Microtiter plate immunoenzymometric assay for estrogen receptor

Valérie Delage,¹* Jean-Marie Teulon,¹ Laurent Bellanger,¹ Patrick Seguin,¹ Françoise Descotes,² and Simone Saez²

The estrogen receptor (ER) status of breast cancer is used both as a prognostic factor and as a predictor of response to endocrine therapy. An immunoenzymometric assay for ER was developed on 96-well microtiter plates (EIA96). This technique involves two monoclonal antibodies directed against different epitopes in the B domain of ER. The two-step protocol (16–18 h and 3 h at 4 °C) requires 100 μL of cytosol. This assay showed a detection limit of 0.58 pmol/L. Intra- and interassay CVs of clinical specimens were ≥5% except for the least concentrated sample (6.5 pmol/L, CV = 6.7%). In a comparison study involving cytosols of breast adenocarcinoma tissue biopsies, we compared the EIA96 with the radioligand assay (RLA) and the Abbott ER-EIA, widely used techniques for determining ER concentration in cytosols of breast cancer tumors. The two EIA showed excellent agreement; however, two samples showed discrepant results by EIA96 and RLA.

INDEXING TERMS: steroid receptor • enzyme immunoassay • monoclonal antibodies • radioligand assay • breast cancer

Many physiological functions, such as the female reproductive system and bone and lipid metabolisms, are controlled by estrogens [1]. Their action is mediated by the estrogen receptor (ER), a member of the steroid/nuclear receptor superfamily of ligand-regulated transcription factors.² ER is a 66-kDa protein made up of six domains (i.e., A to F) responsible for ligand binding, nuclear localization, dimerization, DNA binding, and transcription activation [2].

Besides its traditional physiological roles, ER is involved in various cancers, both in estrogen target tissues (breast, endome-

trium, ovary) and in nonestrogen target tissues (prostate, pituitary, and thyroid glands; digestive tract; urinary tract; and thymus [3]). The presence of ER in breast cancer is a well-established independent prognostic factor [4] and, in association with progesterone receptor (PgR), is a predictor of a tumor's response to hormonal therapy. Patients with ER-positive tumors tend to have a more favorable prognosis than patients with receptor-negative tumors [5]. The disease-free survival of ER+ and PgR+ stage 1 and 2 breast cancer patients is longer than that of patients whose tumors are negative for both receptors [6]. Moreover, 70–80% of breast cancer patients with tumor biopsies containing ER and PgR respond to endocrine treatment, compared with only 10% of patients with ER-negative and PgR-negative tumors [7]. ER status is therefore an important factor in predicting the hormone-responsiveness of a tumor.

Two kinds of techniques are widely used for determining the ER status of a tumor: quantification of ER in cytosol extracts of tumor tissue, and immunohistochemistry with tissue sections. Immunohistochemistry, which involves anti-ER monoclonal antibodies, allows visualization of the tissue distribution and subcellular localization of ER by direct antigenic recognition [8, 9]. Nevertheless, this method has some disadvantages: It is not standardized, and results are at best only semiquantitative. Quantitative techniques performed on cytosol extracts of tumor tissue fall into two classes. The basic method for comparison is the radioligand assay (RLA), which is based on the binding of a specific tritiated ligand to unoccupied ERs, followed by removal of unbound material with dextran-coated charcoal. The displacement of tritiated estradiol by an excess of unlabeled diethylnltilbestril and the introduction of dihydrotestosterone in the incubation mixture allow all specific binding sites to be reached, which obviates interferences from nonspecific binding proteins. The main limitation of the RLA is the detection limit.

When anti-ER monoclonal antibodies became available, an enzyme immunoassay (EIA) of ER was developed (ER-EIA monoclonal kit from Laboratoires Abbott, Rungis, France) [10]. Whereas RLA measures the number of binding sites and their affinity for the ligand, the EIA detects immunoreactive ERs by antigenic recognition. For the ER-EIA, the epitopes detected are localized in domains D and E of the receptor. This technique requires less fresh tissue than RLA. However, al-

¹ CIS Bio International, Division In Vitro Technologies, BP 175, 30203 Bagnols-sur-Cèze cèdes, France.
² Institut Pasteur de Lyon, Unité de Radioanalyse et de Biologie des Tissus Tumoraux, Ave. Tony Garnier, 69365 Lyon cédes 07, France.
*Author for correspondence. Fax Int + 33.66.90.02.70.

