Laboratory Medicine Practice Guidelines

Use of Tumor Markers in Clinical Practice: Quality Requirements

Edited by Catharine M. Sturgeon and Eleftherios Diamandis

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Presents

LABORATORY MEDICINE PRACTICE GUIDELINES

USE OF TUMOR MARKERS IN CLINICAL PRACTICE: QUALITY REQUIREMENTS

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Introduction

The National Academy of Clinical Biochemistry (NACB) Laboratory Medicine Practice Guidelines (LPMG) for Use of Tumor Markers in Clinical Practice: Quality Requirements are intended to encourage more appropriate use of tumor marker tests by primary care physicians, hospital physicians and surgeons, specialist oncologists, and other health care professionals. The background and methodology described below represent the larger undertaking to address tumor markers in clinical practice of which this set of guidelines is a part.

Background to the NACB Tumor Marker Guidelines

Herein we report the updating and extension of practice guidelines first proposed in 2002 (1). Undertaken under the direction of a steering committee appointed by the NACB, the process involved consideration of 16 specific cancer sites and quality requirements for well-established tumor markers and as well as those being developed using new technologies. The draft guidelines were posted on the NACB Website in July 2005 and were presented as an EduTrak at the 2005 Joint AACC/IFCC Annual meeting in Orlando. Informed comment was also actively sought from individuals, organizations, and other interested parties.

NACB Tumor Marker Guideline Development Group

Nineteen Subcommittees developed draft guidelines. Subcommittee members included individuals with extensive expertise in the science, technology and clinical practice of tumor markers in academia, hospitals, and/or industry. In guidelines in which "expert opinion" is incorporated as part of the recommendations, bias, including conflict of interest, may intrude (2). Members of the in vitro diagnostic industry in the subcommittee membership were deliberately included so as to obtain a representative cross-section of experts and perspectives in the field. This major undertaking has involved significant input from approximately 100 scientists and clinicians from more than ten countries and diverse backgrounds.

Methodological Approach

There is extensive literature on the preparation (3, 4) and evaluation (5) of practice guidelines. Many experts have emphasized the importance of a good "evidence base" in developing such guidelines (3, 6) and the challenges of their effective implementation (6–9). Good methodology during guideline development is highly desirable, although it has recently been noted that good reporting of methodological quality does not necessarily lead to more valid recommendations or vice versa (10).

A recent assessment of nine clinical oncology practice guidelines has demonstrated significant heterogeneity in the development, structure, user and end points of these guidelines, which the authors conclude is not detrimental but rather is necessary, in order to meet divergent demands (11). No available guidelines are likely to be perfect in all situations—all have limitations, some of which the NACB guidelines presented here undoubtedly share. However, characteristics identified as critical to the effectiveness of practice guidelines are a clear definition of purpose and intended audience, adherence to methodological standards, and systematic evaluation (audit) of their clinical impact after their introduction (11).

Here a relatively informal methodological approach was adopted and subcommittee chairs were allowed considerable latitude. While some of the diversity evident in the guidelines presented here undoubtedly reflects the predilection and idiosyncrasy of individual subcommittees, much of it arises from the different numbers of tumor markers described for each specific cancer as well as the variable maturity of clinical validation and currently available evidence for these markers. It is therefore not realistic to expect to achieve consistency of approach across the spectrum of cancers examined.

The subcommittees were, however, asked to follow a recommended structure when developing and formulating the guidelines and to consider each of the major potential clinical applications of tumor markers (screening/early detection, diagnosis, prognosis, treatment monitoring and surveillance) in order to achieve a reasonably homogeneous presentation across cancer types. Subcommittees were also strongly encouraged to undertake as thorough a review of the literature as feasible, with particular attention given to reviews (including systematic reviews), prospective randomized trials that included the use of markers and existing guidelines.

Importantly, each subcommittee was asked to compare its guidelines with those of other groups and to present these comparisons in tabular form, elaborating on any differences and also providing estimates of both the level of evidence (LOE) (7) and the strength or grade of recommendation (SOR) (12) (Table A) ascribable to each NACB recommendation. The LOE and SOR respectively reflect the

strength of published evidence supporting the recommendations made and the degree of consensus within the guideline development group, while the tables relating to individual malignancies provide a convenient summary of the relevant NACB guidelines. Where consensus could not be achieved within a subcommittee, this is explained, describing the conflicting views and reasons for these.

The final result is a set of practice guidelines that follow a reasonably homogeneous style and approach. The strength and type of evidence underlying each recommendation is clearly stated, together with an estimate of the confidence with which each recommendation has been made, so the reader can readily discern which are based on incontrovertible clinical evidence and which are based on the expert consensus of committee members.

Review and Refinement of the NACB Tumor Marker Guidelines

Subcommittee chairs reviewed and responded to suggestions and corrections received after posting of the guidelines on the NACB website. These NACB guidelines will inevitably require updating, refinement, and modification in the future, as knowledge and understanding of tumor markers and their biological roles increases. As suggested in the very helpful AGREE document (5), and reflecting work in progress for a number of tumor markers, when the guidelines are next updated it may be possible to include some estimate of the cost-effectiveness of tumor marker use, to take account of patients' views, and to report on audit studies of their effectiveness. For this purpose it would be desirable to use a consultation form similar to that developed by the Scottish Intercollegiate Guideline Network (SIGN) [see eg, (13)].

Implementation of the NACB Tumor Marker Guidelines

Adoption of these guidelines is voluntary, some recommendations may not be appropriate in all settings (eg, clinical trials) and for effective implementation they may require translation and/or other modification in some settings. There is good evidence that “locally owned” guidelines are much more likely to be successfully adopted in routine clinical practice (4). In addition, carefully designed audit studies would be highly desirable before and after introduction of the guidelines (11).

These recommendations, which, to facilitate their dissemination, are being published in electronic form on the NACB web site, should encourage more optimal use of tumor marker tests by clinical and laboratory staff, thereby better informing medical decisions directed toward improved clinical outcome and/or quality of life for increasing numbers of cancer patients.

Table A. Levels of Evidence and Strengths of Recommendation Used to Grade the NACB Guidelines for Tumor Markers

Assessment	Criteria
Level of Evidence (8)	
I	Evidence from a single, high-powered, prospective, controlled study that is specifically designed to test marker, or evidence from a meta-analysis, pooled analysis or overview of level II or III studies.
II	Evidence from a study in which marker data are determined in relationship to prospective therapeutic trial that is performed to test therapeutic hypothesis but not specifically designed to test marker utility.
III	Evidence from large prospective studies.
IV	Evidence from small retrospective studies.
V	Evidence from small pilot studies.
Expert opinion *Strength of recommendation (12)*	
A – High	Further research is very unlikely to change the Panel's confidence in the estimate of effect.
B – Moderate	Further research is likely to have an important impact on the Panel's confidence in the estimate of effect and is likely to change the estimate.
C – Low	Further research is very likely to have an important effect on the Panel's confidence in the estimate of effect and is likely to change the estimate.
D – Very low	Any estimate of effect is very uncertain.

