

Chapter 4
**Bacterial Virus T₄: A Simple
Biological System to Introduce
Genetic Mapping in a Biology
Laboratory Course**

P. J. Valchopoulou-Xanthos

Department of Biology
McGill University
1205 Avenue Docteur Penfield
Montreal, P.Q. H3A 1B1
Canada

J. P. Valchopoulou-Xanthos was born in Greece and studied chemistry and molecular genetics in the Aristotelean University of Thessaloniki, Greece and the University of Toronto, Canada. Her research includes work with bacteriophages λ and T₇. Presently she is Faculty Lecturer in the Department of Biology at McGill University, Montreal, Canada and she is interested in the laboratory teaching aspects of molecular biology.

49

49

49

I Introduction

Bacteriophage T₄ is an attractive organism to use in a teaching laboratory because among other advantages it offers a well-characterized genetic map and availability of conditional lethal mutants together with simple preparation and maintenance of high titer phage stocks (Snustad and Dean 1971). In this laboratory students use a number of point and deletion mutants of bacteriophage T₄ in an effort to map genetically part of the rII region of the chromosome.

The experiment consists of three procedures: a recombination test of about 2 hours duration, a complementation and a deletion test of about 1 hour duration each. Each part may be independent and performed in different lab periods or alternatively all parts might be performed by a group of two to four students in a 3-hr lab period. In this case it is recommended that students start with the recombination test and do the other two tests during the incubation period of the recombination procedure. The experiment also involves overnight incubation of the agar plates and scoring of the results next morning or during another laboratory period.

This exercise is included in the genetics section of a laboratory course in cell and molecular biology for second and third-year students taking the Biological Sciences Core Curriculum at McGill University. There is a lecture before each laboratory session and the prerequisite for this course is a lecture/conference first year course in cell and molecular biology. Original research reports were first adapted for a teaching laboratory session by Dr. J. F. Somers and later modified to the present form by subsequent instructors of the course.

II. Student Materials

Introduction

Bacteriophages, like other viruses, are obligate parasites and require metabolising host cells for their growth. T₄ and related T-even bacteriophages are virulent phages of *E. coli*. When a phage suspension is added to a bacterial culture, the phage particles collide randomly with the bacterial cells, and the tail fibres and tail plate attach to the bacterial wall. The phage DNA (about 200 genes) is injected into the bacterial cell, and the protein coat remains outside. As soon as the phage chromosome gains entry to the cell it directs the production of many copies of itself. At the end of the cycle, the cell bursts and liberates completed free phage. This lytic cycle can be followed by counting the number of virus particles present at each stage from adsorption of phage (to the surface of a cell) to lysis of the cell (Stent and Calendar 1978).

In practice the unit of assay for virus particles is the *plaque-forming unit* (pfu) and this is related to a specific titrating system. If a drop of a dense

bacterial culture is spread over the surface of an agar plate, instead of discrete colonies, a confluent film or “lawn” of bacteria will appear. If some virus particles are incorporated into the bacterial suspension, a virus will enter and lyse a single cell on the agar.

The progeny of this infection will initiate a new round of infection with adjacent bacterial cells. This process of lysis and re-infection will continue on the petri plate until the area of lysis is large enough to appear as a macroscopically visible clearing or “plaque” against the background lawn of bacteria. If only a few plaque-forming units are added to the indicator bacterial culture, the plaques will be discrete and countable just as one would count bacterial colonies (see Figures 4.1 and 4.2). When titrating a concentrated suspension of phage the usual dilution practices are followed.

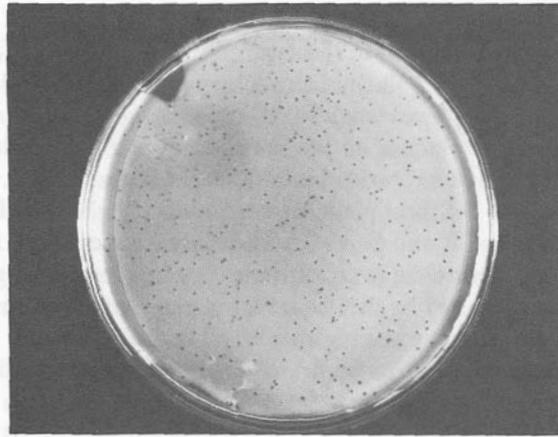


Figure 4.1. Plaques formed by T₄ phage on a lawn of *E. coli* bacteria.



Figure 4.2. Colonies of *E. coli* bacteria.

