# A MEMBRANE-FILTER TECHNIQUE FOR THE DETECTION OF COMPLEMENTARY DNA*

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The technique (Gillespie and Spiegelman, 1965) for detecting RNA complementary to DNA by "hybridizing" the RNA to DNA bound to nitrocellulose membrane filters has been modified to detect complementary DNA. Denatured DNA, but not free RNA, sticks to nitrocellulose membranes (Nygaard and Hall, 1963). We have found that the nonspecific sticking of denatured or single-stranded DNA can be prevented by preincubating the filters in an albumin solution. The specific annealing of denatured DNA to complementary DNA previously bound to the filter is not blocked by the preincubation. With this change all the advantages of the filter paper technique (inexpensive, easy to handle many samples) may be had without recourse to the intermediate step of making RNA complementary to the DNA (Green, 1963). We have used this technique to examine the kinetics of synthesis of single-stranded ØX DNA in ØX-infected cells.

## Materials and Methods

Nitrocellulose Membrane Filters. Schleicher & Schuell Type B-6 and Millipore HAWP 25 mm filters are satisfactory.

DNA. $H^3$ labeled SS-DNA** was extracted from ØX virus particles (Guthrie and

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** Abbreviations used: SS, single-stranded; DS, double-stranded; RF, replicative form.

Sinsheimer, 1963). Lambda DNA ($H^3$ labeled and unlabeled) was a gift from G. Ihler. ØX RF-DNA was a gift from D. Dressler. It had been purified by passage through a methylated albumin column.

SSC. SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0.1X, 3X, 6X, and 10X are 0.1, 3, 6, and 10 times as concentrated.

Attachment of DNA to Filters. DNA was denatured by heating to 100° for 5 min in 6XSSC and quick-cooling. The solution was passed (5 ml/min) through a filter prewashed with 6XSSC. The filter was washed with 5 ml of 6XSSC, dried overnight in a vacuum dessicator, then placed in a vacuum oven at 80° and 29 inches Hg for 2 hours (Gillespie and Spiegelman, 1965). About 95% of the DNA was retained by the filter, and, after drying, less than 1% was released during 24 hours incubation at 65°. The RF DNA used in this work contained single-strand breaks; hence upon denaturation the complementary strands were able to separate.

Preincubation. PM contained 0.02% each of Ficoll (Pharmacia, av. M.W. 400,000), polyvinylpyrrolidone (Sigma, av. M.W. 360,000), and bovine albumin (Armour, Fraction V) in 3XSSC. The filters were rolled up and placed in 1 ml volumetric flasks with 1 ml of PM and incubated for 6 hours at 65°.

Annealing. DS-DNA was denatured either by heating to 100° for 5 min in 6XSSC or by exposure to 0.1 M NaOH for 5 min followed by neutralization with HCl. The denatured DS-DNA or SS-DNA was added to the flasks containing the filters and the incubation at 65° continued for 12 hours more.

Determination of bound DNA. After the 12 hour incubation each side of the filter was washed with 40 ml of SSC. The filters were dried and counted in toluene-liquifluor in a scintillation spectrometer.

## Results

Binding of Single-Stranded DNA. Table 1 shows the effect of no preincubation, or preincubation with different materials. Bovine albumin alone is effective,

Table 1: Effect of Preincubation

| Preincubation Solution[1] | % Input DNA Bound to RF-filters | % Input Bound to Blank |
|---|---|---|
| no preincubation[2] | 65 | 80 |
| 0.1% Ficoll, 0.1XSSC | 7.1 | 2.9 |
| 0.1% Ficoll, SSC | 31 | 5.1 |
| 0.1% Ficoll, 10XSSC | 47 | 5.3 |
| 0.1% Bovine Albumin, SSC | 26.5 | 0.64 |
| 0.1% Polyvinylpyrrolidone, SSC | 31 | 1.1 |
| PM (standard preincubation medium)[3] | 52 | 0.64[4] |

1 Each RF filter carried 0.28 μg of RF. 10 μl ($10^8$ molecules, 5500 cpm) of a $H^3$-SS-DNA stock was added to each flask after the 6 hour preincubation.

