Part B. Isolate and Purify Phage Genomic DNA

Overview

Students will isolate and purify phage genomic DNA from the phage head. Any residual bacterial cell debris and macromolecules (including capsid proteins and bacterial proteins) must be removed. Of all of the bacteria-derived macromolecules, the bacterial nucleic acids, both DNA and RNA, are the most important ones to be removed from solution before the protective coat proteins of the phage head are disrupted. Removing them will reduce the potential complexities of the restriction and sequence analyses.

The initial step is to incubate the intact phage in a solution containing both DNase I and RNase A, two nucleases with nonspecific DNA and RNA hydrolytic activities, respectively. Then the coat proteins and enzymes are denatured (unfolded and/or inactivated). The denaturant in the reagent is guanidinium thiocyanate. While the proteins are being denatured, the phage genomic DNA will bind to the resin. After removal of the denatured proteins and salts from the resin with an aqueous alcohol, isopropranol, the phage genomic DNA is eluted (removed) from the column with hot (80˚C) sterile ddH_20 and is then ready for quantification and analysis.

Key Words and Concepts

Macromolecules, nucleid acids, denaturation, resin.

Supplies

- ☐ A sterile serological pipette (5-mL)
- ☐ Isopropanol (80%) (6mL/sample)
- ☐ DNaseI 2000 units
- ☐ RNaseA 7000 units
- ☐ Promega DNA Clean Up Kit
- ☐ 1.5-mL microcentrifuge tubes (6)
- ☐ A 15-mL conical tubes
- ☐ Sterile dd H_20. 90°C (PCR grade)
- ☐ Gloves (latex or vinyl)

