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Standardization of Steroid Receptor Assays in Human Breast Cancer—I. Reproducibility of Estradiol and Progesterone Receptor Assays

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Abstract—*Four different lyophilized cytosols were analyzed for estrogen and progesterone receptor content by 13 laboratories. Three of the cytosols were assayed on 9 consecutive working days. The fourth cytosol was analyzed 3 times. All laboratories used the same methods of receptor and protein assays. Estradiol and progesterone receptors were measured by Scatchard analysis with centrally provided radioactive ligands and employing charcoal adsorption of free steroid. Protein was assayed by the Bradford technique with the same lots of Coomassie brilliant blue and human serum albumin standard. All participating groups except one produced receptor results* (fmol/ml *cytosol) with less inter-laboratory variation than in previous trials. Recalculation of the raw data of all participants by a common computer program further reduced this inter-laboratory variation. The discrepancies between the reported and recalculated receptor binding data are discussed. The intra-laboratory variation was sometimes surprisingly high and occurred at random. Single-dose saturation assays showed good agreement with multipoint Scatchard assays for the high receptor-positive samples, while poor agreement was observed for the heat-inactivated, receptor-negative sample. The use of common reagents and methodologies diminished the inter-laboratory variation coefficients of the protein assay to 14–17%; however, the protein estimation still needs to be improved.*

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INTRODUCTION

In 1972 the EORTC Breast Cancer Cooperative Group agreed on certain methodological aspects of estradiol receptor (ER) analysis in breast cancer biopsies [1]. In 1979 a second workshop defined adequate and uniform methodological standards for estradiol as well as progesterone receptor (PgR) assays performed by institutions participating in clinical trials of the EORTC Breast Cancer Group [2]. Moreover, the participants agreed on the need of establishing a quality control system. Lyophilized samples were chosen as a reference preparation because of their thermostability [3, 4]. In 1979 and 1980, 7 different lyophilized control samples were analyzed during the three trials conducted [5]. Agreement between laboratories was excellent on the receptor status of lyophilized reference samples having high receptor values. The rate of false negative or false

positive assessments of receptor status on samples with low values or on receptor-negative samples was 14 and 10% respectively. However, large inter-laboratory variations in quantitative receptor values were noted. These variations were at least in part due to variations in the protein values used as reference as well as to methodological differences in the dextran-coated charcoal techniques employed by the participating groups. In order to further standardize the DCC (dextran-coated charcoal) method, the participants agreed that in 1981 all laboratories would perform multipoint (Scatchard) analyses, using the same batch and concentrations of radioactive ligands (estradiol and 16 α-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione, Org 2058), and would determine the non-specific binding with the same compounds (diethylstilbestrol and Org 2058) at the same concentration (10^{-6} M). Moreover, the procedure for charcoal adsorption of free steroid was standardized. Protein was to be assayed by the Bradford technique [6] using human serum albumin as a standard. During this trial special attention was paid to the intra-laboratory reproducibility of the receptor assays. Therefore each of the three different lyophilized reference cytosols, prepared from calf uterine tissue, was to be assayed a total of 9 times on 9 different working days.

MATERIALS AND METHODS

Preparation of lyophilized reference sample

Calf uterine tissue, obtained from a local slaughterhouse, was immediately placed on ice and transported to the laboratory. Both uterine horns were cleaned of fat and adherent connective tissue and washed with cold (0–4°C) physiological saline solution. The uterine tissue was minced on ice with scissors, immediately frozen in liquid nitrogen and pulverized at −196°C to a fine homogeneous powder by means of a Thermovac tissue pulverizer and a Microdismembrator. Cytosol was prepared by stirring the powder with phosphate buffer (0.02 M Na_2HPO_4/NaH_2PO_4, 1.5 mM EDTA, 3.0 mM NaN_3, pH 7.5; w/v = 1:6.5) for 30 min at 4°C and centrifuged at 28.000 *g* for 1 hr at 2°C. Part of the cytosol was placed on ice and the remainder was heated to 60°C for 1 hr. The temperature was again lowered to 4°C and the cytosol that was continuously kept on ice was diluted 2-, 4- and 12-fold with the heat-inactivated cytosol. These 3 diluted samples will be denoted as samples A–C. Cytosols were mixed thoroughly and 5 ml aliquots of samples A–C as well as the undiluted, heat-inactivated cytosol (sample D) were measured into glass vials, placed in liquid nitrogen and lyophilized for 72 hr. Vials were then stoppered under vacuum and stored at 4°C until shipment. The ratio of receptor values of samples A–C is 6 (sample A): 3 (sample B): 1 (sample C), and expressed as fmol/mg cytosol protein the values of sample A are 405 for the estrogen receptor and 1125 for the progesterone receptor (values from reference laboratory in Nijmegen). Small deviations from the calculated ratios can be expected, since the three samples were prepared on different days. This was unavoidable due to the limited capacity of the lyophilization chamber (maximum capacity 126 vials).

