

CHAPTER 14

A New, Rapid Analysis Technique for Quantitation of Radioactive Samples Isolated on a Solid Support

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INTRODUCTION

The quantitation of radioactivity on solid supports has grown extremely rapidly in the past five years due to the increased use of microplate assays. These assays use a special microplate capable of holding 96 samples of volumes up to 300 μL in the 8×12 format. A typical microplate is 3 in \times 5 in. The incubation of cells, DNA, tissues, or other substrate takes place directly in the microplate. Once the incubation has been completed in the presence of a radioactive labeled substrate, the unreacted/unincorporated components must be separated from the incorporated/bound substrate. This is normally done with a cell harvester, which deposits the cell particulate bound material on a filter media, usually glass fiber filter. Alternatively, the DNA can be spotted with a dot blot apparatus in the 8×12 format and hybridization can be performed. A wash solution (buffer or water) removes any unincorporated/unbound radioactive material on the filter. Each of the filters is punched out of the filter mat into individual scintillation vials, 1 to 12 samples at a time. Five mL of scintillation cocktail is added to each of the individual scintillation vials containing the filters. The 96 vials are capped, shaken, and placed in the counting cassettes of a liquid scintillation counter. Each of the 96 samples is counted for 5 to 10 minutes. The data are printed out and transferred to a computer for final data reduction and graphic presentation. As can be clearly seen from this description of the steps involved in harvesting and quantitation, this procedure is not only time consuming but expensive.

Packard has developed an alternative to this procedure that reduces both the time and cost of harvesting and quantifying each sample by a factor of over ten. This procedure uses two new instruments. The first is a special 96 sample cell harvester (Micromate 196). It can harvest all 96 samples from the microplate simultaneously and is as efficient as the manual harvesters. The second is a

special 96 sample radioactivity reader. This Packard Matrix 96 can analyze 96 samples simultaneously using 96 individual detectors using no vial, no cocktail, no bags, and no special filters and causing no destruction to the samples. A description of this harvester and reader will be presented in this article along with applications for which this equipment can be used.

HARVESTING/BLOTTING

Three different methods exist for preparing samples on a solid support. The first is the direct method. This involves spotting the samples in the 8×12 format. The best example of this application is dot blots. For this application the DNA is spotted on a membrane in the microplate format, and ^{32}P or ^{35}S DNA probes are used. A typical DNA hybridization device is shown in Figure 1. The radioisotope, which becomes hybridized to the DNA, is then quantitated using radioautography/densitometry or liquid scintillation counting. This DNA probe hybridization assay has become increasingly popular because of the strong interest in DNA probes and DNA sequencing.

The second method is the use of a cell harvester. This method involves using a special device which is able to aspirate the sample (cell/particulate material) from the wells of the microplate onto a filter media, and wash the wells and the filter to remove any unincorporated/unbound radioactive material. These filter bound samples are punched into liquid scintillation vials, processed, and quantitated in a liquid scintillation counter. With the present manual harvesters, only 6 samples can be harvested, washed or punched out at a time. The new Packard Micromate 196 cell harvester is capable of harvesting and washing all 96 samples simultaneously from a microplate. This is done directly, without any excess tubing in the cell harvester that could become contaminated. This harvester is shown in Figure 2. In order to assess the Micromate 196 cell harvester performance compared to the manual harvester, two separate microplates were prepared using the ^3H -Thymidine cell proliferation assay. These separate plates were analyzed by the manual (6 samples) and the Micromate 196 sample cell harvester. The correlation of the data found an R^2 of 0.949 (Matrix Application Note). From this data it is clear that the new Micromate 196 cell harvester performs as well as the manual harvester but 16 times faster. These samples from the Micromate harvester can either be manually punched out and analyzed by liquid scintillation counter or they can be quantitated directly by the Matrix 96, direct beta counter.