1 Nonstandard abbreviations: ER, estrogen receptor; EIA, enzyme immunoassay; EIA96, microtiter plate EIA for ER; PgR, progesterone receptor; HRP, horseradish peroxidase; RLA, radioligand assay; TEM, Tris/EDTA/molybdate/monothioglyceral; and TEDM, Tris/EDTA/dithiothreitol/molybdate/glycerol.

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though the ER-EIA was well correlated with RLA at the beginning [10], its correlation with RLA is now more controversial [11, 12]. A third technique described recently, enzyme-ligand immunonassay, combines saturation of the receptor by the hormone and immunological determination of the ligand after dissociation from the receptor [13]; this allows simultaneous determination of ER and PgR, but the protocol is complicated to use.

Here, we describe a sandwich immunoenzymometric assay technique (EIA96) for the ER. In this assay, the receptor is recognized by two monoclonal antibodies directed against different epitopes of the protein. Quantification is obtained by reference to a set of dilutions of human recombinant ER. The assay characteristics and data from a comparison study of our method with RLA and Abbott ER-EIA are presented.

**Materials and Methods**

**SAMPLE COLLECTION**

Receptors were measured in breast adenocarcinoma tissue biopsies. The samples were obtained from surgical specimens and immediately frozen in liquid nitrogen before transferal to the laboratory. None of the patients in this study had received chemotherap or radiotherapy before surgical treatment.

**REAGENTS**

We used the following buffers: phosphate-buffered saline, 50 mmol/L Na2HPO4/KH2PO4, 150 mmol/L NaCl, pH 7.4; TEM buffer, 10 mmol/L Tris-HCl, 1.5 mmol/L EDTA, 5 mmol/L Na2HPO4/NaH2PO4 0.1 mL/L monothioglycerol, pH 7.4; TDM buffer, 10 mmol/L Tris-HCl, 1.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 10 mmol/L Na2HPO4/NaH2PO4 100 mL/L glycerol, pH 7.4; buffer A, 50 mmol/L Na2HPO4/NaH2PO4 400 mmol/L KCl, pH 8.0; and buffer B, 50 mmol/L Na2HPO4/NaH2PO4 pH 8.0.

17β-[2,4,6,7-3H]Estradiol, specific activity $3.3 \times 10^{15}$ to $3.9 \times 10^{15}$ Bq/nmol, was purchased from Amersham (Les Ulis, France). Inert steroids and diethylstilbestrol were obtained from Roussel-Uclaf (Romainville, France). The radiolabeled steroid was purified by paper chromatography, eluted with absolute ethanol, and stored under nitrogen in amber vials at -20 °C until use. Inert steroids were prepared as 1 mmol/L solutions and stored at 4 °C.

**CYTOSOL PREPARATION AND RLA METHOD**

Frozen tumor biopsies were pulverized in a tissue dismembrator (Mikro-dismembrator U. B. Braun; Biotech International, Melsungen, Germany) and then homogenized in cold TEDM buffer (10 mL/g tumor). Ultracentrifugation of the homogenate at 105 000g for 70 min at 4 °C (TFT5038 rotor in a Centrifor T-2070 ultracentrifuge; Kontron Instruments, Montigny-le-Bretonneux, France) yielded the cytosol, which we aliquoted and distributed among the wells of 96-well microtiter plates. The presence and the number of ER binding sites was determined by the RLA, according to the recommendations of the European Organization for the Research and Treatment of Cancer [14]. For routine experiments, labeled estradiol was used at a final concentration of 5 nmol/L and displaced by unlabeled steroid in 10-, 50-, 100-, and 200-fold excess. This saturation method allowed us to measure the number of specific binding sites. The results were expressed as femtomoles of bound estradiol per milligram of cytosolic protein (or pmol/g). These RLA results were the reference values for comparison with the other methods' data.

