Pergamon

*European Journal of Cancer* Vol. 32A, No. 8, pp. 1371–1381, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

PII: S0959-8049(96)00118-9

## Original Paper

# Immunoassays (ELISA) of Urokinase-type Plasminogen Activator (uPA): Report of an EORTC/BIOMED-1 Workshop*

Th.J. Benraad,[1] J. Geurts-Moespot,[1] J. Grøndahl-Hansen,[2] M. Schmitt,[3] J.J.T.M. Heuvel,[1] J.H. de Witte,[1] J.A. Foekens,[4] R.E. Leake,[5] N. Brünner[2] and C.G.J. Sweep[1]

[1]532 Department of Experimental and Chemical Endocrinology, St Radboud Hospital, University of Nijmegen, P.O. Box 9101, The Netherlands; [2]The Finsen Laboratory, Rigshospitalet, Copenhagen, Denmark; [3]Frauenklinik der Technischen Universität, Klinikum rechts der Isar, München, Germany; [4]Division of Endocrine Oncology, Department of Medical Oncology, Dr. Daniel den Hoed Cancer Center, Rotterdam, The Netherlands; and [5]Department of Biochemistry, University of Glasgow, U.K.

**The urokinase-type plasminogen activator (uPA) is considered to play a key role in the process of invasion and metastasis. In several independent studies, in a variety of cancer types (e.g. of the breast, colon, stomach, lung, ovary), high antigen levels of uPA in tumour extracts have been associated with rapid disease progression. In these studies, different sets of antibodies and standards (often as commercially available uPA ELISA kits) have been used. The standards provided with the different uPA ELISA kits are different from each other in both composition and source. In addition, the different uPA ELISA kits use antibodies which differ in specificity and affinity for the various forms of uPA including pro-uPA, HMW-uPA, LMW-uPA, the aminoterminal fragment (ATF) and complexes with inhibitors (PAI-1 and PAI-2) and the receptor (uPAR). Further, the composition of tumour tissue extraction buffers differ significantly among the published studies. Thus, it is not surprising that the ranges of cytosolic uPA levels reported differ considerably even when measured within the same tumour type. These discrepancies led the EORTC Receptor and Biomarker Study Group, in conjunction with the BIOMED-1 consortium on 'Clinical Relevance of Proteases in Tumour Invasion and Metastasis', to organise a workshop to study the characteristics associated with six different uPA immunoassays (ELISA) used in clinical studies reported in the literature. Although the absolute uPA antigen values measured with the respective uPA ELISA kits differed, high correlations were obtained for any two of the four uPA ELISA kits finally applied to sets of breast cancer cytosol preparations. The preparations used at present as standards in the various uPA ELISA kits are not representative of actual human breast cancer cytosols. Thus absolute standardisation is only possible by using a common reference sample (breast cancer cytosol) and similarly composed ELISA uPA kits. Then it will be possible to generate comparable data on clinical tissue as well as to check for batch-to-batch variations within particular ELISA kits. Copyright © 1996 Elsevier Science Ltd**

**Key words: uPA, ELISA, breast cancer, standardisation, quality assurance, EORTC**
*Eur J Cancer*, Vol. 32A, No. 8, pp. 1371–1381, 1996

## INTRODUCTION

In BREAST cancer, the selection of high-risk patients has always been difficult due to the unpredictable biological course of the disease. Lymph node status is recognised as one of the best clinical discriminants between good and bad prognosis. Yet almost 30% in node-negative patients will relapse within a few months, while in node-positive patients approximately 25% are free of disease 10 years after primary surgery [1]. Additional prognostic parameters are therefore needed and one approach to elucidate this problem has been the measurement of various biological parameters in primary tumour

Correspondence to Th.J. Benraad
*Supported by the BIOMED-1 project BMH1-CT93-1346.
Received 27 Sep. 1995; revised 19 Jan. 1996; accepted 30 Jan. 1996.

tissue. Early work showed that oestrogen receptor positivity was associated with longer survival [2–4], most probably because oestrogen receptor-positive patients are the ones most likely to respond to steroid hormone therapy. Quantification of oestrogen receptor is important because the chance of patients responding to hormonal therapy is proportional to the oestrogen receptor content in the primary tumour [5–7]. For this reason, determination of biological indices should be quantitative, rather than qualitative. In consequence, quality assurance groups have been established to monitor tumour biology factors, e.g. steroid hormone receptors [8–10].

To increase the knowledge of the biological basis of tumour development, various studies have been undertaken to look into the mechanism of tumour invasion. A major component of tumour invasion is the action of tumour-associated proteases in disrupting the surrounding basement membrane and the adjacent stromal matrix. The action of these proteases is under control of a cascade response which induces activation of proteolytically active urokinase (uPA) from its precursor pro-uPA [11–13]. Recent studies have suggested that the uPA level might be a strong biological indicator of prognosis in breast cancer [14, 15] and of response to endocrine therapy in recurrent breast cancer [16]. There is also broad interest in the relationship of uPA levels with other types of cancer [17–19]. Various ELISAs for uPA have been established and reported in the literature, and some of these are commercially available.

During a workshop held in Nijmegen, The Netherlands, under the auspices of the EORTC and the BIOMED-1 programme supported by the European Community, a number of uPA ELISAs were tested. The present report contains results of uPA measurements obtained during this workshop supplemented with data collected in later studies. Attention was paid to the immunological potencies of various standards, to parallelism, to detection limits and to within- and between-assay variations achievable.

It should be kept in mind that the purpose of this study was not to evaluate the quality of the individual methods, but to better understand the characteristics associated with each of the procedures.

## PATIENTS AND METHODS

*Organisation*

Within the framework of both the Receptor and Biomarker Study Group of the EORTC and a consortium of the BIOMED-1 programme 'Clinical Relevance of Proteases in Tumour Invasion and Metastasis', a workshop was organised in Nijmegen. A number of different ELISA kits were used to assess uPA antigen in primary breast cancer. The companies that generously made their reagents available are American Diagnostica (Greenwich, U.S.A.), Biopool (Umea, Sweden), Oncogene Science (Cambridge, U.S.A.), Sangtec Medical (Bromma, Sweden) and Technoclone (Vienna, Austria). In addition, the group was provided with the relevant reagents to determine uPA by the Finsen Institute (Copenhagen, Denmark), a laboratory with a longstanding experience in the uPA field. Comparable reagents are commercially available from Monozyme (Denmark). Many, but not all, participants had previous experience with these assays. It is reasonable to assume that the experience of the operators will have positively influenced the performance of the assays during the workshop. At the workshop, measurements of PAI-1, a natural occurring inhibitor, and the receptor uPAR, were also investigated and will be the subject of future publications.

*Samples*

Primary breast cancer biopsies from our tumour bank (−80°C) were analysed. Xenografts, raised in nude mice after implanting MDA-NB-231 human breast cancer cells, were kindly provided by the Finsen Institute. Biopsies and xenografts were homogenised (microdismembrator) in EORTC buffer (20 mM $K_2HPO_4/KH_2PO_4$, 1.5 mM $K_2EDTA$, 3 mM sodium azide, 10 mM monothioglycerol, 10% [v/v] glycerol/water, pH 7.4), centrifuged at 800*g*, the supernatant collected and subjected to centrifugation for 1 h at 100000*g* at 4°C. This procedure has for many years been used to make cytosols for measuring the oestradiol and progesterone receptor levels. It is worthwhile mentioning that this procedure was used to collect cytosolic proteins. However, uPA in addition to its free state is also bound to its receptor, uPAR. Therefore, in various published studies, detergent-containing buffers have been used for extraction of uPA. However, it has been the experience of various laboratories that while the uPA content of these extracts are much higher than the values obtained in the EORTC cytosol, there is a close correlation between the values obtained with the two techniques [20] (see also the Results section). Dilution of cytosols was performed applying dilution buffers recommended for that particular assay procedure. The following extraction buffers were used for pellet extraction: (1) Jänicke buffer: 20 mM Tris–HCl, 125 mM NaCl containing 1% Triton X-100, pH 8.5 [20]; and (2) Camiolo buffer: 75 mM potassium acetate, 0.3 M NaCl, 0.1 M L-arginine, 10 mM $K_2EDTA$, 0.25% Triton X-100, pH 4.2 [21].