The third method is the use of special filter bottom plates. These filter bottom plates offer the advantage of being able to incubate, wash, and harvest all in a single microplate. Two different types of microplates are available, those with and those without removable filters. Once the cell harvesting and washing is complete each of the 96 filters can either be punched out into liquid scintillation counting or gamma counting vials. If the membrane can be removed from the microplate, the complete membrane (96 samples) can be

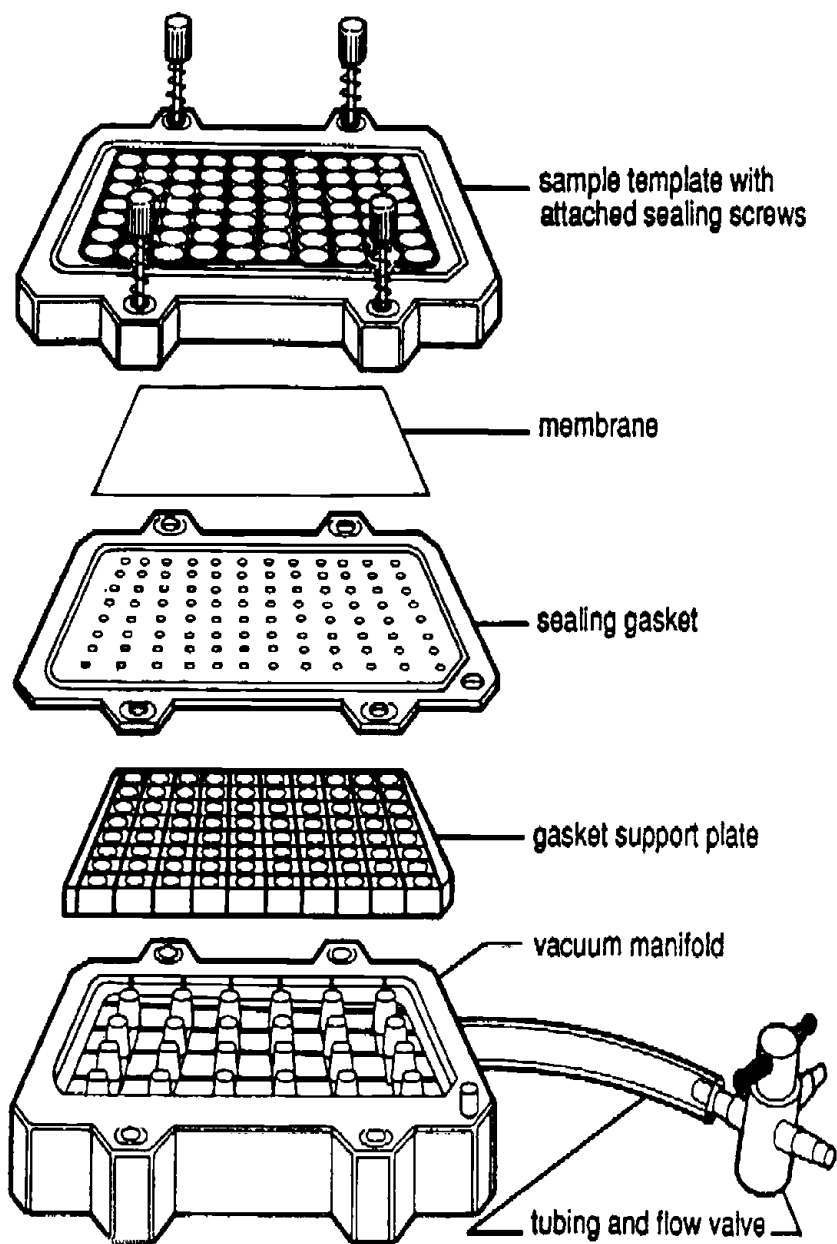


Figure 1. Typical dot blot apparatus.

