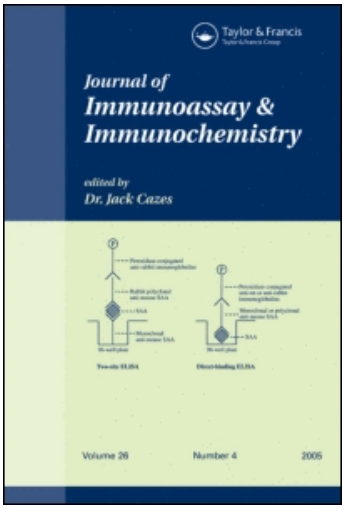

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Quality Control of Estrogen Receptor Assays

William Godolphin[a]; Beryl Jacobson[a]
[a] Department of Pathology, University of British Columbia Vancouver General Hospital, Vancouver, BC, Canada

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QUALITY CONTROL OF ESTROGEN RECEPTOR ASSAYS

William Godolphin and Beryl Jacobson
Department of Pathology, University of British Columbia
Vancouver General Hospital
Vancouver, BC, Canada

ABSTRACT

Four types of material have been used for the quality control of routine assays of estrogen receptors in human breast tumors. Pieces of hormone-dependent Nb rat mammary tumors gave a precision about 40%. Rat uteri and rat tumors pulverized at liquid nitrogen temperature and stored as powder yielded precision about 30%. Powdered and lyophilised human tumors appear the best with precision as good as 17%.

INTRODUCTION

Knowledge of the estrogen receptor status of breast cancer has emerged over the past decade as a powerful predictor of response to hormonal manipulation(1,2). During the same period there has been a proliferation of estrogen receptor assay methods(3) and many centres are performing the analysis as a routine service. There is, however, a dearth of information on quality control of the assay, a need for which has recently been emphasised by the British Breast Group(4) and a National Institutes of Health Consensus Development Conference(5).

The little quality control data that has been published has shown a precision of 15-30%(6-9). However, these figures have come from small series of analyses over short periods of time. This deficiency is a consequence of the lack of good quality control material; namely a plentiful, stable and homogeneous specimen.

We have used several preparations for the quality control of routine estrogen receptor analyses and the most promising appears to be powdered, lyophilised tumors(10).

MATERIALS AND METHODS

Four kinds of material were used for quality control: pieces of rat mammary tumors, powdered rat uteri, powdered rat mammary tumors and powdered lyophilised human breast tumors. Rat uteri and mammary tumors were obtained from Nb rats bearing hormone dependent experimental adenocarcinomas(11). Human breast cancer tissue was obtained by pooling parts of specimens submitted for routine estrogen receptor analysis. Tissue was frozen and transported in liquid nitrogen (-196°C) at the time of excision(12). All subsequent storage in the following experiments was in sealed containers at -85°C.

Pieces from a single, large, solid rat tumor were carefully removed, trimmed to about 200 mg and selected for their uniformity of gross appearance. These were individually stored in small vials (Nunc Cryotubes, Gibco, Calgary, Alta T2G 4B7). An-

other batch of this material was prepared by cutting several cross-sections from the same tumor. The sections were then cut in a grid fashion to yield similarly sized pieces. These were stored as above but neither trimmed nor selected for uniformity.

Either prior to analysis or in preparation of powdered lyophilised specimens all material was thawed at 4°C, cut into 2-3 mm blocks, frozen in liquid nitrogen and placed in a precooled cylindrical teflon mortar. The mortar and a stainless steel ball (1 cm diam) were cooled in liquid nitrogen and subjected to two 45 s bursts at full excursion in a Mikro-dismembrator (B Braun Melsungen AG, W Germany) with cooling between bursts(13).

A pool of powder from many rat uteri was thoroughly mixed (at - 196°C) and ~200 mg amounts were stored in sealed vials. A similar large pool of powdered material was prepared from a single rat mammary tumor. The lyophilised material was prepared by placing tissue powder under vacuum overnight (Freeze Dryer-3, Labconco, Kansas City, Mo 64132). The dry powder was thoroughly mixed at room temperature, aliquoted and stored in sealed vials at -85°C.

