Basics of Molecular Cloning: Instructor's Manual

This instructor's manual is avaliable online only.

1. Purpose and Concepts Covered

This introductory cloning laboratory is for use in courses that cover basic topics in molecular biology and genetics. Molecular cloning is a basic technique used in a molecular biology labs. In this manual, we include a protocol for isolating the luciferase gene from DNA using restriction digestion and cloning it into the multiple cloning region of a vector. The cloned luciferase gene is then expressed in *E. coli*.

2. Protocol for Cloning the Luciferase Gene into the pGEM® Vector

Preparation for the Laboratory

Materials Required

- pGEM®-*luc* DNA (Cat.# E1541)
- pGEM®-4Z Vector (Cat.# P2161)
- BamHI restriction enzyme (Cat.# R6021; 10u/µl)
- SacI restriction enzyme (Cat.# R6061; 10u/µl)
- Buffer E (supplied with BamHI enzyme)
- Acetylated BSA (supplied with restriction enzymes)
- Agarose, LMP, Preparative Grade for Large Fragments (>1,000bp; Cat.# V2831)
- TBE Buffer, 10X, Molecular Biology Grade (Cat.# V4251; or have students make their own buffer)
- Ethidium Bromide Solution, Molecular Biology Grade (Cat.# H5041)
  Note: The gel fragment cleanup system will not work with DNA that has been stained with methylene blue.
- BenchTop pGEM® DNA Markers (Cat.# G7521)
- Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281)
- Blue/Orange Loading Dye, 6X (Cat.# G1881)
- T4 DNA Ligase (Cat.# M1801)
- agarose gel electrophoresis apparatus and power supply
- 37°C water bath
- pipettors (0.5–10µl and 10–200µl) and appropriate sterile pipet tips
- long-wave UV light box and camera or scanner
- 1.5ml sterile tubes
- gloves

(!) Ethidium bromide is a carcinogen. Be sure to follow your institution's safety and disposal instructions when working with ethidium bromide.

2.A. Excising the Luciferase Gene from Surrounding DNA

In this part of the protocol, you will be isolating the gene of interest, the firefly luciferase gene, from surrounding DNA. To remove the luciferase gene, you will be performing a restriction digest using BamHI and SacI. You also will need to perform the same double digest on your target cloning vector (pGEM®-4Z Vector).

You will need to set up your restriction digest in a total volume of **20µl** using the guidelines below:

Restriction Enzyme Buffer E, final concentration 1X
2µg of Acetylated BSA
1µg of DNA
Nuclease-Free Water to a volume of 19.0µl

Mix these reagents by pipetting and then add:

5 units of BamHI in a total of 0.5µl
5 units of SacI in a total 0f 0.5µl

1. Calculate the volume of Buffer E you will need in the reaction to achieve a final concentration of 1X.

   Note to the instructor: Buffer E is supplied as a 10X solution. The students will need to dilute the Buffer E so that its final concentration in the reaction is 1X.

   Using CV = CV,

   (y µl) (10X Buffer E) = (20µl of 1X Buffer E)
   10y = 20
   y = 2µl of 10X Buffer E

2. Calculate the volume of DNA you will need to add to your reaction in order to digest 1µg.

   Note to the instructor: The concentration of the pGEM®-*luc* Vector is provided on the lot-specific product information sheet that accompanies the vector. Using that information, your students can calculate the volume of DNA to add to their reaction.

   They can do the same for the target vector (pGEM®-4Z Vector).

3. Calculate the volume of Acetylated BSA you will need to add to your reactions, so that you have a total of 2µg of Acetylated BSA in your reaction. The Acetylated BSA is supplied at a concentration of 10µg/µl.

   Note: Pipetting volumes smaller than 0.5µl is very difficult. When determining how much Acetylated BSA to add, you may need to dilute the BSA to a lower concentration so that you can pipette a larger volume into your reaction (0.5 or 1.0µl).

4. Calculate the volume of water to add to bring the volume (without enzyme) to a total of 19.0µl. Enter the volumes of each component into the table below:

Reaction Component	Concentration	Volume to Add
Nuclease-Free Water (add to a total volume of 19.0µl)	NA	_____
Restriction Enzyme Buffer E	10X	_____
Acetylated BSA	_____	_____
DNA	_____	_____

5. Add the components, in the order listed, to a sterile 1.5ml tube. Mix them by pipetting.

6. Calculate the amount of each enzyme to add to your reaction. You will need to add a total of 5 units of each enzyme. Using the concentration of each of the enzymes provided to you by your instructor, determine what dilution (if any) you need to make so that 0.5µl of the enzyme contains 5 units.