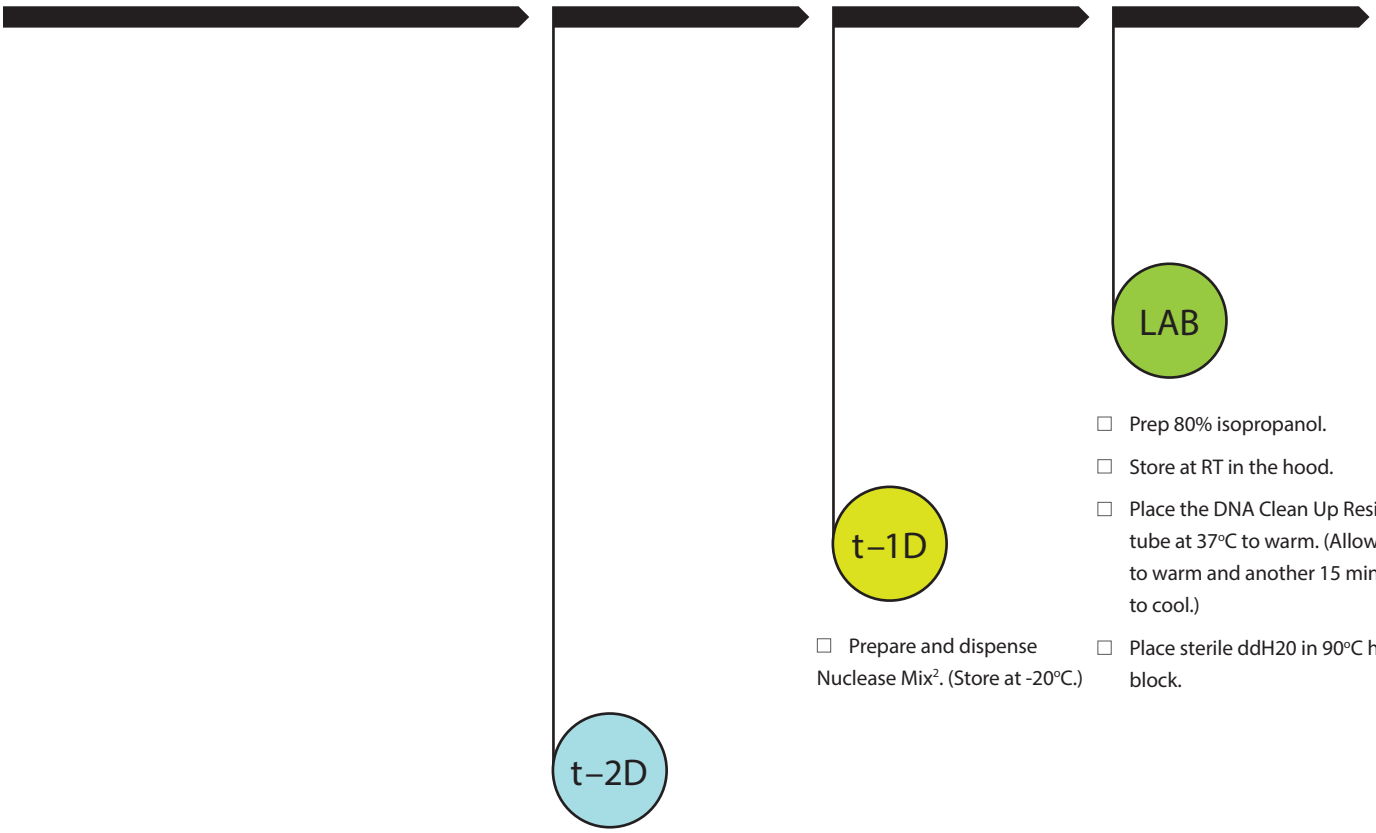
Each student will also need access to

- ☐ A micropipettor and tips

Equipment

- ☐ A microcentrifuge, Set to maximum speed 10,000 x g)
- ☐ 90°C heating block or water bath
- ☐ 37°C water bath
- ☐ A refrigerator
- ☐ –20°C freezer
- ☐ A spectrophotometer, Nanodrop or fluorometer (and accessories) for DNA quantification

Implementation Timeline



LAB: the day of the lab; t–1D: the day before the lab; t–2D: two days before the lab; etc.

Part B. Isolate and Purify Phage Genomic DNA

Overview

There are various ways to extract clean DNA from a high titer phage lysate. This one requires 1 mL of sample, and utilizes a commercial DNA Clean-Up Kit. Phage genomic DNA can be extracted from phage lysates using protein's and DNA's inherent chemical properties, in a method that is a hybrid of batch and column ion exchange chromatography. Before you extract phage DNA from your phage lysate, you will want to degrade the bacterial DNA and RNA using nucleases, then you will want to release phage DNA from its capsids and denature the proteins. Genomic Phage DNA then binds the resin, and finally is eluted from a column using sterile dd H_20.

Objective

The primary objective is to isolate and purify phage genomic DNA in sufficiently high amounts for restriction analysis and sequencing protocols.

Supplies

- ☐ A sterile serological pipette (5-mL)
- ☐ Isopropanol[1] (80%) (6 mL/1 mL lysate)
- ☐ DNaseI 2000 units (Ambion: AM2222)[2]
- ☐ RNaseA 7000 units (Qiagen: 19101)[2]
- ☐ Promega DNA Clean Up Kit (Product # A7280) [3]
  - DNA Clean-Up Resin
  - Minicolumns
  - Syringe barrels
- ☐ 1.5-mL sterile microcentrifuge tubes (6)
- ☐ 15-mL conical tube (1)
- ☐ Syringes, 3-mL or 5-mL
- ☐ pre-warmed (to 95°C) sterile ddH_2O (PCR grade)
- ☐ pre-warmed to 80°C
- ☐ Gloves (latex or vinyl)

Equipment

- ☐ A microcentrifuge (Maximum speed 10,000 x g)
- ☐ Micropipettors & tips
- ☐ 95°C heating block or water bath
- ☐ 37°C water bath
- ☐ A refrigerator
- ☐ –20°C freezer
- ☐ A spectrophotometer, Nanodrop, or fluorometer (and accessories) for DNA quantification

If your lysate has a titer < 5 x 10^9 pfu/mL, do NOT proceed.

Procedure

A. Degrade bacterial DNA/RNA that is in 1 mL of phage lysate.[4 & 5]

1. Transfer 1 mL of the filter-steriled phage lysate into a microcentrifuge tube. Store the remaining lysate at 4°C.
2. Add 1 µL RNaseA and 0.8 µL of DNaseI to your sample. Mix gently but thoroughly by repeated inversions. Do not vortex!
3. Incubate at RT for 30 minutes. You can shorten the incubation time to 10 minutes if you incubate at 37°C.

D. Obtain the DNA-purification kit from your instructor.

E. Uncoat the phage genomic DNA.

1. Put on gloves.

The resin contains guanidinium thiocyanate, a chemical that denatures proteins. Do not get it on your skin!

2. Add 2 mL of pre-warmed (37°C) DNA Clean Up Resin to a 15 mL conical tube.

   Important: Make sure that the bottle of resin is well mixed and that the precipitate has re-dissolved before aliquotting the 2mL. The resin beads tend to settle to the bottom, so make sure you take you 2ml from the bottom of the bottle.

3. Add your nuclease treated phage lysate to the tube of resin. Uncoat the phage particles by gently inverting the tube for 1 minute (minimum). Do not rush this step.

