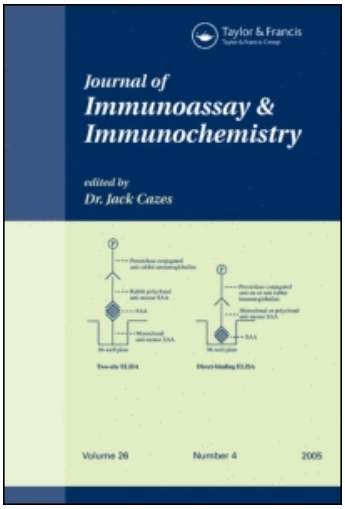

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

ADAPTATION OF COMMERCIAL IMMUNOASSAYS TO ANALYSIS OF COMPLEX BIOLOGICAL SUBSTANCES

Michael J. Lacy[a]; Rong Wang[a]; Valerie Brookshire[a]; Kevin Floyd[a]
[a] Corixa Corporation, Hamilton, MT, U.S.A.

Online publication date: 04 March 2002

To cite this Article Lacy, Michael J. , Wang, Rong , Brookshire, Valerie and Floyd, Kevin(2002) 'ADAPTATION OF COMMERCIAL IMMUNOASSAYS TO ANALYSIS OF COMPLEX BIOLOGICAL SUBSTANCES', Journal of Immunoassay and Immunochemistry, 23: 1, 1 — 32
To link to this Article: DOI: 10.1081/IAS-120002272
URL: http://dx.doi.org/10.1081/IAS-120002272

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 23(1), 1–32 (2002)

ADAPTATION OF COMMERCIAL IMMUNOASSAYS TO ANALYSIS OF COMPLEX BIOLOGICAL SUBSTANCES

Michael J. Lacy,* Rong Wang, Valerie Brookshire, and Kevin Floyd

Corixa Corporation, 553 Old Corvallis Road, Hamilton, MT 59840, USA

ABSTRACT

An Intracellular Adhesion Molecule I (ICAM-1) immunoassay from R and D Systems, and a Melanoma Inhibitory Activity (MIA) immunoassay from Roche Diagnostics were tested for accurate quantitation within complex biological substances such as cell lysates. Prior to assay, lysates of melanoma cells were treated with detergents to obtain soluble antigens. Maximum ICAM-1 and maximum MIA were detected after treatment using 0.8% Triton X-100. Two key aspects of assay accuracy were: 1) determining the dilutions of test sample that provided accurate quantitation (sample range), and 2) performing spiking experiments at these dilutions to determine absence or presence of a "matrix" effect due to biological complexity of the sample. A high degree of accuracy was found by diluting this particular cellular extract 50-fold prior to ICAM-1 assay, or only 5-fold prior to MIA

*Corresponding author.

assay. In addition, the bicinchoninic acid protein assay was analyzed to test the accuracy of protein quantitation of cellular lysates. Precision, limits of detection, and quantitation, robustness, linearity, and specificity also were tested for the immunoassays.

INTRODUCTION

While testing biological substances, immunoassay signals can be lessened due to interference, or immunoassay signals can be increased by amplification of the signal due to aggregation. This is particularly true for complex biological substances such as cellular extracts. Accuracy of the assay can be tested simply by diluting the cellular extract. In turn, when using standard curve analysis, the range of the standard curve must be adjusted to reflect the range of signals from diluted test samples, while maintaining test sample optical density of an optimal intensity that is near the midpoint of the standard curve.

The availability of commercial immunoassay kits is increasing. Two immunoassay kits of application to melanoma studies were purchased. These immunoassays were for quantitation of ICAM-1 and of MIA. ICAM-1 is an 85–110 kDa surface molecule of the adhesion molecule family. Also named CD54, ICAM-1 is bound to the cell membrane and extends into the extracellular environment. ICAM-1 has been identified in melanoma lesions (1–4) and in serum.(5) ICAM-1 expression can be increased by the cell in response to stimulants such as TNF-α, IL-1β, IFN-γ (6–8), but not IFN-α or IFN-β.(6) Because of the capacity of ICAM-1 for altered expression, a relatively constant level of ICAM-1 content would suggest a duplicable process for growth of cells for preparation of a biological product.

Melanoma inhibitory activity (MIA) was described (9) as an autocrine growth-regulatory factor expressed by several malignant melanoma cell lines. An 11 kDa molecule, MIA is secreted into medium during culture and can be collected in supernatant. MIA protein was detected in neoplastic melanocytes and breast cancers (10), in serum of rheumatoid arthritis patients (11), in serum of some gastrointestinal carcinoma patients (12), but not other skin tumors, including basal cell cancers, squamous cell cancers, normal melanocytes, and keratinocytes.(10) MIA mRNA could be induced by treating fibroblasts, keratinocytes or Hela cells with phorbol myristate acetate.(13) An inverse correlation of MIA with pigmentation was reported.(14) Because MIA is secreted, cells obtained from various cultures may contain variable amounts of MIA.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Bicinchoninic acid (BCA) protein assay (15) is based upon development of a colored complex due to reaction with protein components. Substances that interfere with the BCA color signal in the presence of bovine albumin have been reported.(16)

An ICAM-1 immunoassay was adapted for use with the complex biological substance Melanoma Cell Lysate (MCL). An MIA immunoassay was tested also for potential use in a similar manner. BCA protein assay was tested for accuracy when used to determine total protein of this complex cellular lysate. In order to coordinate these assays with a single extraction procedure, detergents were tested for interference in immunoassays, as well as BCA protein assay. The assays were tested for accuracies and inaccuracies using validation procedure guidelines.

EXPERIMENTAL

Assay Kits and Reagents

ICAM-1 commercial immunoassay kits were purchased from R and D Systems (Minneapolis, Minnesota). MIA assay kits were purchased from Roche Diagnostics (Indianapolis, Indiana). BCA protein assay reagents were purchased from Pierce Chemical Co. (Rockford, IL). Lyophilized reagents that required rehydration in water were resuspended into Sterile Water for Irrigation (SWFIr) (Abbott, Abbott Park, Illinois). Colored substrate *o*-phenylinediamine (OPD) was purchased from Abbott Laboratories. Sulfuric acid was diluted from concentrate to 1 N in SWFIr. PBS was diluted from $10\times$ (Mediatech) into SWFIr.

Melanoma Cell Lysate

In order to have a large supply of a complex biological substance, MCL was obtained from the manufacturing facility of Corixa Corporation, Hamilton, MT. MCL consisted of blended homogenates of two melanoma cell lines (17) designated Mel-D and Mel-S. Homogenates had been lyophilized for storage without removal of any cellular components. These lyophilized homogenates constituted a complex biological substance.

Because reconstituted MCL contained particulate materials, detergent treatment, followed by centrifugation, was required to obtain a solution that would be appropriate for immunoassay. After testing for detergent concentration, vigor of vortex treatment, and incubation

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

time in detergent, a standardized procedure for extraction of MCL was developed.

1) To lyophilized MCL, add 1 mL 0.8% Triton X-100 in SWFIr.
2) Vortex 30 s at high speed.
3) Incubate 5 min at 37°C.
4) Vortex an additional 30 s.
5) Aspirate from vial with sterile syringe using 22 gauge needle and transfer to 1.5 mL microtube.
6) Centrifuge 10 min 14 000 to 16 000 × *g*.
7) Remove 860 µL of supernatant for assay.

Equipment

A BioTek EL340 plate reader and a BioTek ELX808 plate reader, BioTek Instruments (Winooski, Vermont) were used. The use of a multiple channel pipet facilitated delivery of any immunoreagent to the entire assay plate within one minute. In consideration of ergonomics, two varieties of multiple channel pipets were used: 1) thumb pipets (Rainin, Eppendorf, Oxford) that required thumb movement, and 2) trigger pipets (Costar) that required index finger movement. Pipet tips were obtained from Fisher, Costar and Eppendorf. For dispensing bulk reagents such as substrate or stop solution using a multiple channel pipet, reagent basins (Labcor) were utilized.

Plate washing was performed by multiple channel pipets that delivered 300 µL of wash buffer per well. Immunoreagents were delivered in replicates to the assay plate after arranging these in the desired position in a deepwell plate. Polypropylene 96-deepwell plates were purchased from Marsh Industries to provide 0.5 mL capacity per deepwell or 1.0 mL capacity per deepwell.

Assay Guidelines

Three replicates were performed for each concentration of standards and for test samples during development of this assay. Late in development, and for the Standard Operating Procedure, test samples were performed as quadruplicates. Replicates were placed horizontally in the assay plate to minimize O.D. variations produced by vertical strips of 8-wells.

Wells that contained SWFIr were included in each assay plate in order to obtain a reproducible machine blank that would not vary due to temperature or other conditions. The "zero standard" was not the machine blank. True background (also called "noise") of the assay was obtained as

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

the increase in optical density of the ''zero standards'' above SWFIr machine blanks.

In general, certain guidelines for pipetting were established. First, assay diluent that contained masking solutions were delivered early to all microtubes that would be used subsequently for dilutions of standards. This procedure increased the possibility of masking the microtube, and decreased the possibility of loss of analyte to the tube wall. Second, to avoid the possibility of creating ''bubbles'' in deepwells, which would obscure visibility, ''blowout'' functions of mechanical pipets were not used. Third, because of the use of multiple channel pipets and deepwell plates, delivery of immunoreagents to an entire assay plate was completed within one minute after initiating delivery to that plate. Fourth, all channels of a multiple channel pipet were utilized for delivery in order to maximize pipetting accuracy. Fifth, when volume adjustments were performed upon the mechanical pipet, the pipet was adjusted to a larger volume and then was adjusted downward to the target volume setting.

