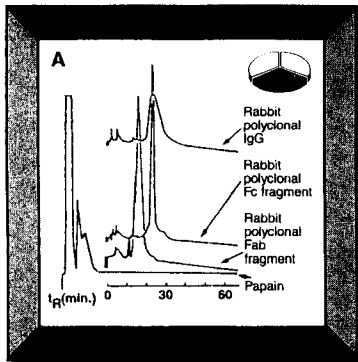


# PORTRAITS IN CHROMATOGRAPHY

## Fragment, Conjugate Separations Ion Exchange HPLC



**Papain Digest of Rabbit Polyclonal IgG on 5 $\mu$ m BAKERBOND ABx\***

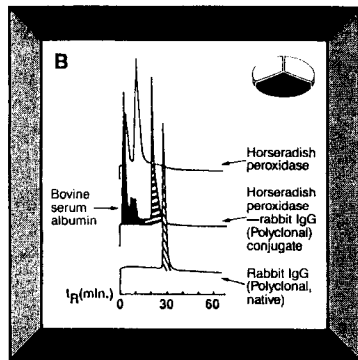
### Analytical Conditions:

#### Portrait A only:

**Mobile Phase:** A = 25 mM MES, pH 5.4  
B = 500 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> plus 20 mM NaOAc, pH 6.7  
**Gradient:** 100% A to 100% B over 60 min.  
**Sample:** 2.0 ml, containing at least 1 mg protein, diluted in A buffer.

#### Common to Portraits A and B:

**Column:** 7.75 x 100 mm, 5 $\mu$ m BAKERBOND ABx (Cat. #7271-07)  
**Flow Rate:** 1.0 ml/min.  
**Pressure:** 200 psi  
**Detection:** UV at 280 nm; 2.0 AUFS



**HRP/IgG Conjugate Separation on 5 $\mu$ m BAKERBOND ABx\***

### Analytical Conditions:

#### Portrait B only:

**Mobile Phase:** A = 25 mM MES, pH 5.2  
B = 500 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> plus 20 mM NaOAc, pH 6.7  
**Gradient:** 100% A for 10 min, followed by a linear gradient of 100% A to 100% B over 30 min.  
**Sample:** 2.0 ml containing at least 1 mg protein in A buffer.

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\*D.R. Nau, *BioChromatography* (submitted.)

# BioFeedback

E4

defined as 50%-observed CPE, a subjective evaluation. Much more accurate would be a signal that disappears at the dilution point corresponding to one unit of the IFN being tested. This can be achieved by using molecular hybridization of a labeled viral probe to the supernatants of a multi-well plate. Initially, the cells are plated and treated with IFN for 6 h according to standard procedures. The challenge virus (EMC) is then added and incubation is continued for 12-18 h. At this point, 200  $\mu$ l of the supernatant of each well is transferred directly to nitrocellulose using a BRL dot-blot apparatus. The plate with monolayer cells still attached can also be stained as a control. In such case, however, there is little or no clear CPE (Figure 1A). The nitrocellulose filter is fixed in 1% glutaraldehyde for 1 h, and viral proteins digested in 20  $\mu$ l/ml proteinase K, as described in Paeratakul et al. (3). The membrane is then subject to pre-hybridization for 2 h at 65 $^{\circ}$  C in 3X SSC, 35% formamide, 0.5% SDS and 1X Denhardt's solution, and viral RNA is hybridized to the NC filters for 12-16 h with a probe encoding for sequences of the challenge virus labeled with <sup>32</sup>P (we normally use the oligonucleotide random primer method described in Paeratakul et al., 1988). The filter is washed sequentially at room temperature with 2X SSC, 0.5% SDS for 15 min, 1X SSC, 0.5% SDS for 15 min and 0.1X SSC, 0.5% SDS at 55 $^{\circ}$  C.

In the assay presented in Figure 1, WISH cells were infected with EMC virus. Recombinant IFN-alpha and -gamma standards were added to the monolayer 6 h prior to virus infection. As shown in Figure 1B, viral signal disappears at the dilution point between 1 and 1/2 unit of IFN (the IFN titer as estimated by NIH standards). The original multi-well plate, shown after staining by conventional techniques (Figure 1A), revealed a very unclear result.

We have found this method to be very convenient when dealing with IFN samples that had previously been difficult to titer. However, a slightly greater amount of work is required using this technique than the CPE assay. We propose this method as an alternative to the CPE assay for all

cases in which high sensitivity is required in the quantitation of IFN activity. This method can be adapted to detect viral RNA from any RNA virus, and may also be used as a method of virus titration.

