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Reliability of Steroid Hormone Receptor Assays: an International Study

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Abstract—*The reproducibility of oestrogen and progestin receptor assays performed by laboratories participating in an international breast cancer treatment trial has been assessed. Three tissue reference powders containing low, medium and high oestrogen receptor levels* (22 ± 4, 88 ± 7, 227 ± 13 fmol/mg *cytosol protein respectively) were prepared in Louisville, KY, U.S.A., assayed repeatedly and multiple samples of each shipped on solid* CO_2 *to the coordination-distribution centre in Berne, Switzerland. Samples were dispatched from Berne to Cantons within Switzerland, to Yugoslavia, South Africa, Australia, New Zealand and also back to the United States for oestrogen and progestin receptor assays. Results were returned to Berne, Switzerland. There was a decrease in the levels of oestrogen and progestin receptors during the time of storage and transit. However, the ability to assign a powder to either the low, medium or high level of oestrogen receptor was not affected. Laboratories also determined progestin receptor. All laboratories clearly identified the powder containing the low level of progestin receptor, but there was poor quantitation with the other two assay standards. It is recommended that clinical hormone receptor laboratories, especially those participating in clinical trials, establish regular quality control procedures for both daily evaluation internally and periodic outside monitoring of interlaboratory variation.*

INTRODUCTION

IT IS now accepted that hormonal sensitivity in breast cancer can often be predicted by the presence or absence of oestrogen receptors in the tumour [1, 2]. However, not all tumours with a detectable level of oestrogen receptor respond to endocrine manipulation. To refine prediction of hormone sensitivity, both oestrogen and progesterone receptor assays are often undertaken as the progesterone receptor is a specific gene product of oestrogen action that may, as a result, indicate a functional oestrogen receptor system [3].

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Justification for patient stratification based on the results of hormone receptor assays in large national and international clinical trials (NSABP, ECOG, SECSG, Ludwig) is often limited by the diverse procedures used to collect tumour tissue after surgery and the variety of receptor assay techniques already established in different centres. In general, assay reliability is assumed rather than critically monitored. However, it is becoming clear that quality control systems are required. Several groups (NSABP, ECOG, SECSG) are instituting a quality control network as this is becoming a requirement for multicentric clinical protocols.

The aims of this study were (1) to test the stability and utility of a tissue powder (not a lyophylized cytosol) for a spot check of quality control and (2) to report our experience in a study

of the reproducibility of oestrogen and progestin receptor assays within laboratories of the Ludwig Breast Cancer Study Group compared with a central reference laboratory.

MATERIALS AND METHODS

Preparation of powders

Various human tissues, such as uterus, leiomyomata, breast carcinoma, muscle and pregnancy serum, as well as animal tissues, including uterus, mammary gland, liver and kidney, have been used as a source of material for the preparation of tissues reference powders [4–6]. To prepare LUD 11, 12 and 13, different preparations of human uterus, breast carcinoma and muscle were frozen in liquid nitrogen, pulverized into fine powders in a mechanical grinding device and stored separately at -80°C. Random samples were assayed at intervals for up to 90 days before dispatch to Switzerland.

Distribution of powders

Sets of standard powders (LUD 11, 12, 13) were shipped air freight on solid CO_2 from Louisville, KY, U.S.A. to Berne, Switzerland. They were stored in Berne at -75°C (17.12.79 until 14.1.80) before being shipped on solid CO_2 to participating laboratories. Upon receipt, each laboratory stored the powders at -70°C before measuring oestrogen and progestin receptor concentrations by their preferred methods. Because of the complex arrangements for transportation it was not possible to plan for laboratories to assay standards at a particular time. Assays were completed by laboratories between 14.1.80 and 6.2.80. One set of standards was shipped back to Louisville, KY, U.S.A. from Berne (25.2.80, received Louisville 29.2.80) on solid CO_2 to assess receptor loss with time during transportation. These powders were assayed with the same standard powders that were stored in Louisville at -80°C throughout the course of the study.