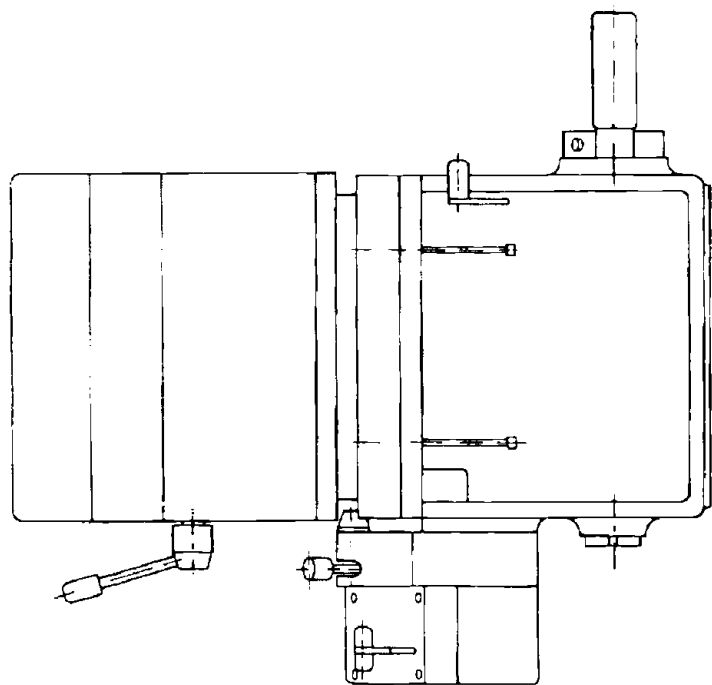
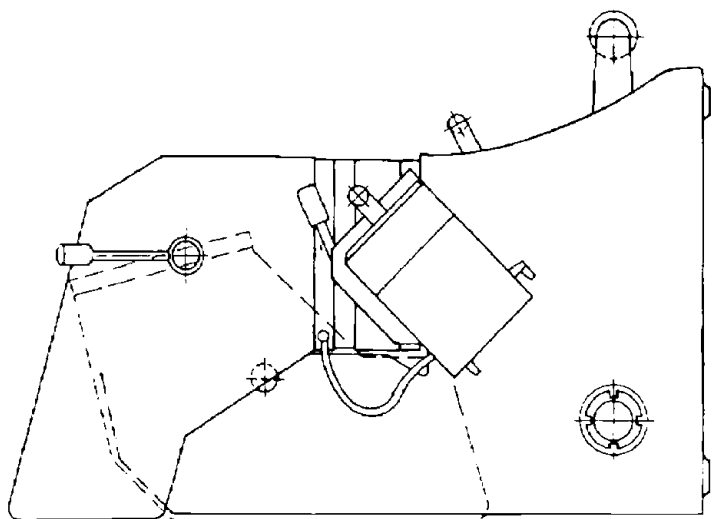


Figure 2. Packard Micromate™ 196, 96 sample microplate harvester.

analyzed on the new Packard Matrix 96 radioactivity reader. This stripping of the membrane from Microplate is illustrated in Figure 3. At present these strippable membrane bottom plates are manufactured by Pall Biosupports.

In summary, three separate methods exist for preparing samples on a solid support media in the microplate format. The first is the direct method of spotting as exemplified by the dot blot application. The second is the harvester applications which aspirate the cell/particulate from the microplate onto a filter media and remove the unincorporated/unbound radioactivity by extensive washing steps. The third is the use of membrane bottom microplates which allow the incubation, harvesting and washing of the assay components in a single microplate. All three methods can be used to prepare samples for quantitation using the Matrix 96 radioactivity reader. (Figure 4).

