Development of Two ELISA for Estrogen and Progesterone Receptor With Sufficient Sensitivity for Fine Needle Aspirate and Core Biopsy

C.-F. Sun,¹ T.-L. Wu,¹ K.-C. Tsao,¹ and J.T. Wu^{2*}

¹Department of Pathology, Chang Gung Memorial Hospital, Taipei, Taiwan

²Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, Utah

The quantitative determination of estrogen and progesterone receptors (PR and ER) in breast tumor cytosol has been routinely performed in clinical laboratories to aid in the selection between hormonal and chemotherapy and also to predict prognosis. However, the small amount of tissue available from the increasingly popular fine-needle aspiration and core biopsies from breast cancer patients requires more sensitive immunoassays for receptor quantification. We have developed two sensitive immunoassays for ER and PR on microplate with the use of recently available anti-ER

and anti-PR antibodies of higher affinity and a powerful signal magnification agent, namely Amdex. The calibrator was a pooled breast tumor cytosol used as calibrator and calibrated against Abbott kits. The protein concentration of the cytosol and the upper normal cutoffs for our assays were reduced to approximately 0.2 mg/mL and 3 fmol/0.2 mg/mL, respectively. Both assays have sensitivities close to 1 fmol/mL, which are sufficiently sensitive for the receptor quantification in fine-needle aspiration biopsies and cord biopsies of breast tumor. *J. Clin. Lab. Anal.* 15:138–143, 2001. © 2001 Wiley-Liss, Inc.

Key words: estrogen receptor; progesterone receptor; ELISA, fine-needle aspiration biopsy; breast tumor cytosol

INTRODUCTION

The measurement of estrogen and progesterone receptors (ER and PR) in breast tumor cytosol has served as a guide to the selection of either endocrine or chemotherapy for breast cancer patients (1,2). ER and PR are also valuable prognostic markers; they can predict the success of endocrine therapy. Positive ER and PR values predict good prognosis and longer disease-free survival time intervals (1,2). Because fine-needle aspiration biopsy (FNAB) has become an increasingly popular method to obtain tissue specimens, it is often regarded as a more accurate tool than imaging or physical examination for the diagnosis of breast lesion (3). Replacing tissue biopsy with FNAB allows patients to be evaluated every 3–6 months. The combination of FNAB and quantitative immunoassay allows not only a frequent assessment but also a more accurate prognosis to be made of the clinical status of a patient (4). However, because the amount of tissue in the FNAB and core biopsy is approximately in the 5- to 50-mg range, it is usually impossible to prepare cytosol at 1 mg/mL protein concentration to measure ER and PR as required by the Abbott kit. In other words, a 3- to 5-fold increase in assay sensitivity is needed if we hope to quantify the receptor concentration in FNAB or cord biopsy.

Recently, new antibodies for ER and PR with higher affin-

ity have become available commercially. Even though these antibodies were originally designed for immunohistochemistry, we were able to establish ER and PR enzyme-linked immunosorbent assay (ELISA) using these antibodies and a new, powerful amplification agent, namely Amdex A/S (Møllehaven, Denmark) with a much higher sensitivity than the commercial Abbott ER and PR assays. We also have further optimized various assay parameters for both ELISA, such as antibody coating, sample volume, incubation time, number of incubations, and concentration of detecting antibody and Amdex, in order to achieve a sufficient sensitivity to quantify both receptors in FNAB and core biopsy.