Prior to analysis tissue powder was reconstituted either directly in the mortar (in the case of tumor pieces) or in a

test tube (in the case of powders) with cold TED buffer (10 mmol Tris-HCl, 1.5 mmol EDTA, 0.5 mmol dithiothreitol per litre, pH 7.5), 5 mL/gm frozen powder or 25 µL/gm lyophilized powder and centrifuged at 39,000 xg, 0°C, for 15 min (rotor model AH 650, Dupont Sorval, Wilmington, Del 19898). The cellulose nitrate centrifuge tubes were punctured above the pellet and cell-protein solution, but none of the top lipid layer was withdrawn. The supernatant protein concentration was estimated spectrophotometrically ($1.55\ A_{280} - 0.74\ A_{260}$ = mg protein/mL) and diluted with TED buffer to yield 2 mg/mL; equivalent to 1 mg/mL in the incubation mixture.

Supernatant (250 µL) was mixed with 250 µL cold TED buffer containing either 150, 200 or 500 fmol 17 β-[2, 3, 6, 7 - ^{3}H] estradiol (New England Nuclear, Boston, Mass 02118, specific activity 100 Ci/mmol) or 200 fmol ^{3}H-estradiol plus 200 pmol diethylstilbestrol (Sigma, Saint Louis, Mo 63178). The earliest assays during which the rat uteri were used for quality control were performed with 100, 150 or 200 fmol ^{3}H-estradiol and 500 pmol nafoxidine ("U-11,100A", Upjohn Co., Kalamazoo, Mich 49001). Blanks of each of the above mixtures, but containing no protein, were also prepared. All tests were performed in duplicate and incubated at 0-4°C for 16-18 hours. Unbound hormone was removed by 30 min incubation with 0.5 mL of a suspension of 0.5% charcoal, 0.05% Dextran 70 in TED buffer

at $4^{o}C$. Dextran-charcoal was removed at 12,000 xg for 4 min in an Eppendorf 3200 centrifuge (Brinkmann Instruments, Rexdale, Ont M9W 4Y5). The supernatant (0.5 mL) was removed, with great care to avoid contamination with charcoal, mixed with 10 mL scintillation cocktail (Scintiverse, Fisher Scientific, Fairlawn, NJ 07410 or Unogel, Schwarz/Mann, Orangeburg, NY 10962) and counted (Model LS-9000, Beckmann Instruments, Irvine, Calif 92713) at 44% efficiency with automatic data reduction to DPM after quench correction.

Blanks were subtracted and nonspecific binding (that which is found in the presence of excess nafoxidine or diethylstilbestrol and assumed to be linear with concentration of estradiol) was extrapolated and subtracted from the total binding determined at various concentrations of ^{3}H-estradiol in the absence of nafoxidine and diethylstilbestrol. The resultant specific binding was expressed in fmol/mg tissue protein. Protein determinations were by the method of Lowry et al standardized with crystalline bovine serum albumin(14). Albumin concentration of the human tumor supernatants were measured by radial-immunodiffusion (Behring, Hoechst Pharmaceuticals, Montreal, Que H4R 1R6). Serum protein (from blood vessels and interstitial fluid) may considerably alter the apparent receptor level when this is expressed in units of estrogen bound per mass of soluble protein in the cytosol preparation (e.g. fmol/mg). The contribution of this serum protein

component can be estimated from the albumin concentration x 1.67. We have found a wide range of albumin concentrations (5-50% of total protein) in human malignant tumors and have routinely applied this correction(15) when dealing with human material and hence expressed receptor content as fmol/mg cytosol protein.

An estimate of the variation in estrogen receptor content to be found in various parts of a tumor was obtained by assaying pieces selected from the peripheral, pericentral and core regions of six large tumors. An additional estimate of precision was obtained by repeating the assay on human tumors which had been stored from one to 19 weeks after the first determination.