   Note: High concentrations of either glycerol or enzyme can interfere with your restriction digestion. Therefore, be sure that you do not add more than 1.0µl total of enzymes to your digest.

7. Add 5 units of BamHI and 5 units of SacI to your restriction digest. Mix gently by pipetting.

8. Close the tube and centrifuge for a few seconds in a microcentrifuge to pull all of the liquid to the bottom of the tube.
9. Incubate at 37°C for 1 hour.

   Note to the students: You should have performed two restriction digestions: one of pGEM®-*luc* DNA to remove the luciferase gene insert, and one of the pGEM®-4Z Vector to prepare it to receive the insert.

2.B. Purifying the Gene Fragment

After your restriction digest is complete, you will separate the luciferase gene fragment (~1,740bp) from the remainder of the DNA using agarose gel electrophoresis. The luciferase gene DNA can be purified from the gel using a commercially available DNA cleanup system. You also will need to gel purify your restricted target vector (pGEM®-4Z Vector), which will be a fragment of ~2,733bp.

1. Determine the appropriate agarose gel composition to use to isolate the luciferase gene from the other DNA in the restriction digest using the table provided at: **www.promega.com/techserv/techref/agarose_polyacryl.htm** (Gel percentages: resolution of linear DNA on agarose gels). You will need to clearly separate the 1,740bp gene fragment from the other fragment that will be created by the digest (3,191bp).

   Note to the instructor: We recommend 1.5% agarose.

(!) Wear appropriate safety glasses when working over a UV light box.

2. Prepare 100ml of 10X TBE Gel Running Buffer using the following guidelines.

   0.89M Tris base
   0.89M boric acid
   20mM EDTA (pH 8.0)

   Notes to the instructor:

   Molecular Weight (MW) Tris base 121.14g/mol; MW boric acid 61.83g/mol

   Recipe for 1L 10X TBE from promega.com: Dissolve 108g of Tris base and 55g of boric acid in 900ml of deionized water. Add 40ml of 0.5M EDTA (pH 8.0), and increase the final volume to 1 liter. Store at room temperature or 4°C.

   Make 1X TBE by diluting the 10X TBE with water. Determine the final volume of 1X TBE needed. Caluculate how much 10X TBE and how much water to mix to make this volume of 1X TBE.

3. Prepare your agarose gel 1.5% weight/volume agarose in 1X TBE (e.g., 1.5g of agarose in 100ml of 1X TBE). Put the agarose and the buffer in a flask that is at least twice the volume of buffer used. Heat the buffer and agarose in a microwave oven or on a hot plate to melt the agarose. (Do not let the buffer boil over. The loss of buffer will change the agarose percentage.) Allow the solution to cool to 50°C (cool enough to hold comfortably in your hand) before pouring the solution into the gel tray. Add ethidium bromide to a final concentration of 5µg/ml to the cooling solution, and swirl the flask to mix. Remember to put in the comb to create loading wells before the agarose solidifies.

2.B. Purifying the Gene Fragment (continued)

Notes to the instructor:

You will need to provide detailed instructions (volume, inserting comb, etc.) for gel preparation based on the particular gel aparatus you are using.

We recommend adding ethidium bromide (final concentration 5µg/ml) to the gel before it is poured rather than using it in the gel running buffer. Although the sizing is more accurate when the gel is stained after running, confining the ethidum bromide to the gel itself helps manage disposal.

4. When the gel has solidified, remove the comb, being careful not to tear the wells. Place the gel in the gel box and cover with 1X TBE buffer.
5. Remove 5µl from each of your restriction digests. Add 1µl of 6X blue/orange loading dye. Add 1µl of dye to 5µl of the uncut DNA.
6. Load digests and run the gel until the markers in the loading dye have migrated to the appropriate point in the gel to have separated the fragments in which you are interested. (See reference material at: **www.promega.com/techserv/techref/agarose_polyacryl.htm** for help determining this).
7. Turn off the electricity flowing to the gel box. Remove the gel, and place it on a long-wave UV box. (Short wave UV can damage the DNA.) Using a scalpel or razor blade, cut the 1,740bp gene fragment from the gel, and place it in a preweighed 1.5ml microcentrifuge tube.
8. Weigh the tube with the gel slice, and determine the weight of your gel slice.

