THE CHINESE UNIVERSITY OF HONG KONG

SCHOOL OF LIFE SCIENCES

LSCI 3000

Synthetic Biology Workshop

Laboratory Manual

2015-16

Time and Venue

Teaching: Mon and Tue, 6 pm – 9 pm (either one), SC 388

Lab: Designated timeslots on booking basis, SC 388/TA's lab

Teaching team

NAME	POST	LAB	EMAIL	PHONE
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Jie Yang	Helper	RRSSB116	Jieyang0622@gmail.com	39431217
Stephen Leung	Guest speaker	---	---	---

Marking

GRADES	REQUIREMENTS
C RANGE	Attend all classes Hand in lab log and assignments Unable to achieve the milestones in core modules
B RANGE	Attend all classes Tidy lab log and finish assignments Good research attitudes Achieve two milestones in core modules
A RANGE	On top of the requirements of getting B grades, students can either: -Achieve optional modules to upgrade (1 sub-grade for completion of each module)

Events	Grading	Descriptions
3 absence	Fail	
2 absence	Min. D	
Full attendance	Min. C-	
Finish Milestone 1	+1 sub-grade	
Finish Milestone 2	+1 sub-grade	
Lab-log	/	No submission/ Low quality
	+1 sub-grade	Acceptable
	+ 2 sub-grade	Perfect
4 Assignments	/	No submission/ Low quality
	+1 sub-grade	Acceptable
Finish Optional project 1	+1sub-grade	
Finish Optional project 2	+1sub-grade	

Teaching Schedule

Date	Content	Submit	Speaker
Week 1 (1 Feb 2 Feb)	Good attitudes General Lab safety Lab Space management iGEM biobrick standard Use of common lab equipment Aseptic techniques Common buffers Introduction to assignments and milestones **Assignment 1**: Choose 1 favourite / best iGEM team from each year between 2010 and 2015, and write down the reasons.		Stephen Asa
Week 2 (15 Feb 16 Feb)	Miniprep DNA content determination DNA gel electrophoresis Optional Modules **Assignment 2**: Given restriction enzymes and plasmid sequence, predict band size.	Assignment 1	Ryan
Week 3 (22 Feb 23 Feb)	Restriction Digestion Gene clean Primer design **Assignment 3**: Design GFP primers in RFC10 standard. Given primers and plasmid sequence, predict band size.	Assignment 2	Asa
Week 4 (29 Feb 1 Mar)	PCR PCR Clean Sequence search: BLAST	Assignment 3	Ryan
Week 5 (7 Mar 8 Mar)	Ligation Transformation Spread plate Site-directed mutagenesis **Assignment 4**: design mutagenesis primer with rationale.	**Milestone 1**	Asa
Week 6 (14 Mar 15 Mar)	Pick clones Streak plate Colony PCR Restriction mapping Analysis of DNA sequencing Harvest cells	New biobrick design (max. 1 A4 paper)	Eddie
Week 7 (21 Mar 22 Mar)	Protein expression SDS-PAGE Preparation of chemical competent cells	Assignment 4	Eddie
(11 Apr)		**Milestone 2**	
(22 Apr)	Teaching deadline for optional modules		
(20 May)		Optional Modules	

* iGEM: international Genetically Engineered Machine.

Core Modules

Milestone 1: DNA (deoxyribonucleic acid) Marker project

From RE (restriction enzyme):
Unknown Plasmid = 8695 bp
Operon = 5936 bp
pRSETA = 2897 bp

From PCR (polymerase chain reaction):
Gene1 + Gene2 =1398 + hanging bp
Gene1 =798 bp
Gene2 =600 bp
Mixture of the above contributes to a DNA ladder

Milestone 2: Cloning and protein expression project

Clone GFP (green fluorescence protein) to pRSFDuet-1 using RFC10 standard (EX-SP) and over-express it

* 200 μL of competent cells are provided

Optional Modules

Project 1: Preparation of competent cells by Calcium chloride method (Ryan)

- Transformation Efficiency of PUC19 (5*10^6 to pass, for *DH5α* only)

Project 2: Production of new biobrick

- Cloned GFP under iGEM provided constitutive promoter from designing primers

Week 1 Laboratory guidelines, rules and regulation

Aim

To equip ourselves with the basic concepts and hints to enter lab and start bench work.

To be familiar with iGEM biobrick standard.

To understand the use of laboratory equipment: micropipettor, alcohol lamp, spectrophotometer, *etc.*

To understand how to prepare common buffers.

General lab safety guidelines

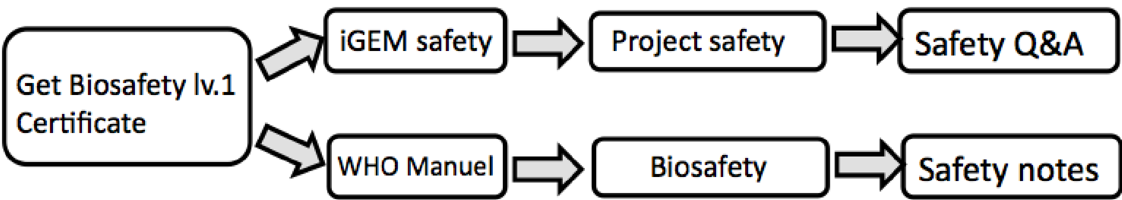
1. Dress properly, where protective gloves or goggles/glasses if needed, wear lab coat and shoes at all time. Tidy up your hair. No slippers or sandal allowed.

2. NO foods, drinks, or chewing gum in the lab.
3. Jokes, games, and pranks are prohibited.
4. DO NOT touch any equipment or chemicals unless you have learned how to and allowed to use it.
5. Keep all lab area tidy and clean at all time (before, during, and after work).
6. Keep good record of your experiments.
7. Label all samples with date and names.
8. Report all accidents if any immediately.

9. Put away all wasted or used materials carefully, bacterial waste to biohazard waste, organic waste to halogenated waste, paper waste to regular waste, pipette tips to sharp wastes, etc.
10. NEVER work alone after normal working hours.

Safety is always important. iGEM stresses its importance by setting up a series of question outline to check if any team cannot fulfill the safety requirements!

Our CUHK team in 2011 answered the safety questions in details. Therefore, there was an email from iGEM HQ to praise and remark our work! And we even aim for Safety Commendation prize in 2012.



What can you prepare for lab safety?

1. Obtain a **safety level 1 certificate.**
2. Read the iGEM safety requirement carefully.
   http://igem.org/Safety/Safety_Form
   http://2014.igem.org/Safety_Hub
3. Read the past iGEM team safety proposal.
4. Read the biosafety Manuel by WHO (Comprehensive Information).
   http://www.who.int/csr/delibepidemics/WHO_CDS_CSR_LYO_2004_11/en/
5. Find the safety information of our project (e.g. the bacterial strain of our project is not harmful, the “biobrick” made from our project contains no viral/toxin sequence which causes potential risks.)

Preparation of buffers

1) 5X TAE (Tris-Acetate-EDTA)

Chemical	Amount / Volume
Tris base	24.2 g
Glacial acetic acid	5.71 ml
0.5 M EDTA (pH 8.0)	10 ml
ddH_2O	up to 1 L
Total Volume	1 L

* EDTA: ethylenediaminetetraacetic acid.

2) 5X TBE (Tris-Borate-EDTA)

Chemical	Amount / Volume
Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
ddH_2O	up to 1 L
Total Volume	1 L

3) 10X PBS (Phosphate-Buffered Saline)

Chemical	Amount / Volume
NaCl	40 g
KCl	1 g
Na_2HPO_4	7 g
KH_2PO_4	1 g
ddH_2O	up to 500 ml
Total Volume	500 ml, pH 7.4, autoclave

4) Antibiotic Ampicillin (stock 50 mg/ml)

Chemical	Amount / Volume
Ampicillin powder	0.5 g
ddH_2O	up to 10 ml
Total Volume	10 ml, filter

Preparation of bacterial culture media

1) LB (Lauria Broth) plates (Antibiotic to be added: 65 mg/L ampicillin)

Chemical	Amount / Volume
LB powder	20 g
Agar powder	15 g
ddH_2O	up to 1 L
Total Volume	1 L, autoclave

2) LB broth

Chemical	Amount / Volume
LB powder	20 g
ddH_2O	up to 1 L
Total Volume	1 L, autoclave

3) SOB (Super Optimal Broth) medium

Chemical	Amount / Volume
SOB powder	25.5 g
ddH_2O	up to 1 L
Total Volume	1 L, autoclave

4) SOC (Super Optimal broth with Catabolite repression) medium

Chemical	Amount / Volume
Autoclaved SOB medium	980 ml
1M Glucose (filtered)	20 ml
Total Volume	1 L

Week 2 Plasmid DNA extraction and agarose gel electrophoresis

Aim

To lyse bacteria (alkali lysis) and isolate the plasmid DNA from the bacterial lysates to extract the amplified DNA.
To use a DNA ladder marker as a reference in agarose gel after electrophoresis to determine the DNA size approximately.

Introduction

Plasmids have been found to be wide distribution in bacteria. They are autonomously replicating extrachromosomal elements which are not essential for the growth of their host cells. However, they may encode a wide range of genetic products which may permit their host to adapt better to adverse conditions, for example, in the presence of antibiotics.
In cloning work, very often the recombinant plasmids have to be isolated from their transformed hosts in order to characterize by restriction analysis and sequencing. The information from these analyses provides a basis for the mode of their presentation to the transformants and the planning of future experiment for the recombinant molecules.
Among the various methods available for the preparation of plasmid DNA for rapid screening, a protocol involving the use of an alkaline solution to lyse the cells, salt precipitation to remove cell debris and chromosomal DNA and application to hibind DNA column to eliminate proteins and other contaminants has been widely employed.
Gel electrophoresis, which is easily performed, rapid, inexpensive and reproducible, has become the most popular resolution technique in nucleic acid research. Gel electrophoresis using agarose, a highly purified linear polysaccharide derived from agar, has been widely used in the detection and characterization of plasmids, also the linear DNA fragments. Plasmids of sizes ranging from less than one kilobase (kb) to over a few hundred kb can resolved by conventional agarose gel electrophoresis.
Since all the DNA molecules have the same charge to mass ration, electrostatic charge is not a factor in electrophoretic mobility. Different DNA molecules will nevertheless move through a gel at different rates on the basis of size and conformation. The electrophoretic mobility of a DNA species through a gel is described in the following equation:
Electrophoretic mobility= d/Et

Where *d* is the distance travelled in cm. *E* is the electric field strength in V/cm and *t* is the time in seconds. The mobility of a DNA band is subject to alterations resulting from variation in voltage supply, gel concentration, ionic strength, pH of the electrophoresis buffer and temperature.