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Fine structure analysis of rII region of phage T₄ genome

One of the important tasks of genetics is to be able to construct a map of the chromosomes in which a sequence of genes is identified. In order to do this, two things are necessary: 1) a series of mutant phenotypes to allow the identification of the allelic states of a gene, and 2) a recombination system—or a system that allows progeny to form different genotypes than those expressed in the parents. This is true for all genetic mapping. The experiments in this section, which are the classic experiments of Seymour Benzer (1962), added new dimensions to genetic analysis by showing that genetic recombination can occur in the smallest units of “living” matter—the viruses, and that recombination could occur not only between gene loci but between point mutations within a gene.

The T₄rII locus and recombination

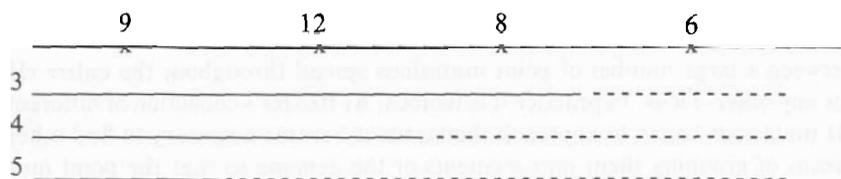
The rII gene is well suited to this type of analysis (Edgar *et al* 1962). rII was originally isolated as a plaque morphology mutant. When plated with *E. coli* C, wild type (rII⁺) gives small fuzzy-edged plaques whereas the mutant (rII) gives large clear plaques on the same strain of *E. coli*. The usefulness of the rII mutant, however, resided in a different characteristic. rII mutants do not grow (i.e. do not form plaques) on *E. coli* K, whereas rII⁺ produces plaques on both *E. coli* K and *E. coli* C. The consequences of this for detecting the frequency of genetic change are obvious. The frequency of a genetic event rII → rII⁺ can be scored by first counting plaques on the permissive strain (*E. coli* C), thus recording the number of rII and rII⁺ phage, and then by counting plaques on the restrictive strain (*E. coli* K) thus recording only wild type (rII⁺). In principle a genetic event (in this case reversion or recombination) can be accurately measured at a frequency as low as 10⁻⁸.

The frequency of recombination between two genes is used to determine the map distance between them. This is based on the statistical assumption that chromosome breakage and rejoining of heterologous strands (crossing over) is more likely to occur between two widely separated points than between two points that are close together. In practice, recombination frequency cannot be used to map genes that are very far apart since multiple crossovers occur with significant frequency between widely separated loci, thus making interpretation of the data impossible.

The genetic map distance is based on the percent recombination. Thus, if two phage each carrying a different point mutation coinfect a host and 1000 of the progeny form plaques on strain K while the total number of progeny (number of plaques counted on strain C) is 10⁷, the percent recombination =

$$\frac{2 \times \text{WT progeny}}{\text{total progeny}} \times 100 : \frac{2 \times 1000 \times 100}{10^7} : 0.02\% \text{ or } 0.02 \text{ map units.}$$

The analysis can be refined still further.



The point mutations 9, 12, 8 and 6 can be ordered on the basis of recombination with a series of extended deletion mutations. For example, strain 6 can recombine with none of the strains carrying extended deletions whereas strain 8 can give recombinants with strain 3 but not with 4 and 5. This allows the assignment of point mutation 8 to a position on the map in a segment of the chromosome between the end of deletion 3 and the end of deletion 4.

Only in those instances where a group of two or more point mutations fall within the same complementation and/or deletion group will it be necessary to use recombination analysis to determine their order.

Procedure—Recombination Test

Each group of students will need the following:

Materials

O/N cultures of *E. coli* K (restrictive) and C (permissive)
 100 ml H broth
 16 large test tubes
 3 small test tubes
 12 tubes soft agar
 12 agar plates prelabelled for each of the 3 crosses 29 × 28 (1–4) 29 × 27 (1–4), 29 × 26 (1–4)
 chloroform
 rII mutants 26, 27, 28, 29 at 10⁹ pfu/ml.

Outline of procedure

T₄rII point mutants will be crossed on *E. coli* C permissive strain. The number of unadsorbed phage particles will be determined by chloroform treatment of aliquots at the end of the adsorption phase. After growth, the progeny from each cross will be titered by plating on *E. coli* C permissive to determine the total progeny and on *E. coli* K to determine the number of wild type recombinants.

