

## LIQUID SCINTILLATION COUNTING IN MOLECULAR BIOLOGY

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**ABSTRACT.** Radioactivity in molecular biology is commonly used for labeling DNA probes with  $^{32}\text{P}$  or  $^{35}\text{S}$  that are hybridized to target nucleic acid sequences and usually visualized by autoradiography. Quantification of autoradiographs can be performed by densitometry, but this technique suffers from poor linearity and gives only a limited range. Phosphorimagers and proportional counters can be used, but these machines have relatively poor counting efficiencies. At present, the use of liquid scintillation counting (LSC) is rare, and is confined to testing a few samples to determine the specific activity of newly synthesized probes, and occasionally, where electrophoretically separated radioactive bands are excised from dried down gels, for conventional LSC in vials with scintillant. The flatbed scintillation counter has been used to quantify a variety of samples produced by molecular biological techniques. I discuss here the methods I have used in developing LSC techniques for research in molecular biology.

### INTRODUCTION

Liquid scintillation counting (LSC) has been essential in the development of biochemistry and other areas of biological science. This is evidenced by the immense range of radioactive compounds available, often with different isotopes used as labels. For diagnosis, radioimmunoassay (RIA) has been invaluable using LSC and gamma counting, although there is a trend toward non-radioactive ELISA methodology. I believe that LSC provides the system of choice in any emerging field of study where sensitive measurement of specific radioactive label is required. Licensing procedures and the potential hazard of radioactive labels make it worthwhile to perfect non-radioactive measurement techniques; this should only be accomplished after initial difficulties in assay techniques are overcome.

Developments in molecular biology over the past decade have followed this scenario. Because very small quantities of nucleic acids are to be detected, maximum sensitivity is required; this has been met by the ubiquitous use of the  $^{32}\text{P}$  label for the phosphate backbone chain of DNA or RNA. Detection of the label has been performed almost entirely by autoradiography; it is surprising that LSC measurement has not been employed more often. Because of two factors, only a few LSC techniques appropriate for the quantification of samples produced by molecular biological methods have been described (Potter & Warner 1989). The first factor relates to the intrinsic properties of data produced by methods used in molecular biology. These methods have been molded by crucial discoveries, such as restriction enzymes from bacteria that can cut DNA at particular base sequences, which allows DNA to be cut into reproducible fragments. Autoradiography is the primary method employed for monitoring the manipulation of DNA fragments and testing for the presence of genetic sequences. Unfortunately, the method suffers from a very restricted measurement range. Much effort has been expended on improving the speed, sensitivity and sophistication of densitometers while the underlying problems in the subject autoradiographic material, particularly where there is a wide range in activity, are ignored.

A second reason for the lack of LSC in molecular biology is that, generally, only qualitative information is needed. Although data output is large, its qualitative nature places molecular biology at the early stages of scientific evolution where data collection predominates. On the other hand, the need for proper quantification in molecular biology has been pointed out by the editor of *Nature* (Maddox 1992):

*Liquid Scintillation Spectrometry 1992, edited by*

*J. E. Noakes, F. Schönhofer and H. A. Polach. RADIOCARBON 1993, pp. 277–283*

. . . molecular biology seems well on the way to becoming a largely qualitative science. The notion that science hangs on measurement seems to have been diminished. . . . Curiously enough, even when molecular biology derives from fields in which there is a strong tradition of measurement . . . attempts to make the arguments quantitative appear to be neglected. More seriously, the neglect of quantitative considerations may well be a recipe for overlooking problems of great importance . . . it would be a worthwhile precaution against the quantitative days that lie ahead that people should make sure that published data are capable of quantitative interpretation . . . (Reprinted with permission from *Nature* ©1992 Macmillan Magazines Ltd.)

Quantitative methods are now needed for measurements of RNA abundance, as these determinations give insight into “switched on” cell functions.

## GEL METHODS

### <sup>32</sup>P Measurement

Some recognition of the needs and difficulties of quantification has resulted in the recent development of instruments for measuring samples containing <sup>32</sup>P as two-dimensional arrays of bands or spots on gels or transfer membranes. Such instruments include improvements on long-established techniques, such as gas flow machines, sometimes as scanners or with multiple detectors on moving heads. The Betascope™ (Betagen, Waltham, Massachusetts USA) has a counting efficiency of 18% for <sup>32</sup>P and 2.5% for <sup>35</sup>S. Microtitration plate 8 × 12 arrays can also be measured by multiple detectors. A recent development is the PhosphorImager™ (Molecular Dynamics, Sunnyvale, California, USA), which collects information on a plate that can be read later by laser scanning. Up to 30 Mb data per image are produced; although a superb analytical machine, it has 4% efficiency for <sup>32</sup>P. For <sup>3</sup>H, the PhosphorImager™ is almost completely ineffective; for <sup>35</sup>S, it gives 0.02% efficiency (Molecular Dynamics Application note #50).

