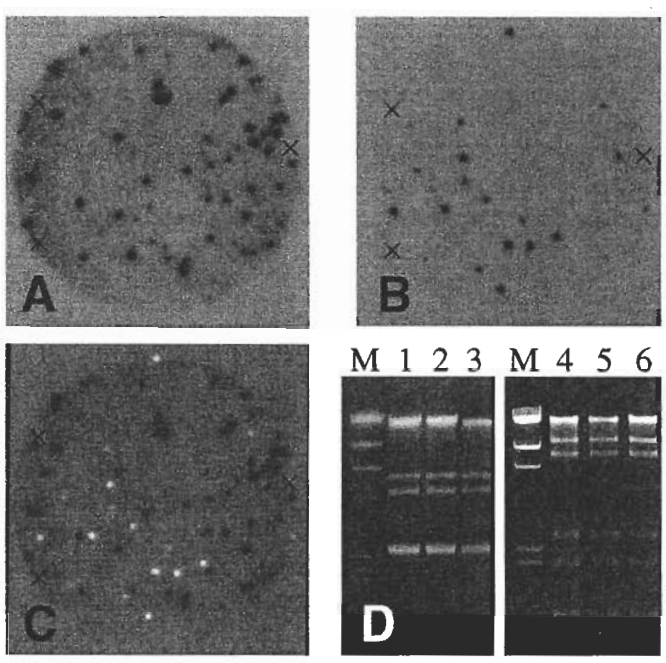

FIG. 1. Analysis of the stability of the DNA from the transformed phages. A DNA mixture from two different phages was electroporated in *E. coli* cells and, after plating and overnight incubation, phage plaques were immobilized onto nitrocellulose filter (original and replica) as described in the text. Filters were hybridized with either probe pBS7e, plasmid containing a 0.7-kb *Not*I repetition unit of the *T. cruzi* histone H2A gene (7) (A) or probe pBg2, plasmid containing a 2.5-kb *Bgl*II repetition unit of the *T. cruzi* Hsp70 gene (6) (B). (C) Superposition of the autoradiographs shown in A and the negative autoradiograph shown in B. (D) Gel electrophoresis of phage *Bam*HI-digested DNAs from phage clones G-4.1 (lanes 1–3) and Tc-70.6 (lanes 4–6). Lanes 1 and 4 show the digestion pattern of the DNAs used in the phage reconstitution experiments. Lanes 2 and 5 show the digestion pattern of the DNAs isolated from phage reconstitution by electroporation. Lanes 3 and 6 show the digestion pattern of the DNAs isolated from phage reconstituted *in vitro* using commercial packaging extracts. Lane M contains *Hin*dIII-digested λ DNA molecular weight marker.

Trypanosoma cruzi H2A and Hsp70 genes, respectively. After plating and overnight incubation of the plates, phage plaques were immobilized on nitrocellulose filters (original and replica) and hybridized using the standard techniques (5). The probes were labeled by the random primer method using [α-^{32}P]dCTP (6). Figure 1 (A and B) shows the results of hybridization of the immobilized phage DNA with probes pBS7e and pBg2, respectively. Figure 1C shows the superposition of both autoradiograms, indicating the absence of recombination between the DNAs used for transformation. In order to determine whether there was any rearrangement in the electroporated DNA, the DNA from two lysis plaques, chosen at random, were purified and digested with *Bam*HI and their restriction patterns compared with those of the DNA obtained from phage controls. The restriction patterns obtained for each one of these clones were identical (Fig. 1D). In conclusion, these data indicate that there had been no rearrangement or recombination between the two DNAs, thus excluding a significant frequency of these events associated with this methodology. Therefore, the described method represent a quick and cheap way to rescue phage clones stored as naked DNAs, an otherwise serious problem in molecular biology laboratories associated to the storage of particular clones. Additionally, this technical approach could be used for the construction of genomic and expression libraries in phage vectors.

Acknowledgements. L. P. and C. M. were supported by a CICYT and PAI Predoctoral Fellowship, respectively. This work was supported by BIO96-0468 Grant from Plan Nacional I + D (CICYT), Spain.