Study protocol

Each participant received 30 vials (9 each of samples A–C, and 3 of sample D) that were mailed to the participants at ambient temperature. In order to monitor the stability of the samples under these conditions a set of samples was returned to the laboratory in Nijmegen via one of the participants in Italy (control of shipping conditions).

Three vials were to be assayed each day on 10 consecutive working days. The sequence was established by throwing dice and was unknown to the participants. Furthermore, the fact that 3 receptor-negative samples (sample D) were included in the series was also unknown to the participants.

Preparation of cytosols

The participants received the following instructions for the preparation of cytosol from lyophilized material: store samples at 0–4°C. On the day of analysis tap vials so that the lyophilized cytosol is on the bottom of the vial, remove caps, place in ice and add 5.0 ml of ice-cold (0–4°C) water containing 10% (v/v) glycerol. Gently dissolve by leaving on ice for 10 min and drawing the solution back and forth into a Pasteur pipet (10 times). Centrifuge at 100,000 *g* for 15 min (0–4°C) and use immediately.

Receptor assay

The specific assay conditions agreed upon for common use were in accord with the previously accepted guidelines [2]. Every participant employed the same batch of [^{3}H]-estradiol (54 Ci/mmol) and [^{3}H]-Org 2058 (45 Ci/mmol) purchased from the Radiochemical Centre, Amersham, U.K. The [^{3}H]-estradiol was purified by paper chromatography before sending to the participants (Max-Planck Institut für Experimentelle Endokrinologie). The following concentrations of radioactive ligands were employed: 1, 1.5, 2, 4, 6 and 8×10^{-9} M. 10^{-6} M diethylstilbestrol or 10^{-6} M Org 2058 was added before or simultaneously with the labeled steroids as

competitive inhibitors. Incubation was overnight at 0–4°C. The final concentration of the charcoal suspension during adsorption was 0.25% charcoal (Norit A, sieved through 25 μm nylon netting) and 0.025% dextran T70 in the phosphate extraction buffer. Each participant used their own standard method to calculate results but additionally supplied raw data to Nijmegen. These were used to recalculate data according to a common method (see below).

Protein assay

The protein content of the cytosol was determined by the Bradford technique (Coomassie brilliant blue; Bio-Rad Laboratories) [6] using human serum albumin (Kabi; Kabi Diagnostica, Stockholm, Sweden) as a standard. Both reagents were supplied from a common stock by the Max-Planck Institut für Experimentelle Endokrinologie.

Recalculation of data

The raw data of all participants were recalculated using a single computer program to investigate whether inter-laboratory variation would decrease. The following equation was used for conversion of number of counts/min bound to number of fmol of hormone bound per ml cytosol:

$$\frac{\text{count/min} \times 100}{\text{counting eff. (\%)}} \times \frac{1}{\text{spec. act. (dpm/fmol)}} \times \frac{\text{incub.} + \text{DCC vol. } (\mu\text{l})}{\text{aliquot counted } (\mu\text{l})} \times \frac{1000}{\text{cytosol vol. in assay } (\mu\text{l})}. \quad (1)$$

