

Radioimmunoprecipitation Assay for Antibodies to Glutamic Acid Decarboxylase and Other Autoantigens in Microplates

Dr. Shahnaz Fida and Dr. Merrill J. Rowley, Department of Biochemistry and Molecular Biology, Monash University, Clayton Victoria, 3168 Australia

Abstract

The radioimmunoprecipitation (RIP) assay has been a sensitive tool to detect antibody interactions in diabetes mellitus.¹ RIP assays have been frequently performed in individual plastic tubes. However, assays done in this manner are time consuming, since each tube must be handled individually.

A new "semi-automated" RIP assay which uses a convenient 96-well microplate format, allows the reduction of the amount of Protein A Sepharose™ (Amersham Pharmacia Biotech, Buckinghamshire, England) without loss of sensitivity, and is suitable for screening large numbers of sera, such as for population studies. Samples are washed and transferred to 96-well Packard UniFilter® GF/C® plates using the Packard FilterMate™ Harvester, and counted on the Packard TopCount® microplate scintillation and luminescence counter, which allows the simultaneous counting of up to 12 wells of a standard 96-well microplate. Results are compared for the radioimmunoprecipitation assay using the traditional plastic tubes and the microplate format.

Introduction

Radioimmunoprecipitation assays involve the binding of antibodies in the serum to radiolabeled antigen and separation of the antibody/antigen complex by precipitation, often with Protein A Sepharose. Radioimmunoprecipitation assays are used to detect autoantibodies in the serum of patients with diabetes.^{2,3} Autoantibodies to glutamic acid decarboxylase (GAD) have been measured using

³⁵S-labeled GAD prepared in rabbit reticulocyte lysate (RRL).⁴ Previously the RIP assays have been performed in plastic tubes with quantification using liquid scintillation by a beta counter in individual tubes. As each tube must be treated individually, this method is time consuming and tedious. A more convenient and efficient procedure is possible using 96-well microplates that contain 1 to 2 mL per well, for the initial reaction. Washing is performed in the same plates using a benchtop centrifuge with a Heraeus Labofuge 400 microplate plate rotor (Heraeus infosystems GmbH, Hanau, Germany), and a FilterMate Harvester. The autoantibody complex-bound Protein A Sepharose is then transferred onto 96-well UniFilter GF/C plates again using the cell harvester. This adaptation has several advantages including decreased sample handling, smaller reagent volumes and efficient counting on the TopCount.^{5,6} Reported herein are the results from a comparison with the traditional method using plastic tubes, and the effect of the use of reduced amount of Protein A Sepharose.

Methods

Radioimmunoprecipitation Assay for GAD and Other Autoantibodies in Microplates

Ninety-six-well microplates with a capacity of 2 mL were used in this assay. Five µL of serum was aliquoted into duplicate wells and incubated with 40,000 DPM of ³⁵S-labeled GAD in 25 µL of 20 mM Tris, 150 mM NaCl, 0.5% Triton-100, pH 7.4 (Tris wash buffer) for 16 hours at 4 °C.

Twenty-five μL of a 50% suspension of Protein A Sepharose in Tris wash buffer was added and incubated at 4 °C for one hour. The Protein A Sepharose pellet was washed three times, each time centrifuging the microplate briefly in a Heraeus microplate centrifuge after addition of 1 mL of Tris wash buffer. Centrifugation of the pellet was used at this stage of the assay instead of harvesting, because the protein stuck to the filters, and blocking and extensive washing did not reduce the sticking. The supernatant was removed by aspiration using the Packard FilterMate Harvester. The pellet was resuspended in Tris wash buffer and the Protein A Sepharose was transferred onto the glass fiber UniFilter GF/C plates using the FilterMate Harvester. Twenty μL of MicroScint™ 40 (Packard Instrument Company) was added to each well since it was available in the laboratory. MicroScint 20 may be used for wet filters, and MicroScint O may be used for dried UniFilters. The plates were sealed with TopSeal™-A (Packard Instrument Company), and read on the TopCount for one minute per well.

Radioimmunoprecipitation Assay in Plastic Tubes

The assay was performed as described above, in 1.5 mL plastic tubes, with the modification that the Protein A Sepharose pellet was washed in individual tubes four times with Tris wash buffer, the tubes were centrifuged at 1000 g for five minutes and the supernatant was removed by aspiration from individual tubes. One mL of scintillant was added to each tube and the samples were counted on a liquid scintillation counter. The results were compared for the two methods by linear regression analysis using Microsoft® Excel® Version 5.0.

Effect of Reduced Protein A Sepharose Volumes

Since we have previously used 50 μL of Protein A Sepharose for all immunoprecipitations in our laboratory^{1,2,3}, a separate experiment was performed in plastic tubes to study the effect of volume of the Protein A Sepharose. Ten human sera were selected with low or high reactivity with GAD 65 and a

radioimmunoprecipitation assay was performed in 1.5 mL plastic tubes as previously described. Additions of 20 μL , 30 μL , 40 μL or 50 μL of Protein A Sepharose were made to each tube and incubated as before. The pellets were washed four times with Tris wash buffer, 1 mL each, and the radioactivity in the pellets was counted as previously described. The CPM precipitated with the various Protein A Sepharose volumes were compared. Samples were tested in duplicates and the experiment was repeated three times.