*Standards*

The NIBSC uPA standard 87/594, consisting of highly purified HMW uPA derived from human urine, was kindly provided by Dr P.J. Gaffney (NIBSC, U.K.). Until now, this standard has not officially been assigned an antigen value. For internal purposes, NIBSC use the value of 32.5 μg/ampoule. This standard for high molecular weight urokinase (HMW-uPA) is alternatively named HMW-two chain uPA (HMW-tc-uPA).

The uPA standard provided with the American Diagnostica ELISA kit, designated as sc-uPA, is a glycosylated pro-uPA, secreted by a cell line and purified from conditioned culture media. The Grünenthal standard (Saruplase®) is a recombinant non-glycosylated pro-urokinase produced by *E. coli*, generously provided by Grünenthal GmbH, Stolberg, Germany. The pro-uPA content has been determined by Grünenthal on the basis of amino acid determination. The Finsen laboratory, in their published studies, used the NIBSC standard as well as the Grünenthal preparation, calibrated against the former [22].

*Analytical methods*

The basic characteristics of the different ELISAs are shown in Table 1, including the type and range of standards used, the type of primary and secondary antibodies employed and information about the forms of uPA recognised by the relevant assay system. This information was provided by the manufacturers.

The ELISAs were all of the sandwich type. The ELISAs were all performed in precoated microtitre plates using spec-

*Table 1. Characteristics of uPA ELISAs*

| Company/institute (trade name) | Type of standard and antibodies (from inserts) | Forms of uPA recognised (from inserts) |
|---|---|---|
| American Diagnostica: (Imubind® uPA) | sc-uPA standard range: 0–1 ng/ml, primary antibody: monoclonal, secondary antibody: monoclonal-biotin, streptavidin-HRP-TMB: 450 nm | sc-uPA, HMW uPA, uPA-uPAR, uPA-PAI-1, uPA-PAI-2 |
| Finsen: Reagents for uPA assay | NIBSC standard, HMW uPA 87/594, range: 0–1 ng/ml, primary antibody: polyclonal, secondary antibody: 3 monoclonals-biotin, streptavidin-HRP-OPD: 490/620 nm | sc-uPA, HMW uPA, uPA-inhibitor, uPA-uPAR, LMW uPA |
| Biopool: (TintElize® uPA) | pro-uPA standard, range: 0–4 ng/ml, primary antibody: monoclonal, secondary antibody: polyclonal-HRP, OPD: 490 nm | sc-uPA, HMW uPA, LMW uPA |
| Sangtec: uPA LIA | HMW uPA standard, range: 0–40 ng/ml, primary antibody: monoclonal, secondary antibody: monoclonal-lumin, luminometer | sc-uPA, HMW uPA, uPA-uPAR, uPA-PAI-1 |
| Technoclone: TC® uPA | uPA standard, range: 0–10 ng/ml, primary antibody: monoclonal, secondary antibody: HRP-antibody, ABTS: 405 nm | sc-uPA, tc uPA, uPA-serpin, complexes |
| Oncogene Science: uPA Elisa | pro-uPA standard, range: 0–350 pg/ml, primary antibody: 2 monoclonals, secondary antibody: polyclonal, final antibody: HRP-antibody, OPD: 490/620 nm | sc-uPA, HMW uPA, LMW uPA, uPA-uPAR, uPA-PAI-1 |

HRP, horseradish peroxidase; TMB, tetramethyl benzidine; OPD, ortho-phenylenediane; ABTS, azido-bis thiazoline-sulphonic acid.

trophotometric determinations, whereas the LIA type of ELISA, using a luminometric detection system was performed in antibody precoated tubes. The ELISAs were used according to the instructions provided by the manufacturers. The ELISA developed in the Finsen laboratory was used as previously described [18]. No major difficulties were noticed while performing the assays.

The lower limit of detection was defined as three standard deviations in the measurement of zero analyte. The overall coefficient of variation (CV) was calculated using the formula overall CV% = square root of $\Sigma$ [CV%]$^2/n$).

## RESULTS

*Standard curves in different assay procedures*

Figure 1 shows representative standard curves obtained using six different uPA procedures and the standards provided by the companies with their different kits. All curves were more or less curvilinear. The Finsen and American Diagnostica procedures (Figure 1a, b) employ a working range from 0 to 1 ng/ml. The lower limits of detection were nearly identical. The overall CVs of the standard points were 8.1 and 7.5%, respectively. These characteristics are in agreement with the information given in the kit-insert.

The standard curve obtained using the Sangtec kit (Figure 1c) shows the relationship between relative light units (RLUs) and the amount of uPA, up to 4 ng/ml. The lower detection limit was calculated to be as low as 0.010 ng/ml (1.0 pg per tube), with an overall CV of 4.4%. The span of the standard curve in the Oncogene Science procedure (Figure 1d) ranged from 0 to 0.35 ng/ml. In this very sensitive assay, the detection limit was 0.002 ng/ml. In contrast to the aforementioned assays, the Biopool uPA assay (Figure 1e) was specifically designed for the quantitative determination of uPA in plasma samples. For this purpose, the detection limit of 0.277 ng/ml is adequate. The Technoclone assay (Figure 1f) is also specifically intended for the determination of uPA in plasma, and the highest standard used is 10 ng/ml. The lower limit of detection was calculated at 0.384 ng/ml, with an overall CV of 4.9%.

In the four most sensitive assays (Finsen, American Diagnostica, Sangtec, Oncogene Science) five different types of standards were tested. Figure 2 shows that the slopes of the curves in all methods varied considerably. Obviously, in all assay methods, the values measured in unknowns depend strongly on the standard applied. It is worth noting that the immunoreactive potencies, as reflected by the absorbance values, of the different standards do not follow the same order in the different assay procedures. For instance, in three out of the four procedures (Finsen, Sangtec, Oncogene Science) the HMW-uPA standard provided with the Sangtec kit, gave the highest signal, whereas the lowest signal was evoked by the Biopool pro-uPA standard. In contrast, in the American Diagnostica procedure little difference in optical density was observed using these two standards.