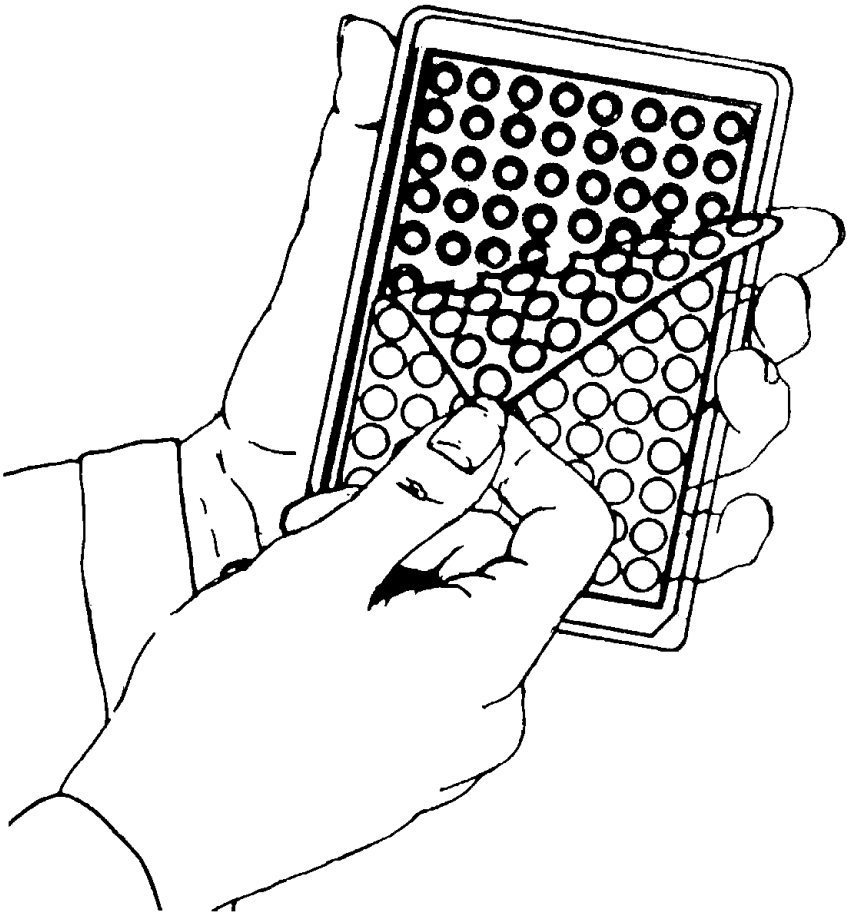


Figure 3. Strippable bottom microplates pall biosupports.



Figure 4. Packard Matrix™ 96, 96 sample direct beta counter.

QUANTITATIVE TECHNIQUES

Four separate techniques for quantitating samples on a solid support exist in the microplate format. These include standard liquid scintillation counting, multi detector liquid scintillation counting, position sensitive proportional counter scanning, and multi-detector (96) avalanche gas ionization detector quantitation. Each technique will be evaluated in detail with the number of steps, time, and cost of each presented. The first technique is liquid scintillation counting. This technique is the gold standard by which all others are measured. This technique involves over 500 steps from the sample harvesting to the actual quantitation. The first step is harvesting the samples from the microplate 6 samples at a time. This procedure is repeated 16 times in order to harvest all of the samples from the 8×12 microplate. Each of the individual samples, which are harvested and washed onto a filter, is punched out into one of the 96 individual scintillation vials which were previously labeled to prevent a sample mix up. Scintillation fluid (4–7 mL) is added to each of the 96 scintillation vials one sample at a time. Each of the 96 samples is manually capped, transferred to the samples cassettes for the counter, placed in the liquid scintillation counter, and analyzed for 1 min each. The data is removed from the counter and processed using an external computer system. The over 500 sample handling steps from harvesting to sample analysis, takes over 3 hr to complete.

The second technique is harvesting the sample on a specially prepared filter

mat using a multidetector (6) liquid scintillation counter. This technique harvests 12 samples at a time using a manual harvester and a special filter mat in the 6×16 format. Thus the harvesting time is similar to that of the liquid scintillation counter technique. Once the samples have been harvested, the entire filter mat is dried and placed into a special chemically resistant plastic bag. To this bag is added 10 to 15 mL of a hydrophobic scintillation cocktail. The bag is sealed to prevent the cocktail from escaping and rolled to insure the cocktail completely saturates the filter mat. This sealed bag is placed into a special holder, and 6 samples are analyzed at a time on the multidetector (6) liquid scintillation counter. This reduces the counting time by one sixth that of conventional liquid scintillation counting. Once the entire plate has been quantitated the data must be converted from the 6×16 format to the 8×12 . This technique reduces the total time of harvesting and analysis to one third that of liquid scintillation counting. It takes over 60 minutes for a microplate of 96 samples and involves 18–20 steps.