Under the influence of an electric field, the motilities of different DNA species through a gel are inversely related to their respective molecular sizes. Therefore, those with larger sized will move more slowly. If it so happens that two DNA species are of the same size, but different conformation, for example a covalently closed circular (CCC) species versus its open circular (OC) counterpart, agarose gel electrophoresis can still be used to separate them as CCC molecules are more compact, so less retarded than those of OC form.

Materials

Miniprep plasmid DNA extraction kit

ddH_2O (deionized, sterile, DNase-free)

DNA ladder (25bp/ 100bp/ 1kb)

6×/10× DNA loading dye

DNA staining dye (SYBR Green/ Red Safe/ Gel Red…DO NOT use Ethidium Bromide)

Agarose gel (use DNA grade agarose)

TAE buffer (Tris-Acetate-EDTA buffer)

Gel imager: UV transilluminator

(Beware of UV damaging your skin, eye and your DNA samples)

Procedures

Plasmid DNA extraction

In this excise, DNA-spin^{TM} Plasmid DNA Purification Kit (Intron) is used. The manual from Intron is given.

PROTOCOL

- Add the dissolved RNase A solution to Resuspension Buffer, mix, and store at 2~8°C.
- Add ethanol (96–100%) to Washing Buffer B before use (see bottle label for volume).
- Check Lysis Buffer and Neutralization Buffer before use for salt precipitation. Redissolve any precipitate by warming to 37°C. Do not shake Lysis Buffer vigorously.
- Close the bottle containing Lysis Buffer immediately after use to avoid acidification of Lysis Buffer from CO_2 in the air.
- Lysis / Neutralization Buffer and Washing Buffer B contain irritants. Wear gloves when handling these buffers.
- Optional: Add the provided LysisViewer to Resuspension Buffer and mix before use. Use one vial of LysisViewer (spin down briefly before use) per bottle of Resuspension Buffer to achieve a 1:250 dilution. LysisViewer provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris.

1. **Pick a single colony from a freshly streaked bacterial plate and use it to inoculate LB (+antibiotics). And then grow at 37 ℃ for 12 ~ 16 hrs with vigorous shaking (OD_{600} = 1.0 ~ 1.5).**

2. **Harvest 3 – 5 ml of bacteria culture by centrifugation at 13,000 rpm for 30 sec at RT and discard supernatant.**
   Note : Drain tubes on a paper towel to remove excess media.

3. **Resuspend pelleted bacterial cell thoroughly in <u>250 µl of Resuspension Buffer</u> by vortexing until no clumps remain.**
   Note : Ensure that RNase A solution has been added to Resuspension Buffer. It essential to completely resuspend the cell pellet. It may affect the lysis efficiency.
   Note : If LysisViewer reagent has been added to Resuspension Buffer, vigorously shake the buffer bottle to ensure LysisViewer particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

4. **Add <u>250 µl of Lysis Buffer</u> to resuspended cells and mix by inverting the tube 10 times. DO NOT VORTEX and incubate for 3 min at RT.**
   Note : The optimal lysis time allows maximum release of plasmid DNA without release of chromosomal DNA, while minimizing the exposure of the plasmid to denaturing conditions. Long exposure to alkaline condition may cause the plasmid to become irreversibly denatured. It is important to proceed to next step immediately after the lysate becomes clear without any cloudy clumps. Do not vortex, it may cause shearing of genomic DNA.
   Note : If the Lysis buffer becomes too cold, SDS precipitation may occur, leading to poor cell lysis. If a precipitate has formed, warm the Lysis buffer to 37°C with gentle shaking.

Note : If LysisViewer has been added to Resuspension Buffer, the cell suspension will turn pale pink after addition of Lysis Buffer. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

5. **Add 350 µl of Neutralization Buffer and gently mix by inverting the tube 10 times then incubate the tube in ice for 5 min.**
   Note : After addition of Neutralization Buffer, the solution should become cloudy and a fluffy white form. Incubation on ice may help precipitating the denatured cell components more efficiently. The precipitated material contains genomic DNA, protein, cell debris, and SDS.
   Note : If LysisViewer reagent has been used, the suspension should be mixed until all trace of pink has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

6. **Centrifuge at 13,000 rpm for 10 min at 4℃. While waiting for the centrifugation, insert a column into collection tube.**

7. **After centrifugation, transfer supernatant promptly into the column.**
   Note : Cell debris, protein , and genomic DNA will form a compact white pellet in the tube. Do not transfer with white pellet.

8. **Centrifuge at 13,000 rpm for 1 min. Remove the column from collection tube, discard filtrate in collection tube. And then place the spin column back in the same collection tube.**

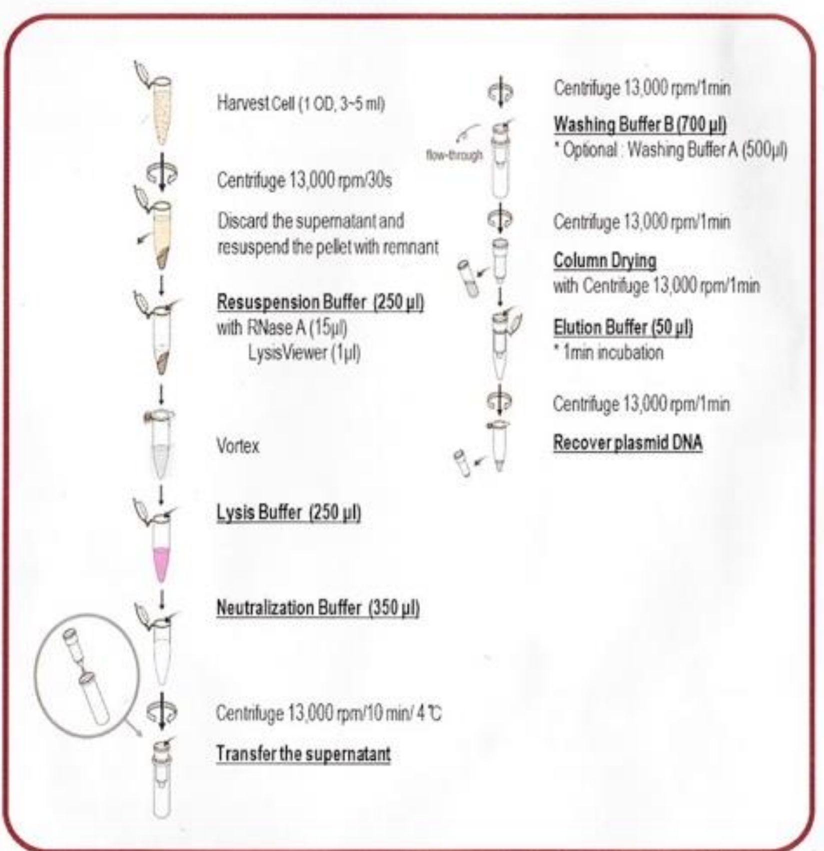
9. **(Optional) Add 500 µl of Washing Buffer A and centrifuge at 13,000rpm for 1 min. Remove the column from collection tube, discard filtrate in collection tube. And then place the spin column back in the same collection tube.**
   Note : This step is necessary to remove trace nuclease activity. endA+ strains, such as BL21, HB101, JM series, or any wild-type strains, have high level of nuclease activity that can degrade plasmids. But endA-strains,such as DH5α, XL1-blue and etc, do not require this additional washing step.

10. **Add 700 µl of Washing Buffer B, centrifuge at 13,000 rpm for 1 min. Discard filtrate in the collection tube and place the spin column back in the same collection tube.**
    Note : If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, repeat the step10 using 500 µl of Washing Buffer B.

11. **Centrifuge at 13,000 rpm for 1 min to dry the filter membrane.**
    Note : Completely remove ethanol. Residual ethanol from Washing Buffer B may inhibit subsequent enzymatic reaction.

12. **Put the column into a clean and sterile centrifuge tube. Add 50 µl of Elution Buffer or distilled water to the upper reservoir of the column, and let it stand for 1min. Then, centrifuge the tube assembly at 13,000 rpm for 1 min.**
Note : It is suggested to use at least 30 µl of the Elution buffer to obtain best result. If the plasmid is low-copy -number or larger than 10 Kb, the yield of plasmid may not be sufficient. In this case, pre-warmed (about 50℃) elution buffer will improve efficiency of elution.

Quick Guide



Agarose gel electrophoresis

Setting up an agarose gel:

1. For a small gel (the one used in our lab), add 20 ml 1×TAE buffer to a conical flask. (If there is none, dilute the 50×TAE buffer by 50 times.)
2. Then, add 0.2 g agarose (1%) to the conical flask and heat it by microwave oven by 30-45 s to dissolve it until it becomes a clear and transparent liquid.
3. Cool it down a little bit by running water for around 15 s.
4. Add about 1 μl (for 20 ml TAE) of DNA staining dye, red safe (20000×).
5. Pour the solution to the white tightened tank with gates to allow it to solidify. Add the gel comb so as to create wells for the gel. Wait >15-30 min until it is gel-like and ready to use.

Running agarose gel:

1. Orient the gel with wells (comb removed) facing the BLACK negative electrode. Check if the gel is covered by TAE buffer in the tank.
2. Add 6×/10× loading dye to the DNA to a total volume of <25 μl (depended on the well) before adding to the wells. Mix loading dye to DNA to make the solution colored.
3. Load the sample to the wells (<25 μl/ well)
4. Add 3-5 μl DNA ladder to a separate well.
5. Connect the electrodes to the power supply with correct color, black to black, red to red. Apply power supply with 120 V. Check if there are bubbles on the negative electrodes.
6. Allow it to run for about ~30 min (the time is variable based on the gel concentration and the size of interested DNA. Be aware the samples run into the gel by checking if the blue band stays on the gel.
7. After electrophoresis for 30 min, disconnect power, take the gel to imager, and turn UV on to observe bands.

**REMINDER:* Never run a gel with >200V, as the heat so generated can melt the gel and also easier to cause electric leakage. Range from 80 – 160 V is acceptable.

Usually it needs at least 100 ng DNA for a band to be seen and visualized on the UV transilluminator.

Lab log requirement

A gel photo to show the plasmids with the correct size and marker. Clear labeling requirement is a must.