Standard assay procedures

The methods for oestrogen and progestin receptor assays for all participating laboratories are provided below.

Frozen pulversized tissue was homogenized in about a 1/5 (w/v) volume of Tris buffer (0.01 M Tris-HCl, pH 7.4, at 0–4°C, 0.0015 M EDTA, 10% glycerol, 0.001 M monothioglycerol) with a Polytron PT-ST homogenizer (Brinkman Instruments, Inc.) at a speed setting of 3.5 for 3 × 10-sec intervals.

The homogenate was centrifuged at 100,000 *g* for 30 min at 0°C. The high-speed supernatant was adjusted to about 2 mg protein/ml with Tris buffer according to a standard dilution scheme (1 g tissue ≃ 20 mg cytosol protein). Protein values were later more accurately determined by the method of Lowry *et al.* [7].

For the oestrogen receptor assay 100 μl of cytosol (in duplicate) were incubated with 100 μl of five separate doses of [^{3}H]-oestradiol (final dose range 0.2–3 nM) with or without a 100-fold excess of diethylstilboestrol prepared in Tris buffer. Progestin receptor was assayed similarly using [^{3}H]-R5020 (final dose range 0.5–5 nM) with or without a 100-fold excess of the nonlabelled competitor R5020.

After overnight incubation at 0–4°C, 500 μl of dextran-coated charcoal suspension was added to each tube. The tubes were vigorously shaken for 5 min (progestin receptor) and 30 min (oestrogen receptor) and then centrifuged for 5 min at 2000 rpm to sediment the charcoal.

A 500-μl fraction from each tube was counted directly in scintillation fluid. The data were analysed by the method of Scatchard [8] to generate dissociate constants and level of binding sites. Data was expressed as fmol receptor per mg cytosol protein and tumor weight.

Deviations from assay procedures

Some of the groups adhered strictly to the assay procedures recommended by the coordinating centre in Berne (C, L1, L4, L6, L7). Others used the following modifications: L2 and 3: performed the progestin receptor assay using a single dose (6 nM) of [^{3}H]-R5020 and a hydroxylappatite assay [9]; L5: used mercaptoethanol in the Tris buffer instead of monothioglycerol, an incubation volume of 100 μl, a final dose range of 0.1–2 nM, [^{3}H]-E2 ± 200-fold excess DES for oestrogen receptor, 0.5–8 nM [^{3}H]-R5020 ± 200-fold excess R5020 for progestin receptor, 4 hr incubation time, and only 200 μl of DCC; L8: assayed oestrogen receptor with a single dose (3 nM) of [^{3}H]-E2 and progesterone receptor with a single dose (6 nM) of [^{3}H]-progesterone in a total incubation volume of 450 μl. One millilitre of DCC was added to the incubates and 1 ml counted for radioactivity.

Protein standardization

To assess differences in receptor content per unit protein, bovine serum albumin was distributed from Berne with the frozen powders. Each group was asked to calculate receptor values per mg protein using both their own protein standard and the one sent from Berne. No significant differences were noted with the two different protein standards using the Lowry method [7].

RESULTS

Assay of powders in Louisville

Three powders were prepared and assayed repeatedly for oestrogen and progestin receptor using a multipoint titration procedure described above. Samples were designed to have low (LUD 13), intermediate (LUD 11) and high (LUD 12) levels for oestrogen receptors.

Oestrogen receptor

Although there was considerable variation within each sample, binding remained within definite regions of low, medium and high oestrogen receptor content (Fig. 1). The mean ± S.E.M. of LUD 11, 12 and 13 is coded as C1 in Table 1. The dissociation constants (K_d), an index of specificity and high-affinity oestradiol binding for all control samples (C1, C2A, C2B) were in the range of $0.1–4.0 \times 10^{-10}$ M.

