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Standardization of Steroid Receptor Assays in Human Breast Cancer—III. Selection of Reference Material for Intra- and Inter-laboratory Quality Control

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Abstract—*A comparison of estrogen and progesterone receptor (ER and PgR, respectively) analyses using minced frozen tissue and lyophilized cytosols of the same samples demonstrated that intra-laboratory variations in assays performed by* 13 *members of the EORTC Receptor Group are lowest using the lyophilized samples. Inter-laboratory variation in receptor values was on the same order of magnitude for both types of samples* (ca 22% *for ER and* 30% *for PgR). There was no correlation between receptor values measured within each laboratory for either ER or PgR in minced tissues compared to lyophilized cytosols, which illustrates that methods of tissue disruption, extraction of receptors, and preparation of cytosol are sources of intra- and inter-laboratory variation. In some laboratories the handling of the tissue was apparently sub-optimal since a slight but significant difference was found in the overall mean concentration of ER in minced tissue compared to lyophilized cytosol samples. It was concluded that lyophilized tissue samples are the material of choice for routine intra- and inter-laboratory quality controls. However, differences in methods of handling tissue to obtain cytosol should not be disregarded since they lead to increased intra-laboratory variation. A difference was demonstrated between use of a common batch of isotope and the different batches concurrently employed in the laboratories, but the differences were not large enough to warrant use of a common batch for routine inter-laboratory comparisons. Differences in methods used to convert* cpm *to* dpm *did not appreciably affect the results when counting samples of tritium containing from* 30,000 *to* 105,000 dpm.

INTRODUCTION

ESTROGEN receptor (ER) content of breast cancer tissue is both an important prognostic factor as well as an important determinant in predicting the response to endocrine therapy in advanced disease. It is therefore essential that intra- and inter-laboratory receptor analyses yield reliable, reproducible data, and that control analyses should be routinely run to validate assay results from patient samples [1,2]. These controls should give results that are representative of those obtained using patient tissue and they should, preferably, be easy to handle. Some of the receptor study groups have chosen frozen tissue powders as the control material of choice [3,4], while others have chosen lyophilized cytosols (for review, see [5]). All of our own earlier EORTC Receptor Group trials to evaluate reproducibility of ER and progesterone receptor (PgR) analyses have utilized lyophilized cytosols [6,7].

Use of lyophilized cytosols for quality control purposes evades, however, methodological points such as homogenization of tissue, extraction of receptors, and method of cytosol preparation that can influence assay results. A trial was therefore conducted to investigate whether use of different methods to prepare cytosol affects the inter-

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laboratory variation.

Other potential sources of inter-laboratory variation were also investigated. All receptor methods using radioactive ligands rely upon the ability to correctly convert the measured number of counts per min (cpm) to disintegrations per min (dpm) so that the concentration of hormone bound to receptor can be calculated from the specific activity of the isotope. Since many different methods of converting cpm to dpm are currently in use, these calibration methods were investigated with ready-to-use internal standard capsules as well as with a lyophilized [^{3}-H]-estradiol–BSA solution. Finally, it has been reported that different batches of isotope may consistently give different receptor values if tumor samples are assayed simultaneously [8]. To clarify this point, we investigated whether use of a common batch of [^{3}-H]-estradiol for receptor analysis yields significantly different results from those obtained using the random batches employed at any given time in the different laboratories.

MATERIALS AND METHODS

Tissue. Calf uterine tissue was used as minced tissue, and lyophilized cytosols were prepared from the same material as previously described [5–7]. In order to assure homogeneity, the calf uterine tissue was mechanically minced before homogenization.

Chemicals. A common batch of [^{3}-H]-estradiol (batch No. 84, TRK. 322, specific activity = 101 Curies/mmol) was procured from Amersham International, U.K.

Commercially available capsules (Lumac Instand, Lumac Systems, AG, Basel) were used for the estimation of liquid scintillation counting efficiency. Furthermore, vials containing 2.0 ml of a lyophilized [^{3}H]-estradiol–BSA solution were distributed from the central laboratory.