Room temperature conditions were utilized for ICAM-1 immunoassay. Although shaking was recommended by the manufacturer for MIA immunoassay, 37°C without shaking provided reproducible conditions for assay incubations.

Commercial immunoassay used proprietary detergents permissive for binding of antibodies to their respective antigens. Additional detergents were tested for 1) ability to extract antigen from the complex biological matrix, and 2) effects upon antibody binding. The detergents were CHAPS (Pierce), Brij 35 (Pierce), Brij 58 (Pierce), octyl beta-glucoside (Pierce) and Triton X-100 (Pierce).

Accuracy: Range of the Sample and Linearity of the Sample

For accurate sample ranges (range of the sample), total analyte content of samples were compared at different dilutions. This comparison was performed visually by graphing dilution factor (reciprocal of dilution) on the *X*-axis, and total analyte content on the *Y*-axis. Within a range of dilutions in which the total analyte content is nearly equal, %C.V. can be expected to be very low. Total samples within an accurate range were expected to result in a %C.V. less than 5 or 10%, depending upon the assay.

Sample dilutions that provided linear content values (linearity of the sample) were determined by graphing dilution factor on the *X*-axis and analyte content of that dilution on the *Y*-axis. Within an accurate range, a straight line (r^2 0.98 or 0.99) was formed.

For ICAM-1 assays, MCL was diluted 1/30, 1/40, 1/50, 1/60, 1/70, 1/80, and 1/90 and ICAM-1 concentration was plotted against dilution

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

factor (30, 40, 50, 60, 70, 80, and 90). For MIA assays, MCL was diluted to achieve dilution factors of 3, 4, 5, 6, 7, and 8. For BCA protein assays, MCL was tested at dilution factors of 4, 5, 6, and 7.

Accuracy: Spiked Recovery and Standard Addition

Assessment of accuracy by spiking was achieved by two processes: 1) spiked recovery of purified analyte added to the crude substance, and 2) standard additions of purified analyte added to the extracted test sample.

Ideally, spiked recoveries are performed by placing purified antigen into a substance that possesses a matrix identical to the test substance but does not possess that antigen. Because it was not possible to obtain a biological sample that had been prepared identically, but without analyte, spiking of purified recombinant ICAM-1 into MCL was performed. Such spiked recoveries, therefore, were actually spiked additions. Known amounts of purified, carrier-free, recombinant ICAM-1 (R and D Systems) were added to crude MCL, the extraction procedure was performed, and the soluble fraction was diluted and assayed. At least three different concentrations of purified ICAM-1 were added to separate samples of MCL for each test. Total recovered ICAM-1 in ng/mL was plotted against concentration added, and the line was extended to zero using linear regression. The *Y* intercept corresponded to a basic sample without a spike. Simultaneously, unspiked sample was tested. Percent recovery was calculated by dividing *Y* intercept by unspiked ICAM-1 content.

For standard additions to extract of MCL (the test sample), ICAM-1 or MIA that was calibrated previously by the manufacturer was added to the diluted test sample prior to adding the test sample to the immunoassay plate. In practice, if the test sample were to be diluted 50-fold, and if the targeted spike amount were 8 ng/mL, then a 25-fold dilution of test sample was mixed with an equal volume of 16 ng/mL purified analyte. The test mixture then was added to the immunoassay plate. Three or more concentrations were tested. In addition, unaltered samples with no spiked addition were tested. A range of 80 to 120% recovery was required for designation of an "accurate" dilution. For the BCA assay, bovine albumin was added to MCL samples.

Standard Curve

Standard curves for each immunoassay were composed of six non-zero equidistant points. The range of concentrations of the standard curve was determined by the most accurate signals of the test samples which were

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

configured near the center of the standard curve, ranging from around 0.4 to around 0.6 O.D. units. Points of the standard curve were manipulated until they spanned a range of concentrations useful for test samples whose analyte content might vary. In addition, the range of the standard curve could be extended so that it was sufficient to accommodate tests of "spiked" additions of standards to the samples for accuracy measurements.

The zero standard for the standard curve consisted of commercial assay diluent alone, and was positioned on the assay plate with the standards of the standard curve. The zero standard (assay diluent) was distinguished from the machine blank (SWFIr). When the data were "blanked" against SWFIr, the "zero standard" provided a background optical density that was a useful measurement for signal/noise calculations.

For ICAM-1, three ranges of standard curves were tested, and the ICAM-1 standard curve selected for use was composed of 6 equidistant non-zero concentrations from 3 through 18 ng/mL. Three MIA standard curves were tested also, and an MIA standard curve consisting of 6 non-zero points: 2, 5.6, 9.2, 12.8, 16.4 and 20 ng/mL, was selected for final testing. Standard curve for the BCA assay consisted of 100, 280, 460, 640, 820, and 1000 μg/mL solutions.

Precision: Repeatability, System Precision, Intermediate Precision

Three elements of precision were analyzed: sample repeatability, system precision (intra-assay precision), and intermediate precision (inter-assay precision). Three to six separate vials of MCL were assayed at the same time to determine sample repeatability. Dilutions were compared to the standard curve, and multiplied by dilution factor for total ICAM-1 content. For System Precision, 12 identical samples of MCL were tested, as well as a standard solution of commercial antigen, for each immunoassay and %C.V. was calculated. Intermediate precision was determined over a period of months, using at least two standard curve ranges, by testing a single lot of MCL using a new assay kit for each test and by calculating %C.V. An additional analysis of Intermediate Precision involved three days of testing identical samples (samples were stable to one freeze/thaw cycle) using three separate immunoassay kits.

Limit of Detection and Limit of Quantitation

A lower limit of concentration of ICAM-1 that could provide an optical density greater than background optical density was determined

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

empirically by diluting ICAM-1 standard to very low concentrations and testing them using the ICAM-1 immunoassay. A concentration that consistently developed an optical density that was three-fold greater than the background optical density of the ''zero standard'' (assay diluent) was considered above the limit of detection (LOD). A lower limit of quantitation (LOQ) was determined similarly. An acceptable accurate range of values for each LOQ sample was 80 to 120 percent of the concentration tested.

Robustness: Variations and Parameters

''Front to back'' variations were due to the length of time required to load an assay plate which, if lengthy, could lead to variations in optical density of a sample loaded first compared to that loaded last. After reading an ICAM-1 immunoassay plate, the plate was turned 180° and read again. Results were calculated for each orientation. Similarly, plate reader variations were determined with 8-well strips by carefully repositioning strips of wells. After performing a front to back test, the right column of wells was carefully dislodged from the assay plate. Each column of 8-well strips was moved one column to the right, leaving the first column empty. Results were calculated for both orientations. This was called a ''column shift'' test.

Time of incubation, number of wash cycles, and temperature of incubation were investigated. Extraction procedures were tested in order to obtain complete extraction of antigen from MCL. Effects of detergents were tested using Triton X-100, Brij 35, Brij 58, CHAPS, and octyl beta-glucoside.

Stability

Stability was determined by submitting biological substances to multiple freeze/thaw conditions or to 4 to 37°C storage of MCL extracts. Test samples of MCL, as well as positive control samples of ICAM-1 and MIA, were subjected to freezing to −20°C for 15 min, followed by thawing at 37°C or room temperature.

Micro-BCA Protein Assay

Protein content was determined in a 96-well microplate format using a wavelength of 550 nm in a microplate reader, using the Pierce microwell assay. Wavelength 550 nm was selected from the outset because most

plate readers possess a filter enabling optical density determination at this wavelength. Volume of the sample or protein standard per well was 25 μL added to 200 μL of BCA reagent mixture. Briefly, bovine albumin was used to generate a standard curve using 6 non-zero standards that provided equidistant spacing along a straight line from 100 to 1000 μg/mL: 100, 280, 460, 640, 820, and 1000 μg/mL. The most reproducible signals were obtained using bovine albumin as a 2 mg/mL solution in normal saline (Pierce). Unlike the standards, which contained normal saline, MCL contained PBS and Triton X-100. To equalize NaCl, buffer, and detergent conditions, equal volumes of standards were mixed with equal volumes of PBS in Triton X-100 solution. Test samples were brought to equality for NaCl, phosphate buffer, and Triton X-100 concentration by adding equal volumes of normal saline. Standardized final concentrations were $0.5 \times$ PBS, $1 \times$ normal saline, and 0.13 or 0.16% Triton X-100.

RESULTS

Accuracy: Range of the Sample and Linearity of the Sample

A range of sample dilutions were tested to determine any patterns of variation that may have occurred due to sample matrix, or dilution manipulations. It is noteworthy that the mean ICAM-1 content per dilution, when plotted, provided no strong pattern for assessing an appropriate range of dilutions of MCL samples, as shown in Figure 1, although a trend toward lessened values was visible at the highest dilution. Interestingly, mean ICAM-1 content for each day, regardless of dilution, when calculated per experiment, had 1.5 to 10.6%C.V., which included 12 ranging experiments, each using multiple dilutions. This suggested that the ICAM-1 immunoassay could be accurate when used with a wide range of sample dilutions. Linearity testing, however, defined a much narrower range of sample dilutions that would be accurate. In 5 sample linearity experiments, dilutions of 1/50, 1/60, and 1/70 provided a straight line with r^2 greater than 0.98 for all 5 tests, and r^2 greater than 0.99 for 4 of the 5 tests.