Reference

eshop.intronbio.com (2012). *DNA Prep :: Plasmid & Probe DNA*. Retrieved January 15, 2016 from
http://eshop.intronbio.com/Product/View.asp?pIdx=1&pageno=1&MainItem=A&SubItem=A

Week 3 Restriction digestion, gel extraction and primer design

Introduction

Molecular cloning requires manipulation of nucleic acid, including copying (synthesize), cutting (digest) and pasting (ligate). In bacteria, it is usually done in plasmid form. To insert your target gene into a vector, you need to digest the gene and the vector to produce joining sites. Restriction enzymes are needed because it recognizes and digests DNA at specific site. According to the cleavage end, it can be divided into two groups, blunt end and staggered end (or sticky end); According to recognition mechanism and cleavage sites, it can be classified into 5 types. The common restriction endonucleases we use belong to type II. It requires no ATP, but Mg^{2+} as co-factor. It recognizes and cleaves at the same palindromic sites, i.e. same sequence from either strand of DNA from 5' to 3' (e.g. GAATTC).

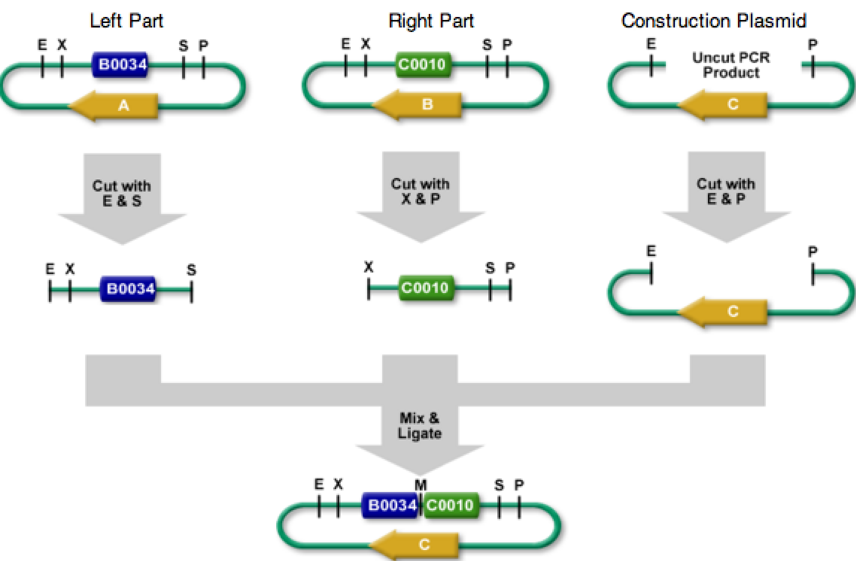
Depending on the digestion template and enzymes used, following step may be different. If only 1 restriction enzyme is used to linearize a circular DNA, heat inactivation of enzymes is enough. If 2 or more restriction enzymes are used at a distance from each other to cut out a DNA fragment, agarose gel electrophoresis is required to separate the DNA fragments and then recover the desired one from gel.

Blunt end	Staggered end
GAA/TTC	G/AATTC
CTT/AAG	CTTAA/G

DNA Digestion by Restriction Enzyme

Aim

This step is to generate sticky ends or blunt ends of the 5' end/ 3' end of the DNA, or to change a circular plasmid into a linear form. The figure below illustrates double enzyme digestion of DNA for biobrick construction.



Materials

Restriction enzyme(s)

100 × BSA (bovine serum albumin)

10 × NEB buffer

ddH_2O (deionized, sterile, DNase-free)

DNA template

37 °C incubator or dry bath

Procedure

1. Place the required reagents on ice. Label them.
2. Follow the table below to prepare restriction digestion reaction mixture:

Reaction Volume	**10 µl**	**25 µl**	**50 µl**
DNA template	0.1 µg	0.5 µg	1 µg
10X Buffer (1-4, according to enzymes)	1	2.5	5
100X BSA	0.1	0.25	0.5
Enzyme	1 U	5 U	10 U

* *Enzymatic units differ among enzymes and companies. Refer to the documents of the supplier to calculate the needed amount.*

3. Pipette the solution up and down to ensure all reagents are mixed well.
4. Place the reaction mixture at 37 ^{o}C incubato or dry bath for 2-4 hours.
5. Purify the DNA by PCR purification kit/gel extraction kit for downstream process.

Notes

- Water is always added first.
- Buffer is always added before that of enzyme.
- Make sure that you use the relevant buffer.
- Beware of the temperature for optimal enzyme digestion and DO NOT over-digest your DNA.
- Enzyme dissolved in glycerol sticks to the sides of the pipette tip. Therefore, just touch the pipette tip to the surface of the enzyme solution to ensure correct volume of the enzyme being pipetted.
- Total enzyme volume should be lower than 10 % of the reaction volume to avoid star activity

DNA Purification from Gel by spin column

Aim

This step is to purify the DNA (70 bp – 10 kb) by removing the nucleotides, enzymes and ions from solution, and separate different pieces of DNA for target DNA selection. The purified DNA is suitable for downstream process.

Materials

Gel clean kit

ddH_2O (deionized, sterile, DNase-free)

60 ^{o}C dry bath

Procedures

(Modified from QIAGEN Gel clean kit protocol)

All centrifugation steps are carried out at 10,000 – 13,000 rpm (17,900 x g) in a conventional tabletop micro centrifuge at room temperature.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by trimming off extra agarose gel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (300 μl QG to 100 mg gel). For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg.
3. Incubate the tube at 60°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by inverting the tube every 2 min during the incubation. Solubilize agarose completely. For >2% gels, increase incubation time.
4. If the color of the mixture is orange or violet, add 10 μl of 3 M sodium acetate, pH 5.0, and mix the reagents. The color of the mixture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator, which is yellow at pH ≤7.5 and orange or violet at higher pH.
5. Add 1 gel volume of isopropanol to the sample and mix the reagents well. For example, if the agarose gel slice is 100 mg, add 100 μl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min. The maximum volume of the column reservoir is 800 μl. For sample volumes of more than 800 μl, simply load and spin again.
8. Discard flow-through and place QIAquick column back in the same collection tube. It can be reused to reduce plastic waste.
9. Add 0.75 ml of Buffer PE to wash the QIAquick column, and centrifuge for 1 min. If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifugation.
10. Discard the flow-through and centrifuge the QIAquick column for one more min. Residual ethanol can inhibit downstream process. Ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
11. Place QIAquick column into a clean 1.5 ml micro centrifuge tube.

12. To elute DNA, add 30-60 μl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) (pre-warmed at 60 °C) to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge the column for 1 min.

Notes

- One advantage using Gel purification kit over PCR purification kit is that it can purify single type of DNA when there is any other suspected DNA contamination (such as primer-dimer, non-specific amplification, etc).

You don't have to use QIAquick from Qiagen, other brand names will do just fine but you need to follow their protocols carefully.

Primer design

To initiate nucleotide synthesis, primers are needed to specify the location. Especially in PCR, there is no topoisomerase to stabilize unwinding double helix, the specificity is defined by the complementarity of primer sequence and the annealing temperature of the primer:template duplex. Following requirements are suggested for good primer design:

- **GC content**: 40 – 60%. As GC pair has 3 hydrogen bonds, while AT pair has only 2, higher GC content means higher binding strength of primer to the template. However, the primer may lose its specificity if its GC content is too high.
- **Length**: A primer usually has around 15 bp, but it can be as long as 30 or even more in some cloning method, e.g. overlapping PCR. The longer the length, the higher the binding strength and melting temperature.
- **Melting temperature (Tm)**: The temperature at which 50% of the oligonucleotide and its perfect complement are in duplex. Wallace rule gives the most basic calculation for short oligonucleotides at 0.9 M NaCl, $Td = 2°C(A+T) + 4°C(G+C)$. More accurate thermodynamic calculation gives the nearest neighbour melting temperature. The Tm difference between primer pair should be within 5˚C.
- **Secondary structure**: Primers are single-stranded. It tends to for secondary structure, e.g. hairpin, to stabilize itself. PCR will fail if formation of primer hairpin is more preferable than primer:template duplex. Some online tool can do prediction, e.g. sequence manipulation suite.

- **Self-annealing**: Primers have chance to form dimer, or even multi-mer in some cases. It may not be "lethal", but better avoid it.
- **Runs of A/T**: It may cause frameshift.
- **GC clamp**: 2 G/C at the 3'-end is essential to ensure the primer the tightly bound to the template for elongation. More than 3 G/C at the 3'-end may cause non-specific binding.

Homework

Double digest the unknown plasmid with *EcoRI* and *XbaI*.

Single digest the unknown plasmid with either *EcoRI* or *XbaI*.

You may use heat inactivation or PCR clean or gel extraction depending on the purity of your digestion reaction.

Lab log requirement

Include gel photos showing digested products and clean bands of final products.

Week 4 Polymerase Chain Reaction (PCR) and DNA Purification

Aim

To introduce basic principle of Polymerase Chain Reaction (PCR).

To amply target gene by using PCR.

To purify the DNA fragment from excess nucleotides, enzymes and ions using PCR purification kit.

Polymerase Chain Reaction (PCR)

Introduction

Polymerase Chain Reaction was first envisaged in 1984 by Kary Mullis. PCR allows the amplification of exceedingly small amount of DNA *in vitro*, without prior transfer into living cells. Because of PCR, "insufficient nucleic acid" is no longer a limitation in molecular biology research.

PCR requires a pair of short DNA fragment called primers, which is complementary to and flank the target DNA sequence to be amplified, in a series of DNA polymerase-catalyzed reactions. First, DNA containing the sequence to be amplified is heat-denatured and then annealed to the primers, which are present in excess (Step 1 and 2). Next, polymerase chain reaction is carried out from the primer 3' termini (Step 3). Then a second cycle of heat denaturation, primer annealing and extension of the annealed primers is carried out.

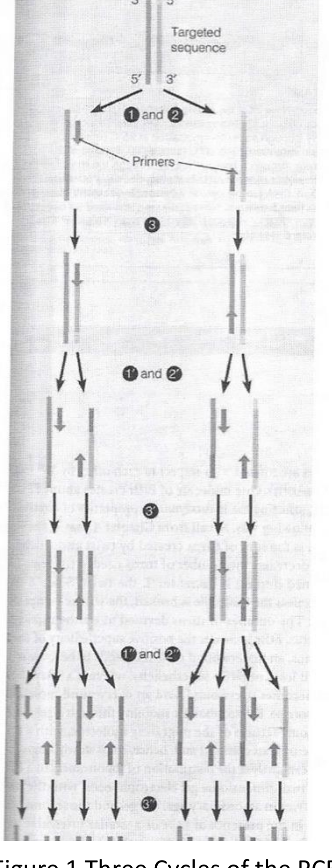


Figure 1 Three Cycles of the PCR

Using a thermo stable form of polymerase, for example, *Taq* polymerase, form *Thermus aquaticus*, a bacterium that live in high temperature (hot spring), and *Pfu* DNA polymerase, form *Pyrococcus furiosus,* avoids the need to add more polymerase at each cycle due to the enzyme is not inactivated at DNA-denaturing temperature. The extension

products synthesized in a given cycle can serve as a temple in the next cycle, so the number of target DNA copies approximately doubles every cycle.