Progestin receptor

Progestin receptor concentrations were more variable. There was an obvious loss of receptor over the 5-month period (Table 1, C1 vs C2B). Dissociation constants (K_d for samples LUD 12 and 13) were quite uniform and of high affinity throughout the assay period (0.31–0.72 nM and 0.42–0.481 nM, respectively). Dissociation constants for samples LUD 11 were more variable over the assay period. Lower binding affinity was consistently associated with a higher binding capacity. The mean K_ds for samples on days 1–10, 11–39, 75–89 and 139–152 (i.e. samples C2B in Table 1) were 2.84 nM (range 1.8–3.7 nM),

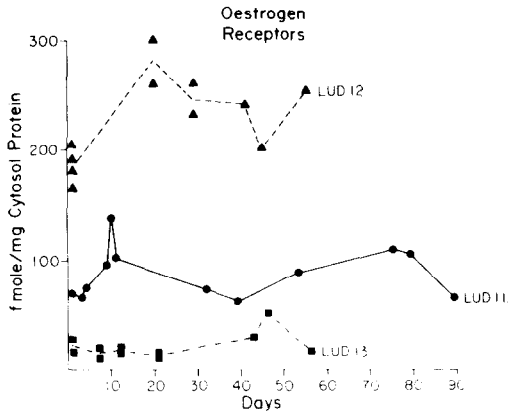

Fig. 1. Control oestrogen receptor analysis. Oestrogen receptor was assayed periodically in samples LUD 11, 12 and 13 over a 90-day period by methods described in the text. Each point represents the result of a single assay by Scatchard analysis and are the individual results of the C_1 means in Table 1.

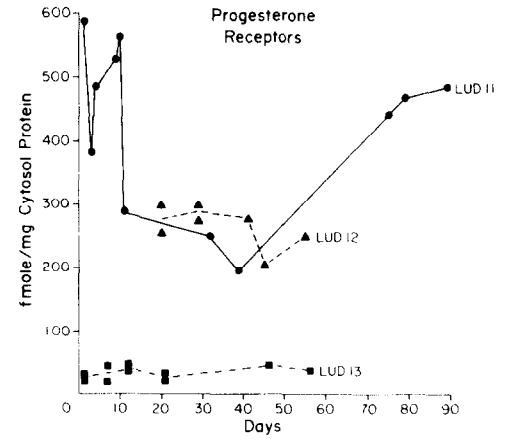

Fig. 2. Control progesterone receptor analysis. Details are the same as for Fig. 1.

Table 1. Oestrogen and progestin receptor analysis

Coded laboratory	Oestrogen receptor (*n*)	11	12	13	Progestin receptor (*n*)	11	12	13
C1	(12, 11, 11)	88 ± 7	227 ± 13	22 ± 4	(11, 7, 10)	422 ± 39	263 ± 12	34 ± 4
C2A	(1, 2, 2)	47	156, 195	11, 15	(1, 2, 2)	236	137, 138	27, 29
C2B	(6, 6, 5)	48 ± 5	170 ± 12	14 ± 1	(6, 6, 5)	218 ± 26	138 ± 11	28 ± 3
L1	(5)	57 ± 2	154 ± 6	6 ± 3	(5)	147 ± 38	222 ± 17	0
L2	(3)	46 ± 3	126 ± 8	7 ± 1	(3)	53 ± 7	52 ± 1	6 ± 1
L3	(3)	45 ± 4	130 ± 5	0	(2)	62 ± 10	60 ± 1	0
L4	(4)	47 ± 2	117 ± 3	10 ± 1	(4)	257 ± 53	143 ± 16	21 ± 1
L5	(3)	16 ± 1	43 ± 4	15 ± 1	(3)	41 ± 4	41 ± 8	14 ± 1
L6	(1)	17	137	6	(3)	207 ± 2	189 ± 2	17 ± 5
L7	(6)	63 ± 5	149 ± 18	6 ± 1	(6)	28 ± 9	33 ± 7	6 ± 2
L8	(6)	70 ± 4	175 ± 5	7 ± 2	(6)	85 ± 6	72 ± 6	8 ± 4
Total means*		51 ± 3	143 ± 7	8 ± 1		126 ± 16	109 ± 11	12 ± 2
(*n*)		(38)	(39)	(38)		(36)	(40)	(39)

Oestrogen and progestin receptors were assays in samples 11, 12, and 13 by the control centre in Louisville, KY before shipping to Berne for distribution (C1), after receiving samples returned from Berne (C2B) and after continuous storage in Louisville for a time period equal to the age of samples C2B (C2A). Assay values for oestrogen and progesterone receptors (mean ± S.E.M. fmol/mg cytosol protein) for samples 11, 12 and 13 are represented as L1–L8 for the coded participating laboratories. *n* represents the number of assays performed by each centre.

