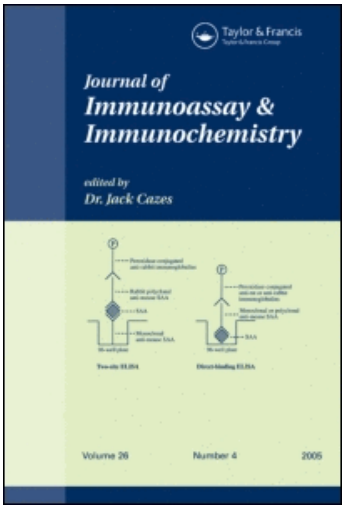

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A New Competitive Enzyme Linked Immunosorbent Assay (MRP83-CA15-3) for MUC1 Measurement in Breast Cancer

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Abstract: A new competitive enzyme linked immunosorbent assay was developed in this study. Monoclonal antibody (PR81) against the tandem repeat of the core protein was prepared, characterized, purified, and conjugated to HRP. This antibody exhibited no cross reactions with proteins such as bovine serum albumin, keyhole limpet homocyanin, human serum albumin, casein, human milk fat globin (HMFG), and peptone. The native cancerous MUC1 protein was purified from ascites fluid of a patient suffering from small cell lung carcinoma by immunoaffinity chromatography and used as a standard preparation in the assay buffer. The standard curve was constructed following a competitive procedure in the range of 0–200 U/mL. The level of MUC1 in normal and cancerous samples was compared following this procedure and using available CA15-3 EIA (Can Ag), as well as LIAISON CA15-3 commercial kits. The correlation coefficient between the procedure reported in this work (MRP83-CA15-3) and CA15-3 EIA (Can Ag) was 0.68 and was 0.95 with the LIAISON CA15-3 kit. We concluded that the present assay can detect MUC1 in breast cancer patients with great sensitivity and accuracy.

Keywords: MUC1, ELISA, Breast cancer

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INTRODUCTION

The MUC1 gene encodes a type–I transmembrane glycoprotein that is expressed on the apical surface of most simple epithelia, including mammary glands, female reproductive tract, lung, kidney, stomach, gall bladder, and pancreas, as well as some non epithelial cell types.[1] Functions attributed to MUC1 include those generally associated with mucins, such as lubrication and hydration of cell surfaces, as well as protection from microorganisms and degradative enzymes.[2] MUC1 is anchored to the cell surface in an elongated form, extending beyond most other cell surface proteins. It was also described as a normal component in human milk fat globulins.[3] Another name for MUC1 is episialine, polymorphic epithelial mucine (PEM), CA15-3, CA27.29, and a few others.[4–6] This glycoprotein has a tandem repeat region of 20 amino acids with polymorphism in the number of repeats and the extent of O-linked carbohydrates rich in galactosamine, glucosamine, galactose, fucose, and sialic acid.[7,8] In normal breast tissue, MUC1 is expressed on the apical surface of epithelial cells in the ducts and acini from which the molecule is shed via milk fat globules and in soluble form into milk. In the case of tumor cells, polarization is lost and this altered cell surface expression, coupled with the disruption of the normal tissue architecture caused by the growing tumor, allows the MUC1 mucine to be shed into the circulation, where it can be measured by means of immunoassays.[9] The measurement of MUC1 is not recommended as a screening procedure to detect or confirm breast cancer in the general population. However, it is used to detect distant recurrence after primary treatment in breast cancer patients, where it often increases before clinical symptoms become evident.[10–12] In an ISOBM MUC1 workshop, 30 monoclonal antibodies (mAb) directed against the hydrophilic epitope (PDTR) and mainly carbohydrate independent epitope of MUC1 were reported and described. However, five monoclonal antibodies were directed against other epitopes in the tandem repeats, while sixteen mAbs were carbohydrate dependent and, finally, two mAbs were shown to be bound to the non-repeat peptide core.[13] Further investigation by Norum et al. showed that only few antibodies prepared against MUC1 may successfully be used in immunoassays.[14] In this study we set up an enzyme linked immunosorbent assay for MUC1 using monoclonal antibody (PR81) which recognized the tandem 20 amino acid repeat of MUC1.[15]