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Adaptation of the Protein Kinase Filter Paper Assay to a 96-Well Microtiter Format

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Received June 29, 1998

The most widely used method for assaying protein kinase activities involves incorporation of radioactive phosphate into a protein or peptide substrate with subsequent binding or precipitation of the radiolabeled substrate onto filter paper squares. We have adapted this assay for use with readily available 96-well microtiter plate technologies. The sensitivity and reproducibility of the modified 96-well protein kinase assay are comparable to standard

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Analytical Biochemistry **267,** 235–238 (1999)
Article ID abio.1998.2985
0003-2697/99 $30.00

filter paper assay methods, but assay throughput is much greater and reagent costs are significantly reduced. This technique has been used to assay several different protein kinase activities using both P81 phosphocellulose and 3MM filter papers. The general methods described here could be readily adapted for use with other kinds of filter materials (e.g., nitrocellulose, polyvinylidene fluoride, and nylon) or used with automated assay systems.

Protein kinases, the enzymes that catalyze protein phosphorylation, form the largest known superfamily of enzymes, with approximately 1% of the mammalian genome estimated to be protein kinase genes (1). Because of their involvement in a variety of physiological and pathophysiological processes, there is significant interest in being able to rapidly assay protein kinase activities *in vitro* to determine kinetic properties, substrate specificities, potential endogenous modulators and cofactors, and potential inhibitors that might be useful lead compounds for drug development. A variety of radioactive and nonradioactive protein kinase assays have been developed over the years. One of the most widely used assays involves precipitating ^{32}P-labeled peptides and proteins on filter paper squares (2–5). As originally described and as currently used in many laboratories, the technique involves spotting a small aliquot of a protein kinase reaction mixture on a 2 × 2-cm filter paper square and extensively washing the filter paper square to remove nonpeptide bound radioactivity (3, 4). The filter paper square is then placed in a scintillation vial and counted to determine the amount of radioactivity incorporated into the peptide or protein substrate. Although accurate and relatively precise, the technique is labor-intensive, relatively slow, and uses significant quantities of filter paper, scintillation cocktail, and scintillation vials. The labor- and reagent-intensive nature of this assay format limit its use in high-throughput screening applications.

With the development and widespread use of 96-well microtiter assay technologies, several protein kinase assays have been developed based on this format that significantly increase the number of protein kinase reactions that can been completed in a day (6–13). Some of these assays are rather labor-intensive and involve processing and counting many pieces of filter paper. Others require the use of proprietary or unusual materials (filters and/or substrates) that are significantly more expensive than the materials used in the standard filter paper assay. The 96-well protein kinase assay system described here is an economical and labor-saving adaptation of the standard filter paper assay that uses equipment and reagents already available in most biochemistry and molecular biology laboratories. The procedure can be easily modified to use most any filter material and could be readily automated.

The equipment required for this assay includes a dot blot apparatus such as the Miniblot I microsample filtration manifold (Schleicher & Schuell), an adjustable (10–50 μL) eight-channel pipettor (Rainin Instrument or Brinkmann), a 96-well format scintillation counter such as the TopCount (Packard Instrument Co.), and filter paper carriers suitable for use in a 96-well scintillation counter such as the FlexiFilter tray and carrier system (Packard Instrument Co.). Phosphorylation reactions (50 μL total volume) are performed in 0.2-mL conical bottom polypropylene thin-wall PCR tubes (Out Patient Services). The tubes are used in an eight-strip format to facilitate rapid transfer of tubes between ice bath and heated water bath. These tubes are inexpensive and easily handled. Because the tubes are relatively short (2 cm long) and have conical bottoms, sampling of reaction mixtures using standard multichannel pipettors is easily accomplished.

Development of the methods described here was done using a protein kinase reaction for myosin light-chain kinase similar to that described by Dasgupta *et al.* (14). Reaction mixtures contained bovine calmodulin (a range of concentrations were used), 50 μM synthetic peptide substrate (KKRPQRATSNVFS-amide), 50 mM 4-morpholinepropanesulfonic acid, pH 7.0, 1 mM dithiothreitol, 10 mM magnesium acetate, 0.2 mM calcium chloride, 16 nM rabbit skeletal muscle myosin light chain kinase, and 0.1 mg/mL bovine serum albumin. Reaction mixtures were prepared fresh and kept on ice for up to 2 h before use. Five minutes before starting the reactions, tubes containing reaction mixtures were transferred to a 30°C water bath. Reactions were started by adding 10 μL of [γ-^{32}P]ATP (1 mM final concentration; 100–500 cpm/pmol) using an eight-channel multipipettor. An 8-tube strip of reactions was started every 30 s. To obtain two time points from each reaction, a maximum of 48 tubes (six 8-tube strips) was used per reaction set. The left-hand 48 wells of the filter paper could then be used for the first set of time points, and the right-hand 48 wells for the second set. Samples of each reaction (20 μL) typically were removed at 5 and 15 min with an eight-channel pipettor and spotted on an 8 × 12-cm sheet of P81 phosphocellulose paper (Whatman) mounted in a 96-well Miniblot I microsample filtration manifold. To facilitate subsequent handling, the sheet of P81 paper was precut to fit the FlexiFilter carrier assembly and the four corner wells were marked with pencil to assure proper alignment with the wells of the filtration manifold. Immediately after spotting samples of reactions on the P81 paper, 500 μL of 75 mM phosphoric acid was added to each well of the filtration apparatus and allowed to soak through the P81 paper without vacuum. The filter