NOTE: Adapted from Hayes et al (8) and Atkins et al (12).

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Chapter 1

Quality Requirements for the Use of Tumor Markers

Herein, further developing previous recommendations of the NACB and European Group on Tumor Markers (1), we present quality requirements relevant to all tumor marker measurements under the following broad headings

- Pre-analytical requirements: choice of tumor marker, specimen type, specimen timing, sample handling.
- Analytical requirements: assay standardisation, internal and external quality control, interferences.
- Post-analytical requirements: reference intervals, interpretation, and reporting of tumor marker results.

Finally, some of the clinical issues relevant to enhance the clinical utility of tumor marker testing, both now and in the future, are briefly considered.

PRE-ANALYTICAL QUALITY REQUIREMENTS

Reporting of erroneous tumor marker results is more likely to cause undue alarm to patients than is the case for many other laboratory tests. As well as adhering to general pre-analytical recommendations applicable to all diagnostic tests (2) and encouraging appropriate test requesting (1, 3, 4), the laboratory must exercise extra vigilance in ensuring that correct results are reported (5). Errors reportedly occur more often in the pre-analytical than analytical phase [30% to 75% and 13% to 31% respectively as quoted in one review (6) and 10 times as often in a transfusion medicine study (7)]. As for other analytes, the majority of pre-analytical errors for tumor markers will be simple specimen handling errors (eg, inappropriate sampling handling, hemolyzed specimens, insufficient specimens, incorrect specimens and errors at data entry) and their occurrence should be minimized by adherence to good laboratory practice and assessment in an effective audit cycle. As outlined in Tables 1 and 2, there are a number of additional circumstances in which misleading results may be obtained, particularly for PSA and CA125. Implementing the NACB recommendations that are presented in the following articles and are currently available on the web (8)—in particular by discouraging inappropriate test requesting (9, 10), ensuring appropriate specimen timing and requesting confirmatory specimens when required—should reduce the risk of causing patients unwarranted distress and the likelihood of unnecessary clinical investigations.

With the advent of electronic health records (EHRs), every effort should be made to link the tumor marker ordering process with pre-analytical precautions available through Clinical Decision Support (CDS) databases (11) such as that recently made available through the American Association of Clinical Chemistry (12). These databases are in the process of standardization to produce common conventions for the reference knowledge and means of accessing and using it (13).

ANALYTICAL QUALITY REQUIREMENTS

The almost universal use of automated immunoassay analyzers for many commonly requested tests means that responsibility for analytical quality now rests largely with the diagnostic industry, which must meet quality requirements defined by national or international regulatory authorities [eg, US Food and Drug Administration (FDA) regulations, European Commission in Vitro Diagnostics Directive (IVDD)]. It is nevertheless crucial for satisfactory measurement of any analyte that laboratories independently monitor their own performance carefully, both to ensure that analyzers are being used appropriately and to confirm that individual methods are performing according to specification. This is best achieved by implementation of rigorous internal quality control (IQC) and participation in well-designed proficiency testing (PT) [external quality assessment (EQA)] programs (1, 14). It is crucial of course that laboratories participate in such programs and take appropriate action to investigate the cause of unsatisfactory results immediately.

NACB recommendations for both IQC and PT are presented in Table 2. Most of these are common to all analytes, but several have particular relevance to tumor markers. Specimens for both IQC and PT should always resemble clinical sera as closely as feasible. Where clinical decision points are commonly employed, it is important to ensure stable and consistent performance, and inclusion of IQC specimens at concentrations close to such decision points is highly desirable. This is especially critical when screening asymptomatic individuals [eg, for prostate cancer using PSA (15)], or where chemotherapy may be instituted on the basis of a rising tumor marker level in the absence of other scan evidence [eg, when monitoring testicular cancer patients with α-fetoprotein (AFP) or human chorionic gonadotropin (hCG) (16)]. The functional sensitivity [ie, lowest result that can be reliably reported, best defined as the concentration at which the day-to-day coefficient of variation is <20%] is also very important for certain tumor marker applications [eg, when using prostate specific antigen (PSA) to monitor prostate cancer patients after radical prostatectomy or thyroglobulin or calcitonin to monitor

Table 1. NACB Recommendations: Quality Requirements in the Pre-Analytical Phase

Requirements	Recommendations	Comments/Specific Examples	References
Analyte related			
Type of specimen	Requirements should be checked in the product information supplied with the kit. It is the laboratory's responsibility to provide clear advice about the appropriate tube type for each test, thereby ensuring that manufacturers' instructions are always followed.	Serum or plasma are usually (but not always) equally appropriate. Gel tubes may not be suitable for some assays (44). Where feasible using primary tubes for analysis minimizes the risk of identification errors that occur when serum aliquots are prepared. Anti-coagulating agents such as ethylene- diamine tetraacetic acid (EDTA) may interfere in some detection methods.	(16)
	Standardized conditions of specimen collection and fixation are crucial for immunohistochemical analyses.	Immunohistochemical studies with tumor markers that do not define the type of specimen and fixative used definitely prejudice the value of the results.	(45)
Specimen stability	Tumor markers are generally stable, but serum or plasma should be separated from the clot and stored at 4°C (short-term) or –30°C (long-term) as soon as possible. For longer term storage, specimens should be stored at –70°C. Stability to repeated freeze-thawing is likely to be analyte (and possibly sample-) dependent.	The stability of total and free PSA under different storage conditions is especially critical in the context of a screening program (1, 46). Measurements of AFP, CEA, and CA125 have been shown to be more readily affected by long-term frozen storage compared with frequent freeze thawing, while CA19-9 is relatively unstable under both conditions (47).	(1, 46)
	Heat treatment (eg, to deplete serum complement components or to inactivate human immunodeficiency virus [HIV]) should be avoided, particularly for hCG and PSA. At high ambient temperature the potential influence of transit time on analyte results should be considered	HCG may dissociate at elevated temperature to form its free α and β-subunits.	(16)
Patient related			
Test selection	Ordering of tumor marker tests should be according to locally agreed protocols, based on established national and international guidelines.	Previous NACB recommendations for use of tumor markers in routine clinical practice and those of other international groups have previously been reviewed elsewhere (48). Updates to the NACB recommendations, are now available on the AACC website (8), and are being published in the present series of articles. Abbreviated versions of such recommendations, tailored for local practice [eg, as by the Association of Clinical Biochemists in Ireland (49) or in user-friendly laboratory handbooks], are likely to be most effective.	(48, 49)
	Although in certain circumstances tumor markers may aid in diagnosis, speculative measurement of panels of tumor markers (“fishing”) should be discouraged, with the possible exception of patients with known malignancy of unknown origin (50).	PSA should never be measured routinely in females. CA125 should never be measured routinely in males. CA15-3 should only be measured routinely in males with an established diagnosis of breast cancer.	