Correction for non-specific binding is in accord with the principle and method described by Chamness and McGuire [7]. Briefly, it is based upon the assumption that the non-specific binding is directly proportional to the concentration of the free ligand, i.e. the ratio of B/F is a constant. To obtain specific binding, the non-specific binding is subtracted from total binding in each incubation according to the equation:

$$B_{\text{spec.}} = B_{\text{total}} - F \times (\lim_{B \to \infty} {}^{B}/_{F}), \quad (2)$$

i.e. where $(\lim_{B \to \infty} {}^{B}/_{F})$ is determined in the presence of an approximately 100-fold excess of non-radioactive competitor. It may be noted that:

$$F = \text{ligand}_{\text{added}} - B_{\text{total}}. \quad (3)$$

RESULTS

Stability of powders

Details of the stability of powders when stored under different conditions in Nijmegen have previously been published [3, 4], while the data shown in Table 1 show that the mailing of samples had no deleterious effect on either ER or PgR. With one exception (lab. B) all analyses were performed within a 7-week period, and during this time both ER and PgR remained stable (data not shown). Laboratory B performed 4/10 analyses within the 7-week period, while 6/10 analyses were performed 1½–2½ months later. While the first set of lab. B's PgR results were comparable to the results of the other participants, the results of later PgR assays were lower. Whether this decrease in progesterone receptor binding was due to decay of the receptor in these lyophilized cytosols or to some unknown problem remains unsettled. Analysis of the same batch of samples in the reference lab. (Nijmegen) at approximately the same time as the second set of lab. B's analyses gave values that were only slightly lower than those initially observed (data not shown).

Table 1. Steroid receptor measurements in lyophilized reference cytosols mailed without temperature control and in identical samples continuously stored at 4°C

	Samples stored at 4°C (9 vials/sample)	Samples posted for 13 days (3 vials/sample)
Sample A:		
ER	1369 ± 65	1381 ± 175
PgR	3786 ± 201	3732 ± 218
Sample B:		
ER	678 ± 25	693 ± 28
PgR	1644 ± 64	1669 ± 47
Sample C:		
ER	230 ± 22	239 ± 15
PgR	577 ± 41	572 ± 65

The results are given as the mean ± S.D. and all values are expressed as fmol/ml cytosol.

Estradiol receptor

The results reported by the 13 participating groups are summarized in Table 2. This table gives the mean, standard deviation and range of values of each laboratory after elimination of the outlyers of each sample by the test of Doornbos [8].

Although it was agreed that 6-point Scatchard analysis would be performed, two of the participants performed single-dose saturating assays using 5.0 (lab. C) or 8.0 nM [^{3}H]-estradiol (lab. G). The results of these two laboratories

Table 2. Summary of estradiol receptor results

Participating laboratories	Sample A Mean ± S.D.	Sample A Range	Sample B Mean ± S.D.	Sample B Range	Sample C Mean ± S.D.	Sample C Range
A	93 ± 15	77–118	59 ± 29	32–106	—*	—
B	1113 ± 181	830–1396	559 ± 75	431–634	180 ± 23	159–215
C	1129 ± 49	1100–1200	516 ± 29	490–570	168 ± 12	150–180
D	1151 ± 35	1091–1206	548 ± 38	502–615	175 ± 10	161–188
E	1212 ± 161	930–1450	599 ± 56	520–710	251 ± 32	210–300
F	1369 ± 65	1295–1475	678 ± 25	640–720	230 ± 22	195–270
G	1425 ± 236	1066–1718	607 ± 54	501–675	212 ± 22	176–236
H	1438 ± 510	813–2133	721 ± 277	393–1033	—†	—
I	1497 ± 79	1400–1606	682 ± 43	640–743	256 ± 30	214–295
J	1503 ± 288	1090–1940	710 ± 33	670–760	272 ± 28	240–320
K	1518 ± 49	1438–1580	841 ± 70	730–944	557 ± 70	486–668
L	1564 ± 143	1360–1820	722 ± 54	620–800	240 ± 39	200–300
M	1599 ± 148	1356–1846	807 ± 58	744–895	275 ± 33	235–320

All results are given as fmol/ml cytosol.
*Six of the vials were scored as receptor-negative.
†Too few reliable results.

remained within the range of values reported by the others performing Scatchard analysis (Table 2) and are included in the ensuing discussion. Two other laboratories (labs B and I) performed predominantly Scatchard analyses but included a few single-dose saturating assays. Since the results of the two types of assays overlapped, they are also included. Laboratory A reported estradiol receptor values that were 5- to 10-fold lower than the results of the others and therefore, unless otherwise indicated, their results have been excluded from the following discussion.

Since statistical evaluation showed constancy of receptor values with time, this trial gives an estimate of the degree of variance of single laboratories. Table 2 demonstrates that the day-to-day variation differed enormously within certain laboratories. Reasons for this were not always clear, although in one case (lab. H) this was subsequently found to be due to differences in [^{3}H]-estradiol solutions. Omitting lab. H, the interassay coefficients of variation for each sample were, with one exception (lab. J, sample A), all less than 17%. The median value was 10%.