MATERIALS AND METHODS

The rabbit anti-ER polyclonal antibodies and biotin-mouse-anti-ER monoclonal antibody were from Zymed lab (South San Francisco, CA). Mouse monoclonal anti-ER antibody (MU 368-UC) and anti-PR antibody were also obtained from Biogenex (San Ramon, CA). Rabbit polyclonal anti-PR an-

*Correspondence to: James T. Wu, ARUP, 500 Chipeta Way, Salt Lake City, Utah 84108. E-mail: wuj@arup-lab.com

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tibodies (A0098) and mouse monoclonal anti-PR antibody (clone PR 636) for detection were purchased from DAKO Corp. (Carpinteria, CA). Amdex HRP conjugate (including high-performance streptavidin HRP conjugate or anti-mouse IgG HRP conjugate were all from Amersham Life Science (Arlington, IL). The One-Step TMB substrate was purchased from Neogen Co. (Lexington, KY). Commercial ER-EIA and PR-EIA were purchased from Abbott laboratories (Abbott Park, IL). The BCR protein assay kit was obtained from Pierce (Rockford, IL).

Pulverization

Tissue was pulverized in liquid nitrogen with a mortar and pestle, and a suspension was made by adding homogenization buffer to the pulverized powder (buffer/tissue = 1 mL/20 mg). The mixture was centrifuged in a refrigerated Beckman L7 ultracentrifuge for 30 min at 120,000g. Protein concentration of the supernatant (cytosol) was determined using a BCA protein assay kit.

A list of the composition of various buffers and solutions used in the current investigation follows.

Coating Buffer: 0.1 mol/L sodium carbonate buffer, pH 9.6;

Sample Diluent: PBS containing 1% BSA, pH 7.2;

Washing Solution: 0.05% Tween 20 in PBS, pH 7.2. Washing was always carried out with 250 μ L washing buffer three times after sample incubation, incubation with detecting antibody, and addition of signal antibody;

Blocking Solution: PBS containing 1% BSA, 0.05% casein, and 0.05% Tween 20, pH 7.2;

Homogenization Buffer: 10 mM Tris, 1.5 mM EDTA, 5.0 mM Na_2MoO_4 , and monothioglycerol (1:10 diluted, 1 μ L/1 mL buffer), pH: 7.4 ~7.5. Monothioglycerol was freshly prepared and added.

Preparation of Antibody-Coated Microplate

We usually added 100 μ L of antibody at 2 μ g/mL concentration in 0.1 mol/L sodium carbonate buffer, pH 9.6 to each well and allowed the microplate to be incubated overnight at 4°C with the cover on. After emptying the antibody solution, the microplate was washed once with 200 μ L washing solution, followed by the addition of 200 μ L blocking solution to each well. Incubation was carried out at least 6 hours at room temperature before use; otherwise, the blocking solution was left in the well during storage. The blocking solution was discarded and the plate was washed twice immediately before the assay.

ER and PR ELISA on Microplate

Assay parameters for ER ELISA are outlined in Table 1; assay parameters for PR ELISA are in Table 2. Briefly, for the PR ELISA, DAKO polyclonal antibody A0098 was used

TABLE 1. Outline of assay parameters for ER

Major step	Material	Method
Coating Ab	Zymed poly anti-ER (TE 61-0174)	100 μ L, 2 μ g/mL, 4°C overnight
Sample or calibrator + detecting Ab	90 μ L cytosol (0.2 mg/mL) + 10 μ L anti-ER (1:200) mAb from Biogenex (MU 368-UC)	Overnight, rt.
Detecting Ab	10 μ L Biogenex mAb anti-ER (1:500) + 100 μ L blocking solution	1 h, rt.
Signal Ab	100 μ L Amdex (Anti-mouse IgG-HRP) (1:3000)	1 h, rt.
Substrate	100 μ L TMB (K-blue)	10 min, rt.

Ab, antibody; rt, room temperature.

for coating the microplate and monoclonal antibody clone 636 from DAKO was used as the detection antibody. For the ER ELISA, polyclonal TE 61-0174 from Zymed was used for coating and monoclonal antibody MU368-UC from Biogenex was used as detection antibody. Amdex-HRP (anti-mouse IgG) from Amersham was used for signal amplification for both the ER and PR assay. It is important to note that instead of adjusting the cytosol protein concentration to 1 mg/mL as suggested in the traditional Abbott assays, we adjusted the protein concentration of extract from FNAB or cord biopsies to 0.2 mg/mL.

