperature	Time	Purpose	Cycle
94	5min	pre-degeneration	
94	30s transgender		32 cycles
Annealing temperature gradients on	1min	annealing	32 cycles
72	2min	expansion	32 cycles
72	10min		
4			save

3. DNA agarose gel electrophoresis

1. Seal the two ends of the gel-forming modelwith medical tape properly, positioned horizontally, posit the selected comb properly, leave1mm space between bottom between the comb and the model.

2.Weight 0.24g agarose for DNA electrophoresis, put it in into 250ml erlenmeyer, add 50ml of 1xTAE buffer, after mixing, put the on erlenmeyer in the oven, heated to boiling until the agarose is completely dissolved.

3.Turn off the microwave, remove the Erlenmeyer at room temperature until gelatin completely solidified, it takes about 30 minutes, remove the tape, pull out the comb, put the plastic sheet into the electrophoresis tank.

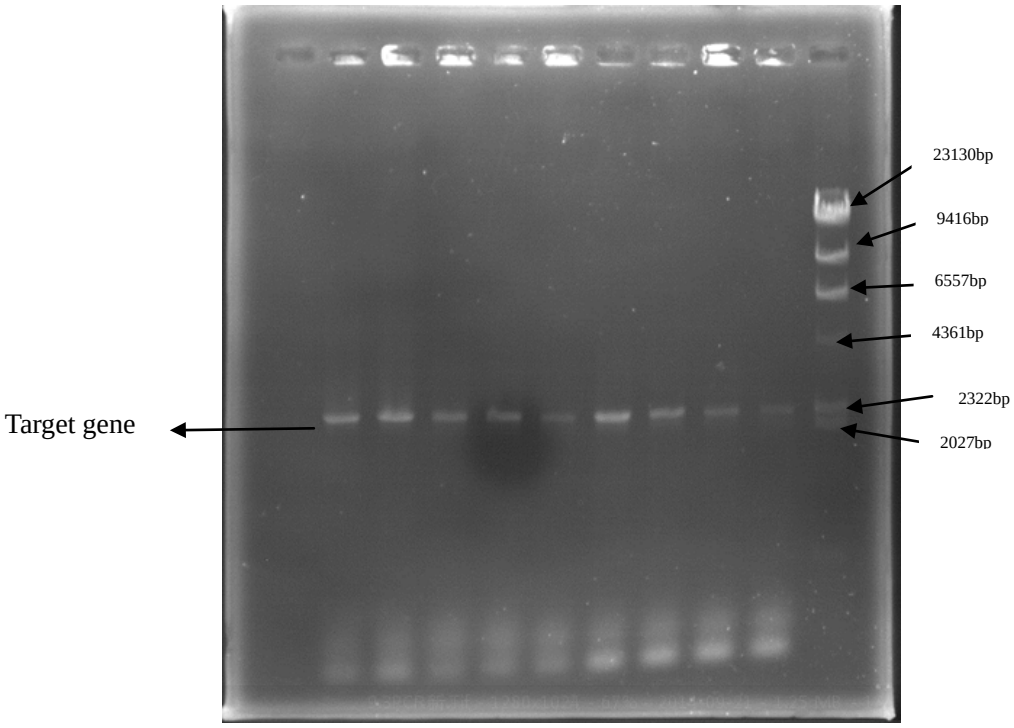
4.Add 1xTAE buffer into the electrophoresis tank above gel surface around high 2mm.

5.Using transfer pipe to transfer the sample. Add 5 ul of DNA sample and DNA:10x loading

buffer (5:1) to spotting holes.

9.Powered on, and adjust the voltage to 80 volts, electrophoresis for 30 minutes, remove the gel plate, staining in ethidium bromide solution for 20min. observe the result under UV light.

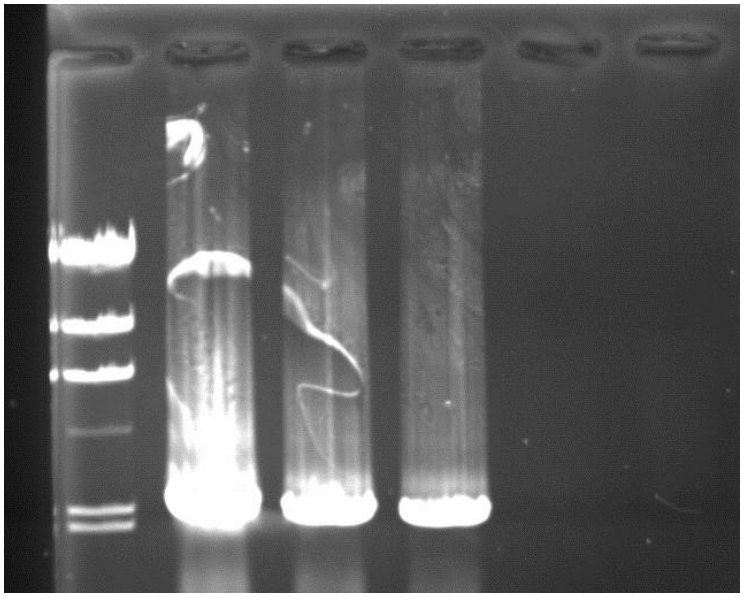
The results:



