

## Translational Crossroads for Biomarkers

Robert C. Bast, Jr.,<sup>1</sup> Hans Lilja,<sup>2</sup> Nicole Urban,<sup>3</sup> David L. Rimm,<sup>4</sup> Herbert Fritsche,<sup>1</sup> Joe Gray,<sup>5</sup> Robert Veltri,<sup>7</sup> George Klee,<sup>10</sup> Andrew Allen,<sup>11</sup> Nam Kim,<sup>6</sup> Steven Gutman,<sup>8</sup> Mark A. Rubin,<sup>12</sup> and Andrew Hruszkewycz<sup>9</sup>

**Abstract** A group of investigators met at a Specialized Programs of Research Excellence Workshop to discuss key issues in the translation of biomarker discovery to the development of useful laboratory tests for cancer care. Development and approval of several new markers and technologies have provided informative examples that include more specific markers for prostate cancer, more sensitive tests for ovarian cancer, more objective analysis of tissue architecture and an earlier indication of response to treatment in breast cancer. Although there is no clear paradigm for biomarker development, several principles are clear. Marker development should be driven by clinical needs, including early cancer detection, accurate pretreatment staging, and prediction of response to treatment, as well as monitoring disease progression and response to therapy. Development of a national repository that uses carefully preserved, well-annotated tissue specimens will facilitate new marker development. Reference standards will be an essential component of this process. Both hospital-based and commercial laboratories can play a role in developing biomarkers from discovery to test validation. Partnering of academe and industry should occur throughout the process of biomarker development. The National Cancer Institute is in a unique position to bring together academe, industry, and the Food and Drug Administration to (a) define clinical needs for biomarkers by tumor type, (b) establish analytic and clinical paradigms for biomarker development, (c) discuss ways in which markers from different companies might be evaluated in combination, (d) establish computational methods to combine data from multiple biomarkers, (e) share information regarding promising markers developed in National Cancer Institute–supported programs, and (f) exchange data regarding new platforms and techniques that can accelerate marker development.

At the 12th Annual Specialized Programs of Research Excellence (SPORE) Investigators' Workshop, in July 2004, a special session, titled "Translational crossroads for biomarkers," addressed several key issues in the translation of biomarker discovery to the development of useful and robust laboratory tests. Robert Bast, who moderated the session, indicated that new biomarkers are being developed to identify individuals at risk for cancer, to

detect disease earlier, to determine prognosis, to detect recurrence, to predict response to particular agents and to monitor response to treatment. Discovery, testing and validation of clinically appropriate and commercially useful tumor markers should permit individualization of therapy. In February 2004, a meeting was held to review and to prioritize 162 candidate markers identified by SPORE investigators. Twelve of these markers were considered to have promise for clinical use and to merit high priority for advanced development. However, several gaps in commercial marker development were identified.

To address existing gaps in the paradigm of biomarker translation from the research laboratory to potential commercial utility, four speakers shared their experience with the development of new technologies. They discussed circulating molecular and cellular markers, quantitative image analysis of tissue sections, and the simultaneous evaluation of multiple markers to assess disease status. A panel of biomarker experts with representatives from academia, industry, and the Food and Drug Administration (FDA) then addressed a number of related questions.

**Authors' Affiliations:** <sup>1</sup>University of Texas M.D. Anderson Cancer Center, Houston, Texas; <sup>2</sup>Memorial Sloan Kettering Cancer Center, New York, New York; <sup>3</sup>Fred Hutchinson Cancer Center, Seattle, Washington; <sup>4</sup>Yale University School of Medicine, New Haven, Connecticut; <sup>5</sup>Berkely-Livermore National Laboratory, Livermore; <sup>6</sup>diaDexus, South San Francisco, California; <sup>7</sup>Johns Hopkins University Hospital, Baltimore; <sup>8</sup>United States Food and Drug Administration, Rockville; <sup>9</sup>National Cancer Institute, Bethesda, Maryland; <sup>10</sup>Mayo Clinic, Rochester, Minnesota; <sup>11</sup>Abbott Laboratories, Abbott Park, Illinois; <sup>12</sup>Dana-Farber Cancer Institute, Boston, Massachusetts

Received 10/29/04; revised 5/2/05; accepted 6/1/05.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Dr. Rimm is the scientist founder of HistoRx, the exclusive licensee of the AQUA™ technology developed in his lab at Yale. He is a stockholder and consultant to HistoRx.