Protein analysis. The Bradford technique (Coomassie brilliant blue; Bio-Rad Laboratories) [9] was used with human serum albumin (Kabi; Kabi Diagnostica, Stockholm, Sweden) as the standard.

Study protocols

(a) Investigation of minced tissue vs lyophilized cytosol. Each participant received 5 vials of the minced calf uterine tissue on solid CO_2 and 5 vials of the lyophilized cytosol at ambient temperature. All 10 assays were performed on the same day in the same analysis within 1 week after arrival. Complete results were returned from 14 laboratories. The results from one laboratory that was participating for the first time were excluded due to use of incubation conditions, tritiated ligands, and a protein assay that differed from those of all other participants.

The methodology for tissue homogenization (Table 1) were those routinely employed in each laboratory with the exception that a tissue : buffer ratio of 1 : 20 was employed.

Lyophilized cytosols were suspended with 5 ml ice-cold (0–4°C) water containing 10% glycerol (v/v) and gently dissolved after standing on ice for 10 min by drawing the solution back and forth into a Pasteur pipette 10 times. Reconstituted cytosol was used directly without centrifugation.

One laboratory (lab 6) performed agar-gel electrophoresis, and one laboratory (lab 1) performed single dose saturating analysis. The remaining 11 laboratories performed multipoint titration assays with Scatchard analysis of the binding data. All laboratories used [^{3}-H]-estradiol for the ER assay and all laboratories except lab 13 used ORG-2058 for the PgR assay. In accord with the previously described guidelines of this Receptor Study Group [10] all 12 laboratories performed the assay using overnight equilibration with ligand at 4°C. Subsequent adsorption of unbound hormone with DCC was performed for 10 or 15 min. The majority of the laboratories (7/13) used a charcoal concentration within the range of 0.23–0.28%. The remaining 5 laboratories used concentrations of 0.125, 0.16, 0.3 and 0.5%. Two participants employed a ratio of charcoal : dextran of 1 : 100, while the remaining laboratories used a ratio of 1 : 10.

(b) Investigation of the effect of batch of isotope used for ER analysis. Three vials each of three different lyophilized human breast tumor cytosols were distributed to each participant, one of which was an ER and PgR negative control. All nine vials were analyzed on the same day; one set was to be analyzed using the batch of radioactive estradiol routinely in use in the laboratory, while the other set was to be analyzed using the common batch of radioactive estradiol. Nine of the 11 laboratories performed multipoint titration analyses and Scarchard analysis of the data, while the remaining two laboratories performed single dose saturation analyses.

(c) Calculation of dpm in unknown radioactive samples. Two capsules each of a hydrophilic and a lipophylic commercial standard as well as a single vial containing lyophilized [^{3}-H]-estradiol–BSA were sent to each participant. Two ml of distilled water was used to dissolve the [^{3}-H)-estradiol–BSA. Two aliquots of the same volume as that used for routine counting of samples were removed from the [^{3}-H]-estradiol–BSA and pipetted into counting vials. In preparation for counting, the same volume of buffer that is routinely used for counting of samples was added to each of the 2 hydrophilic standard capsules. The volume of scintillation counting liquid routinely used for assay samples was added to each vial, and the samples were

Table 1. *Methodology used to prepare cytosols*

Lab	Tissue disruption	Tissue suspension	Centrifugation $g \times 10^{-3}$	Centrifugation Time (min)
1	Dismembrator	Spatula	80	30
2	Dismembrator	Pasteur pipette	100	30
3	Dismembrator	All glass homogenizer	105	90
4	Dismembrator	Magnetic stirrer	40	60
5	Dismembrator	Pasteur pipette	100	60
6	Dismembrator	Pasteur pipette	100	30
7	Dismembrator	Vortex	105	45
8	Dismembrator	Pasteur pipette	50	60
9	Ultraturrax	Pasteur pipette	30	30
10	Dismembrator	Pasteur pipette	105	45
11	Dismembrator	Pasteur pipette	40	90
12	Ultraturrax	All glass homogenizer	6	10
13	Spex freezer	Polytron	105	60