EXPERIMENTAL

Production of mAb PR81

The hybridoma producing PR81 mAb was developed according to the method of Kohler and Milstein,[16] using homogenized malignant breast

tissue (stage I, II) as an immunogen in female Balb/c mice. Hybridomas were established from the fusion of mouse spleen lymphocytes and sp2/0 myeloma cells. The PR81 clone produced an IgG1 antibody directed towards the tandem repeat (20 amino acid) of MUC1. This antibody was found to be highly reactive towards native MUC1, as well as cell line expressing MUC1.[15]

Large Scale Production and Purification of mAb PR81

The hybridoma cell line was cultured in RPMI1640 medium; supernatants of this hybridoma cell line were collected and the mAb PR81 was concentrated, partially, by ultrafiltration, salting out with ammonium sulfate and, finally, purified by affinity chromatography (protein G coupled to CNBr activated sepharose). mAb PR81 was also produced on a large scale by using a Cell Line1000 (integra bioscience) bioreactor. This method does not require any down stream processing, such as tedious purification steps; the yield in this method was found to be around 10 mg of pure protein per week.[17]

Working Standards

To prepare the standard MUC1 solution, the native cancerous antigen was purified from ascites fluid of a patient with an aggressive small-cell lung carcinoma and metastasis to the peritoneum, by an antibody-sepharose affinity (immunoaffinity) column as follows. The immunoaffinity column was prepared by coupling 2 mg of polyclonal anti MUC1 peptide antibody to 0.8 g of cyanogen bromide-activated sepharose, as described by the manufacturer. The cancerous MUC1 was purified from diluted ascitic fluid using this column, eluted by acidic pH. The concentration of MUC1 in the eluted marerial was measured by LIAISON CA15-3 and was diluted in assay buffer to concentrations of 12.5, 25, 50, 100, and 200 U/mL and were used as working standards.

Reactivity of mAb PR81 Towards MUC1

Purified MUC1 was coated onto the wells of microtiter plates (0.2, 0.4, and 0.6 μg/well) maintained at 37°C, overnight. Bovine serum albumin was used as negative control. The contents of the wells were emptied, washed, and blocked with a 1% solution of BSA in PBS (10 mM, pH 7.2) for 1 h at 37°C. At the end of the incubation time, the wells were washed and added with dilutions of mAb PR81, and was incubated at 37°C for 2 h. The contents of the wells were emptied, wells were washed, added with proper dilution of rabbit anti mouse conjugated to HRP, and incubated at 37°C for

1 h. Finally, wells were washed, 50 μL of substrate tetramethylbenzidine (TMB) was added, and the mixture was incubated for 5–10 min. The enzyme reaction was terminated and the color development was measured at 450 nm.

Titration of mAb PR81 and MUC1

Purified MUC1 (7, 15, 30, 60, 125, and 250 ng/well) and BSA (250 ng/well) were coated onto the wells of microtiter plates, in duplicate, at 37°C overnight. The contents of the wells were emptied, washed, and blocked with a 1% solution of BSA in PBS (10 mM, pH 7.2) for 1 h at 37°C. At the end of the blocking time, the wells were washed and 50 μL of mAb PR81- HRP conjugated (in different dilutions 1:100, 1:250, 1:500, and 1:1000) were added to each well. The contents of the wells were incubated at 37°C for 1 h, washed, and mixed with 50 μL of substrate TMB and was further incubated for 5–10 min. The enzyme reaction was terminated and the color development was measured at 450 nm.

Competitive ELISA for Standard Curve

The MUC1 ELISA assay was the competitive assay based on inhibition of antibody binding to immobilized MUC1. In order to evaluate the standard curve, purified MUC1 was coated onto the wells of microtiter plates (15 ng/well) in PBS (10 mM, pH 7.2) at 37°C overnight. The contents of the wells were emptied, washed, and blocked with a 1% solution of BSA in PBS (10 mM, pH 7.2) for 1 h at 37°C. At the end of incubation time, wells were washed and 50 μL/well of MUC1 standards (0, 12.5, 25, 50, 100, and 200 U/mL) and 50 μL/well of 1:250 diluted mAb PR81-HRP conjugate prepared in EIA buffer (PBS 10 mM, 0.1% BSA) was added. The contents of the wells were incubated at 37°C for 45 min, washed, mixed with 50 μL of substrate TMB, and incubated for 5–10 min. The enzyme reaction was terminated and the color development was measured at 450 nm.

