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ABSTRACT

A method is described for the preparation and assay of T4 bacteriophage. This description is of sufficient detail to allow those unfamiliar with virology techniques to successfully utilize and study this phage system.

Key Words: Preparation - Assay Bacteriophage

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INTRODUCTION

Viruses are the smallest and simplest biological systems to which the term "living" can be ascribed. In their ability to reproduce their own kind, to multiply, to be capable of mutation, and to exchange genetic material provided by more than one parent, they exhibit the most salient characteristics of living organisms. Yet they can be isolated in pure, frequently crystalline forms, stored, examined, analyzed, manipulated and, in short, treated as distinct chemical entities. Thus, although viruses form part of living nature, they have also provided investigators with easily comprehensible models for life processes which are characteristic of more complex forms. For these reasons viruses are eminently suitable in investigations on the molecular basis of life.

Bacteriophages, viruses that employ bacteria as hosts for reproduction, have been the subject of much concentrated study ever since d'Herelle's incisive experiments allowed him to recognize the essential features of the bacteriophage life cycle. In 1926 he summarized (1) his observations as follows: "The first act of bacteriophagy consists in the approach of the bacteriophage corpuscle toward the bacteria, then in the fixation of the corpuscle to the latter... The bacteriophage corpuscle penetrates into the interior of the bacterial cell. When, as a result of its faculty of multiplication, the bacteriophage corpuscle which has penetrated into the bacterium forms a colony of a number of elements, the bacterium ruptures suddenly, liberating into the medium young corpuscles which are then ready to continue the action." This process of phage infection, multiplication, and subsequent bacterial host cell lysis serve as the basis for the assay procedure to be described in this document. Further studies, particularly over the past twenty years, have delineated in greater detail the parasitic life cycle of bacteriophage and, in addition, have elucidated the structure and genetic map of T4 phage.

Such research efforts have resulted in the publication of numerous papers dealing with bacteriophages, and much of this information has been compiled into several excellent texts (2, 3) on bacterial viruses. However, there apparently is no detailed description of how one prepares and actually carries out an assay on viral activity. The purpose of this document is to outline, in sufficient detail, how such an assay may be accomplished.

DISCUSSION

A) Obtaining phage and bacterial host.

An initial supply of both phage and suitable bacterial host is obviously essential. Possible sources of these materials include the American Type Culture Collection[1], the International Registry of Microbial Genetic Stocks[2], or a local university, hospital, or research institute. Once obtained, these stock cultures should be kept under refrigeration (4°C.) until used. The preparation and assay of only T4 bacteriophage and its bacterial host, *E. coli* B, will be considered in this document.

B) Preparation of *E. coli* and T4 phage working stocks.

Larger amounts of T4 phage and *E. coli* B must be cultured from the initial supply of phage and bacterial host. The following materials are to be sterilized for use in preparing *E. coli* B and T4 phage working stocks:

1. one liter of 3% (wt./vol.) T.S.B.[3] in a two liter Erlenmeyer flask which is sealed with aluminum foil.
2. rubber stopper which is suitable for (1).

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[1]American Type Culture Collection, c/o Order Dept., 12301 Parklawn Drive, Rockville, Maryland 20852

[2]*Experiments in Microbioal Genetics* (ed. R. C. Clowes and W. Hayes, John Wiley & Sons, Inc., New York, 1968) contains the Registry under Appendix C.

[3]Abbreviations used:

T.S.B.	-	Trypticase Soy Broth, BBL, Division of BioQuest
T.S.A.	-	Trypticase Soy Agar, BBL, Division of BioQuest
N_o	-	initial phage concentration
N	-	phage concentration after a given treatment
TN	-	too numerous to count

3. two Erlenmeyer flasks (1 liter) with rubber stoppers.

4. one liter Millipore suction flask sealed with aluminum foil.

5. one liter beaker covered with aluminum foil.

6. centrifuge tubes.