In contrast, a narrower range of accurate dilutions (range of the sample) was detected using the MIA immunoassay. Dilution factors of 3, 4, and 5 resulted in a general plateau as shown in graphical form in Figure 2, with a mean value of 52 ng/mL, 10.1%C.V. using 25 data points. On the same graph, dilution factors of 6, 7, and 8 formed a lower bench-like pattern in the curve. Values obtained using dilution factor 6 (mean of 47 ng/mL) were the highest of that "low" group, yet only decreased the mean by 1 ng, and increased %C.V. to 11.3%. Next, linearity of the sample for MIA

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

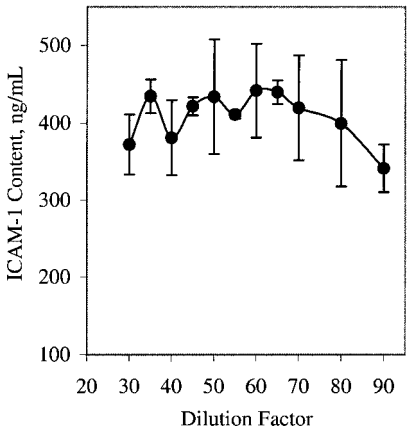

Figure 1. Mean ICAM-1 (ng/mL) content data were obtained for seven different dilutions of MCL. Using the accepted standard curve of 3 ng/mL through 18 ng/mL, MCL was diluted 1/30 through 1/90, tested, and mean ICAM-1 content (ng/vial) was plotted. A weak "plateau" can be visualized between dilutions 1/45 through 1/70. Dilutions of 1/50, 1/60 and 1/70 were tested 11 or 12 times each. Other dilutions were tested 3 to 7 times.

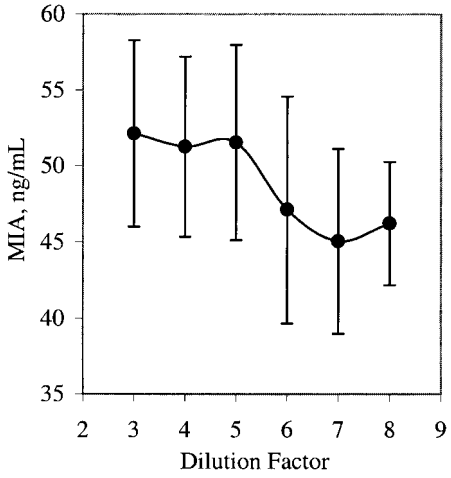

Figure 2. Mean MIA concentration (ng/mL) was calculated for six different dilutions of MCL. Visual inspection suggests that a degree of similar accuracy exists for samples at dilutions of 1/3, 1/4, and 1/5. Each mean was obtained using 5 to 14 data points.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

immunoassay was tested using dilution factors of 3, 4, 5, 6, 7, and 8. Using five sets of experimental data, linearity (r^2) was calculated resulting in r^2 of 0.91–0.93 for dilution factors 3 through 8, r^2 of 0.96–0.97 for dilution factors 3 through 6, r^2 of 0.89–0.98 for dilution factors 4 through 7, and r^2 of 0.99 for dilution factors 4, 5, and 6. Combined data were interpreted to mean that dilution factors of 4 and 5 were most accurate for testing MCL, and that dilutions of 1/6 would be slightly less accurate.

Range of the sample for the BCA protein assay was determined with dilution factors of 4, 5, and 6, and resulted in a %C.V. of 3.2. A similar %C.V. (3.7) was compared by analysis of all data points obtained with dilution factors 4, 5, 6, and 7. Linearity of the sample for the BCA protein assay was tested using MCL dilutions factor of 4, 5, 6, and 7. Dilution factors of 4, 5, and 6 resulted in a line with r^2 of 0.99 (two tests), and dilution factors 4, 5, 6, and 7 resulted in r^2 values of 0.97 (two tests). Combined data for these two tests predicted, therefore, that the most accurate range of dilution factors of MCL for the BCA protein assay would be 4, 5, or 6. MCL dilutions of 1/7 would be less accurate.

Accuracy: Range of the Standard Curve and Linearity of the Standard Curve

Each standard curve consisted of six non-zero concentrations placed equidistantly along a straight line. The range of concentrations was an extension of data that placed the most accurate test sample dilution near or slightly below the midpoint of the standard curve. For the ICAM-1 SOP, standard curve 3 ng/mL through 18 ng/mL was combined with the use of 1/50 dilutions of MCL in order to position MCL values slightly lower than the midpoint of the standard curve. This strategy was used for all three assays described in this report. In addition, linearity of each standard curve was assessed by calculating r^2. Linearity of the ICAM-1 standard curve (3 to 18 ng/mL) was 0.9946, the mean r^2 of 12 curves.

Range of the MIA standard curve was 2 to 20 ng/mL, which had a mean r^2 of 0.9906 (range 0.9825–0.9993) for 16 determinations. Range of the BCA protein assay was 100 to 1000 μg/mL, which had a mean r^2 of 0.9947 (range 0.9899 to 0.9972 for 5 determinations).

Accuracy: Spiked Recovery

Recombinant human ICAM-1 was purchased from R and D Systems, diluted into 0.8% Triton X-100 in SWFIr, and added to MCL. Vials of

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

MCL were treated according to the assay procedure and total recovered ICAM-1 was compared to a separate vial of Melanoma Lysate to which no recombinant ICAM-1 was added. Spiked recovery, as 3 additions ranging from 133 to 436 ng/mL, using the preferred 3 through 18 ng/mL standard curve, resulted a mean recovery of 98.6% (range 69.8 to 132%) using 1/50 dilutions of test substance, and 87.7% (range 52.4 to 118%) using 1/60 dilutions of test substance. Figure 3 provides a representative graph of ICAM-1 spiked recoveries.

Accuracy: Standard Additions

Standard additions were performed by adding commercial standards to equal volumes of MCL. Several dilutions of MCL were tested. ICAM-1 concentrations of 50 to 225% of endogenous ICAM-1 content for that dilution, were added to diluted MCL test sample. Accuracies of 102 to 108% (3 experiments) were obtained for dilutions of 1/60 and 1/70. For 1/50 dilutions, accuracy was 95.4% (range 84.8 to 119%, 4 tests) for

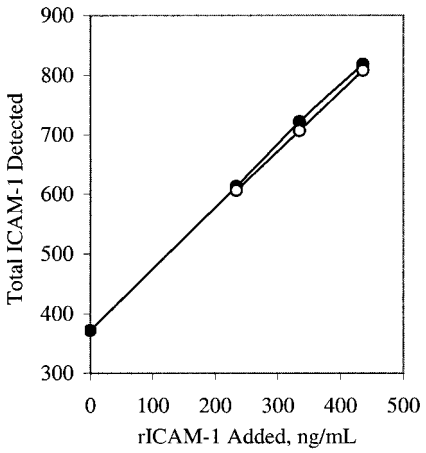

Figure 3. Spiked Recovery of recombinant human ICAM-1 added to MCL was tested for 1/50 dilutions of MCL. A representative graph is shown. Three concentrations of recombinant human ICAM-1 were added to separate samples of MCL, recovered, and tested. Detected ICAM-1 (filled circles) was plotted against expected ICAM-1 (empty circles). Recoveries were 101 to 102% for these three concentrations tested. Calculated *Y* intercept was 372.8 ng/mL, which represented unspiked MCL, the actual ICAM-1 content of which was 394.1 ng/mL.

additions of 4 ng/mL, 94.2% (range 74.3 to 119%, 6 tests) for additions of 6 ng/mL, and 91.2% (range 84.8 to 100%, 4 tests) for 8 ng/mL. Endogenous ICAM-1 content of a 1/50 dilution was about 8 ng/mL.

For the MIA immunoassay, spiked additions were performed using dilution factors of 4, 5, and 7 for MCL. Precalibrated MIA standards from the manufacturer were used: 5.4, 8.6, and 12.5 ng/mL. A mean accuracy of 99.0 to 111.4% was obtained using 1/5 dilutions (8 tests) of MCL, but 109.5 to 119.0% accuracy was obtained using 1/7 dilutions (7 tests) of MCL. Dilutions of 1/4 had lower accuracy ranging from 73.7 to 93.7%. Dilutions of 1/5 were selected to be most accurate.

Standard additions of bovine albumin (250 to 500 μg/mL) were accurately detected by the BCA protein assay within diluted MCL. MCL dilutions of 1/6 had less accurate (75.7 to 92.4%) detection of bovine albumin, than did MCL dilutions of 1/7 (90 to 100%), in two tests. Additions above 325 μg/mL had 80% or above accuracy for both dilutions of MCL.

Precision: Repeatability, System Precision, and Intermediate Precision

To assess Repeatability, six separate vials of MCL (lot 1) were assayed for ICAM-1 content on the same assay plate, using a standard curve of 3 through 18 ng/mL, and 1/50 dilutions of MCL. Mean ICAM-1 content had a 4.5%C.V. The range of ICAM-1 content varied by 92.8 to 104% of the mean value. On a separate occasion, six separate vials of MCL (lot 1) had a 3.8%C.V., and MCL (lot 2) had a 6.4%C.V. Percent C.V. for assays using only three vials of test substance varied from 3.3 to 9.9%.

To determine system precision, 12 identical samples of ICAM-1 commercial standard were tested, resulting in a 9.1%C.V.