**Requests for reprints:** Robert C. Bast, Jr., Box 355, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-7743; Fax: 713-792-7864; E-mail: rbast@mdanderson.org.

© 2005 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-04-2213

### Prostate-Specific Antigen as a Model for Marker Evolution and Development

Hans Lilja of Memorial Sloan Kettering Cancer Center reviewed progress in prostate cancer markers as an example of

the potential value, as well as the complexity of biomarker development. Prostate-specific antigen (PSA) has had a major impact on detection of prostate cancer. PSA is sufficiently sensitive to detect disease at an early stage, but can be elevated both by benign and malignant prostatic disease. Recent developments have focused on improving PSA specificity to avoid unnecessary biopsies. PSA is a protease, 1 of 15 human kallikreins, and antiproteases regulate extracellular exposure to the functional enzyme. Active PSA forms complexes with antiproteases such as  $\alpha$ -1-anti-chymotrypsin and  $\alpha$ -2-macroglobulin. The native conformation of the PSA- $\alpha$ -2-macroglobulin complex shields access to most PSA epitopes. Several independently accessible epitopes are available to detect the PSA- $\alpha$ -1-anti-chymotrypsin complex and some uniquely accessible epitopes on free PSA permit selective detection of this PSA fraction (1). Thus, free PSA and total PSA can be measured with appropriate immunoassays. When receiver-operating characteristic curves are compared, the percentage of free PSA yields ~20% greater specificity than total PSA at 95% sensitivity and can be used to distinguish invasive prostate cancer from benign disease in the typical diagnostic gray zone where total PSA levels are  $\leq 10$  ng/mL (2). Thus, 20% of men without cancer might be spared biopsy, if the free PSA assay were measured consistently.

Release of other kallikreins might complement the ability of PSA to detect invasive features of prostate cancer (3, 4). Recent studies of human glandular kallikrein 2 (hK2) use an innovative assay design that provides a coefficient of variation of <20% at hK2 levels <5 pg/mL and that exhibits minimal cross-reaction with PSA, despite the 80% sequence identity to hK2. Levels of hK2 are independent predictors of extra-prostatic growth. Pretreatment levels of free hK2, free PSA, and total PSA, combined with Gleason grade and clinical stage, improve prediction models that identify men at increased risk for biochemical recurrence of prostate cancer.

### Multiple Markers for Assessment of Disease Status in Ovarian Cancer

Use of multiple markers is also being explored in sera from patients with ovarian cancer. Nicole Urban of the Fred Hutchinson Cancer Center reviewed the simultaneous use of multiple biomarker tests to improve assessment of disease status. Dr. Urban's group has developed panels of markers for early detection and for prognostication of ovarian cancer. For early detection, they discovered genes on cDNA expression arrays that were up-regulated in ovarian cancers, but not in normal ovarian cells. Overexpression was confirmed by reverse transcription-PCR.

Two genes, *WFDC2* (HE4) and *Mesothelin*, were strongly expressed in ovarian carcinomas but not in normal tissues. The *WFDC2* gene was initially identified in epithelial cells of human epididymis and was referred to as an epididymis-specific fertility-related protein, HE4 (*Homo sapiens* epididymis-specific). Although the function of the HE4 protein is unknown, it is a member of a family of stable 4-disulfide core proteins that are secreted at high levels. An ELISA developed from mouse monoclonal antibodies reactive with HE4 has similar sensitivity to the standard CA125 assay, but shows improved specificity in distinguishing malignant from benign ovarian disease (5). A similar ELISA for soluble mesothelin-

related proteins complements CA125 in that a combination of the two markers produces an improved receiver-operating characteristic curve relative to either marker alone (6). Statistical methods have been developed to combine markers at one point in time. The resulting composite marker can improve sensitivity without losing specificity (7, 8).