Linear regression analysis of the data of each individual laboratory (dilution factor vs fmol/ml cytosol) demonstrated correlation coefficients ranging from 0.952 (lab. J) to 0.997 (lab. D) (lab. H was omitted from this analysis since no values were reported for sample C). As reported earlier [5], most laboratories reported values that were consistently high or low in comparison to the overall group results. Only results from lab. K demonstrated significant effects of dilution. This relative increase of measured receptors by lab. K in the more dilute samples (ratio A:B:C = 6.0:3.3:2.2) was due to incorrect calculation of non-specific binding (see Discussion).

The reported K_d values varied widely (Fig. 1) both from lab.-to-lab. and within any one laboratory (up to 5-fold). The majority of K_ds reported for ER were from 1 to 4×10^{-10} M. Values exceeding 1 nM were infrequently reported.

Sample D, the heat-inactivated cytosol, was analyzed three times by each laboratory. The results of these analyses are shown in Table 4, where the classification of the sample as positive, borderline or negative has been assigned according to the usual classification criteria and cut-off levels in each individual laboratory.

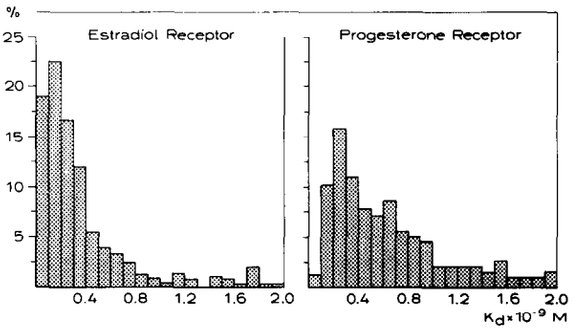

Fig. 1. Estradiol and progesterone receptor analysis of lyophilized calf uterine cytosols (samples A–C) by 13 laboratories participating in the 1981 trial of the EORTC Receptor Group: frequency distribution of reported dissociation constants (K_d).

Progesterone receptor

Twelve of the 13 laboratories performed PgR analyses (Table 3). Laboratory A again reported much lower values than the rest of the participants, and data from this lab. are excluded in the following discussion unless otherwise stated. The results of lab. B are excluded from the

table for reasons already stated (see Stability of Powders). One participant (lab. G) performed single-point assays using 8 nM [^{3}H]-Org 2058 with and without a 100-fold excess of Org 2058.

Again, the intra-laboratory variations were considerable and also varied from sample to sample. The lowest and highest calculated intra-laboratory coefficients of variation were 4 and 28% respectively. The mean values of most laboratories agreed reasonably well. The higher values reported by labs H, K and M resulted from incorrect calculation of data (see below). From Tables 2 and 3 it can be seen that laboratories observing high or low estradiol receptor values did not necessarily also report respectively high or low progesterone receptor values.

Non-linear Scatchard plots with a horizontal component at low steroid concentrations were often observed in sample A and these often resulted in high K_d values, which are sometimes associated with unusually high receptor values.

Greater variation in K_d values were reported for PgR than ER (Fig. 1). Almost all values were less than 2.0×10^{-9} M, while a few were as high as 6.0×10^{-9} M (not shown in figure).

The results of the analysis of sample D are shown in Table 4.

Protein measurement

All participants used the same batch of Coomassie brilliant blue and human serum albumin. The results of the individual participants are given in Table 5, while Table 6 shows calculations based solely upon the mean values of the various laboratories. For all three samples a considerable range of mean values was observed. The ratio between the highest and the lowest mean value was approximately 1.6 (Table 6). With regard to this range of values, the various labs reported rather consistently high, intermediate or low protein results for the various samples analyzed. For example, lab. J reported the

