HZAU-2013iGEM common experiment protocol

(1) Electroporation

1. Add 10ul ddH_20 into the wells and pipette up and down several times,let sit for a few minutes.
2. To use frozen electrocompetent cells.Store the tubes at an ice bath to make the cells thawed.
3. Pipette some volumes of the thawed electrocompetent cells(DH-5ａ) into appropriate number of bacterial electroporation cuvettes.
4. Add 1ul plasmid DNA into the bacterial electroporation cuvettes.Tap the solution to ensure that the suspension of bacteria and DNA sits at the bottom of the cuvette.
5. Set the electroporation apparatus to deliver an electrical pulse of 1.8kV and place the cuvette in the electroporation device.A time constant of 4-5milliseconds with a field strength of 1.8kV should register on the machine.
6. As quickly as possible after the pulse,remove the electroporation cuvette and add 600ul of LB medium at room temperature.
7. Transfer the cells to a 1.5ml centrifuge tube and incubate the cultures with gentle rotation for 1 hour at 37℃.
8. Plate 100ul of the electroporated cells onto LB agar medium containing right antibiotic.

(2)Minipreparetion of Plasmid DNA by Alkaline Lysis with SDS

Buffers and Solutions:Alkaline lysis solution I、II、III，Antibiotic for plasmid selection.Ethanol chloroform: isoamyl alcohol (1:1,v/v)

METHOD:

1. Inoculate 5ml of rich medium containing the appropriate antibiotic with a single colony of transformed bacteria.Incubate the culture overnight at 37℃ with vigorous shaking.
2. Pour 2 ml of the culture into a microfuge tube. Centrifuge at 12000 r for 1 minutes at 25℃.
3. Remove the medium by aspiration,leaving the bacterial pellet as dry as possible.
4. Resuspend the bacterial pellet in 200ul of Alkaline lysis solution I by vigorous vortexing.
5. Add 400ul of freshly prepared Alkaline lysis solution II to each bacterial suspension.Close the tube tightly,and mix the contents by inverting the tube rapidly five times.Do not vortex!
6. Add 300ul of Alkaline lysis solution III.Store it at -20℃ for 10min.
7. Centrifuge the bacterial lysate at 12000r for 5 mintues.Transfer the supernatant to a fresh tube.
8. Add an equal chloroform: isoamyl alcohol.Add 2/3 isopropanol of the total volumes,Mix it by vortexing centrifuge it for 10 min at -20℃ and 12000r.
9. Remove the supernatant and add 700ul-1ml 70% ethanol to wash the precipitated,invert the closed tube several times,Recover the DNA by centrifugation at 12000r/min,5min.
10. Remove all of the supernatant by gentle aspiration, store the open tube at 37℃ until the ethanol has evaporated and no fluid is visible in the tube.
11. Dissolve the nucleic acids in 20ul ddH_20 containing 20ug/ml DNase-free RNase and store it at -20℃.
12. Test the nucleic acids by electrophoresis and sequencing.

⑶Minipreparetion of Plasmid by Miniprep Kit 50-prep.

Today we use Axyprep Minipreparetion of plasmid by Miniprep Kit 50-prep to extract plasmid.

Before use,add all RNase A to Buffer S1 and store it at 4 ℃， and add 56ml ethanol to Buffer W2 concentrate.

1. Inoculate 5ml of rich medium containing appropriate antibiotic with a single colony of transformation bacterial. Incubate the culture overnight at 37℃ with vigorous shaking.
2. Pipette 1-4ml of bacterial to microfuge tube,centrifuge it 12000r for 1 min at room temperature.
3. Resuspend the bacterial pellet in 250 ul Buffer S1.(if extracted Plasmid from bacillus subitilis , lysozyme should be added 30ul after S1 and then incubate at 37℃ for 30min or 4℃ overnight, the following is the same with Ecolic)
4. Add 250ul Buffer S2 to each bacterial suspendsion.mix the contents by inverting the tubes softy 4-6 times until it's to be transparent.This step should better not over 5min.
5. Add 350ul Buffer S3,mix the contents by inverting the tubes softy 6-8 times and then centrifuge it 12000r for 10 min at tp.
6. Pipette the supernatant to mini-prep column(over in the 2ml microfuge tube), centrifuge it 12000r for 1min then threw the filtrate.
7. Put mini-prep column into 2ml microfuge tube, and add 500ul Buffer W1, centrifuge it 12000r for 1min,threw the filtrate.
8. Put mini-prep column into 2ml microfuge tube, and add 700ul Buffer W2,centrifuge it 12000r for 1min,threw the filtrate.
9. Repeat step 8 again and centrifuge the empty mini-prep column 12000r for 2min.
10. Store the open tube at 37℃ until no fluid is visible in the tube.
11. Put min-prep column into a new 1.5ml microfuge tube, add 60ul ddH_2O(65℃) to mini-prep column and let stand for 1 min and then centrifuge it 12000r for 1 min.
12. Pipette the nucleic acids to mini-prep column and centrifuge it 12000r for 1 min.
13. Store the nucleic acids at -20℃.