At Oxford University, we have attempted to develop straightforward procedures for sensitive measurement of samples in gels and on nylon membrane. We have used the flatbed scintillation counter, because it is ideally suited to counting sample arrays.

One gel method in use at Oxford is that for RNA protection assays. A short sequence specific to a particular RNA is synthesized, labeled with <sup>32</sup>P and hybridized to an unseparated mRNA sample in solution. Digestion with RNAase leaves short double-stranded labeled molecules, which are then electrophoresed. Often, we included a sequence with constant cellular expression as an internal control. We developed the following measurement procedure (Potter, Tan & Ratcliffe 1991): the radioactive band was located by autoradiography on the gel, which had been dried onto filter paper and excised. Initially, the band was placed in a shallow plastic well and a drop of liquid scintillant was added. This appeared quite successful, but the pulse occasionally gave counts at odds with the appearance of the autoradiograph. Examination of the pulse-height spectrum showed it to be multimodal, with a low-energy crosstalk peak (Fig. 1). If each band was placed on sticky sample areas on the outside of a bag containing a glass fiber filter (plus 10 ml BetaplateScint™), the spectrum was satisfactory; crosstalk was reduced by restricting the lower part of the spectrum without losing significant <sup>32</sup>P counts. Crosstalk could be reduced further when samples were placed at alternate sample positions on the nylon membrane; staggered photomultiplier tubes (PMT) match these positions, so no counting time was wasted by counting empty sample areas. The bags had photomount adhesive sprayed through a mask to produce the adhesive patches to which the gel fragments were attached. A second bag and filter was placed over the first, and the “sandwich” mounted in the cassette for counting (Fig. 2). Meltilex™ sheets could also be employed, but were more cumbersome to use. We achieved good linearity and sensitivity (Potter, Tan & Ratcliffe 1991). The background count rate was about 3 cpm for our quartz PMT; lower detection limit was

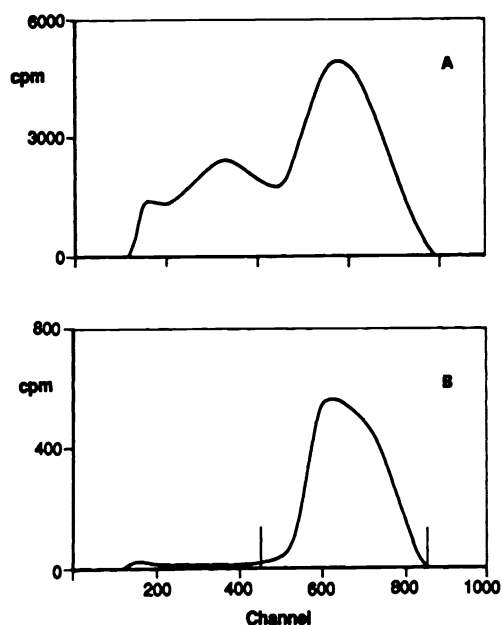


Fig. 1. Typical pulse-height spectra (A) for a gel fragment counted with liquid scintillant in a shallow tray; (B) for a (different) gel fragment, sandwiched between two glass fiber filters, each bagged and sealed with liquid scintillant. Reprinted by permission of Academic Press ©1991, from Potter, Tan and Ratcliffe (1991).

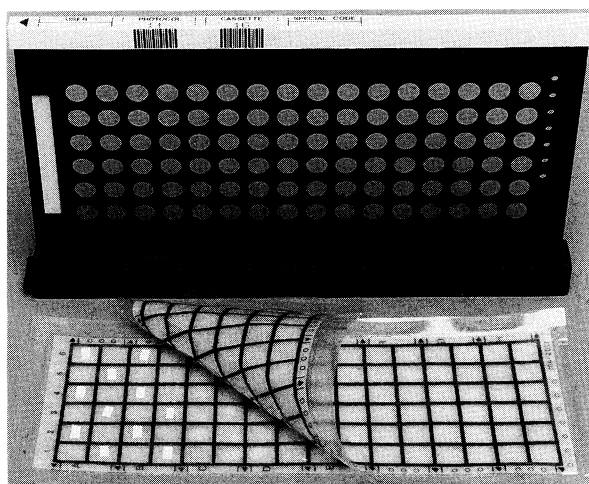


Fig. 2. Placement of gel fragments between two filters, each bagged with liquid scintillant before mounting in cassette. Reprinted by permission of Academic Press ©1991, from Potter, Tan and Ratcliffe (1991).