Method

1. Dilute O/N strain C 1 ml + 4 ml broth and use for adsorption of phage particles by the bacteria.
The following steps are procedures for *each* cross. After mixing rII 29 with the appropriate mutant strain in 3 separate adsorption tubes, keep the crosses separate.
2. *At time = 0 mins Adsorption:* transfer 0.5 ml of diluted permissive bacteria C to 3 small test tubes. Add 0.25 ml of rII 29 to each of the 3 tubes, and 0.25 ml of one of rII 28, rII 27, rII 26 to each of the tubes. Incubate at 37°C with gentle shaking to facilitate adsorption.
3. *At time = 10 mins Terminate adsorption:* dilute 0.1 ml of each cross 10^{-4} (2×10^{-2} -fold) into broth to terminate adsorption. Use the final 3 dilution tubes as the only *growth tubes* for the rest of the experiment.
4. Start incubation of *growth tubes* at 37°C and immediately remove samples to titer unadsorbed phage as follows: Pipette 0.5 ml from growth tube into 4.5 ml broth. Add a few drops of chloroform to kill infected bacteria containing phage at early stages of development. Mix well. Allow chloroform to settle, and add 0.1 ml from the upper phase to a soft agar tube. Add 2 drops *E. coli* C (permissive) to the soft agar tube, vortex, and pour (Plate 1).
5. Continue to incubate the *growth tube* at 37°C with gentle shaking.
6. *At time = 90 mins Titre progeny:* add a few drops of chloroform to the *growth tube*. Vortex, and allow chloroform to settle.
 - a) In order to titer the wild type recombinant progeny, plate* two samples from the growth tube with 2–3 drops of restrictive strain K. One sample (0.1 ml) is taken directly from the growth tube and plated with *E. coli* K (plate 2). The second sample is diluted 10-fold and then 0.1 ml is plated with *E. coli* K (plate 3).
 - b) From the growth tube make a 10^{-3} -fold (10^{-1} and 10^{-2}) dilution. In order to titer the total progeny (WT and mutant) plate 0.1 ml from the dilution tube with 2–3 drops of permissive strain C (plate 4).

Procedure—Complementation Test

Each group of students will need the following:

Materials

7 agar plates

7 soft agar tubes

O/N culture *E. coli* K (restrictive strain)

rII mutants 24, 25, 26, 27, 28, 29, all at 1×10^8 pfu/ml for spots, and at 1×10^9 pfu/ml for background.

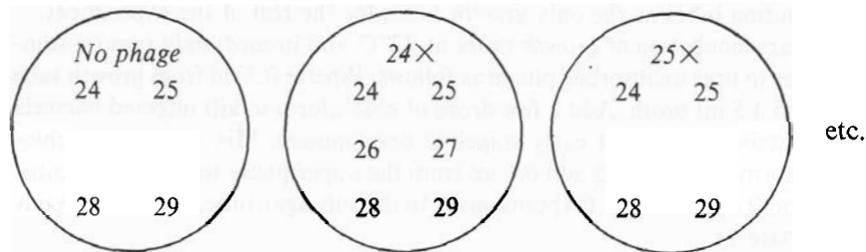
*For plating procedure see Appendix I.

Outline of procedure

The mixing of the two phage and the host cells will be done on the petri plate. One of the rII mutant phage will be mixed with indicator bacteria (*E. coli* K) in soft agar and poured out onto the surface of the agar plate. The crosses will be done by placing a drop of *each* suspension of the six point mutant strains at each of six different places on the surface of the agar. A control plate will contain indicator bacteria with no phage mixed in, but will contain six spots where the point mutant suspensions have been applied. This plate will serve as a control for reversion.

Method

1. Prelabel the base of each plate.



Refer to Appendix I for general plating instructions.

2. Add 1 drop of O/N *E. coli* K (restrictive) to each soft agar tube.
3. Pour the first soft agar tube onto the plate 'no phage'.
4. Add 0.1 ml of rII 24 at 1×10^9 pfu/ml to the second soft agar tube. Mix and pour onto plate 24X. Repeat for the other mutants, pouring onto the appropriately labelled plates. Allow agar to set.
5. Using a sterile (flamed) loop, spot twice a drop of each phage suspension (one drop on top of another and flame your loop in between) at 1×10^8 pfu/ml onto the relevant position on each plate.

Caution

- a. The spots must be well spaced, or they will coalesce.
- b. Do not disturb the plates after spotting until the spots have dried.