RESULTS

All of the prepared quality control material appeared stable over periods up to a year with no statistically detectable change in trend of the mean of receptor content. The frozen powdered uteri yielded better precision than tumors prepared the same way (Table 1). Analyses of pieces of tumor were least precise (Table 2) even when carefully selected for uniformity. This variation of estrogen receptor content in different parts of a tumor is emphasised by the analyses performed on specimens deliberately chosen from different regions of large tumors (Table 3). Similar precision was demonstrated by repeat analyses of 25 spec-

TABLE I

Estrogen Receptor Quality Control Data for Powdered Tissue Pools Prepared from Rat Uteri or Rat Mammary Tumors

Assay type	n	Mean (fmol/mg tissue protein)	C.V. (%)
(a) Uteri			
single dose assay (0.4 nM E_2)	49	68	25
Scatchard analysis	34	134	26
(b) Tumors			
single dose assay (0.4 nM E_2)	19	12	37
Scatchard analysis	18	26	20

Tissues were powdered in the Mikro-dismembrator at liquid nitrogen temperature, mixed thoroughly, aliquoted into single assay amounts and stored at -85°C.
The uteri pool(a) was analyzed over a period of one year and the tumor pool(b) over a period of two months, by three analysts.

imens of human breast cancer with from one to 19 weeks time lapse (mean = 5), between the first and second analysis. Estrogen receptor content ranged from 1 to 456 fmol/mg cytosol protein (mean = 135) and the difference between duplicates yielded a precision of 41%. Powdered and lyophilised human breast tumors have resulted in the best precision data (Table 4) so far.

DISCUSSION

Ideal quality control material for the receptor assay should be as similar as possible to that routinely analysed i.e. fresh

TABLE 2

Estrogen Receptor Quality Control Data for Pieces from a Single Large Rat Mammary Tumor

Assay type	n	Mean (fmol/mg tissue protein)	C.V. (%)
(a) Carefully selected and trimmed			
single dose assay (0.4 nM E_2)	17	21	41
Scatchard analysis	15	25	36
(b) Randomly selected, untrimmed			
single dose assay (0.4 nM E_2)	11	7.8	41
Scatchard analysis	6	11	32

Pieces of tumor (~200 mg) were either(a) carefully trimmed and selected for their uniform appearance and consistency or(b) diced from cross-sections of tumor with no selection for uniformity. All specimens were stored separately in sealed vials at -85°C. Analyses took place over a period of five months and were performed by two analysts.

TABLE 3

Estrogen Receptor Content in Different Parts of the Same Tumor (fmol/mg cytosol protein)

Tumor	Site within Tumor		
	peripheral	random or pericentral	central core
#R-6-72	7	7	11
# 122	47	25	21
# 149	166	128	114
# 199	160	69	46
# 237	367	287	413
# 564	219	397	360

TABLE 4

Estrogen Receptor Quality Control Data for Powdered Lyophilised Human Breast Tumors

Assay type	n	Mean (fmol/mg cytosol protein)	C.V. (%)
Pool A			
single dose assay (1nM E_2)	26	169	32
Scatchard analysis	24	195	30
Pool B			
single dose assay (1nM E_2)	26	45	17
Scatchard analysis	25	50	17

Several tumors were powdered in the Mikro-dismembrator at liquid nitrogen temperature, mixed thoroughly, lyophilised, aliquoted in single assay amounts and stored in sealed vials at -85°C. Pool A was analyzed over a period of four months by three analysts and Pool B over a period of five months by four analysts.

or frozen human tumors. However, human tumors large or homogeneous enough to provide sufficient material are rare or nonexistent. The rat tumors used in this work were remarkably homogeneous in appearance, yet they resulted in poor precision. Homogeneity can be improved by pulverising the tissue and mixing it. However, this approach has the disadvantage of not controlling the first step of a routine assay (powdering or homogenising the tissue). Even better homogeneity can be assured if a large pool of soluble tissue protein (the "cytosol" fraction) is prepared. However, in this form the receptors are particularly labile and the initial two steps of analyses are not controlled.

Koenders et al(10) discovered that estrogen receptors in powdered lyophilised tumors were relatively stable, even at 4°C or room temperature. Our first batch of lyophilised material yielded precision no better than that obtained with powdered rat uteri (Table 1 and 4). However, the uteri data were produced by experienced analysts whereas much of Pool A of the lyophilised material was analysed by analysts just learning the technique. We believe the data of lyophilised Pool B to be a better reflection of the true precision of the assay.

The lyophilised material is relatively easy to prepare, has excellent stability and seems to be the material of choice for both intra- and inter-laboratory quality control programmes.

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Correspondence should be addressed to Dr. W. Godolphin, Division of Clinical Chemistry, Vancouver, General Hospital, Vancouver, B.C. V5Z 1M9.

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