iGEM 2017: Laboratory BootCamp

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Students:

Introduction

This guide contains the instructions and reference information for the iGEM 2017 Laboratory Bootcamp. The purpose of this bootcamp or workshop is to prepare students unfamiliar with the Kinsgborough laboratory and protocols with some basic training in the lab. At the same time, you will be completing an ancillary project to the main iGEM goal, and might generate data that will be useful for the team's presentation.

The goal of the project is to demonstrate the effectiveness of a particular strategy for assembling different DNA fragments together, the so-called "Kingsborough Assembly". This assembly approach relies on restriction endonucleases (enzymes that cut DNA at specific internal sites) and DNA ligase (enzyme that joins DNA fragments). Most iGEM teams using a restriction enzyme based assembly (there are newer techniques that rely on different enzymes) in the past have utilized an approach known as 3A or 3 antibiotic assembly. The Kingsborough Assembly technique, if effective, eliminates some limitations and steps from the traditional approaches.

3A Assembly and the Limitations

The 3A assembly approach is the standard restriction enzyme cloning strategy for assembly of DNA parts, or BioBricks, in the iGEM competition. In short, two BioBricks, both sourced from plasmids with different antibiotic resistance genes, are combined into another plasmid backbone with yet another, third antibiotic resistance gene (hence the name of the assembly method). This assembly method was designed to eliminate the need for gel purification of DNA fragments, which is laborious and introduces unwanted complications. Restriction digestion of an iGEM plasmid using EcoRI/SpeI or XbaI/PstI combinations will release the gene or DNA of interest, and generate a separate backbone fragment. Insufficient digestion or religation of this fragement and backbone will generate unwanted products, leading to lower cloning efficiency and/or false positives. Use of plasmids with different antibiotic resistance markers at the very least will remove false positives, as religated or uncut plasmids will be unable to grow if grown on an antibiotic corresponding to one of the other plasmids in the assembly.

Despite obviating the need for gel purification of DNA fragments, 3A assembly has limitations. First, it does not prevent creation of unwanted side products (such as religated plasmids), but rather eliminates any bacteria that accept these plasmids during the transformation. Thus, there should be room for improvement in cloning efficiency (that is, amount of desired plasmid constructs and colonies per amount of DNA). Moreover, the necessity of having 3 plasmids, all with different antibiotic resistance genes, limits plasmid choices.

Kingsborough Assembly (3RE Assembly?)

The Kingsborough assembly method seeks to improve upon 3A assembly. Instead of involving different antibiotic resistance genes and a separate acceptor plasmid backbone, the Kingsborough approach features triple digestion of the iGEM plasmids that contain the two DNA sequences that you wish to combine. This is illustrated in the appendix.

As shown, triple digestion with wither EcoRI/SpeI/PstI or EcoRI/XbaI/PstI will generate, for each plasmid, three fragments: 1) A plasmid backbone with EcoRI and PstI 'sticky' ends, 2) A DNA fragment of interest, where 'sticky' end is compatible with the plasmid backbone but the other is compatible only with the fragment from the other reaction and 3) a very short DNA fragment representing the other 'sticky' end for the backbone. This smallest fragment is removed by column purification, which typically excludes any DNA fragments that are smaller than 40bp.

This approach has a number of advantages over typical restriction enzyme cloning and 3A assembly. 1) No gel purification is necessary 2) There is no restriction on plasmid choice, no requirement for different antibiotic resistance. 3) Only two restriction digests are necessary – a third reaction for the backbone is not required, thus saving on tubes, tips, buffer and enzyme.

However, there are potential complications. The two plasmid backbones generated can ligate with each other, forming a large plasmid containing two antibiotic resistance genes (either for some antibiotic or two different antibiotics, depending on plasmid choice) and two origins of replication. This should not be any more problematic than the normal amount of backbone 'doubling' that can occur in a typical restriction digest reaction. Nonetheless, it is important to establish the efficiency of this method. This will be your task in the workshop.

In this series of experiments, you will combine genes from two different iGEM plasmids. These gens encode chromoproteins which will cause the bacteria form colored colonies – an easy visual output that indicates the presence or absence of these plasmids and genes. Moreover, you will use plasmids for different chromoproteins / colors, featuring different antibiotic resistance genes. Although using different antibiotic resistance on the two plasmids is not necessary for this type of assembly, doing so here will enable us to track / determine how efficient the cloning is – for example, the creation of a large plasmid from interaction between the two backbones can easily be determined by selecting for the two different antibiotics.

