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# HPLC Micromethod for Simultaneous Measurement of Estradiol, Progesterone, Androgen and Glucocorticoid Receptor Levels. Application to Breast Cancer Biopsies

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**Abstract**—*Estradiol (ER), progesterone (PR), androgen (AR) and glucocorticoid receptor (GR) levels were assayed in 25 breast cancer tumors. The tissue was pulverized and homogenized in buffer, then divided into two parts: one was assayed by the standard dextran-coated charcoal method (DCC), with Scatchard plot analysis, the other was assayed by a micromethod developed in our laboratory, as described below:*

*—incubation of the cytosol with several ligands (labelled and unlabelled) selected to avoid unwanted cross-reactions*

*—DCC separation, followed by extraction of all receptor-bound steroids by precipitation of proteins with methanol/TCA*

*—separation of these steroids on a high pressure liquid chromatography (HPLC) column using a methanol/water solvent*

*—collection of the fractions of the column outlet and counting.*

*Use of three labelled ligands and appropriate unlabelled ligands allowed assays of the four receptors. This micromethod was highly correlated with the standard method: ER = 0.985 ($P < 0.001$); PR = 0.999 ($P > 0.001$); AR = 0.989 ($P < 0.001$); GR = 0.867 ($P < 0.001$). Thresholds of positivity were not modified. This micromethod allowed simultaneous measurement of several receptors in 40 mg biopsy specimens and can be applied to other hormone-dependent tissues.*

## INTRODUCTION

STEROID HORMONE receptors have been found in numerous cancers, and particularly in human breast malignancies, which can contain estradiol (ER) [1, 2], progesterone (PR) [3, 4], androgen (AR) [5] and glucocorticoid (GR) [6] receptors. Steroid hormone receptor assays are an important means for evaluating malignant mammary tumors, with results serving as the bases for therapeutic strategies and/or prognosis. In particular, the role of estrogen and progesterone receptor assays is well established in the management of breast cancer. Several studies have demonstrated that the presence of both receptors has a predictive value, and treatment as a function of assay findings has led to response rates of 75% following endocrine therapy.

While androgen and glucocorticoid receptor assays have been performed for some years, their importance remains uncertain. However, androgen receptors seem to be a significant predictor of survival [7], and a considerable proportion of human breast tumors are glucocorticoid-dependent [8, 9]. Nevertheless, the rate of response to endocrine therapy seems higher in patients in whom AR and GR are associated with ER and PR.

Receptor assays are advisable for all breast tumors, whether or not surgery is performed, so that the patient can be offered the best possible therapeutic strategy. The widely used standard DCC method requires a large amount of biological material [8]; often, mutilating surgery is necessary to obtain a sufficient amount of material. To eliminate this constraint, thereby allowing use of more conservative therapy, several authors have tested single-point assays and assays on microsamples:

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tru-cut, biopsies, needle aspirates [10–22]. These different techniques primarily concern ER and PR measurement, as these are the only receptors with therapeutic applications at the present time.

We previously described [23] a microsample assay method combining the dextran-coated charcoal (DCC) technique and high pressure liquid chromatography (HPLC). The present study involved coevaluation of four receptors (ER, PR, AR, GR) in tru-cut drill biopsies. Micromethod results were compared with the standard DCC method examined by Scatchard plot analysis, and correlations and reproducibility were determined for each receptor.

## MATERIALS AND METHODS

*Labelled steroids*

[$^3$H]ORG 2058 (sp. act.: 51 Ci/mmol) and [$^3$H]-dexamethasone (sp. act.: 45 Ci/mmol) were purchased from Amersham-Searle Corp. (Arlington Heights, U.S.A.). [$^3$H]R1881 (methyltrienolone, sp. act.: 86 Ci/mmol) and [$^3$H]R2858 (moxestrol, sp. act.: 87 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.).

*Unlabelled steroids and other products*

Diethylstilboestrol (DES), dexamethasone, cortisol and phenylmethyl sulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ORG-2058 and R1881 were obtained from the same supplier that provided the corresponding labelled hormones.

Charcoal (Norit A) and dextran T70 were purchased from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade.

*Buffer solutions*

Extraction buffer TED: Tris–HCl: 10 mM; EDTA: 1 mM; DTT: 0.5 mM; sodium molybdate: 10 mM; pH 7.4.