Procedure—Deletion Test

Each group of students will need the following:

Materials

- O/N cultures *E. coli* K (restrictive) and *E. coli* C (permissive)
- 24 sterile small tubes
- 24 tubes 9.9 ml broth (make up yourself)
- 1 tube 12 ml broth (make up yourself)

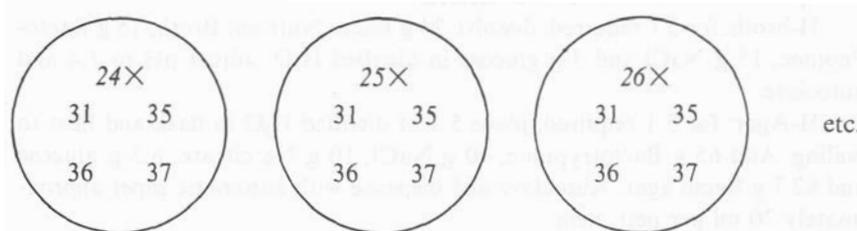
6 soft agar tubes
 6 agar plates
 rII mutants 24, 25, 26, 27, 28, 29, at 10^9 pfu/ml (point mutants)
 rII mutants 31, 35, 36, 37 at 10^9 pfu/ml (deletion mutants)

Outline of Procedure

Recombination of strains carrying point mutations with strains carrying deletion mutations will be done in test tubes using permissive host infected with pairwise combinations of these two types of mutants. A drop of the mixture is then spotted on petri plates spread with restrictive host.

Method

1. Label 24 small sterile tubes with the numbers of strains to be mixed, i.e. 24×31 , 24×35 , 24×36 etc. so that each point mutant is crossed with each deletion.
2. Prelabel the base of the plates,



3. Dilute the O/N permissive host C 3 ml + 12 ml broth.
4. Add 0.25 ml of each of the appropriate strains of phage to each tube.
5. Add 0.5 ml of diluted permissive strain to each tube. The adsorption time will be 12 minutes, so it will be necessary to add the bacteria at 30-second intervals and, in the same order, terminate adsorption in each tube at 30-second intervals.
6. Terminate adsorption by diluting a 0.1-ml aliquot of each tube into 9.9 ml of broth.
7. Add one drop of *restrictive* host K to each of 6 soft agar tubes. Mix and pour onto the prelabelled petri plates.
8. Use a sterile (flamed) loop to spot the 100-fold dilutions twice (one drop on top of another and flame the loop in between) on the appropriate plates in the appropriate positions.

Caution

- a. The spots must be well spaced, or they will coalesce.
- b. Do not disturb the plates after spotting until the spots have dried.

III. Instructor's Materials

The quantities of materials given below are for twenty students working in groups of two and performing all three parts of the experiment.

Equipment

Autoclave, pH meter, balance, 4–1 flasks and automatic pipette for agar are necessary for the preparation of cultures and media. Two waterbaths with shaker (37°, 45°), 10 bunsen burners, 37° incubator, 1–2 colony counters, 20 bacteriological loops, 250 H-agar plates, 1- and 10-ml pipettes (800 and 120 respectively), small empty test tubes (300), small H-soft agar test tubes (250), regular size test tubes for dilutions (350), racks for test tubes.

All media and glassware must be sterile.

Media

Most ingredients of media used for growth of bacteria and bacteriophage were purchased from Difco Laboratories.

H-broth: for 3 l required, dissolve 24 g Bacto Nutrient Broth, 15 g Bacto-Peptone, 15 g NaCl and 3 g glucose in distilled H₂O, adjust pH to 7.4 and autoclave.

H-Agar: for 5 l required, place 5 l of distilled H₂O in flask and heat to boiling. Add 65 g Bactotryptone, 40 g NaCl, 10 g Na citrate, 6.5 g glucose and 62.7 g Bacto agar. Autoclave and dispense with automatic pipet approximately 20 ml per petri dish.

H-Soft Agar: for 800 ml required, dissolve 6.4 g NaCl, 1.6 g Na citrate, 2.4 g glucose, 5.2 g Bacto agar and 10.4 g Bactotryptone in distilled H₂O, autoclave and dispense approximately 3 ml per tube. Before use, heat to melt and keep at 45°.

Preparation and Storage of Bacterial Stocks

Escherichia coli C 11303 is used as the permissive host and *E. coli* K 10798 is the restrictive host for T₄rII mutants.

To prepare pure bacterial stocks it is recommended to start from single cell cultures as follows: a concentrated bacterial culture is serially diluted with H-broth or saline to approximately 10³ cells/ml, and 0.1 ml of this suspension is plated on an agar plate and allowed to grow into visible colonies. One colony is then selected, resuspended into appropriate media, and an aliquot taken from it for further culturing or storage. At the same time another aliquot from the same colony is tested to verify the genotype of the colony, in this case if the cell is permissive or restrictive for growth of T₄rII mutants.