Schedule in Brief, Summary

Day 1: Setup a triple digestion of the two Bio-brick plasmids to be used. Allow incubation overnight. Preparation and sterilization of LB plates for Day 3.

Day 2: Run the digestion reaction on an electrophoresis gel to determine quantity and quality of the DNA. Purify the remaining DNA from each reaction (using spin column / mini-prep kit). Setup a ligation between the two reactions with appropriate controls. Incubate overnight.

Day 3: Transformation of ligation reactions into competent bacteria.

Day 4: Collection of results, clean up and preparation / replacement of materials

Day 1-A: Mini-prep of bacterial plasmid DNA

In this step, you will extract plasmid DNA from pellets of bacteria grown previously by Professor Camenares. Each group will be given two pellets – each containing a different plasmid, with different chromoproteins. Please follow the modified QIAgen protocol or EZ protocol (provided in the appendix) for completing this task. The modifications will be indicated by the instructor, but will most likely include:

1. Using a solution of 3M Potassium Acetate and 0.9M Glacial Acetic Acid instead of buffer N3 (QIAgen kit)

2. Precipitation of the DNA by adding an equal volume of 95% ethanol after the centrifugation that follows the neutralization step (i.e. before addition to the column). This and the proceeding changes are done to circumvent problems we have had with Guanidine hydrochloride, a standard component of the QIAgen kit.

3. Note: You will set aside any used spin columns and collection tubes – do not discard. These will be cleaned for further reuse later in the bootcamp.

Mini-prep protocol (solutions I, II and III, as well as other materials, will be provided). Annotations in Italics, minutes indicated as ‘ marks, seconds as “ marks.

1. Centrifuge 1 to 1.5mL of the bacterial culture in a microfuge tube at max speed for 30”

2. Aspirate supernatant by pipetting into waste container. Repeat steps 1 and 2 until entire bacterial culture has been pelleted

3. Resuspend pellet in 100uL of ice-cold Solution I

4. Add 200uL of room temperature (RT) Solution II

This step will partially dissolve the bacterial membrane, allowing small proteins, RNA, and plasmid DNA to escape, but leaving genomic DNA and other high molecular weight molecules behind.

5. Invert the tube rapidly five times, incubate on ice until solution is homogenized (3-5’)

6. Add 150uL of Solution III, incubate on ice 3-5’

This step will precipitate all high molecular weight macromolecules and cell debris

7. Centrifuge for 5’ at max speed. Transfer supernatant to fresh tube (pipette carefully, do not disturb pellet!)

8. Add 2 volumes of RT 100% ethanol (or isopropanol). Incubate 2’ at RT

This step will precipitate nucleic acids and prepare them to bind the spin column

9. Load mixture onto a QIAgen mini-prep spin column (or similar column). Load a maximum of 750uL, centrifuge for 1' at max speed, and discard flow-through. Repeat until the entire contents of the tube at step 9 is transferred and centrifuged.

10. Add 750uL of 70% Ethanol. Centrifuge 1' at max speed, discard flow-through.

11. Centrifuge and additional minute to remove any residual ethanol.

12. Transfer the spin column to a fresh 1.5mL micro-centrifuge tube (set previous collection tube aside). Add 30uL to 50uL of dH20 (for metal sensitive applications) or TE (pH 8) to the column.

This step releases DNA bound to the spin column – the flow through generated will be saved. Label this tube appropriately.

13. Centrifuge for 1' at max speed. Remove spin column, store flow through / centrifuge tube in at -20C.

Day 1-B: Setting up the Restriction Digestion reaction

In this step you will mix the plasmid DNA from the above mini-prep (1-A) with restriction enzymes so that the plasmid will be cut into fragments, producing a plasmid backbone to accept new fragments as well as the chromoprotein gene on a separate fragment.

Setup two separate 500uL tubes, labelling in some way to differentiate between the two plasmids you will be using (i.e. mark the top of one with a blue dot, the other with a red dot.) Make sure you make clear on this document or in your notes how you are labelling your tubes.

Reaction setup (last four columns are restriction enzymes, to be provided by the instructor when you are ready):

Reaction / Label	**DNA**	**10X Cutsmart Buffer**	**dH20**	**EcoRI**	**PstI**	**XbaI**	**SpeI**
Plasmid #1 Color:	25uL	5uL	17uL	1uL	1uL	1uL	0
Plasmid #2 Color:	25uL	5uL	17uL	1uL	1uL	0	1uL

Make sure that you add the enzymes last, after all the other components are added. Place the tubes at 37C for 1 to 3 hours and then hold at 4C or -20C until the next day.