Intermediate Precision was tested in three ways. First, vials of MCL were assayed over a period of months to determine variations of the immunoassay on a day-to-day basis. For ICAM-1, 8 vials on 8 separate days had 10.4%C.V. with a 1/50 dilution and an 11.1%C.V. with a 1/60 dilution. Dilutions of 1/50 resulted in a range of values from 86.4 to 115% of the mean value and dilutions of 1/60 ranged from 86.1 to 118% of the mean value. For MIA, intermediate precision was 19.8% using 1/5 dilutions and was 16.6% using 1/7 dilutions.

Second, Intermediate Precision was tested by assaying aliquots of the same sample on different days. Because ICAM-1 is stable to more than one freeze/thaw cycle (see Stability, below), a large sample of undiluted MCL extract was divided into aliquots followed by freezing of all aliquots

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

simultaneously. The aliquots were stored at −20°C. On three separate days, four aliquots were thawed and tested for ICAM-1 content resulting in a combined if 3.2%C.V.

Third, Intermediate Precision of the ICAM-1 immunoassay was tested using ten separate sample vials of MCL, five each from two different production lots, tested each day for three days, totaling 30 sample vials. For lot 1, %C.V. per day were 3.9, 5.2 and 3.6%, with 4.4%C.V. overall. For lot 2, %C.V. per day were 6.8, 10.7 and 4.8%, with 7.3%C.V. overall.

Limit of Detection (LOD)

LOD was defined as the concentration of ICAM-1 that provided an optical density that was three-fold the background. Background was obtained from the optical density of a zero-standard. A zero-standard was a sample that contained all other immunoreagents except ICAM-1. In order to obtain a background optical density from a zero-standard, the immunoassay plate was designed to include several wells of SWFIr to which each plate reader was blanked. Optical densities obtained from a zero-standard were very low, in most cases 0.03 optical density units or less. For each assay, mean optical density of LOD samples was divided by the mean optical density of the zero standard samples. The resulting value was plotted against concentration of ICAM-1 tested. This method produced similar data irrespective of the standard curve in use at the time. ICAM-1 at a concentration of 1 ng/mL provided an optical density that reproducibly was three-fold the optical density of background. LOD data for ICAM-1 are shown in Figure 4.

Limit of Quantitation (LOQ)

Concentrations of ICAM-1 standard lower than the lowest point of the standard curve, 3 ng/mL, were tested for accuracy of quantitation. Using cumulative evidence from accuracy measurements, LOQ calculated values were considered accurate at 80 to 120% of the tested concentration. This 20% variation was an allowance that was calculated around mean spiked recoveries using 1/50 dilutions which, although they had accurate mean recovery (98.6%), still had a 19.5%C.V. around that mean.

Calculated values for each LOQ sample were divided by the concentration tested, and plotted against the concentration tested. Extensive preliminary testing provided evidence that as test concentrations approached

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

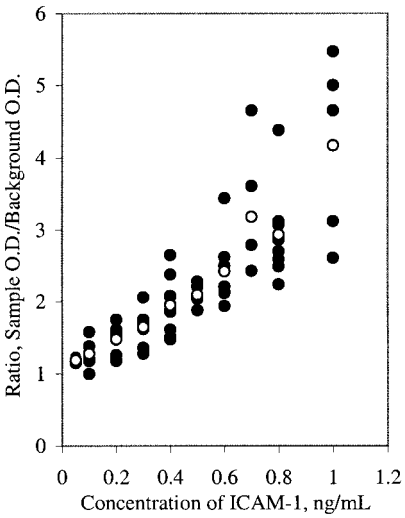

Figure 4. Lower Limit of Detection (LOD) for ICAM-1. For LOD calculations, low concentrations samples were assayed and optical density was obtained. The optical density of each low concentration sample was divided by the optical density of the zero-standard (background) for that assay. The resulting ratio was the signal-to-noise ratio and was plotted against concentration tested. Average three-fold background was reached with 0.7 ng/mL sample, lost at 0.8 ng/mL, and regained at 1.0 ng/mL.

2 ng/mL, ratios of calculated/test concentrations approached 1.0 (top panel of Figure 5). Further testing was performed (lower panel of Figure 5), resulting in mean ratios for LOQ tests: 1.6 ng/mL, 0.54; 1.8 ng/mL, 0.69; 2.0 ng/mL, 0.77; 2.2 ng/mL, 0.72; 2.4 ng/mL, 0.86; 2.6 ng/mL, 0.83; 2.8 ng/mL, 0.81. A concentration above 2.4 ng/mL consistently provided accuracy with a mean value greater than 0.80, or 80% of the tested concentration.

Specificity Determined by Parallel Line Analysis

Parallel line analysis provided a means of determining that the immunoassay recognized native ICAM-1 similar to recombinant ICAM-1 standards, thereby offering a type of specificity. Spiked additions of up to six concentrations (2 through 9 ng/mL) of purified recombinant ICAM-1 were delivered into MCL extract. Unspiked and additive values of ICAM-1

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

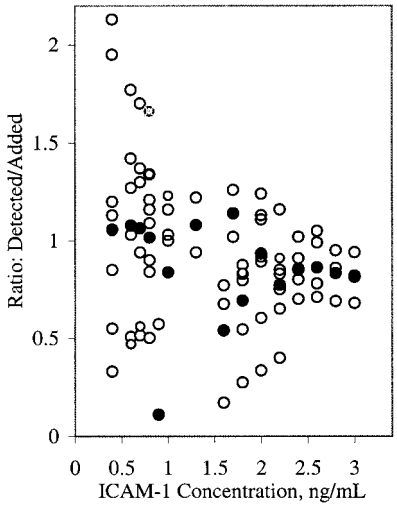

Figure 5. Limit of Quantitation (LOQ) was determined empirically by testing low concentration samples of purified ICAM-1. For each concentration tested (3 ng/mL or less), a ratio of calculated results to actual concentration was calculated. At the LOQ, this ratio was within the assay accuracy of 80 to 120%. Mean (filled circles) ratios (detected/added) were above 0.8 for ICAM-1 samples with concentrations above 2.4 ng/mL. The LOQ, therefore, was 2.4 ng/mL.

content were calculated and graphed. Calculations using the method of Massart et al., 1989 (18), determined that the slopes of the two lines were equivalent.

Robustness: Periods of Mixing, Cycles of Washing, Pipet Brand, and Front-to-Back Variations

In order to determine the effects of mixing by vortex upon the detection of ICAM-1, four samples of test substance were extracted with no mixing by vortex, 15 s mixing by vortex, 30, or 45 s. ICAM-1 content of each sample was 457, 483, 478, and 470 ng/vial, respectively. Mixing was better, therefore, than not mixing, but any difference between 15 to 45 s had only a 1.4%C.V. Accidental alteration of the time period for mixing by mechanical vortex would not alter subsequent detection substantially.

3, 6, and 9 cycles of washing were compared to determine any loss of signal due to excessive washing of the ICAM-1 assay plate. Background

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

optical densities of the zero standard for all washing variations had a 5.6%C.V., and the maximum signals were similar with a 2.3%C.V. Furthermore, 3, 4, and 5 washes of the MIA assay plate were compared. Linearity of the standard curve was unaffected. Highest optical density of the standard curve was achieved with 3 washes, with 4 washes having 88.3% of the signal of that of 3 washes, and 5 washes having 92.1% of the O.D. of 3 washes.

Two types of mechanical pipets were tested for delivery to the assay plate: Benchmate (Oxford) 8 channel pipet, and Pipetman P-100 (Rainin) single channel pipet (Experiment #W020100A), both pipets fitted with Costar tips. Standard curves were performed in triplicate. Mean %C.V. for all concentrations of standards for 8-channel pipet was $4.2 \pm 2.2\%$ with a median of 3.7%. For a single channel pipet, mean %C.V. was $6.9 \pm 3.0\%$, with a median of 6.4%. Standard curves had r^2 values of 0.993 and 0.989 for 8 channel and single channel pipets, respectively. No variations due to the plate reader were detected using "front to back" re-orientation of the plate or using "column shift" re-organization of strips.

Robustness: Incubation Time and Temperature

As expected, increased incubation time of antibodies resulted in increased optical density signals. For the MIA immunoassay, heightened optical densities were obtained from 90 through 150 min incubations. Equivalent quantitation of MCL was obtained for 90 and 120 min incubations. Using a 150 min incubation, however, a slight increase in MIA was obtained for each dilution factor tested, (5 and 7), resulting in about 5 ng increase (after adjustment to undiluted product) per sample, which was 10% of the average final value.

For the ICAM-1 immunoassay, no advantage was found by performing the assay at the higher temperature. For the MIA immuno-assay, 37°C temperature was advantageous. MIA standard curves were tested using three conditions: shaking the plate at room temperature (as per manufacture recommendations), no shaking at room temperature, no shaking at 37°C. Standard curves for all three conditions had r values of 0.99, and Y intercepts of 0.02 to 0.06. Maximum optical densities, however, were significantly lower (0.86) when antibody incubations were performed at room temperature without shaking. Although highest optical density was achieved with shaking at room temperature (1.56), an intermediate optical density of 1.08 was obtained under 37°C conditions, without shaking. Because shaking vigor (RPM), was a condition for which calibration was not available, 37°C condition without shaking was selected for antibody incubation during the MIA immunoassay.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Robustness: Substrate for the MIA Assay

Substrate supplied in the MIA assay kit was 2,2′-azino-di-[3-ethyl-benzthiazolin-sulfonate] (ABTS), which did not have qualities we desired for an endpoint assay using sulfuric acid as a stop solution. Because the commercial MIA immunoassay utilized an HRP-labeled immunoreagent, OPD (Abbott) substrate was substituted for ABTS, and was tested for endpoint optical density without drifting. Stable endpoints were established by adding equal volumes (100 μL) of 1 N sulfuric acid to OPD, after OPD had developed. This change of substrate also necessitated investigation of optimum incubation time of OPD. Increased optical densities were obtained by incubating for 12 to 15 min, but no change occurred between 15 and 20 min incubations.