Incubation buffer TEDG: Tris–HCl 10 mM; EDTA: 1 mM; DTT: 0.5 mM; sodium molybdate: 10 mM; glycerol 20% (v/v); pH 7.4.

*Charcoal suspensions*

DCC-A: Tris–HCl: 10 mM; Norit A: 0.7%; dextran T70: 0.07%; BSA: 1 mg/ml; pH 8.0.

DCC-B: Tris–HCl: 10 mM; Norit A: 1.4%; dextran T70: 0.14%; BSA: 1 mg/ml; pH 8.0.

*Collection of samples*

Following surgical excision, tumor specimens were examined histologically, trimmed of excess fat and appendage tissues, cut into strips, identified and frozen in liquid nitrogen until assayed.

*Cytosol preparation*

Frozen tissues were pulverized to a fine powder in a liquid nitrogen-cooled Thermovac tissue pulverizer (Ind. Corp., Copiague, New York, U.S.A.). The resulting powder was weighed and homogenized in 10 vol. TED buffer using a Polytron PT 10–20 homogenizer (Brinkman Instrument Inc., Lucerne, Switzerland) at a speed setting of 5 for three 5-s intervals in an ice bath. For small samples, the powder obtained by pulverization was weighed and homogenized with 0.5 ml TED buffer in polycarbonate ultracentrifuge tubes using a Polytron homogenizer.

PMSF was added to all homogenates at a final concentration of 1 mM in order to inhibit protease activity. Homogenates were then centrifuged for 1 h at 105,000 ***g*** (0°C) (Kontron Ultracentrifuge Unit, France). The supernatants were collected (cytosols) and used for steroid receptor and protein assays.

Protein measurements were performed using Coomassie G-250 (Protein Assay Reagent, Biorad Ltd., U.K.) with bovine serum albumin fraction V (Sigma Chemical Co.) as standard [24].

*Standard receptor assay*

A DCC competitive binding assay was performed for all receptors. In the few cases in which the amount of available cytosol was too small, one or two receptors were measured by a saturating single dose assay. In brief, 100 µl of cytosol were incubated overnight with 100 µl of labelled steroid prepared in TEDG buffer (total binding). Nonspecific binding was determined by parallel incubation of cytosol with labelled steroid and a 200-fold excess of competitive ligand, expressed as dry extract. At the end of incubation, 0.5 ml of DCC-A was added. After agitation in a horizontal shaker for 15 min at 0°C, the tubes were centrifuged (15 min, 2800 ***g***, 0°C). A 0.5 ml aliquot of the supernatant was counted after addition of 4.5 ml ACS (Aqueous Counting Scintillant, Amersham-Searle) using a Packard TriCarb 460 liquid scintillation counter. Specific binding for each receptor was determined by Scatchard plot analysis and expressed as fmol/mg protein.

*ER assay*

ER was assayed using [$^3$H]R2858 (0.10–9 nM) and unlabelled DES as competitor.

*PR and AR assays*

PR was assayed by the DCC method, using [$^3$H]R1881 (0.15–8 nM) and unlabelled ORG-2058 as competitor. 200 times more cortisol was added to all tubes to saturate GR.

R1881 was usually used for androgen receptor assays, but showed strong affinity for progesterone receptor [25]. Ekman *et al.* [15] used R1881 to

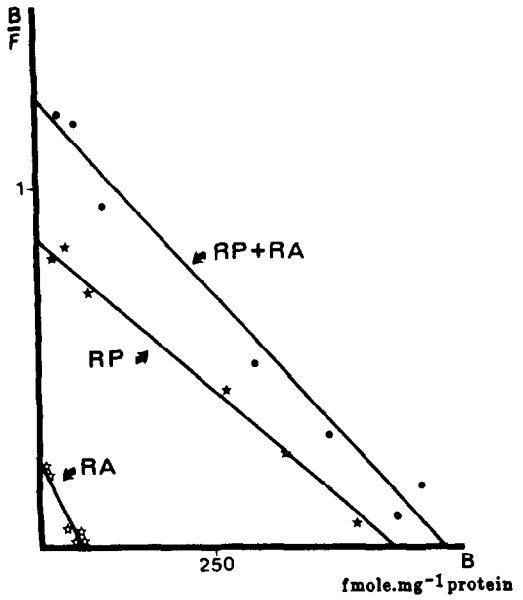


*Fig. 1. Scatchard plots for simultaneous measurement of AR and PR with* [$^3$H]*R1881.*

measure PR and AR in human prostate biopsies, and it seemed interesting to use R1881 for progesterone receptor assays in human breast carcinoma. In order to utilize this ligand in the micromethod for simultaneous determination of PR and AR, the results obtained using [$^3$H]R1881 first had to be compared with those obtained by our standard method using [$^3$H]ORG 2058.