*The total means are calculated from C2A, C2B and L1–8, i.e. all the data collected after the dispatch of samples from Louisville (C1).

Table 2. Pooled standard deviations of replicate assays based on transformed data

Coded laboratory	Oestrogen receptor		Progestin receptor	
	All samples	LUD 11 and 12	All samples	LUD 11 and 12
C	0.25 (19)*	0.28 (13)	0.20 (19)	0.21 (13)
L1	0.78 (12)	0.15 (8)	0.31 (13)	0.40 (8)
L2	0.18 (6)	0.19 (4)	0.20 (6)	0.22 (4)
L3	0.14 (6)	0.17 (4)	0.19 (3)	0.23 (2)
L4	0.12 (9)	0.11 (6)	0.33 (9)	0.40 (6)
L5	0.14 (6)	0.16 (4)	0.27 (6)	0.32 (4)
L6	— (0)	— (0)	0.29 (4)	0.02 (3)
L7	0.49 (15)	0.57 (10)	0.74 (13)	0.60 (8)
L8	0.51 (15)	0.18 (10)	0.89 (15)	0.20 (10)

*Figures in parentheses represent degrees of freedom.

1.12 nM (range 0.87–1.14 nM), 3.9 nM (range 2.6–5.4 nM) and 1.4 nM (range 1.1–1.6 nM) respectively. As one can see from Fig. 2 and Table 2, this fluctuation coincides with the rise and fall of progestin binding capacities for these samples.

Samples returned to Louisville two months after dispatch to Berne (Table 1, C_2B) were reassayed with equivalent samples that had remained in Louisville (Table 1, C_2A). In general, there was a decrease in both oestrogen and progestin receptor concentrations with storage. The values of oestrogen receptor (LUD 11) and progestin receptor (LUD 11 and LUD 12) were significantly decreased ($P < 0.001$ by Student's t test).

Assay of powders in laboratories

Oestrogen receptor determinations for all participating laboratories are compared with the original mean values from Louisville before (Table 1, C_1) and after (Table 1, C_2B) dispatching to centres. Laboratories assayed the powders more than 6 weeks after dispatch from Louisville and in general were in good agreement with each other. L5 and L6 declared technical difficulties with the assays when the results were returned. L5 standards thawed before cytosol preparation and this was reflected in low oestrogen and progesterone receptor levels. L6 discovered major deterioration of the [^{3}H]-oestradiol. All laboratories other than L5 could identify the low, middle and high concentrations of oestrogen receptors. Results were consistent with the values of powders obtained upon return and reassay in Louisville (Table 1, C_2B). Comparison of laboratories (excluding L5 or 6) with C_2B by Student's t test showed LUD 11 to be consistent for all laboratories. The control was significantly higher than L4 when measuring LUD 12 and was significantly higher than L2, L3 and L8 when measuring LUD 13 ($P < 0.001$). A rigorous statistical consideration is made in the following section.

Dissociation constants (K_d) ranged between 10^{-11} M and 10^{-10} M for all oestrogen receptor samples, indicating the presence of high-affinity oestradiol binding.

Progestin receptor determinations were more variable (Table 1), both within the Louisville control (Fig. 2) and the participating laboratories (Table 1). All laboratories could detect LUD 13 as the low range powder, but LUD 11 and 12 were as often as not identified within the same range.