In the following exercise, you will learn the basic of PCR, the procedure to set up the PCR reactions, including the PCR reaction mixture and PCR thermo cycle. The PCR products form the exercise will be finally characterized by electrophoresis in order to reveal the effect of annealing temperature on yield and quality of PCR product. Electrophoresis is also a purification step, to limit the side product and primer-primer dimer. Finally, the PCR product of interest will be collected form the gel for later cloning step.

The PCR condition should follow the manual recommended by the polymerase producer. In general, a PCR mixture contains components as follows:

1. DNA polymerase
2. DNA polymerase buffer
3. dNTPs (deoxynucleotide)
4. Forward and Reverse Primers
5. DNA template
6. DNase-free ddH2O

Typically, PCR include following steps:

1. Initial denaturation: it is only required for DNA polymerases that require heat activation by hot-start PCR). × 1 cycle.
2. Amplification (to produce the product) × 30 cycles
   1) Denaturation: it causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
   2) Annealing: let the primer anneal to template.
   3) Extension: DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs.
3. Final extension: it ensures that any single strand DNA produced is completely extended. × 1 cycle.

In each step, duration and temperature are different. The denaturation and extension temperature depends on the DNA polymerase used. Annealing temperature depends on the primers.

Materials

Phusion DNA polymerase

5X Phusion HF Buffer
10mM dNTPs
10uM Forward and Reverse Primers
DNA template (10 pg- 10 ng)
ddH2O (deionized, sterile, DNase-free)
PCR (200 µl) tubes
Thermocycler

Procedures

1. In this practice, the Phusion® High-Fidelity DNA Polymerase (NEB) will be used and the manual of this polymerase is given in the attachment. Based on the information, please calculate the volume of each component to be added and draft out thermal cycle details by filling in the following form.

PCR mixture	
Component	Volume (µl)
DNA polymerase	
DNA polymerase buffer	
dNTPs	
Forward Primer	
Reverse Primer	
DNA template	
DNase-free ddH_2O	
Total	

PCR thermal cycle			
	Temperature (°C)	Duration	Cycle
Initial denaturation			1
Denaturation			

Annealing			
Extension			
Final extension			1
Sample keeping	4	∞	1

2. Add the DNase-free ddH_2O in PCR tube.
3. Add the other Component in the tube. If the added volume is less, pipetting up and down is necessary.
4. Mix reagents in tubes by pipetting the solution up and down slowly.
5. Quick spin the PCR tube to ensure the mixture is in the bottom of the tube.
6. Put it in the thermocycler (PCR machine) and set the cycle information.
7. Start the cycle and wait till it is finished.
8. During the waiting time, the gel can be made for the characterization and purification.

DNA Purification

Introduction

There are two methods, or Kits, for DNA purification, PCR purification kit and Gel clean kit (purification from Gel). The main difference is running the gel (electrophoresis) of not. The selection of these two kits is based on the purpose. If you want to remove excess nucleotides, enzymes and ions only, PCR purification kit is used. If you want to separate different pieces of DNA for target DNA selection, purification from Gel is the only option.

Materials

Intron PCRquick-spin(TM) PCR Product Purification Kit

ddH_2O (deionized, sterile, DNase-free)

Procedures

For research purpose only. Not for use in diagnostic procedures for clinical purposes. For IN VITRO USE ONLY.

ISO 9001/14001 Certified Company

PCRquick-spin™ PCP Product Purification Kit

Cat. No.	17201	50 columns
Cat. No.	17202	250 columns

DESCRIPTION

DNA fragments for probe DNA or ligation must be separated and purified from other DNA fragments. Normally, purification method for DNA fragments from agarose gel is used silica adsorption. Silica adsorption depends on the tendency of nucleic acids to adsorb to silica (glass) in the presence of a chaotropic salt. This tendency was discovered by Vogelstein and Gillespie who found that DNA fragments adsorbed to powdered flint glass in the presence of saturated NaI. Different types of nucleic acid adsorb more or less tightly to glass depending on the ionic strength and the pH of the surrounding solution. A low salt buffer or water is always used to elute the nucleic acid from glass.

PCRquick-spin™ PCR Product Purification Kit employs a column method to purify target DNA and is designed to direct purification of double- or single-stranded PCR products (100bp-10kb) from amplification reactions and DNA cleanup from other enzymatic reactions. The column method uses a highly concentrated salt solution to keep the target DNA bound to the column membrane.

PCRquick-spin™ PCR Product Purification Kit's buffers are optimized for efficient recovery of DNA and removal of contaminants. Since the binding process is specific for nucleic acids, the bound material can be separated and purified from impurities e.g. salts and proteins, by a simple washing step. Nucleic acids elute from the column membrane in a low salt buffer or water.

PCRquick-spin™ PCR Product Purification Kit promises a high yield of purification up to 70-95%. Furthermore, the purification process is quick and simple to perform. Using the 15 min direct purification method, PCR products are effectively purified from contaminants, including primer dimers. But to separate PCR product from nonspecific amplification products, use the agarose method (MEGA-spin™ Agarose Gel DNA Extraction Kit, Cat.No.17181, 17183).

KIT CONTENTS AND STORAGE

Label	Contain 50 Columns	Contain 250 Columns
Binding Buffer	30 ml	150 ml
Washing Buffer(concentrate)[1]	10 ml	50 ml
Elution Buffer	20 ml	20 ml
PCRquick-spin™ column (Blue)	50 columns	250 columns
Collecting tube	50 tubes	250 tubes

[1] Washing Buffer is supplied as concentrates. Add 40 ml of ethanol (96~100%) according to the bottle label before use.

CONSIDERATIONS BEFORE USE

1) Selective binding

PCRquick-spin™ PCR Product Purification kit system combines the convenience of spin-column method with selective binding properties of a silica-gel membrane. Special buffers provided with this kit are optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are efficiently washed away.

2) Efficient recovery of large fragments of DNA

The efficiency with which DNA is recovered from amplification reactions is a function of its molecular weight. As the size of the DNA fragments increases, the yield progressively decreases. This kit gives reasonable yields of DNA fragments that are less than 7-10 kb in length (70-95%).

3) Efficient recovery of small amounts of DNA

The smaller the amount of DNA applied to amplification PCR product, the lower the yield of purified fragments.

CONSIDERATION BEFORE USE

Preparation of kit working solutions

Add absolute EtOH to Washing buffer before use (see page or bottle label for volume).

Preparation of sample material

Normally use 20-50 μl of PCR product. It is possible of purifying up to 100 μl of PCR product. Useful Binding buffer volume according to volume of PCR product, it describes "Procedure, Step 1"

PROTOCOL

1. Add 500 μl of Binding buffer to PCR product (up to 20–50 μl) in 1.5 ml microcentrifuge tube and mix well. It is necessary not to remove mineral oil or kerosene.
   Note : (Optional) Add 150 μl of isopropanol to the step 1 tube and mix well by pipetting. Do not centrifuge. This step is necessary to increase the yield of ≤200 bp DNA fragment (70-200 bp DNA fragment).
   Note : If the PCR product is 20 μl-50 μl, add 500 μl of Binding buffer and 150 μl of isopropanol. When the 500 μl of Binding buffer is good for 20–50 μl of PCR product (not including oil). If more than 50 μl of PCR product is processed, add 700 μl of Binding buffer. When adding the isopropanol and mixing well by pipette, small white pellet and clump should be form. But never mind, go the following step.
2. After mixing, incubate at room temperature for 1 min.
3. During the incubation time, place a spin column in a provided 2 ml collection tube.
4. Load the sample to the spin column and centrifuge at 13,000 rpm for 1 min. Discard the flow-through after centrifuging and place the spin column back in the same 2 ml collection tube.
   Note : The maximum volume of the column reservoirs is 800 μl. For larger volume, sample reload and spin again.
5. Add 700 μl of Washing buffer to column and centrifuge at 13,000 rpm for 1 min. Discard the flow-through after centrifuging and place the spin column back in the same 2 ml collection tube.
   Note : If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, repeat the step 5 using 500μl of Washing Buffer.
6. Centrifuge for 1 min at 13,000 rpm to dry the spin membrane.
   Note : It is important to dry the spin membrane since residual ethanol may interfere with other reactions.
7. Put the column into a clean and sterile centrifuge tube. Add 50 μl of Elution Buffer or distilled water to the upper reservoir of the column, and let it stand for 1 min. Then, centrifuge the tube assembly at 13,000 rpm for 60 sec.
   Note : It is suggested to use at least 20 μl of the Elution buffer to obtain best result.

Lab log requirement

A gel photo which includes all the PCR products with bands at correct size and marker. Clear labeling requirement is a must.

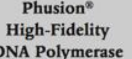
If Multi band exits, the gel purification is needed and re run the gel for showing the single band.

Reference

neb.com (2016). *Phusion® High-Fidelity DNA Polymerase*. Retrieved January 15, 2016 from https://www.neb.com/products/m0530-phusion-high-fidelity-dna-polymerase#tabselect2

eng.intronbio.com (2004). *PCRquick-spin(TM) PCR Product Purification Kit*. Retrieved January 15, 2016 from http://eng.intronbio.com/product/PCRquick-spin.htm

Attachment: Phusion polymerase manual



1-800-632-7799
info@neb.com
www.neb.com

M0530S

100 units	2,000 U/ml	Lot: 0031206
RECOMBINANT	Store at –20°C	Exp: 6/13

Description: High Fidelity DNA Polymerases are important for applications in which the DNA sequence needs to be correct after amplification. Manufactured and quality controlled at New England Biolabs, Thermo Scientific® Phusion High-Fidelity DNA Polymerase offers both high fidelity and robust performance, and thus can be used for all PCR applications. Its unique structure, a novel *Pyrococcus*-like enzyme fused with a processivity-enhancing domain, increases fidelity and speed. Phusion DNA Polymerase is an ideal choice for cloning and can be used for long or difficult amplicons. With an error rate 50-fold lower than that of *Taq* DNA Polymerase and 6-fold lower than that of *Pyrococcus furiosus* DNA Polymerase (1), Phusion is one of the most accurate thermostable polymerases available. Phusion DNA Polymerase possesses 5´→ 3´ polymerase activity, 3´→ 5´ exonuclease activity and will generate blunt-ended products.

Phusion DNA Polymerase is supplied with standard 5X Phusion HF Buffer, as well as 5X Phusion GC Buffer, which can be used for complex or GC-rich templates. Each of these buffers contains $MgCl_2$ (1.5 mM at the final [1X] reaction concentration). Reactions can also be optimized using the provided DMSO or $MgCl_2$ solutions.