⑷PCR reaction and system.

If colony PCR, the pre-degeneration should be at least 5 min and it would be better to boil the colony with 20ul ddH2O and add 1-3ul as template.

Reaction:

TaKaRa LA Taq	0.25ul
dNTP	2-3ul
Template	1ul
Primer F	1-2ul
Primer R	1-2ul
10×Buffer	5ul
ddH2O	
Total	50ul

The most used system:

95℃,2min、

95℃，30S

50℃-70℃，30S、34cycle
72℃,1min40s
72℃,5min
4℃：∞
Lip temperature 105℃

(5)Gel extracted by TIANgel Midi Purification Kit.

Before use ,add 60ml ethanol to wash Buffer PW.

1. add 500ul Balanced solution BL to Spin column CA2 and put CA2 column into 2 ml microfuge tube ,centrifuge it 12000 r for 1 min, threw filtrate away and put the column into microfuge tube again.
2. Get the aimed DNA from agarose gel and put the gel into microfuge tube.
3. Add three times as much agarose gel of Buffer PN into the microfuge tube， put it into waterbath at 50℃ for 30 min and shake it softy every 5 min to make sure the gel is completely dissolved.
4. Add all of the step 3 solution to Spin column CA2,let stand for 2 min and centrifuge it 12000r for 30-60sec,threw the filtrate away and put the column again into microfuge tube.
5. Add 600ul wash buffer PW,centrifuge it 12000r for 30-60sec,threw the filtrate away and put the empty column again into microfuge tube.
6. Do step 5 again.
7. Put Spin column CA2 into microfuge tube, centrifuge it 12000r for 2 min and let stand for several min until no fluid is visible in the tube.
8. Put Spin column CA2 into a microfuge tube and add a little of Buffer EB into the column,let stand for 2 min at rp and centrifuge it 12000r for 2 min to get the DNA.

(6)Extracted the PCR production of G-protein again by Axygene DNA gel extracted Kit.

Before use add 56ml ethanol to Buffer W2 concentrate and prepare waterbath at 75℃。

1. Cut off the gel including aimed DNA in UV-light and put it into a microfuge tube,calculate the weight and exchange it to volumes (1mg=1ul)
2. Add three times as much gel of Buffer DE-A into the tube and heat the gel at 75℃ waterbath for 6-8min,shake it every 2-3min to make sure the gel is dissolved.
3. Add Buffer DE-B which volumes is half of Buffer DE-A, mix it.
4. Pipette the volumes of step 3 and transform it into DNA column tube,centrifuge it 12000r for 1 min, threw the filtrate away.
5. Put the column tube into microfuge tube and add 500ul Buffer W1,centrifuge it 12000r for 30S, threw the filtrate away.
6. Put the column tube into microfuge tube again and add 700ul Buffer W2,centrifuge it 12000r for 30S,threw the filtrate away.
7. Do step 6 again except for the time of centrifuge is 1 min.
8. Centrifuge the empty column tube 12000r for 2 min.
9. Put the column tube into 1.5ml microfuge tube and add 25-30ul Eluent or ddH20 ,let stand for 1 min and centrifuge it 12000r for 1 min to wash DNA.

(7). The digestion system of double enzyme cutting.

All HZAU-2013iGEM enzymes were bought from TaKaRa company. The following is the

digestion system we used.

1. EcoRI and PstI

DNA	≤1ug
EcoRI	1ul
PstI	1ul
10×H Buffer	2ul
ddH2O	up to 20ul

2.EcoRI and XbaI

DNA	≤1ug
EcoRI	1ul
XbaI	1ul
M Buffer	2ul
BSA	2ul
ddH2O	up to 20ul

3.EcoRI and SpeI

DNA	≤1ug
EcoRI	1ul
SpeI	1ul
H buffer	2ul
ddH2O	up to 20ul

4.SpeI and PstI

DNA	≤1ug
SpeI	1ul
PstI	1ul
Buffer H	2ul
ddH2O	up to 20ul

5.XbaI and PstI

DNA	≤1ug
XbaI	1ul
PstI	1ul
Buffer M	2ul
BSA	2ul
ddH2O	up to 20ul

(8)3A assembly system for ligation standard part.

Part I

EcoRI	1ul
SpeI	1ul
Buffer H	2ul
ddH2O	up to 20ul

Part II

EcoRI	1ul
SpeI	1ul
Buffer H	2ul
ddH20	up to 20ul

Ligation system

Part I	some volumes
Part II	some volumes
T4 DNA ligase	1ul
Buffer	2ul
ddH20	up to 20ul