The third technique is position sensitive proportional counter scanning. The scanners were originally designed to detect radioactivity on a flat surface for such thing as TLC or paper chromatography. The technique uses a position sensitive wire detector to count 12 samples (in a single row) simultaneously and scans lane by lane over the 8×12 matrix (8 separate scans). This method has three major disadvantages.

First, the detectors are not highly sensitive to low energy beta emitter (i.e., ^3H). Second, the detectors are subject to high amounts of cross talk when high energy isotopes (i.e., ^{32}P) are analyzed. Third, the detectors are not uniform across the entire length of the wire. This is critical because the wire detectors must be able to locate and quantitate the radioactivity on the solid filter matrix. These factors make the technique unsuitable for quantitative applications.

The fourth technique is quantitating with a specially designed 96 detector quantitation system. It uses avalanche gas amplification detectors with collector/cathode voltage bias operating in the Geiger-Muller voltage region. This system is capable of analyzing 96 samples simultaneously with 96 individual detectors in the 8×12 format. The technique uses an open ended avalanche gas ionization detector and is capable of quantitating ^3H , ^{32}P , ^{35}S , ^{14}C , ^{125}I and many other isotopes which produce ionizing radiation. The only steps involved in using this technique is harvesting 96 samples simultaneously with the Micro-mate 196, drying the sample filter, and quantitating all 96 samples simultaneously in the Matrix 96. This entire process from harvesting to quantitation, requires less than 12 min/microplate of 96 samples, and there is no liquid radioactive waste to dispose of at the end of the experiment, only a filter membrane. This technique does not destroy the sample either, so the sample can be removed from the filter mat and analyzed further (NMR, Mass spectrometry, DNA sequencing, etc.). The filter mat can then be analyzed by the Matrix 96 and placed in a plastic bag for storage or liquid scintillation counting for quantitation at a later date.

Table 1. Cost of Analysis of 500 Microplate/Year for Mixed Lymphocyte Cultures Assays by Various Methods

	LSC ^a	MD-LSC ^b	MD-AGD ^c
1. Scintillation Vials	\$2500	0	0
2. Scintillation Cocktail	\$2500	\$250	0
3. Glass Fiber Filters	\$350	\$1000	\$350
4. Special Sample Filter Bags	\$0	\$350	\$0
5. Cost/Technician Time (sample preparation, harvesting, counting)	\$25,000	\$8500	\$1700
6. Waste Disposal Costs	\$500	\$100	\$10
TOTAL	\$30,850	\$10,250	\$2060

^aLSC = liquid scintillation counting.

^bMD-LSC = multi-detector LSC.

^cMD-AGD = multi-detector avalanche gas detector.

Now that the steps involved in each technique have been shown, what about the cost involved in analyzing a series of microplates? If 10 microplates/week were analyzed over the period of a year then approximately 500 plates would be quantitated in one year. The cost for each of these three methods is based on the time required to prepare and analyze the samples and the cost of chemicals and supplies required. The first technique of liquid scintillation counting requires scintillation vials, scintillation cocktail, filter mats, and time to prepare and dispose of the 96 samples obtained from each plate. If 500 plates were analyzed by this method it would require over \$30,000/year as shown in Table 1. For the second technique of multidetector liquid scintillation counting, the scintillation vials have been eliminated, but more expensive special filter mats are required. The cost of the filter bags, cocktail, special filter mats, and labor to prepare and dispose of these samples is over \$10,000 for the same 500 plates (Table 1). For the third technique which uses the Micromate 196 cell harvester, to harvest all 96 samples simultaneously, and the Matrix 96 reader, to quantitate all 96 samples simultaneously, the only cost is the filter mat (the same type used on the manual harvester) and the labor costs. The cost of labor and materials is approximately \$2000 for the 500 microplates or 15 times less than the standard liquid scintillation counting technique.

APPLICATIONS

Several applications exist involving radioactivity quantitation on a solid support in a microplate format. These include dot blots, ³H-thymidine cell proliferation assays, receptor binding assays, radioimmunoassays, DNA polymerase spot assays, broken cell assays (fungi), and many others. Three specific assay types will be evaluated in detail with reference to the three quantitation techniques described earlier.