To aid in selecting the best combination of biomarkers, multiplex testing with high-throughput technology is being developed that conserves valuable serum specimens and could ultimately lead to more convenient assays of multiple markers. The implementation of multiple marker testing is, however, legally complex when markers are developed by different companies. New statistical paradigms must also be employed to facilitate multiple marker analysis and improve clinical performance compared with the evaluation of individual markers.

### Quantitative Pathology for Objective Analysis of Tissue Specimens

David Rimm of the Yale University Medical School discussed applications of a commercially available quantitative pathology to analyze cancer tissue. Dating back to the 1960s, architecture of tissue specimens has been quantified with different systems. A new molecular microassay considers features based on molecular interactions rather than only tissue morphology (9). The system described by Dr. Rimm (called AQUA) uses fluorescence probes rather than the brown stain of conventional immunoperoxidase immunohistochemistry. These techniques extend the principles of flow cytometry to tissue sections where spatial and architectural information is retained. Automated quantitative imaging software uses two algorithms to define a histologic region of interest, to define tissue and cellular compartmentalization, and to define signal localization. This pathologist-free image analysis depends upon quantization of expression within molecular compartments rather than on morphologic features or subjective criteria. Binary probe gating of high-molecular weight cytokeratin staining of epithelium separates tumor from stroma. Cellular compartment-specific fluorescent probes distinguish nuclei, cytoplasm, and membranes. A rapid exponential subtraction algorithm abstracts information from markers, optimizing an image. Using these techniques, subgroups of cancers can be defined that affect analysis of the prognostic significance of HER-2 (10),  $\beta$ -catenin expression, and a number of other markers. Ratios between markers can be calculated with great precision. Diagnosis of prostate cancer has also been achieved with this quantitative approach to morphology (11). The increase in commercially available clinical laboratory-based tissue reading machines and software is increasing opportunities for quantitative tissue diagnostics.

Image-based quantitative pathology can offer a dramatic improvement over the subjective method that represents the current standard. However, a series of critical issues must be addressed with any antibody-based assay. Table 1 illustrates these problems and their solutions, which are especially important in the context of the added rigor of quantitative pathology (12, 13). Many of these variables can be addressed through use of rigorous validation and quality control procedures.

**Table 1. Problems with immunohistochemical analysis and possible solutions**

Immunohistochemistry Problem	Solution
Inadequate antibody validation	Test antibodies by Western blot, immunoprecipitation, transfected cell lines, or other methods to confirm antibody specificity
Lot to lot antibody variability	Use monoclonal antibodies that have consistent reactivity, but still require validation as above
Variable fixation, including under- or over-fixation	Use antibodies against normal components (like keratin or vimentin) to assess tissue quality
Variable oxidation of sections after microtomy	Stain immediately after sectioning or store slides dipped in paraffin under nitrogen
Variable methods for antigen retrieval and user-specific slide to slide variability in staining	Use 2- to 3-fold redundant tissue microarrays with a series of 10 to 12 cell line controls on each slide for normalization between slides

### Circulating Breast Cancer Cells to Assess Prognosis and Treatment Response

Herbert Fritsche of University of Texas M.D. Anderson Cancer Center discussed the introduction of an assay for circulating tumor cells (CTC) in breast cancer patients, which isolates CTC and then identifies them using a fluorescent image-based technology that is proprietary and commercially available. For the assay, tumor cells are isolated and identified with antibodies against epithelial cell adhesion molecule, cytokeratins, and CD45. In a recent report (14), breast cancer patients with more than five CTCs prior to the administration of chemotherapy for metastatic disease exhibited a significantly shortened progression-free and overall survival compared with patients with less than five CTCs. A decrease in CTC levels over 4 to 8 weeks on chemotherapy predicted progression-free survival.