RESULTS

Selection of Antibody

Several new antibodies of higher affinity for both receptors, which were designed for immunohistochemistry, became available recently from Zymed laboratories, Biogenex, and DAKO. We coated commercially available polyclonal anti-ER and anti-PR antibodies on microplates because higher assay sensitivity is always associated with coating polyclonal antibody rather than with coating monoclonal antibody (Fig. 1). For the detecting antibody we decided to use monoclonal antibody for providing assay specificity. Even though all monoclonal antibodies were designed for immunochemistry, they all worked well for ELISA format as detecting antibodies for ER and PR assay. Based on the result of our comparison studies, as illustrated in Figure 2A, we chose DAKO monoclonal antibody for the PR assay as the detecting anti-

TABLE 2. Outline of assay parameters for PR

Major step	Material	Method
Coating Ab	100 μ L DAKO polyclonal anti-PR antibody (A0098)	100 μ L, 2 μ g/mL, 4°C overnight
Sample mAb from Biogenex	100 μ L cytosol (0.2 mg/mL)	Overnight, rt.
Detecting Ab	100 μ L DAKO monoclonal anti-PR antibody (PgR 636) (1:500)	1 h, rt.
Signal Ab	Amdex (Anti-mouse IgG-HRP) (1:2000)	1 h, rt.
Signal	100 μ L TMB (K-blue)	15 min., rt.

Fig. 1. ELISA test formats for ER and PR.

body (Fig. 1). In fact, satisfactory assay sensitivity can also be established if Biogenex monoclonal antibody is to be used. Similarly, we chose monoclonal anti-ER antibody from Biogenex as the detecting antibody for the ER ELISA.

Coating Antibody Concentration

For both ER and PR ELISA we coated various concentrations of polyclonal antibodies from 1 to 5 $\mu\text{g/mL}$ in order to find out which concentration would provide the highest assay sensitivity. (Fig. 2B). The slightly higher sensitivity shown at coating 3 $\mu\text{g/mL}$ antibody appeared to be due solely to the increase of the assay background. There was no difference between calibration curves when coating from 3 or 5 $\mu\text{g/mL}$. Similar results were found with the PR assay. Therefore, coating 2 $\mu\text{g/mL}$ antibody concentration was employed for both assays.

Sample Incubation Time

It is well known that a longer sample incubation time usually increases the sensitivity of an immunoassay. We found that incubation overnight provided the highest assay sensitivity for both ER and PR assays. We tried not to incubate receptors at elevated temperatures to avoid any possibility of denaturation, because both receptors, especially PR, are heat labile.

Number of Incubation

In our experience, higher assay sensitivity can be achieved in ELISA by incubating the sample and detecting antibody at

Fig. 2. Assay optimization.

the same time in the antibody-coated well, thereby reducing two incubations (sample and detection antibody) to one. Such streamlining can also eliminate one washing step, further reducing the overall assay time. Surprisingly, we observed higher assay sensitivity in the one-incubation procedure only for ER ELISA. The regular two-incubation procedure in PR ELISA resulted in a higher sensitivity than did one incubation (Fig. 2C). Therefore, we adopted the one-incubation procedure only for the ER ELISA and kept the traditional two-incubation method for PR ELISA.

Sample Volume

In the ER assay, with only one incubation, the volume of sample and detection body was limited to a total volume of 100 μ L. For this reason, we applied only 10 μ L detecting antibody in order to leave more room for cytosol application. Using a much more concentrated detecting antibody, we were able to reach the same final concentration of detecting antibody even though only 10 μ L was applied. This left 90 μ L for cytosol specimen and provided sufficient sensitivity for the assay. For the PR assay we were able to apply 100 μ L cytosol because we added cytosol and detecting antibody in two separate steps for two separate incubations. In the second incubation, 100 μ L detecting antibody was added to the antigen-antibody coated well.