could also be prewet with phosphoric acid, but this did not seem to have any significant effect on assay results. For washing, the sheet of P81 paper was removed from the filtration apparatus and placed in a 600-mL beaker containing 400 mL of 75 mM phosphoric acid. A stainless steel wire mesh basket was used to prevent the stir bar from contacting the sheet during the phosphoric acid washes. This method of washing the filter sheet was found to be more effective than washing the filter in place on the dot blot apparatus and also reduced the volume of radioactive waste in the reservoir of the dot blot apparatus. The sheet was washed with 75 mM phosphoric acid a total of four or five times (10–20 min each time) with gentle stirring. The P81 sheet was then soaked briefly in 95% EtOH and dried under a stream of warm air. The sheet was placed in a FlexiFilter tray and carrier assembly before adding 10 μL of MicroScint-O (Packard Instrument Co.) scintillation cocktail to each well. The top of the FlexiFilter tray was covered with a clear adhesive plastic film then counted in a TopCount scintillation counter configured for counting 96-well microtiter plates.

Using the 96-well protein kinase assay described here it is possible to greatly increase sample throughput with minimal modification of the standard filter paper assay method. This is due to the significant amount of time spent handling and processing the many small pieces of filter paper in the standard filter paper assay. Using the standard assay with two time points per reaction, it is difficult for one person to finish many more than about 100 reactions (two time points per reaction) in a day. Moreover, handling many small filter papers is tedious and the chance of human error is high. Using the 96-well filter paper protein kinase assay described here, one person can routinely complete 300 or more reactions in a single day. Data handling can also be expedited since data from the TopCount scintillation counter can be transferred to a floppy disk for import directly into a spreadsheet program for data analysis.

The costs of scintillation counting reagents and supplies are significantly lower with the 96-well format compared with the standard filter paper assay. Based on a 48-reaction assay (two time points per reaction), the cost of the standard assay is approximately \$12.50, whereas with the 96-well assay using the FlexiFilter system, the cost of filter paper, carrier, and scintillation cocktail is approximately \$7.40, with the major cost being that of the FlexiFilter tray and carrier assembly (approximately \$6.75 per assembly). This expense can be substantially reduced if the FlexiFilter assembly is washed and reused (it is possible to reuse the FlexiFilter apparatus many times if it is not exposed to high temperatures). In addition to the cost-savings associated with scintillation counting materials, there are also potentially significant savings associated with low-level radioactive storage and disposal costs since the volume of waste generated using the 96-well format is a fraction of that generated using the standard filter paper protein kinase assay. An alternative to the FlexiFilter system is the Multiscreen (Millipore Corporation) phosphocellulose filter system which consists of an integral filter-carrier assembly. The Multiscreen filter could provide comparable labor-saving advantages to the FlexiFilter system, but at a significantly higher materials cost (\$12.80 per filter assembly).

An important question regarding the 96-well assay is whether it yields accuracy, sensitivity, and reproducibility comparable to the standard protein kinase assay. Variability among replicate samples within a given assay was found to be comparable for the 96-well assay and the standard assay (approximately 5–10% (data not shown)). To determine whether the 96-well system provides counting efficiency and reproducibility similar to that of the standard filter paper assay, myosin light chain kinase reactions were run using the 96-well assay and samples spotted on sheets of P81 paper as described above. The P81 paper sheets were processed and counted in a TopCount 96-well scintillation counter, after which each filter was removed from the FlexiFilter carrier assembly and cut into its 96 component squares and the squares were counted individually in plastic minivials containing 0.5 mL OptiFluor (Packard) using a Packard 2000A Tri-Carb liquid scintillation counter. Figure 1 shows a comparison of the counts obtained with the TopCount instrument compared to the corresponding counts obtained by conventional liquid scintillation counting. Overall there is excellent agreement between the two counting methods. Linear regression analysis indicates excellent linearity over a wide range of counts (from less than 100 cpm to at least 30,000 cpm), a y-intercept of 105 counts (indicating a very low level of background counts in the TopCount system), and a slope of 0.87 (indicating the counting efficiency of the TopCount instrument is approximately 87% that of the liquid scintillation counter). The observed counting efficiency is in excellent agreement with product specifications provided by Packard using MicroScint-O and counting ^{32}P. It should be noted that the counting efficiency obtained with the TopCount instrument was dependent on the choice of scintillation cocktail and whether a black or a white FlexiFilter was used. Packard recommends the use of MicroScint-O for counting ^{32}P with the TopCount for optimal counting efficiency. Early experiments in our laboratory indicated significantly lower efficiency using OptiFluor compared to MicroScint-O in the TopCount, but the relative efficiency was not determined. The FlexiFilter tray and carrier assembly can