2 DNA in 2XSSC was added directly to a dry filter.

3 All other substances tested (including pyrophosphate, sucrose, polyvinylsulfate, and polyuridylic acid) were less satisfactory.

4 0.9% bound to a filter carrying 1 μg of E. coli DNA.

although at concentrations above 0.05% it reduces the specific binding. With no preincubation much of the ØX DNA will stick to the filter. After preincubation with PM for 6 (at least 3) hours less than 1% of the input SS-DNA will stick to blank filters or to filters carrying E. coli DNA, while more than 50% will stick to RF-filters.

The 6 hour preincubation followed by 12 hours of incubation with DNA at 65° gives the best results. There is a small increase in the amount of radioactivity binding to both RF-filters and blank filters if the incubation exceeds 12 hours.

The relation between the amount of SS-DNA annealed to the RF-filters and the amount added to the flask is shown in Fig. 1. For a fixed amount of RF on the filter, a constant fraction of the input DNA binds over the range tested. Fig. 2 indicates the amount of SS-DNA annealed to RF-filters with different amounts of RF attached; with sufficient RF on the filter more than 50% of the added DNA will specifically anneal to the DNA-filter.

As a simple application of this technique we examined the kinetics of synthesis of SS-DNA in ØX-infected cells. Fig. 3 contains plots of 1) the number of phage, 2) the number of infectious SS-DNA molecules, and 3) the

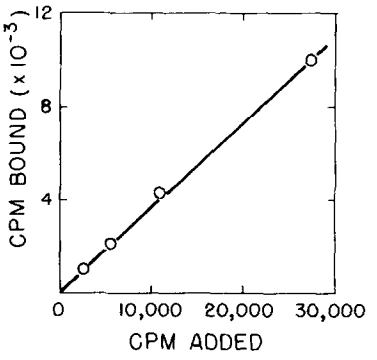


Fig. 1. Filters carrying 0.28 μg of RF ($5x10^{10}$ molecules) were incubated with various amounts of SS-DNA (1 cpm = $10^4$ molecules).

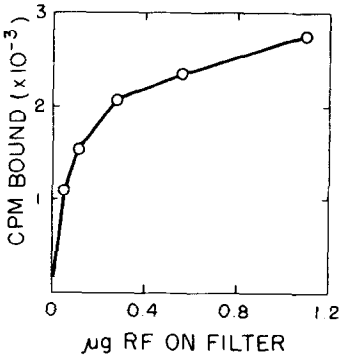


Fig. 2. Filters carrying the indicated amount of RF were incubated with SS-ØX-DNA (5500 cpm, $6x10^7$ molecules).