Signal Amplification Agent

As mentioned here, one of the major reasons for us to be able to establish such sensitive ELISA for receptors was the use of Amdex, a synthetic molecule with multiple horse radish peroxidase (HRP) components covalently coupled to a chemically activated dextran molecule. As many as 80 HRP are coupled to each dextran molecule. The activated labeled dextran is also coupled directly to specific binding reagents, such as anti-mouse IgG or anti-rabbit IgG antibody or avidin, allowing them to bind to various detecting antibody or biotin conjugated antibodies in an ELISA assay. Because of the number of HRP attached to a single dextran molecule, the Amdex enzyme conjugate is able to greatly magnify the enzyme signal and produce a very high assay sensitivity. For our assay development for receptors, the incorporation of Amdex in our assays provided sufficient assay sensitivity for ER and PR in a small amount of tissue or cells from the breast FNAB and core biopsy.

Effect of Cytosol Protein Concentration

Abbott immunoassays for ER and PR require that the protein concentration of the cytosol preparation be adjusted to 1 mg/mL. This is because the amount of ER and PR in the cytosol at 1 mg/mL in general is within the detection limit of the Abbott assay. In FNAB the protein concentrations of the cytosol were usually much below 1 mg/mL. The average pro-

tein concentration in the cytosol was close to 0.2 mg/mL when we homogenized 10 mg tissue in 1 mL buffer. Conceivably, the concentration of ER and PR in cytosol at protein concentration of 0.2 mg/mL will be much less than cytosol adjusted at 1 mg/mL. This is also the primary reason for the need of much more sensitive assays that can accommodate these low receptor concentrations. We need assays capable of detecting less than 3 fmol receptor per mL. As shown in the following calibration curves (Fig. 3, Table 3) our newly developed assays have a sensitivity of less than 1 fmol/mL. In other words, our assay should be able to quantify receptors in cytosol concentrations of 0.2 mg/mL or lower. We should be able to quantify FNAB even with a total of 5 mg cells. The sensitivity of our assay should have no difficulty quantifying ER and PR in core biopsies, because those biopsies usually contain tissue close to 50 mg.

Coating Antibody Volume

We found that the sensitivity of the assay could be further improved if the volume of the antibody coating was increased from 100 to 150 μ L. Increasing coating antibody allows more antigen to be captured during sample incubation, thus binding more signal antibody and eventually producing a higher

Fig. 3. Calibration curves.

TABLE 3. Assay sensitivity

Receptor	Background noise (fmol/mL)	Assay sensitivity (fmol/mL)
ER	0.1	0.5
PR	0.02	0.5

enzyme signal and higher assay sensitivity. As shown in Figure 2D, when we increased our coating antibody from 100 to 150 μ L without increasing the sample, we were still able to increase the assay sensitivity slightly.

Calibrator and Calibration Curve

We pooled the breast tumor tissue cytosol for calibrator. Values of both calibrators were calibrated against Abbott ER and PR assay, respectively. The concentration ranges of both our calibration curves ranged from 0 to 20 fmol/mL. Because we expressed the receptor concentration based on 0.2 mg/mL protein concentration of the cytosol, which was 5 times less than the traditional 1 mg/mL protein concentration, the upper normal cutoffs for ER and PR were also reduced 5 times. In other words, they were reduced from 15 fmol/mg to 3 fmol/0.2 mg. In this case, a much lower concentration range of receptors is needed for both calibration curves.

Assay Sensitivity

By assaying the calibrator at various dilutions we found that our assays were capable of detecting both ER and PR at concentrations less than 1 fmol per mL (Table 3). We were not able to differentiate receptor concentrations below 0.5 fmol/mL. Our assays should have sufficient sensitivity even to quantify receptors below the upper normal limit.