Robustness: Detergent

Detergent compatibility was tested first in the ICAM-1 immunoassay with known standards, and next for extraction of natural ICAM-1 from MCL. Five different detergents (CHAPS, Brij 35, Brij 58, octyl-glucoside, and Triton X-100) were tested for potential interference with antibody detection within the proprietary buffer system of the ICAM-1 and MIA immunoassays. In general, most of the detergents induced no significant alteration of detection of ICAM-1 standards within the range of about 0.01 to 0.1% detergent as shown for CHAPS and Brij 35 (Figure 6), Brij 58 and octyl beta-glucoside (Figure 7), and Triton X-100 (Figure 8). Close agreement was found for all ICAM standards tested, in the presence of Triton X-100 at final concentrations of 0.003 to 0.02% detergent in the immunoassay wells (Figure 8).

Brij 35 and Triton X-100 were compared further for ability to solubilize ICAM-1 within the crude MCL matrix. Results are shown in Figure 9, which illustrate the superiority of Triton X-100 over Brij 35 for solubilizing natural ICAM-1 in MCL, followed by ICAM-1 immunoassay.

A working concentration of Triton X-100 that provided optimum detection of ICAM-1 in MCL then was determined as shown in Figure 10, resulting in the selection of 0.8% Triton X-100 for development of a standard procedure to prepare MCL for immunoassay. When diluted 1/50, a 0.8% Triton X-100 extract of MCL results in a 0.016% solution of Triton X-100 in the assay well, which was within the range of 0.003 to 0.02% described in Figure 8.

Triton X-100 was tested also for interference with antibody binding in the MIA immunoassay (Figure 11). Very little interference was found.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

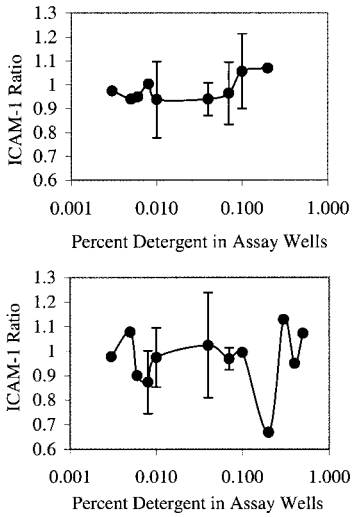

Figure 6. CHAPS and Brij 35 were tested for alteration of detection of ICAM-1 molecules in immunoassay format. CHAPS detergent and Brij 58 were mixed with ICAM-1 standards and were tested for interference with detection. Because three different concentrations of ICAM-1 standards were tested, results were normalized by comparing the calculated results to concentration of ICAM-1 standard tested. Roughly, a region of lesser interference can be seen at detergent final concentrations between 0.01 and 0.1% (concentrations within the assay well). Top panel, CHAPS; bottom panel, Brij 35.

The final concentration of Triton X-100 in assay wells of the MIA assay was 0.16% after a 1/5 dilution of 0.8% Triton extract. In the MIA immunoassay, variation due to 0.16% Triton X-100 in assay wells was 2.4%. All concentrations tested of Triton X-100 caused 6.6% or less variation in MIA assay signal.

Extraction of antigen from MCL by detergent, followed by centrifugation, resulted in an insoluble pellet and a soluble supernatant. To test completeness of extraction, the pellet was treated again using 0.8% Triton X-100 and retested for residual ICAM-1 antigen using this ICAM-1 immunoassay. No ICAM-1 could be reclaimed from the pellet.

Stability to Conditions of Storage and Cycles of Freeze/Thaw

Test samples of MCL were treated with 0.8% Triton X-100, centrifuged, and supernatants were subjected to freezing from 1 to 4 cycles using

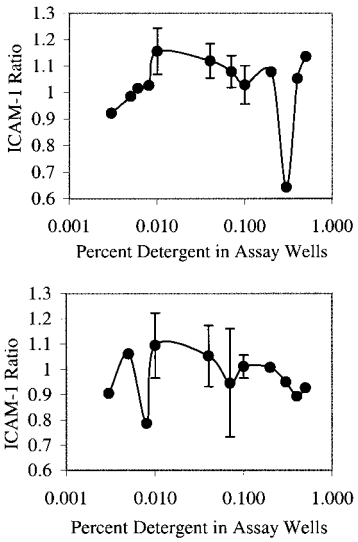

Figure 7. Brij 58 and octyl beta-glucoside resulted in scattered results when ICAM-1 standards were assayed in the presence of various concentrations of those detergents. As for Figure 6, detergents were diluted into ICAM-1 standards at three concentrations of standard, and tested by immunoassay. Mean values are shown. Detergent concentrations were those within assay wells. Top panel, Brij 58; bottom panel, Octyl beta-glucoside.

−20°C to room temperature conditions. Detectable amount of ICAM-1 in MCL was lessened consistently with increasing cycles of freeze/thaw, as shown in Figure 12. Similarly, an ICAM-1 commercial standard (13.8 ng/mL) lost detection from 12.3 ng/mL at one cycle to 10.8 ng/mL at the second cycle of freeze/thaw. Similarly, a large sample of MCL extract was divided into 12 aliquots and frozen at −20°C. Four aliquots each were thawed and tested at three different time points. MCL extract aliquots tested after 24 h frozen storage had a mean ICAM-1 content of 434.6 ng/vial with a %C.V. of 1.9%. Similarly, after 48 h storage, 4 separate aliquots had a mean ICAM-1 content of 439.9 ng/vial with a %C.V. of 2.3%. After 144 h of storage, 4 aliquots had a mean of 448.5 ng ICAM-1 per vial with 4.5%C.V. The mean of all twelve aliquots tested at three storage times was 441 ng/vial with a combined %C.V. of 3.2%. ICAM-1 in MCL extract was stable at −20°C over at least 144 h, thereby allowing tests of intermediate precision over a period of six days using identical samples.

Furthermore, extracts in 0.8% Triton X-100 were obtained from a single MCL sample and stored at room temperature, at 4, or 37°C, for

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

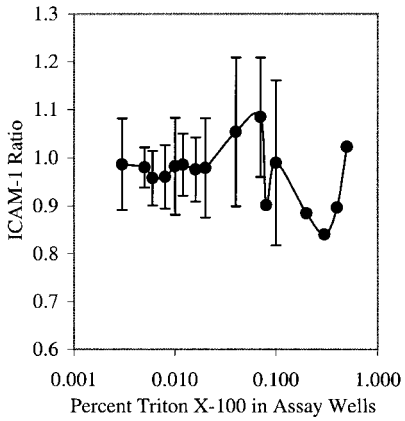

Figure 8. Triton X-100 was tested for interference with detection of ICAM-1 standards in immunoassay. Close agreement was found for all ICAM standards tested, regardless of ICAM-1 concentration, in the presence of Triton X-100 at final concentrations of 0.003 to 0.02% in the immunoassay wells. When compared to the adopted procedure of treating Melanoma Lysate with 0.8%, Triton X-100 and diluting 50 fold for assay, the final concentration of Triton X-100 in the assay well would be 0.016% which is within the range of accuracy.

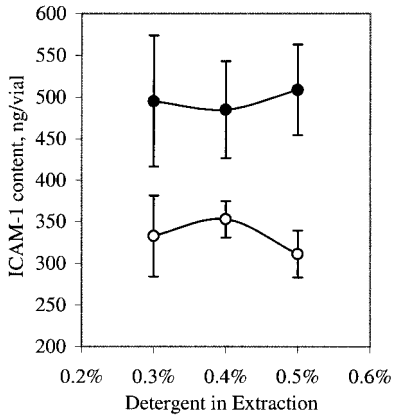

Figure 9. Triton X-100 is superior to Brij 35 for solubilization of ICAM-1 in MCL. Three different concentrations of Brij 35 (empty circles) and Triton X-100 (solid circles) were compared for effectiveness of solubilization of ICAM-1 in MCL followed by detection in ICAM-1 immunoassay. Data points represent mean values of at least four experiments and are shown with error bars.

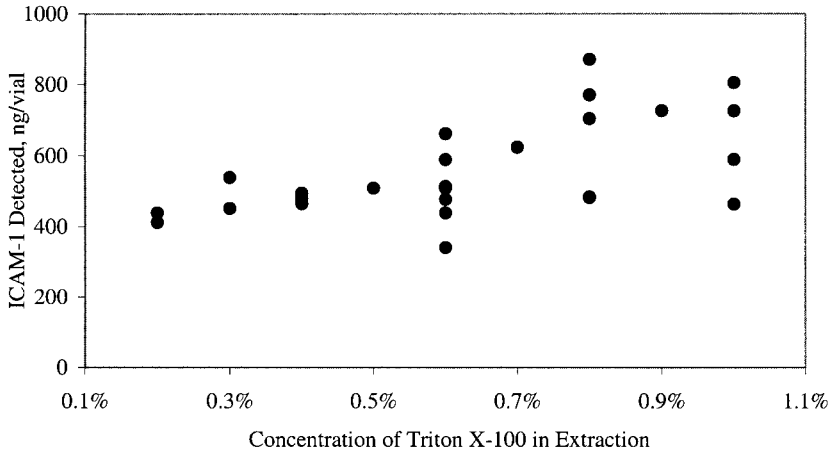

Figure 10. Treatment of MCL with 0.8% Triton X-100 results in optimum detection of ICAM-1 by immunoassay. Data from 10 experiments were combined. Mean ICAM-1 content per extraction was plotted, regardless of dilution after extraction by Triton X-100. Each mean was obtained from 4 or more values of MCL. Triton X-100 concentration (horizontal, *X* axis) refers to the concentration used for extracting ICAM-1 from MCL.