**MICROTITTER PLATE EIA96**

**Assay protocol.** All steps of this sandwich technique were performed at 4 °C in 96-well microtiter plates (Greiner-Labortechnik, Frickenhausen, Germany). The wells were coated with a mouse monoclonal antibody (B10, IgG2a [15]) directed against amino acids 151-165 of the human ER (B domain [16]). Calibrated solutions of recombinant human ER expressed in yeast were used as standards. In each well 100 μL of cytosol (pure or diluted in TEM buffer) or 100 μL of standard (lyophilized standard reconstituted in deionized water and diluted in TEM buffer) was incubated with 100 μL of buffer A. After 16- to 18-h incubation at 4 °C, during which the ERs bound to the antibodies, the contents of the wells were aspirated and the wells were washed (3 × 0.3 mL of 1 mL/Tween 20 in deionized water) to remove unbound material. We then added to each well 200 μL of horseradish peroxidase (HRP)-conjugated antibody diluted in buffer B, incubated the plate for 3 h at 4 °C, and then aspirated and washed the wells as before. The HRP-conjugated antibody was a mouse anti-ER monoclonal antibody (AER314, IgG1; Bioprobe B.V., Amstelveen, The Netherlands) [17] directed against an epitope distinct from B10 in the B domain (region 121-168). We then added 100 μL of the enzyme substrate solution (hydrogen peroxide and o-phenylene-diamine-2HCl), incubated this for 30 min at room temperature in the dark, and stopped the enzymatic reaction by adding 100 μL of 1 mL/L sulfuric acid. The intensity of the coloration at 492 nm, which was proportional to the amount of receptor, was measured with a Spectra spectrophotometer (SLT Labinstruments, Grödig, Austria). The ER concentration of the cytosol was directly determined from a calibration curve of absorbance vs concentration, constructed with a set of ER standards ranging from 0 to 250 pmol/L.

**Production and extraction of recombinant human ER.** The yeast strain TGY14 (Mat a, ura3-251-373-328, leu2, pep4-3), transformed by pYHER2, was kindly provided by P. Chambon (Strasbourg, France) [18]. Cells were incubated in a final volume of 45 L of YCG medium (6.7 g/L yeast nitrogen base, 5 g/L casamino acids, and 10 g/L D-glucose, pH 6.0) at 30 °C with constant agitation. Yeast nitrogen base and casamino acids were purchased from Difco Labs. (Molesey, UK) and D-glucose was from Sigma Chimie (Saint Quentin Fallavier, France). The absorbance at 600 nm was monitored; when it reached 1.0, the cells were sequentially concentrated (final volume, 0.5 L) by cycles of tangential filtration on a 0.45-μm (pore-size) Filtron cartridge (Filtron Scandinavia, Bjarrant, Sweden) and suspended in phosphate-buffered saline. The concentrated cells were centrifuged for 15 min at 3500g and 4 °C (Jouan LR5.22 centrifuge; Jouan, Saint Nazaire, France), and the pellet was then resuspended in TEM buffer before the cells were disrupted.
with glass beads in a bead-beater (Biospec Products, Bartlesville, OK). The beads were retained in a 45-μm pore-size nylon filter (Btyrel TI45; Fytis-UGB, Lyon, France) and washed with TEM buffer. The crude extract was recentrifuged for 35 min at 3500g at 4°C (Jouan LR5.22), and the supernate was lyophilized and used as calibrator (standard 250).

**ABBOTT ER-EIA**

ER was assayed with the Abbott ER-EIA monoclonal kit, according to the manufacturer’s instructions. We incubated 100 μL of cytosol (diluted in TEM buffer to a protein concentration of 1–2 g/L) with 100 μL of sample diluent and an anti-ER monoclonal antibody-coated bead for 18 h at 4°C. The calibrators (5, 25, 100, and 250 pmol/L) were prepared by diluting the lyophilized standard 500 (after reconstitution with reconstitution buffer) with the lyophilized standard 0 (reconstituted with reconstitution buffer). We incubated 200 μL of each calibrator with one bead for 18 h at 4°C, then washed the beads (with a Pentawash, from Abbott) and incubated each with 200 μL of an anti-ER monoclonal antibody conjugated to HRP for 1 h at 37°C. The beads were washed and transferred into tubes. After a 30-min incubation at room temperature in the dark with 300 μL of the enzyme substrate solution (hydrogen peroxide and o-phenylenediamine-2HCl), the enzymatic reaction was stopped by adding 1 mL of 0.5 mol/L sulfuric acid. The intensity of the color developed at 492 nm was measured with a Photocis IV spectrophotometer (CIS Bio International, Gif-sur-Yvette, France). The ER concentration of each tumor cytosol assayed was calculated by determining the corresponding concentration on the calibration curve and multiplying the value by a factor of 2.

**PROTEIN CONCENTRATION**

The protein concentration of cytosols was determined with the BCA protein assay kit (Pierce Europe, Oud Beijerland, The Netherlands), according to the manufacturer’s instructions. Bovine serum albumin (Sigma; Cohn Fraction IV) was used as the calibrator.