1 cpm over 60-min count time. We counted six samples in coincidence simultaneously with satisfactory results (Ratcliffe *et al.* 1990; Campbell *et al.* 1991; Tan, Eckardt & Ratcliffe 1991).

### <sup>35</sup>S Measurement

We hoped that the same technique would be useful for <sup>35</sup>S. However, few counts were obtained unless “Amplify” (Amersham International plc) was used to permeate the gel before drying. Because the paper onto which the gel was dried is a barrier to both electron and light emissions from <sup>35</sup>S, we found that an “open sandwich” gave better results. This was improved further by making the paper translucent with oil or a drop of BetaplateScint™. This method (Elvin *et al.* 1992) was useful in quantifying the interrelation of assembly of the major histocompatibility complex; on T cells, the complex holds fragments of foreign proteins for binding to other lym-

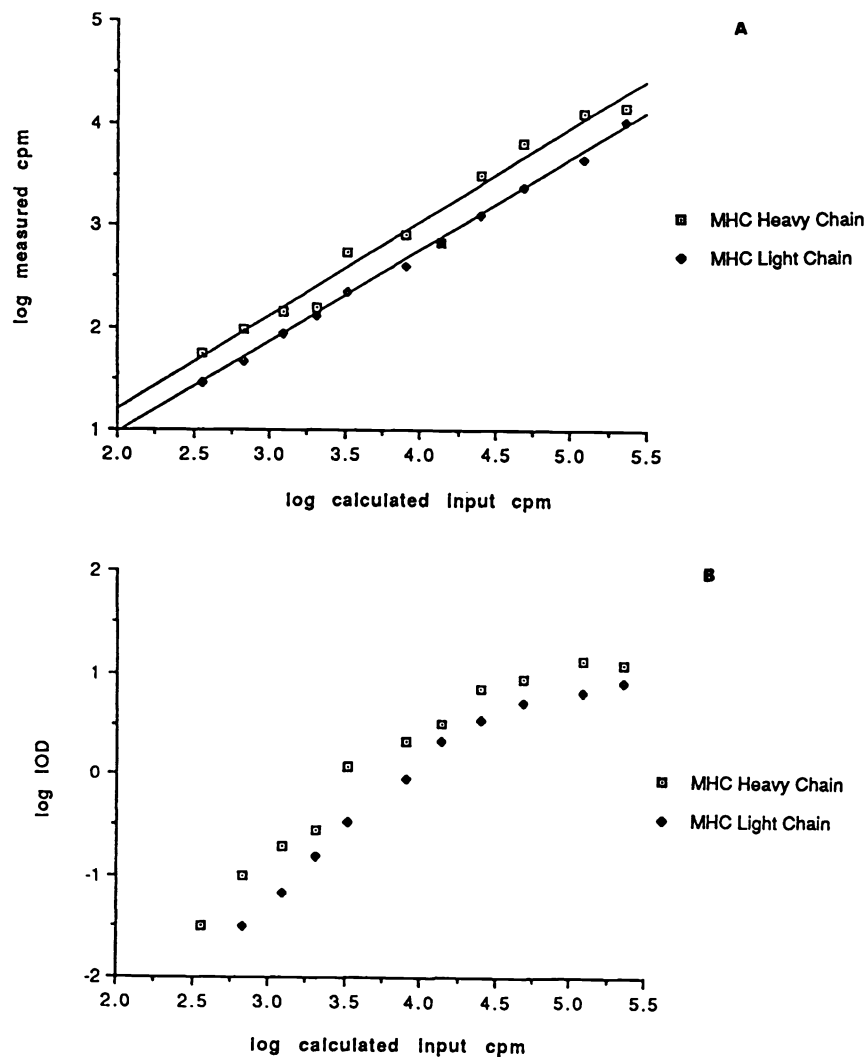


Fig. 3. Different amounts of  $^{35}\text{S}$ -labeled MHC light and heavy chain proteins in acrylamide gels, measured (A) by counting radioactivity of gel fragments and (B) by densitometry of the autoradiograph. Reproduced by kind permission of Dr. J. Elvin and Prof. Alain Townsend.

phocytes so that the immune response can be triggered (Elvin *et al.* 1991; Elliot *et al.* 1991). Dilution curves (Fig. 3A), rather than densitometry (Fig. 3B) showed the improvement of counting.