   Note: At this point you will use the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) to extract your insert DNA from the gel slice. Detailed instructions are available in Technical Bulletin #TB308, available at: **www.promega.com/tbs/**
9. Add 10µl of Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C for 10 minutes or until gel slice is completely dissolved.
10. Insert the SV Minicolumn into a Collection Tube from the Wizard® SV Gel and PCR Clean-Up System.
11. Transfer the dissolved gel mixture to the Minicolumn assembly. Incubate at room temperature for 1 minute.
12. Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
13. Add 700µl of Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

    Note: You can have students calculate the RPM of the centrifuge to achieve a target RCF (in this case the target is 16,000 × *g*) for your particular microcentrifuge using the radius of the rotor. There is an online calculator at: **www.sciencegateway.org/tools/rotor.htm**, but if you prefer, your students can use this equation:

    $g = (1.118 \times 10^{-5}) RS^2$

    Where *g* is the relative centrifugal force, R is the radius of the rotor in centimeters, and S is the speed of the centrifuge in revolutions per minute.

Note: The Wizard® SV Gel and PCR Clean-Up System recommends spinning down the minicolumn for one minute to help with ethanol evaporation. We eliminated this direction from Step 15 because of safety concerns. Instructors should use their discretion about recommending this procedure for students.

14. Repeat Step 13 with of 500µl Membrane Wash Solution. Centrifuge at 16,000 × *g* for 5 minutes.
15. Empty the Collection Tube, place the open column in a stand on the lab bench to evaporate the ethanol. There should be no longer an odor of the Column Wash buffer.
16. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
17. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × *g* for 1 minute.
18. Discard Minicolumn and store DNA at 4°C or –20°C.

2.C. Ligating the Insert into the Vector

Determining the Vector:Insert Ratio

Note: To estimate DNA concentration, students should prepare a second gel and load it with 10µl of the digests and 500ng of Lambda Hind IIIDNA markers to estimate the concentration. Alternatively this can be done using a spectrometer if one is available.

After the insert DNA has been prepared for ligation, estimate the concentration by comparing the staining intensity with that of DNA molecular weight standards of known concentrations on an ethidium bromide-stained agarose gel. If the vector DNA concentration is unknown, estimate the vector concentration by the same method. Test various vector:insert DNA ratios to determine the optimal ratio for a particular vector and insert. In most cases, either a 1:1 or a 1:3 molar ratio of vector:insert works well. The following example illustrates the calculation of the amount of insert required at a specific molar ratio of vector.

[ng of vector × size of insert (in kb)] ÷ size of vector (in kb) × molar amount of (insert ÷ vector) = ng of insert

Example: How much 500bp insert DNA needs to be added to 100ng of 3.0kb vector in a ligation reaction for a desired vector:insert ratio of 1:3?

[(100ng vector × 0.5kb insert) ÷ 3.0kb vector] × (3 ÷ 1) = 50ng insert

1. Briefly centrifuge the pGEM®-4Z Vector and the DNA insert tubes to collect contents at the bottom of the tube. (**Note:** Detailed information about the vector is provided in Technical Bulletin #TB033, available at: www.promega.com/tbs .)
2. Set up ligation reactions as described below. Vortex the T4 DNA Ligase 10X Buffer vigorously before each use. Use 0.5ml tubes known to have low DNA-binding capacity.

Component	Standard Reaction	Negative Control
T4 DNA Ligase 10X Buffer	1µl	1µl
Vector DNA	50ng (Xµl)	50ng (Xµl)
Insert DNA	Xng (Xµl)	None
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl
Nuclease-Free Water to a volume of	**10µl**	**10µl**

Note: If digest yields are low, it may be necessary to increase the overall volume of the reaction. Remember to maintain rations of volume is increased.

2.C. Ligating the Insert into the Vector (continued)

3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature. Alternatively, incubate the reactions overnight at 4°C for the maximum number of transformants.