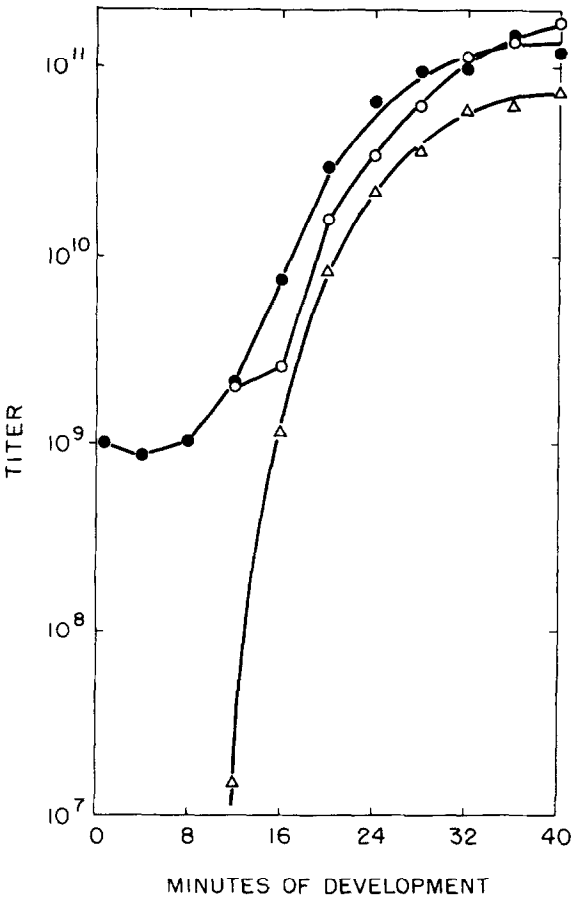


Fig. 3. Titer of phage Δ-Δ, SS-DNA molecules measured by infectivity ●-●, and SS-DNA molecules O-O, in ØX-infected cells at various stages of development. Starved E. coli CR ($3x10^8$ cells/ml) were infected with a multiplicity of 3 of $ØXρ^-$, and growth was initiated synchronously (Denhardt and Sinsheimer, 1965) by the addition of nutrient at t=0. The only thymine available to the thymine-requiring cells was present as thymidine at a specific activity of 1.9 c/mmole (Schwartz). At intervals aliquots of the culture were chilled and lysed with lysozyme-versene. The aliquots were assayed for phage. Then the DNA was extracted with phenol at 55°. The amount of infectious DNA was determined using the spheroplast technique of Guthrie and Sinsheimer (1963). The number of SS-DNA molecules was determined from the amount of $H^3$ that would specifically bind to RF filters.

number of SS-DNA molecules in the culture at various times. We wanted to know whether a pool of noninfectious SS-DNA molecules existed in the cell. The data in Fig. 3 indicate that the total number of SS-DNA molecules in the cell is comparable to the number of infectious SS-DNA molecules. It appears that as the SS-DNA molecule is synthesized (or very quickly thereafter) it becomes infectious and is incorporated into a phage particle.

Binding of Double-Stranded DNA. The data of Table 2 demonstrate that this technique will work for denatured DS-DNA as well as for SS-DNA. In this case the efficiency of detection of the complementary DNA is reduced by the renaturation of the DNA in solution. This competing reaction may be minimized by 1) adjusting the ratio of DNA on the filter to DNA in solution at as high a value as possible and 2) fragmenting the DS-DNA prior to denaturation.

Two advantages of this method over the DNA-agar method (Bolton and

Table 2: Annealing of Double-Stranded DNA to DNA-Filters

| Amount of DNA on the filter | DNA in the solution to be annealed to the DNA-filter | % DNA Annealed | % Bound to Blank |
|---|---|---|---|
| 1.5 μg lambda | whole undenatured lambda[1] | 0 | 0 |
| " | whole alkali-denat. lambda | 15 | 1.4 |
| " | sonicated[2] alkali-denat. lambda | 38 | 3.1 |
| " | sonicated heat-denat. lambda | 41 | 1.5 |
| 0.5 μg coli | $10^{-3}$ μg sonicated heat-denat. coli[3] | 18 | 0 |
| 0.5 μg coli | $10^{-2}$ μg sonicated heat-denat. coli | 2.4 | 0.2 |
| 4 μg coli | $10^{-2}$ μg sonicated heat-denat. coli | 5.0 | 0.2 |

1 The lambda DNA-filters were exposed to 0.06 μg (850 cpm) of lambda DNA.
2 The DNA was exposed to the maximum output of an MSE Ultrasonic Disintegrator for 30 sec.
3 Specific activity of 320,000 cpm/μg

McCarthy, 1962; McCarthy and Bolton, 1963) are to be noted. There is no loss of DNA from the filters, whereas 10% or more of the DNA is lost from the agar (Cowie and McCarthy, 1963). The separation of the bound from unbound DNA is achieved merely by removing the filter from the solution and washing it.

References

Bolton, E.T., and McCarthy, B.J., (1962). Proc. Natl. Acad. Sci. U.S. 48, 1390.
Covic, D.B., and McCarthy, B.J., (1963). Proc. Natl. Acad. Sci. U.S. 50, 537.
Denhardt, D.T., and Sinsheimer, R.L., (1965). J. Mol. Biol. 12, 641.
Gillespie, D., and Spiegelman, S., (1965). J. Mol. Biol. 12, 829.
Green, M.H., (1963). Proc. Natl. Acad. Sci. U.S. 50, 1177.
Guthrie, G.D., and Sinsheimer, R.L., (1963). Biochim. Biophys. Acta 72, 290.
McCarthy, B.J., and Bolton, E.T., (1963). Proc. Natl. Acad. Sci. U.S. 50, 156.
Nygaard, A.P., and Hall, B.D., (1963). Biochem. Biophys. Res. Comm. 12, 98.