A QUANTITATIVE ASSAY FOR DNA-DNA HYBRIDS USING MEMBRANE FILTERS.

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In this paper a rapid and sensitive method to assay for DNA-DNA hybridization is presented.
The method is inspired on the recently published membrane filter technique of Gillespie and Spiegelman (1965) for detecting RNA-DNA hybridization.
In our procedure advantage is taken of the observation of Klamerth (1965) that single stranded DNA can be eluted from nitrocellulose with buffers of low ionic strength and high pH, whereas the DNA hybridized can not.

While this manuscript was in preparation another modification of Gillespie and Spiegelman's technique to detect DNA-DNA hybridization was published by Denhardt (1966).

This method makes use of a preincubation of the filters with albumin solution to eliminate the absorption of non hybridized DNA to the filters.

In our method there is no need for this preincubation and the background levels of non specifically bound DNA are about one order of magnitude lower than in the method of Denhardt.

Materials and Methods.

Preparation of membrane filters was carried out as described by Gillespie et al. (1965). The filters used are Millipore H.A. and type M.F.30 from the Membrane Filter Gesellschaft, Göttingen, both of 22 mm diameter.
T4 DNA was prepared as described by Mandell and Hershey (1960). We wish to thank Dr.G.Veldhuisen, T.N.O. Rijswijk, for his generous gift of ^{32}P labeled T4 phage.

E.Coli RNA was prepared from E.Coli following the method of Bautz and Hall (1960) using hot phenol extraction as described by Scherrer and Darnell (1962).
Calf Thymus DNA was purchased from Sigma Chemical Co.. 1 x SSC buffer contained 0.15 M NaCl and 0.015 M sodium-citrate, 2 x SSC contained two times this concentration etc.
Hybridization was carried out in screw capped vials containing, unless stated

otherwise, 3.2 ml 1.25 x SSC pH 7.0 buffered with 10^{-2} M tris HCl, 1 µg of ^{32}P labeled, sonified and denatured T4 DNA, and a filter prepared as described by Gillespie et al. (1965), loaded with varying amounts of T4 DNA or Thymus DNA. After incubation at 60°C the filters were removed and rinsed briefly in 3.10^{-3}M tris-HCl buffer pH 9.4. The non hybridized radioactive DNA was removed by washing the filters by suction on both sides with 100 ml of 3.10^{-3} M tris-HCl buffer pH 9.4. The "noise" level of non specifically bound radioactive DNA could thus be reduced to less than 0.04 percent of the total radioactive DNA added with the Millipore filters, and to less than 0.1 percent with the MF30 filters. With MF30 filters the "noise" when the filters were loaded with calf thymus DNA, was less than with blank filters.
All experiments were carried out in duplo.

Results.

In fig. 1 the amount of hybrid formed is plotted against the time of incubation at 60°C.

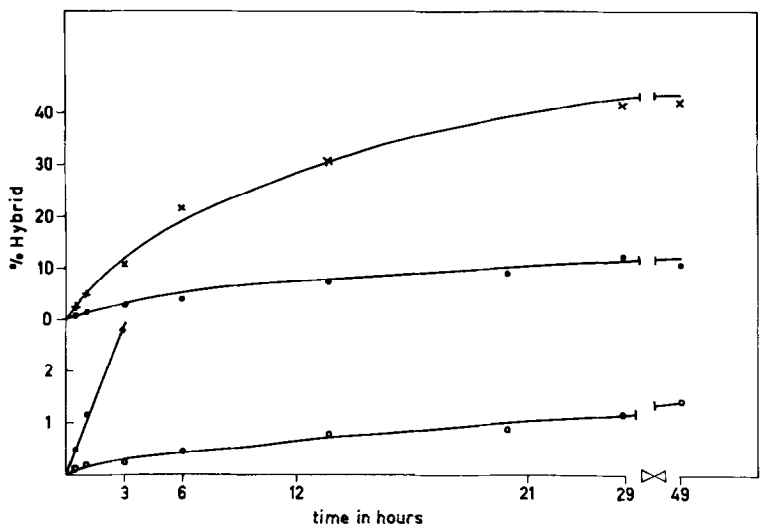

Fig.1. Mean percentages of (^{32}P) T4 DNA hybridized versus time of incubation at 60°C. x, • and o respectively: 40, 4 and 0.4 µg T4 DNA fixed on Millipore filters.

It can be seen that the hybridization reaction under these conditions is a slow process taking about 24 hours to reach a plateau level.
In the case of RNA-DNA hybridization Gillespie et al. (1965) found that a plateau was reached in a period ranging from 2 to 10 hours.
The influence of variations in the pH and the temperature during incubation on the extent of hybridization is shown in figures 2 and 3.
It can be seen that increasing the pH above 7.0 or the temperature above 60°C decreases the yield of hybridization.

The effect of increasing the volume in the incubation was to decrease the yield of ^{32}P labeled DNA complexed to the DNA on the filter, possibly as a result of increased

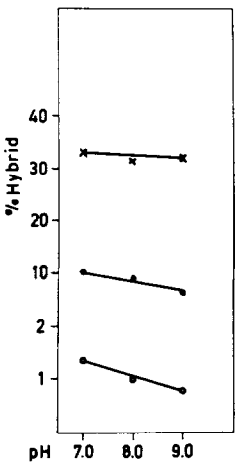

Fig. 2. Mean percentages of (^{32}P) T4 DNA hybridized, versus pH during incubation. x, • and o respectively: 40, 4 and 0.4 μg of T4 DNA fixed on Millipore filters. Incubation was for 17 hours at 60°C.