Source: An *E. coli* strain that carries the Phusion DNA Polymerase gene.

Applications:

- PCR
- Cloning
- Long or Difficult Amplification
- High-throughput PCR

Supplied in: 20 mM Tris-HCl (pH 7.4 @ 25°C), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, stabilizers, 200 µg/ml BSA and 50% glycerol

Reagents Supplied with Enzyme:
5X Phusion HF Buffer
5X Phusion GC Buffer
100% DMSO
50 mM $MgCl_2$ Solution

Reaction Conditions: 1X Phusion HF or GC Buffer, DNA template, 0.5 µM primers, 200 µM dNTPs (not included), 3% DMSO (optional) and 1 unit of Phusion DNA Polymerase in a total reaction volume of 50 µl.

Unit Definition: One unit is defined as the amount of enzyme that will incorporate 10 nmol of dNTP into acid insoluble material in 30 minutes at 74°C.

Unit Assay Conditions: 25 mM TAPS-HCl (pH 9.3 @ 25°C), 50 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 200 µM dNTPs including [^{3}H]-dTTP and 400 µg/ml activated Calf Thymus DNA.

Heat Inactivation: No

Quality Control Assays

7.5 kb Genomic DNA PCR: 30 cycles of PCR amplification in a 50 µl reaction containing 50 ng genomic DNA with 1.0 unit of Phusion DNA Polymerase in the presence of 200 µM dNTPs and 1.0 µM primers in Phusion HF Buffer results in the expected 7.5 kb product.

20 kb Lambda DNA PCR: 22 cycles of PCR amplification in a 50 µl reaction containing 10 ng Lambda DNA with 1.0 unit of Phusion DNA Polymerase in the presence of 200 µM dNTPs and 1.0 µM primers in Phusion HF Buffer results in the expected 20 kb product.

Endonuclease Activity: Incubation of a 50 µl reaction in NEBuffer 2 containing a minimum of 10 units of Phusion DNA Polymerase with 200 µM dNTPs and 1 µg of supercoiled ϕX174 DNA for 4 hours at either 37°C or 72°C results in < 10% conversion to the nicked form as determined by agarose gel electrophoresis.

PCR

The following guidelines are provided to ensure successful PCR using Phusion DNA Polymerase. These guidelines cover routine PCR reactions. Amplification of templates with high GC content, high secondary structure, low template concentrations or long amplicons may require further optimization.

Reaction Setup: We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the 3´→ 5´ exonuclease activity. Phusion DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors. **Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.**

COMPONENT	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
5X Phusion HF or GC Buffer	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
DMSO (optional)	*(0.75 µl)*	*(1.5 µl)*	*(3%)*
Phusion DNA Polymerase	0.25 µl	0.5 µl	1.0 units/ 50 µl PCR
Template DNA	variable	variable	<250 ng
Nuclease-Free Water	to 25 µl	to 50 µl	

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling:

Thermocycling Conditions for a Routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	5–10 seconds
	45–72°C	10–30 seconds
	72°C	15–30 seconds/kb
Final Extension	72°C	5–10 minutes
Hold	4°C	

General Guidelines:

1. Template:
   Use of high quality, purified DNA templates greatly enhances the success of PCR reactions. Recommended amounts of DNA template for a 50 µl reaction are as follows:

DNA	AMOUNT
Genomic	50 ng–250 ng
Plasmid or Viral	1 pg–10 ng

   If the template DNA is obtained from a cDNA synthesis reaction, the volume added should be less than 10% of the total reaction volume.

2. Primers:
   Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 (http://frodo.wi.mit.edu/primer3) can be used to design or analyze primers. The final concentration of each primer in a PCR reaction using Phusion DNA Polymerase may be 0.2–1 µM, while 0.5 µM is recommended.

3. Mg^{++} and additives:
   Mg^{++} is critical to achieve optimal activity with Phusion DNA Polymerase. The final Mg^{++} concentration in 1X Phusion HF and GC Buffer is 1.5 mM. Excessive Mg^{++} can prevent full denaturation of DNA as well as cause non-specific binding of primers. The optimal Mg^{++} concentration is affected by dNTP concentration, the template being used and supplements that are added to the reaction. If chelators (e.g. EDTA) are present, it may be necessary to increase the Mg^{++} concentration. Mg^{++} can be optimized in 0.5 mM increments using the $MgCl_2$ provided.

   Amplification of difficult targets, such as those with GC-rich sequences or secondary structure, may be improved by the presence of additives such as DMSO (included). A final concentration of 3% DMSO is recommended, although concentration can be optimized in 2% increments. It is important to note that if a high concentration of DMSO is used, the annealing temperature must be lowered as it decreases the primer T_m (2). Phusion DNA polymerase is also compatible with other additives such as formamide or glycerol.

4. Deoxynucleotides:
   The final concentration of dNTPs is typically 200 µM of each deoxynucleotide. Phusion cannot incorporate dUTP and is not recommended for use with uracil-containing primers or template.

5. Phusion DNA Polymerase Concentration:
   We generally recommend using Phusion DNA Polymerase at a concentration of 20 units/ml (1.0 units/50 µl reaction). However, the optimal concentration of Phusion DNA Polymerase may vary from 10–40 units/ml (0.5–2 units/50 µl reaction) depending on amplicon length and difficulty. Do not exceed 2 units/50 µl reaction, especially for amplicons longer than 5 kb.

(see other side)

CERTIFICATE OF ANALYSIS

6. Buffers
   5X Phusion HF Buffer and 5X Phusion GC Buffer are provided with the enzyme. HF buffer is recommended as the default buffer for high-fidelity amplification. For difficult templates, such as GC-rich templates or those with secondary structure, GC buffer can improve reaction performance. GC buffer should be used in experiments where HF buffer does not work. Detergent-free reaction buffers are also available for applications that do not tolerate detergents (e.g. microarray, DHPLC).

7. Denaturation:
   An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 minutes) for templates that require it.

   During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

8. Annealing:
   Annealing temperatures required for use with Phusion tend to be higher than with other PCR polymerases. **The NEB T_m calculator (www.neb.com/TmCalculator) should be used to determine the annealing temperature when using Phusion.** Typically, primers greater than 20 nucleotides in length anneal for 10–30 seconds at 3°C above the T_m of the lower T_m primer. If the primer length is less than 20 nucleotides, an annealing temperature equivalent to the T_m of the lower primer should be used. A temperature gradient can also be used to optimize the annealing temperature for each primer pair. For two-step cycling, the gradient can be set as high as the extension temperature.

   For high T_m primer pairs, two-step cycling without a separate annealing step can be used.

9. Extension:
   The recommended extension temperature is 72°C. Extension times are dependent on amplicon length and complexity. Generally, an extension time of 15 seconds per kb can be used. For complex amplicons, such as genomic DNA, an extension time of 30 seconds per kb is recommended. Extension time can be increased to 40 seconds per kb for cDNA templates, if necessary.

10. Cycle number:
    Generally, 25–35 cycles yields sufficient product.

11. 2-step PCR:
    When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol is recommended.

    Thermocycling Conditions for a Routine 2-Step PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	5–10 seconds
	72°C	15–30 seconds/kb
Final Extension	72°C	5–10 minutes
Hold	4°C	

12. PCR product:
    The PCR products generated using Phusion DNA Polymerase have blunt ends; if cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, then DNA should be purified prior to A-addition, as Phusion DNA Polymerase will degrade any overhangs generated.

    Addition of an untemplated -dA can be done with *Taq* DNA Polymerase (NEB #M0267) or Klenow exo⁻ (NEB #M0212).

References:

1. Frey, B. and Suppman, B. (1995) *BioChemica*, 2, 34–35.
2. Chester, N. and Marshak, D.R. (1993) *Analytical Biochemistry*, 209, 284–290.

Companion Products Sold Separately:

Phusion HF Buffer Pack
#B0518S 6.0 ml

Phusion GC Buffer Pack
#B0519S 6.0 ml

Detergent-free Phusion HF Buffer Pack
#B0520S 6.0 ml

Detergent-free Phusion GC Buffer Pack
#B0521S 6.0 ml

Phusion High-Fidelity PCR Kit
#E0553S 50 reactions
#E0553L 200 reactions

Phusion High-Fidelity PCR Master Mix with HF Buffer
#M0531S 100 reactions
#M0531L 500 reactions

Phusion High-Fidelity PCR Master Mix with GC Buffer
#M0532S 100 reactions
#M0532L 500 reactions

Deoxynucleotide Solution Set
#N0446S 25 μmol of each

Deoxynucleotide Solution Mix
#N0447S 8 μmol of each
#N0447L 40 μmol of each

Magnesium Chloride ($MgCl_2$) Solution
#B9021S 6.0 ml

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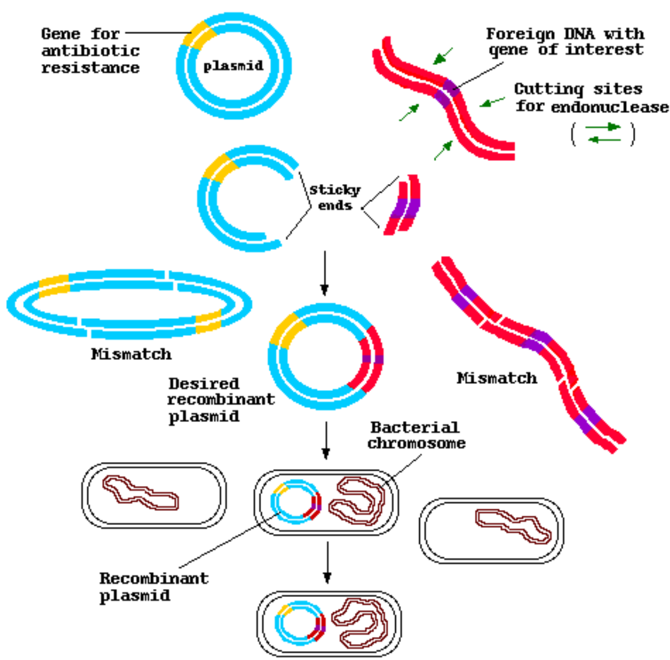
Week 5 Ligation, Transformation, Spread plate and Site-directed mutagenesis

Introduction

With the production of DNA fragments with joining sites from restriction digestion, a clone can be made by ligating the gene of interest with the vector (antibiotic resistant gene-containing). The reaction mixture is then transformed into competent cells. Heat shock is thought to generate pore on cell membrane and calcium ions is thought to facilitate intake of DNA due to chare-charge interaction.



http://www.biologyjunction.com/molecular biology.htm

T4 ligase catalyzes the formation of phosphodiester bond between 3'-hydroxyl group and 5'-phosphate group with ATP (adenosine triphosphate) as energy source. However, it is non-specific to DNA sequence, choice of restriction enzymes in previous steps is important. Blunt end ligation is non-directional, while sticky end ligation after double digestion is directional. The reaction follows collision theory that the higher concentration of DNA, the higher the reaction rate. Unfortunately, self-ligation is a serious problem. The more DNA population, the more impure the products are and also the lower the yield of your target DNA.