*Correlation and comparison between cytosolic uPA assay methods*

Figure 3a shows uPA values measured in 117 breast cancer cytosols both with the American Diagnostica procedure and the Finsen assay method (both methods used in previous publications). The values obtained by the American Diagnostica kit ranged from 0 to 8.14 ng/ml; the values obtained by the Finsen procedure ranged from 0.16 to 9.05 ng/ml. In the Finsen procedure the pro-uPA from Grünenthal was used as a standard and in the American Diagnostica assay the sc-uPA standard provided with the kit. Least squares regression analysis of the data gave the relationship $y = 1.13x + 0.17$. Although the strength of relation is very high ($r = 0.950$), this is not an appropriate indicator of agreement between the methods. For an adequate assessment of how closely the two methods agree, a so-called Difference Plot [23] was produced (Figure 4a). This figure reveals the true extent of the bias especially at low uPA concentrations. In 5 of the 117 samples,

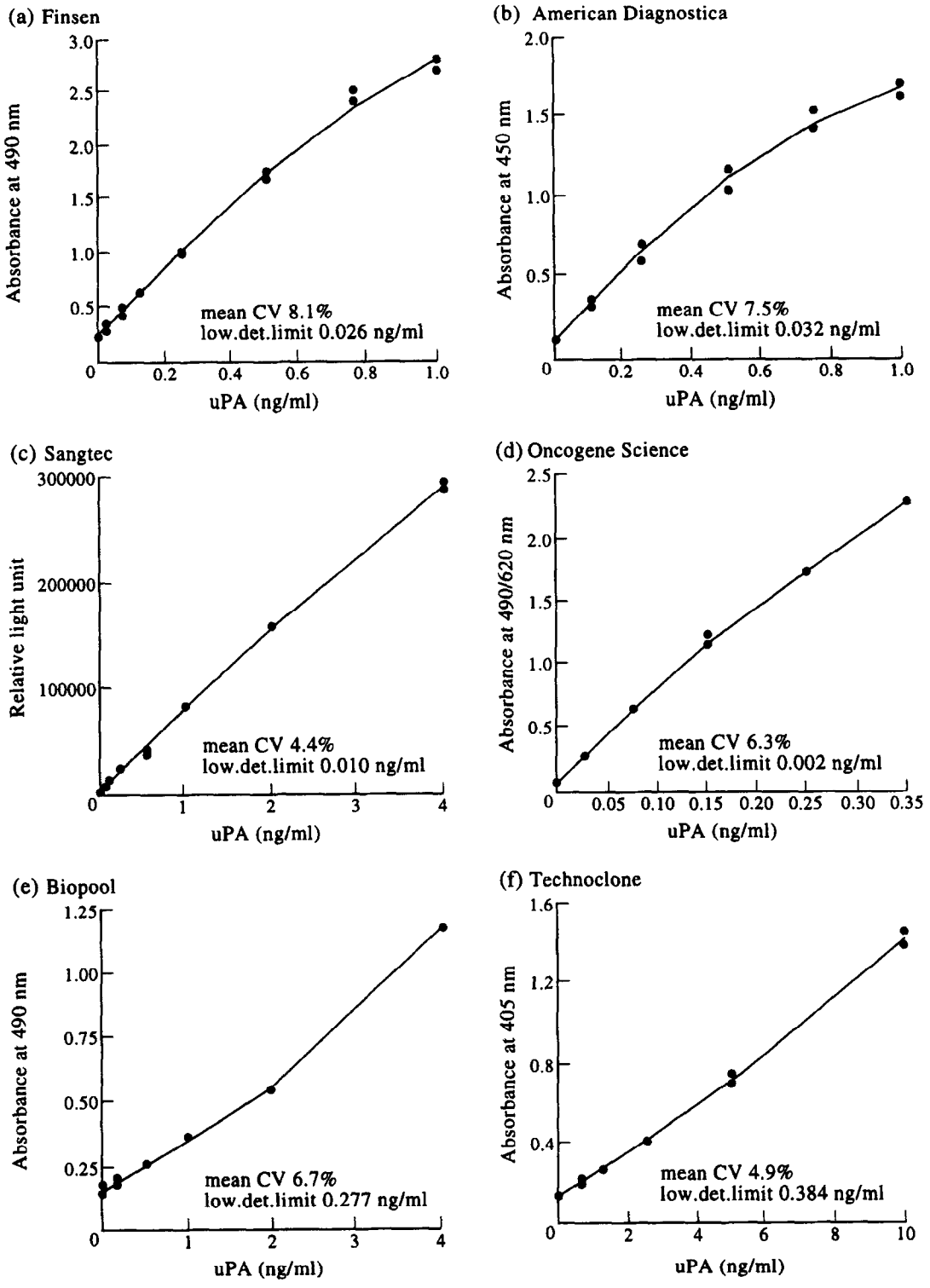


**Figure 1. Representative standard curves for six different uPA assays. Measurements were performed in duplicate (low. det. limit, lower limit of detection).**

the uPA values measured with the American Diagnostica procedure were lower than the detection limit, whereas in the Finsen procedure the values were low but measurable. These five samples were excluded from the Difference Plot. Of the American Diagnostica values 87 were lower, 1 was equal and 29 were higher than in Finsen procedure.

Breast cancer cytosols ($n = 18$) were also analysed with both the Sangtec procedure and the American Diagnostica kit. In both assays the standards provided by the companies were used. The values obtained by the American Diagnostica procedure ranged between 0.82 and 8.58 ng/ml; in the Sangtec procedure the values were between 0.06 and 4.57 ng/ml. These values were highly correlated (Figure 3b). The relation between the Sangtec values and the American Diagnostica values is defined by $y = 0.60x - 0.36$. The respective Difference Plot is shown in Figure 4b. Although the number of observations is rather small, there is a tendency for a decrease in percentage difference at higher uPA concentrations.

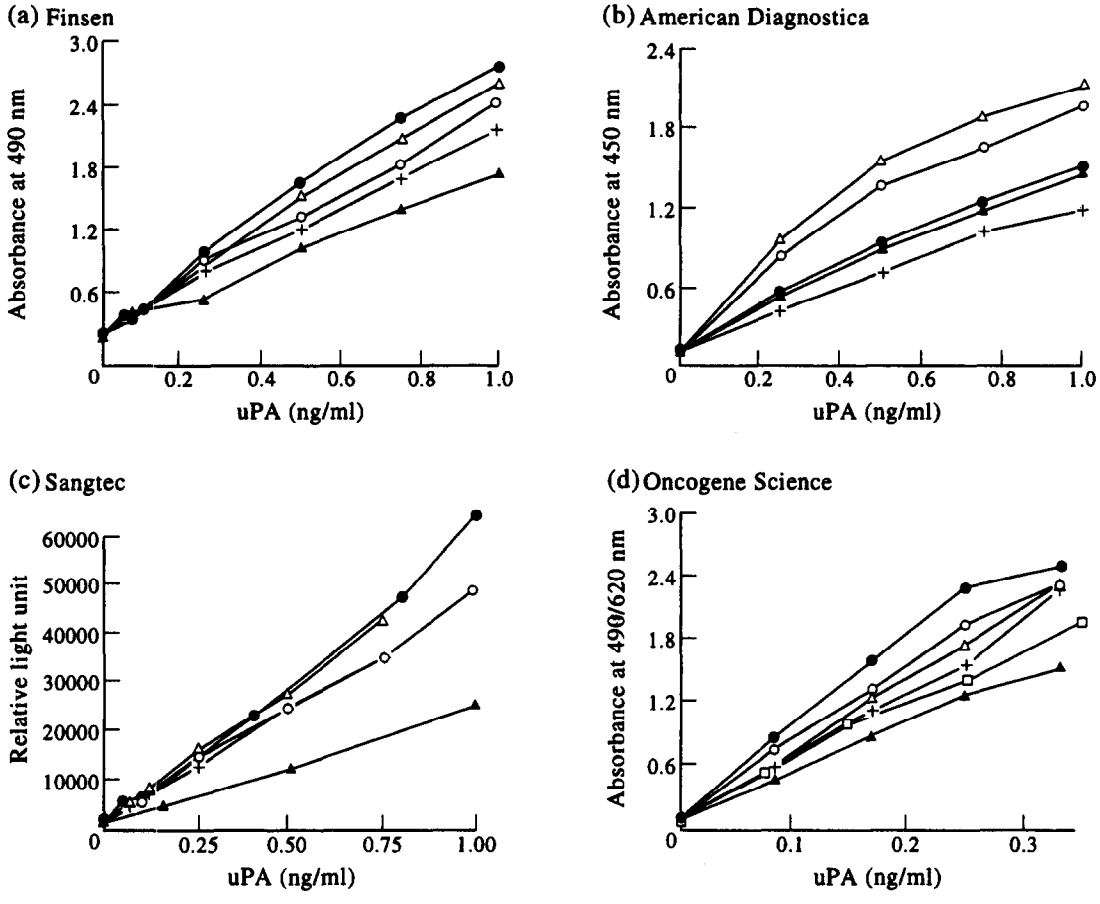


**Figure 2. Standard curves constructed by using various uPA standard preparations (Sangtec (●), pro-uPA Grünenthal (△), sc-uPA American Diagnostica (○), NIBSC 87/594 (+), Biopool (▲) and Oncogene Science (□)), and employing four different uPA assays.**