The first application is dot blots. This technique specifically identifies a sequence of DNA or RNA of interest in a specific disease or DNA/RNA fragment. The technique involves the following basic steps. The DNA or RNA

of interest is bound to a special membrane in a dot blot device (Figure 1). A special radiolabeled DNA/RNA probe, complementary to the region of DNA/RNA of interest is prepared. The radiolabeled probe is added to various DNAs to locate the sequence of interest. The noncomplementary DNA is removed from the membrane by washing, and the radioactivity on the membrane is quantitated. This quantitation is normally accomplished by using X-ray film exposure, because the radionuclide is high energy ^{35}S or ^{32}P . The radioactivity on the film is determined by densitometry. The alternative method is the use of the Matrix 96 direct beta counter for these samples. A comparison of the quantitation of the densitometry and the Matrix 96 is shown in Figure 5. As can be clearly seen, the densitometry has a small dynamic range because the X-ray film becomes saturated (50 to 100 fold range). On the other hand the Matrix 96 has a dynamic range of over 10^5 for the ^{32}P dot blots. In addition to

MATRIX 96 VS DENSITOMETRY 32P DOT BLOT

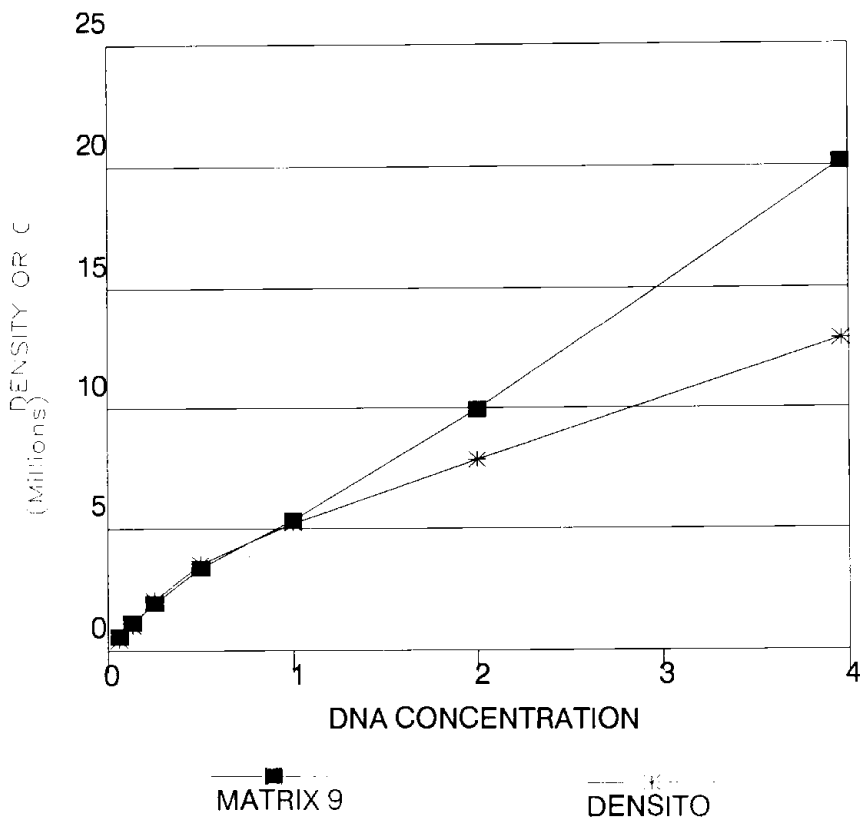


Figure 5. Comparison of Matrix 96 and densitometry for blot applications.

the larger dynamic range the Matrix 96 is able to analyze the dot blots in 1/50 to 1/100th the time of the X-ray film method. A correlation between the Matrix 96 and liquid scintillation counting on the same dot blot samples was determined to be an R^2 of 0.949 (Matrix Application Note).