*P.R. De Stasio is supported by the Istituto Pasteur-Fondazione Cenci-Bolognetti and M.W. Taylor by USPHS grant AI 21898.*

*Address correspondence to M.W. Taylor.*

### REFERENCES

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2. Langford, M.P., D.A. Weigent, G.J. Stanton and S. Baron. 1981. Virus plaque-reduction assay for interferon: Microplaque and regular macroplaque reduction assays. *Meth. Enzymol.* 78:339-346.
3. Paeratakul, U., P.R. De Stasio and M.W. Taylor. 1988. A fast and sensitive method for detecting specific viral RNA in mammalian cells. *J. Virol.* 62(4).

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## Radiochemical Degradation of Nucleic Acids by 32-Phosphorus

Nucleic acids labeled internally with <sup>32</sup>P are subject to radiochemical degradation of the sugar-phosphate backbone with stoichiometric efficiency, resulting in shortened single-strands (6,8), partial double-strand breakage (5,7), and inactivation of biological activity (3).

Despite this apparent disadvantage of <sup>32</sup>P, it remains the isotope of choice in many applications due to its short half-life, high  $\beta$ -emission energy (16.7 MEV, which allows easy detection by a variety of methods), relatively low

cost, and ready availability in a growing number of useful compounds.

Because of its potential importance in hybridization and cloning, radiochemical degradation was investigated by electrophoresis of denatured plasmid pBR322 probe samples labeled with the oligonucleotide priming procedure (2,4) to produce strands containing one labeled nucleotide precursor (dCTP, 3,200 Ci/mmole). Figure 1 shows rapid radiochemical degradation of  $^{32}\text{P}$  labeled strands at various times after synthesis. Densitometric size measurements from autoradiograms were adjusted for size distortion (1) (a random sized population of macromolecules has a number-average size of 1/2 the observed weight-average size) and plotted together with a theoretical determination of radiochemical degradation derived from the equation:

$$L_t = L_0 / (1 + N_x(1 - e^{-kt}))$$

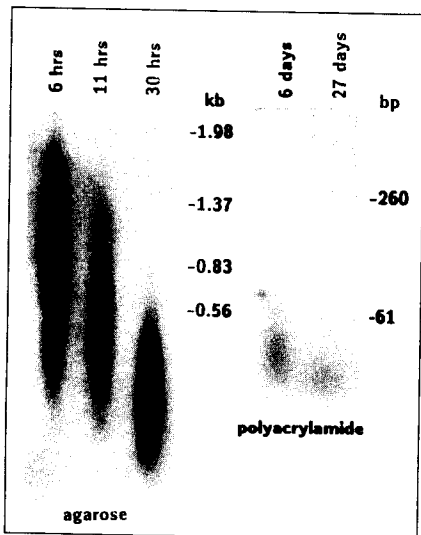


Figure 1. Radiochemical degradation of  $^{32}\text{P}$  labeled DNA strands over time.

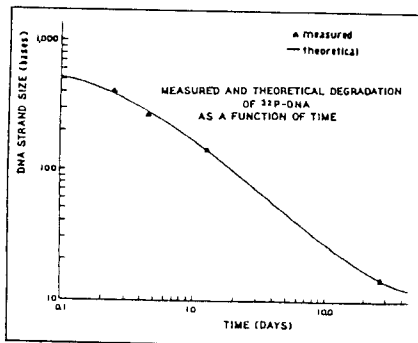


Figure 2. Measured and theoretical degradation of  $^{32}\text{P}$  DNA as a function of time.

where  $L_t$  is the number-average strand length at time  $t$ ;  $L_0$  is the initial number average length;  $N_x$  is the total number of labeled loci per initial number-average strand;  $k$  is the rate constant for  $^{32}\text{P}$  (0.0487/day) and  $t$  is the time (see Figure 2). The close correlation between observed and calculated values demonstrates the usefulness of the equation in predicting radiochemical decay.

A simple computer program (NEWDEG, written in IBM-BASICA and compiled for IBM-compatible computers) based upon the above relationship was devised to provide degradation tables and graphs for the spectrum of specific activities and initial lengths that may be encountered in diverse laboratory situations.