Dr. Fritsche outlined the role of the hospital clinical laboratory in converting a research assay into a standardized, reproducible, and cost-effective test that provides consistent and accurate results on a day-to-day basis. In order to develop a biomarker such as CTC for routine clinical use, quality controls need to be established and the reproducibility of a test must be determined. Interpretive criteria need to be developed for objective assessment of tumor cell identity and the proficiency of each technologist performing the assay needs to be assessed. Preanalytic factors, such as specimen collection and processing, need to be standardized and physiologic variation within patients needs to be measured. Also, an external proficiency program must be established to improve lab-to-lab concordance and achieve compliance with Clinical Laboratory Improvement Act guidelines. Once the test has been confirmed as a clinical assay, it should be certified by appropriate laboratory testing agencies. Not until such authorization is obtained can the test be considered as a reimbursable clinical procedure with defined clinical utility.

### Panel Discussion

The speakers joined a panel that further explored issues raised in each of the previous presentations. They also discussed the general question of how best to bring together the efforts of National Cancer Institute (NCI)-sponsored investigators with industry and the FDA.

*Is there a standard paradigm for biomarker development?* Despite much thought regarding the phases of biomarker development (15), at present, there is a lack of a consensus on a paradigm for development of newly discovered markers. Following discovery, clear criteria must be established for the development of a new marker. Each assay must be optimized and then validated in retrospective and prospective clinical trials, while adhering to good manufacturing and laboratory practice.

*Why are so few markers worthy of regulatory approval?* Given the large number of candidate markers, it is remarkable that only a limited number have been approved by the FDA. Panelists suggested that this reflects the lengthy process of assay development and validation, lack of reproducible data supporting clinical application, as well as limited support by industry for these efforts, particularly with regard to the performance of prospective clinical trials. In addition, many markers that correlate with disease statistically may not prove to be useful clinically (16).

*What change in FDA regulations could accelerate effective marker development?* Panelists felt that the FDA's approach to approving assays had evolved and that the agency was not slowing the process of marker development unduly. Information on current FDA review processes can be found at <http://www.fda.gov/cdrh/oivd/>. There are, however, ways in which the FDA could further accelerate biomarker development.

- Establishment of specialized groups to handle: (a) molecular-based diagnostic technology; (b) multiplex diagnostic testing that includes algorithms; and (c) quantitative pathology.
- Clarification and increased use of the new category of diagnostic products that have "orphan" status to encourage development of oncology biomarkers with small market sizes.

*What are the barriers to marker application?* One potential barrier to biomarker development and application is the availability of clinical specimens that permit rapid optimization and validation of new assays. The National Biospecimen Network is a large-scale effort to develop a systematic nationwide collection of human tissue samples to accelerate cancer research with a sophisticated carefully annotated database. Additional information regarding the National Biospecimen Network Blueprint can be found at: [http://www.ndoc.org/about\\_ndc/reports/NBN\\_comment.asp](http://www.ndoc.org/about_ndc/reports/NBN_comment.asp) or [http://www.ndoc.org/about\\_ndc/reports/pdfs/FINAL\\_NBN\\_Blueprint.pdf](http://www.ndoc.org/about_ndc/reports/pdfs/FINAL_NBN_Blueprint.pdf). Critical issues being confronted in developing the National

Biospecimen Network relate to the accessibility of well-annotated specimens, compliance with Health Insurance Portability and Accountability Act regulations, and protection of intellectual property associated with the research.

Once assays for new biomarkers are developed, neither industry nor regulatory agencies may be prepared to evaluate, approve, and then market the tests. Development and validation of multiple biomarkers can be even more difficult when more than one company has rights to the markers in a panel and where issues of intellectual property and licensing must be resolved. Several additional barriers to biomarker commercialization were identified (Table 2). As a result of these barriers to commercialization, there continues to be limited support for biomarker development in academic institutions.