The second application is ^3H -Thymidine cell proliferation assays. These assays use the tritiated thymidine which becomes specifically incorporated into DNA as a measure of cell growth or proliferation in culture. This proliferation assay is used to test toxic substances, potential cancer drugs, AIDS drugs, and other important naturally occurring and synthetic substances. The conventional method of analysis is cell harvesting with a manual harvester and analysis by liquid scintillation counting. A series of samples with various radioactivity incorporated into the cellular DNA are analyzed using both the Matrix 96 reader and the conventional liquid scintillation counting techniques. The data from this experiment is shown in Figure 6. The correlation of the data was performed and an R^2 of 0.999 was calculated (Matrix Application Note). This clearly demonstrates that this technique provides results which are as accurate as the liquid scintillation counting technique in one tenth the time and at one tenth the cost.

The third application is the radiolabeled receptor binding assays. This technique involves using either a specific type of cell culture or a tissue homogenate preparation of a specific animal tissue which contains the receptors of interest. The substrate for the receptor is radiolabeled and a competitive binding assay is performed with unlabeled substrate. The number of receptors and the binding constant can be determined using this technique. The application can be analyzed by one of the three quantitative methods described earlier. If the multidetector liquid scintillation counter is compared to the Matrix 96 radioactivity reader, the results of the two techniques can be correlated. The R^2 for these two techniques was calculated to be 0.993 (Figure 7). A similar correlation for liquid scintillation counting and the Matrix 96 was also obtained. In addition to performing receptor binding assays with ^3H , the radionuclide ^{125}I can be performed and quantitated on the Matrix 96 (Matrix Application Note).

Several other applications (Matrix Application Notes) can be performed which use quantitation on a solid support in the microplate format. These include radioimmunoassays with either ^3H or ^{125}I . Special DNA polymerase reactions which using spotting in a microplate format can be used. Initial experiments indicate that chromium release studies can be performed with the Matrix 96 detectors.

SUMMARY

This article presents three different methods for preparing samples on a solid support in the microplate format. These include dot blotting or direct spotting, cell harvesting in a manual or Micromate 196 96 sample microplate