Assay Precision

Results of all within-day and day-to-day precision studies are shown in Table 4. Each assay was done at two different receptor concentrations. One of the difficulties of studying the day-to-day precision for ER and PR receptors has to do with how labile they are, especially PR. This also explains the poor CV that we obtained for day-to-day precision for PR. Otherwise the precision of both assays is acceptable.

TABLE 4. Assay precision

Receptor	Within day	Day to day
ER	Mean $\pm$ SD = 9.1 $\pm$ 0.82	Mean $\pm$ SD = 8.9 $\pm$ 1
	CV = 9% (N = 24)	CV = 11.3% (N = 6)
	Mean $\pm$ SD = 4.2 $\pm$ 0.4	Mean $\pm$ SD = 3.8 $\pm$ 0.54
	CV = 9.7% (N = 24)	CV = 14.1% (N = 6)
PR	Mean $\pm$ SD = 5.8 $\pm$ 0.23	Mean $\pm$ SD = 5.3 $\pm$ 0.77
	CV = 3.9% (N = 23)	CV = 14.5% (N = 4)
	Mean $\pm$ SD = 2.8 $\pm$ 0.13	Mean $\pm$ SD = 2.8 $\pm$ 0.72
	CV = 4.8% (N = 24)	CV = 26.2% (N = 4)

N, number of repeats.

Comparison Assay Parameters for In-House and Abbott Immunoassay

In Table 5 assay parameters of our assay were compared with those of the Abbott kit. Most noticeable is the difference in the amount of tissue required by the two different procedures. Our assays can quantify receptors in as little as 10 mg of tissue or cells from fine-needle aspirate biopsy.

DISCUSSION

In recent years clinical laboratories received less and less breast tumor tissue for ER and PR receptor determinations. The amount of tissue or cells from the fine-needle aspiration biopsy and core biopsy are so small that the traditional assays using the Abbott kits no longer have the sensitivity for the quantification of these receptors. Most FNAB had to be sent away for qualitative estimation of these receptors based on immunohistochemistry. Therefore the success of our development of sensitive ELISA for both receptors would mean that once again the quantitative measurement could be made of ER and PR from small amounts of tissue from FNAB and core biopsy. These sensitive ELISAs can also compliment the immunohistochemical techniques when there is any doubt as to whether the negative immunohistochemical staining is a result of improper sampling.

Many commercial antibodies, including both polyclonal and monoclonal anti-ER and anti-PR antibodies, became available only in recent years. These antibodies were mainly designed for immunohistochemical staining. However, we found it was possible to use them in the ELISA format. These commercial antibodies—from Dako, Zymed, and Biogenex—were all very similar in their affinity against ER and PR. Almost all of them can be used in the ELISA format for measuring ER and PR in small amounts of tissue. The antibodies we selected in our assays were antibodies demonstrating slight higher sensitivity at the same dilution. Therefore, price is the major consideration for determining the selection.

It is well known that the hook effect occurs more frequently with one incubation procedure (5). Elimination of the washing step following sample incubation may produce a hook effect when excess antigen saturates both coating and detecting antibodies. However, such situations only occur with

TABLE 5. Comparison in-house and Abbott immunoassay for ER and PR determination

Assay parameter	Old (Abbott kit)	New (in-house kit)
Tissue required	100 mg	10 mg
Homogenization buffer	1 mL	1 mL
Cytosol protein conc.	2–5 mg/mL	Approximately 0.2–0.5 mg/mL
Aliquot of Cytosol for assay	0.1 mL	0.1 mL
Upper normal cutoff	15 fmol/mg	3 fmol/0.2 mg
ELISA	Antibody-coated bead	Antibody-coated microplate
Calibrator conc. range	0 to 250 fmol/mL	0 to 20 fmol/mL

highly elevated concentrations of analyte, several thousand-fold higher than the upper normal cutoff. Because such elevated receptor concentrations are rarely encountered in breast tumor cytosol, we do not anticipate that hook effect will be a problem in our ER assay.

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