   Note to the Instructor: Because the target vector was cut with two different enzymes that do not generate compatible ends, the vector should not spontaneously religate. You might discuss with the students what precautions they would have to take if the enzymes produced "compatible ends" or if they were only cutting the vector with a single enzyme (i.e., removing phosphate groups from the end of the vector so that the ligation reaction could not be catalyzed between the two ends).

2.D. Transforming Bacteria with Your Clone

Materials Required

- Single-Step KRX Competent Cells (Cat.# L3002; three tubes per lab group)
- vector with ligated insert
- uncut vector positive transformation control
- cut vector negative transformation control
- 17 × 100mm polypropylene tubes (BD Falcon Cat.# 352059) or 1.5ml microcentrifuge tubes
- ice bath
- 42°C water bath
- 37°C shaking water bath or incubator
- pipettors (0.5–10µl and 10–200µl) and appropriate sterile pipet tips
- LB/ampicillin/IPTG/X-Gal plates
- SOC medium
- glass "hockey stick" for plating cells on selective plates

1. Prepare LB/ampicillin/IPTG/X-Gal plates (see Section).
2. Centrifuge the ligation reactions briefly.
3. Place the high-efficiency Single-Step KRX Competent Cells in an ice bath until just thawed (5 minutes). Mix cells by gently flicking the tube.
4. Add the 2µl of the ligation reaction to one tube of thawed cells. Add 0.1ng of uncut plasmid to a second tube of thawed cells for the positive control tube. Add 0.1ng of cut plasmid to a third tube of thawed cells for the negative control. Gently flick the tubes, and incubate on ice for 5 minutes.
5. Heat-shock the cells for 15–20 seconds in a water bath at exactly 42°C. DO NOT SHAKE. Immediately return the tubes to ice for 2 minutes.
6. Add 950µl of room temperature SOC medium to the ligation reaction transformations and 900µl to the tranformation control tube. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
7. Plate 100µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC is recommended prior to plating.
8. Incubate plates overnight at 37°C. Select white colonies.

2.D. Transforming Bacteria with Your Clone (continued)

Calculation of Transformation Efficiency

For every transformation with competent cells, we recommend performing a transformation control experiment using a known quantity of a purified, supercoiled plasmid DNA (e.g., pGEM®-4Z Vector, Cat.# P2161). Calculate the transformation efficiency as described below.

transformation efficiency (cfu/µg) = (cfu on control plate ÷ ng of supercoiled vector plated) × (10^3ng/µg) × final dilution factor

cfu = colony forming units

Example:

A 100µl aliquot of competent cells is transformed with 1ng of supercoiled pGEM®-4Z Vector DNA. Ten microliters of the transformation reaction (0.1ng total DNA) is added to 990µl of SOC medium (1:100 dilution). Of that volume (1,000µl), a 100µl aliquot is plated (1:1,000 final dilution), and 100 colonies are obtained on the plate. What is the transformation efficiency?

(100cfu ÷ 0.1ng of supercoiled vector plated) × (10^3ng/µg) × 1,000 = 1 x 10^9 cfu/µg

Note to the Instructor: If you have your students plate 10µl, 100µl, 500µl of the uncut positive transformation control, they can practice calculating the transformation frequency for their experiment using the above equation.

2.E. Expressing Luciferase in *E. coli*

- Terrific Broth + ampicillin (100µg/ml) (fresh)
- Luria Broth + ampicillin (100µg/ml) (fresh)
- 20% solution of Rhamnose (L-rhamnose Monohydrate, Cat.# L5701)
- 37°C shaking incubator for liquid culture
- 15–25°C shaking incubator for liquid culture
- Luciferase Assay Reagent (LAR; Cat.# E1483)
- 450µl 100mM sodium citrate (pH 5.5)
- pipettors (0.5–10µl, 10–200µl and 1ml) and appropriate sterile pipet tips

White colonies should have the luciferase gene cloned in the correct orientation in the pGEM®-4Z Vector to be expressed from the T7 promoter on the vector. Since the KRX strain of *E. coli* contains the necessary information to express a gene from the T7 promoter, your students should be able to see "glowing" cells when the cells are provided with the appropriate substrates for the luciferase reaction.

1. From your transformation plates, use a sterile innoculating loop to pick four or five white colonies into indivdual 5ml liquid cultures of LB Broth + Amp using aseptic technique and grow overnight at 37°C with shaking*. (Seal your original plates and store them, inverted at 4°C.)