Table 3. Summary of progesterone receptor results

Participating laboratories	Sample A		Sample B		Sample C	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
A	634 ± 47	546–688	228 ± 18	208–257	113 ± 37	76–177
B	—*	—	—*	—	—*	—
C	not done		not done		not done	
D	3487 ± 315	3044–4100	1464 ± 114	1259–1632	521 ± 45	446–574
E	3062 ± 515	2170–3705	1445 ± 211	1145–1680	573 ± 105	425–685
F	3786 ± 201	3550–4130	1644 ± 64	1530–1740	577 ± 41	525–635
G	3327 ± 385	2900–4061	1622 ± 195	1345–1991	549 ± 35	496–603
H	6737 ± 982	5333–8400	2296 ± 145	2067–2500	754 ± 80	627–840
I	4087 ± 351	3602–4418	1927 ± 105	1806–2017	835 ± 53	775–871
J	4022 ± 560	3300–4890	1591 ± 372	1120–1990	633 ± 25	599–670
K	4717 ± 737	3730–5683	1907 ± 109	1719–2037	1108 ± 179	818–1402
L	3609 ± 231	3180–3920	1540 ± 85	1440–1660	447 ± 114	300–600
M	—†	—	2687 ± 754	1504–3639	917 ± 187	631–1181

All results are given as fmol/ml cytosol.
*Values varies with time. Did not follow agreed time protocol.
†Too few reliable results.

Table 4. Results obtained on the heat-inactivated calf uterine cytosol (sample D)

Classification assigned by individual laboratories	No. assays in stated classification	
	Scatchard analysis	Single-dose assay
Estradiol receptor:		
positive	1*	4†
borderline	1	1
negative	30	2
Progesterone receptor:		
positive	2‡	5§
borderline	1	
negative	23	

*6 fmol/mg protein with K_d value of 2.9×10^{-9}M.
†The values ranged between 17 and 27 fmol/mg protein.
‡12 (K_d:2.5×10^{-9}M) and 18 fmol/mg protein (K_d:3.6×10^{-9}M).
§The values vary between 11 and 80 fmol/mg protein.

highest protein values on three occasions and participants D, E and H consistently obtained the lowest values (Table 5). Moreover, highly significant Spearman rank correlation coefficients were obtained between the protein values of the various participants: samples A–B, $r = 0.84$; A–C, $r = 0.91$; and B–C, $r = 0.90$, with P values less than 0.002. The reproducibility of the protein assay differed considerably from one laboratory to another. However, without exception, the intra-laboratory variation coefficient within labs was less than 15%, the median value being 7–8%. In comparison, the variation coefficient between labs ranged from 14 to 17% of the mean value (Table 6).

Table 6. Summary of protein measurements

Sample	Range of mean values (mg/ml cytosol)	Mean	S.D.	V.C. (%)
A	3.22–4.86	3.81	0.55	14.4
B	3.07–4.91	3.75	0.52	13.9
C	2.47–4.04	3.09	0.53	17.2

Recalculation of data

Recalculation of the data from the individual labs was performed using a single method that is in accord with the equations and principles discussed in Materials and Methods. A comparison of reported and recalculated data from 11/13 labs for the ER determination and 10/13 labs for the PgR determinations are shown in Tables 7 and 8 respectively. It can be seen that although the mean values were hardly affected, both the range of values and the variation were considerably reduced using a common method of calculation. This effect was most apparent in the sample with lowest receptor content.

The reasons for these differences in reported and recalculated results are mainly (a) incorrect calculation and correction for non-specific binding; (b) incorrect estimation of concentration of steroid in each incubation; and (c) errors in correction for aliquots used. Two different errors were encountered in the correction for non-specific binding. In some laboratories specific binding was calculated as the difference between binding in the presence of an excess of non-radioactive competitor and the total binding in absence of the competitor. Another error was incorrect interpretation of equation (2) (Materials and Methods). The ratio of B/F in the presence of an excess of non-radioactive competitor at saturating concentrations of the labeled ligand was multiplied by B_{total} rather than the free ligand concentration (F). This resulted in a systematic overestimate of the number of receptors, which was inversely related to number of receptors present.

The two labs experiencing problems with estimation of ligand present during binding assays had both relied on the information given pertaining to specific activity of the ligand and the dilutions performed from the stock solution of concentrated ligand to calculate the amount of steroid bound in each incubation. In both cases the actual number of counts/min incubated was lower than the expected number, provided that the counting efficiency of the scintillation counter and the series of dilutions were correct. This resulted in overestimation of specific binding, which was especially apparent in PgR determinations.