Bacterial stocks may be stored on agar slants for 1–4 months at 4°. To prepare add 6 ml sterile nutrient agar in a sterile 15-ml screw-cap vial and allow it to set on a slant. Use a bacteriological loop to pick up some cells from a liquid culture and transfer them to the agar slant, gently streaking the loop over the surface of the agar. Incubate overnight at 37° and store at 4°.

To prepare overnight bacterial culture for the experiment, simply resuspend aseptically a loopful from the appropriate slant into liquid medium and incubate with shaking, at 37°, overnight.

Preparation of Bacteriophage Stocks

Bacterial and bacteriophage strains can be obtained from research laboratories or biological supply houses such as American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MA 20852. The numbers 24–29 and 31, 35, 36, 37, referring to the T₄rII mutants used in this experiment, correspond to the ATCC call number: 11303–B₂₄ to 11303–B₂₉, 11303–B₃₁, etc.

High-titer bacteriophage stocks for this experiment can be obtained by standard phage preparation on agar plates or in liquid media. T₄rII point mutants seem to have substantially lower reversion frequency when they are grown on plates. Deletion rII mutants give high-titer stocks in liquid media. In any case, for genetic experiments it is advisable to use single clonal phage stocks for all mutants. Procedures for isolation of single plaques and two methods for large scale preparation are described below.

Isolation of a single plaque

Using 0.05 ml of a phage stock, prepare serial dilutions (10⁻², 10⁻⁴, etc.), plate 0.1 ml of each on *E. coli* C 11303 (permissive strain) and incubate the plates at 37° overnight. The following morning choose one plate on which the plaques are well spaced and using a loop, transfer a small bit of soft agar containing one plaque to about 2 ml of H-broth. Add one drop of chloroform, mix very well and allow to sit on the bench for a few hours. Prepare dilutions of the single plaque suspension 10⁻¹, 10⁻², 10⁻³, 10⁻⁴; plate 0.1 ml of each dilution as well as 0.1 ml of the undiluted suspension. Refrigerate the suspension. Incubate the plates overnight at 37°. The following day, screen the plates. Determine the titre of the single plaque suspension, i.e. count the number of plaques. Multiply this number by the dilution and a volume correction (10). This gives the titre of the single plaque suspension.

Preparation of high titer phage stocks on H-agar plates

It is strongly recommended to use freshly poured, i.e. less than 24 hrs old, H-agar plates for this part of the experiment. Dilute the single plaque suspension to titre 2 × 10⁵ phage/ml. Mix 0.1 ml of the diluted phage and 0.05 ml of an *E. coli* C overnight in molten soft agar; pour over the surface of a

fresh plate; allow to set and incubate overnight at 37°. The following morning the plate should exhibit confluent lysis, i.e. plaques over the entire surface with virtually no visible bacteria. Add 5.0 ml H-broth to the plate. Using a flamed spreader, gently scrape the soft agar and broth off the plate and into a centrifuge tube (or bottle). Vortex the contents of the centrifuge tube very well. Spin at 5000 rpm for 20 minutes. Carefully decant the supernatant containing the phage into a flask. Add a few drops of chloroform; shake well. Spin again at 6000 rpm for 15 minutes. Decant supernatant into a sterile bottle. Add a few ml chloroform. Store in the refrigerator. An average titer of a phage stock prepared by this method is approximately 10¹⁰ pfu/ml.

Preparation of high-titer phage stocks in liquid media

In order to use this method of preparation it is required to have an initial clonal phage stock of about 1–2 × 10⁹ pfu/ml.

An overnight culture of *E. coli* C 11303 is diluted 100-fold into 100 ml of H-broth and the cells are grown with shaking in a 2–1 conical flask at 37° until they reach a concentration of approximately 1–2 × 10⁸ cells/ml (bacterial cell growth may be followed spectrophotometrically or roughly until the culture becomes slightly turbid). Then 10 ml of phage at 1–2 × 10⁹ pfu/ml are added and shaking of the culture is increased to the maximum possible. Growth is continued for 2½ to 3 hrs during which time the turbid culture will often “clear”. 1–2 ml of chloroform are then added, the lysate is well shaken, allowed to stand for 10 min and decanted into centrifuge tubes. Bacterial debris are pelleted at low speed and the phage suspension may then be stored at 4°.