#### APPLICATIONS

Earlier, we measured probes labeled with  $^3\text{H}$  (as well as the usual  $^{32}\text{P}$ ), to measure the amount of human B19 parvovirus in serum spotted out on nylon membrane (Fig. 4) (Potter & Anderson 1987). The nylon was dried and placed in a bag with scintillant; this constitutes the essentials of the flatbed Betaplate<sup>TM</sup> scintillation system. For  $^{32}\text{P}$ , the crosstalk between samples was a problem, as both electrons and light can be viewed at a low level from adjoining samples. We tried printing a grid on the nylon, known to reduce crosstalk, but the original print composition interfered with

the hybridization process. Cerenkov counting was possible; because the count rate on many samples was very low, we did not pursue this method.

Since then, the printing problem has been resolved. We then turned to optimizing counting for RNA samples on nylon membrane that remained water-wet, but sealed in a plastic bag. We found the answer in the careful use of a manifold (Schleicher & Schuell) to collect the sample, then counting by using the sandwich technique with two glass fiber filters, each bagged with 10 ml scintillant placed above and below the nylon. The count rate was then nearly as high as with scintillant inside the bag with a dried nylon membrane, but quenching was greatly reduced (Potter & Le Jeune 1991). This permitted restriction of the pulse-height spectrum to reduce crosstalk to a low level.

We measured mRNA in cells from breast tumors to correlate its abundance with the rate of disease progression; further study is in progress. The technical value of this method is that the samples are kept in good condition so that the radioactive probes can be removed by boiling water, and the samples reprobed to look for and quantify other sequences of interest (Fig. 5). Repeated hybridization gave a good correlation with the first experiment.

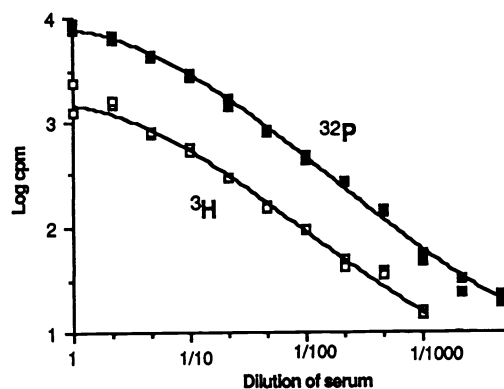


Fig. 4. Dilution series dot blot hybridization for human parvovirus B19 detected by counting either  $^{32}\text{P}$ - or  $^3\text{H}$ -labeled probes

The current method suffers only from the possibility of occasional unreliable comparison between activity of successive probes, because some nucleic acid may be removed during the stripping process. Thus, we have developed a double-label procedure for simultaneous hybridization of nucleic acids with probes labeled with  $^{35}\text{S}$  and  $^{32}\text{P}$ . Scintillant must be used to detect the  $^{35}\text{S}$ , which is easily distinguished from the  $^{32}\text{P}$  if the sandwich preparation is employed (Fig. 6). The signals are separated by conventional double-labeling procedures, complicated in the Betaplate™ by small differences in the pulse-height spectra and relative counting efficiencies of the six PMT pairs; this is corrected by a suitable normalization procedure. The probe for  $\beta$ -actin labeled with  $^{35}\text{S}$  was chosen as the internal control, as there are several copies of the gene (some inactive); this ensures a good signal for the lower-specific-activity  $^{35}\text{S}$  labeling. In this way, mRNA can be measured with an internal control. Using this method, we measured 35 individuals' DNA for an oncogene (Int-2) on a supposed 1- $\mu\text{g}$  DNA per sample spot. The probe activity varied over a five-fold range, showing the difficulty of DNA quantification in solution by the standard spectrophotometric method. However, the ratio of  $^{32}\text{P}$  to  $^{35}\text{S}$  had a coefficient of variation of about 7%. Comparison of 35 breast tumor DNAs from the same individuals showed that about 40% had extra copies of the oncogene (Stickland *et al.* 1993). Such "amplification" may have diagnostic significance. We hope that the loss of genes that control cell division may also be revealed by this technique; several other applications that measure gene dosage are expected to be useful for prenatal diagnosis and