PR was measured in a second manner, using labelled (0.15–7 nM) and unlabelled ORG-2058 as competitor. A 200-fold excess of cortisol was added to all tubes to saturate both corticosteroid binding globulin (CBG) and glucocorticoid receptors. This manipulation was performed to validate PR determination using [$^3$H]R1881 as the labelled ligand.

AR was assayed using [$^3$H]R1881 (0.15–8 nM) and unlabelled ORG-2058 as competitor. A 200-fold excess of ORG-2058 was added to all tubes to block progesterone receptors; a 200-fold excess of cortisol was added to all tubes to saturate the glucocorticoid receptor.

In the presence of cortisol, [$^3$H]R1881 binds to both AR and PR, and total binding is given by AR + PR + NS* = (1).

In the presence of ORG-2058 and cortisol, [$^3$H]R1881 binds to AR; total binding is given by AR + NS = (2).

In the presence of R1881 and cortisol, [$^3$H]R1881 is not specifically bound: NS (3).

The difference (1) − (2) gives specific progesterone receptor binding: the difference (2) − (3) gives specific androgen receptor binding (Fig. 1).

Use of [$^3$H]R1881 allowed determination of PR and AR levels with a single labelled ligand and carefully selected unlabelled ligands.

*NS: nonspecific binding.

*GR assay*

GR was assayed using labelled (3–50 nM) and unlabelled dexamethasone as competitor. A 200-fold excess of ORG-2058 was added to all tubes to saturate mineralocorticoid receptors.

*Micromethod*

The micromethod required 400 µl of cytosol, and was performed in glass tubes. Table 1 shows the distribution of labelled and unlabelled ligands in each tube.

*Principle*

This technique involves four steps:

1. Incubation of the cytosol with labelled and unlabelled ligands and separation of free steroids by DCC
2. Protein precipitation and extraction of bound steroids
3. Separation of labelled steroids by HPLC
4. Counting of the radioactivity of the fractions collected at the HPLC column outlet, and calculation.

*Step* 1

The tubes were incubated as described (final incubation volume was 200 µl) (Table 1). After overnight incubation at 0°C, 100 µl of DCC-B were added. After shaking (15 min at 0°C), the tubes were centrifuged for 15 min at 2800 ***g***, 0°C.

*Step* 2

200 µl of absolute ethanol containing DES 1 µM, ORG-2058 1 µM, R1881 1 µM, dexamethasone 10 µM and 20 µl of an aqueous solution of 10% trichloracetic acid were added to 200 µl of the supernatant. The tubes were capped and vigorously shaken for 2 h at 0°C, then centrifuged (15 min, 2800 ***g***, 0°C). The final supernatant was then available for HPLC separation.

*Step* 3

The HPLC system used included a 6000 A pump, a U6K injector, a model 440 absorbance detector fitted with a 254 nm interferential filter and a data module integrator (Waters Assoc., Milford, MA, U.S.A.).

Chromatographic separation of the steroids was performed with a radial compression system (RCM 100), using Rad-Pak cartridges filled with 5 µm microparticles of reversed phase $C_{18}$ (Waters).

Prior to injection of the supernatants from extraction, unlabelled aliquots of dexamethasone, R2858 and 1881 ($10^{-4}$ M) were injected to characterize the retention time of these steroids in this system. The UV detector and the recorder were then disconnected and 150 µl of each supernatant from extraction were injected.