First, the E. coli B working stock is prepared by inoculating the 3% T.S.B. solution with the initial bacterial culture. The flask is resealed with the sterile aluminum foil and shaken gently at room temperature until the solution is just visibly turbid (10^7 cells/ml). In our laboratory we inoculated the T.S.B. solution with 5 ml of a bacterial culture containing 10^8 cells/ml. This inoculum was visibly turbid after shaking for 5 hours.

When turbidity is reached, pour approximately 500 ml of the E. coli B suspension into a sterile one liter Erlenmeyer flask, seal with a rubber stopper, and store under refrigeration (4°C.). This mixture will serve as the E. coli B working stock and should be so labeled.

The remaining 500 ml of the suspension is inoculated with T4 phage (approximately 10^8 phage particles), re-sealed with the sterile foil cap, and allowed to shake overnight (~18 hrs.). Addition of the phage to the bacterial suspension is routinely accomplished in this laboratory by first adding the phage to 100 ml of 3% T.S.B. and then slowly adding this phage mixture to the bacterial suspension while gently swirling. One should avoid too low a ratio of bacteria to phage because of the possibility that the phage could lyse the bacteria without first undergoing phage multiplication (the so-called "lysis from without"). Hence, a ratio of at least ten bacteria per phage particle is desired at this phage inoculation step.

After shaking for approximately 18 hours, the turbid suspension is centrifuged at 3000 rpm for 10 minutes and the phage-containing supernatant is stored in a sterile one liter flask. Further removal of particulate matter is achieved by filtration of the supernatant under vacuum through a 0.45 micron filter into a sterile suction flask. A wire grid is used to support the membrane filter during this process. A sintered glass support was not used because of the fear that it might entrap or damage the phage particles during filtration. The filtrate, which contains the T4 phage, is then chilled to 4°-5°C.

After chilling, the clear filtrate is transferred to a sterile one liter beaker and the solution is acidified to pH 3.9-4.0 with 0.1 N HCL (approx. 25 ml). The solution is swirled during the addition of the acid, and the resulting cloudy suspension is allowed to stand overnight at 4°C. The precipitated phage is isolated by centrifugation at 3000 rpm for 10 minutes. The resulting pellet is resuspended in a minimal volume of 1% T.S.B. to yield a white opalescent suspension which is stored at 4°C. This suspension is the T4 phage working stock and should be so labeled. Heriott and Barlow (4) utilized this acid precipitation technique to prepare and concentrate T2 bacteriophage. Table 1 indicates the efficiency of the isolation procedure and the increase in phage concentration during isolation.

TABLE 1

Purification of T4 Phage

Treatment	Volume (ml)	Titer/ml	Total Titer	Recovery (%)
Lysate	1000	1.0×10^{10}	1×10^{13}	100
Centrifuge (Supernatant)	1000	8.0×10^{9}	8×10^{12}	80
Millipore Filtration (Filtrate)	1000	6.2×10^{9}	6.2×10^{12}	62
pH 4 (Precipitate)	8	4.12×10^{11}	3.3×10^{12}	33

C) Assay of T4 bacteriophage.

This phage assay is based on the lysis of a suitable bacterial host after phage infection and multiplication. For clarity, a brief summary of the assay protocol is presented.

An aliquot of the E. coli B working stock is gently shaken at room temperature two hours prior to the actual phage assay. This yields a suspension which contains approximately 10^8 cells per ml. For efficient phage multiplication, the bacterial host should be undergoing logarithmic growth. The two hour incubation period fulfills this requirement. Once the E. coli B have reached this level of growth, a certain amount of the E. coli B suspension is added to a special agar-nutrient mixture referred to as soft agar. After mixing, 9.5 ml of the bacteria-soft agar suspension is pipetted into vials. These vials, containing the bacteria and soft agar, are maintained at 42°C. This temperature keeps the agar in a liquid state and is not deleterious to the bacteria.