Results and Discussion

Antibodies to GAD 65, in the serum of 20 normal human controls and 140 patients with insulin dependent diabetes, have been tested using the microplate assay, and the results were compared with those obtained using immunoprecipitation in individual tubes. The experiments clearly showed that the results obtained on the TopCount were similar to those obtained on the traditional liquid scintillation counter. In every case, the two methods gave very similar results, with a correlation coefficient for linear regression of 0.91 (Figure 1).

When the effect of using different amounts of Protein A Sepharose was assessed, 20 μL of Protein A Sepharose was as effective as higher amounts of Protein A Sepharose when using 5 μL serum (Figure 2).

The assay performed in the microplates was very reproducible. Replicates of control sera with low, medium or high levels of reactivity included in each assay showed an inter-assay coefficient of variation of 12%, 12% and 6% (n=11) respectively.

Conventional β -counting required 1 mL of scintillant per tube, whereas using the UniFilter plates, 20 μL of scintillant was used, a 50-fold reduction in the volume.

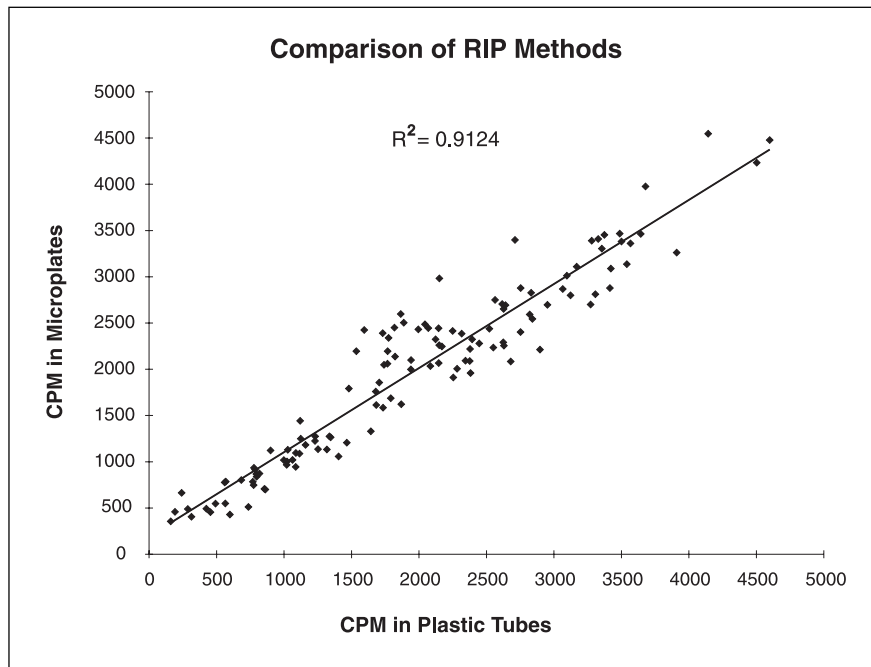


Figure 1.

Comparison of reactivity of 20 normal sera and 140 insulin dependent diabetes mellitus (IDDM) sera after immunoprecipitation with ^{35}S GAD 65 in plastic tubes and microplates. The linear regression line is indicated.

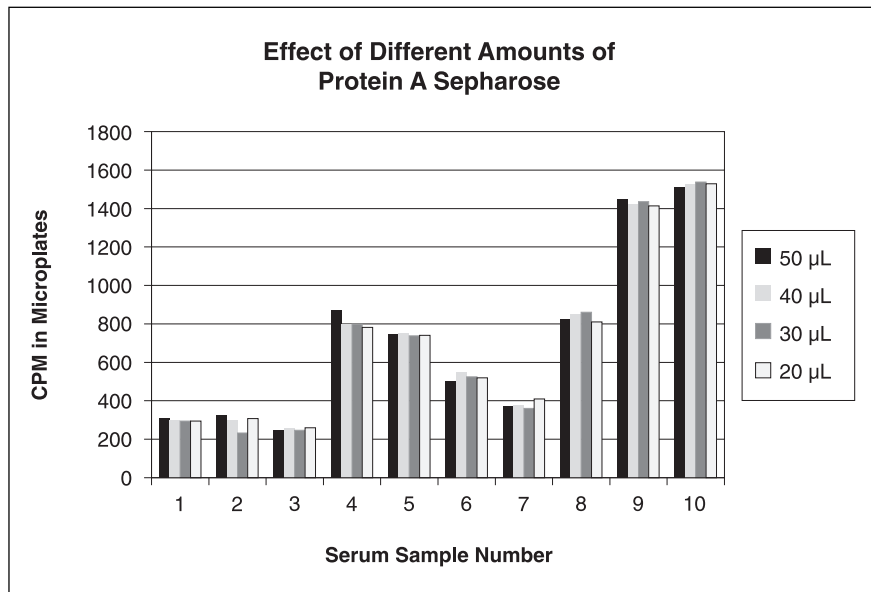


Figure 2.

Comparison of the effect of reducing amount of Protein A Sepharose 50 µL, 40 µL, 30 µL and 20 µL and on the precipitation of immune complexes from 10 sera after addition of ^{35}S GAD 65.

Conclusion

The use of microplates for radioimmuno-precipitation assays, and automation using the TopCount microplate scintillation counter and FilterMate Harvester can reduce manual labor and costs by decreasing sample handling, volumes of reagents used, radioactive waste disposal costs and increasing throughput. Thus, performing radioimmunoprecipitation assays for GAD and other autoantibodies in microplates is the preferred alternative to the more conventional radioimmuno-precipitation assay.

References

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