To solve this problem, people use excess DNA insert than linear vector to lower the chance of self-ligated empty vector and to increase the yield. Cell population transformed with self-ligated inserts are removed by antibiotic selection. In reality, since sticky ends are susceptible to damage and DNA fragments may be digested if the sample is not clean, colonies may be found to have flaws or even fake (empty vector). Therefore, controls including self-ligation of single digested and double digested vector are essential as a quality check.

T4 DNA Ligation

Aim

This step is to ligate (join) the sticky end or blunt end of DNA segments after digestion by restriction enzymes.

dephosphorylated vector

Materials

T4 DNA ligase
10X T4 DNA ligase buffer
ddH_2O (deionized, sterile, DNase-free)
DNA with restriction cut
16 °C dry bath/ 4 °C fridge

Procedure

1. Place the reagents on ice.
2. Prepare the reaction mixture with the steps in the following table (recommended by NEB)

Total volume	**10 µl**	**20 µl**
DNA (10-200 ng) + ddH_2O	8.5	17
10 × T4 DNA Ligase Reaction Buffer	1	2
T4 DNA Ligase (400,000 units/mL)	0.5	1

* Ligation can be performed in any NEB restriction buffers if it is supplemented with 1 mM ATP. ATP decomposes quickly at room temperature. It is preferable to aliquot the buffer into 10 µl/tube, and do not re-freeze the aliquot tubes after used.

* One unit is defined as the amount of enzyme required to give 50% ligation of HindIII fragments of λ DNA (5′ DNA termini concentration of 0.12 µM, 300- µg/ml) in a total reaction volume of 20 µl in 30 minutes at 16°C in 1× T4 DNA Ligase Reaction Buffer.

3. Allow the ligation to take place at 4 °C over-weekend; or 16 °C overnight; or 22-25 °C for 10 minutes.
4. 5 µl of the ligated sample should be used for agarose gel electrophoresis to confirm whether ligation has occurred.

Notes

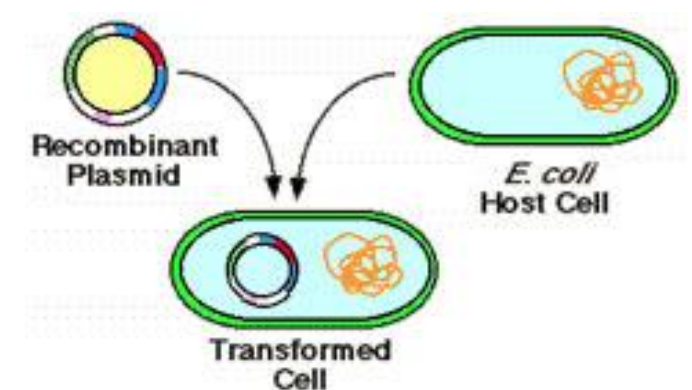
- Insert/'Vector mass' ratio varies with protocols (1:1, 3:1, 6:1). The basic principle is the amount of insert should be more than that of vector, and the total amount should be <100 ng (Too many DNA leads to self-ligation/ inhibition of ligation).

Bacterial Transformation

Aim

Two transformation methods are available, electroporation and chemical transformation. This lab uses chemical and heat shock to open the bacterial membrane with pores to let circular DNA plasmid enter the bacteria. Supercoiled plasmid DNA can enter the cells efficiently, while linearized DNA do not.

Materials



Competent bacterial cell (*DH5α*)
42 °C water bath/ dry bath
LB medium/ SOC medium
37 °C incubator
LB plate with appropriate antibiotics
Plasmid DNA

Procedures

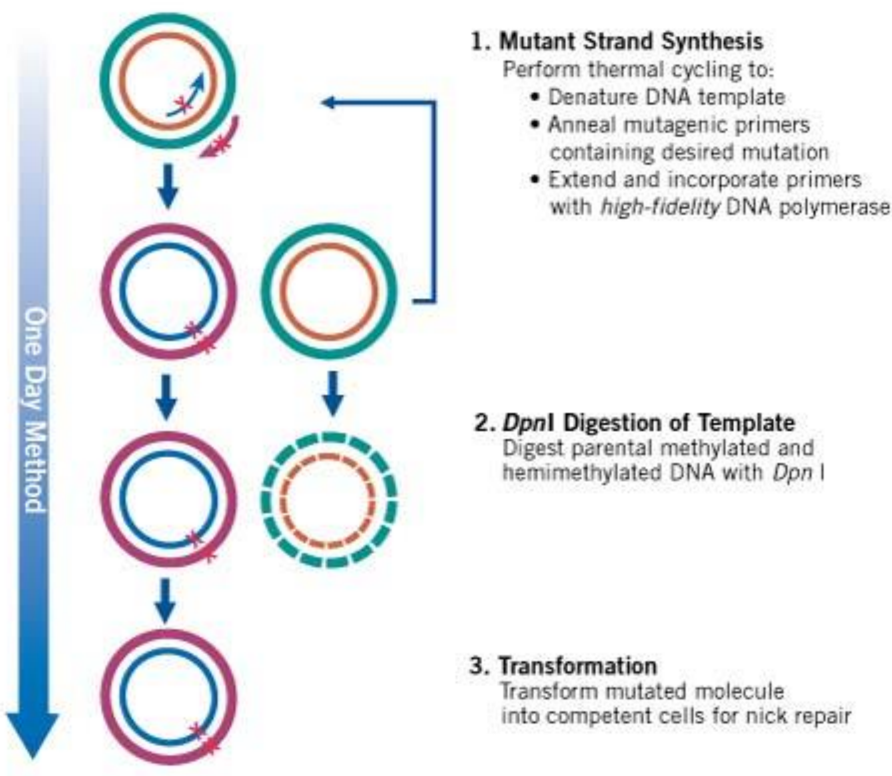
1. Thaw a tube of competent cells (usually 100 µL) on ice, and use as soon as possible.
2. Pipette 50 ~ 100 ng DNA (<10 µL) to the solution surface of competent cells. (The amount varies according to the efficiency of the cells)
3. Put the tube on ice for 15 – 30 min.
4. Heat shock: Put the tube at 42 °C for 45 s to 2 min.
5. Put the tube in ice for 5 min.
6. Transfer the cells to a 1.5 mL or 2 mL microfuge tube
7. Add 1 ml LB broth or SOC medium.
8. Incubate the tube at 37 °C shaker for 45 – 90 min with shaking (~ 250 rpm).
9. Spread 3 dilution of cells (10-fold serial dilution) onto the pre-warmed agar plate (with suitable antibiotics), each in 50 – 100 µl.
10. Incubate the agar plates upside down at 37 °C incubator overnight (~16 hrs).
11. Wrap the plates with parafilm and store at 4 °C for further use.

Notes

- The cells should be in 0.5 mL microfuge tube before recovery due to better heat transfer across thin wall.
- Heat shock temperature must be accurate.
- During spread plate, cool down the hot spreader to prevent it from killing the cells or degrading the antibiotics on plate.
- The plates can be stored up to two weeks without loss of plasmid DNA.

Site-directed mutagenesis

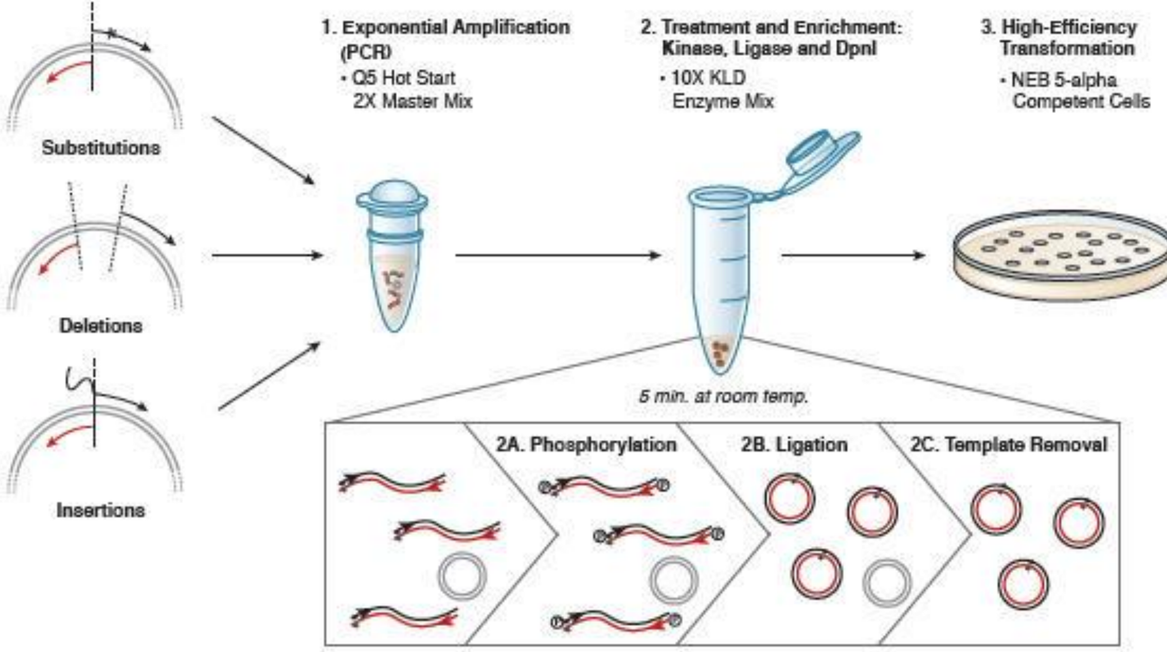
When there are illegal restriction sites in your target gene, you may want to remove it by introducing silent mutation; or one may want to make a point mutation to make a mutant protein. Traditional method Quikchange makes use of a mutant primer pair overlaps to produce mutant plasmid under thermal cycles. However, unlike PCR where the product increases exponentially, the mutant plasmid cannot be used as template for further PCR since it is nicked at different positions on the two strands, therefore the product increases linearly. It is followed by DpnI digestion to remove template plasmid, which is methylated during propagation in bacterial host.