Concurrent with preparation of the vials containing bacteria and soft agar, the phage samples are diluted to a countable concentration. A small amount of (0.5 ml) of the phage, appropriately diluted, is added to each of the vials containing the bacteria-soft agar suspension. Once the phage and bacteria have been mixed, the processes of phage infection and multiplication are initiated. A small amount of the bacteria and phage mixture is then pipetted onto a petri dish. The E. coli B grow rapidly producing a dense lawn covering the agar surface. The clear areas in this bacterial lawn, plaques, result from the lysis of the phage-infected bacteria. Each phage-infected bacteria gives rise to a single plaque. Therefore, by counting the number of plaques one can quantitate the number of phage particles present at a given dilution. This brief outline of the assay protocol emphasizes the need for adequate preparation and planning prior to the assay.

The amount of material needed for the bacteriophage assay depends on the scope of the experiment. The following sample defines the materials needed for the determination of a T4 phage survival curve after heating at 66°C. for one hour. All materials must be sterilized prior to use:

1. Sample-containing test tubes:

    control - 2 (Duplicate samples are run per data point.)

    30 min. - 2

    60 min. - 2

    6 total tubes with foil covers

2. Each sample test tube requires appropriate dilution containers, 50 ml beakers and 99 ml dilution bottles. Four containers are usually sufficient for the necessary dilution of the contents of each test tube assayed.
3. Each dilution container requires a sterile virus dilution vial.
4. 9.5 ml of soft agar are needed for each dilution vial.
5. Each virus dilution vial requires two petri dishes containing 10 ml of 4% T.S.A.[3]

6. <u>E. coli</u> B in logarithmic phase of growth.

7. 1 ml, 5 ml, and 10 ml pipettes.

In summary, the following sterile materials are needed to conduct this assay: 6 test tubes with lids, 24 dilution containers, 24 dilution vials with screw caps (size 25mm, ID, 95mm, ht), 230 ml of soft agar, 48 petri dishes containing agar (size 20mm x 100mm, Falcon plastic disposable), <u>E. coli</u> B and pipettes.

Prepare the dilution bottles with 1% T.S.B., autoclaving the bottles and stoppers separately. After cooling, adjust the volume in each bottle to the 99 ml mark and add the stopper. The 50 ml dilution beakers are also sterilized but contain no T.S.B. medium. Nine ml of sterile 1% T.S.B. will be added just before the assay to each beaker needed.

The amount of soft agar needed depends on the number of virus vials used, since each vial contains 9.5 ml of this agar. Therefore, 24 vials require approximately 230 ml of soft agar. The soft agar is prepared in the following manner: weigh out 7.5 gm of T.S.B. and 2.0 gm agar-agar and add 250 ml dionized water. This solution is 3% (wt./vol.) in T.S.B. and 0.8% (wt./vol.) in agar. It is autoclaved, cooled, and maintained at 42°C.

Prepare petri dishes by adding 10 ml of 4% T.S.A. to each dish and allowing to cool. No petri dish is used if it has been prepared more than 24 hours previously, since drying of the agar must be avoided if good plaque size is to be obtained. In this laboratory, the petri dishes are prepared on the day of the viral assay. If streaks in the bacterial lawn are to be prevented, heavy condensation of water vapor on the top of the petri dish as the agar solidifies also must be avoided. Reduction of the condensate can be achieved by cooling the agar to 50-60°C. prior to pouring the plates and by stacking them at least ten high after the agar has been delivered to the petri dishes. Those plates with little condensate are selected for the assay.

An aliquot of *E. coli* B working stock is placed on a shaker at room temperature two hours prior to the actual phage assay. The amount of *E. coli* B needed depends on the size of the experiment. Consider the above example where 230 ml of soft agar are required. 3.5 ml of *E. coli* B working stock are used for each 100 ml of soft agar. Hence, approximately 8 ml of *E. coli* B are necessary for the 230 ml of soft agar $\left(230 \times \frac{3.5}{100}\right)$. After two hours of shaking, this suspension contains approximately 10^8 cells/ml. Such a concentration of bacteria yields a dense lawn in which the smallest plaques can be easily counted.

