CANCER GENOMICS & PROTEOMICS *2*: 37-42 (2005)

Review

Protein Microarrays – A Promising Tool for Cancer Diagnosis

NICOLE SCHNEIDERHAN-MARRA[1,2], ANETTE KIRN[2], ANETTE DÖTTINGER[2], MARKUS TEMPLIN[2], GEORG SAUER[1], HELMUT DEISSLER[1] and THOMAS O. JOOS[2]

[1]*University of Ulm Medical School, Department of Obstetrics and Gynaecology, Frauensteige 14, 89075 Ulm;*
[2]*NMI Natural and Medical Sciences Institute at the University of Tübingen, Markwiesenstraße 55, 72770 Reutlingen, Germany*

Abstract. *Within recent years, protein microarrays have been developed to quantify a large number of parameters present in a given sample simultaneously. Such miniaturised and parallelised sandwich immunoassays are of general interest for all proteomic and diagnostic approaches in which several parameters have to be determined from small samples, e.g. biopsy material. In addition to planar microarray-based approaches, bead-based flow cytometry is quite suitable for the multiplex detection of target molecules, especially when a limited number of parameters are to be analysed. Appropriate sensitivity, reproducibility and robustness have to be demonstrated before protein microarray technology can be used to characterise clinical samples and generate reliable data sets. As a model system to analyse these issues, a set of multiplexed sandwich immunoassays based on Luminex beads were developed to screen clinical samples for the presence or absence of marker proteins indicative of prognosis or response to therapeutic options.*

The basic principles of miniaturised and parallelised ligand binding assays were described in the early 1980s by Roger Ekins' ambient analyte theory (1). Today, DNA microarrays are well-established high-throughput hybridisation systems that enable the exploration of the whole transcriptome in a single experiment (2). However, there is no absolute correlation between mRNA expression level and the corresponding protein level and the function of the proteins cannot be deduced exclusively on mRNA or protein expression data sets (3). Therefore, additional high-throughput technologies, deciphering direct protein expression and providing functional analysis, are needed to overcome the gap between genomics and proteomics. Microarray technology has already been expanded beyond the level of DNA chips. Within the last few years, a large variety of different types of protein array-based approaches have been demonstrated. Such microarray based assays include antibody microarrays that are used to identify and quantitate target proteins of interest and protein affinity assays that are used to study the interactions between proteins and immobilized binding molecules such as proteins, peptides, low molecular weight compounds, oligosaccharides, or DNA (4, 5). These miniaturised and parallelised assay systems have great potential as replacements of singleplex analysis systems.

Particularly, in medical research, protein microarrays can accelerate and improve diagnostics by looking at a large number of relevant parameters simultaneously. Today, sample volume can be the limiting factor, as it is in the case of the analysis of multiple tumor markers from biopsy material. Protein microarrays provide an appropriate solution to generate the required data from a limited amount of sample. Therefore, protein microarrays have the potential to offer new possibilities with respect to therapy-relevant classification of patients and patient monitoring during treatment.

Different approaches have been described to use protein microarrays for the parallel study of expression of a number of proteins. Similar to dual-colour labelling used to visualise differential mRNA expression, antibody microarray approaches have been undertaken to analyse the differential display of proteins using two protein samples which were labelled with two different fluorophores. Equal amounts of total protein were mixed and incubated on the antibody microarray. The differences in the concentrations of the target proteins in each capture spot can be detected *via* dual wavelength fluorescence (6). Results gained with such a type of protein microarray must, however, be evaluated carefully

Correspondence to: Thomas O. Joos, NMI Natural and Medical Sciences Institute at the University of Tübingen, Markwiesenstraße 55, 72770 Reutlingen, Germany. Tel: +49 7121 51530 844, Fax: +49 7121 51530 16, e-mail: joos@nmi.de

Key Words: Protein microarray, bead-based technology, sandwich immunoassays, breast cancer.

since proteins are often assembled in multiprotein complexes. As a consequence, a strong signal can either result from a large amount of target protein or from the capture of a huge complex of different proteins bound to the captured target. Nevertheless, field applications have proved the general applicability of this technology (7-11).