**STATISTICAL ANALYSIS**

The precision profile was established by using the Variance Function Package program [19]. Statistical analysis of the data was performed according to the method of Passing and Bablok [20, 21].

**Results**

**ASSAY CHARACTERISTICS OF MICROTITER PLATE EIA96**

**Calibration curve.** Fig. 1 shows the calibration curve. The 250 pmol/L calibrator was obtained by reconstitution of a lyophilized recombinant human ER solution in distilled water (see above). The others (5, 25, and 100 pmol/L) were prepared by diluting the 250 pmol/L calibrator in TEM buffer. TEM buffer alone was the 0 pmol/L control. For each calibrator, the absorbance at 492 nm is greater than that observed with Abbott ER-EIA, indicating a slightly higher nonspecific binding of the HRP-conjugated antibody in EIA96; EIA96 A492 = 0.011; ER-EIA A492 = 0.002.

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**Table 1. Intra- and Interassay repeatability.**

<table>
<thead>
<tr>
<th>Pool</th>
<th>ER conc., pmol/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraassay (n = 15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.5</td>
<td>0.4</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>28.8</td>
<td>1.1</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>163.9</td>
<td>7.4</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Interassay (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10.2</td>
<td>0.4</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>28.8</td>
<td>1.4</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>145.5</td>
<td>6.5</td>
<td>4.4</td>
<td></td>
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</table>

**Fig. 1.** Typical calibration curves from EIA96 (■) and ER-EIA (□).
and Abbott ER-EIA). The PgR status—as determined by RLA or by Abbott PR-EIA—was known for each sample. The results were expressed in fmol ER per mg of cytosolic protein and the relations between the data obtained by both EIAs and RLA were analyzed by the Passing–Bablok method (20, 21) (Fig. 3). As none of the variables could be assumed to be free of error, and as the variance of the measurement errors increased with the magnitude of the measurements, we used a linear regression procedure with no special assumptions regarding the distribution of the samples or of the measurement errors. The hypothesis slope = 1 and intercept = 0 was accepted for EIA96 vs ER-EIA (Fig. 3, top), because the values of 1 and 0 were enclosed in the confidence intervals for the slope and the y-intercept, respectively. For EIA96 vs RLA (Fig. 3, middle) and ER-EIA vs RLA (Fig. 3, bottom), the hypothesis was rejected.

Discrepancies in terms of positivity/negativity of the results were observed in three samples (Table 3). We used a cutoff value of 15 fmol/mg protein, as in the ER-EIA kit. Cytosol A was then negative by both EIAs and slightly positive by RLA, whereas cytosol B was ER-negative by ER-EIA and ER-positive by RLA and EIA96. Cytosol C was negative by RLA and positive by both EIAs. In addition, one sample (cytosol D) showed a wide variation in ER values, although it was classified as ER-positive by all three methods: RLA, 16.0 fmol/mg protein; EIA96, 40.9 fmol/mg protein; and ER-EIA, 110.3 fmol/mg protein. The PgR concentration of cytosol D was 32 fmol/mg protein.

**Discussion**

Using two monoclonal antibodies directed against different epitopes in the B domain of the estrogen receptor, we developed an immunoenzymometric assay for ER. The sample concentration is read on a calibration curve constructed with a set of dilutions of a recombinant antigen standard solution (range 0–250 pmol/L) calibrated by reference to cytosols of mammary tumors assayed by RLA. Unlike the Abbott ER-EIA technique, EIA96 processes samples and standards in the same way (same first incubation volume and buffer). The calibration curve is linear, giving an absorbance at 492 nm of 2.014 for the 250

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**Table 2. Dilution test on three pools of cytosols of mammary tumors.**

<table>
<thead>
<tr>
<th>Pool (and ER content)</th>
<th>Diln. factor, n-fold</th>
<th>ER conc., pmol/L</th>
<th>% difference from undiluted pool$^b$</th>
<th>Mean$^c$ ER, pmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 (53.1 pmol/L)</td>
<td></td>
<td>Measured</td>
<td>Corrected$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>5.7</td>
<td>45.6</td>
<td>-14.1</td>
<td>51.1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>12.9</td>
<td>51.6</td>
<td>-2.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>27.0</td>
<td>54.0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>7 (40.2 pmol/L)</td>
<td>16</td>
<td>2.2</td>
<td>35.2</td>
<td>-12.4</td>
<td>37.6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>4.8</td>
<td>38.4</td>
<td>-4.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>9.1</td>
<td>36.4</td>
<td>-9.5</td>
<td></td>
</tr>
<tr>
<td>8 (178.8 pmol/L)</td>
<td></td>
<td>22.2</td>
<td>177.5</td>
<td>-0.8</td>
<td>176.0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>43.8</td>
<td>175.3</td>
<td>-2.0</td>
<td></td>
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<tr>
<td>2</td>
<td></td>
<td>86.3</td>
<td>172.5</td>
<td>-3.5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Corrected for dilution factor.