The degradation profiles calculated by NEWDEG for kilobase-sized nucleic acids labeled with various specific activities of  $^{32}\text{P}$  precursors are shown in Figure 3. For example, 1 kb DNA labeled as in Figure 1 degrades to 100 base pairs in 2.3 days. Degradation rate is highly length dependent. Since degradation affects both hybridizability and cloning efficiency of labeled strands, it is important to have at least a

general idea of how much degradation occurs at various specific activities and times.  $^{32}\text{P}$  induced damage may not always be apparent, since visual evidence of strand breakage is often obscured by hydrogen bonding on non-denaturing gels. In addition, double-stranded molecules (such as cDNA) experience twice the number of breaks per unit length shown here for single-strand probe fragments. The specific activity of  $^{32}\text{P}$  should be limited in cDNA cloning since radiochemical degradation results in selection for small cDNAs. Likewise, high specific activity hybridization probes may be disadvantageous if probes are to be reused, sold, or used at a later date, since hybridization rate and melting temperature are size dependent.

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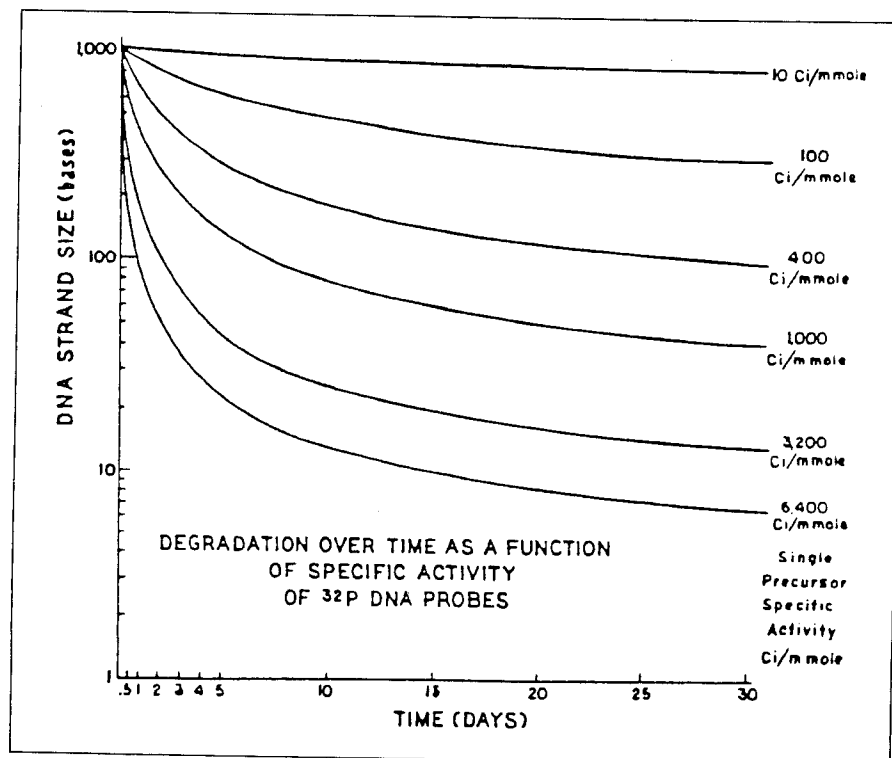


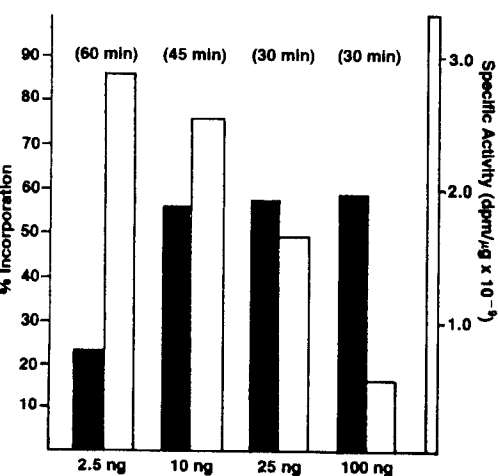
Figure 3. Degradation over time as a function of specific activity of  $^{32}\text{P}$  DNA probes.

# Optimized Random Primed Labeling:

## Influence of template concentration and size

### Introduction

DNA probe synthesis using the random-primed method of Feinberg and Vogelstein (1,2) has become an important technique for molecular biologists. When performed properly, the method can produce high specific activity radiolabeled probes with speed (30 minute reaction time) and efficiency (specific activity  $> 1.6 \times 10^9$  dpm/ $\mu$ g). We have investigated some of the critical parameters of random primed labeling reactions. In this report, we describe our results regarding the influence of template concentration and template size (length) on probe specific activity.