*Table 1. Distribution of labelled and unlabelled ligands in each tube using the micromethod*

| No. | Cytosol volume | Labelled ligand final concentrations | | Unlabelled ligand final concentrations (dry extract) | | Corresponding receptors |
|---|---|---|---|---|---|---|
| 1 | 100 | 100 µl | [$^3$H]R2858 | 9 nM | DES | 1.8 µM | ER |
| | | | [$^3$H]R1881 | 8 nM | | | PR + AR |
| | | | + cortisol | 1.8 µM | | | |
| 2 | 100 | 100 µl | [$^3$H]R1881 | 8 nM | R1881 | 1.6 µM | PR + AR |
| | | | [$^3$H]dexamethasone | 50 nM | Dexamethasone | 10 µM | GR |
| 3 | 100 | 100 µl | [$^3$H]R2858 | 9 nM | | | ER |
| | | | [$^3$H]R1881 | 8 nM | ORG 2058 | 1.6 µM | PR + AR |
| | | | + Cortisol | 1.8 µM | | | |
| 4 | 100 | 100 µl | [$^3$H]dexamethasone | 50 nM | ORG 2058 | 1.6 µM | GR |

*Table 2a. Correlation of PR measurements with the two ligands ([$^3$H]R1881 vs [$^3$H]ORG 2058)*

| | Number of cases | Slope | Coefficient of correlation | |
|---|---|---|---|---|
| Values ≤ 20 fm/mg | 6 | 1.032 | $r = 0.845$ | $P < 0.05$ |
| Values > 20 fm/mg | 12 | 0.922 | $r = 0.985$ | $P < 0.001$ |
| All values | 18 | 0.924 | $r = 0.990$ | $P < 0.001$ |

*Table 2b. Values of* $K_d$

| Ligand | $K_d$ (mean ± S.D.) (nM) |
|---|---|
| ORG 2058 | 0.233 ± 0.177 |
| R1881 | 0.188 ± 0.122 |

*Step* 4

Aliquots (900 µl) were collected at the column outlet (one fraction every 30 s) using a Gilson 202 fraction collector (Gilson Medical Electronics, France); Amersham Counting Scintillant (4.5 ml) was added and the radioactivity was measured.

At the retention time for a ligand, specific binding was evaluated by subtracting the radioactivity obtained by incubation in the presence of labelled and unlabelled ligands (nonspecific binding) from the radioactivity obtained by incubation in the presence of labelled ligand alone (total binding). The results are expressed in fmol/mg protein.

## RESULTS

*Validation of the PR assay using [$^3$H]R1881 as ligand instead of [$^3$H]ORG 2058*

Results (Table 2a) revealed a highly significant correlation (slope = 0.924; $r = 0.990$; $P < 0.001$) for all values taken together as well as for low and high values considered separately. The mean $K_d$ value for ligands were not significantly different (Table 2b). In view of these results, PR assays were performed using [$^3$H]R1881 as the ligand.

*Comparison of the micromethod with the standard receptor assay*

Figure 2 is a chromatogram, obtained after injection of a mixture of unlabelled moxestrol, dexamethasone and methyltrienolone, giving the retention time of each steroid at a flow rate of 1.8 ml/min with 65% methanol/water (v/v) as the eluent.

The radioactive elution profile of the four incubation tubes is given in Fig. 3:

—The 1st profile (tube 1) represents the total binding AR + PR and the nonspecific binding $ER_{NS}$ (a)

—the 2nd profile (tube 2) represents the nonspecific binding $AR_{NS}$ and the nonspecific binding $GR_{NS}$ (b)

—the 3rd profile (tube 3) represents the total binding of ER and AR; it also represents the nonspecific binding of $PR_{NS}$ (c)

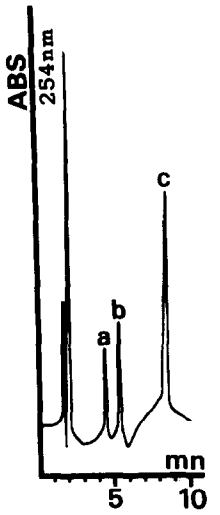


*Fig. 2. HPLC separation of unlabelled ligands:* (a) *dexamethasone* (b) *R*2858 (c) *R*1881.