Specimen schedule	The following baseline concentrations are of particular importance: A pre-operative concentration and a nadir concentration. A pre-follow-up concentration.	Interpretation of subsequent results is aided by knowledge of the pre-treatment “baseline” level.	(16)
Specimen timing	No strong evidence of diurnal variation for most markers, so specimens can be taken at any time of day.		
	Blood for PSA should be taken before any clinical manipulation of the prostate. Any measurements taken too soon should be repeated.	Prostatic biopsy, transurethral resection of the prostate, or traumatic catheterization may markedly elevate serum PSA and/or free PSA. [See (46) and Prostate Cancer Section for details of recommended delay in sampling after such intervention.]	(16, 46)
	Blood for CA125 should not be taken during menstruation, which may increase the serum concentration two to three-fold. A confirmatory specimen avoiding sampling during menses should be requested.		(51)
Clinical conditions	Generally liver and renal disease as well as inflammation and infection may cause elevated tumor marker concentrations. Benign diseases of tumor marker—producing tissues [eg, ovarian cysts, thyroid disorders, renal stones and sarcoidosis (52)] also frequently cause elevated concentrations.	Awareness of these caveats is essential for proper interpretation.	(1)
	Renal failure is most likely to cause inappropriately elevated results for CEA and cytokeratins. For patients in this category, this should be noted on the clinical report. Impaired renal clearance has also been reported to cause such results for hCG in some assays, depending on the molecular forms of hCG measured (53).	Awareness of these caveats is essential for proper interpretation.	(16)
	HCG may be persistently raised in menopausal women and/or women with a high normal level.	Awareness of these caveats is essential for proper interpretation. Elevated serum and urinary hCG levels in healthy women should be investigated systematically to exclude an underlying malignant process and to avoid inappropriate surgical and medical intervention. Long-term follow-up is required as tumors may not become apparent for many months or years (54).	(54–56)
	CA125 may be mildly elevated in endometriosis and the first two trimesters of pregnancy, and markedly raised in any patient with benign (non-tumoral) ascites. Levels >1,000 kU/L have been reported in a patient with hypothyroidism and ascites (57). Careful interpretation of results for patients with these conditions is essential, and their implications should be noted on the clinical report.	Awareness of these caveats is essential for proper interpretation.	(1, 57–60)
	CA125 may be markedly raised in patients with benign ovarian tumors or other benign pelvic pathology. CA125 levels ranging from 42 to 7,000 kU/L have been reported in Meigs' syndrome (61). A CA125 of 9,739 kU/L has been reported in a patient with a final diagnosis of benign clear-cell adenofibroma of the ovary (62).	Awareness of these caveats and that “it is important to remember that a pelvic neoplasm in a woman presenting with hydrothorax, ascites and elevated CA-125 levels might be benign and that this condition can rapidly be resolved with surgical removal” (61) is essential for proper interpretation in combination with ultrasound results (63).	(63–65)
	CA125 may be markedly elevated in patients with heart failure. CA125 levels have been reported to be significantly correlated with the severity of left ventricular systolic dysfunction (66).	Awareness of this caveat is essential for proper interpretation.	(64, 65, 67)
	CA125 may be elevated in patients with liver cirrhosis and chronic active hepatitis [35% and 10% of patients respectively in one study (68)].	Awareness of this caveat is essential for proper interpretation.	(68)

(Continued)

Table 1. NACB Recommendations: Quality Requirements in the Pre-Analytical Phase *(Cont'd)*

Requirements	Recommendations	Comments/Specific Examples	References
	CA125 may be increased in tuberculosis and in tuberculosis peritonitis, which is not due to renal insufficiency.	Awareness of this caveat is essential for proper interpretation.	(69, 70)
	Hypereosinophilia may be associated with increased levels of CEA [a serum level of 85 μg/L has been reported in one patient (71)] which normalize after treatment.	Awareness of this caveat is essential for proper interpretation.	(71)
	MUC-1 antigens (eg, CA-15.3 and BR27.29) may be increased in some non-breast pathologies – both malignant (eg, ovary, lung, myeloma) and non-malignant (eg, dermatological conditions, colitis, benign hepatitis).	Awareness of this caveat is essential for proper interpretation.	(72)
	Markedly elevated levels of CA125 may be associated with recurrent ischemic strokes in patients with metastatic cancer.	Awareness of this caveat is essential for proper interpretation.	(73)
	Urinary tract infections and prostatitis may increase PSA markedly, and confirmatory specimens should be taken after successful antibiotic treatment.	Serum PSA usually falls relatively rapidly but results may take more than a month (up to 9 months in one study) to return to within normal limits.	(46, 74)
	CA19.9 may be increased in common benign gynecologic diseases including endometriosis and leiomyoma.	Awareness of this caveat is essential for proper interpretation.	(75)
	CA19-9 is frequently increased in patients with chronic viral hepatitis and chronic hepatitis [46% and 54% respectively in one study (76)].	Awareness of this caveat is essential for proper interpretation.	(76)
	Cholestasis may markedly increase CA19.9 concentrations. For patients in this category, this should be noted on the clinical report.	Awareness of this caveat is essential for proper interpretation.	(16)
Medication/other treatment/lifestyle	5α-reductase inhibitors [eg, Finasteride (Proscar®; Propecia®), Dutasteride (Avodart®)] cause a median decrease in PSA concentration of ≈50%. For patients in this category, this should be noted on the clinical report.	Awareness of this caveat is essential for proper interpretation.	(16, 46)
	Transient increases in tumor marker concentrations may occur after chemotherapy.	Awareness of this caveat is essential for proper interpretation.	(16)
	Cannabis may transiently increase hCG.	Awareness of this caveat is essential for proper interpretation.	(77)
	Smoking may slightly increase apparent CEA levels in some immunoassays.	Awareness of this caveat is essential for proper interpretation.	(16)
	Alternative complementary treatments may affect tumor growth and alter tumor marker levels (78). Such medication should be noted on the clinical report.	Awareness of this caveat is essential for proper interpretation.	(16)
Specimen contamination	Salivary contamination can markedly increase apparent concentrations of CEA, CA19.9, and tissue polypeptide specific antigen (TPS). If contamination is suspected a repeat specimen should be requested. [Where procedures are fully automated this is unlikely to be a problem.]	Awareness of this caveat is essential for proper interpretation.	(16)

Table 2. NACB Recommendations: Quality Requirements for Assay Validation, Internal Quality Control, and Proficiency Testing