The assay procedure begins by removing the *E. coli* B suspension from the shaker and adding it to approximately 230 ml of soft agar. This mixture is swirled and held at 42°C. 9.5 ml of this bacteria-soft agar mixture is then delivered to each of the 24 virus dilution vials. These vials are then capped and maintained at 42°C.

The following phage dilution procedure is done concurrently with the addition of the bacteria to the soft agar and the distribution of the agar-bacteria mixture into the virus dilution vials. This dilution schedule produces the indicated phage concentration per dilution container if one assumes a concentration in the control assay test tube of 1×10^8 phage/ml.

Sample	Dilution	Container	Dilution Required	Phage Concentration/Dilution Container
Control	A	dil. bottle	1 ml assay + 99 ml 1% T.S.B.	10^6/ml
	B	beaker	1 ml dil. A + 9 ml 1% T.S.B.	10^5/ml
	C	dil. bottle	1 ml dil. A + 99 ml 1% T.S.B.	10^4/ml
	D	beaker	1 ml dil. C + 9 ml 1% T.S.B.	10^3/ml
	E	dil. bottle	1 ml dil. C + 99 ml 1% T.S.B.	10^2/ml
	F	beaker	1 ml dil. E + 9 ml 1% T.S.B.	10/ml

The phage assay is carried out by first deciding which dilutions will insure a countable number of phages per petri dish. In the above example, dilutions C through E may be used. Remove 0.5 ml from the selected dilution containers and add to the appropriately labeled virus dilution vials which contain 9.5 ml of the E. coli B - soft agar mix. This results in a twenty-fold dilution of the phage concentration for a given dilution container. The vial is capped, inverted ten times to mix in the virus, and placed in a 42°C. water bath until all pipettings and mixings have been accomplished. A 5 ml blow-out pipette is then used to deliver 2 ml (blow out) of the contents of each vial into each two appropriately labeled petri dishes. The dish is quickly rotated to spread the agar over the surface before it hardens. Since the experiment is performed with duplicate samples, the second virus vial holding the duplicate phage sample is pipetted according to the same scheme. A fresh sterile 5 ml pipette is used to remove the two 2 ml portions from this second vial.

The petri dishes are placed in a 37°C. incubation chamber overnight (~18 hours) and the plaques are counted the following day. Table 2 indicates the type of data obtained from such a protocol. This data is presented in Figure 1 as part of the survival curve data for the synergistic inactivation of T4 phage by thermoradiation.