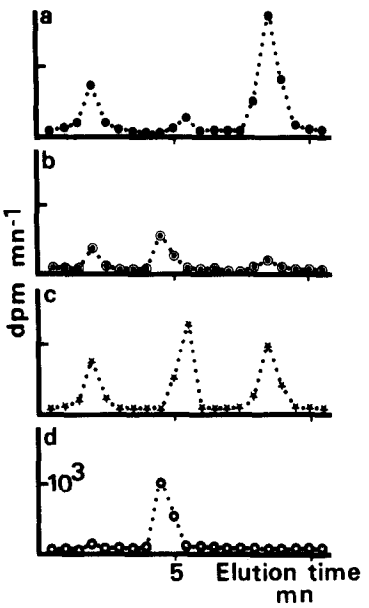


*Fig. 3. Radioactive HPLC profiles of the four extracted cytosols.*

—the 4th profile corresponds to the total binding of GR (d).

Specific estradiol receptor binding is thus given by the difference of the radioactivity peaks (c) − (a) obtained for the retention time of moxestrol (5.5 min).

Specific progesterone receptor binding is given by the difference of the radioactivity peaks (a) − (c) for the retention time of methyltrienolone (8.5 min).

Specific androgen receptor binding is given by the difference of the radioactivity peaks (c) − (b) for the retention time of methyltrienolone.

Specific glucocorticoid receptor binding is given by the difference of the radioactivity peaks (d) − (b) for the retention time of dexamethasone (4.5 min).

All of these specific bindings are expressed in dpm, and must be converted to fmol/mg protein,

*Table 3. Correlation between the two methods*

| | Number of cases | Coefficient of correlation | |
|---|---|---|---|
| *Values* ≤ 20 fm/mg | | | |
| ER | 12 | $r = 0.837$ | $P < 0.001$ |
| PR | 7 | $r = 0.959$ | $P < 0.001$ |
| AR | 9 | $r = 0.954$ | $P < 0.001$ |
| *Values* > 20 fm/mg | | | |
| ER | 13 | $r = 0.982$ | $P < 0.001$ |
| PR | 18 | $r = 0.999$ | $P < 0.001$ |
| AR | 16 | $r = 0.979$ | $P < 0.001$ |
| *All values* | | | |
| ER | 25 | $r = 0.985$ | $P < 0.001$ |
| PR | 25 | $r = 0.999$ | $P < 0.001$ |
| AR | 25 | $r = 0.989$ | $P < 0.001$ |
| GR | 25 | $r = 0.867$ | $P < 0.001$ |

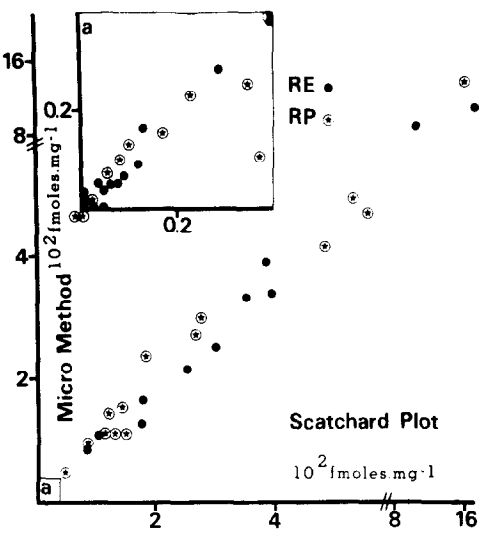


*Fig. 4. Correlation between the two methods for ER and PR.*

taking into account the specific activity of each labelled ligand, the protein content of the cytosol, and assay dilution.

The results obtained by each method for all receptors were highly correlated (Table 3, Figs. 4, 5), for high and low values as well as for all values together.

The reproducibility of the micromethod is very satisfactory; the coefficient of variation is under 10% even if greater dispersion was seen for AR than for ER and PR (Table 4).

Although the correlation coefficient for GR is satisfactory, micromethod findings were usually lower than those obtained by Scatchard plot analysis. It is important to note that all values were of the same order, except for one, greater than 20 fmol/mg, so that no correlations were possible for high and low values. One value that was positive with the Scatchard plot analysis become negative with the micromethod.