A number of other breast cancer cytosols ($n = 34$) were analysed with both the Oncogene Science and the American Diagnostica kit. In this series the span of uPA values obtained with the latter ranged between 0.08 and 4.97 ng/ml and the Oncogene Science values ranged between 0.21 and 10.6 ng/ml. Figure 3c shows the linear relation with a high correlation coefficient. The relation between uPA values obtained with the Oncogene Science and the American Diagnostica kit is defined by $y = 2.10x + 0.38$. The Difference Plot is shown in Figure 4c, implying that over the whole range of uPA concentrations the difference between the results obtained with both procedures is remarkably constant.

The repeatability of the three assay procedures is reflected by the CVs shown in Table 2. The uPA values measured were, for each procedure, divided into four quartiles. The CVs calculated from duplicates were averaged and are shown in the last column of the table. All coefficients of variation calculated were acceptable. The CV calculated from the 18 duplicate Sangtec measurements was 14.6%. The kits from Biopool and Technoclone, designed for uPA measurement in plasma, were not included in these experiments because they would require undiluted cytosol samples.

*Dilution of cytosolic samples*

The different assay procedures recommend that samples be diluted to within a specified range of protein content prior to assay. Values corrected for dilution should give the same result irrespective of the extent of dilution. Dilution experiments were carried out using both the Finsen assay and the American Diagnostica procedure. Such experiments are often referred to as parallelism studies because dilutions of sample should parallel the standard curve. In Table 3 the results obtained with the Finsen procedure are displayed. A human breast cancer cytosol (no. 1), prepared according to the EORTC guideline, was diluted with the appropriate dilution buffer obtaining dilutions from 1/4 to 1/64. The actual uPA concentrations measured in the diluted samples ranged from 0.067 to 0.866 ng/ml. The corresponding CVs calculated from the duplicates ranged from 0.9 to 6.3%. Multiplying these concentrations by the appropriate dilution factor will give the actual uPA value in the undiluted sample. The mean of these uPA values was 3.7 ng/ml and the CV was calculated to be 9.4%. Evidently, no systematic error could be detected, i.e. a perfect parallelism was observed. Three different 'xenograft cytosols' (nos 2, 3 and 4) with high uPA concentrations were diluted even further, obtaining dilutions down to 1/400. The CVs observed analysing uPA in the dilutions were <5%. Again, no systematic error was observed and parallelism was ascertained. In another set of dilution experiments performed with the American Diagnostica procedure, comparable results were obtained (see Table 4). No systematic decrease or increase of uPA levels was seen upon dilution.

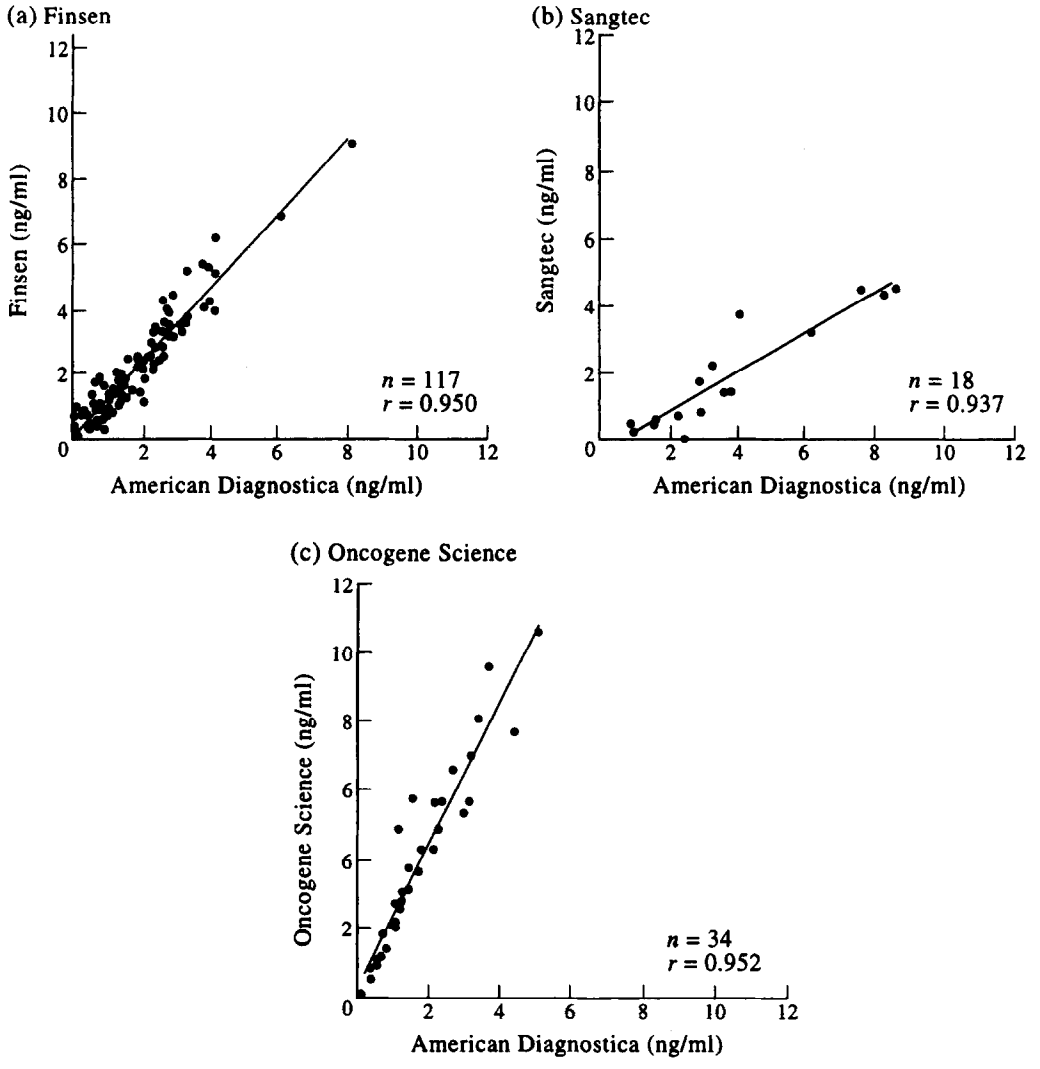


**Figure 3. Correlation of uPA levels in human primary breast tumour cytosols assayed with the American Diagnostica kit and the Finsen procedure (a), Sangtec kit (b) and the Oncogene Science kit (c).**

*uPA in tissue pellet extracts*

In the experiments described so far, uPA was assessed in cytosolic extracts employing the routine EORTC buffer as originally described for determination of steroid hormone receptors. However, uPA was also measured in tumour pellet extracts. Ten mammary tumours were homogenised in the EORTC buffer and the homogenates were pooled and divided into two equal parts. After centrifugation at 100000*g* the cytosol was separated from the pellets. One pellet was extracted with a buffer previously described by Jänicke and colleagues [20] which contains the non-ionic detergent Triton X-100 (pH 8.5). The other pellet was extracted with a buffer described by Camiolo and coworkers [21]. This latter buffer has a high ionic strength and a low pH (pH 4.2). Both extracts were diluted to various extents not with the extraction buffers but with the appropriate dilution buffers belonging to the particular assay procedure. uPA was measured with six different assay procedures.