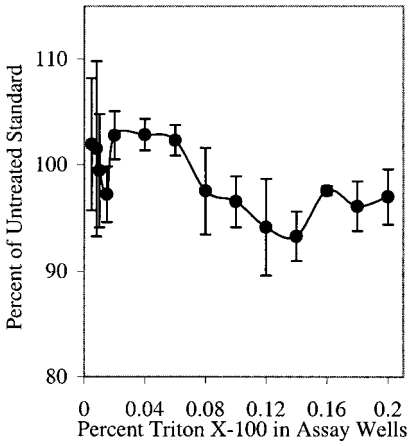

Figure 11. Effects of Triton X-100 Detergent concentrations upon fixed concentrations of MIA standards were tested. Data shown are means of three experiments. The largest variation found due to detergent was 6.6%. At the concentration of Triton X-100 found in the assay well (0.16%), $97.6 \pm 0.4\%$ accuracy was found.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

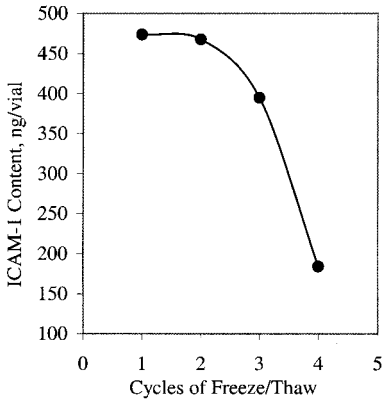

Figure 12. ICAM-1 within MCL is susceptible to freeze/thaw denaturation. MCL was solubilized in 0.8% Triton X-100, divided into equal volumes and each aliquot was submitted to cycles of freezing and thawing. Loss of ICAM-1 was noted after 2 cycles of freeze/thaw.

5 h or overnight. All samples were then tested on the same assay plate. Results were calculated relative to room temperature conditions, resulting in identical (100 and 98% agreement) quantitation for 4° storage, and loss (up to 12% loss) of signal due to 37°C conditions of storage for 5 h or overnight. Storage of MCL extract at room temperature did not alter, significantly, the detectable ICAM-1 level.

MIA standards were subjected to one or two cycles of freeze/thaw. All curves, regardless of freeze/thaw treatment, had r values of 0.99, and Y intercepts of 0.07 to 0.09. Optical density of highest standard of once-frozen standards was 96.2% of fresh standards, and optical density of highest standard of twice-frozen standards was 99.2% of fresh standards.

Conditions for Optimizing BCA Accuracy in the Presence of Detergents

In general, increasing the concentrations of Triton X-100, Triton X-114, Brij 35, and Brij 58, from 0.1 to 0.8%, using bovine albumin as a test sample, caused 6 μg increase in BCA assay results prior to adjustment for dilution factor. Using 62.5, 125, and 250 μg test samples of bovine serum albumin, concentrations of 0.1 and 0.2% detergents resulted in losses or gains for Triton X-100 (gain of up to 17 μg), Brij 35 (loss of 6 μg to gain of 3 μg), Brij 58 (gain of 3–4 μg), CHAPS (gain of up to 40 μg), and octyl

beta glucoside (loss of 27 μg to gain of 15 μg) of the expected quantitation prior to adjustment for dilution factor. Using 125 and 250 μg test samples of BSA, concentrations of 0.1 and 0.2% Triton X-100 in the diluted sample resulted in 100% (identity) to 107% (up to 17 μg increase) of the expected quantitation prior to adjustment for dilution factor.

MCL extracts were standardized using 0.8% Triton X-100 for the BCA protein assay also, in order to coordinate BCA assays with immunoassays. Using MCL dilutions of 1/6, Triton X-100 concentrations in the test sample would be within 0.1 to 0.2% detergent concentrations. Effects by residual detergents in MCL tested using the BCA assay were 7% or less.

Standard Operating Procedures for these Assays

Standard Operating Procedures (SOPs) were developed. The ICAM-1 and MIA immunoassays shared an identical procedure for antigen extraction from MCL, but diverged in the procedure for antigen detection. Briefly, a sample of MCL averaging about 3.0 mg/mL protein content lyophilized in PBS, was treated with 0.8% Triton X-100 in SWFIr, vortexed 30 s, incubated 37°C for 5 min, vortexed again for 30 s, then centrifuged in a microtube at 14 000 to 16 000 × *g* for 8 min. From the supernatant, 860 μL were removed from the microtube and submitted to immunoassay.

For ICAM-1 immunoassay, a standard curve of 6 non-zero equidistant points (3 to 18 ng/mL) in triplicate was made by diluting only the highest concentration standard of the commercial ICAM-1 immunoassay kit. MCL extract was diluted 1/50 in commercial assay diluent for addition to the assay plate. The MCL test sample was incubated at ambient temperature for 2 h with capture antibody, followed by 5 washes, followed by 2 h incubation at ambient temperature with detecting antibody, 5 washes, and 20 min of OPD color development, followed by equal volume (100 μL) of stop solution. Excel spreadsheets were developed to analyze data imported from the plate reader.

Similarly, the MIA immunoassay proceeded by diluting the highest concentration standard to make a standard curve of 6 non-zero points, equidistant on the curve from 2 to 20 ng/mL. Test sample (1/5 dilution of MCL) was added in a 20 μL volume with 180 μL detecting antibody and incubated for 120 min at 37°C. Following 4 washes, color was developed for the MIA immunoassay using 100 μL OPD for 15 min followed by equal volume of 1 N sulfuric acid stop solution. Excel spreadsheets were developed to analyze data imported from the plate reader.

A standard operating procedure for BCA protein assay was also developed. Bovine albumin in saline was diluted to make a two-fold concentration of each standard curve concentration. Equal volumes of each two-fold

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

standard and a standards diluent (0.266% Triton X-100 in 1 × PBS) then were mixed. MCL was prepared as 0.8% extract in Triton X-100. One part MCL extract was added to 2 parts PBS. That test sample was mixed 1 : 1 with normal saline, resulting in dilution to 1/6. To 200 µL BCA reagent mixture was added 25 µL standards or test sample. Solutions were incubated 30 min at 37°C. O.D. was determined at 550 nm using a plate reader. Suggested dilutions were based upon an MCL protein content of about 3 mg/mL. Excel spreadsheets were developed for data analysis.

DISCUSSION

Biological products may be processed from biological substances that are complex in composition. Biological complexity can exist at steps during the production process, and can persist at its end. Assays to monitor the presence and quantity of a specific component within a complex biological substance include immunoassays. Indeed, ICH guidelines of 1999 for test procedures for biological products state that "immunochemical properties may be used to quantify a protein," and referred to ELISA or Western blot procedures.(19) An ELISA is an assay whose components are biological products, whose specificity relies upon immunochemical recognition. Like other assays, an ELISA possesses elements that can be tested for agreement with a previous standard or agreement with historical information, or agreement with a large number of samples. These elements are tested to determine if an assay is appropriate for its purpose. Such testing is called validation.

Although previously validated by their manufacturers for use with culture supernatants or with serum, we wished to assess two immunoassays for use with MCL, a complex biological product. At the outset, we wished to determine a dilution of sample that 1) gave a reasonably strong signal using the immunoassay, and 2) gave accurate results with a minimum of matrix effect. Assessments of accuracy, therefore, were most valuable for our efforts, and were a natural starting point. We assessed accuracy using several steps: 1) determine a dilution that provides an assay signal that is high enough to be near the midpoint of a linear standard curve, and that, simultaneously, is low enough to allow addition of purified analyte (for spiking studies); 2) determine an expanded range of sample dilutions that might provide such a signal; 3) determine the presence or absence of a matrix effect at these sample dilutions by adding purified analyte and testing for percent recovery or percent detection.

Accuracy was calculated using four measurements: spiked recovery, spiked additions, range of the sample, and linearity of the sample. It was important to determine the ability of antibodies used in these two

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

immunoassays to detect, equally, the natural analyte possessed by the test sample, as well as to detect purified analyte used for each standard curve. Spiking experiments determined that purified recombinant analyte could be detected within the matrix in which the natural analyte was detected, but that the measurement was most accurate using a particular dilution of the complex biological substance.