Meanwhile, the first commercial antibody microarrays consisting of several hundred monoclonal antibodies have entered the market. However, these antibody microarrays still have to be improved substantially to become a global discovery tool for proteomic research comparable to a whole genome DNA chip. In addition, the complex direct labelling approach has severe limitations with respect to sensitivity and sample throughput. As an alternative to the array-based proteomics approach, miniaturised and multiplexed sandwich immunoassays provide an excellent solution for applications in which several parameters have to be determined simultaneously from a limited amount of sample material. In addition to planar microarray-based systems, which are perfectly suited to screening for a large number of target proteins, bead-based assays are a very interesting alternative to planar microarrays, as these assay systems have already demonstrated their appropriate robustness, sensitivity and sample throughput. Bead-based assay systems employ different color-coded microspheres as the solid support for the capture molecules and a flow cytometer, which is able to identify each individual type of bead and quantify the amount of captured targets on each bead. Their sensitivity, reliability and accuracy are similar to those observed with standard microtiter ELISA procedures. Color-coded microspheres can be used to perform up to a hundred different assay types simultaneously (LabMAP system, Laboratory Multiple Analyte Profiling, Luminex, Austin, TX, USA) (12-16). For example, microsphere-based systems have been used to determine the concentration of cytokines or antibodies in biological samples such as patient serum or cell culture supernatant (13, 14, 17-20).

Diagnostic potential of protein microarrays – investigation of breast tumor specimens as an example

Our research efforts aim at the characterisation of breast tumor-derived lysates in terms of differential expression of the potentially informative threshold concentration of markers. In this context, we have developed a variety of miniaturised and parallelised sandwich immunoassays using the bead-based Luminex technology. For the multiplexed set-up, capture and detection antibodies have to be carefully validated with respect to specificity and cross-reactivity. Cross-reactivity of the used antibodies is the most critical issue for multiplexing sandwich immunoassays. Thus, it is necessary to have well characterised antibodies binding to defined epitopes.

The selection of the parameters included in our assays is based the assumption that relative amounts of these proteins may be informative in breast cancer diagnosis, prognosis and help to decide between therapeutic options.

To date, the consensus-statements of the AGO [http://www.ago-online.de], St. Gallen (2001, 2003 NIH 2000) [http://consensus.nih.gov/cons/114/114_statement.pdf], American Association of Clinical Oncology (ASCO 1996) and EORTC Receptor and Biomarker Group (1995) recommend the following prognostic factors for determination of mamma carcinoma in routine clinical laboratories: tumor size, lymph node state, histopathological typing, grading, and steroid hormone receptors, namely estrogen receptor (ER) and progesterone receptor (PR). Also Her-2/c-erbB2 is of interest, but still needs further evaluation.

Patients with positive hormone receptor status have, in general, a better prognosis with respect to overall survival as well as relapse-free time and these patients can be selected for endocrine therapy. In contrast, an overexpression of Her-2 indicates, in general, a more aggressive tumor and often correlates with the loss of ER and PR expression, but also provides a target for therapeutic intervention with anti-Her-2 antibodies.

To date, the gold standard analysis for these receptor proteins is immunohistochemistry (IHC). Only a minority of clinical laboratories use EIA or ELISA for the detection of these prognostic and predictive factors. IHC provides the clinicians not only with the number of positive stained tumor cells, their local staining intensity and local distribution, but also with an evaluation of the invasiveness of the tumor. However, IHC staining is a labor-intensive and time-consuming method. To allow the fast analysis of numerous parameters, we are developing miniaturised sandwich immunoassays for the detection of the established prognostic indicators and predictive factors, *e.g.* the hormone receptors ERα and PR and the cell surface receptors Her-2 and EGFR. Additional sandwich immunoassays are included to analyse parameters involved in tumor proliferation, tumor vascularisation and metastatic potential.

EGF and EGFR family members, *e.g.* both Her-2/EGFR2 and EGFR (Her-1/EGFR1), are known to be involved in the regulation of cell cycle progression and cell survival of breast epithelial cells. Excessive activation of signaling pathways downstream of EGFRs has been linked to breast cancer development and resistance of cancerous cells to cytotoxic drug therapy. In addition, overexpression of both receptors are related to a short overall and disease-free survival (21). This provides a rationale for the development of sandwich immunoassays allowing measurements of EGFR and Her-2 in patient-derived samples.