The various dilutions in which uPA was assayed are shown in Table 5. The table shows that for the various assay procedures the dilutions varied, taking into account the different sensitivities of the asssay procedures. With each assay procedure, five different dilutions of both the Camiolo and Jänicke extracts were analysed and each was measured five times. The results are presented in Table 6. It is noteworthy that the uPA values in the Jänicke extracts were lower than the levels present in the Camiolo extracts. This holds for all six assay procedures. Obviously, because of the use of different internal standards and different antibodies, a large variation was observed between the uPA values obtained employing the different assay procedures.

The degree of parallelism, which can be deduced from the uPA values measured in the different dilutions, was higher when the pellet extraction was performed with the Jänicke buffer. This holds for three of the assay procedures (Finsen, American Diagnostica and Sangtec). The strongest parallelism was observed using the Oncogene Science kit. In those assay procedures which were developed for uPA analysis in plasma samples, less diluted tumour extracts could be used. In those cases, matrix effects resulted in non-parallelism.

*uPA values in cytosol fractions versus corresponding pellet extracts*

Nine primary breast cancers were homogenised in EORTC buffer and uPA was measured in the cytosols. The 100000*g* pellets were extracted with the Camiolo buffer and uPA was measured in these extracts (Figure 5). There was a strong

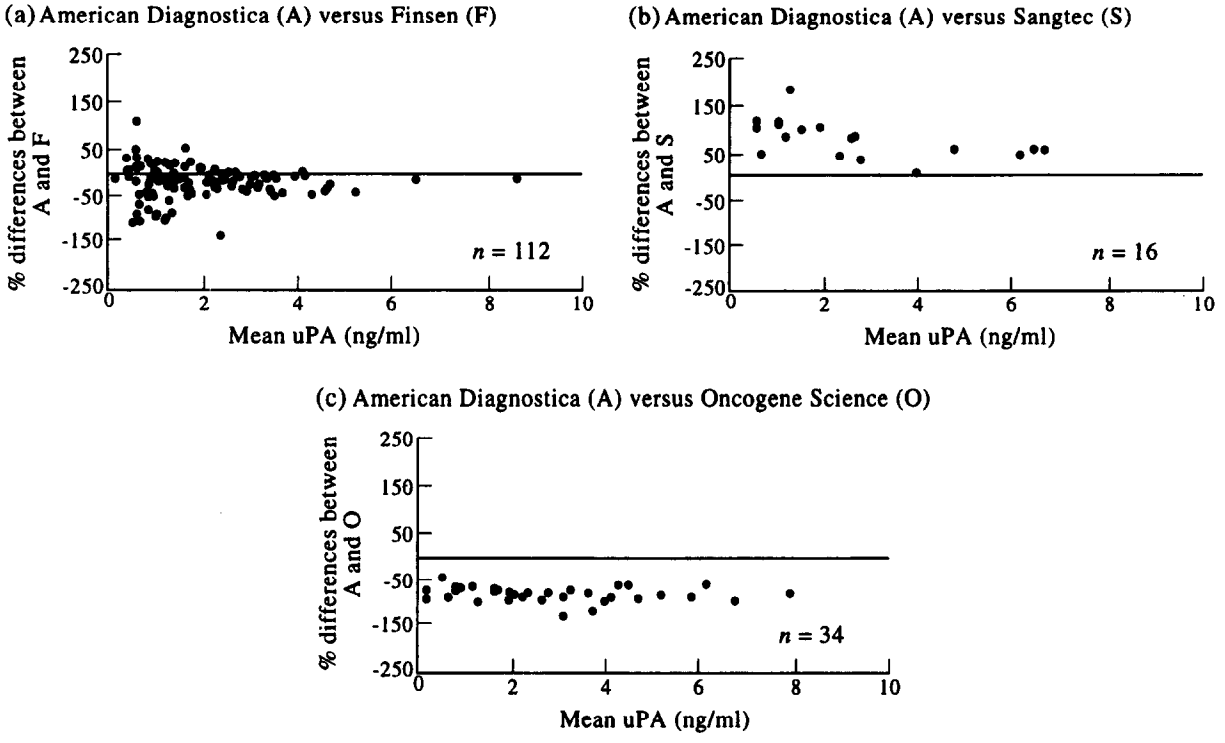


**Figure 4. Difference plots of measured uPA levels in breast tumour cytosols with the American Diagnostica kit (A) compared with (a) the Finsen procedure (F); (b) the Sangtec kit (S) and (c) the Oncogene Science kit (O). The percentage difference between two assays is plotted as the function of the level of uPA [22].**

*Table 2. Coefficients of variation based on duplicate measurements obtained at various uPA concentration ranges*

| uPA ranges | Number of duplicates | Number under detection limit | Average CVs (%) |
|---|---|---|---|
| American Diagnostica | 160 | 21 | |
| 0.16–0.38 ng/ml | | | 11.7 |
| 0.39–0.60 ng/ml | | | 12.0 |
| 0.62–1.10 ng/ml | | | 10.2 |
| 1.11–3.45 ng/ml | | | 7.6 |
| Finsen | 130 | 0 | |
| 0.24–0.90 ng/ml | | | 9.0 |
| 0.94–1.64 ng/ml | | | 9.4 |
| 1.68–2.92 ng/ml | | | 3.2 |
| 3.02–9.00 ng/ml | | | 2.7 |
| Oncogene Science | 34 | 0 | |
| 0.21–1.48 ng/ml | | | 4.0 |
| 1.86–3.17 ng/ml | | | 2.9 |
| 3.73–5.68 ng/ml | | | 5.6 |
| 5.70–10.64 ng/ml | | | 3.3 |

correlation between the soluble and detergent-extracted values. The Camiolo extracts contained 50 times higher uPA concentrations compared with the values in the corresponding cytosols. The uPA values presented in the figure are expressed per mg protein present in the samples. When the uPA concentrations were expressed per ml of sample, the Camiolo extracts contained 10 times higher uPA values than the corresponding cytosols ($y = 10.48x - 5.57$, $r = 0.924$).

*Analysis of a lyophilised quality control sample*

To be able to perform adequate internal and external quality control on the assays, a representative and stable control sample is needed. Therefore, cytosols prepared from primary breast cancer tissues were pooled and the pool was spiked with recombinant pro-uPA and lyophilised. This control sample was analysed on 26 separate days divided over a period of 18 months (Figure 6). Three operators performed the assays using various batches of the uPA kit from American Diagnostica. The mean of 26 uPA concentrations was 10.9 ng/ml with an overall CV of 11.5%. No statistically significant trend was observed.