Temperature in the order from left to right, is 50.9 ° c 51.8 ° c, 52.7 ° c, 53.6 ° c, 54.5 ° c and 55.5 ° c, 56.4 ° c, 57.3 ° c, 58.2 ° C,59.1 ° c. the results show that ten can be amplification of target gene, but the 50.9 ° c, 51.8 ° c and 55.5 ° c temperature Strip indicating maximum amplification of target gene products. Soselect 55.5 ° c as the annealing temperature.

4. Cut the plastic recycling

Once again the PCR, gene recycling purpose.



For cutting plastic recycling purpose stripe .

5.Building efficient replicated vector

Connected with the t-carrier system

Reagent	/μL
T-carrier (PMD19T)	1ul
InsertDNA	4ul
Solution Ⅰ	5ul

Response 4 ° c overnight.

Seven: the recombinant plasmids into cells: (heat-shock method)

1. From-80 c freezer remove divide into cells, incubated on ice.

2. Reformer tubes linking 3ul DNA solution into 30ul cells, mix gently.

3. On ice for 30 min

4. Add 42 deg c in the water bath, heat-shock 90 seconds

5. Quickly insert on the ice and leave for 3 minutes;

6. Add 400 μl of SOC medium to a centrifuge tube, standing in the 37 c water bath 10min.Turbulence Training

7.150rpm,37 ℃ 1 hour (13);

8.2500rpm, centrifugal 5min, 100ul lower 100ul coated in 100mg/ml Amp/LB on the culture plate.

9.37 c training upside down overnight.

6. Construction of expression vector

1. connect the enzyme product carrier PET29a and lab simultaneously double digestion, respectively.

Double digestion system is as follows 40ul

Reagent	/μL
EcoR I enzyme	-4
Nde I enzyme	-4
GSHf-18T / PET29a	24
10XH	4
Dd water	4

37 ° c, the reaction 3H.

2. after the double digestion, denaturation of the enzyme at 65 ° c for 10min.

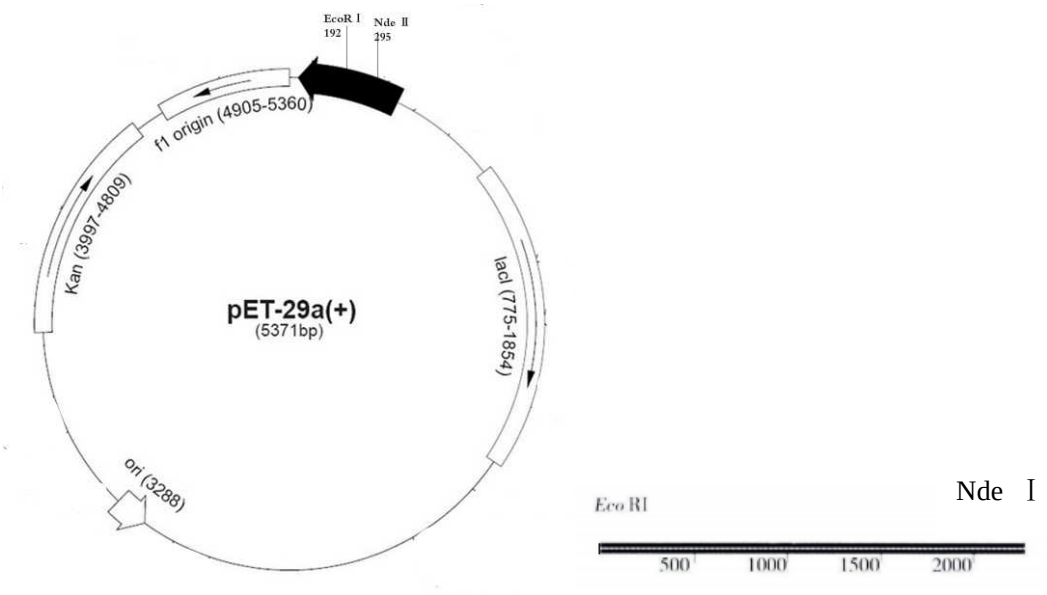
connect with enzyme.