The DNA resin is a slurry solution. Warm until the preciptate goes back into solution. Mix well (by inversion) before aliquotting your 2 mL.

F. Isolate the phage genomic DNA.

1. Label 2 Wizard Kit columns with your phage name. Attach each column to a 3cc syringe barrel. Make sure you remove the plungers before you attach the syringe barrel.
2. Apply 1.5 mL of phage DNA/resin solution to each column using a pipette. Follow the steps below for each column:

3. Using the plungers from the 3cc syringes, carefully push all the liquid through. Collect the flow-through in an Eppendorf tube. Use a steady, gentle, UNRELENTING downward movement. It is really important to maintain even pressure, and not let the plunger move back out of the barrel. When you finish expelling all the liquid, dry the last drop by touching the column to a KimWipe. Unscrew the column, and set it into a new Eppendorf tube. THEN you can let go of the syringe plunger.

4. Remove the plunger from the syringe barrel, then reattach the syringe barrel to the column.

5. Wash the salts from the DNA (now in the column) by carefully adding 2mL 80% isopropanol to each column. Use the plunger to push the Isopropanol through the columns. Collect the wash in an Eppendorf tube. Maintain even pressure and dry the last drop as above in step 3. Remove the syringe barrel from the column.

6. Repeat steps 4 and 5 twice more for a total of three washes (this removes extra guanidine).

7. Put each column in a fresh 1.5mL microcentrifuge tube. Spin at 10,000 x g (NOT top speed, and no more than 10,000 x g) for 5 min to remove residual isopropanol. (Your two

Food for Thought

Why are you unconcerned that the DNase you added to your sample might destroy your phage DNA?

columns can balance each other).

8. Take the columns out of the 1.5mL microcentrifuge tubes and put them in the 90°C heat block for 30 sec-1min. This evaporates the very last bit of isopropanol. Do not leave your columns in too long, the DNA will denature!

9. Put the columns in fresh 1.5mL microcentrifuge tubes. Apply 50µL of 90°C sterile ddH_20 directly to the column (don't take the water out of the heat block. That way it stays hot!).

Do not allow the water to cool below 80°C before it is applied to the column.

10. Incubate column at 1min at Room Temperature.

11. Spin at 10,000 x g for 1 min.

If you exceed 10,000 x g in the centrifuge the columns may break.[5]

12. Combine the products from both tubes into one tube; this is your first elution.

13. Repeat steps 9-12 twice more (for a total of three elutions per column). Each time, combine the products from each pair of tubes. You will end up with a total of 3 tubes – one for each elution. Generally, the majority of the DNA comes off the column during the first elution, but not always. Store at 4°C.

G. Determine the concentration of your DNA.

Using a spectrophotometer (fluorometer, or Nanodrop) and a protocol from your instructor, quantify your DNA. Store at 4°C. or -20°C long term.

Questions

1. What is the property of DNA that allows it to be separated from the denatured proteins and other components of original sample?

2. An aqueous alcohol solution is used to "wash" the salts from the column. Why not just use straight alcohol?

Procedural Notes

1.

Make the 80% isopropanol using a fresh bottle of reagent-grade isopropanol that has been stored at room temperature. As alcohols absorb water from the air, their concentration changes. The phage genomic DNA sticks to the resin under conditions of high salt or high alcohol. The DNA will come off the column in solutions that lack salt or alcohol. This means that if the isopropanol is not actually 80%, the DNA will be washed off the column at this step and thrown away.

2.

These brands of DNAseI and RNaseA are listed because they will be needed if protein work is done. Actually any DNase1 or RNaseA should work for this extraction. Note that the volumes are drastically reduced from years past. You can create a master mix of the nucleases and distribute .

3.

It is a good idea to read the Promega Kit insert. You will note that the binding capacity of each column is 12-15µg of DNA under ideal conditions. Overloading the column can be as bad or worse than under-loading, in terms of DNA yield.

4.

Students must use their filter-sterilized phage lysates for this step; any other sample will clog the column. If there is doubt that the sample was filter-sterilized before storage or if particulate matter is apparent, repeat the filter-sterilization step. If lysate clogs the sterile filter, centrifuge the lysate for 20 minutes at 10,000xg, decant the supernatant into a clean 50-mL screw-cap tube, and refilter.

5.

If phage lysate titer is low ($<5 \times 10^9$ PFU/mL) you can use one of two methods to concentrate the phage:

1. Pellet the phage by high speed centrifugation
2. PEG (Poly-Ethylene Glycol) precipitate the phage

Protocols for each method are found at PhagesDB: Resources: Protocols: Amplification