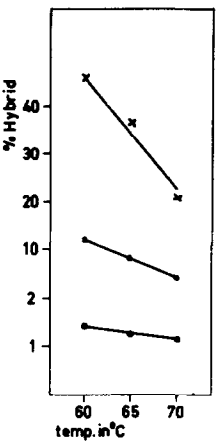

Fig. 3. Mean percentages of (^{32}P) T4 DNA hybridized, versus temperature during incubation. x, • and o respectively: 40, 4 and 0.4 μg of T4 DNA fixed on Millipore filters. Incubation was for 40 hours.

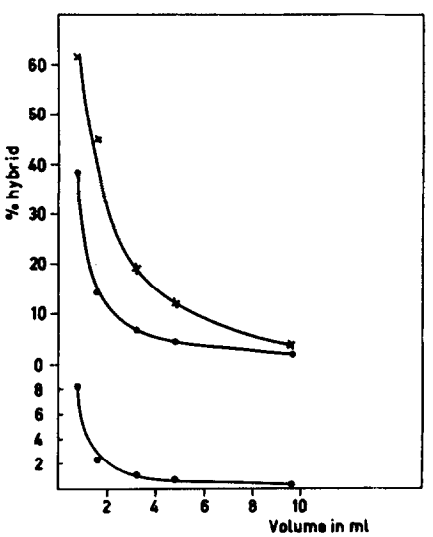

Fig. 4. Effect of varying the volume in the incubation. The points refer to the mean percentages of (^{32}P) T4 DNA hybridized. x, • and o respectively: 40, 4 and 0.4 μg of T4 DNA fixed on MF30 filters. Incubation was for 26 hours at 60°C in 1 x SSC.

self annealing of the ^{32}P labeled DNA. See fig. 4.

We did an experiment to try to saturate the DNA fixed on the filter with radioactive DNA. Under our experimental conditions the DNA on the filters was still far from saturated at a ratio of $\frac{(^{32}P)\ T4\ DNA}{T4\ DNA\ on\ filter}$ of 87.5 (see figure 5) .

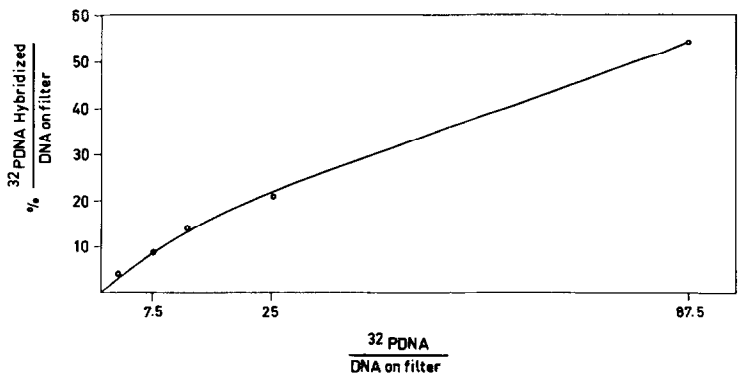

Fig.5. Saturation of DNA on Millipore filters with (^{32}P) T4 DNA. Incubation was carried out in 1 ml 1 x SSC for 17 hours at 60°C. The filters were loaded with 0.4 μg T4 DNA.

Gillespie et al. showed that essentially all of the DNA fixed on the filters can anneal with RNA.

We then assessed the influence of unrelated nucleic acids. The results are summarized in tables I and II. From table I it can be seen, that in solution an excess of thymus DNA of 400 times the amount of (^{32}P) T4 DNA added, had no important effect on the extent of the hybridization reaction.
Similarly an excess of thymus DNA fixed on the filter of 100 times the amount of T4 DNA fixed had no important effect (table II) .
It will be noticed from table I that as little as 10 μg of E.Coli RNA strongly depressed the amount of hybrid formed when the filters were loaded with 40 ug of T4 DNA. This effect was not seen with filters loaded with 4 or 0.4 μg T4 DNA. No explanation can be offered for this phenomenon.

TABLE I	Amount of T_4 DNA fixed on filter			
RNA or denatured DNA added to solution in vial	40 μg	4 μg	0.4 μg	no DNA
---	38.5	12.4	1.8	0.1
10 μg E Coli RNA	24.3	11.9	1.5	
100 μg E Coli RNA	23.3	13.4	1.5	0.1
10 μg Thymus DNA	44.1	13.7	1.8	
100 μg Thymus DNA	46.6	13.6	2.5	0.06
400 μg Thymus DNA			2.2	

The numbers in the table are the percentages of (^{32}P) T4 DNA hybridized. Filters used are type MF30. Incubation was carried out for 64 hours at 60°C.

TABLE II	Amount of T_4 DNA fixed on filter		
Denatured Calf Thymus DNA fixed on filter	4 μg	0.4 μg	no DNA
---	5.2	0.8	
10 μg	8.9	1.2	0.04
40 μg	6.2	0.95	0.04

The numbers in the table are the percentages of (^{32}P) T4 DNA hybridized. Filters used are Millipore H.A. Incubation was carried out for 45 hours at 60°C.

References.
E.F.K.Bautz and B.D.Hall, Proc.Natl.Acad.Sci., 48, 400 (1962)
D.T.Denhardt, Biochem.Biophys.Res.Commun., 23, 641 (1966)
D.Gillespie and S.Spiegelman, J.Mol.Biol., 12, 829 (1965)
O.Klamerth, Nature, 5017, 1318 (1965)
J.D.Mandell and A.D.Hershey, Anal.Bioch., 1, 66 (1960)
K.Scherrer and J.E.Darnell, Biochem.Biophys.Res.Commun., 7, 486 (1962)