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DNA Ligation

Teacher's Guidebook

(Cat. # BE-308)

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MATERIALS INCLUDED

This kit has enough materials and reagents for 24 students (six groups of four students).

- 1 vial λ DNA (digested)
- 1 vial T4 DNA Ligase Buffer (4X)
- 6 vials T4 DNA Ligase Enzyme
- 1 vial LabSafe™ Nucleic Acid Stain
- 1 vial Sterile Water
- 1 pack Agarose
- 1 vial DNA Ladder (1kb)
- 1 vial DNA Loading Buffer (6X)
- 1 bottle TAE Buffer (50X)
- 60 Centrifuge Tubes (2ml)

SPECIAL HANDLING INSTRUCTIONS

- Store λ DNA, T4 DNA Ligase Buffer and Enzyme, DNA Ladder and DNA Loading Buffer frozen at -20°C
- Store LabSafe™ Nucleic Acid Stain at 4°C.
- All other reagents can be stored at room temperature.

ADDITIONAL EQUIPMENT REQUIRED

- Agarose Electrophoresis Equipment
- Washing trays 12cm x 12cm
- UV Light box or transilluminator

TIME REQUIRED

- **Day 1**: 30 minutes
- **Day 2**: 1-2 hours

OBJECTIVES

- Learn how to join pieces of DNA.
- Ligate DNA fragments into single DNA piece.

BACKGROUND

DNA ligation is an important technique in molecular cloning and in the generation of recombinant DNA. DNA ligation is the act of joining together DNA strands with covalent bonds with the aim of making new viable DNA or plasmids.

The enzyme routinely used is the T4 bacteriophage T4 DNA ligase that creates a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another (Figure 1). This reaction requires adenosine triphosphate (ATP). DNA ligases from bacteria can be used; however these are not as common and require NAD (Nicotinamide Adenine Dinucleotide) as a cofactor.

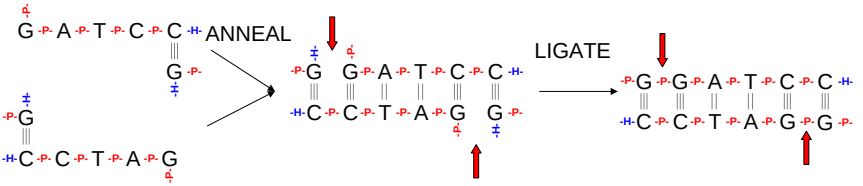


Figure 1: Formation of phosphodiester bonds during ligation.

T4 DNA ligase is able to ligate overhanging, cohesive (sticky) ends and blunt ends, although a higher concentration of enzyme is required. The major reason being that the complementary ends aid in ligation by bringing matching DNA ends together, in the case of blunt ended DNA there is no initial attraction of the two strands to be joined. There are three crucial components to a ligation reaction:

1) Two or more fragments of DNA that have compatible ends
2) A buffer that has 0.25-1mM ATP (adenosine triphosphate) to provide the necessary energy for the reaction
3) The T4 DNA ligase itself.

The ideal temperature for the T4 DNA ligase reaction is 16°C, however it works over a range of temperatures, so 4°C or room temperature can also be used. The optimal temperature for a ligation is a balance between the optimal temperature for T4 DNA Ligase enzyme activity (16°C) and the temperature necessary to ensure annealing of the fragment ends (the melting temperature), which can vary with the length and base composition of the overhangs. For most sticky ends 90-100% ligation is achieved at 16ºC after a 16 hour ligation.

The ideal ratios for ligating insert to vector for sticky end ligations ranges between 1:1 and 3:1, where as for blunt ended ligations the insert/vector ration should be at least 10:1.

This kit is supplied with all the reagents necessary to ligate multiple DNA fragments into a single piece of DNA that is easily visualized on an agarose gel.

This kit is also designed to be used with the DNA fragments produced with the “DNA Restriction Digestion” kit and purified with the “Purification of a Gene”. Simply follow the modified protocol using your DNA fragments; the larger fragment is the vector. This will result in the generation of a viable plasmid.

TEACHER'S PRE EXPERIMENT SET UP

Preparation of agarose gel

Each group of students will require 5 sample wells, so for 6 groups, there needs to be 30 wells for the samples, plus a well for the reference 1kb DNA ladder. For optimal results, the capacity of each well should be up to 30µl.

Make 1-2 hours before the experiment.

Wear heat protective gloves throughout the agarose melting and pouring procedure

1. Prepare running buffer: In a clean two liter container, add the entire contents of the 50X running buffer and add two liters of ultra pure water to make a 1X running buffer solution. Stir until thoroughly mixed.

2. Prepare agarose: In a clean, glass 1000ml container add the entire contents of the agarose pack and add 625ml of the running buffer from step 1.

3. Heat the solution in a microwave on full power, using 10 second bursts, or use a boiling waterbath. Check to see if all the agarose has dissolved. Continue until agarose has dissolved.

DO NOT BOIL. The agarose gets very hot, very quickly and can cause severe burns. Wear heat protective gloves throughout.

4. Once the agarose has cooled to the point it can be held comfortably in your hand, add the entire contents of the LabSafe™ Nucleic Acid Stain to the agarose and swirl to mix.

5. Pour the agarose into the gel casting mould as per the manufacturer's instructions.

6. Once the gels have set, remove the comb, transfer to the running apparatus and cover with the running buffer until ready to use.