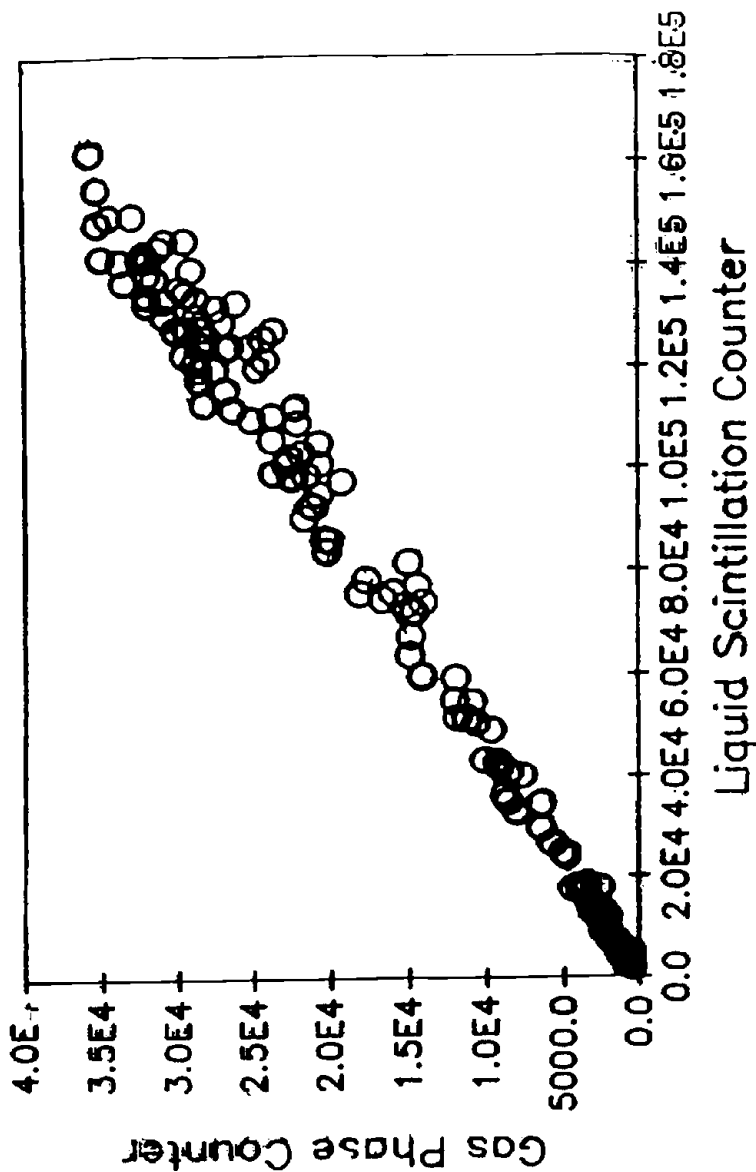


Figure 6. Comparison of MLC samples analyzed on Matrix 96 and liquid scintillation counter.

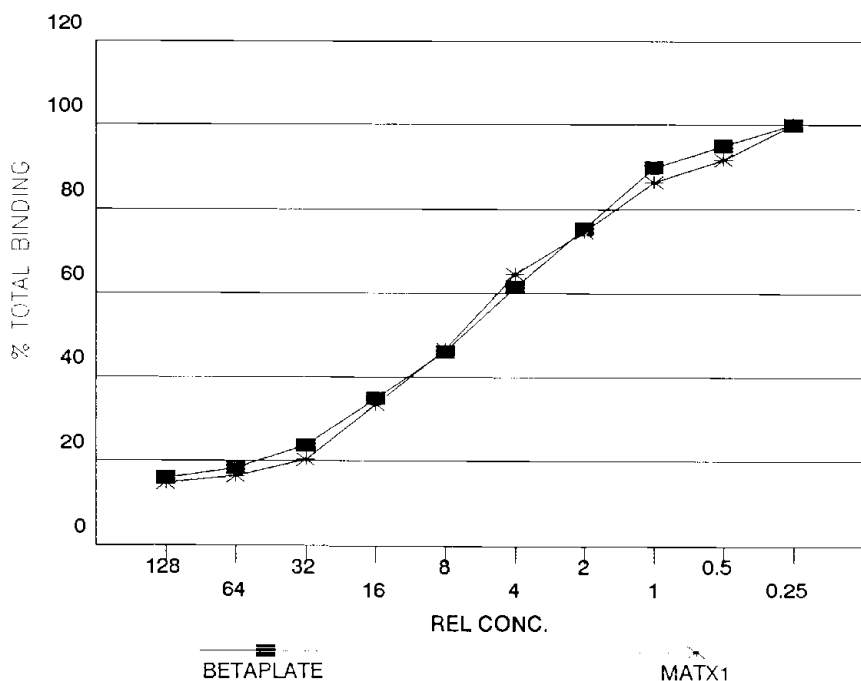


Figure 7. Receptor binding application comparison of Matrix 96 and betaplate.

harvester, and the use of a membrane bottom strippable microplate which allows incubation, washing, and harvesting in a single microplate. The strippable bottom allows the membrane to be analyzed directly on the Matrix 96 radioactivity reader.

Four separate methods of quantitating these harvested samples are presented and compared. The liquid scintillation counting technique requires about 3 hours of sample preparation/microplate and cost about \$60/plate. The second technique is multidetector liquid scintillation counting which requires about 60 min/microplate and cost about \$20/plate. The third technique is scanning with position sensitive proportional counter. This method improves the throughput but suffers from the disadvantages of cross talk, low ^3H -efficiency, and wire detector nonuniformity. The fourth technique, and the most efficient, is the use of the Micromate 196 96 sample cell harvester and the Matrix 96 96 detector direct beta counter. This technique requires about 12 min/microplate and cost \$4/microplate. In summary, this new technique provides a method of rapidly analyzing 96 samples simultaneously from microplates into a solid support, and quantitating the radioactivity directly, without any liquid scintillation counting waste. The Matrix technique uses *No Vials, No Cocktail, No Bag, No Sample Destruction, No Special Filters, and No Waste* (liquid) to analyze the 96 samples on the 8×12 Microplate.