CA15-3 EIA (CanAg)

The CA15-3 EIA is a manual enzyme immunometric assay (sandwich principles), using antibody M695 which is coated onto the solid phase, acting as capture antibody and HRP labeled antibody M552 which is used as the tracer antibody. Serum samples were tested as recommended by the manufacturer; working standards ranged from 0 to 250 U/mL. Serum samples of the values above 250 U/mL were diluted with sample dilution buffer and MUC1 concentration was measured.

LIAISON CA15-3

LIAISON CA15-3 is a two-site immunoluminometric assay (sandwich principles). The two different highly specific monoclonal antibodies were 115D8 coated onto the solid phase magnetic particles acting as capture antibody and DF3 antibody labeled with isoluminol acting as tracer antibody. Serum samples were tested as recommended by the manufacturer; the working standards ranged from 0.3 to 1000 U/mL.

MUC1 Concentration Measurement by Competitive ELISA (MRP83-CA15-3)

A total of 83 serum samples (72 breast cancer patients and 11 normal female blood donors) were analyzed, having been diluted to 1 : 200 using dilution buffer containing 0.001 M 8-anilino-naphthalenesulphonic acid (8-ANS) by the methods presented in this work, as well as by CA15-3 EIA (CanAg). Another 34 serum samples were analyzed by our method, as well as by LIAISON CA15-3, and the results were compared.

RESULTS

Figure 1 shows the reactivity of mAb PR81 towards the native cancerous MUC1 purified from metastasis ascites fluid, following the ELISA procedure represented here. mAb PR81, produced from hybridoma cell line, showed high immunoreactivity towards MUC1 protein. The highest optical density difference from that of the nonspecific (reactivity of anti MUC1 with corresponding concentration of BSA) was found at an antibody concentration of

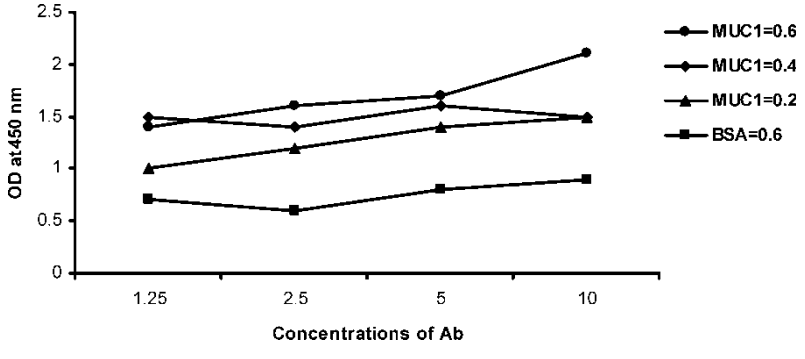

Figure 1. Reactivity of mAb PR81 towards cancerous MUC1 purified from ascites fluid. All concentrations (antibody, MUC1 and BSA) are in μg/well, Assays were performed in duplicate.

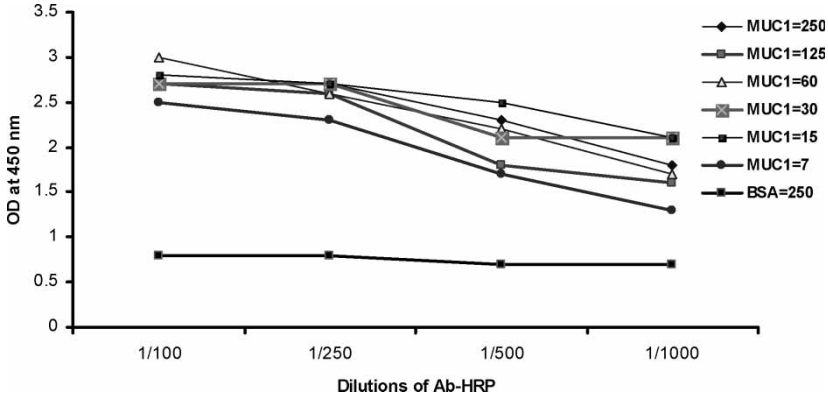

Figure 2. Titration of PR81 mAb – HRP and MUC1 by ELISA, (MUC1 and BSA concentrations are μg/well). All assays were performed in duplicate.

10 μg and antigen concentration of 0.6 μg MUC1 per well. Titration analysis of HRP labeled mAb PR81 and MUC1 was performed using an ELISA procedure and is shown in Figure 2. The proper concentration of MUC1 was found to be 15 ng/well and the proper concentration of mAb-HRP was found to be 1 : 250. Figure 3 shows the specificity of mAb PR81 that was examined by a competitive ELISA procedure using several common proteins (BSA, casein, KLH, gelatin, HMFG, and peptone). No significant cross reactivity of mAb PR81 with these proteins could be detected in the ELISA experiments.