   Note: Better growth was observed when culture tubes were incubated at an angle during overnight shaking.

2. Dilute your overnight cultures 1:100 into new, labeled tubes of Terrific Broth + Ampicillin. Grow cultures at 37°C with shaking at 275rpm until they reach an $O.D._{600}$ of 0.8–1.0.

3. Shift cultures to a second incubator shaker set at 15–25°C and continue shaking until cells reach an $O.D._{600}$ of 1.0–1.5.

4. Induce protein expression by adding rhamnose to a concentration of 0.1%. (1:200 dilution of a 20% rhamnose solution).

5. Grow cultures overnight at 15–25°C with shaking at 275rpm.

6. Transfer 500µl of each your cultures to labeled 1.5ml centrifuge tubes. Pellet the cells by centrifugation at 10,000 × *g* for 3 minutes in a microcentrifuge.

7. Aspirate the supernatant and resuspend the cell pellet in 450µl 100mM sodium citrate + 50µl Luciferase Assay Reagent.

7. Take the tube into a dark room. Vigorously shake the tube to break up the clumps of cells and to introduce oxygen into the reaction.

8. Are any of the cells in your tubes "glowing"?

   Note: The luminescence will be very faint to the naked eye. Comparison to blank tubes helps with visual dectection of luminescence.

3. Composition of Buffers and Solutions

IPTG stock solution, 0.1M

1.2g IPTG (Cat.# V3955)

Add water to 50ml final volume. Sterilize through a 0.2µm filter unit, and store at 4°C for up to 60 days or store in aliquots at –20°C for up to six months.

LB medium with or without antibiotic

10g/L Bacto®-tryptone
5g/L Bacto®-yeast extract
5g/L NaCl

Adjust the pH to 7.5 with NaOH. Autoclave to sterilize. Allow the autoclaved medium to cool to 55°C, and add antibiotic to a final concentration shown in the table below.

Antibiotics Useful for Plasmid Selection in KRX.

Antibiotic	Plasmid Marker	Final Concentration
Ampicillin	*bla*, Amp^r	100µg/ml
Carbenicillin	*bla*, Amp^r	50µg/ml
Kanamycin	Kan^r	30µg/ml
Tetracycline	Tet^r	12.5µg/ml
Chloramphenicol	Cm^r, Cam^r	34µg/ml

For LB plates, include 15g agar prior to autoclaving.

rhamnose, 20% (w/v)

10g L-rhamnose monohydrate (Cat.# L5701)

Add distilled water to 45ml, sterilize through a 0.2µm filter unit and store in aliquots at –20°C.

sodium citrate

To prepare a sodium citrate solution at pH 5.5 you would weigh the appropriate amount of trisodium citrate dihydrate solid (m.w. 294.12 g/L) depending the desired molarity. Dissolve it in deionized water and titrate the pH to 5.5 using citric acid monohydrate (m.w. 210.14 g/L). Note: There are 3 types of sodium citrate salts: monosodium citrate, disodium citrate, and trisodium citrate. Disodium citrate and trisodium citrates are commonly used in the lab.

SOC medium

2.0g Bacto®-tryptone
0.5g Bacto®-yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml Mg^{2+} stock solution, 2M
1ml 2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave, and cool to room temperature. Add sterile 2M Mg^{2+} stock and 2M glucose stock, each to a final concentration 20mM. Bring to 100ml with distilled water. Filter through a sterile 0.2µm filter unit.

Terrific Broth

12.0g Bacto®-tryptone
24.0g Bacto®-yeast extract
4ml glycerol
100ml potassium phosphate, 0.89M

Add Bacto®-tryptone, Bacto®-yeast extract, glycerol to 750ml distilled water. Stir to dissolve, then bring the volume to 900ml with distilled water. Autoclave, and cool to 60ºC. Add 100ml of sterile 0.89M potassium phosphate to a final volume of 1,000ml. Filter through a sterile 0.2µm unit.

X-Gal

Available from Promega (Cat.# V3941) at a concentration of 50mg/ml in dimethylformamide.

Note for all filter sterilized solutions: Filter-sterilizing units should be prerinsed with distilled water before use to remove any toxic material.