Table I. *Frozen breast tumor and corresponding normal tissue from 5 different patients were pulverized in a Mikro-Dismembrator U (Braun/Satorius, Germany) at full speed for 1 min. Proteins were extracted with a buffer containing 50 mM Tris pH 7.5/ 400 mM NaCl/ 1 mM $CaCl_2$/ 1 mM $MgCl_2$/ 1% Triton X-100/ Phosphatase-Inhibitor I and II (Sigma) 1% each/ 1x Complete Protease Inhibitor (Roche). Measurements were done using bead-based sandwich immunoassays on the Luminex100 IS. Median fluorescence intensities (MFI) are listed and log2 ratios of tumor versus normal tissues were calculated. (A) ERa was measured in a 1-plex, VEGF and Her-2 were measured together in a 2-plex assay and VEGFR 1-3 and EGFR were measured in a 4-plex assay. For each assay, 10 μg total protein were used. (B) Measurement of MMPs from the same tissue lysates in a 7-plex using 5 μg total protein.*

Table IA.

Analyte	Patient	Tissue		log2 Ratio [Tumor/ Normal]
		Normal	Tumor	
Her-2	1	152	101	-0.59
	2	83	2001	4.59
	3	114	1474	3.70
	4	162	2226	3.78
	5	222	2022	3.19
VEGF	1	21	194	3.24
	2	15	81	2.44
	3	20	158	3.01
	4	21	150	2.85
	5	35	747	4.44
VEGFR1	1	61	388	2.67
	2	63	274	2.12
	3	32	249	2.96
	4	64	268	2.06
	5	36	600	4.06
VEGFR2	1	20	23	0.20
	2	22	20	-0.14
	3	19	20	0.07
	4	23	20	-0.20
	5	17	18	0.08
VEGFR3	1	11	16	0.54
	2	13	11	-0.24
	3	10	13	0.38
	4	12	10	-0.26
	5	10	10	0.00
EGFR	1	442	347	-0.35
	2	499	416	-0.26
	3	173	204	0.24
	4	597	559	-0.09
	5	345	496	0.52
ERa	1	17	22	0.37
	2	27	16	-0.75
	3	41	325	2.99
	4	23	30	0.38
	5	23	10	-1.20

Table IB.

Analyte	Patient	Tissue		log2 Ratio [Tumor/ Normal]
		Normal	Tumor	
MMP 1	1	17	103	2.59
	2	16	136	3.09
	3	17	45	1.40
	4	17	54	1.67
	5	18	69	1.93
MMP 2	1	13	15	0.16
	2	13	14	0.11
	3	12	20	0.74
	4	11	17	0.63
	5	12	16	0.42
MMP 3	1	24	51	1.07
	2	27	93	1.78
	3	20	29	0.54
	4	28	55	0.97
	5	24	68	1.50
MMP 7	1	29	83	1.52
	2	40	54	0.43
	3	28	93	1.73
	4	31	142	2.20
	5	66	176	1.42
MMP 8	1	140	119	-0.24
	2	79	65	-0.28
	3	67	496	2.89
	4	49	82	0.74
	5	104	140	0.43
MMP 9	1	55	127	1.21
	2	30	165	2.46
	3	29	216	2.92
	4	21	133	2.66
	5	41	227	2.47
MMP 13	1	14	27	0.95
	2	14	19	0.44
	3	15	19	0.34
	4	13	355	4.77
	5	15	36	1.26

Angiogenesis is a complex process leading to the formation of new blood vessels from a pre-existing vascular network. Growth, invasion and metastasis of many cancers depend on angiogenesis. In particular, neovascularisation is a prerequisite in solid tumors to grow beyond a size of 1 mm^3. This is only possible if the supply of oxygen and nutrients is assured (22-24). Several anti-angiogenic drugs targeting the VEGF/VEGFR are already in the later stages of clinical trials. Thus, detection of vascular endothelial growth factor (VEGF-A), VEGF receptor 1 (VEGFR1/Flt-1), VEGF receptor 2 (VEGFR2/ KDR/Flk-1) and VEGF receptor 3 (VEGFR3/Flt-4) is of great interest (Table IA and Figure 1A).