To construct the standard curve, MUC1 standards were prepared and tested by the competitive ELISA procedure. MUC1 standards were prepared in a 1 : 200 diluted normal male serum that was immuno stripped off from possible endogenous MUC1.[18] The standard preparations were adjusted,

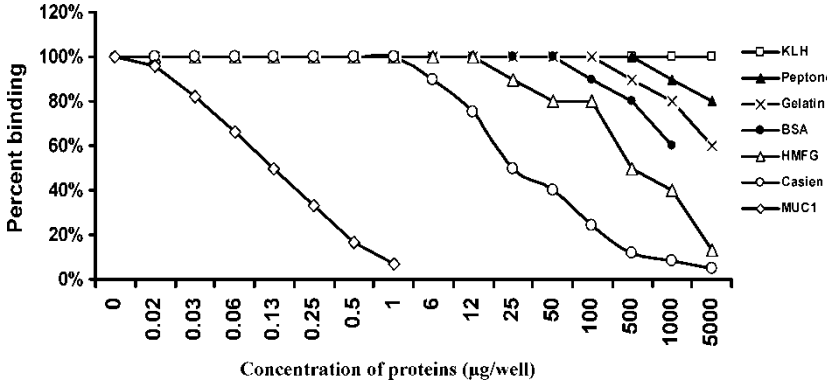

Figure 3. Cross reactivity of PR81 mAb with proteins (BSA, casein, KLH, gelatin, HMFG, and peptone). All assays were performed in duplicate.

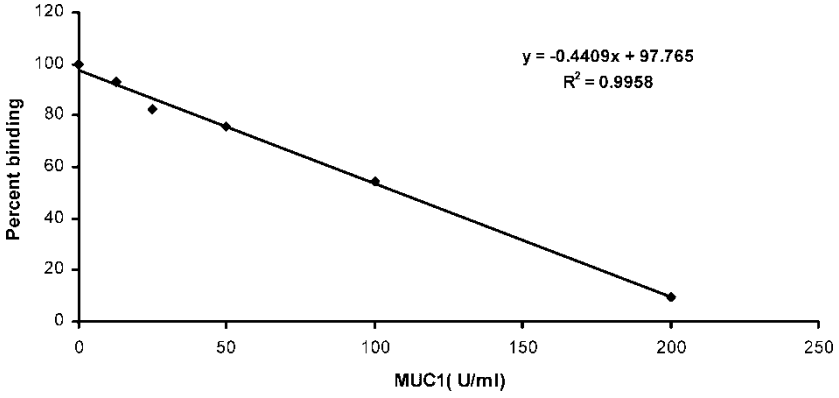

Figure 4. The pooled result from five-standard curve for MUC1 by competitive ELISA.

and the concentrations were compared following a LIAISON CA15-3 assay. The results are shown in Fig. 4, where the standards ranged from 0 to 200 U/mL in a linear manner. The affinity constant of mAb PR81 towards MUC1 was also calculated according to the method described by Beaty et al.;[19] it was found to be $Ka = 2 \times 10^8\,M^{-1}$. To obtain the proper dilution of serum, three predetermined samples in the range of 30, 56, and 83 U/mL were selected. MUC1 contents of these samples were measured using our competitive ELISA, after diluting them with 8-ANS (10^{-3} mol in 10 mM PBS, pH = 7.2). The greatest difference in optical densities were obtained in the 1:200 dilution of serum sample shown in Figure 5.

For the quality control of this new assay, we examined inter- and intra-assay variation and parallelism. For the inter- and intra-validation, three