Day 1-C: Preparation of sterile LB plates with appropriate antibiotics

In this step, you will prepare plates of nutrient agar (LB agar) containing appropriate antibiotics (Chloramphenicol, among others) that will be used on Day 3 to select for antibiotic resistant bacteria. Follow the **Autoclave** use protocol for proper use of the autoclave, as the instructor indicates, and prepare 500mL of LB Agar. Once the LB agar has cooled to the point where it is very warm, but not hot, you will add the antibiotic, swirl the flask, and pour the plates as indicated by the professor. (If time does not permit, the professor may pour the plates).

As indicated in the protocol, the LB plates and agar are made of 25g/L of LB broth powder and 15g/L agar. They will contain the antibiotic Chloramphenicol, to be prepared by the instructor.

Day 2-A: Casting the gel

In order to determine if the restriction digest was successful, and to qualitatively measure the concentration of the DNA, you will be using gel electrophoresis to separate by size the DNA mixtures (plasmid preps, digestion reactions). This procedure has been split among several protocols to indicate that you should perform other activities while waiting for several of these steps. Your instructor will indicate how many gels should be made per student or group (several gels for a large group of students – for a smaller set, 1 gel to be shared may suffice. Each student needs between 2 and 4 lanes, and there are 10 and 14 well combs available.)

- If necessary, prepare 500mL of 1X $NaBO_4$ running buffer by mixing 50mL of 10X buffer with 450mL of dH20. Use a 500mL or greater beaker or flask.

- In a separate, smaller flask, mix 50mL of the 1X running buffer with 0.5g of Agarose (weigh using a balance and weighing dish). Setup the gel apparatus to receive the gel (i.e. create a reservoir by taping the ends or through other means, and locate an appropriate comb.

- Heat the flask in the microwave for approximately 2 minutes at medium power, or until all the agarose is dissolved. Caution! The resulting mixture will be hot! Pour the molten agarose mixture into the gel casting tray / reservoir. Allow the gel to cool for 15-20 minutes.

Day 2-B: Column purification of Digest reactions

In this step, you will purify the DNA from the digestion reaction, removing any buffering salts and the restriction enzymes. This step is a necessary precursor to mixing the DNA from each ligation reaction and ligation of them together. Continued presence of the restriction endonucleases will interfere with this step. This step is very similar to the last half of the mini-prep protocol referenced above, and follows exactly the protocol provided by Bio-Basic. Here is a brief summary, with volumes adjusted for this workshop:

- Add 150uL of Binding Buffer II to the restriction digestion mixture. Pipette up and down several times to mix.

- Transfer the entire volume of the digestion / binding buffer mixture (~200uL) to the provided spin columns (Bio-Basic kit). Centrifuge 1' at max speed (12500 rpm is OK) and discard flow through.

- Wash the column with 750uL of Wash solution containing Ethanol. Centrifuge 1' at max speed and discard flow through.

- Centrifuge the column an additional 1' to remove any residual Ethanol.

- Place the spin column in a fresh 1.5mL micro-centrifuge. Add 30uL of dH20 to the spin column, incubate 1', and centrifuge for 1'. Remove the spin column – the flow through contains your DNA, and the micro-centrifuge tube should be labelled appropriately.

Day 2-C: Loading and running the gel

Once the gel has cooled / solidified, and the digest DNA has been column purified, you can load the digests on the gel. The order of samples to be loaded on the gel will depend upon how many gels were cast, how many samples are present, and will be determined by your instructor. Carefully remove the comb and make sure the gel is oriented appropriately. It is also standard practice to submerge the gel in 1X running buffer (prepared in the earlier step) before loading each well, but you may load the gel dry and carefully add the running buffer afterwards if more convenient.

For each well, you will load 5uL of DNA from either a control (a molecular weight marker or ladder), the plasmid mini-prep, and/or the purified digests. For each 5ul of sample, it must first be mixed with 1uL of 6X dye. The recommended way to do this is as follows: take a piece of wax paper / parafilm, upon which you will place several spots containing 1uL of dye. To each spot, you will add 5uL of the appropriate DNA sample, mix by pipetting up and down, and then load the entire mixture (5-6uL) to the appropriate well on the gel.