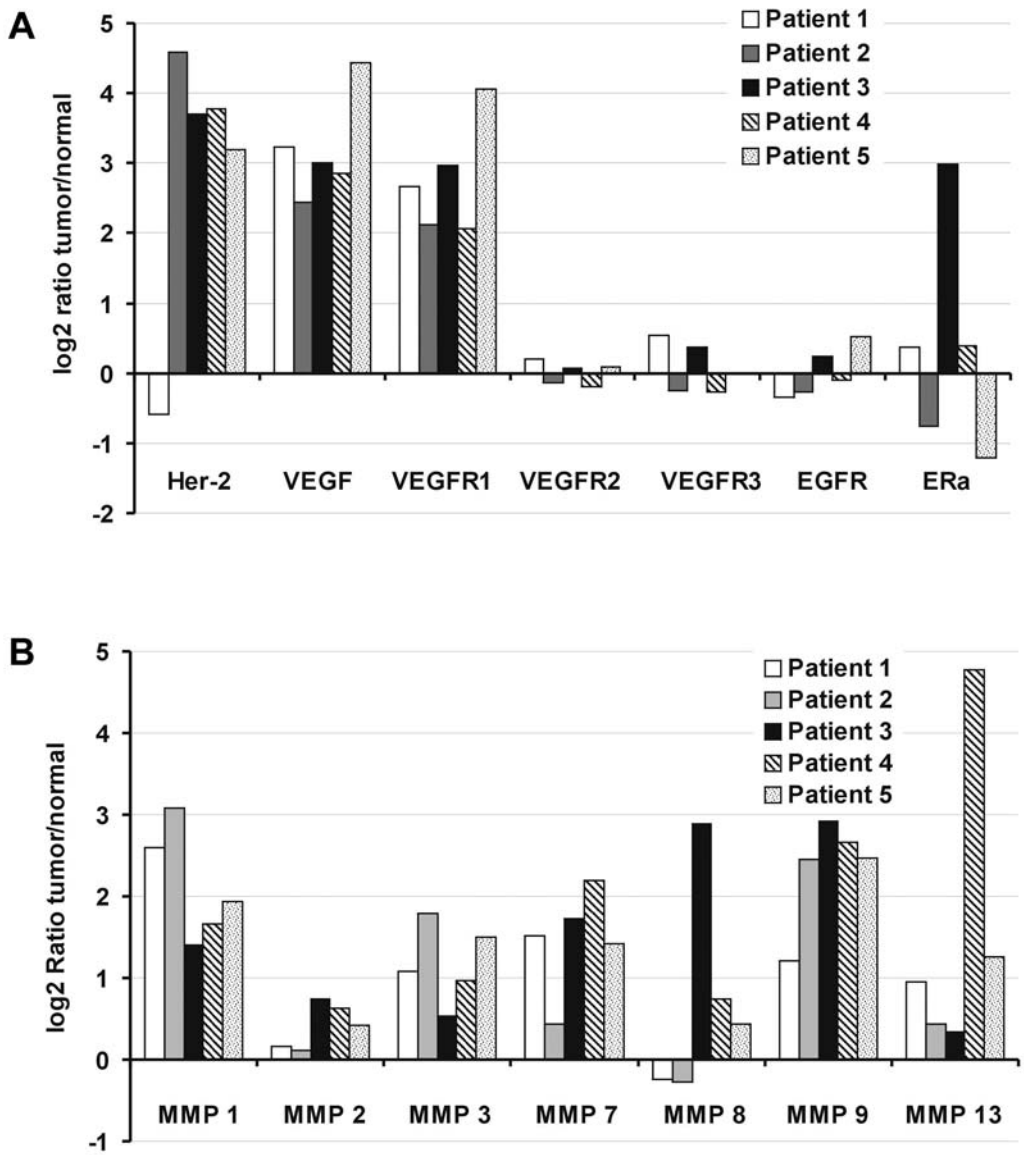

Figure I. *Log2 ratios of tumor versus normal values shown in Table I A and B from ERα, VEGF, Her-2, VEGFR1-3, and EGFR measurements are plotted in (A) and MMP 1, 2, 3, 7, 8, 9 and 13 in (B).*

The family of matrix metalloproteases (MMPs) is discussed in the context of tumor invasion and metastasis and is currently made up of 26 related gene products (25). These zinc proteases are shown to play an essential role in breaking down the extracellular matrix (ECM). ECM-degrading enzymes, such as matrix metalloproteases, are frequently up-regulated in tumor cells during progression of the disease. To get a more detailed insight into the MMP expression profile, we have developed a set of sandwich immunoassays for the multiplex detection of 7 different secreted MMPs (Table IB and Figure 1B). All ELISAs detect pro-, mature-, and TIMP complexed forms of the MMPs. In some cases, not the total amount of protease may be of interest. It is more likely that statements on MMP activity will give more useful information. The creation and validation of such multiplex detection systems of MMP activities is more complicated and takes more time than that of simple ELISAs.