Mean ICAM-1 accuracy using spiked recoveries was 98.6% using 1/50 dilutions, but had a range of 70 to 133%. Accuracy measurements were obtained, also, by adding ICAM-1 standards to diluted MCL extract (Standard Addition), resulting in a mean 93.7% accurate detection, within an actual range of 74.3 to 119%. Accuracy of such recovery experiments have been reported by other investigators and include 113 to 136% in plasma (20), $99.3 \pm 1.5\%$ in plasma (21), 81 to 101% and 85 to 122% in rat serum (22), $109 \pm 11\%$ in whole blood (23), and 92 to 104% in serum.(24) ICAM-1 tests using different dilutions of MCL (Range of the Sample) were expected to point to a narrow range of sample dilutions that would be accurate, and that range of dilutions was expected to agree with data using Spiked Recovery. Range of the Sample was tested using dilutions of 1/30 to 1/90, none of which dramatically altered the quantity of ICAM-1 detected, and ranged from 90 to 115% of the mean of those values. Sample linearity, however, detected greatest linearity for 1/50, 1/60, and 1/70 dilutions of MCL. Adaptation of ELISA by other investigators have involved fewer dilutions, such as evaluation of two dilutions (1/3 and 1/10) for antibiotic residue in ground beef using an ELISA developed for milk.(25) Combined Accuracy measurements, therefore, led to the selection of 1/50 dilutions of MCL for use in the ICAM-1 immunoassay SOP. In turn, the O.D. of 1/50 dilution MCL was the single largest factor for determining the range of ICAM-1 standard curve concentrations, following the logic that a 1/50 dilution MCL sample should lie near the midpoint of the standard curve. It is noteworthy that Triton X-100 concentrations within a wide range did not affect significantly the quantitation of ICAM-1 by immunoassay, which provided accuracy for other measurements within that acceptable range of detergent concentration. CDER guidelines (26) include "±15% of normal value" for accuracy. Guidelines for complex biological products have not been suggested. As a result of these investigations, we determined that 80 to 120% of the target value, reflecting the range of recoveries, was an accurate range of quantitative detection for the ICAM-1 immunoassay when used with the complex biological product MCL.

Second, we determined precision. The two commercial immunoassays tested herein possessed precision when used with MCL. Using an assay procedure that we propose for the ICAM-1 immunoassay, precision was determined using a combination of measurements: repeatability, system pre-

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

cision, and intermediate precision. Measurements of precision are reflected in %C.V. of the mean of results.(26,27) For ICAM-1 content of MCL extracts, precision as %C.V. was 3.3 to 9.9% (repeatability), and 10.4% (intermediate precision). This precision compares favorably with intermediate precisions obtained by other investigators using ELISA methods, including 0.39 to 11.1% for a plasminogen activator ELISA (28), 7.6 to 14.8% for human epidermal growth factor (29), 4.4 to 7.7% for thrombospondin ELISA (20), 6.4 to 10.7% for an osteocalcin ELISA in rat serum (22), or 2.9 to 4.2% in a plasma homocytesine ELISA.(21) Our highest ICAM-1 C.V. was 10.4% which is considerably less than the 20% recommended by Findlay (30), and within the 15% upper limit suggested by CDER (26) for bioanalytical methods.

System precision (intra-assay precision) of ICAM-1 immunoassay was measured at 9.1%, which is slightly higher than other reported CVs for system precision, which include 5.16 to 7.24% for a plasminogen activator ELISA (28), less than 7.5% for thrombospondin ELISA (20), 4.6 to 6.1% for an osteocalcin ELISA in rat serum (22), or 0.3 to 2.8% for a plasma homocytesine ELISA.(21)

Limits for detection of ICAM-1 were tested for agreement with manufacturer's data. LOD was 1.0 ng/mL. LOQ was determined using an acceptance criterion of 80 to 120% accuracy. Samples of 2.4 ng/mL achieved 80% accuracy. Using other elements of validation, the ICAM-1 immunoassay was robust as judged by temperature of incubation, time period of mixing by vortex, type of mechanical pipet used, and cycles of washing the assay plate.

Other elements of assay validation were investigated. Our calibration curves (standard curves) were linear, although some ELISA standard curves are non linear.(30) Linearity of our standard curves may have been a result of a lessened range of concentrations for both the ICAM-1 and MIA ELISAs. For stability, room temperature storage of MCL in 0.8% Triton X-100, after centrifugation, provided an ICAM-1 assay result similar to that of storage at 4°C. This stability of epitopes ensures accurate quantitation over the period of 5 h tested. CDER guidelines (26) suggest that stability to freeze-thaw cycles can be determined by submitting the test substance to −20°C then thawing for up to 3 cycles, followed by testing. For MCL; quantitation of ICAM-1 in MCL was lessened after the second cycle of freeze/thaw. Even more sensitive to freeze/thaw cycles was commercial recombinant ICAM-1, the detection of which was lessened after the first freeze/thaw cycle.

For the MIA immunoassay, accuracy was measured by testing the range of sample dilutions, linearity of sample dilutions, and by spiking, using the technique of standard addition. Similar to the strategy for adaptation of the ICAM-1 immunoassay, a range of sample dilutions was tested

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

for evidence of accurate immunodetection within the MCL matrix. The most linear range of MCL dilutions that of DF 4, 5, and 6, had an r^2 of 0.99. Standard additions had most accuracy (99.0 to 111%) using a 1/5 dilution of MCL. A dilution to 1/5, therefore, became the preferred dilution of MCL for MIA immunoassay.

MIA precision was based upon an intermediate precision (%C.V.) of 19.8%, which is a value very close to the 20% limit C.V. recommended by Findlay (30), and greater than the 15% upper limit suggested by CDER (26) for bioanalytical methods.

If it is desired that antigen content be related to protein content, we found that the BCA protein assay is accurate using the same Triton X-100 extracts that were tested by immunoassays. The BCA protein assay (15) quantifies color formation due to dye complexes with cysteine, cystine, tryptophan, tyrosine, and peptide bonds (16), but can suffer interference due to compounds which include the reducing agent dithiothreitol. Minimal alteration of BCA signal by Triton X-100 has been described.(31) Spiking of bovine serum albumin into Triton X-100 extracts confirmed that Triton X-100 did not alter accuracy of the BCA assay. Our MCL test product had about 2.3 mg/mL protein. Although dilutions of 1/4 through 1/7 resulted in similar total protein content with a 3.7%C.V., we found that high concentration spikes ($>325\,\mu g/mL$) of BSA were detected more accurately than low concentration spikes into more diluted 1/7 than less diluted 1/6 samples. It is possible that lipid or other cellular constituents may interfere with accurate detection of added protein.

We found that Triton X-100 was permissive for binding of antibodies to ICAM-1 and to MIA over a range of concentrations. For our purposes, Triton X-100 was selected after testing several detergents. Triton X-100 binds hydrophobically to proteins (32), but does not promote denaturation.(33) Triton X-100 has allowed native ligand binding in biological systems such as serotonin receptors in the range of 0.01 to 0.1% Triton X-100 (34), gamma-aminobutyric acid (GABA) receptors (35), and estrogen receptors (36) without increasing non-specific binding. Concentrations above 0.1% have caused loss of binding (36,37), and 0.2% Triton X-100 solutions have lessened insulin receptor binding.(38) Triton will solubilize eukaryotic membranes.(37)

In summary, during the developmental stage of immunoassay adaptation for use with a complex biological substance, we found that tests of accuracy had the greatest value. Not only were several dilutions of MCL tested (range of the sample), but ''spiking'' experiments were performed to determine which of those dilutions would be most accurate. In fact, four aspects of accuracy were tested for ICAM-1 immunoassay, and three for MIA: spiked recovery (ICAM-1 only), standard additions, range of the

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

sample, linearity of the sample. After accurate dilutions were determined, other features of the assay could be solidified, such as range of the standard curve, precision, establishment of detergent concentrations permissive for antibody binding at the most accurate dilution of sample, stability, and robustness.

ACKNOWLEDGMENTS

The authors would like to thank Vicki Archer for her skills and patience in the preparation of this manuscript and William Pfeffer for parallel line analysis.

REFERENCES

1. Johnson, J.P.; Stade, B.G.; Holzmann, B.; Schwable, W.; Riethmuller, G. De Novo Expression of Intercellular-Adhesion Molecule 1 in Melanoma Correlates With Increased Risk of Metastasis. Proc. Natl. Acad. Sci. USA **1989**, *86* (2), 641–644.
2. Denton, K.J.; Stretch, J.R.; Gatter, K.C.; Harris, A.L. A Study of Adhesion Molecules as Markers of Progression in Malignant Melanoma. J. Pathol. **1992**, *167* (2), 187–191.
3. Altomonte, M.; Gloghini, A.; Bertola, G.; Gasparollo, A.; Carbone, A.; Ferrone, S.; Maio, M. Differential Expression of Cell Adhesion Molecules CD54/CD11a and CD58/CD2 by Human Melanoma Cells and Functional Role in Their Interaction With Cytotoxic Cells. Cancer Res. **1993**, *53* (14), 3343–3348.
4. Kageshita, T.; Yoshii, A.; Kimura, T.; Kuriya, N.; Ono, T.; Tsujisaki, M.; Imai, K.; Ferrone, S. Clinical Relevance of ICAM-1 Expression in Primary Lesions and Serum of Patients With Malignant Melanoma. Cancer Res. **1993**, *53* (20), 4927–4932.
5. Becker, J.C.; Dummer, R.; Schwinn, A.; Hartmann, A.A.; Burg, G. Circulating Intercellular Adhesion Molecule-1 in Melanoma Patients: Induction by Interleukin-2 Therapy. J. Immunother **1992**, *12* (2), 147–150.
6. Garbe, C.; Krasagakis, K. Effects of Interferons and Cytokines on Melanoma Cells. J. Invest. Dermatol. **1993**, *100* (2 Suppl), 239S–244S.
7. Maio, M.; Gulwani, B.; Langer, J.A.; Kerbel, R.S.; Duigou, G.J.; Fisher, P.B.; Ferrone, S. Modulation by Interferons of HLA Antigen, High-Molecular-Weight Melanoma Associated Antigen, and Intercellular Adhesion Molecule 1 Expression by Cultured Melanoma