Requirements	Recommendations	Comments/Specific Examples	References
Assay validation			
Well-characterized methods	Prior to their introduction in routine clinical practice, both immunoassay and immunohistochemical methods must be validated by defined and well characterized protocols that meet regulatory guidelines [eg, Food and Drug Administration (FDA) approval in the United States, European Community (CE) marking in Europe].	It is critically important that methods are properly validated prior to their introduction to avoid misleading reports both in routine clinical practice and in the scientific literature. Failure to have done so accounts for some of the past issues with diagnostic tests, particularly for immunohistochemistry and fluorescence in situ hybridization (FISH) testing.	(45)
	Methods for immunohistochemistry should be particularly carefully described if appropriate high-quality reference materials are not available.	For example, tissue arrays with variable tumor marker amounts can be used.	(45)
	Where available, internationally recognized guidelines for the performance of immunohistochemical tests should be adopted.	The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have recently developed such guidelines for the tissue measurement of HER2.	(79)
Internal Quality Control			
Assessment of reproducibility	Within-run variability <5%; between-run variability <10%.	Manual and/or research assays may be less precise but PT data suggests these targets should be readily achievable for most analytes.	(1, 18)
Established objective criteria for assay acceptance	Limits for assay acceptance should be pre-defined and preferably based on logical criteria such as those of Westgard. [For further detailed discussion and examples, see (80, 81).]	IQC data should be recorded, inspected, and acted upon (if necessary) prior to release of any clinical results for the run.	(1, 17)
Appropriate number of IQC specimens	The number of IQC specimens included per run should allow identification of an unacceptable run with a given probability acceptable for the clinical application.		(1, 17)
Specimens closely resembling authentic patient sera	QC material not provided by the method manufacturer is preferable. At least one authentic serum matrix control from an independent source should be included in addition to any QC materials provided by the method manufacturer.	Kit controls may provide an overly optimistic impression of performance, particularly if they are prepared by adding standard to an artificial matrix.	(1, 82)
IQC specimens of concentrations appropriate to the clinical application	Negative and low positive controls should be included for all tumor markers. The broad concentration range should also be covered and IQC specimens should ideally include a high control to assess the accuracy of dilution.	Where clinical decision points are commonly employed (eg, PSA, 0.1 and 3 or 4 μg/L; AFP, 5-8 kU/L; hCG, 5 U/L), IQC specimens of these concentrations should be included.	(1)
Proficiency Testing			
PT specimens of appropriate analyte concentration	Concentrations should assess performance over the working range.	Distribution of occasional specimens of high concentration to check linearity on dilution and of specimens containing analyte-free serum to check baseline security for certain analytes (eg, AFP, hCG) is desirable.	(1)

(Continued)

Table 2. NACB Recommendations: Quality Requirements for Assay Validation, Internal Quality Control, and Proficiency Testing ***(Cont'd)***

Requirements	Recommendations	Comments/Specific Examples	References
PT specimens closely resembling authentic patient sera	PT specimens should ideally be prepared from authentic patient sera, which for tumor markers may require dilution of high concentration patient sera into a normal serum base pool.	PT specimens prepared by spiking purified analyte into serum base pools provide an overly optimistic impression of between-method performance (eg, for CEA, mean CVs of 14% cf 20% for pools containing patient sera)	(1, 82)
PT specimens that are stable in transit	Evidence of the stability of PT specimens in transit should be available.	Stability in hot climates is particularly relevant for hCG and free PSA, but reliable data should be available for all tumor markers.	(1, 46)
Accurate and stable target values	The validity of the target values (usually consensus means) should be demonstrated by assessing their accuracy, stability, and linearity on dilution.	Accuracy should be assessed by recovery experiments with the relevant International Standard [see Table 2], stability by repeat distribution of the same pool, and linearity by issue of different dilutions of the same sera in the same serum base pool. Issue of PT specimens containing the IS can also for some analytes elucidate the extent to which different methods recognise different isoforms (eg, hCG, PSA).	(17)
Assessment of assay interferences	Occasional specimens should ideally be issued to check for interference (eg, from heterophilic and other antibodies, high-dose hooking).	The volume of sera required may preclude undertaking this for all participants, but by distributing such specimens to a limited number of users of different methods, valuable information about method robustness can be obtained and the results subsequently provided to all participants.	(1)
Evaluation of interpretation as well as technical results is required for PT of immunohistochemical tests	The interpretation of the pathologist as well as the technical aspects of the test must be evaluated.	Since immunohistochemistry reports routinely include an interpretive comment, the accuracy of these should be rigorously and independently assessed.	(45)
Interpretative exercises and surveys	Occasional surveys are desirable to compare practice in difference laboratories.	PT schemes can make a powerful contribution to national audit by highlighting differences in reference intervals, reporting practice, and interpretation of clinical results, particularly when the ethos of the schemes is educational rather than regulatory.	(1, 19)
Provision of relevant educational updates to all participants	Incorporating regular updates to participants on new developments relevant to provision of a tumor marker service is desirable and can be conveniently done in Comments sections accompanying reports.	Surveys of recent literature can provide a helpful monthly addition to PT reports.	(1, 19)

patients with thyroid cancer or medullary carcinoma of the thyroid after total thyroidectomy]. Similarly, by issuing specimens of the same low concentration pool repeatedly, PT schemes can provide valuable complementary information about the stability of results over time (17). Because cancer patients are often monitored using tumor markers over months or years, similar assessment of long-term assay stability is also desirable at other analyte concentrations.

Long-term monitoring presents major challenges as patients may change hospital and laboratories may change the tumor marker methods during the relevant time period. While ideally results obtained in different methods would be fully interchangeable, data from PT schemes confirm that this is not the case, with between-method coefficients of variation in excess of 20% still observed for some tumor markers (18). Major causes of observed between-method variation for these complex analytes include poor calibration, differences in the specificity of antibodies used, and differences in method design (19).

It should be possible to achieve reasonably standardized and accurate calibration, but only for those analytes for which a recognized international standard (IS) or reference reagent (IRR) is both available (Table 5) and universally adopted by diagnostic manufacturers for primary calibration of their methods. Unfortunately, as yet there are no IS for any of the important CA series of tumor markers, a major gap that should be addressed urgently. Where relevant IS or IRR are available, recovery experiments undertaken by PT schemes (Table 2) provide (together with linearity and stability studies) the independent validation of consensus target values that is essential in a well-designed PT scheme. Conveniently, since PT schemes should be working toward improving between-method agreement, the same experiments also permit assessment of the correctness of calibration of individual methods and identification of those methods requiring improvement (eg, methods over- or under-recovering the relevant IS by more than 10%). Long-term PT scheme data can also confirm the effect of successful introduction of a new IS. Data from the UK National External Quality Assessment Service (UK NEQAS) for PSA, for example, confirm that mean geometric coefficients of variation (which reflect scatter) decreased from 21.9% in 1995, before the first IRR was introduced, to 8.7% in 2007 (20). Carefully designed experiments with the IRR for PSA and free PSA have enable assessment of the calibration and equimolarity of assays for PSA. The need to ensure clinical as well as analytical accuracy [eg, by selecting optimized pairs of assays for "free" and "total" PSA and using method-appropriate cut-offs] has been highlighted by a number of authors (21–24)] as particularly critical in the context of prostate cancer screening.