All established assays were used to screen a first set of operated breast tumor specimens and corresponding normal breast tissues from the same patients. In brief, 40 to 80 mg frozen (-70˚C) tissues from 5 different patients were pulverized in a Mikro-Dismembrator U (Braun/Satorius, Germany) at full speed for 1 min. Proteins were extracted

with a 10-fold excess of solubilisation buffer: 50 mM Tris pH 7.5/ 400 mM NaCl/ 1 mM $CaCl_2$/ 1 mM $MgCl_2$/ 1% Triton X-100/ Phosphatase-Inhibitor I and II, Sigma, 1% each/ 1x complete protease inhibitor, Roche. After 1-h incubation on ice, cellular debris was removed by centrifugation at 16,000 x g for 5 min. The total amount of solubilised protein was determined by the Bradford assay.

The assays were designed as a 7-plex for the MMPs, a 4-plex including VEGFR 1-3 and EGFR, a 2-plex for the measurement of VEGF and Her-2, and a singleplex for ERα determination. Five μg (for MMP multiplex) or 10 μg (all others) of total solubilised protein were diluted in 30 μl assay buffer and mixed with 30 μl bead suspension mix in a 96-well filter plate. After incubation overnight at 4˚C in a plate shaker, unbound proteins were washed away and beads were resuspended in 30 μl of a mixture of biotinylated detection antibodies. After 2-h incubation at room temperature, excessive antibodies were removed. Bound biotinylated antibodies were detected by R-phycoerythrin labelled streptavidin (S-PE). Excessive S-PE was removed and beads were resuspended in 100 μl assay buffer. The beads were then analysed in a Luminex100 IS and at least 100 beads were counted. The resulting median fluorescence intensities (MFI) are presented in Table 1 and Figure 1.

The up-regulation of several investigated proteins was observed and individual characteristics of the different patients revealed. In addition, the measured expression of ERα and Her-2 correlated well (100%) with the IHC findings of the clinical routine laboratory.

Following the protocol described above, the amount of extracted protein from tumor tissues ranged between 17 and 48 μg protein per mg tissue and from normal tissue between 2 and 16 μg protein/mg tissue. This means, 40 mg tumor tissue gives rise to approximately 700 μg total solubilised protein of which 5 μg or 10 μg are needed per well.

Discussion

As an initial step in the development of a multi-parametric assay to determine the amounts of informative proteins in tumor-derived samples, we have established a set of sandwich immunoassays for the simultaneous detection of relevant marker proteins using the Luminex xMAP system. The performance of our set-up was tested with clinical samples. In a first screening round, 5 breast tumor specimens and the corresponding normal tissue from the same patient were screened for expression of 14 different parameters. The different protein expression in the tumor and normal tissues is shown in Figure 1. The relative amount of 14 (see Table I and Figure 1) proteins can be determined from a minute amount of tissue lysate. Furthermore, our measurements of the expressions of ERα and Her-2 were in perfect accordance (100%) with conventional IHC.

The number of proteins to be analysed can easily be increased by adding additional sandwich immunoassays to the current set-up. New sandwich immunoassays are set up on a different bead type, screened for cross-reactivity with the other sandwich immunoassays, before they are combined in the whole set-up. If cross-reactivity is observed, the bead-based technology allows at least the performance of this assay in an additional well separated from the other sandwich immunoassays. Due to miniaturisation of the approach, only 5 to 10 μg of solubilised proteins are used in a single assay. Therefore, this approach is perfectly suited to analyse relevant proteins in small samples like fine-needle biopsies, which are important in the initial diagnosis of breast cancer.

These miniaturised and parallelised assay systems have reached a sufficient level of sensitivity and, hence, have the potential to replace singleplex analysis systems. Several companies already offer a steadily growing list of ready-to-use multiplexed sandwich immunoassays to quantify *e.g.* cytokines, metabolic markers, cell signaling molecules and their phosphorylation, or do isotyping of antibodies (26).

The miniaturised and parallel detection of dozens of proteins from a minute amount of sample is one step in the direction of an individualised therapy. However, as long as only a few parameters have to be analysed from the same sample, the emerging multiplexing technologies are to date no competitive solution for routine diagnostics, especially with respect to costs involved. New instruments in combination with new assay formats always involve huge investments. It will be much more economical to increase the throughput of the currently available highly robust and automated diagnostic analysers. Therefore, new diagnostic parameters have to be identified whose expression is of therapeutic or prognostic relevance. A number of potentially informative proteins are currently being validated and for their combined diagnostic assessment the multiplexing approach is certainly a very interesting alternative.

Acknowledgements

This work is supported by the German Ministry of Education and Science (BMBF), PepArt, FKZ0312879A. This work was presented at the 7th International Conference of Anticancer Research, 25-30 October 2004, Corfu, Greece.