**How can clinical laboratories facilitate biomarker validation?** The academic clinical laboratory plays a key role in moving a biomarker from initial discovery into clinical practice. First, such a laboratory has the expertise to validate analytic methods, ensuring the accuracy and precision of biomarker tests consistent with Clinical Laboratory Improvement Act requirements. Second, the academic clinical laboratory offers a standardized infrastructure for the collection of blood, fluid, and tissue samples, performing the assays under the strictest of laboratory regulations, including the development and implementation of rigid quality control procedures with appropriate documentation. Third, the academic clinical laboratory offers a professional staff for developing laboratory and clinical studies to validate the clinical claims of a new biomarker test. This includes assessment of biomarker stability, within-individual variation of the biomarker, assessment of factors that interfere with the assay, expected values, and development of interpretive criteria. When the biomarker has been clinically validated, the clinical laboratory can make the test available for patient care on a fee-for-service basis. Reimbursement of a new laboratory test is a reflection of its acceptance and helps to set the community standard for the clinical use of a new biomarker.

**What is the role of standard reference materials and reference methods in biomarker assay development?** The development of analytic and clinical performance criteria for new assays is critically dependent on traceable reference standards. Without uniform standards, clinical assay test results will vary considerably between methods and within methods over time. A lack of standardization can impede translation of results from clinical validation trials to patient care in the community.

The panelists proposed that the NCI facilitate the development of reference standards for all new biomarkers developed for clinical use by the SPOREs and other NCI-sponsored programs. Establishing standards could potentially be done in collaboration with industry and with other government agencies, such as the National Institute of Standards and Technology.

**When is a marker ready for translation? At what point in marker development should industry be involved?** Translation is a multistep process. In general, decisions to continue biomarker development are largely based on a marker's potential to contribute cost-effectively to management of disease. Prediction of a biomarker's potential is usually based, in turn, on data derived from statistically significant studies. A biomarker is ready for prospective testing in the clinic when retrospective studies at more than one institution consistently confirm the

**Table 2. Barriers to the application of biomarkers**

Status of intellectual property protection
Availability of standard reference materials for the assay
Complexity of assay format and determination of reproducibility and accuracy
Implementation of quality control to assure reproducibility and accuracy
Sufficient market testing size to assess methods of commercialization
Lack of clear guidelines for good manufacturing/laboratory practice and quality control requirements for all phases of biomarker development
Cost and effort required to accumulate clinical data under appropriately designed, Institutional Review Board – approved prospective trials
The interval required for resolution of patent issues, assay standardization, validation, testing, and regulatory approval

ability of the biomarker to perform at the requisite levels of sensitivity and specificity to aid in patient care.

In the case of early detection, retrospective analysis of stored serum samples is essential to justify the expense of a prospective trial. Industry should be involved in the process of marker development as early as possible, but certainly during assay optimization with Good Manufacturing Practices level reagents and during multi-institutional confirmatory studies. A reasonable point for industry to enter the biomarker validation process is after favorable results are obtained at the completion of retrospective clinical studies. At this stage, industry can assist in clarifying clinical application of the potential test, help design key clinical trials, formulate an FDA approval strategy, and assist in developing a robust and reproducible assay with an appropriate level of quality control. Industry also has extensive experience in assay development and kit manufacture. In addition, they potentially have the resources to support clinical trials.

**What kinds of companies are involved in marker development?** Companies fall into several categories. One group of companies includes large entities that tend to have diagnostic and therapeutic divisions with separate management teams. They often possess diverse instrument platforms that permit both immunologic and molecular testing. Such companies have experience in research and development, product development, regulatory compliance, marketing, and sales of numerous diagnostic products, as well as a commitment to serve a significant segment of the healthcare market. Some large companies develop diagnostic markers to track the activity of their therapeutic agents. Large diagnostic companies tend to seek large market opportunities with low risk through their licensing and business development departments.