Once the gel is loaded, connect the electrodes to the power supply (make sure the negative electrode is near the end of the gel that has the DNA – the DNA will migrate towards the positive end). The gel should be run at a voltage of 120 – 200 volts (the higher, the fast the gel runs) for about 30 to 60 mins. Be careful to make sure that the dye front moves in the correct direction and has travelled at least halfway down the gel. Running at too high a voltage will cause the gel to heat up, and excessive temperatures will melt the agarose and ruin the experiment. Periodically checking the temperature if running at a high voltage is a good idea.

Day 2-D: Setting up the ligation reaction

In a ligation reaction, different DNA fragments with compatible ends are mixed together and permanently joined by DNA ligase to form a new recombinant molecule. The resulting molecule is usually not utilized directly or purified from the reaction, but rather is transformed in bacteria and selected for afterwards. Moreover, having insufficiently cut DNA, or the presence of several fragments that can recombine in several ways makes analysis of a ligation reaction difficult. Thus, it is normally necessary to include controls, such as samples that do not have ligase, to determine what DNA present at the end is actually recombinant DNA created by the ligation reaction (as opposed to uncut plasmid DNA).

The following protocol is based upon the fast E-Z ligation kit from Bio-Basic. You will need to get 5 new 500uL tubes, and label either with a color or letter / number designation, which you can indicate below. For Plasmids 1 and 2, you will use the plasmids that you digested and purified in earlier experiments (Days , respectively).

Reaction / Label	**Plasmid 1**	**Plasmid 2**	**dH20**	**2X Ligation Mix**	**Purpose**
A	2.5uL	0	7.5uL	0	Uncut Control
B	2.5uL	0	2.5uL	5uL	Self-ligation Control
C	0	2.5uL	7.5uL	0	Uncut Control
D	0	2.5uL	2.5uL	5uL	Self-Ligation Control
E	2.5uL	2.5uL	0	5uL	Desired Product

The above reaction should be incubated for at least 10 minutes at room temperature – but for optimal results, can be left at room temperature for up to an hour. Afterwards, the mixture should be stored at -20C.

Day 2-E: Staining the gel

In order to visual the DNA on the gel, you must apply a stain that will specifically bind nucleic acid. The stain most commonly used is known as Ethidium Bromide (EtBr). It must be handled with care, and your instructor will handle all steps involving EtBr. All liquids and solids that contain EtBr must be considered EtBr waste and be disposed of appropriately (do not discard in the normal trash or down the sink). The dangerous of EtBr are sometimes exaggerated, but it is better to be safe than sorry, and it is imperative to follow the prescribed safety procedures.

- Turn off the voltage and remove the gel from the electrophoresis apparatus and place in a staining tray or similar container. Add approximately 50mL of dH20 (enough to cover the bottom of the tray).

- Add ~5uL of EtBr stain, and place on an orbital platform to shake for about 10 minutes.

- Discard the staining solution into EtBr liquid waste container, and add another ~50mL of dH20 to the gel to remove excess stain. Place on the shaking platform for an additional 5 minutes.

- Discard the destaining solution as EtBr liquid waste. Place the now stained gel on the UV transilluminator to visual the EtBr stained DNA bands (they will appear orange). Caution! Place the plastic shield over the transilluminator so that you are not exposed to the UV rays.

- For your records, it is a good idea to take a picture of the gel. It is helpful to turn off the room lights when observing the gel, or to place the camera shield over the gel before taking a picture (the shield with the red/orange filter, located underneath the transilluminator, will be particularly helpful as it will filter out the UV lights from the camera image.

Day 3-A: Transformation of chemically competent cells

In this step, you will use chemically competent cells, prepared by the instructor, to transform the five ligation reactions setup on Day 2, in addition to a control (Labelled as: _____) to ensure that the bacteria are accepting DNA efficiently. The protocol is found in the appendix, and is in general as follows:

- Before beginning, have ready an ice bucket and set a heating block to 42C. Also, obtain 8 LB plates with chloramphenicol, and label them with the date and an indicator matching the transformation it will be used for: A, B, C, D, or E0, E1, E2, and E3 (for replicates of the final transformation / ligation mixture).

- Add between 2 – 4uL of the ligation reaction to the competent cell mixture

- Incubate on ice for 30 minutes. It is critically important that the competent cells are kept cold until the next step – be careful when handling them!