Table 2. *Summary of estrogen and progesterone receptor results*

Lab	Minced tissue ER*	Minced tissue PgR*	Lyophilized cytosol ER*	Lyophilized cytosol PgR*
1	590 ± 69	1080 ± 84	602 ± 30	883 ± 52
2	478 ± 34	2382 ± 257	747 ± 54	1590 ± 47
3†	672 ± 82	1486 ± 278	627 ± 58	1396 ± 85
4	874 ± 110	1881 ± 241	1020 ± 180	2141 ± 386
5	604 ± 67	1070 ± 187	674 ± 42	1276 ± 79
6‡	554 ± 56	974 ± 115	643 ± 26	1095 ± 58
7	705 ± 200	1491 ± 363	1017 ± 58	1993 ± 75
8	513 ± 53	2003 ± 470	622 ± 95	1332 ± 131
9	433 ± 60	1072 ± 154	1006 ± 34	2030 ± 190
10	661 ± 115	1496 ± 139	553 ± 79	1023 ± 81
11	516 ± 90	1407 ± 123	577 ± 27	1357 ± 65
12	704 ± 203	1253 ± 248	883 ± 163	1088 ± 122
13	525 ± 22	851 ± 110	586 ± 15	960 ± 90
Range	433–874	851–2382	553–1020	883–2141
Mean	602	1419	735	1397
S.D.	119	447	180	423
c.v. (%)	19.8	31.5	24.5	30.3

*fmol/mg cytosol protein.
†2/5 values obtained for minced tissue were < 30 fmol/mg cytosol protein and have been excluded from the analysis of the data.
‡Agar-gel electrophoresis.

counted in a routine manner. Conversion from cpm to dpm was performed according to each laboratory's usual procedure. The following methods were used to convert cpm to dpm: external channel ratio, sample channel ratio, internal standard and H-factor.

Statistical analysis. The Wilcoxon test for pair differences, the Students *t*-test distribution and Spearman's Rank Correlation coefficient analysis have been applied to the data.

RESULTS

Minced tissue vs lyophilized cytosol

The mean values of the five determinations of ER and PgR expressed as fmol/mg cytosol protein for each of the 13 laboratories are shown at the bottom of Table 2. The overall averages of the laboratories for ER and PgR are similar for analysis of both minced tissue and lyophilized cytosols. While significantly lower ER results are obtained

in the individual laboratories using minced tissue than lyophilized cytosols, ($t = -2.29$, $d.f. = 24$, $0.025 < P < 0.05$), the magnitudes of ranges of ER contents reported are the same. There is no significant difference in the mean PgR concentrations in the two types of tissue preparations. A significant correlation was found between concentration of ER and PgR determined in lyophilized cytosols ($r_s = 0.70$; $0.01 < P < 0.02$), i.e. some laboratories consistently determine high receptor concentrations while others consistently determine low concentrations. This correlation is absent in samples from minced tissue ($r_s = 0.11$). Furthermore, there is no significant correlation between receptor values obtained in lyophilized cytosols and minced tissues for either ER ($r_s = 0.31$) or PgR ($r_s = 0.46$).

Figure 1 shows the coefficients of variation of the individual laboratories for minced and lyophilized tissue for both ER and PgR. In most laboratories, the coefficient of variation is lower for lyophilized cytosol than for minced tissue (median c.v. for ER: 6 vs 12% and for PgR: 7 vs 13%, respectively). This difference is significant at the 5 and 1% level for ER and PgR determinations, respectively. The decrease in the coefficients of variations exceeds 10% in one group of four laboratories for ER determinations (labs 7,9,11 and 12) and another group of four for PgR determinations (labs 3,5,7 and 8). The only laboratory that is included in both groups is lab 7 where the decrease in the coefficient of variation is approximately 22% for both determinations.