$^b$ Difference between corrected value and ER content in undiluted pool.

$^c$ Mean of the values for the undiluted sample plus those for three dilutions.
pmol/L standard. All signals are superior to those obtained by ER-EIA, with a slightly greater nonspecific absorbance for the HRP-conjugated antibody for EIA96. A dilution test carried out with three pools of cytosols of mammary tumors (Table 2) shows linearity between the concentrations and the dilution factors. This test reveals a major element for the reliability of the calibration curve, namely, the similarity of behavior in the assay between ERs in the calibrators and ERs in the tumor samples. Reproducibility of results was good for cytosols of mammary tumors in three different concentration ranges, with CVs of ≤5.0% except for the least concentrated pool. EIA96 is a very sensitive assay, the detection limit being established at 0.58 pmol/L. This sensitivity, combined with the good precision of the assay of tumor samples, is particularly important clinically for reliability of discrimination between ER+ and ER− samples.

Results for 50 cytosols of mammary tumors show excellent agreement between EIA96 and ER-EIA. The Passing–Bablok data analysis highlights at least a proportional difference between EIA96 and RLA, and, more slightly, between ER-EIA and RLA. These results can be explained by the very different principles on which the techniques are based: Both EIAs quantify precise regions of ER by immunological recognition (B domain for EIA96, D and E regions for ER-EIA), whereas RLA measures the number and affinity of estradiol-binding sites, i.e., the functionality of the E domain.

As for the samples that gave discrepant results by the three assays, many variant forms of ER have been described in human mammary tumors [23] as well as in normal breast tissue [24]. The vast majority of these variants arise from an alternative splicing of the mRNA, leading to deletion of whole exons, i.e., exons 2, 3, 4, 5, and 7. The proteins encoded by aberrantly spliced transcripts are truncated or internally deleted. Study of their functional behavior has shown most notably the existence of dominant-positive receptors, which are transcriptionally active in the absence of estrogen (e.g., an ER encoded by an exon 5-deleted mRNA [25]), and a dominant-negative receptor (ER encoded by an exon 7-deleted mRNA [26, 27]), which is transcriptionally inactive and prevents the action of wild-type ER. The discrepancies found could be explained by the presence of variant forms of the receptor, which would be recognized differently by the three methods, depending on the localization of the mutation. The dispersion of the values obtained for cytosol D could result from the presence of a great quantity of estradiol, which, on the one hand, would not be totally displaced by RLA, and, on the other hand, would not inhibit the receptor recognition by EIAs. Thus, for EIA96, the epitopes are not in the E domain, and, for ER-EIA, the localization of one epitope

![Graphs showing correlation between EIA96 and ER-EIA](image)

**Table 3. Cytosols giving discrepant results in ER determinations.**

<table>
<thead>
<tr>
<th>Cytosol</th>
<th>ER conc., pmol/L</th>
<th>ER conc., fmol/mg</th>
<th>PgR conc., fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLA</td>
<td>EIA96</td>
<td>ER-EIA</td>
<td>RLA</td>
</tr>
<tr>
<td>A</td>
<td>12.8</td>
<td>0.0</td>
<td>3.7</td>
</tr>
<tr>
<td>B</td>
<td>156.3</td>
<td>158.5</td>
<td>9.4</td>
</tr>
<tr>
<td>C</td>
<td>14.1</td>
<td>76.7</td>
<td>83.2</td>
</tr>
</tbody>
</table>

Equations for curves obtained by Passing–Bablok method (see text) are shown for each panel. Confidence intervals for the slope and y-intercept, respectively, are: (top) 0.943–1.299 and −1.377 to 0.506; (middle) 1.050–1.490 and 0.920–2.300; and (bottom) 1.002–1.407 and 1.350–2.850.
in the E domain does not inhibit the recognition of liganded ERs [8].

In conclusion, this characterization of EIA96 shows it to be a very sensitive, reproducible assay. Moreover, its excellent agreement with Abbott ER-EIA is important clinically, for determination of the hormone responsiveness of a tumor.

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References