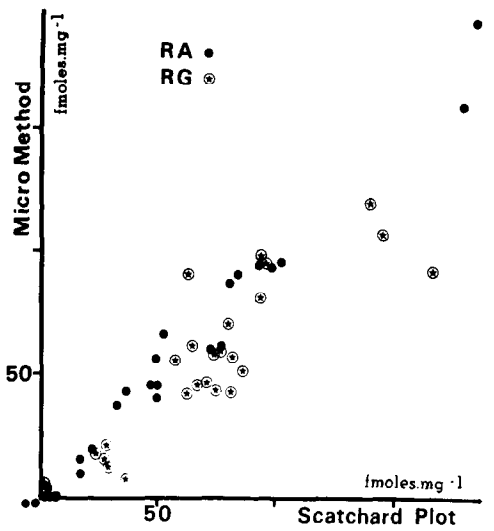


*Fig. 5. Correlation between the two methods for AR and GR.*

column outlet further automates counting and the calculation.

In this study, cytosol protein levels were always above 1 mg/ml in the incubation medium [26]. Correlations between the two methods were very satisfactory for all receptors measured, implying that the thresholds of positivity established with the DCC method remained unchanged with the micromethod.

Furthermore, all coefficients of variation were under 10%. However, this micromethod, like single-point assays, can overestimate receptor levels.

Biopsies are often contaminated by blood, and assay results can be vitiated by nonspecific fixation to serum proteins. Use of the synthetic ligands: R2858 (moxestrol) and R1881 (methyltrienolone) allowed the reduction of these effects as these ligands have no affinity for blood transport proteins [25].

As the cellular heterogeneity of breast cancer is well established [27, 28], the quality of biopsy

*Table 4. Reproducibility of the micromethod*

| Receptor | Results Five measurements of the same cytosol (fmol/mg) | | | | | Mean | S.D. | CV (%) |
|---|---|---|---|---|---|---|---|---|
| Estrogen | 83 | 87 | 83 | 86 | 81 | 84.00 | 2.45 | 2.92 |
| Progesterone | 298 | 295 | 299 | 296 | 280 | 293.60 | 7.77 | 2.65 |
| Androgen | 96 | 88 | 79 | 94 | 101 | 91.60 | 8.44 | 9.21 |
| Glucocorticoid | 120 | 112 | 94 | 113 | 110 | 109.80 | 9.60 | 8.75 |

2 × 5 aliquots of the same cytosol were used to determine specific binding.
CV = coefficient of variation.

## DISCUSSION

The micromethod allowed simultaneous measurement of four receptors (ER, PR, AR, GR) in tumoral microsamples weighing at least 30–40 mg (400 μl of cytosol).

The technique combines the standard DCC method with HPLC, and uses [$^3$H]R1881, allowing coevaluation of two receptors and reducing the number of samples needed.

The measurement of estradiol and glucocorticoid receptor levels with two ligands which were not completely separated by HPLC in our working conditions was possible by avoiding the simultaneous presence of the two ligands in the same incubation medium.

In the second incubation tube, the presence of two ligands ([$^3$H]R1881 and [$^3$H]dexamethasone) that cross-react with the receptors evaluated is no problem, because this sample is used to determine nonspecific AR and GR binding after HPLC.

Moreover, after DCC separation, this technique is relatively rapid: 2 h for extraction, 15 min for HPLC elution and collection of fractions. The use of a continuous flux radioactivity detector at the

samples must be carefully controlled by histologic and cytologic methods. We assayed the DNA level of each tumor sample; a DNA ≥ 500 mg/g of tumor represented the threshold for validation of receptor levels, and was obtained for all samples in our study (unpublished data).

Although the role of androgen and glucocorticoid receptors in mammary carcinoma is not well established, this micromethod allows development of a tumor receptor panel which may provide additional useful information about the responses that can be expected from endocrine therapy. Receptor levels can be monitored during treatment by obtaining several biopsies at specific times. Assays of recurrent tumors just before therapy may provide the most accurate index of response to hormonal treatment.

Development of this micromethod using breast tumor material as part of a separate multiparametric study was facilitated by the large amount of tissue available and the simultaneous presence of the four receptors. This method can also be used for other types of tumors in which certain of these receptors have been observed, and which are amenable to biopsy (endometrium, prostate, liver, lar-

yngeal mucosa etc.). Depending on requirements (target tissue, number of receptors to be assayed), the number of incubation tubes and thus the cytosol samples can be reduced so as only to measure those receptors of interest.

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