Laboratories C, L1, L4 and L5 adhered to the standard assay procedures, and with the exception of L5, all reported the most consistent binding values. Very high binding affinities reported by L5 ($\sim 10^{-10}$) relative to the other groups ($\sim 10^{-9}$ M) may reflect one of the reasons why L5 reported very low PgR values.

Laboratories L2 and L8 used only a single saturating dose of ligand to measure progestin receptor. Their progestin receptor binding values were internally quite consistent (Table 1) but much lower than the values relative to the other groups using the multiple dose assay. Laboratory L8 used the low-affinity ligand [^{3}H]-progesterone rather than the high-affinity ligand [^{3}H]-R5020, which might account for an underestimate in progestin receptor levels.

Overall statistical considerations

In the analysis of general performance all the data from the laboratories (L1–L8) and the control laboratory (C2A and C2B) was pooled and is represented graphically in Fig. 3 (a and b) for the oestrogen and progestin receptors respectively. The samples are arranged so that the average assay value increases from left to right. It is clear that the variability of assay values among replicates tends to increase as the average of replicates increases (Fig. 3 and Table 1).

Within-laboratory variation

In order to compare the laboratories on the basis of their internal variability, the individual

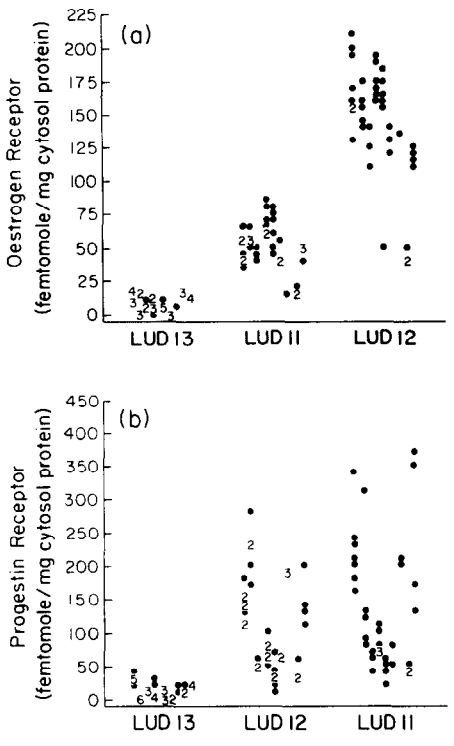

Fig. 3. The individual oestrogen (a) and progestin (b) receptor values for samples LUD 11, 12 and 13. The results are shown in ascending order of receptor concentration.

data have been transposed to a single value for a laboratory. The transposition used [$\sqrt[3]{(OER)}$ value or ln (PgR value +1)] reduced the trend in sample variability mentioned above. The transformed values are shown in Fig. 4 (a and b). The

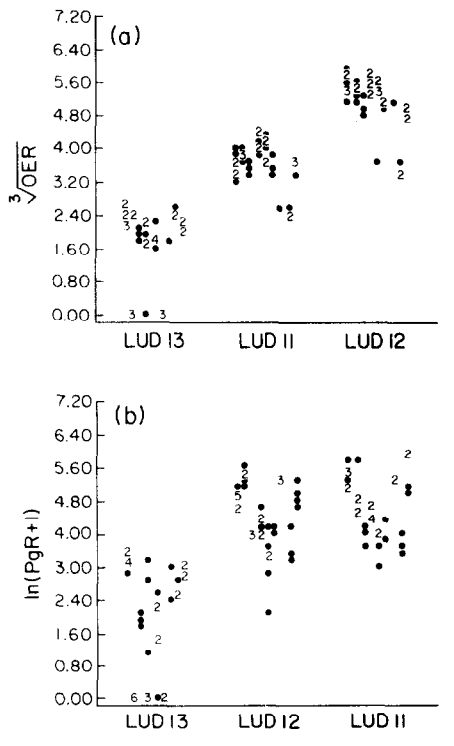

Fig. 4. The transformed oestrogen (OER) (a) and progestin (PgR) (b) receptor values for samples LUD 11, 12 and 13.

reporting of a zero value by a laboratory makes a substantial contribution to both the within-laboratory and between-laboratory variation. In Table 2 the within-laboratory standard deviation for each laboratory is shown. The pooled estimate was obtained by combining the standard deviations (based on the transformed data) of the replicates for each sample. For the oestrogen receptor assays, most of the differences in within-laboratory variability are due to the zeros reported by L1 and L8 for LUD 13 and to a single very low measurement of LUD 12 by L7. If the single measurement was omitted, then the pooled standard deviation would be 0.23 over all samples and 0.22 for samples LUD 11 and LUD 12.