Prepare the reference markers

1. Add 25µl ultra pure water to the lyophilized DNA ladder (1kb), dissolve by gently pipetting up and down 5-6 times.

2. Add 5µl DNA Loading Buffer (6X), mix by gently pipetting up and down 5-6 times.

3. Load 10µl into each reference well.

Reconstitute DNA & Aliquot Reagents

 *Once the reagents have been thawed and/or resuspended they must be kept on ice. The reagents must remain on ice throughout the experiment.*

If using the fragments purified with the "Purification of a Gene" then the λ DNA (digested) does not need to be resuspended; however it can also be used as a positive control.

1. Transfer 150µl sterile water to the λ DNA (digested). Resuspend the DNA by gently pipetting up and down.

2. Label six tubes with "λ DNA digested". Transfer 25µl λ DNA from step 1 into the bottom of each tube. Supply each group with a single tube.

3. Label six tubes with "4X Buffer". Transfer 25µl T4 DNA Ligase Buffer (4X) into the bottom of each tube. Supply each group with a single tube.

4. Label six tubes with "T4". Transfer 25µl T4 DNA Ligase Enzyme into the bottom of each tube. Supply each group with a single tube.

Bacterial Transformation & Confirmation of Ligation

This kit contains all the reagents necessary to ligate DNA fragments produced with the "DNA Restriction Digestion" (Cat. # BE-307) kit and purified with the "Purification of a Gene" (Cat. # BE-306) to form a functional plasmid. To visualize this ligation, the reaction needs to be transformed into bacteria. We recommend using the "Bacterial Transformation" kit (Cat. # BE-309).

MATERIALS FOR EACH GROUP

Supply each group with the following components.

- 25µl λ DNA Digested
- 25µl 4X Ligation Buffer
- 1 vial T4 DNA Ligase
- Sterile Water
- 1 vial DNA Loading Buffer (6X) (to be shared with class)
- 5 Centrifuge Tubes (2ml)

 *If using digested plasmid and insert from the DNA Restriction Digestion and Purification of a Gene Kit, follow the notes in italics.*

PROCEDURE

1. Each student sets up their own ligation reaction and as a group sets up the negative control.

2. For the ligation reaction, aliquot the following into their labeled tube. Use a clean pipette tip for each reaction to prevent cross contamination.

    a. 5µl λ DNA Digested

    b. 5µl Sterile Water

    c. 5µl 4X Ligation Buffer

    d. 5µl T4 DNA Ligase

     *If using insert DNA, replace "λ DNA Digested" with digested plasmid and the "Sterile Water" with the insert DNA.*

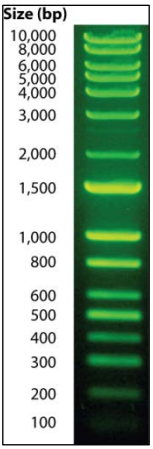
3. Incubate each tube at room temperature for 10 minutes.

    If using plasmid and insert DNA, a suitable number of ligations should have occurred during the 10 minute ligation and the reaction can be used in a bacterial transformation, however for greater success continue with step 4.

4. The ligated λ DNA molecule is >45,000bp and due to the lengths of the molecule and the fragments the ligation reaction requires a longer incubation time. Place the ligation reaction at 4°C, in a fridge, overnight.

5. Following ligation of the "λ DNA Digested", visualize the ligation on a 0.8% agarose gel. Add 4µl DNA loading buffer (6X) to each student sample and load 24µl. Compare to the unligated "λ DNA Digested" as a negative control. Add 1µl of DNA loading buffer to the 5µl unligated "λ DNA Digested" and load 6µl.

6. The agarose gels should have been prepared by your teacher/supervisor. Each student takes turn in loading a sample into a well. Your teacher/ supervisor will load a reference lane to determine the size of the DNA (see image for reference markers).



7. Once the samples are all loaded, apply a current and migrate at 12-15V/cm. For an 8cm long gel run at 96-120 volts.

8. Once the blue dye front has migrated ¾ the length of the gel, turn off the power and carefully transfer the gel to a UV Light box.

 *CAUTION: Wear UV Safety Specs when working around the UV Light Box.*

 *If plasmid and insert were ligated together then use in the bacterial transformation kit as per your teacher's instructions.*

RESULTS, ANALYSIS & ASSESSMENT

This is dependent on how the ligation was confirmed, whether by bacterial transformation or agarose electrophoresis. Design questions dependent on the choice of technique used.

For the λ DNA, fragments of ~3.5, 4.9, 5.6, 5.8, 7.4 and 21.2kbp should be seen in the unligated lines and a major large band of ~48.5kbp in the ligated lane. Minor bands may appear as complete ligation has not been achieved. The large band appears "fuzzy" as it is a mix of large DNA fragments.

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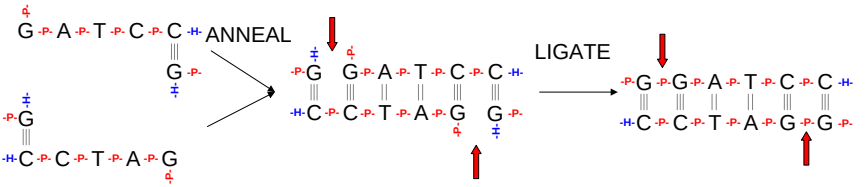

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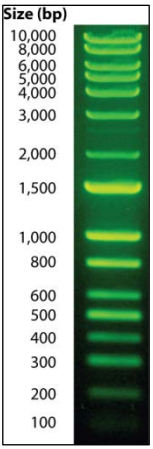

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RESULTS, ANALYSIS & ASSESSMENT

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