A second group includes mid-sized biotechnology companies that have reached profitability based on one or more products that they have discovered, developed, and have had approved by the FDA. Such companies are usually publicly held, have a cash reserve, and tend to be focused on diagnostics that have potential for a moderate to large market. They will also tend to license-in existing approved products to compete in large markets. Such companies will have experienced management in

all areas of business development, R&D, regulatory compliance, marketing, and sales.

A third group of companies are start-ups that have not yet gone public or have just done so, but have new and innovative assay delivery platforms, and/or a disease-focused strategy. Often, such companies are run by entrepreneurial scientists with or without the collaboration of an executive experienced in the growth and development of start-ups. Many such companies in this category have limited funding, but tend to license early stage, high-risk markers to break out of their start-up mode. They communicate quite well with academic scientists who are at the cutting edge of discovery. Where the first two groups companies tend to pass over high-risk technologies with incomplete intellectual property protection or a requirement for additional clinical trials, the third group companies can support such opportunities with funding for research in the discoverer's laboratory with a promise for payment to the sponsoring institution if and when the technology reaches the marketplace. A goal of many third-tier companies is to be bought out by a larger company. They may, however, aid in bringing markers to the market through clinical trials.

**What are the strengths of industry for marker development?**

When engaged, industry can bring many strengths and resources to marker development. These are enumerated in Table 3.

**What are the strengths of SPOREs?** SPOREs are ideally positioned to recognize clinical needs and to discover new markers. Consortia, such as the SPOREs, can also provide an excellent mechanism for clinical evaluation, as they include multidisciplinary teams with leading experts in the development of new laboratory correlates for clinical care. SPOREs have several strengths that can contribute to biomarker development (Table 4).

**What can we do to accelerate marker development among the SPOREs?** The panel suggested that a new paradigm was needed for marker development. Closer collaboration of translational clinical research teams with the academic

**Table 4. Strengths of SPOREs for biomarker development**

Broad coverage of cancer disease sites with a commitment to translational research for diagnostics, chemoprevention, and therapeutics
Cutting-edge discovery of novel targets and biomarkers
Opportunity to discover clinically useful biomarkers in cancers of relatively low prevalence with high mortality rates
Ability to evaluate a new tumor biomarker across multiple tumor types, if appropriate
Banks for well-annotated clinical specimens to validate newly discovered biomarkers
Ability to conduct clinical trials through the network of academic institutions that treat patients with different types of cancer
Expertise in academic pathology, clinical trials, and use of the hospital clinical laboratory to evaluate new clinical assays and technology

hospital clinical laboratory and with industry is desirable to define clinical needs. The NCI could sponsor joint panels to identify clinical problems and opportunities in cancers at different organ sites where early detection might impact on clinical outcomes, where predictive tests could aid clinicians and their patients in choosing among active drugs and different modalities, and where prognostic tests could permit patients to avoid toxic therapy. Early discussion of strategy with the FDA would also be important to determine how best to show clinical validity and utility of a marker. At present, there is no NCI-funded mechanism to support the development of biomarkers comparable to the Rapid Access to Interventional Development (RAID) program for the development of drugs. Similarly, there is no ongoing mechanism to identify and to prioritize biomarkers for further development. Consequently, the panelists proposed that a working group be formed to advise the NCI on how best to develop a pathway for the clinical application of promising biomarkers. A "biomarker development primer" might be constructed to provide critical information regarding assay development, analytic, and clinical validation, and the protection of intellectual property. A web site could be established to share information on new biomarkers among SPORE investigators. In addition, this working group might organize a forum that would bring together representatives of industry, National Institute of Standards and Technology, NCI (including biomarker development experts of the SPOREs, Early Detection Research Group (EDRN), and others) and the FDA to (a) define clinical oncology patient needs, (b) recommend an analytic paradigm for the translation of biomarker assays toward clinical validation and use in clinical trials and clinical care, (c) discuss ways biomarkers from different companies might be used in combination, (d) share information regarding promising biomarkers developed in NCI programs, and (e) become more familiar with platforms and techniques being developed in the private sector. The establishment of a working group that would include biomarker experts from the NIH, FDA, and industry could bring new solutions to cancer marker development and accelerate implementation for cancer care.