References

1 Ekins RP: Multi-analyte immunoassay. J Pharm Biomed Anal *7(2)*: 155-68, 1989.
2 Chipping Forecast II. Nat Genet *32 Suppl*: 461-552, 2002.
3 Gygi SP, Rochon Y, Franza BR and Aebersold R: Correlation between protein and mRNA abundance in yeast. Mol Cell Biol *19*: 1720-1730, 1999.

4 MacBeath G: Protein microarrays and proteomics. Nat Genet *32 Suppl*: 526-532, 2002.
5 Templin MF, Stoll D, Schwenk JM, Potz O, Kramer S and Joos TO: Protein microarrays: promising tools for proteomic research. Proteomics *3*: 2155-2166, 2003.
6 Haab BB, Dunham MJ and Brown PO: Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. Genome Biol in press.
7 Knezevic V, Leethanakul C, Bichsel VE, Worth JM, Prabhu VV, Gutkind JS, Liotta LA, Munson PJ, Petricoin EF 3rd and Krizman DB: Proteomic profiling of the cancer microenvironment by antibody arrays. Proteomics *1*: 1271-1278, 2001.
8 Sreekumar A, Nyati MK, Varambally S, Barrette TR, Ghosh D, Lawrence TS and Chinnaiyan AM: Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated proteins. Cancer Res *61*: 7585-7593, 2001.
9 Sreekumar A and Chinnaiyan AM: Using protein microarrays to study cancer. Biotechniques *Suppl*: 46-53, 2002.
10 Miller JC, Zhou H, Kwekel J, Cavallo R, Burke J, Butler EB, Teh BS and Haab BB: Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. Proteomics *3*: 56-63, 2003.
11 Miller JC, Butler EB, Teh BS and Haab BB: The application of protein microarrays to serum diagnostics: prostate cancer as a test case. Dis Markers *17*: 225-234, 2001.
12 Dasso J, Lee J, Bach H and Mage RG: A comparison of ELISA and flow microsphere-based assays for quantification of immunoglobulins. J Immunol Methods *263*: 23-33, 2002.
13 Carson RT and Vignali DA: Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. J Immunol Methods *227*: 41-52, 1999.
14 Dunbar SA, Vander Zee CA, Oliver KG, Karem KL and Jacobson JW: Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. J Microbiol Methods *53*: 245-252, 2003.
15 Joos TO, Stoll D and Templin MF: Miniaturised multiplexed immunoassays. Curr Opin Chem Biol *6*: 76-80, 2002.
16 Prabhakar U, Eirikis E and Davis HM: Simultaneous quantification of proinflammatory cytokines in human plasma using the LabMAP assay. J Immunol Methods *260*: 207-218, 2002.
17 De Jager W, Te Velthuis H, Prakken BJ, Kuis W and Rijkers GT: Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol *10*: 133-139, 2003.
18 Chen R, Lowe L, Wilson JD, Crowther E, Tzeggai K, Bishop JE and Varro R: Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. Clin Chem *45*: 1693-1694: 1999.
19 Fulton RJ, McDade RL, Smith PL, Kienker LJ and Kettman JR Jr: Advanced multiplexed analysis with the FlowMetrix system. Clin Chem *43*: 1749-1756, 1997.
20 Bellisario R, Colinas RJ and Pass KA: Simultaneous measurement of antibodies to three HIV-1 antigens in newborn dried blood-spot specimens using a multiplexed microsphere-based immunoassay. Early Hum Dev *64*: 21-25, 2001.
21 Abd El-Rehim DM, Pinder SE, Paish CE, Bell JA, Rampaul RS, Blamey RW, Robertson JF, Nicholson RI and Ellis IO: Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma. Br J Cancer *91*: 1532-1542, 2004.
22 Risau W: Mechanisms of angiogenesis. Nature *386*: 671-674, 1997.
23 Folkman J: What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst *82*: 4-6, 1990.
24 Hanahan D and Weinberg RA: The hallmarks of cancer. Cell *100*: 57-70, 2000.
25 Parks WC, Wilson CL and Lopez-Boado YS: Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol *4*: 617-629, 2004.
26 Templin MF, Stoll D, Bachmann J and Joos TO: Protein microarrays and multiplexed sandwich immunoassays: what beats the beads? Comb Chem High Throughput Screen *7*: 223-229, 2004.

Received November 30, 2004
Accepted December 20, 2004