The recently established IRR for isoforms of hCG (25) [developed under the auspices of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)] and PSA (26) provide additional tools for elucidating method-related differences associated with the second major cause of method-related differences, namely, antibody specificity. Experiments currently in progress with six recently established IRR for hCG isoforms provide valuable information about what currently available methods for hCG really "measure" (25, 27, 28), an issue of major importance for oncology applications where recognition of a broad spectrum of hCG-related molecules is recommended (29). Complementary epitope-mapping projects such as those carried out under the auspices of the International Society for Oncodevelopmental Biology and Medicine (ISOBM) may enable broad recommendations to be made regarding the most clinically appropriate antibody specificities for some tumor markers, with some progress toward this objective already having been made for hCG (29). Further progress will be accelerated by rapid adoption of clear IFCC nomenclature for the hCG isoforms (25), reducing the risk of confusion such as that recently reported (30).

The results of such studies may lead to better understanding of optimal method design for the complex tumor markers, thereby addressing the third major cause of method-related differences. Differences in method design are likely to contribute both to the numerical differences in results observed, and to differences in method robustness to clinically relevant interferences, the most important of which are described in Table 3. Maintaining vigilant awareness of these is essential. Ultimately, the most effective way of minimizing the risk of such interference leading to serious clinical errors is to promote regular dialogue between laboratory and clinical staff, encouraging early discussion and investigation of any results that are not in accord with the clinical picture (16).

POST-ANALYTICAL QUALITY REQUIREMENTS

Provision of helpful reports following the NACB recommendations in Table 4 encourages good communication between laboratory and clinic, which is highly desirable to achieve best use of tumor marker tests. Clinical biochemistry laboratories should be prepared to engage more actively in the interpretation of tumor marker results, ensuring that appropriately validated reference intervals are provided (taking account of age and/or sex where relevant), and incorporating estimates of analytical and biological variation, as well as taking account of other factors specific to particular tumor markers and malignancies such as tumor marker half-lives (31) and kinetics (32–34). It is also helpful if clinical and laboratory staff alert each other to relevant trends in results for other relevant tests [eg, declining hemoglobin, rising ESR, and LDH and other metabolites (35)]. In this context it should be recognized that evaluating and assessing the contribution of tumor markers to the evolving health status of the patient requires specific identification of correlated observations from defined data elements in the patient care record. Tumor marker results, together with all other observations made during patient care, can be contained in electronic health records (11, 36). These data should be used to determine the baseline tumor marker level for individual cancer patients during periods of remission, facilitating earlier diagnosis of progression. Clinical laboratory studies of each marker should explicitly identify the electronic health record data elements that contain observations used in

Table 3. NACB Recommendations: Quality Requirements for Minimizing Risk of Method-Related Errors in Tumor Marker Results

Type of Interference	Recommendations	Comment	Reference
Cross-reaction of closely related molecules	Manufacturers should provide clear information about the specificity of the antibodies used in their methods and data on cross-reactivity that is readily comparable with that of other methods. Users should be aware of the characteristics of the methods used.	Often helpful (e.g. when measuring PSA or hCG) but differences in recognition of the cross-reacting isoforms are likely to contribute to numerical differences in results.	(16)
High-dose hook effect	Laboratories should have in place defined protocols for identifying specimens that have "hooked".	Tumor marker concentrations range over several orders of magnitude and for any tumor marker may exceed the capacity of the solid phase. For conditions that are potentially fatal, but curable (eg, childhood hepatoblastoma and gestational trophoblastic neoplasia), failure to recognise extremely high tumor marker concentrations (AFP and hCG, respectively) constitutes a critical clinical error (83, 84).	(16, 49)
Specimen carry-over	Laboratories should occasionally check the vulnerability of their tumor marker methods to carry-over from a preceding high concentration specimen.	Potentially a problem whenever very high concentration specimens are assayed.	(16, 85)
Interference from heterophilic or human anti-mouse antibodies (HAMA)	Laboratories should be aware of the possibility of interference from heterophilic or human anti-mouse antibodies, particularly when results are not in accord with the clinical picture. Where interference is suspected this should be investigated (eg, by re-assaying the specimen) 1. After treatment with commercially available antibody blocking tubes 2. After addition of further immobilized normal, non-immune serum, Protein A or Protein G 3. After precipitation of immunoglobulins with polyethylene glycol (PEG) 4. In a different method, preferably using a different methodology (eg, radioimmunoassay) 5. At several dilutions to assess linearity on dilution.	Falsely high or low results may be obtained for patient specimens containing anti-IgG antibodies capable of reacting with antibodies used in the assay. Such antibodies may be of particularly high titre in patients who have undergone treatment with mouse monoclonal antibodies for imaging or therapeutic purposes. Serious clinical errors as a result of failure to recognize such interference have been most frequently reported for hCG and CA125. A high degree of suspicion is usually required for identification, which is facilitated by good communication between clinical and laboratory staff.	(16, 85)

Table 4. NACB Recommendations: Quality Requirements in the Post-Analytical Phase

Requirements	Recommendations	Comments/Specific Examples	Reference
Factual requirements			
Clinical information from the requesting doctor	Brief clinical information indicating the source of the suspected/diagnosed malignancy and the treatment stage (eg, pre-op, post-op, pre-chemotherapy) should accompany the specimen.	Such information is essential if any laboratory interpretation is to be made and may help to identify occasional laboratory errors (eg, mis-sampling on an analyser).	(1)
Availability of appropriate reference intervals	Reference intervals should be appropriately derived using an appropriate healthy population.	Group reference intervals are usually most relevant for cancer patients pre-treatment, after which the patient's own individual "baseline" provides the most important reference point for interpretation of marker results. If this is well established, increases even within the reference interval may be significant. Even if the marker is below the normal or usual cut-off value any sustained increase must be treated as a possible relapse, provided the measurement procedure is the same (86). Reporting of critical increases of tumor marker concentrations, taking both the analytical performance of the test and the individual reference intervals (under specified clinical conditions; eg, post-chemotherapy) into consideration contributes to earlier diagnosis of relapse.	(1, 86)
	Reference intervals must be included when reporting results for immunohistochemical markers.	Reference intervals may include internal reference materials (eg, normal ducts in breast tissue), and specify what staining they should have.	(45)
Interpretation criteria for immunohistochemical tests	Interpretation criteria must be well documented and must be clearly stated in the clinical report.	For Her-2/neu testing by FISH, for example, it is essential to define clearly what the appropriate ratio of Her-2 signals/CEP 17 signals is for classification as unamplified (eg, 2.0 for Vysis probes), borderline amplified, etc.	(45, 79)
Knowledge of what constitutes a significant change	The percentage increase or decrease that constitutes a significant change should be defined and should take account of both analytical and biological variation (80, 87–89). Laboratories should be willing and able to advise on this issue.	The percentage increase or decrease that constitutes a significant change varies between tumor markers mainly due to the differences in the magnitudes of their biological variation (88–92). A confirmed increase or decrease of ± 25% is frequently considered to be of clinical significance (16) but more work is required in this area, the importance of which has recently been illustrated for PSA (17).	(1, 91, 92)

(Continued)

Table 4. NACB Recommendations: Quality Requirements in the Post-Analytical Phase *(Cont'd)*