Enzymes connected system as follows, 10 UL.

Reagent	/μL

GSHf-18T	6
PET29a	2.5
T4 ligase	0.5
Buffer（10X）	1

16 ° c, the reaction for 12H.



Seven: the recombinant plasmids into cells: (heat-shock method)

1. retreat the competent cells (BL21, DH5 Alpha) from-80 c freezer and incubate on ice.
2. reformer tubes linking 3ul DNA solution into 30ul cells, mix gently.
3. put in on ice for 30 min
4. put into 42 ℃ c in the water bath, heat-shock 90 seconds
5. Quickly put in the ice for 3 minutes;
6. add 400 μl of SOC medium to the centrifuge tube, put in the 37 c water bath for 10min
7.centirfuge 150rpm, 37 ℃ for 1 hour (13);
8. centrifuge in 2500rpm for 5min, 100ul lower 100ul coated in 100mg/ml Amp/LB on the culture plate.
9.cultivate upside down overnight at 37 ℃.
10. pick colonies, each feeling State picked six of the colonies, in Knm/LB liquid medium cultures, overnight.

Eight, plasmid extraction

1. the columns balanced steps: add 500 μl of balanced liquid CBS to adsorption column (column into the collection tube) and centrifuge at 12000 RPM (~13400g) for 1 min, pour away the waste ,

put adsorption column back in the collection tube.

2. take 5-15ml bacterial solution into the centrifuge tube, centrifuge at 12000 RPM (~13400g) for 1 min and remove supernatant.

3. Add 250 μl Solution Ⅰ in centrifuge tube, using the pipet or Vortex oscillator to precipitate the bacteria cells.

4. Add 250 μl Solution II in centrifuge tube and gently flip upside down for 6-8 times to make sure the germ is full cracked.

5. Add 350 μl Solution III, gently flip upside down for 6-8 times mix until white, flocculent precipitate appears and centrifuge at 12000rpm (~13400g) for 15min and precipitation will appear at the bottom of the centrifuge tube.

6. The supernatant collected in the previous step added filtration column (filter columns into a collection tube), 12000rpm (~13400g) for 2 min, carefully collected tube after centrifuge the solution obtained in time to join in adsorption column (column into the collection tube),

7. Centrifuge at 12000rpm (~13400g) for l min, pour away the waste collection tube and put the adsorption column into the collection tube.

8. Add 700 μl WASH to the adsorption column, centrifuge at 12000rpm (~13400g) for l min, pour away the waste in the collection tube and put the adsorption column into the collection tube.

9. Add 500ulWASH, to the adsorption column, centrifuge at 12000rpm (~13400g) for l min, pour away the waste in the collection tube and put CP4 adsorption column into the collection tube.

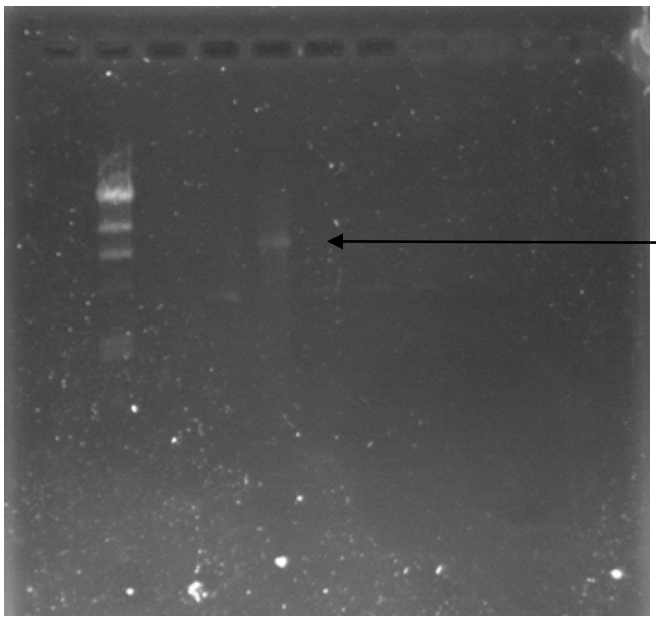
10. Add 500ulWASH, o the adsorption column, centrifuge at 12000rpm (~13400g) for l min, pour away the waste in the collection tube.