For the progestin receptor assays, differences in within-laboratory variability can again be attributed to the zeroes reported by L1, L3, L7 and L8. Also, it should be pointed out that one analysis of LUD 12 by L7 which was equivalent to the low result obtained for the oestrogen receptor determination was also very low for the progestin receptor result. It seems possible that the handling of this particular assay standard was inappropriate either during the preparation of the vial in the U.S.A. or during cytosol preparation. Omitting this value gives L7 a pooled standard deviation of 0.68 over all samples and 0.41 for samples LUD 11 and 12. Finally, L6 has very low variability in replicate measurements of LUD 11 and 12; however, the number of measurements is too small to conclude that this laboratory had less internal variability.

Between-laboratory variation

For the oestrogen and progestin receptor assays we have compared the results with each other rather than compared each laboratory with the control. The results C2A and C2B were combined in the analysis and are referred to as C. To determine differences for the oestrogen and progestin receptor levels in LUD 11, 12 and 13, laboratories were compared two at a time using the Kruskal–Wallis multiple comparisons procedure. The results of these tests, carried out at a 5% significance level separately for each sample, are summarized in Fig. 5. Each point represents the mean of the laboratory with which it is labelled. The height of a point above the horizontal scare axis is proportional to the number of replicates in the mean. Pairs of laboratories that are significantly different are joined by the starred brackets. Thus, for PgRs in LUD 12, L1 is significantly different from each of laboratories L2, L5 and L7, and L7 significantly differs from L1, L6 and C. For the oestrogen receptor assays there is some suggestion that L5 and perhaps C and L8 may be more variable in

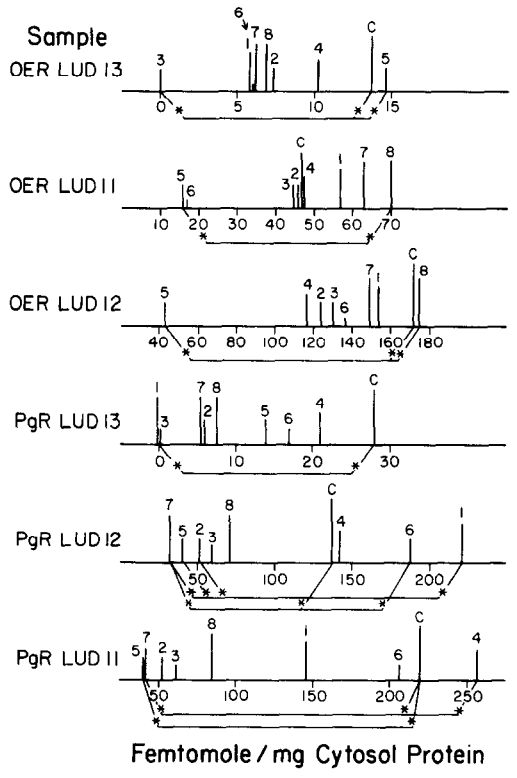

Fig. 5. The laboratory means for oestrogen (OER) and progestin (PgR) receptor measurements for LUD 11, 12 and 13. The significantly different laboratory pairs are indicated by the crossed connecting lines. Significance was determined by Kruskal-Wallis multiple comparisons at the 5% level.

their assays based upon their tendency to have extreme values among the nine laboratories. For the PgR assays the laboratories seem to fall into two groups, particularly for LUD 11 and 12: laboratories L2, L3, L5, L7 and L8 are in the lower group and laboratories L4, L6 and C are in the upper group. L2 was the most variable. Overall, the observations concerning L5 are not surprising as this laboratory declared a technical difficulty (sample thawing) that would be expected to reduce the absolute level of receptors that could be detected.