Requirements	Recommendations	Comments/Specific Examples	Reference
Defined protocol when changing methods	Laboratories should have a defined protocol when changing tumor marker methods. Communication with the main users should always be sought before a changeover of method is undertaken. Ideally, laboratories should avoid changing tumor marker methods unless essential.	This may necessitate analyzing the previous specimen by the new method or requesting a further specimen to re-establish the baseline and/or confirm the trend in marker level. If the results are significantly different it may be necessary to run old and new methods in parallel for a defined changeover period, an approach that also helps clinicians become accustomed to the new values.	(1)
Knowledge of tumor marker half-lives	Laboratories should be able to provide calculated tumor marker half-lives or doubling-time for the markers for which these are relevant (eg, AFP, hCG, PSA, CA125).	Half-lives are defined as the time to 50% reduction of circulating tumor marker concentration following complete removal of tumor tissue. [Calculation of tumor marker half-lives may be irrelevant if a 50% reduction does not represent a significant change.]	(1)
Objective audit of tumor marker utility	Laboratories should be involved in ongoing audit of the clinical utility of the results they provide. Published audits suggest considerable variation in practice (10, 93, 94). Missed results may occur more frequently than is generally appreciated, suggesting a need for improved systems for clinical data management, as has been suggested for PSA (95).	This remains a priority and is being considered by a number of professional organizations.	(1, 9)
Reporting requirements			
Cumulation of tumor marker results	Laboratories should provide fully cumulated tumor marker results. User-friendly graphical representations may also be helpful.	Helpful reports facilitate interpretation and communication between laboratory and clinic. Reports should incorporate any brief and relevant clinical information available, particularly dates of operation etc.	(1)
Tumor marker method used	Laboratories should indicate the method used on the report form and highlight whether any change of method is likely to have affected interpretation of the trend in marker result.	As above.	(1)
Recommendations as to the appropriate frequency of tumor marker measurements	Laboratories should be willing to advise on the frequency of monitoring and the need for confirmatory specimens.	An apparent rise in marker concentration should always be confirmed by repeat measurement at a defined interval.	(1)
Communication between laboratory and clinical staff	Laboratories should always welcome and encourage good communication with clinical users of the service.	Good communication facilitates appropriate use of these (and other) tests. Telephoning results to the relevant clinician may in some cases be desirable.	(1)

Table 5. WHO International Standards (IS) and Reference Reagents (RR) for Major Tumor Markers

Tumor Marker	Code	Year Established	Description	Reference
AFP	IS 72/225	1972	Crude cord serum (50%)	(96)
CA125	-	-	-	-
CA15-3	-	-	-	-
CA19-9	-	-	-	-
CA72-4	-	-	-	-
CEA	IRP 73/601	1973	CEA purified from liver metastases to primary colorectal cancer	(97)
hCG	IS 75/589*	1975	Purified urinary hCG, contaminated with hCGβ and hCGn	(98)
hCGα	IS 75/569	1975		(98)
hCGβ	IS 75/551	1975		(98)
hCG	IRR 99/688	2001	Highly purified urinary hCG, free from nicked forms and free subunits	(25)
hCGn	IRR 99/642	2001	Highly purified urinary hCG, partially degraded, missing peptide bonds in the hCGβ-40-50 region	(25)
hCGα	IRR 99/720	2001	Highly purified urinary hCGα, dissociated from hCG	(25)
hCGβ	IRR 99/650	2001	Highly purified dissociated urinary hCGβ, free from intact dimeric hCG, hCGα and hCGβn	(25)
hCGβn	IRR 99/692	2001	Partially degraded hCGβ, missing peptide bonds in the hCGβ-40-50 region	(25)
hCGβcf	IRR 99/708	2001	Residues hCGβn-6-40, joined by disulphide bonds to hCGβn-55-92	(25)
PSA	IRR 96/670	2000	90:10 ratio of bound : free PSA	(26)
fPSA	IRR 96/668	2000	Purified free PSA	(26)

*Essentially equivalent to hCG IS 75/537 which IS 75/589 replaced.

International Standards and Reference Reagents are available from the National Institute for Biological Standards and Control, Potters Bar, Herts, UK [http://www.nibsc.ac.uk/catalog/standards/preps/sub_endo.html]. For the CA antigens, no international standard established at this time.

Abbreviations: hCG, human chorionic gonadotropin; IS, International Standard; hCGβ, hCG β-subunit; IRP, International Reference Preparation; hCGα, hCG β-subunit; hCGn, nicked hCG; hCGβn, nicked hCG β-subunit; and hCGβcf, hCG β-core fragment; PSA, prostate specific antigen; fPSA, free prostate specific antigen.

evaluating the changes in patient health status, together with contributions of laboratory measurements to either diagnosis or treatment of the individual patient. The electronic health record definition has matured both nationally and internationally to a sufficient degree that these attributes can now be documented in a common fashion,(36) so that patient data can be effectively related to the knowledge structures used in CDS components in health enterprise information architectures to help guide tumor marker data interpretation for the requesting practitioner (13).

CLINICAL ISSUES THAT ENHANCE THE RELIABILITY AND UTILITY OF TUMOR MARKERS

As with all diagnostic tests, tumor markers are surrogate indicators that can be used clinically to increase or decrease the clinician's suspicion that a future clinically important event, such as a new cancer, recurrence, progression, or death will or will not happen, and/or that a specific treatment will reduce that risk. Markers can be used to determine risk, screen for early cancers, establish diagnosis, estimate prognosis, predict that a specific therapy will work, or monitor for disease recurrence or progression (37–39). The value of tumor markers is that they permit more efficient application of therapies, which should result in applying the therapy to those patients most likely to benefit while reducing exposure to toxicities for those patients who would not benefit (40).

Tumor markers are only useful if three circumstances pertain:

- The marker results are appropriate precisely for the required application (ie, risk assessment, screening, diagnosis, prognosis, prediction, or post-treatment monitoring).
- The marker results separate patients into two or more populations whose outcomes differ so strikingly that they and their caregiver would treat one group differently than another. [This consideration depends on several factors, including the end point in question (patients might be more willing to accept therapy for very small mortality reductions but not for similar reductions in occurrence of a new cancer), the toxicity of the therapy (patients are more likely to accept a therapy with small benefits if the toxicities are few), and the cost of the therapy.]
- The estimate of the separation in outcomes for marker positive and negative is reliable.

These issues are inter-related. For example, studies of the prognostic value of a marker that do not consider the manner in which the study populations were treated are not helpful to the clinician trying to decide whether to apply treatment. Indeed, in breast cancer, one might conclude that HER2 overexpression is associated with a poor prognosis, a favorable prognosis, or not associated with either if one studied a patient population that had been variably treated in either the adjuvant or metastatic setting with different types of chemotherapies, different types of hormone therapies, and trastuzumab (41). These variable conclusions might be reached because HER2 is a weak or moderately unfavorable prognostic factor in patients who receive no therapy. It appears to predict weakly or moderately for resistance to chemotherapy regimens that do not contain anthracyclines or taxanes, but it may predict for sensitivity to chemotherapy regimens that do contain anthracyclines or taxanes. HER2 appears to predict for resistance to selective estrogen receptor modulators like tamoxifen and for sensitivity to estrogen ablation strategies like aromatase inhibitors, and it is a very strong predictor of response and benefit from the anti-HER2 humanized monoclonal antibody, trastuzumab.