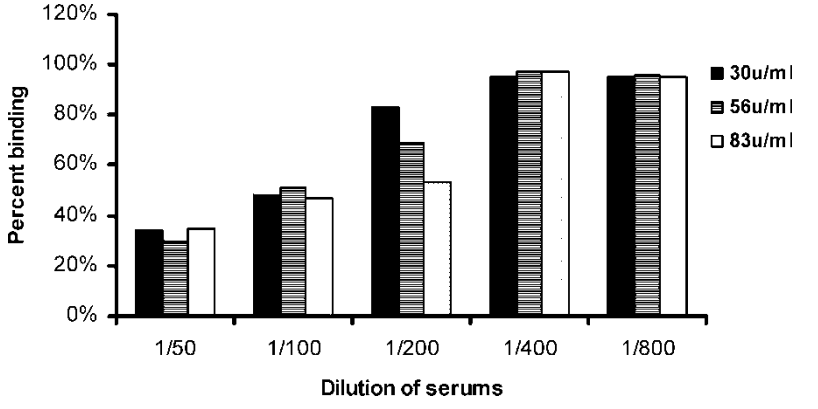

Figure 5. Determination of proper dilution of serums in competitive ELISA. Three serum samples, 30, 56, and 83 were tested. All assays were performed in duplicate.

Table 1. Inter- and intra-assay validation for three samples (low, medium and high) measured for concentration of MUC1 in number of occasions

EN	N	Low mean ± SD	CV (%)	Medium mean ± SD	CV (%)	High mean ± SD	CV (%)
Intra-assay							
1	4	20.25 ± 1.5	7.4	56.5 ± 2.3	4	103.5 ± 2.7	2.6
2	4	21.5 ± 1.8	8.3	55 ± 1.8	3.2	100 ± 5.8	5.8
3	4	20.5 ± 1.9	9.2	52.5 ± 2.7	5.1	103.5 ± 2.9	2.8
4	4	22 ± 1.5	6.8	54 ± 1.8	3.3	100 ± 3.16	3.16
Inter-assay							
	4 × 4	21 ± 1.7	8	54.5 ± 2.1	3.9	101.75 ± 3.6	3.5

samples, containing MUC1 of high, medium, and low concentrations, i.e., 104, 52, and 23 U/mL, respectively, were selected, and MUC1 contents of these samples were measured using our competitive assay (four times in four different days). Results shown in Table 1 indicated that CVs for intra- and inter-assays were not more than 10. For assessment of parallelism, a test was performed in this assay with a sample containing 150 U/mL MUC1; it was diluted to 1 : 2, 1 : 4, and 1 : 8. The MUC1 contents of these dilutions were measured using our competitive assay and the results were compared with expected values. Figure 6 shows the standard curve and sample dilutions; the two lines are parallel ($\alpha 1 = 0.44$ and $\alpha 2 = 0.45$). Figures 7 and 8 show the comparison of CA15-3 EIA (Can Ag) and LIAISON CA15-3 with the new competitive assay. There was a good correlation between the new competitive assay and LIAISON CA15-3 with a Spearman rank correlation coefficient of 0.95. However, the correlation between CA15-3 EIA (Can Ag) and new competitive assay reported here was lower (with a Spearman rank correlation coefficient of 0.68).

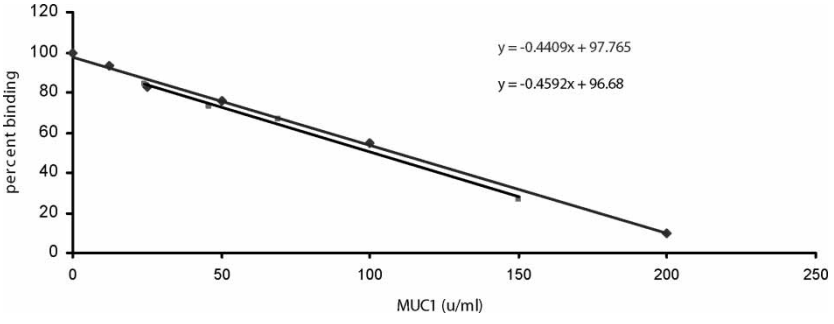

Figure 6. Parallelism test for standards and serial dilution of serum sample 150 U/ml. Lines are parallel ($\alpha 1 = 0.44$ for standards and $\alpha 2 = 0.45$ for serum sample 150 U/mL). All assays were performed in duplicate.