The phage titer obtained by this method is 10¹⁰ – 10¹¹ pfu/ml.

Note: All phage stocks must be tested for reversion after each step of preparation by simply spotting approximately 0.05 ml of 10⁸ pfu/ml of each point mutant to a lawn of *E. coli* K restrictive bacteria.

References

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2. Edgar, R. S.; Feynman, R. P.; Klein, S.; Lielausis, I.; Steinberg, C. M. Mapping experiments with r mutants of bacteriophage T₄D. *Genetics* 47: 179–186; 1962.
3. Snustad, D. P.; Dean, D. S. *Genetics Experiments with Bacterial Viruses*. San Francisco: W. H. Freeman and Co.; 1971.
4. Stent, G. S.; Calendar, R. *Molecular Genetics*. 2nd ed. San Francisco: W. H. Freeman and Co.; 1978.

APPENDIX I
Plating Procedure

0.1 ml of phage suspension or infected bacterial culture is added to a tube containing 2–3 ml soft agar kept molten at 45°C. Two drops of host bacterial culture are added to the agar, the tube is vortexed *gently*, and the contents poured evenly over an agar plate. The soft agar containing the phage and bacteria is allowed to set before the plates are moved.

Agar tubes must be kept at 45° until the moment of pouring.

Phage and bacteria should not be added to the soft agar until ready to pour. Once the phage and bacteria have been added and the soft agar tube removed from the 45° bath the soft agar should be poured and the plate rotated quickly to spread the soft agar evenly across the surface, as it solidifies very quickly.

APPENDIX II
Scoring Results

This part may be normally used as a data sheet for students' results. For the instructor's own use, a set of ideal data is shown.

1. *Complementation Analysis*: Score for complementation (+ or –); i.e. the presence of a clear area or many plaques in the place where each phage was spotted relative to the control plate (no background phage) or control spot (same phage spotted as background phage). The control checks that each mutant *alone* does not form plaques on the restrictive *E. coli* K.

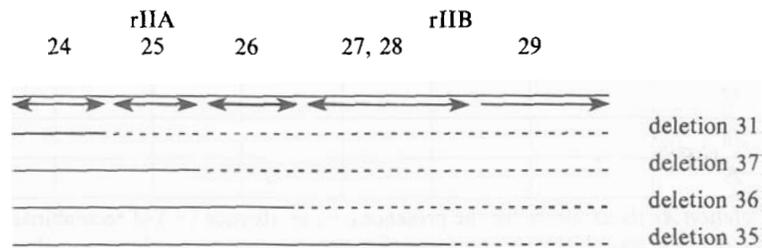
<i>rII</i> point mutants	control	24	25	26	27	28	29
24	–	–	–	–	+	+	+
25	–	–	–	–	+	+	+
26	–	–	–	–	+	+	+
27	–	+	+	+	–	–	–
28	–	+	+	+	–	–	–
29	–	+	+	+	–	–	–

<i>mutants</i>						
	24	25	26	27	28	29
31	+	–	–	–	–	–
35	+	+	+	+	+	–
36	+	+	+	–	–	–
37	+	+	–	–	–	–

3. *Recombination Analysis*: Record the pfu count and calculate the recombination frequency of each cross.

Cross	plate #	Dilution	# of plaques counted	Titer	Recombination Frequency
29 × 26	1	10 ⁻²	50	5 × 10 ³	0.85%
	2	10 ⁻¹	800	8.5 × 10 ³	
	3	10 ⁻²	90		
	4	10 ⁻⁴	200	2 × 10 ⁶	
29 × 27	1	10 ⁻²	50	5 × 10 ³	0.5%
	2	10 ⁻¹	350	4 × 10 ³	
	3	10 ⁻²	45		
	4	10 ⁻⁴	200	2 × 10 ⁶	
29 × 28	1	10 ⁻²	50	5 × 10 ³	0.15%
	2	10 ⁻¹	110	1.5 × 10 ³	
	3	10 ⁻²	20		
	4	10 ⁻⁴	200	2 × 10 ⁶	

4. Draw a fine structure map of the rII region, placing point mutants 24, 25, 26, 27, 28 and 29 in their relative position on the map. Indicate map units where possible.



5. Are the results of the mapping experiments consistent with each other, i.e., if you have assigned a mutant to a particular cistron on the basis of complementation tests, do the results of your deletion analysis also place the mutant in the same cistron? Having placed each mutant in a particular segment by deletion analysis, are these results consistent with the results of the recombination analysis?