Furthermore, while statistical analysis is, of course, important to estimate the reliability of how likely two marker-identified groups might be different, the p-value alone does not indicate clinical utility. If a study is sufficiently powered, a small difference in outcomes of two groups separated by marker results (positive vs. negative) might be statistically significant. Too often an investigator will conclude that a marker is clinically useful because a derived p-value is <0.05. Rather, it is more important, for clinical utility, that one population (marker positive or negative) does extremely well while the other does very poorly, so that one group might accept the therapy of interest while the other would elect not to. In this case, it is imperative that the p-value does suggest statistical significance, but it is not the determining factor for clinical utility. Finally, a single study does not establish a scientific fact. Rather, secondary validation of the results of an interesting study in a subsequent data set is imperative, and the validation study should use the same assay and the same cut point(s). In addition, the patient population must be very similar to that of the preceding study. These requirements are among those highlighted in the excellent reporting recommendations for tumor marker prognostic studies recently developed and published as the REMARK guidelines (42), complementing previous broader statements on the Standards for Reporting of Diagnostic Accuracy (43).

In summary, acceptance of a tumor marker for clinical utility requires careful and thoughtful study design so that the results are meaningful in the clinical setting. Unfortunately, most tumor marker investigations have been studies of convenience, using archived samples that happen to be available (37). Such studies [level of evidence (LOE) III] are useful to generate hypotheses, but as in all science, without careful investigational planning and design the results cannot be accepted as fact. Indeed, LOE II studies, in which the marker is considered prospectively as a secondary objective in a clinical trial, or better yet, LOE I studies in which the marker question is the primary objective, are much more likely to yield acceptable results. In other words, it is better to ask the question and get an answer, rather than to get an answer and then ponder the question. Such evidence-based considerations are particularly important when patient lives are at stake and should be remembered whenever a tumor marker test is requested.

Chapter 2

Microarrays in Cancer Diagnostics

BACKGROUND

Genomic microarrays were first introduced in 1996 by Affymetrix (99). Gene chip protein microarray technology successfully exploits the principles of the ambient analyte ligand analysis first described by Ekins and co-workers in the early 1980s (100, 101) and further developed by others [eg, (102)]. Briefly, Ekins' Ambient Analyte Theory concept recognizes that a minute amount of binding material (eg, antibody or other receptor) does not significantly change the sample concentration and can give much higher sensitivity than assay formats using 100 or 1,000 times the amount of binding material (100, 103). In particular, the use of microscopic spots of "binding agent" located at high-surface density on a solid support (coupled with the use of very high specific-activity labels, such as fluorescent labels) can yield higher sensitivity and shorter incubation times than conventional ligand assay methods, especially so-called non-competitive assay methods. A comprehensive review of the principles of this technology has recently been published (104).

Although still generally restricted to research use, the versatility of microarrays—depending on the biomolecule immobilized on the surface, these devices are commonly known as biochips, DNA chips, protein chips, or cell chips—is such that the major potential clinical applications of the technology in the field of oncology were immediately recognized. Disease classification, prognosis, monitoring, and prediction of therapeutic response are some of the areas where microarrays have the potential to become routine diagnostic tools. This technology enables substitution of linear studies of individual events to parallel and simultaneous analysis of complex systems and pathways. Regardless of the application, the resulting information comprises thousands of individual measurements and provides an intricate and complex snapshot of biological properties of the cell, tissue, organ, or fluid. Microarrays relevant to cancer diagnostics have now been commercially introduced and/or are being developed by Affymetrix (Genechip[R] technology) (99), Randox (Evidence[R] technology) (105) and other major manufacturers.

PRINCIPLES OF MICROARRAYS

A microarray is a compact device that contains a large number of well-defined immobilized capture molecules (eg, synthetic oligos, PCR products, proteins, antibodies) assembled in an addressable format. The best-known microarrays, DNA biochips, are miniature arrays of oligonucleotides attached to a glass or plastic surface. These chips are used to examine gene activity (expression profiling) and identify gene mutations or single nucleotide polymorphisms (SNPs), by hybridization between the sequences on the microarray and a labelled probe (the sample of interest). There are two major methods for microarray fabrication: a) photolithography, as is used in the Affymetrix system (400,000 spots in a 1.25 × 1.25 cm area), and b) mechanical deposition or printing on glass slides and membranes as originally developed by Boehringer-Mannheim and now adopted by Roche Diagnostics (106). [In this context, it is important to note that at least 1,000-fold greater sensitivities are required in the case of protein as compared with gene microarrays.]

Major potential advantages of microarray-based assays include high sensitivity, small amounts of binding reagents required, their independence of sample volume, decreased incubation times, minimal wasted reagents, simultaneous access to many genes or proteins, massive parallel information, automation, and potentially quantification. More detailed information on the subject is readily available in specialized books and reviews (104, 107, 108) and an entire issue of *Nature Genetics* (109).

TISSUE MICROARRAYS

High-throughput analysis of tissues is facilitated by new technologies, such as multi-tissue northern blots, protein arrays, or real-time PCR (110–113). However, the problem with these methods is that tissues are disintegrated before analysis, preventing identification of the cell types expressing the gene of interest (114). These and other shortcomings can be overcome by tissue microarray (TMA) techniques (115). TMAs consist of up to a 1,000 tiny cylindrical tissue samples (0.6 mm in diameter) assembled on a regular-sized routine histology paraffin block. Sections are cut from TMA blocks using standard microtomes. TMA sections allow simultaneous analysis of up to a 1,000 tissue samples in a single experiment. The technique is therefore cost-effective. Despite the small size of arrayed samples, TMA studies generally provide reasonably representative information. TMAs are applied over a broad range of cancer research: prevalence TMAs (116–118), progression TMAs (115, 119–121), prognostic TMAs, and TMAs composed of

experimental tissues, such as cell lines (122, 123) or xenografts (120).

APPLICATIONS OF MICROARRAYS

Microarrays have been successfully applied in a variety of settings including

- Gene expression profiling (the most popular application)
- Detection of single nucleotide polymorphisms (SNPs) (pharmacogenetics)
- Sequencing by hybridization (genotyping/mutation detection)
- Protein expression profiling
- Protein-protein interaction studies
- Whole genome biology experiments

Cancer is a heterogeneous disease in many respects, including its cellularity, different genetic alterations, and diverse clinical behavior. Many analytical methods have been used to study human tumors and to classify patients into groups with similar clinical behavior. Most methods require specialized pathologist interpretation; yet none of the classifications are homogeneous enough. It has been hypothesized that the genetic heterogeneity and clinical behavior of cancer could be better assessed by studying genome-wide gene expression profiles by microarrays (124). Although the potential of microarrays is yet to be fully realized, these tools have shown great promise in deciphering complex diseases including cancer (124). A partial list of applications of microarrays in cancer is presented in Table 6. As is often the case with new technologies, microarrays have many shortcomings, some of which are briefly discussed in the following section.