## DISCUSSION

The proteolytic enzyme uPA, which activates plasminogen to the protease plasmin, plays an important role in cancer invasion and metastasis. In a preliminary report, in 1988, evidence was presented that high uPA enzyme activity in breast carcinoma extracts correlated with a shortened disease-free interval for the patients [14]. Two years later, in 1990, two groups of investigators, Jänicke and coworkers and Duffy and coworkers independently reported measurements of uPA antigen content in breast cancer tissues [15, 24]. Applying completely different uPA ELISAs, these authors reported that a high level of immunoreactive uPA is significantly correlated with high relapse rate and associated with poor prognosis. These observations were later extended and confirmed in other studies [25–27]. The uPA ELISA kits employed differed in the type of antibodies used, in the type of standards and in the instructions on how the tissue extracts had to be prepared. Notwithstanding these differences in analytical features, causing different ranges of values and cut-off points, the same clinical relevance for uPA was observed in all these studies.

The different sandwich-type uPA ELISAs used in those particular studies were included in the experiments of the present study, along with reagent kits marketed more recently or designed for uPA measurement in plasma. It was found

*Table 3. Parallelism study by dilution of cytosolic samples (Finsen procedure)*

| Cytosol nr | Dilution factor | uPA (ng/ml) measured in diluted sample; mean of duplicates | % CV from duplicates | uPA (ng/ml) calculated in undiluted sample | | |
|---|---|---|---|---|---|---|
| 1* | 4 | 0.866 | 0.9 | 3.5 | | |
| | 8 | 0.458 | 2.1 | 3.7 | mean | 3.7 ng/ml |
| | 16 | 0.213 | 3.7 | 3.4 | SD | 0.35 ng/ml |
| | 32 | 0.116 | 5.7 | 3.7 | CV | 9.4% |
| | 64 | 0.067 | 6.3 | 4.3 | | |
| 2† | 100 | 0.704 | 1.6 | 70.4 | | |
| | 150 | 0.473 | 4.1 | 71.0 | | |
| | 200 | 0.354 | 3.2 | 70.8 | mean | 71.1 ng/ml |
| | 300 | 0.225 | 6.4 | 67.5 | SD | 2.91 ng/ml |
| | 400 | 0.189 | 6.1 | 75.6 | CV | 4.0% |
| 3† | 100 | 0.928 | 2.1 | 92.8 | | |
| | 150 | 0.614 | 3.1 | 92.1 | | |
| | 200 | 0.455 | 1.1 | 91.0 | mean | 92.6 ng/ml |
| | 300 | 0.312 | 2.4 | 93.6 | SD | 1.10 ng/ml |
| | 400 | 0.234 | 3.6 | 93.6 | CV | 1.2% |
| 4† | 100 | 0.956 | 3.0 | 95.6 | | |
| | 150 | 0.696 | 4.2 | 104.4 | | |
| | 200 | 0.538 | 0.1 | 107.6 | mean | 101.2 ng/ml |
| | 300 | 0.328 | 0.1 | 98.4 | SD | 4.79 ng/ml |
| | 400 | 0.250 | 0.2 | 100.0 | CV | 4.7% |

*Mammary tumour cytosol. †The cytosols were prepared from three different xenografts raised in nude mice after applying MDA-NB-231 human breast cancer cells.

*Table 4. Parallelism study by dilution of cytosolic samples (American Diagnostica)*

| Cytosol nr | Dilution factor | uPA (ng/ml) measured in diluted sample; mean of duplicates | % CV from duplicates | uPA (ng/ml) calculated in undiluted sample | | |
|---|---|---|---|---|---|---|
| 5* | 20 | 0.119 | 5.1 | 2.4 | | |
| | 50 | 0.050 | 8.8 | 2.5 | mean | 2.6 ng/ml |
| | 100 | 0.030 | 5.0 | 3.0 | SD | 0.26 ng/ml |
| | 200 | 0.013 | 5.5 | 2.6 | CV | 10.0% |
| 6* | 50 | 0.132 | 1.0 | 6.6 | | |
| | 100 | 0.068 | 18.5 | 6.8 | mean | 7.0 ng/ml |
| | 200 | 0.039 | 10.4 | 7.8 | SD | 0.54 ng/ml |
| | 400 | 0.017 | 2.9 | 6.8 | CV | 7.7% |
| 7† | 50 | 0.612 | 5.0 | 30.6 | | |
| | 100 | 0.328 | 1.2 | 32.8 | mean | 30.9 ng/ml |
| | 200 | 0.139 | 6.6 | 27.8 | SD | 2.28 ng/ml |
| | 400 | 0.081 | 0.0 | 32.4 | CV | 7.4% |
| 8‡ | 10 | 1.023 | 4.7 | 10.2 | | |
| | 20 | 0.572 | 11.4 | 11.4 | mean | 10.5 ng/ml |
| | 40 | 0.289 | 9.1 | 11.6 | SD | 0.97 ng/ml |
| | 80 | 0.120 | 5.4 | 9.6 | CV | 9.2% |
| | 120 | 0.080 | 7.1 | 9.6 | | |

*Mammary tumour cytosol. †Colon carcinoma cytosol. ‡Mammary tumour cytosol spiked with 8.4 ng/ml pro-uPA.

that those kits developed for the analysis of tumour cytosols, all reached sensitivities of less than 32 pg uPA per ml, which is satisfactory. The two uPA ELISAs, initially developed for measuring uPA in plasma, did not reach the desired sensitivity, but recently these assays have been redesigned and are now available for tumour extract analysis. The within-assay CVs observed, employing the various assay methods, were acceptable, and all kits designed for tumour analysis displayed an acceptable to excellent degree of parallelism.

When, during our workshop, sets of breast cancer cytosol preparations were analysed applying different uPA ELISAs, different absolute uPA values were observed, although high correlations (Pearson) were obtained for any two of the four uPA ELISAs applied. It is acknowledged that, strictly speaking, the Pearson correlation coefficient is a poor index of the degree of agreement between two methods [23]. How closely two methods agree is a question which is more accurately answered by employing a calculation of the differences

*Table 5. Dilutions of the pellet extracts analysed*

| Pellet extracted with Jänicke buffer* | American Diagnostica | Sangtec | Finsen | Biopool | Oncogene Science | Technoclone |
|---|---|---|---|---|---|---|
| > | 1:20 | 1:1 | 1:30 | 1:10 | 1:90 | 1:1 |
| >> | 1:40 | 1:2 | 1:60 | 1:20 | 1:180 | 1:2 |
| >>> | 1:60 | 1:4 | 1:90 | 1:30 | 1:270 | 1:5 |
| >>>> | 1:80 | 1:8 | 1:120 | 1:40 | 1:360 | 1:10 |
| >>>>> | 1:120 | 1:16 | 1:150 | 1:50 | 1:450 | 1:20 |
| Camiolo buffer* | | | | | | |
| > | 1:10 | 1:1 | 1:20 | 1:10 | 1:60 | 1:1 |
| >> | 1:20 | 1:2 | 1:40 | 1:20 | 1:120 | 1:2 |
| >>> | 1:40 | 1:4 | 1:60 | 1:30 | 1:180 | 1:4 |
| >>>> | 1:60 | 1:8 | 1:80 | 1:40 | 1:240 | 1:8 |
| >>>>> | 1:80 | 1:16 | 1:120 | 1:50 | 1:360 | 1:16 |

*Extracts were diluted with the appropriate dilution buffer.
>, lowest dilution; >>>>>, highest dilution.