**Figure 1.** Bar graph showing the effect of labeling different DNA template concentrations. Four different quantities of linear pBR322 (2.5 ng, 10 ng, 25 ng, 100 ng) were denatured by boiling and then labeled in parallel reactions using the BMB Random Primed Labeling kit. Aliquots of the reactions were removed at 15 minute time intervals. The data presented is from a time point showing peak incorporation; the time is indicated above the bars in parentheses ( ). Percent incorporation (solid bars) was determined by dividing TCA-precipitable counts by total counts. Specific activities (open bars) were calculated by dividing the amount of incorporated radioactivity by the total amount of DNA present at the end of the reaction (starting amount plus newly synthesized). The incorporated radioactivity (dpm) was calculated by multiplying the percent incorporation by the number of  $\mu$ Ci added to the reaction (50  $\mu$ Ci), and then multiplying by  $2.2 \times 10^6$  dpm/ $\mu$ Ci. The amount of newly synthesized DNA was calculated by multiplying the percent incorporation by the number of picomoles of labeled nucleotide added, multiplying by 4, and then dividing by 3 (3 picomoles dNTP = ng).

### Template concentration

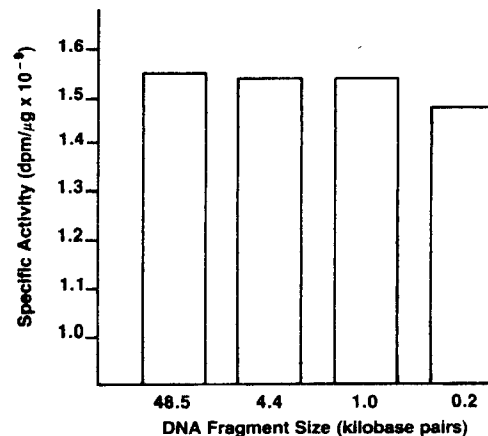
An important characteristic of the random primed labeling method is that only small amounts of DNA template are required to achieve high specific activity probes. Figure 1 shows the results of an experiment in which four different quantities of template were labeled using the BMB Random Primed Labeling Kit. Both percent incorporation and specific activity were determined. Note that, for the smaller quantities of DNA (2.5 ng, 10 ng), specific activities of greater than  $2.5 \times 10^9$  dpm/ $\mu$ g were achieved. Percent incorporation is low because the limiting component under these conditions is the template concentration. Essentially, both strands of the template DNA have been primed and duplicated in these reactions, and a large proportion of the isotope remains unutilized.

The situation changes when higher DNA concentrations are labeled. Figure 1 shows that for template amounts  $\geq 25$  ng, the specific activity of the resulting probes is lower. This occurs because, under conditions of excess template, the limiting reaction component becomes the isotopically-labeled nucleotide. In this situation, the majority of the isotope becomes incorporated into labeled probe. Further incorporation is hindered because the remaining isotope concentration is so low that enzymatic utilization is kinetically difficult. Including excessive amounts of template DNA in the labeling reaction does nothing to increase the percent incorporation, and only serves to dilute the final specific activity of the probe.

Our experiments also showed that the reaction kinetics change when different template concentrations are used. The lower concentrations (2.5 ng, 10 ng) required 45–60 minutes to reach peak incorporation while the higher template concentrations were complete after approximately 30 minutes.

### Template size

It is desirable to have a method capable of labeling DNA fragments of a variety of sizes. It is known that the nick translation labeling procedure first described by Rigby *et al.* (3) is inefficient for labeling smaller DNA fragments. In the experiment



**Figure 2.** Bar graph showing the effect of labeling different sized DNA templates. Four different template sizes were used (48.5 kb lambda DNA, 4.4 kb linear pBR322 DNA, 1.0 kb pBR322 restriction fragment, 200 bp pBR322 restriction fragment). 25 ng of each template was labeled to peak specific activity (30 min.) in parallel reactions. The specific activity was calculated as described in Figure 1 above. Note that all the fragments were labeled with nearly the same efficiency.

shown in Figure 2, we assayed the ability of the random primed labeling method to label templates ranging in size from 48,500 bp to 200 bp. The results showed very little difference in the final specific activity of the probes. The ability of the random primed method to efficiently label DNA's of a variety of sizes is a major advantage of the technique.