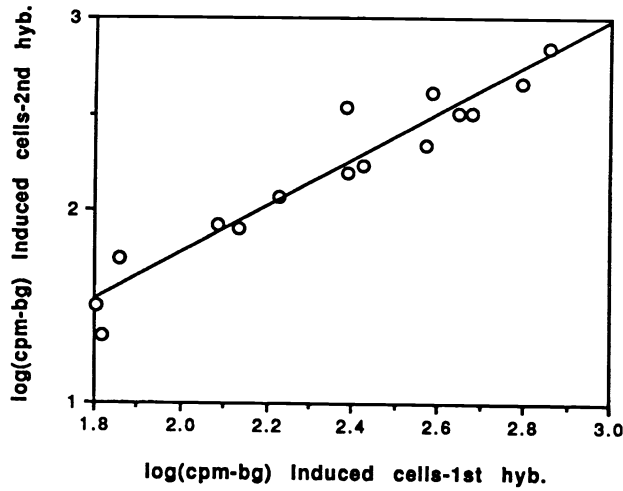


Fig. 5. Radioactivity of dot blots of RNA from tumor cells, hybridized to <sup>32</sup>P-labeled amphiregulin; comparison of count rates for initial hybridization and after stripping the filter and rehybridizing with the same probe. Reprinted by permission of Academic Press ©1991, from Potter and Le Jeune (1991).

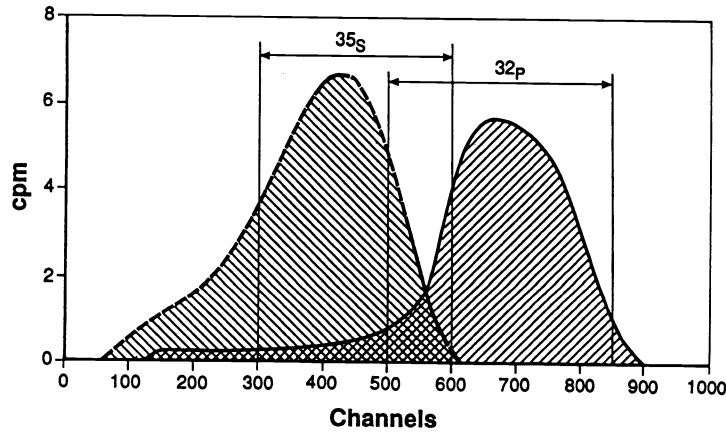


Fig. 6. Dual-label calibration pulse-height spectra of standards for <sup>35</sup>S and <sup>32</sup>P, using labeled probes separately spotted on nylon membrane and counted with liquid scintillant and with the filter sandwiched between two glass fiber filters, each bagged with scintillant from oncogene

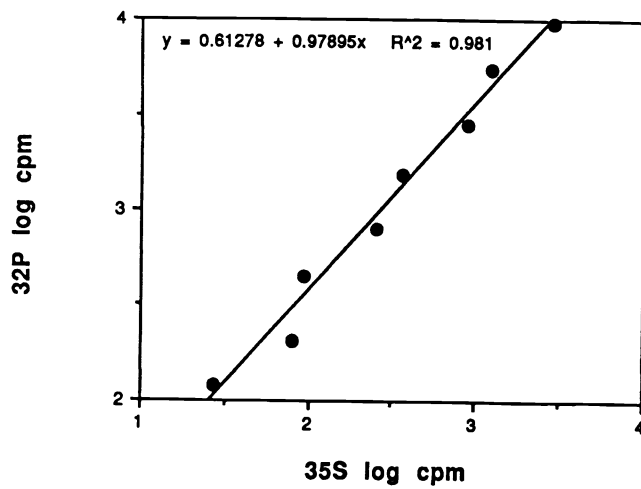


Fig. 7. Dual-labeled dot blots for comparison of <sup>32</sup>P-labeled HPV16 and <sup>35</sup>S-labeled actin in CaSki cells, (known to contain 300-500 copies of virus/cell)

research. Other approaches by restricting fragment polymorphism are applicable only in 40–70% of cases, making surveys and possibility for diagnosis most uncertain.

Hyypia (1990) showed that it was possible to detect the human papilloma virus (HPV) in cervical cells using the flatbed counter. We have since used the double-labeling technique for a study involving cell lines previously known to contain HPV16 (Fig. 7) (Potter *et al.* 1993). A  $^{32}\text{P}$ -labeled probe to HPV16, hybridized to extracted cervical DNA, showed some obviously positive samples; otherwise, it was difficult to determine whether a negative sample was due to the absence of all DNA, *i.e.*, a false negative. Data from double labeling made it clear that this occurs in several cases that would need re-analysis. We are pursuing this work at Oxford now.

#### CONCLUSION

One result, or possibly, cause, of the present preponderance of densitometry for quantification is that workers in molecular biology are often unfamiliar with LSC concepts. I have tried to show here that it is possible to use LSC in several molecular biological techniques; its versatility may be of value when a great deal of quantitative data is required.

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