**Table 3. Strengths of industry for biomarker development**

Experienced business and technical management teams
An international marketing scope for medium-sized and large companies
Experience in development, optimization, manufacture, and regulatory approval of critical raw materials (e.g., antibodies, recombinant proteins, etc.) and assays
Experience in manufacturing reproducible assays and kits that can be used in key clinical studies, and as commercial products
Experience with all levels of diagnostic products including validation of final assay format, formulation of clinical applications, and commercialization plans, development and execution of FDA-approved strategy, instrumentation, and FDA-approved reagents and kits using Good Manufacturing Practices
Demonstrated in-licensing success with key diagnostic products, not only for oncology, but also for cardiovascular disease, diabetes and other areas
Experience in the area of intellectual property and licensing

## References

1. Pettersson K, Piironen T, Seppälä M, et al. Free and complexed prostate-specific antigen (PSA): *In vitro* stability, epitope map, and development of immunofluorometric assays for specific and sensitive detection of free PSA and PSA- $\alpha$ 1-anti-chymotrypsin complex. *Clin Chem* 1995;41:1480–8.
2. Aus G, Becker C, Franzen S, Lilja H, Lodding P, Hugosson J. Cumulative prostate cancer risk assessment with the aid of the free-to-total prostate specific antigen ratio. *Eur Urol* 2004;45:160–5.
3. Piironen T, Villoutreix BO, Becker C, et al. Determination and analysis of antigenic epitopes of prostate-specific antigen (PSA) and human glandular kallikrein 2 (hK2) using synthetic peptides and computer modeling. *Protein Sci* 1998;7:259–69.
4. Haese A, Graefen M, Becker C, et al. The role of human glandular kallikrein 2 (hK2) for prediction of pathologically organ-confined prostate cancer. *Prostate* 2003;54:181–6.
5. Hellstrom I, Raycraft J, Hayden-Ledbetter M, et al. The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma. *Cancer Res* 2003;63:3695–700.
6. McIntosh MW, Drescher C, Urban N, Scholler N, Hellstrom I, Hellstrom K. Combining CA125 and SMR serum markers for diagnosis and early detection of ovarian carcinoma. *Gynecol Oncol* 2004 Oct;95:9–15.
7. McIntosh MW, Pepe MS. Combining several screening test; optimality of the risk score. *Biometrics* 2002 Sep;58:657–64.
8. Etzioni R, Kooperberg C, Pepe M, Smith R, Gann PH. Combining biomarkers to detect disease with application to prostate cancer. *Biostatistics* 2003 Oct;4:523–38.
9. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med* 2002;8:1323–7.
10. Camp RL, Dolled-Filhart M, King BL, Rimm DL. Quantitative analysis of breast cancer tissue microarrays shows that both high and normal levels of HER2 expression are associated with poor outcome. *Cancer Res* 2003;63:1445–8.
11. Rubin MA, Zerkowski MP, Camp RL, et al. Quantitative determination of expression of the prostate cancer protein  $\alpha$ -methylacyl-CoA racemase using automated quantitative analysis (AQUA): a novel paradigm for automated and continuous biomarker measurements. *Am J Pathol* 2004;164:831–40.
12. DiVito KA, Charette LA, Rimm DL, Camp RL. Long-term preservation of antigenicity on tissue microarrays. *Lab Invest* 2004;84:1071–8.
13. Shi S-R, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J Histochem Cytochem* 2001;49:931–8.
14. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004;351:781–91.
15. Pepe MS, Etzioni R, Feng Z, et al. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst* 2001;93:1054–61.
16. Katton MW. Judging new markers by their ability to improve predictive accuracy. *J Natl Cancer Inst* 2003;95:634–5.