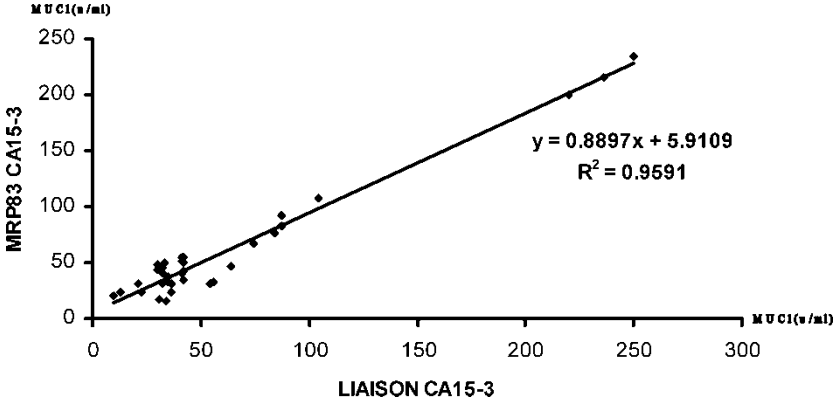

Figure 7. Correlation of the results obtained by measuring MUC1 in 32 serum samples by LIAISON CA15-3 & MRP83 CA15-3 kits (r = 0.95).

DISCUSSION

A new competitive enzyme linked immunosorbent assay using a monoclonal antibody which is specific for the 20 amino acid tandem repeat of MUC1 was developed in this work. Few commercial immunoassays for MUC1 using sandwich or competitive format have been reported. The sandwich assays are mainly based on coating the so called capture antibody directly or indirectly (via biotin-avidin interaction) onto the wells of a microtiter plate. The antigen is then added, followed by the addition of antibody labeled enzyme. The labeled antibody, in some cases, is the same as the capture antibody.[14] However, mainly antibodies against a different epitope, either protein or glycan of MUC1, are used to detect the captured antigen. The combinations of capture and labeled antibodies were tested by Bjerner et al.[7] The

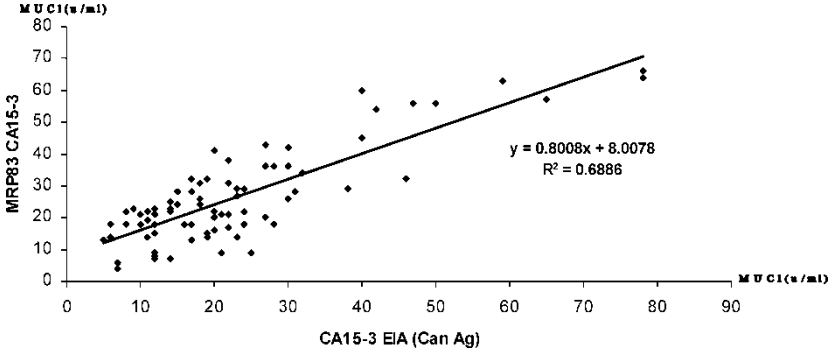

Figure 8. Correlation of the results obtained by measuring MUC1 in 83 serum samples by CA15-3 EIA (CanAg) & MRP83 CA15-3 kits (r = 0.68).

main finding of this study was that three combinations of antibodies (Ma695/Ma552, 115D8/DF3, and BC2/G.P1.4) would best suit the MUC1 detection in a sandwich immunoassay. These three combinations were mainly used in commercially available kits for MUC1 measurement. In the case of inhibition assays, mainly antibodies such as BC2, Ma695, and B27.29 were used.[14] A recent finding suggested that the sensitivity and stage dependency of MUC1 immunoassays is very much dependent on the quality of antibody used in assay design.[14] In this study, a novel antibody (PR81), which was developed and characterized by us, was used.[15] The antibody showed high binding affinities towards the MCF-7 cell line, cancerous tissues,[15] and native MUC1 purified from ascites fluid of a patient suffering from small cell lung carcinoma ($Ka = 2 \times 10^8\,M^{-1}$). Our initial experiments showed that, in order to accurately measure the content of MUC1 in samples, they should be diluted to omit the contribution of the high protein content of serum. However, due to the high sensitivity (5 U/mL), normal samples were accurately measured and found to be within the limit of 30 U/mL. For patients' samples, however, higher values were correctly measured. Validation criteria, such as inter- and intra-assay validation, test of parallelism, and recoveries in three ranges of low, medium, and high MUC1 containing samples were all within the limit of acceptable ranges. Finally, our test was compared with two commercially available tests (CA15-3 EIA (Can Ag) and LIAISON CA15-3). The result of correlation studies showed a high correlation between the values obtained from LIAISON CA15-3 measurement, which is essentially a immunoluminometric assay, with higher sensitivity compared to CA15-3 EIA (Can Ag) ($r^2 = 0.95$). It was concluded that the present competitive assay would be successfully used for detecting MUC1 in serum samples of patients suffering from breast cancer.

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