Cells With Different Metastatic Potential. Cancer Res. **1989**, *49* (11), 2980–2987.
8. Scheibenbogen, C.; Keilholz, U.; Meuer, S.; Dengler, T.; Tilgen, W.; Hunstein, W. Differential Expression and Release of LFA-3 and ICAM-1 in Human Melanoma Cell Lines. Int. J. Cancer **1993**, *54* (3), 494–498.
9. Blesch, A.; Bosserhoff, A.K.; Apfel, R.; Behl, C.; Hessdoerfer, B.; Schmitt, A.; Jachimczak, P.; Lottspeich, F.; Buettner, R.; Bogdahn, U. Cloning of a Novel Malignant Melanoma-Derived Growth-Regulatory Protein, MIA. Cancer Res. **1994**, *54* (21), 5695–5701.
10. Bosserhoff, A.K.; Moser, M.; Hein, R.; Landthaler, M.; Buettner, R. In Situ Expression Patterns of Melanoma-Inhibiting Activity (MIA) in Melanomas and Breast Cancers. J. Pathol. **1999**, *187* (4), 446–454.
11. Muller-Ladner, U.; Bosserhoff, A.K.; Dreher, K.; Hein, R.; Neidhart, M.; Gay, S.; Scholmerich, J.; Buettner, R.; Lang, B. MIA (Melanoma Inhibitory Activity): A Potential Serum Marker for Rheumatoid Arthritis. Rheumatology (Oxford) **1999**, *38* (2), 148–154.
12. Wagner, V.; Rudi, J.; Naher, H.; Stremmel, W. Seropositivity for MIA and S100 in Patients With Gastrointestinal Carcinomas (In Process Citation). Med. Oncol. **2000**, *17* (1), 35–38.
13. Bosserhoff, A.K.; Hein, R.; Bogdahn, U.; Buettner, R. Structure and Promoter Analysis of the Gene Encoding the Human Melanoma-Inhibiting Protein MIA. J. Biol. Chem. **1996**, *271* (1), 490–495.
14. van Groningen, J.J.; Bloemers, H.P.; Swart, G.W. Identification of Melanoma Inhibitory Activity and Other Differentially Expressed Messenger RNAs in Human Melanoma Cell Lines With Different Metastatic Capacity by Messenger RNA Differential Display. Cancer Res. **1995**, *55* (24), 6237–6243.
15. Smith, P.K.; Krohn, R.I.; Hermanson, G.T.; Mallia, A.K.; Gartner, F.H.; Provenzano, M.D.; Fujimoto, E.K.; Goeke, N.M.; Olson, B.J.; Klenk, D.C. Measurement of Protein Using Bicinchoninic Acid (published erratum appears in Anal. Biochem. 1987 May 15; *163* (1), 279). Anal. Biochem. **1985**, *150* (1), 76–85.
16. Wiechelman, K.J.; Braun, R.D.; Fitzpatrick, J.D. Investigation of the Bicinchoninic Acid Protein Assay: Identification of the Groups Responsible for Color Formation. Anal. Biochem. **1988**, *175* (1), 231–237.
17. Mitchell, M.S. Perspective on Allogeneic Melanoma Lysates in Active Specific Immunotherapy. Semin. Oncol. **1998**, *25* (6), 623–635.
18. Massart, D.L.; Smeyers-Verbeke, J.; Rius, F.X. Method Validation: Software to Compare the Slopes of Two Calibration Lines With Different Residual Variance. Trends Anal. Chem. **1989**, *8* (2), 49–50.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

19. *ICH Expert Working Group. Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*. ICH Steering Comm. 1999.
20. Hayden, K.; Tetlow, L.; Byrne, G.; Bundred, N. Radioimmunoassay for the Measurement of Thrombospondin in Plasma and Breast Cyst Fluid: Validation and Clinical Application. Ann. Clin. Biochem. **2000**, *37* (Pt 3), 319–325.
21. Marangon, K.; O'Byrne, D.; Devaraj, S.; Jialal, I. Validation of an Immunoassay for Measurement of Plasma Total Homocysteine. Am. J. Clin. Pathol. **1999**, *112* (6), 757–762.
22. Srivastava, A.K.; Bhattacharyya, S.; Castillo, G.; Wergedal, J.; Mohan, S.; Baylink, D.J. Development and Application of a Serum C-Telopeptide and Osteocalcin Assay to Measure Bone Turnover in an Ovariectomized Rat Model. Calcif. Tissue Int. **2000**, *66* (6), 435–442.
23. MacFarlane, G.D.; Scheller, D.G.; Ersfeld, D.L.; Shaw, L.M.; Venkatarmanan, R.; Sarkozi, L.; Mullins, R.; Fox, B.R. Analytical Validation of the PRO-Trac II ELISA for the Determination of Tacrolimus (FK506) in Whole Blood. Clin. Chem. **1999**, *45* (9), 1449–1458.
24. Fraunberger, P.; Pfeiffer, M.; Cremer, P.; Holler, E.; Nagel, D.; Dehart, I.; Thein, M.; Walli, A.K.; Seidel, D. Validation of an Automated Enzyme Immunoassay for Interleukin-6 for Routine Clinical Use. Clin. Chem. Lab. Med. **1998**, *36* (10), 797–801.
25. Mitchell, J.M.; Yee, A.J.; McNab, W.B.; Griffiths, M.W.; McEwen, S.A. Validation of the LacTek Test Applied to Spiked Extracts of Tissue Samples: Determination of Performance Characteristics. J. AOAC Int. **1999**, *82* (1), 79–84.
26. *Guidance for Industry Bioanalytical Methods Validation for Human Studies*. U.S. Department of Health and Human Services-Food and Drug Administration Center for Drug Evaluation and Research (CDER), 1998.
27. Little, L.E. Validation of Immunobiological and Biological Assays. Biopharm. Drug Dispos. **1995**, *8* (9), 36–42.
28. Stouffer, B.; Habte, S.; Vachharajani, N.; Tay, L. Validation of an ELISA for the Quantitation of Lanoteplase, A Novel Plasminogen Activator. J. Immunoassay **1999**, *20* (4), 237–252.
29. Sizemore, N.; Dudeck, R.C.; Barksdale, C.M.; Nordblom, G.D.; Mueller, W.T.; McConnell, P.; Wright, D.S.; Guglietta, A.; Kuo, B.S. Development and Validation of Two Solid-Phase Enzyme immunoassays (ELISA) for Quantitation of Human Epidermal Growth Factors (hEGFs). Pharm. Res. **1996**, *13* (7), 1088–1094.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

30. Findlay, J.W.; Smith, W.C.; Lee, J.W.; Nordblom, G.D.; Das, I.; DeSilva, B.S.; Khan, M.N.; Bowsher, R.R. Validation of Immunoassays for Bioanalysis: A Pharmaceutical Industry Perspective. J. Pharm. Biomed. Anal. **2000**, *21* (6), 1249–1273.
31. Gates, R.E. Elimination of Interfering Substances in the Presence of Detergent in the Bicinchoninic Acid Protein Assay. Anal. Biochem. **1991**, *196* (2), 290–295.
32. Robinson, N.C.; Tanford, C. The Binding of Deoxycholate, Triton X-100, Sodium Dodecyl Sulfate, and Phosphatidylcholine Vesicles to Cytochrome b5. Biochemistry **1975**, *14* (2), 369–378.
33. Makino, S.; Reynolds, J.A.; Tanford, C. The Binding of Deoxycholate and Triton X-100 to Proteins. J. Biol. Chem. **1973**, *248* (14), 4926–4932.
34. Une, T.; Furukawa, K.; Komiya, M. Improvement of 5-HT3 Receptor Binding Assay: Enhancement of Specific [3H]quipazine Binding With Triton X-100-Treated Membranes From Rat Cortex. Jpn. J. Pharmacol. **1991**, *57* (2), 197–203.
35. Ishige, K.; Ito, Y.; Fukuda, H. Differential Effects of Triton X-100 on Ligand Binding to GABAB Receptors in Mouse Cerebral Cortex, Cerebellum and Whole Brain. Gen. Pharmacol. **1993**, *24* (6), 1533–1540.
36. Pavlik, E.J.; Rutledge, S. Estrogen-Binding Properties of Cytoplasmic and Nuclear Estrogen Receptors in the Presence of Triton X-100. J. Steroid Biochem. **1980**, *13* (12), 1433–1441.
37. Duncan, R.; Ferruti, P.; Sgouras, D.; Tuboku-Metzger, A.; Ranucci, E.; Bignotti, F.A. Polymer-Triton X-100 Conjugate Capable of pH-Dependent Red Blood Cell Lysis: A Model System Illustrating the Possibility of Drug Delivery Within Acidic Intracellular Compartments. J. Drug Target **1994**, *2* (4), 341–347.
38. Hwang, D.L.; Tay, Y.C.; Barseghian, G.; Roitman, A.; Lev-Ran, A. Effect of Triton X-100 on Insulin and Epidermal Growth Factor Receptor Binding and Autophosphorylation in Golgi Fractions and Partially Purified Receptors From Rat Liver. J. Recept. Res. **1985**, *5* (5–6), 367–380.

Received July 19, 2001
Accepted August 22, 2001
Manuscript 3046

Request Permission or Order Reprints Instantly!