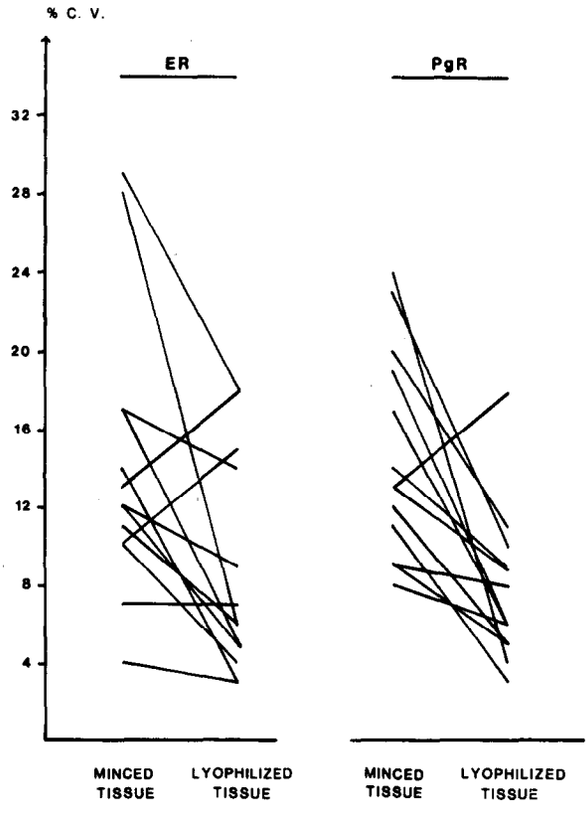

Fig. 1. The coefficients of variation observed within each laboratory in analysis of ER and PgR in minced tissues and lyophilized cytosols are shown. All points represent the c.v. of 5 determinations with the exception of lab 3 as noted in the footnote to Table 2.

Use of a common lot of [^{3}-H]-estradiol

Within individual laboratories, receptor values obtained using random batches of isotope ranged from 82 to 128% of the values obtained using the common batch of [^{3}-H]-estradiol. The mean values from each laboratory (average of triplicate determinations) were used to calculate the inter-laboratory means shown in Table 3. While the inter-laboratory coefficients of variation are 5–7% lower for both receptor positive samples using the common isotope, the mean values are the same.

The receptor negative tumor cytosol was classified as ER negative using both batches of isotopes by all participants.

Calculation of dpm in unknown samples

Ten laboratories reported results (Table 4). Despite the many methods used to estimate counting efficiency of liquid scintillation counters in the different laboratories, the ratio of the highest to the lowest reported dpm for the 10 laboratories is 1.2 and the variation around the mean values calculated from data reported by all laboratories is acceptably low (6–8% c.v.). One of the 10 laboratories reported the dpm of the unknown samples using counting efficiencies of a single counter calibrated in the three following different ways: sample channel ratio correlated to a quench correction curve generated from the quenching of [^{3}-H]-hexadecane with chloroform, counting of internal standard capsules for hydrophilic samples, and counting of internal standard capsules for lipophylic samples. The counting efficiency of unquenched tritium for the same counter (LkB Wallac, 1216 RackBeta) was 49.0, 50.4 and 53.4%, using the three methods, respectively. The coefficient of variation for the mean of the three values for each of the three unknowns was 7.0, 4.3 and 6.1%, which is the same order of magnitude as the inter-laboratory coefficient of variation observed using different methods in different counters.

DISCUSSION

The most significant difference seen between receptor analyses of the minced tissue vs lyophilized cytosols of the same tissue is in the intra-laboratory coefficient of variation. The variation found for analysis of lyophilized cytosols is about two-fold lower than that found for minced tissue for both ER and PgR. Greatest significance is seen with regard to the PgR analysis. Although most laboratories observed lower coefficients of variation

for lyophilized cytosols in comparison to minced tissue samples, only one laboratory (lab 7) experienced dramatic decreases in c.v. (> 10%) for both ER and PgR analyses. This laboratory was also the only one employing a vortex mixer for homogenization of the tissue, but it is not known whether this was the cause of the decrease. Intra-laboratory comparisons of the mean values of the assays showed that the range and standard deviations of the ER and PgR analyses for minced tissues and lyophilized cytosol samples are of the same order of magnitude in both cases. Nevertheless, the finding of a lower content of ER in minced tissue compared to lyophilized cytosol while no difference was observed for PgR analysis is puzzling. Although the differences in ER contents reported in minced and lyophilized samples vary for individual laboratories from being negligible to more than 50%, the trend was observed in 11 of the 13 laboratories. Within the framework of the present data, none of the procedures used for tissue disruption, homogenization, or preparation of cytosol could be singled out as being responsible for these differences. The lower ER value obtained with the minced tissue may be a reflection of less than optimal conditions during handling of the tissues in some laboratories. The similar PgR values simultaneously observed in minced and lyophilized tissues could conceivably be explained if the ability of PgR to bind hormone is better preserved in intact tissue than in lyophilized cytosols.