- Heat shock at 42C for 30 seconds
- Incubate on ice for an additional 2 minutes
- Add 600UL of fresh sterile LB or SOC solution
- Incubate for at least an hour at 37C, shaking if possible. Alternatively, shaking at room temperature is acceptable, especially if additional time can be allotted (~30 mins). This recovery step is necessary to allow the bacteria time to express genes located on the plasmid (such as the selectable marker) before they are applied to a selective media.
- Pipette bacteria (no more than 100uL) onto LB agar petri dish with the appropriate antibiotic, spread evenly using a sterile spreading tool. For transformations A-D, add to the plate of the same name. For transformation E, add to plate E0. Allow the plate to dry and place at 37C overnight.
- Normally, you would store any remaining bacterial mixture in the refrigerator (4C) – this is what you will do with transformations A-D. However, in this experiment you will, for reaction E, add 10uL of X-gal / IPTG solution (to be provided by instructor) to the remaining bacterial mixture and again pipette 100uL of the mixture onto an LB agar petri dish (E1). Repeat this procedure (an addition 10uL of X-gal / IPTG) two more times for plates E2 and E3. Spread bacteria evenly and allow the plate to dry. Place at 37C overnight.

As stated above, it is imperative in this protocol that you keep the bacteria on ice when indicated! Pre-mature thawing of the bacteria can greatly reduce the efficiency at which it accepts foreign DNA. In addition, the final hour incubation is necessary so that the bacteria have time to express the antibiotic resistance gene.

An explanation regarding the last step: The Xgal-IPTG solution contains 10mg/mL of Xgal and 0.5M IPTG in 50% DMF. The working concentration is typically 20ug/mL Xgal and 1mM IPTG. When mixed and ultimately diffused onto the ~20mL agar plate, the concentrations will approximate those listed in the table on Day 4, with some different allowed for a higher local concentration on the surface of the plate.

Day 3-B: Clean-Up of Spin Mini-prep columns from Days 1 and 2

This begins a set of procedures is designed to clean-up or regenerate some of the materials that were used. This is an important step to carry out as you complete different experiments in order to provide ample materials for the next experiment.

This is very simple: assemble used spin columns with collection tubes, and add 500uL of 1M Hydrochloric Acid to each spin column. All the column to incubate for at least 1 hour, or even overnight, at room temperature.

Day 4-A: Recording Results

The results of the above experiments will be observed on this day. The results expected or to be measured will be the number and appearance of the colonies from each transformation.

The number of colonies observed on plates A – D will indicate how much uncut or self-ligated plasmid is present in each plasmid digestion that was prepared. Bacteria that have the genes from plasmid 1 will appear red. Bacteria that have genes from plasmid 2 will appear blue if grown in the presence of a sufficient amount of Xgal and IPTG (the first is the molecule that is blue when cleaved by the LacZ enzyme – the second molecule of that pair is the chemical that induces LacZ expression).

Successful ligation of the two genes from each plasmid together will result in colonies that have a combination of a blue and red hue. Ligation of the plasmid backbones together will result in colonies that have no color. The ratio between the different types of colonies that are observed will indicate how successful this strategy is for combining two genes, and how many unwanted side products are typically produced.

You may record the results in the following table:

Plate	**Beige Colonies**	**Red Colonies**	**Blue Colonies**	**Red/Blue Colonies (Purple)**	**Approx Xgal/IPTG amount (ug/mL, mM)**
A					--
B					--
C					--
D					--
E0					--
E1					8 ug/mL 0.5 mM
E2					18 ug/mL 1 mM
E3					30 ug/mL 1.5 mM

Day 4-B: Regenerating materials

This is a continuation of the spin column clean-up begun on Day 3 (Activity B). In addition to the column regeneration procedure below, you should also restock tip and tube containers and autoclave sterilize them. You should also clean and sterilize the spreaders used, and discard any other leftover materials.

- Step 1: For column clean up, locate the spin columns that have been incubating with 500uL of hydrochloric acid.

- Step 2: Centrifuge the columns for 1' at max speed, discard flow through.

- Step 3: Add 1mL of dH20 to each column

- Repeat steps 2 and 3 an additional 4 times.

- Centrifuge the columns 2 additional times for 1'. Place columns in laminar flow hood, open, for 15 minutes – 1 hour to ensure the columns are dry.

- Store columns in a bag or container that indicates they are regenerated – not new, but ready to be used again.