*Table 6. uPA values in different dilutions of pellet extracts**

| Pellet extracted with Jänicke buffer | American Diagnostica | Sangtec | Finsen | Biopool | Oncogene Science | Technoclone |
|---|---|---|---|---|---|---|
| > | 6.1† (5%)‡ | 8.2 (5%) | 13.7 (4%) | OF | 17.3 (5%) | 11.0 (10%) |
| >> | 5.1 (12%) | 7.5 (2%) | 12.9 (5%) | OF | 17.7 (5%) | 15.4 (17%) |
| >>> | 4.8 (6%) | 7.7 (3%) | 11.3 (4%) | 12.2 (86%) | 17.7 (15%) | 18.3 (7%) |
| >>>> | 4.5 (7%) | 7.9 (3%) | 12.2 (3%) | 13.1 (4%) | 16.7 (8%) | 31.9 (16%) |
| >>>>> | 5.0 (7%) | 9.0 (4%) | 12.7 (7%) | 14.6 (9%) | 16.6 (7%) | 37.3 (19%) |
| Camiolo buffer | | | | | | |
| > | 13.8 (3%) | OF | 16.3 (12%) | OF | 23.8 (9%) | OF |
| >> | 12.9 (2%) | 12.5 (20%) | 16.9 (1%) | 43.8 (6%) | 24.0 (8%) | 21.3 (7%) |
| >>> | 11.2 (6%) | 18.3 (21%) | 18.9 (4%) | 49.2 (11%) | 25.6 (14%) | 41.2 (14%) |
| >>>> | 10.2 (3%) | 14.7 (5%) | 20.2 (2%) | 65.2 (32%) | 25.4 (16%) | 40.0 (8%) |
| >>>>> | 9.7 (3%) | 15.0 (0%) | 22.1 (2%) | 49.4 (22%) | 24.5 (13%) | 42.2 (12%) |

*Values measured five times; †ng uPA/ml. ‡Coefficient of variation. >, lowest dilution; >>>>>, highest dilution; OF, overflow.
Homogenates of ten mammary tumours were pooled. After centrifugation the pellet was divided into two parts. One part was extracted with Jänicke buffer, the other part with Camiolo buffer.

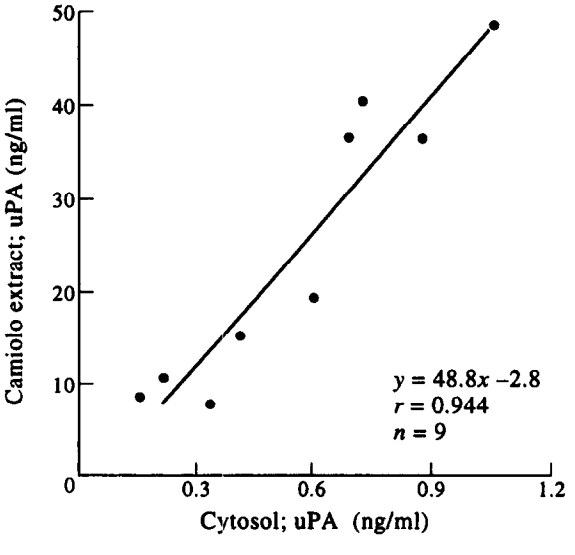


**Figure 5. Comparison of uPA levels measured in breast cancer cytosols compared with uPA levels measured in their paired pellet extracts. Pellets were extracted with the Camiolo buffer, and uPA was determined using the Finsen procedure.**

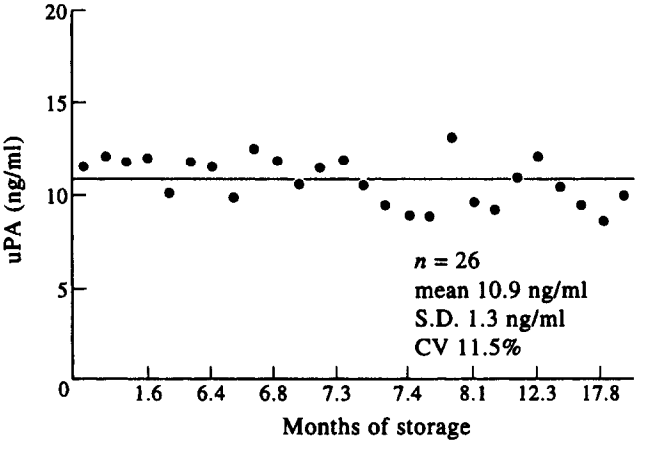


**Figure 6. Effect of storage on the stability of uPA levels in a lyophilised spiked breast cancer cytosol. Breast cancer cytosol (212 ml) was spiked with 1778 ng recombinant pro-uPA and lyophilised in 0.5 ml aliquots. Vials containing the lyophilised residue were stored at 4°C.**

between measurements. Scatter plots of the per cent differences versus the mean of the two methods provide a clear visual indication of the outliers which occur especially at low concentrations.

It is important to note that the uPA values measured with the various uPA ELISAs will depend on the efficiency with which the different sets of uPA antibodies employed detect the various forms of uPA present in the tissue cytosol. Indeed, uPA can occur *in vivo* in different molecular forms: as a single chain pro-enzyme (pro-uPA); as a high molecular weight two chain molecule HMW-uPA; and as a low molecular weight LMW-uPA. Besides these different forms, complexes of pro-uPA and HMW-uPA with the receptor for uPA,uPAR, are present. In addition, HMW-uPA, its receptor-bound form, and LMW-uPA can be complexed with the inhibitors PAI-1 or PAI-2, complexes in which uPA-epitopes may be hidden, due to steric hindrance. Taken together, this implies that, in cytosols, uPA may be present as a mixture of various molecular forms and weights with different structures.

According to Büessecker and colleagues [28], polyclonal antibodies to uPA react with the distinct molecular forms of uPA to a similar degree when tested by ELISA. In contrast, monoclonal antibodies, due to their high epitope-specific recognition, may not *a priori* allow detection of distinct uPA forms and complexes. All the uPA ELISAs tested in the present study use monoclonal antibodies, and the information given by the manufacturers with the kit claim that most, if not all, of the uPA forms are recognised. However, no detailed studies regarding this aspect are available and therefore it has to be emphasised that the efficiencies with which the various forms are detected are reportedly unknown and remain to be determined.

The relative level of each molecular form of uPA present in cytosol may vary between different cytosols. The results of the present study suggest that this variation will be even greater when different ways of extraction are employed, e.g. including or excluding non-ionic detergents in the extraction buffer. Our results confirm earlier findings [25] which indicated that the uPA content in tumour tissue extracts, obtained by treating fresh tumour tissues with a Triton X-100 containing buffer, is much higher than the uPA level in extracts prepared by using the detergent-free EORTC buffer.

As far as the standards provided with the different kits are concerned, these are different in structure (e.g. glycosylated versus non-glycosylated) and in source (e.g. recombinant versus cell-culture supernatants). The results obtained during the workshop disclosed large differences in immunological potencies of the different standard preparations, when they were tested in the various kits. The discrepancies observed cannot solely be ascribed to differences in calibration of the standards because the immunoreactive potencies, as reflected by the different absorbance values generated by different standards, do not follow the same order in the different uPA ELISAs. For example, when a particular standard in one of the uPA ELISAs evoked the highest absorbance value compared with other standards, the same standard evoked only an intermediate signal in another uPA ELISA. It is plausible to assume that the differential specificity of the different sets of antibodies is involved in causing these discrepancies. For proper evaluation of the uPA content determined in tumour cytosols, applying different uPA ELISAs, it should be kept in mind that these kits contain standards which may differ in composition, use antibodies which often differ in specificity but quantify an analyte which is heterogeneous in nature. Thus, it is hardly surprising that the ranges in uPA levels, measured with different ELISAs, vary considerably. However, it is not possible retrospectively to relate the absolute uPA values reported in the literature with those obtained with different ELISAs.