It has previously been reported that laboratories consistently report high or low ER and PgR values when different samples are analyzed in one assay [3,8,11]. It is remarkable that while a correlation was found between ER and PgR in the lyophilized samples, no such correlation was found between either ER or PgR values when comparing minced tissue and lyophilized cytosols. These observations indicate that while the intra-laboratory receptor analyses themselves seem to be consistent, the differences in tissue disruption, extraction of receptors, and preparation of cytosol do result in variations. Raam *et al.* [12] also observed that the tissue homogenization step significantly contributes to inter-assay variation for the estrogen receptor assay in pulverized calf uterine tissue powders stored as small aliquots at −80°C. Moreover, Wagner and Jungblut [13] concluded that standardization of receptor extraction is at least as important as standardization of the receptor binding assay itself.

Up to 28% difference in receptor values was observed if the same samples were assayed simultaneously with two different batches of [^{3}-H]-estradiol. However, although less variation in results is observed with the common batch of isotope, the difference is not large enough to warrant use of a common batch of isotope for routine inter-laboratory comparisons.

Conversion of cpm to dpm appears to be relatively independent of method of calibration of counting efficiency of tritium in liquid scintillation counters for the three types of radioactive standards used here. The important point is to regularly check counting efficiency and to recall that efficiencies differ for different compositions of the counting sample.

In summary, the range and standard deviations of the analyses for minced tissues and lyophilized cytosol samples are of the same order of magnitude. Analyses of lyophilized cytosols led, however,

Table 3. Comparison of ER values obtained using different batches of radioactive estradiol vs a common batch

Sample	*n*	Own isotope $\bar{x} \pm$ S.D. (c.v. %)	Common isotope $\bar{x} \pm$ S.D. (c.v. %)
A	11	91 ± 29 (32%)	92 ± 25 (27%)
B	11	314 ± 83 (26%)	316 ± 61 (19%)

Results expressed as fmol/ml.
Receptor content sample A = 31 fmol/mg cytosol protein.
Receptor content sample B = 92 fmol/ml cytosol protein.

Table 4. Survey of counting results of radioactive standards

	Capsules for aqueous solvents (dpm/capsule)	Capsules for organic solvents (dpm/capsule)	[^{3}H]-estradiol–BSA solution (dmp/0.1 ml)
Reported by supplier	105,600	89,400	–
Range	98,895–118,045	87,308–107,174	31,072–37,523
Mean dpm $n = 10$	107,293	96,221	33,721
S.D.	7157	7707	1846
c.v. (%)	6.7	8.0	5.5

to significantly better intra-laboratory reproducibility. Thus, as has been concluded earlier [6,12,14] lyophilized cytosol rather than minced tissue appears to be the tissue of choice for inter-assay or inter-laboratory quality control investigations. Since lyophilized cytosols are relatively thermostabile [15,16] their ease of distribution favours their use for quality control purposes. The difference in mean values of ER in the two types of preparation and the poor correlation between ER determined in minced tissue and lyophilized cytosols in the different laboratories is disturbing. These two observations indicate that while there is good inter-laboratory reproducibility and consistency when receptor analyses are performed on lyophilized cytosols, differences in handling of the tissues apparently result in greater intra- and inter-laboratory differences. There is a move towards pooling patients from different centers for clinical trials. If receptor analyses are to be used in such trials, either the handling of tissue must be more strictly standardized, or the ranking of laboratories relative to each other to normalize results may also have to be conducted on the basis of minced tissue rather than on lyophilized cytosols alone.

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