TABLE 2

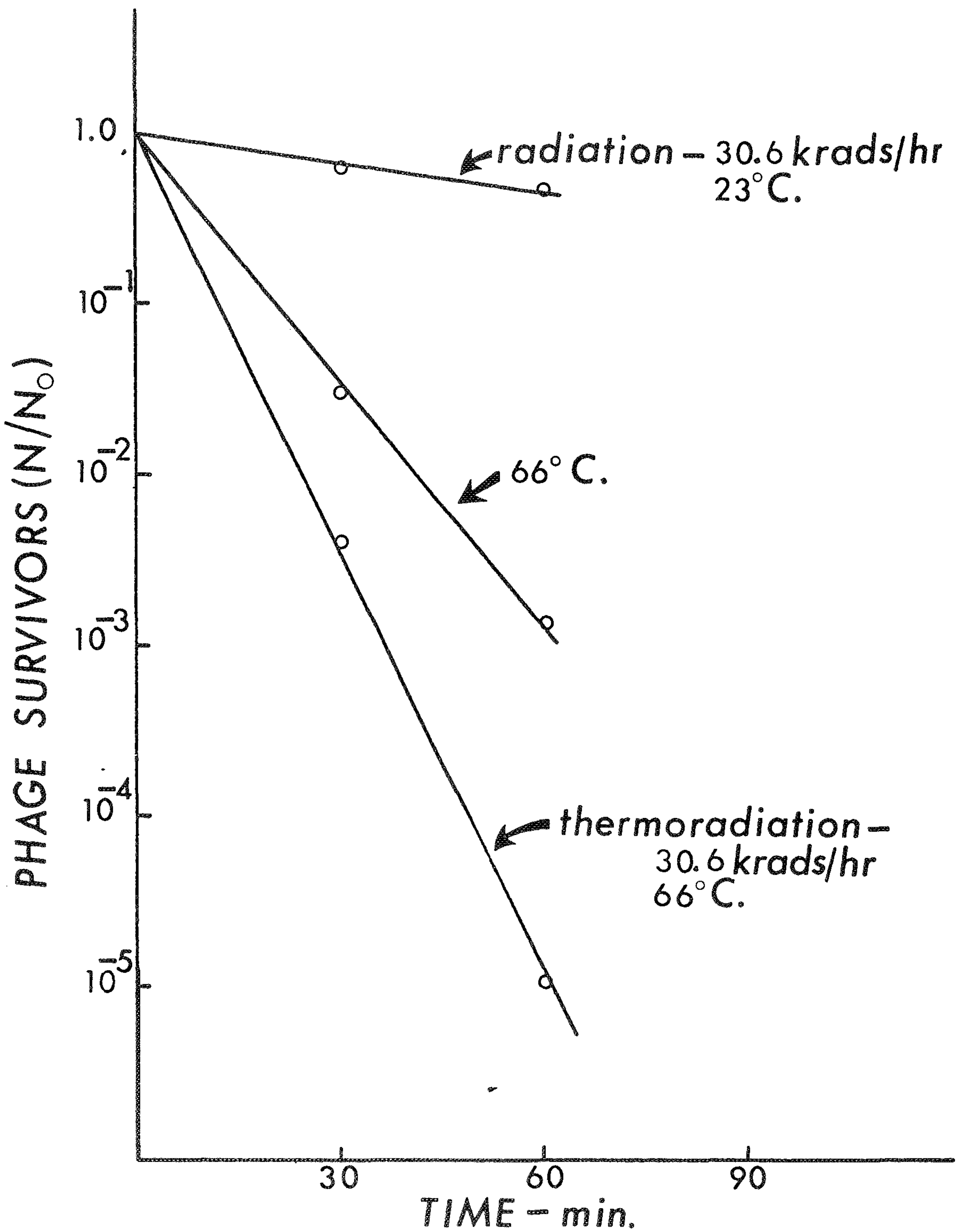
Survival Data for T4 Phage at 66°C.

Treatment	Dilution	Sample A	Sample B	Phage/ml*	N/N_o [3]
Control (No Heat)	E	42,56	45,42	4.9×10^6	1
	D	TN,TN[3]	TN,TN[3]		
30 min. heat	E	1,5	0,2		
	D	16,20	15,17		
	C	142,155	153,160	1.53×10^5	3.13×10^{-2}
60 min. heat	D	0,1	1,1		
	C	1,6	6,9		
	B	68,71	60,68	6.7×10^3	1.37×10^{-3}

*In the course of the assay 0.5 ml of phage, diluted appropriately, was added to 9.5 ml after bacteria-soft agar suspension. One must correct for this dilution in order to obtain a corrected phage concentration. For example, the control sample yields 4.9×10^6 phage/ml

$$4.9 \times 10^6 \frac{\text{phage}}{\text{ml}} \times \frac{10}{0.5} = 9.8 \times 10^7 \frac{\text{phage}}{\text{dil. container}}$$

FIGURE 1
SYNERGISTIC INACTIVATION
OF T4 PHAGE



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