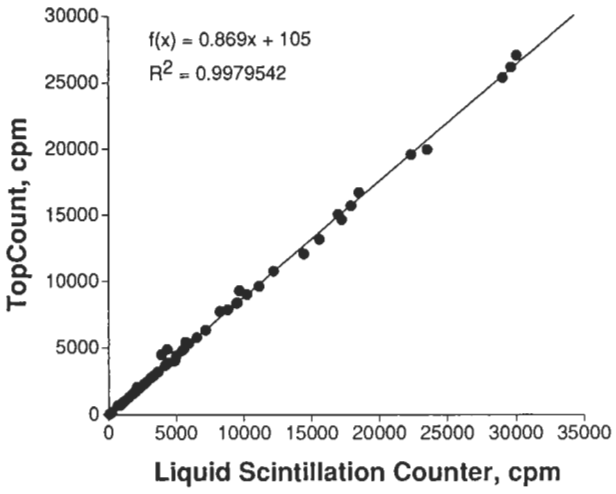

FIG. 1. Comparison of counts obtained using the Packard TopCount and Tri-Carb 2000A liquid scintillation counters. Sheets of P81 paper from two protein kinase assays performed according to the method described herein for the 96-well protein kinase assay were first counted in a TopCount scintillation counter using a white FlexiFilter carrier and tray assembly and OmniScint-O scintillation cocktail. Each filter was then cut into 96 individual squares, placed in plastic minivials with 0.5 mL OptiFluor, and counted in a Packard Tri-Carb 2000A liquid scintillation counter. Counting times were 1 min per sample with both counters. Counts were corrected for decay, as necessary. Linear regression analysis was performed to compare the linearity, efficiency, and reproducibility between the two methods of counting.

be purchased in either black or white. We found the efficiency of the white FlexiFilter (87%) to be significantly better than the black (60%), even though the black FlexiFilter system had somewhat lower background counts. Another potential artifact of the TopCount system when using high-energy emitters such as ^{32}P is spillover or crosstalk between adjacent wells on the filter. Spillover is typically only about 0.3%, but even this low percentage of counts could be a significant problem when a high-count well is situated next to a low-count well. However, spillover can be easily avoided by locating potential high-count wells away from background or low-count wells.

The FlexiFilter 96-well assay method is now routinely used in this laboratory to assay a variety of protein kinase activities. It has successfully been used to assay protein kinase activities that use peptide substrates and P81 phosphocellulose filter paper including cAMP-dependent protein kinase, protein kinase C, phosphorylase kinase, and calmodulin-dependent protein kinase II. The method has also been used to assay several protein kinase activities that use protein substrates and 3MM chromatography paper (Whatman) using trichloroacetic acid precipitation as originally described by Corbin and Reimann (4). A variety of other filter materials can be used in protein kinase assays including nitrocellulose, polyvinylidene fluoride, ferric adsorbent paper, Nytran, and glass fiber filters (6, 13, 15–17). It should be a straight-forward matter to adapt the 96-well assay described here for use with any of these filter materials or to modify the assay for use with many of the 96-well robotics systems now available. It is estimated that upward of sixty 96-well plates could be processed in a 20-h period using modular robotics systems such as the one currently available at the University of Utah Functional Genomics Core Facility (Dr. James Metherall, personal communication). This is approximately 30 times more protein kinase assays per day than is possible for one person to perform using the standard filter paper method.

Acknowledgments. The authors acknowledge Fouad Faour, Joseph Yeh, and Dr. Charles Galea, who have used the 96-well filter assay for assaying other protein kinase activities in this laboratory and who have offered many helpful suggestions to improve the assay and the manuscript. We are also grateful to the Packard Instrument Co. for providing prototype versions of the FlexiFilter carrier and tray assembly during early stages of this work. This work was supported by a grant from the University of Utah Research Committee.

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