DISCUSSION

This study documents an attempt to monitor the reproducibility of hormone receptor assays by clinical laboratories in different countries. Previous studies have used lyophilized cytosols as an assay standard, but we decided to use tissue powders to monitor not only the assay procedure but also to consider the variability of the homogenization step before cytosol preparation. Care was taken to maintain coding of the assay standards, and individual laboratories in the study reported the results directly to the coordinating centre in Berne, Switzerland. However, the code was not altered on samples returned to Louisville. Nevertheless, the assay laboratory in Louisville, KY, reported the values for hormone receptor standards before and after transit to Berne without any knowledge of the results generated by the other participating laboratories. It is clear that extensive transportation in solid CO_2 and storage results in a gradual degradation of oestrogen and progestin receptors. Be that as it may, and despite the technical difficulties in laboratories L5 and L6, there was good agreement between laboratories concerning the classification of the three powders based on oestrogen receptor concentration.

Other reports that compare the reproducibility of oestrogen receptor assay laboratories have been published [10, 11]. In the study conducted among 5 laboratories in Great Britain [10], reproducibility was consistent, although the design which divided a tumour specimen between participating institutions was not optimal since intratumour variations, i.e. differences in oestrogen receptor concentration within the tumour, were not standardized. In the study conducted within 6 laboratories of the Eastern Cooperative Oncology Group [11], participating laboratories were asked to discriminate between samples with an apparently narrow range of receptor values. In this case there was variation in reported oestrogen receptor values, with all but one of the laboratories able to distinguish the high-level powder from medium- or low-level powders. Like the present study, the intralaboratory variation was less than interlaboratory variability.

In the present study reproducibility of progestin receptor assays is clearly a problem between routine assay laboratories. Other studies have not usually attempted to compare the reproducibility of progestin receptor assays, so it is unclear whether the difficulties are widespread or confined only to our group. However, our observation is supported by the finding in an assurance programme used by the Southeastern Cancer Study Group (SECSG) that progestin receptor assays are very unreliable [6]. The problem may not be confined to interlaboratory variation since the variability of assay data for LUD 11 by the control laboratory was large. It must be stressed, however, that the variability was not seen in either samples LUD 12 or 13. The progestin receptor results of the participating laboratories for LUD 11 and 12 were generally lower than the reference laboratory in Louisville. This could be accounted for by (a) the labile nature of the progestin receptor during cytosol preparation; (b) different degrees of purity of [^{3}H]-R5020 used for the assay; and (c) incorrect interpretation of Scatchard plots resulting in lower binding capacities.

Based on the findings in the present study, we

recommend that laboratories devise quality control checks for reproducibility so that clinical studies of correlations of hormone receptors with clinical response to therapy amongst cooperative groups can be evaluated critically. The quality control should be organized on a daily basis within individual laboratories so that the steps in the assay, contamination of buffers and reagents and the biochemical integrity of the radiolabelled ligands can be monitored. Periodic quality control from outside, impartial organizations would also be an advantage to maintain accuracy. Be that as it may, it cannot be stressed sufficiently that the critical step in the whole process of determining hormone receptors in an established, quality-controlled laboratory is the integrity of the tissue specimen sent to the laboratory from surgery. It is essential that an efficient local system is operating to freeze tumour tissue rapidly after surgery (within 30 min) before transportation to the laboratory. Without the guaranteee of a suitable tumour sample, all attempts at laboratory quality control will be invalidated. All laboratories in the Ludwig Breast Cancer Study Group have been visited by representatives of the coordinating centre in Berne to evaluate and improve tissue collection procedures. The information gathered in this study has been used to improve the quality of routine receptor assays in the participating laboratories that serve the clinical participants of the Ludwig Breast Cancer Study Group.

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