### Conclusions

Our experiments indicate that random primed labeling reactions containing small quantities ( $\leq 25$  ng) of DNA template produce the highest specific activity probes. If limiting template concentrations are used, "hot" probes can be produced even though percent incorporation values are low. Using small quantities of template necessitates that slightly longer incubation times be employed. Template size does not significantly influence the efficiency of random primed labeling reactions.

Product	Cat. No.	Size	Price
Random Primed Labeling Kit	1004 760	50 reactions	\$163.00

### References

1. Feinberg, A. and Vogelstein, B. (1983) *Anal. Biochem.* **132**:6
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3. Rigby, P. *et al.* (1977) *J. Mol. Biol.* **113**:237

Product Description

# Prime-a-Gene® Labeling System

## OLIGOLABELING SCHEMATIC

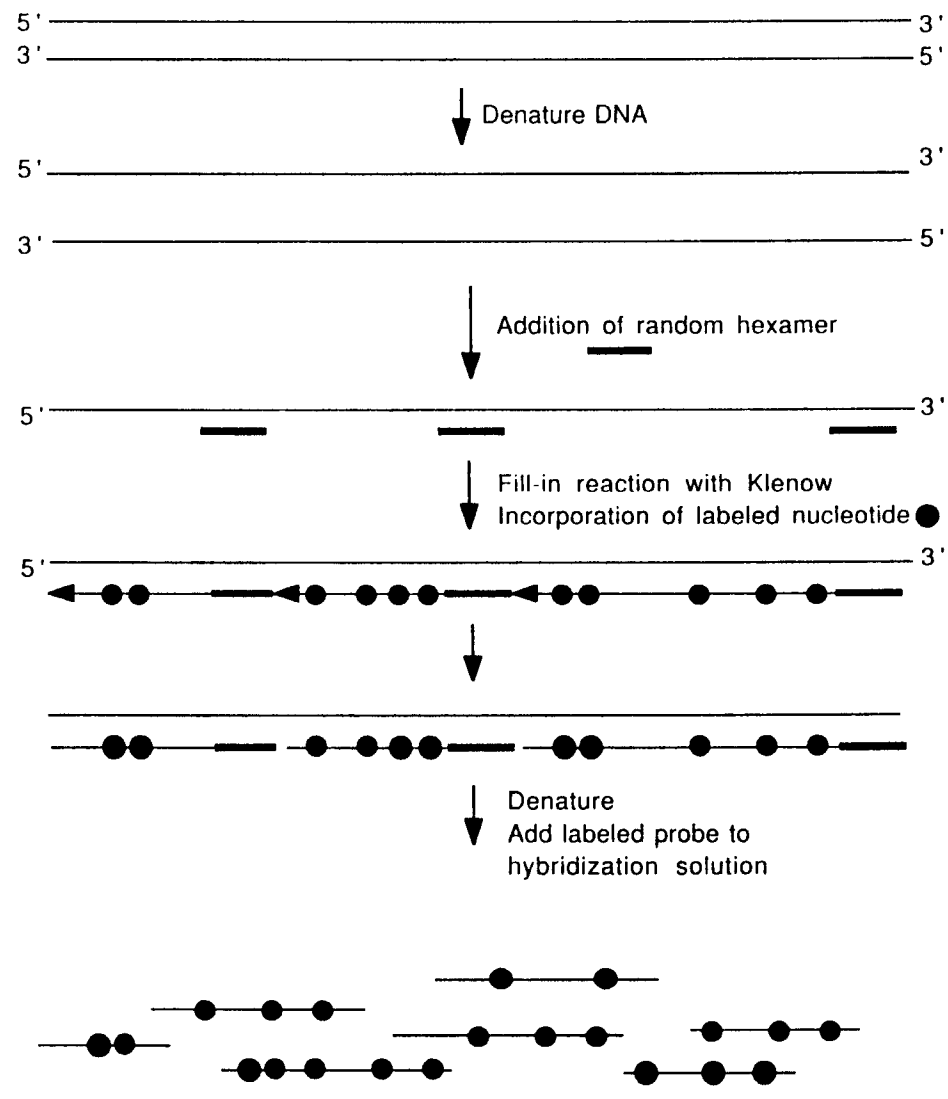


Figure 1. Schematic Diagram of Prime-a-Gene® System.



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