DISCUSSION

The conditions used in this trial have been such that optimum uniformity of results should be obtained. Use of common lyophilized cytosols, lots of radioactive ligands, competitors, Coomassie brilliant blue and protein standard as well as

Table 5. Protein results reported by individual laboratories

Participating laboratories	Sample A		Sample B		Sample C	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
A	3.53 ± 0.07	3.4–3.6	3.51 ± 0.11	3.3–3.7	2.87 ± 0.14	2.7–3.1
B	4.20 ± 0.06	4.1–4.3	4.06 ± 0.13	3.9–4.3	3.65 ± 0.25	3.2–4.0
C	4.41 ± 0.38	3.9–5.0	4.41 ± 0.37	3.7–5.0	3.18 ± 0.32	2.6–3.5
D	3.22 ± 0.12	3.0–3.4	3.22 ± 0.13	3.1–3.3	2.47 ± 0.16	2.3–2.8
E	3.22 ± 0.12	3.1–3.4	3.22 ± 0.08	3.0–3.4	2.53 ± 0.17	2.3–2.7
F	3.36 ± 0.11	3.1–3.5	3.28 ± 0.12	3.1–3.5	2.57 ± 0.10	2.4–2.7
G	4.44 ± 0.28	4.0–4.7	3.79 ± 0.41	3.1–4.4	4.91 ± 0.12	3.8–4.1
H	3.32 ± 0.09	3.2–3.5	3.07 ± 0.16	2.9–3.4	2.50 ± 0.18	2.3–2.8
I	3.92 ± 0.15	3.7–4.2	3.78 ± 0.52	3.1–4.8	3.20 ± 0.19	3.0–3.5
J	4.86 ± 0.54	4.2–5.9	4.91 ± 0.42	4.3–5.7	4.04 ± 0.43	3.4–4.7
K	3.22 ± 0.46	2.4–3.9	3.72 ± 0.31	3.3–4.2	2.89 ± 0.28	2.5–3.3
L	3.90 ± 0.37	3.3–4.4	3.82 ± 0.21	3.4–4.2	3.04 ± 0.13	2.8–3.2
M	3.91 ± 0.18	3.6–4.1	3.98 ± 0.25	3.7–4.4	3.38 ± 0.28	3.1–3.9

All values are given as mg protein/ml cytosol.

Table 7. Summary of reported and recalculated estradiol receptor data (fmol/ml *cytosol*)

Sample	Range of mean values	Mean*	S.D.	V.C. (%)
A:				
reported	1113–1599	1399	168	12.0
recalculated	1212–1577	1413	129	9.2
B:				
reported	548–841	679	95	14.0
recalculated	599–728	659	47	7.2
C:				
reported	175–557	265	108	40.9
recalculated	198–257	232	25	11.0

The results of 11 out of 13 participants are included in this table. Excluded are participant C, who did not send raw data, and participant A, who consistently reported estradiol and progesterone receptor values much lower than all other participants.

*Linear regression of mean values (sample A:B:C = 6:3:1): reported data: $r = 0.999$, $y = 218x + 22$; recalculated data: $r = 0.999$, $Y = 237x - 23$.

Table 8. Summary of reported and recalculated progesterone receptor data (fmol/ml *cytosol*)

Sample	Range of mean values	Mean*	S.D.	V.C. (%)
A:				
reported	3062–6737	4092	1103	26.9
recalculated	3062–4377	3668	421	11.5
B:				
reported	1445–2687	1812	403	22.5
recalculated	1445–1933	1666	158	9.5
C:				
reported	447–917	691	208	30.0
recalculated	440–830	593	100	16.8

The results of 10 out of 13 participants are included in this table. Excluded are participant C, who did not perform progesterone receptor assays, participant B, who reported progesterone receptor values that varied as a function of time, and participant A, who reported unusually low results.