Taken together, this implies that multicentre studies with the goal of measuring uPA antigen in biological material in different laboratories should only be performed if the same type of uPA ELISA is used by all participating laboratories. In that case, the use of a stable reference material (tumour tissue extract) is very useful for normalisation of absolute values. In addition, such normalisation of values should result in lower interlaboratory CVs, but this will occur only if the intralaboratory variation of the determination of uPA by ELISA is acceptably low. If such a reference sample is used throughout the study and the values reported assessed centrally, not only changes within any laboratory but also any changes between two batches of uPA ELISA will be quickly detected. Therefore, it is strongly recommended that a reference tumour tissue extract sample is applied in order to assure a high standard of quality control. From the present study, it appears that lyophilised breast cancer cytosols are stable over a long period of time and can thus serve as an ideal reference sample for the assessment of uPA antigen in breast cancer tissue extracts.


1. Valagussa P, Bonadonna G, Veronesi U. Patterns of relapse and survival following radical mastectomy. *Cancer* 1978, **41**, 1170–1178.
2. Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. *Natl Cancer Inst Monogr* 1971, **34**, 55–70.
3. Walt AJ, Singhakowinta A, Brooks SC, Cortez A. The surgical implications of estrophile protein estimations in carcinoma of the breast. *Surgery* 1976, **80**, 506–512.
4. Bishop HM, Blamey RW, Elston CW, Haybittle JL, Nicholson RI, Griffiths K. Relationship of oestrogen-receptor status to survival in breast cancer. *Lancet* 1979, **2**, 283–284.
5. Jensen EV. Steroid receptors in breast cancer: historical perspective. *Cancer* 1980, **46**, 2759–2761.
6. Lippman ME, Allegra JC. Quantitative estrogen receptor analyses: the response to endocrine and cytotoxic chemotherapy in human breast cancer and the disease-free interval. *Cancer* 1980, **46**, 2829–2834.
7. Foekens JA, Portengen H, Look MP, *et al.* Relationship of PS2 with response to tamoxifen therapy in patients with recurrent breast cancer. *Br J Cancer* 1994, **70**, 1217–1223.
8. Revision of the standards for the assessment of hormone receptors in human breast cancer; report of the second EORTC workshop, held on 16–17 March 1979, in the Netherlands Cancer Institute. *Eur J Cancer* 1980, **16**, 1513–1515.
9. Wittliff JL, Brown AM, Fisher B. Establishment of uniformity in steroid receptor determination for protocol B-09 of the National Surgical Adjuvant Breast Project. In Sarfaty GA, Nash AR, Keightly DD, eds. *Estrogen Receptor Assays in Breast Cancer. Laboratory Discrepancies and Quality Assurance.* New York; Masson, 1981, 27–39.
10. Koenders A, Benraad ThJ. Standardization of steroid receptor analysis in breast cancer biopsies: EORTC receptor group. *Recent Results Cancer Res* 1984, **91**, 129–135.
11. Danø K, Andreasen PA, Grøndahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. *Adv Cancer Res* 1985, **44**, 139–266.
12. Liotta LA, Stetler-Stevenson WG. Tumour invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res* 1991, **51**, 5054s–5059s.
13. Mignatti P, Rifkin DB. Biology and biochemistry of proteinases in tumor invasion. *Physiol Rev* 1993, **73**, 161–195.
14. Duffy MJ, O'Grady P, Devaney D, O'Siorain L, Fennelly JJ,

Lijnen HJ. Urokinase-plasminogen activator, a marker for aggressive breast carcinomas: preliminary report. *Cancer* 1988, **62**, 531–533.
15. Jänicke F, Schmitt M, Hafter R, *et al.* Urokinase-type plasminogen activator (u-PA) antigen is a predictor of early relapse in breast cancer. *Fibrinolysis* 1990, **4**, 69–78.
16. Foekens JA, Look MP, Peters HA, van Putten WLJ, Portengen H, Klijn JGM. Urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1 predict poor response to tamoxifen therapy in recurrent breast cancer. *J Natl Cancer Inst* 1995, **87**, 751–756.
17. Ganesh S, Sier CF, Griffioen G, *et al.* Prognostic relevance of plasminogen activators and their inhibitors in colorectal cancer. *Cancer Res* 1994, **54**, 4065–4071.
18. Pedersen H, Brünner N, Francis D, *et al.* Prognostic impact of urokinase, urokinase receptor, and type 1 plasminogen activator inhibitor in squamous and large cell lung cancer tissue. *Cancer Res* 1994, **54**, 4671–4675.
19. Hasui Y, Marutsuka K, Nishi S, Kitada S, Osada Y, Sumiyoshi A. The content of urokinase-type plasminogen activator and tumor recurrence in superficial bladder cancer. *J Urol* 1994, **151**, 16–20.
20. Jänicke F, Pache L, Schmitt M, *et al.* Both the cytosols and detergent extracts of breast cancer tissues are suited to evaluate the prognostic impact of the urokinase-type plasminogen activator and its inhibitor, plasminogen activator inhibitor type 1. *Cancer Res* 1994, **54**, 2527–2530.
21. Camiolo SM, Siuta MR, Madeja JM. Improved medium for extraction of plasminogen activator from tissue. *Prep Bioch* 1982, **12**, 297–305.
22. Rosenquist C, Thorpe SM, Danø K, Grøndahl-Hansen J. Enzyme-linked immunosorbent assay of urokinase-type plasminogen activator (uPA) in cytosolic extracts of human breast cancer tissue. *Breast Cancer Res Treat* 1993, **28**, 223–229.
23. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986, **1**, 307–310.
24. Duffy MJ, Reilly D, O'Sullivan C, O'Higgins H, Fennell JJ, Andreasen P. Urokinase plasminogen activator: a new and independent prognostic marker in breast cancer. *Cancer Res* 1990, **50**, 6827–6829.
25. Foekens JA, Schmitt M, van Putten WL, Peters HA, Bontenbal M, Jänicke F, Klijn JG. Prognostic value of urokinase-type plasminogen activator in 671 primary breast cancer patients. *Cancer Res* 1992, **52**, 6101–6105.
26. Spyratos F, Martin PM, Hacène K, *et al.* Multiparametric prognostic evaluation of biological factors in primary breast cancer. *J Natl Cancer Inst* 1992, **84**, 1266–1272.
27. Grøndahl-Hansen J, Christensen IJ, Rosenquist C, *et al.* High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. *Cancer Res* 1993, **53**, 2513–2521.
28. Büessecker F, Reinartz J, Schirmer U, Kramer MD. Enzyme-linked immunosorbent assays for plasminogen activators. *J Immunol Meth* 1993, **162**, 193–200.

**Acknowledgements**—This work is supported by the BIOMED-1 project 'Clinical Relevance of Proteases in Tumour Invasion and Metastasis' (BMH1-CT93-1346). The following manufacturers kindly provided us with kits or reagents: American Diagnostica Inc., 222 Railroad Ave, P.O. Box 1165, Greenwich, Connecticut 06836-1165, U.S.A. Biopool, P.O. Box 1454, S-90124 Umea, Sweden. Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, DK-2100 Copenhagen, Denmark. Oncogene Science, 80 Rogers Street, Cambridge, Massachusetts 02142, U.S.A. Sangtec Medical AB, BX 20045, S-16102 Bromma, Sweden. Technoclone GmbH, Müllnergasse 23, A-1090 Vienna, Austria. Dr. P.J. Gaffney from the National Institute for Biological Standards and Control (NIBSC), Potters Bar, Hertfordshire, U.K., is acknowledged for providing us with the uPA standard 87/594.