Schematic diagram of Quikchange (From Agilent technology)

Another method developed recently by NEB is called Q5 mutagenesis kit. The magical part is on back-to-back primer design, generating nicks at the same position on both strands for exponential increase in product. By manipulating the primers, one can do mutation, insertion and deletion using this kit. The idea is to PCR nicked mutant plasmid with Q5 polymerase, then phosphorylate the 5'-end of primers, ligate them and digest out the

template plasmid by *Dpn I* in a KLD reaction mixture, finally take a small portion of the product and transform.



Schematic diagram of the principle of Q5 mutagenesis kit (From NEB)

Homework

Digest pRSFDuet-1 and your PCR product (GFP) with whatever appropriate restriction enzymes, ligate them and transform into *E. coli* DH5α.

Lab log requirement

Please include the following items with proper controls:

- Gel photos of restriction digestion.
- Gel photo of ligation product (before and after).
- Photos of the transformed LB plates.

Week 6 Selection of positive clones

Aim

To screen for clones containing the inserts.

Introduction

After ligation and transformation, there will be some colonies appearing on the agar plate. However, not all clones would be the correct one. In the past lab, you have included negative controls, which would most likely still give out colonies. Those are usually due to the unlikely even of plasmid self-ligation. While theoretically the same would also happen in your experimental plate, it is possible to screen among the clones to determine which contains the correctly ligated insert. Another possible mistake could be that the plasmid or the insert were wrongly propagated, which might cause mutations. All these false positives will be screened away in this lab session with colony PCR, restriction mapping and DNA sequencing.

Materials

NEB standard Taq with buffer

Prefix primers and Suffix reverse complement primers

ddH_2O

dNTPs

Agarose

TAE buffer

200ml flask

DNA gel dye

Ampicillin

LB

Miniprep

Restriction enzymes: *EcoR I*, *Pst I*

NEB Buffer 3

pRSFDuet empty plasmid

Procedures

Signal to noise ratio

1. Count colonies on each control plate.
2. Divide number of colonies of the ligation product plate by that of the no-pcr plate.
3. Decide number of clones to pick.

Colony PCR and DNA sequencing

1. Setup 1X PCR reaction as follow:
   10X Standard Taq Reaction Buffer: 2.5ul
   10mM dNTPs : 0.5ul
   10uM Forward Primer : 0.5ul
   10uM Reverse Primer : 0.5ul
   Taq DNA Polymerase : 0.125ul
   Nuclease-free water : to 25ul
2. Divide an agar plate into 16 squares with markers.
3. Pick a single colony, streak into a square in the ampicillin agar plate and then in a 1X reaction, and repeat as needed, incubate at 37C overnight.
4. Setup PCR routine as follow:
   Initial Denaturation: 95 °C 30 s
   30 Cycles: 95 °C 30 s
   60 °C 60 s
   68 °C 60 s
   Final Extension: 68 °C 5 min
   Hold: 4 °C
5. Run 1% agarose gel to confirm existence of correct band.
6. Pick 2 clones with correct band in agarose gel into 5 ml LB culture with ampicillin and shake it overnight at 37 °C, 220 rpm.
7. Aliquot 1 ml of overnight culture to sequencing.
8. Mini-prep according to the manufacturer's protocol.
9. Take OD 260 and OD 280, 260/280 ratio and DNA concentration.

Restriction mapping

1. Digest the plasmid using *EcoR I* and *Pst I*, with uncut and single cut controls.
2. Run DNA gel with ladder, uncut, *EcoR I*, *Pst I*, Double cut, PCR product, single cut empty vector.

Lab log requirement

1. Signal to noise: Calculation of signal to noise with step.
2. Restriction mapping: DNA gel photos with ladder, both single cut controls, double cut where bands of inserts and vector are both found,.
3. Colony PCR: gel photos with ladder, samples from each clone, and a positive control using the insert as template.
4. Miniprep: OD 260, OD 280, 260/280 ratio, DNA concentration.

Week 7 Protein expression

Aim

To express the protein encoded in the newly cloned biobricks.

Introduction

After checking which clone were correct and prepared plasmid from the clone, we need to start characterizing the biobricks. One of the very first way to characterize is to overexpress the protein, and a common is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). On a SDS-PAGE gel it is at least possible to trace if the biobrick was well expressed. In order to express the protein after T7-promoters, IPTG (isopropyl β-D-1-thiogalactopyranoside), a galactose homolog, will be used to induce the expression of the proteins.

Materials

BL21 cells

Ice

Agar plates with ampicillin

Alcohol lamps

LB

Ampicillin

SDS-PAGE equipment

Separating buffer

Stacking buffer

40% 1:19 acrylamide

TEMED (tetramethylethylenediamine)

1% APS (ammonium Persulfate)

10X SDS buffer

SDS dye

IPTG

Coomassie Blue solution

Gel boxes

Procedures

Transformation

**REMINDER: All steps in this protocol is to be performed under aseptic conditions!*

1. Thaw competent cells (*BL21*) on ice until totally melted.
2. Aliquot 1 ng of DNA into the 100 ul of cells.
3. Store on ice for 30 min.
4. Heat shock the cells at 42 °C for 2 min sharp.
5. Cool on ice for 2 min.
6. Aliquot 800 ul of LB/SOB/SOC/KB (king's broth) into a 1.5 ml centrifuge tube. Add all cells into the tube. Shake in 37 °C and 250 rpm for 45 min to 1.5 h.
7. Centrifuge at top speed briefly and remove 800 ul of supernatant.
8. Re-suspend the cells in the remaining medium.
9. Spread cells on agar plates with ampicillin.
10. Incubate the plate in 37 °C overnight.

Expression

1. Pick a single colony into 5 ml LB with ampicillin and grow in 37 °C overnight.
2. 1% inoculation into 50 ml LB with ampicillin in 200 ml flask. Shake in 37 °C and 250 rpm.
3. Track OD 600 every 30 min until OD reaches ~0.5 A. This takes about 3-4 h depending of strains.
4. Collect 0.5 ml un-induced sample and record the OD, centrifuge at top speed for 1 min, and re-suspend in 50 ul of water. Add 50 ul of SDS-dye, and boil in 95 °C for 15 min.
5. Add 0.1% IPTG into culture, and shake in 37 °C and 250 rpm for 4h.
6. Take 0.1 ml induced sample and record OD. Dilute appropriately, and then add 50 ul of SDS-dye, and boil in 95 °C for 15 min.

SDS-PAGE

1. Cast 4 ml separating 12.5% SDS gel with 1.7 ml water, 1ml 4×separating buffer, 1.25 ml 1:19 40% acrylamide, 4 ul TEMED, and 50 ul 1% APS.
2. Add a few drops of isopropanol on top, and wait for 10 min.
3. Pour isopropanol, absorb remaining isopropanol with filter paper stripes.

4. Cast 2 ml stacking gel on top with 1 ml water, 0.22 ml 1:19 40% acrylamide, 0.45 ml 4× stacking buffer, 4 ul TEMED, 25 ul 1% APS. Put on comb quickly
5. Run SDS-PAGE with 4 ul markers, 10 ul of un-induced, and induced samples at 50 mA for 30 min.
6. Stain gel with Coomassie Blue solution for 15 min after heating.
7. Destain for a few hours after heating.

Lab log requirement

1. Expression: SDS-PAGE gel photo stained with Coomassie Blue, with molecular markers, uninduced and induced samples, showing the protein were well expressed.
2. All gel photos well labeled with all necessary information.

Optional module 1

Preparation of chemical competent cells

Aim

To produce the competent cell of *E.coli* which is suitable to store and ready to be transformed.

Introduction

The cells which are able to take up extracellular DNA are said to be "competent". Under artificial treatment, bacterial cells which cannot undergo transformation under natural conditions may be transiently permeable to DNA and thus inducible. For example, *E.coli* may be inducible by high concentrations of $CaCl_2$ to attain artificial competence. The exact action of $CaCl_2$ in bringing competence to cells and the mechanism by which DNA, such as plasmids, enter competent cell are not clear. Most bacterial strains remain in a competent state for 1-2 days at 4°C subsequent to their exposure to agents that can enhance DNA uptake. However, many of them may be kept as frozen competent stocks upon special treatment and stored at very low temperatures such as -70°C.

Materials

0.6 M $CaCl_2$
0.5 M PIPES pH7.0
Glycerol
H_20
Syringes and 0.22 μM filter
Two flasks of 100 ml LB (autoclaved)

Procedures

Preparation of competent cells

1. Spread the bacterial cell (*DH5α*) for plasmid replication on the LB agar and incubate 37 °C overnight.

2. Pick single colony in 5 ml LB culture medium and grow overnight at 37 °C by shaking at ~220 rpm.
3. Add 1ml overnight culture each to two of 100 ml flasks and grow the cell culture to achieve OD 600 = 0.25-0.4 (~ 3 h)
4. While the cell is growing, mixing the the following reagents to be the Ca/glycerol buffer.

0.6 M $CaCl_2$	10 ml
0.5 M PIPES PH7.0	2 ml
Glycerol	15 ml
H_2O	73 ml
Total	100 ml

5. Clean the Ca/glycerol buffer by flowing the buffer through 0.22 µM filter.
6. Transfer the cell culture (total 200 ml) to four of 50 ml sterile centrifugation tubes.
7. Collect the cell by centrifuging at 1000 g for 10 min at 4°C.
8. Gently resuspend the cell in each tube with 10 ml ice-old Ca/glycerol buffer. Keep the solution ice-cold.

   ** Cells must remain clod for the rest of the procedures!*
9. Collect the cell by centrifuging at 1000 g for 10 min at 4 °C.
10. Gently resuspend the cell with 10 ml ice-old Ca/glycerol buffer.
11. Incubate the cells in Ca/glycerol buffer on ice for 30 min.
12. Collect the cell by centrifuging at 1000 g for 10 min at 4 °C.
13. Gently resuspend the cell in each tube with 1.25 ml ice-old Ca/glycerol buffer.
14. Transfer all cells to one tube.
15. Dispense 100 µl aliquots of competent cells into each Eppendorf.
16. Store the tubes at -80 °C.
17. Test the competent Cell.

Transformation Efficiency of PUC19

1. Transform 1 pg, 10 pg and 100 pg PUC19 in to the 100 µl competent Cell separately (also transform some water to create a control).
2. Spread on the ampicillin agar plates and grow at 37 °C overnight.
3. Court the number of colonies (colony forming units, CFU) on the plates.
4. Calculate the transformation efficiency, the standard is there are 5 x10^6 CFU by using 1 µg

plasmid with 100 μg cell.

Lab log requirement

Transformation efficiency of PUC19 ≥ 5 x 10^6 cfu with 100 μL of cells density of 1.6 – 2.6 x 10^{10} cells/mL.
Hand in competent cell stock.