*Linear regression of mean values (sample A:B:C = 6:3:1): reported data: $r = 0.997$, $y = 687x - 90$; recalculated data: $r = 0.998$, $Y = 619x - 88$.

agreement on a common procedure are all elements contributing to this end. The results reported are better than those experienced earlier among the participants [5], and are generally better than those seen in quality control programs that have been initiated in several countries [9–14]. All laboratories, with one exception (lab. A, see under Results), classified the three samples (A–C) containing ER and PgR as being receptor-positive. Inter-assay coefficients of variation within the individual laboratories for these three samples expressed per ml cytosol were less than 17% for ER, while for PgR a few coefficients were as high as 26%. It may be noted, however, that the upper value of the 95% confidence limits for the distribution of the inter-laboratory variation coefficients for both receptor assays was lower than 15%. If only the recalculated data is considered, the range of inter-laboratory coefficients of variation decreases to 7–17%, thus emphasizing the importance of using the same method of calculation, as has already been noted by Braunsberg and Hammond [15]. It should be noted that had results been expressed in the usual manner, as fmol/mg cytosol protein, the intra- and inter-laboratory variation would have been much greater due to the large variation seen in protein analysis. Reasons for the occasional random outliers have not been found.

Although agreement on receptor positivity was

unanimous for samples A–C, there were discrepancies in classification of sample D, the heat-inactivated cytosol. These discrepancies were especially apparent in the results generated from single-dose saturation analysis. Only 3/58 Scatchard (multipoint) analyses of this receptor-negative cytosol but 9/12 single-dose saturation analyses were reported as being receptor-positive. In the three multipoint analyses classified by the reporting laboratories as positive, none of the three Scatchard plots convincingly demonstrated specific steroid receptor binding. In all three instances the K_d values were higher than 2×10^{-9} M and the correlation between bound/free and bound hormone was not significant. While investigators performing Scatchard analysis have the option of taking the K_d value and presence/absence of a significant linear correlation between B/F vs B into consideration as further criteria when judging whether a particular cytosol should be classified as negative or positive, those performing single-dose saturating analyses must rely entirely on the difference in binding in the presence and absence of an excess of non-radioactive ligand and cannot, therefore, assess whether the saturable binding observed is of high or low affinity. While the former method of classification tends to become subjective and the latter remains objective, the crux of the problem is that different results are obtained using the different methods.

The major achievement of recalculation of the data was in detection of errors either in the interpretation of how to correct for non-specific binding or in the techniques and/or computations in the assay. Correction of these errors led to more similar results in the group of participants, and the coefficient of variation of mean values was, therefore, reduced. The erroneous correction for non-specific binding by direct subtraction of binding observed in the presence and absence of non-radioactive competitor was frequently observed. Especially at low ligand concentrations, the free ligand concentrations in parallel incubations with identical concentrations of tritiated ligand with and without an excess of competitor are not equivalent, and this method of correction for non-specific binding is, therefore, not correct. When non-specific binding is low, the difference between this correction method and the one used for recalculation of data is small. However, in these lyophilized cytosols non-specific binding was substantial. The magnitude of the error is inversely proportional to the concentration of the radioactive ligand.

The error in estimation of concentration of radioactive ligand in the binding assay was probably due to adsorption of the ligand to surfaces of the vials used. Other possible sources of this type of error are errors in dilution of the stock solution of radioactive ligand and incorrect estimation of counting efficiency of the liquid scintillation counter used. The possibility of adsorption problems and dilution errors can be avoided by taking aliquots for counting from vials that have been set up in parallel with those used for incubation of cytosol. Thus the number of counts/min available for binding to receptors is known and accessible for conversion to fmol ligand.

Although inter-laboratory variation in protein determinations is lower in the present trial (c.v. = 14–17%) in comparison with earlier trials (c.v. = 15–31%), the ratio between highest and lowest means of the participants is 1.6, which is unacceptably high. Since a common method of determination was agreed upon and the same lots of Coomassie brilliant blue and serum albumin standard were used in the individual laboratories, there is no self-evident explanation for these differences. Intra-laboratory variation was also considerable; in some cases the coefficient of variation was as high as 15%.

Lyophilized calf uterine cytosols appear to be well suited for intra- and inter-laboratory quality control of steroid receptor analysis. Shipment without temperature control followed by prolonged storage at 4°C (for up to 2 months) had no deleterious effect on the receptor binding activity for any of the 13 participating laboratories. Recently Godolphin and Jacobsen compared four types of material for quality control of routine assays of estrogen receptors in human breast tumors [16]. They concluded that lyophilized material is relatively easy to prepare, has excellent stability and seems to be the material of choice for quality control purposes.

In a subsequent trial lyophilized uterine cytosols with low receptor-binding capacities (<30 fmol/mg protein) were used to elucidate the gravity of the problem of classification of a given cytosol as being receptor-negative or -positive. The results are presented in the accompanying paper.

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