11. Put the adsorption column back in the collection tube and centrifuge at 12000rpm (~13400g) for l min

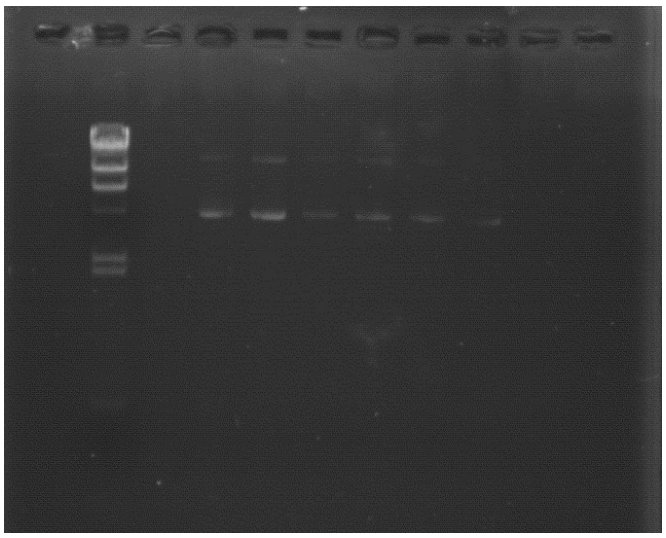
12. Put the adsorption column into a clean centrifuge tube, add 30--50 μ L of Elution buffer Solution Buffer to the film center loft, put in room temperature for 2 min and centrifuge at12000rpm (~13400g) for l min, collect the plasmid solution in centrifuge tubes.

13.Reserve the plasmid at -20 ° C.Extraction results are list below, the size of our genes is 2265bp, the size of PET29a is 5371bp, so on average the enzyme the size is 7636bp.

DH5α



BL21



P*vgb* Protocol

1. Cutting by endonuclease to get *vgb* from part BBa_K561001 and get GFP reporter from part BBa_E0840, using TAKARA's Spe Ⅰ,Xba Ⅰ and Pst Ⅰ cutting 3 hours under 37℃ circumstances more than 3 hours;
   40uL System:

DNA Sample	10uL
Enzyme 1	2uL
Enzyme 2	2uL
H/M Loading Buffer	4uL
ddH_2O	12uL

2. Constructing the *vgb* – GFP plasmid (BBa_K1555003) by TAKARA's T4 ligase under 4℃ overnight;
   20uL System:

Fragment	6uL
Vector	2uL
T4 Ligase	1uL
Buffer	2.5uL
ddH_2O	8.5uL

3. Plasmid transformed into DH5α, streak the plate with *E.coli* and incubated about 16 hours and take a portion of single white colonies into 5mL liquid LB tube(with 5uL chloromycetin), put it in the cradle under 37℃, 160rpm,8 hours and test the florescence under the fluorescence microscopy;
4. Getting 65uL of the *E.coli* and LB liquid into 12 beakerflasks which capacity could reach 100mL(with 100uL chloromycetin) to fill all of the beakeflasks, sealed 11 of it and put in the cradle under 37℃, 160rpm again;
5. The rest one use to test the florescence intensity for starters, get 3 samples and 1 pure LB to test OD600, florescence intensity under 509nm luminescence and wavelength at 475nm and 395nm separately and taking everage, using microplate reader for ELISA(TECAN's Infinite

LA200), test the concentration of oxygen by dissolved oxygen detector;

6. Every two hours, take one bottle of it and comparison(LB), testing the concentration of oxygen, OD600 and florescence intensity, regulating and making average;
7. Deal with the data to calculate and analyses the result.

P*vgb* Protocol

BBa_J23100 (constitutive promoter) + N20sgRNA processing

1. Two designed N20 single stranded fragments are combined into double chain by the way of annealing with PCR Amplifier
2. To synthesize sgRNA, we use sgRNA F and sgRNA R plasmid pCB003 as primer and as a template for PCR

The length of PCR product is approximately 100 bp

3. Step 1 (N20) product and step2 (sgRNA) product as a template and to OLF and sgRNAR as overlap PCR primers are to synthesize N20 and sgRNA fragment
4. Cut the overlap of PCR products with XbaI and SpeI enzyme digestion, enzyme cut over 85 ℃ for 20 min for inactivation
5. Cut BBa_J23100 plasmid with SpeI enzyme and then make the product to take off the phosphorylation
6. Combine step4 product with step5 product using T4 ligase
7. Purify the step6 product
8. Use colony PCR to verify the enzyme product
9. Pick a single colony to culture and then use XbaI & SpeI enzyme to cut the mention of plasmid
10. Sequence the final product (BBa_J23100 + N20sgRNA plasmid)