Reference

sigmaaldrich.com, Competent Cell Compendium,4. Retrieved January 18, 2016 from https://www.sigmaaldrich.com/ifb/life-science/comp-cell-compend/files/assets/basic-html/page4.html

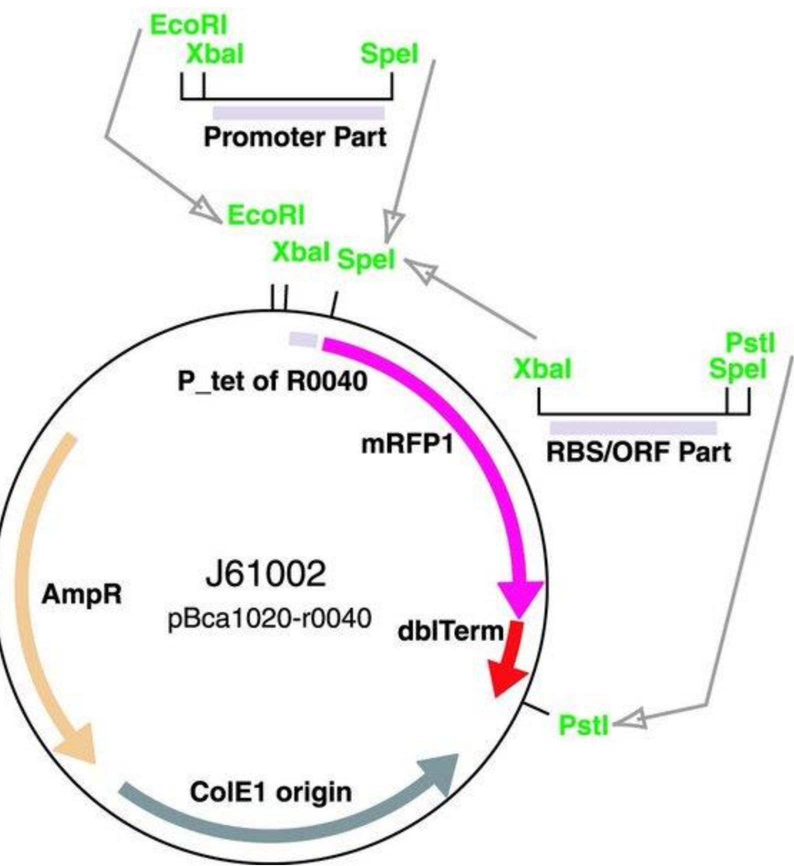
Optional module 2

Production of new biobrick

Clone GFP (BBa_E0040) into any J23100 series constitutive promoters.

Information can be found on iGEM parts registry.

Hand in: clone in plasmid, DNA sequencing file.



Assignment 1

Goal: Select 1 favourite/the best iGEM team from 2010 to 2015. Put down the reasons on the following table.

Date of submission: 15 Feb 2016

	Team	Novelty	Impact	Feasibility	Others
2010					
2011					
2012					
2013					
2014					
2015					

Assignment 2

Goal:

Predict the size of the resultant DNA fragments of the unknown plasmid digested by *EcoR I* and *Xba I*. Also draw a figure to show the expected position of bands in gel using λDNA *Hind III* digest as DNA ladder.

Date of submission: 22 Feb 2016

Unknown plasmid sequence:

GATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCC
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAACTCACCCCA
AAAGAGTTAGACAAGTTGATGCTCCACTATGCTGGAGAATTGGCTAAAAAACGCAA
AGAAAAAGGCATTAAGCTTAACTATGTAGAAGCGGTAGCTTTGATTAGTGCCCATAT
TATGGAAGAAGCGAGAGCTGGTAAAAAGACTGCGGCTGAATTGATGCAAGAAGGG
CGCACTCTTTTAAAACCGGATGATGTGATGGATGGCGTGGCAAGCATGATCCATGAA
GTGGGTATTGAAGCGATGTTTCCTGATGGGACAAAACTCGTAACCGTGCATACCCCT
ATTGAGGCCAATGGTAAATTAGTTCCTGGTGAGTTGTTCTTAAAAAATGAAGACATC
ACTATCAACGAAGGCAAAAAAGCCGTTAGCGTGAAAGTTAAAAATGTTGGCGACAG
ACCGGTTCAAATCGGCTCACACTTCCATTTCTTTGAAGTGAATAGATGCTTAGACTTT
GACAGAGAAAAAACTTTCGGTAAACGCTTAGACATTGCGAGCGGGACAGCGGTAAG
GTTTGAGCCTGGCGAAGAAAAATCCGTAGAATTGATTGACATTGGCGGTAACAGAA
GAATCTTTGGATTTAACGCGTTGGTTGATAGGCAAGCAGACAACGAAAGCAAAAAA
ATTGCTTTACACAGAGCTAAAGAGCGTGGTTTTCATGGCGCTAAAAGCGATGACAAC
TATGTAAAAACAATTAAGGAGTAAGAAATGAAAAAGATTAGCAGAAAAGAATATGT
TTCTATGTATGGCCCTACTACAGGCGATAAAGTGAGATTGGGCGATACAGACTTGAT
CGCTGAAGTAGAACATGACTACACCATTTATGGCGAAGAGCTTAAATTCGGTGGCG
GTAAAACCCTGAGAGAAGGCATGAGCCAATCCAACAACCCTAGCAAAGAAGAATTG
GATCTAATCATCACTAACGCTTTAATCGTGGATTACACCGGTATTTATAAAGCGGAT
ATTGGTATTAAAGATGGCAAAATCGCTGGCATTGGTAAAGGCGGTAACAAAGACAT
GCAAGATGGCGTTAAAAACAATCTTAGCGTAGGTCCTGCTACTGAAGCCTTAGCCGG
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CCAACAAATCCCTACAGCTTTTGCAAGCGGTGTAACAACCATGATTGGTGGCGGAAC
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TAAAATCCACGAAGACTGGGGCACCACTCCTTCTGCAATCAATCATGCGTTAGATGT
TGCAGACAAATACGATGTGCAAGTCGCTATCCACACAGACACTTTGAATGAAGCCG
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CTGAAGGTGCTGGCGGCGGACACGCTCCTGATATTATTAAAGTAGCTGGTGAACAC
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GCATGACATGGGGATTTTCTCAATCACCAGCTCTGACTCTCAAGCTATGGGTCGTGT
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GAACGCTTCTATCCCTACCCCACAACCAGTTTATTACAGAGAAATGTTCGCTCATCA
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CGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCG
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CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTC
TTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTG
ATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGC
GGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATG
CAG

Assignment 3

Goal:

- Design a pair of primers to insert GFP device (BBa_0040) in RFC10 standard into pRSFDuet-1 vector. Show all the information about the primers according to the checklist in lab manual in week 3. Predict the PCR product size.
- Predict the PCR band size using primer 1 and 2 as a set, and primer 1 and 3 as the other set on the unknown plasmid given last time.

Submission date: 29 Feb 2016

Vector sequence of pRSFDuet-1:

GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAAT
AAGGAGATATACCATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGA
ATTCGAGCTCGGCGCGCCTGCAGGTCGACAAGCTTGCGGCCGCATAATGCTTAAGTC
GAACAGAAAGTAATCGTATTGTACACGGCCGCATAATCGAAATTAATACGACTCAC
TATAGGGGAATTGTGAGCGGATAACAATTCCCCATCTTAGTATATTAGTTAAGTATA
AGAAGGAGATATACATATGGCAGATCTCAATTGGATATCGGCCGGCCACGCGATCG
CTGACGTCGGTACCCTCGAGTCTGGTAAAGAAACCGCTGCTGCGAAATTTGAACGCC
AGCACATGGACTCGTCTACTAGCGCAGCTTAATTAACCTAGGCTGCTGCCACCGCTG
AGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGC
TGAAACCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTCCGGTAGTCAATAAAC
CGGTAAACCAGCAATAGACATAAGCGGCTATTTAACGACCCTGCCCTGAACCGACG
ACAAGCTGACGACCGGGTCTCCGCAAGTGGCACTTTTCGGGGAAATGTGCGCGGAA
CCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAATTAATT
CTTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTAT
CAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGC
AGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACAT
CAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCAC
CATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTTCCAGA
CTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAAC
CGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGGTCGCTGTTAAAAG
GACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCA
ACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGG
GGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATG
GTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACA
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CCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTA
TACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTAGAGCAAGACGTT
TCCCGTTGAATATGGCTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGG
GTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAG
GCATGCAGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTACGCTCGGTCGTTCGACT

GCGGCGAGCGGTGTCAGCTCACTCAAAAGCGGTAATACGGTTATCCACAGAATCAG
GGGATAAAGCCGGAAAGAACATGTGAGCAAAAAGCAAAGCACCGGAAGAAGCCAA
CGCCGCAGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGAC
GCTCAAGCCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCC
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CAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCA
GCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACA
CGACTTATCGCCACTGGCAGCAGCCATTGGTAACTGATTTAGAGGACTTTGTCTTGA
AGTTATGCACCTGTTAAGGCTAAACTGAAAGAACAGATTTTGGTGAGTGCGGTCCTC
CAACCCACTTACCTTGGTTCAAAGAGTTGGTAGCTCAGCGAACCTTGAGAAAACCAC
CGTTGGTAGCGGTGGTTTTTCTTTATTTATGAGATGATGAATCAATCGGTCTATCAAG
TCAACGAACAGCTATTCCGTTACTCTAGATTTCAGTGCAATTTATCTCTTCAAATGTA
GCACCTGAAGTCAGCCCCATACGATATAAGTTGTAATTCTCATGTTAGTCATGCCCC
GCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGAT
CCCGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCT
TTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGG
GAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGG
GCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCC
ACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATA
TAACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATGTCCGCACCAACG
CGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCA
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GTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGG
GCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCA
GTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGA
CATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCC
TGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTG
TGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACG
CTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGC
GTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCA
GTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTT
TTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTG
ATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCAC
CACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCG
CCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGA
AATTAATACGACTCACTATA

Primer 1
5'-TTACCAGACTCGAGTCAAACCTTTTGCGTGGT-3'
Primer 2
5'-GAGACACACATATG-ATGAACACTTACGCTCAAG-3'
Primer 3
5’-AATCTCAAATTGAAAGGAATTTTATGGTAAAAATTGGAGTTTGTG-3’ or
5’-TGGAATTCGCGGCCGCTTCTAGATGGTAAAAATTGGAG-3’

Assignment 4

Goal:

- Design a pair of Quikchange primers and Q5 primers to do a point mutation on GFP from *Aequorea Victoria* to abolish its fluorescence. State your rationale.

Submission date: 21 Mar 2016