LIMITATIONS OF MICROARRAYS

Microarray technologies are still evolving and this presents difficulties for standardization and consensus development. There are no gold standards, such as reference reagents or bioinformatics algorithms. These standards are essential for comparison of data between laboratories and on different platforms (125). Recent reports suggest that microarray data are noisy and not reproducible (126, 127). Furthermore, bias poses a significant threat to the validity of data generated by such technologies (128).

KEY POINTS: MICROARRAYS IN CANCER DIAGNOSTICS

There is little doubt that microarrays will eventually become routine diagnostic tools, and the first commercial devices are already on the market (Table 7). However, this is still a relatively new technology and several procedures need to be further optimized and validated prior to the implementation of microarrays into routine clinical practice. These include selection of optimal capture molecules, standardized hybridization protocols, and standardized data collection and interpretation. For DNA and protein microarrays to be reliable tools, they must possess probe sequences that hybridize with high sensitivity and specificity, thereby allowing specific detection of their intended targets. Results must become more reproducible, more robust, and more readily interchangeable between laboratories, and stringent quality control and quality assurance systems must be established (125). Determining the appropriate level of analytical and clinical validation needed for each application raises new challenges for scientists in industry, academia, and regulatory agencies (129).

Two important issues need to be considered when evaluating microarray expression data: whether the results are valid or accurate for the particular biological system under study, and whether the data fundamentally describe the phenomenon being investigated (130).

Introduction of artefacts is possible at any time during an array experiment, therefore, each component of the procedure must be carefully considered. The validation process can be divided into three areas: experimental quality control, independent confirmation of data, and universality of results (130). Furthermore, before implementation of microarrays into routine practice, it will be preferable to automate the process to minimize variability and increase robustness. Array production, like any other diagnostic device, must meet minimum criteria set by the Food and Drug Administration (FDA) (131). The International Meeting on Microarray Data Standards, Annotations Ontologies and Databases (MGED) focuses on standardization of biochips and proposes appropriate guidelines (132, 133). Despite widespread applications of microarrays in research, the level of evidence of these studies for clinical application, as described by Hayes et al (37), is Level V (evidence from small pilot studies that estimate distribution of marker levels in sample population). Based on the information above, the NACB Panel has formulated the recommendations outlined in Table 8.

Table 6. Microarray Applications in Cancer Diagnostics

Microarray Technology	Application	Cancer	Reference
Comparative genomic hybridization	Classification	Breast	(134, 135)
cDNA tissue expression profiling	Classification	Breast	(136)
	Therapeutic response	Lymphoma	(136, 137)
	Molecular profiling	Prostate	(138)
Gene expression profiling	Prognosis	Breast	(139,140)
	Classification	Breast	(139,141)
	Diagnosis	Ewing sarcoma	(141)
	Diagnosis	Rhabdomyosarcoma	(141)
	Diagnosis	Burkitt lymphoma	(141)
	Diagnosis	Neuroblastoma	(141)
	Diagnosis	GI tumor	(142)
	Diagnosis	Prostate	(143)
	Prognosis	Prostate	(144, 145)
	Diagnosis	Bladder	(143)
	Treatment tailoring	Breast	(143)
	Classification	Colorectal	(143)
	Classification	Gastroesophageal	(143)
	Classification	Kidney	(143)
	Prognosis	Kidney	(146)
	Classification	Ovarian	(143)
	Classification	Pancreas	(143)
	Classification	Lung	(143, 147, 148)
	Molecular profiling	Prostate	(149)
	Development stages	B-cell lymphomas	(150, 151)
	Mutations	BRCA 1 (breast, ovarian)	(140, 152, 153)
Prognostic signature	Prognosis	Breast	(154)
		Lung	(155)
Genome mining	Biomarker discovery	Ovarian	(156)

Table 7. Some Commercially Available Cancer Diagnostic Devices Based on Microarray Technology

Name	Intended Use	Manufacturer
1. Amplichip CYP450	Identifies variations in genes CYP2D6 and CYP2C19 for pharmacogenomics	Roche (www.roche.com)
2. GeneChip Mapping 100K	Whole genome SNP analysis (100,000 SNPs) for establishing disease predisposition	Affymetrix (www.affymetrix.com)
3. MammaPrint	70-gene signature for breast cancer prognosis	Agendia (www.agendia.com)
CupPrint	Identifying the primary tumor	
4. p53 GeneChip	Sequencing of p53 gene for identifying mutations	Affymetrix
5. Tumor PSA Array	tPSA, fPSA, CEA	Randox (www.randox.com)
Tumor Monitoring Array	CEA, AFP, hCG, CA19-9, CA125, CA15-3	
Colorectal Cancer DNA Array	TP-53, APC, K-ras, BRAF	
cDNA Expression Array	Ovarian, Breast cancer	

Table 8. NACB Recommendations for Use of Microarrays in Cancer Diagnostics

1. Gene expression microarrays are new and promising devices used for cancer diagnosis, prognosis, prediction of therapeutic response, and monitoring and selection of therapy. The level of evidence from most published studies, according to Hayes et al (37) is level V [lowest category]. Consequently, microarrays should continue to be used as research devices, but not as tools for making clinical decisions.
2. Standardization and clinical validation of expression microarrays is warranted.
3. Quality control and quality assurance programs for expression microarrays need to be further developed.
4. Microarray automation is encouraged for improving reproducibility, throughput, and robustness.
5. Tissue microarrays are devices suitable for high-throughput analysis of large numbers of samples and are recommended for use in clinical trials and retrospective studies for evaluating and validating new tumor markers by immunohistochemical analysis.
6. Use of microarrays for single nucleotide polymorphism analysis is recommended for establishing haplotypes and for correlating these haplotypes to disease predisposition.
7. Use of microarrays is recommended for high-throughput genotyping and mutation/sequence variation detection for cancer diagnostics and pharmacogenomics. More validation is necessary to ensure equivalent results between standard technologies (such as DNA sequencing) and microarray analysis.
8. Protein microarrays and other similar technologies are recommended as research tools for multiparametric analysis of large numbers of proteins. The level of evidence is not as yet high enough for clinical applications.
9. Standardized protocols should be developed for sample collection, handling, and processing.

Chapter 3

Mass Spectrometry in Cancer Diagnostics

BACKGROUND

Despite impressive scientific, medical, and technological achievements over the past few decades, cancer is still a leading cause of death, largely because most cancer patients are diagnosed when disease is advanced. Accumulating evidence suggests that in the case of many cancers, early detection is associated with improved survival rates (157). Mass spectrometry (MS) has the potential to revolutionize cancer diagnostics by facilitating biomarker discovery, generating proteomic profiles as cancer signatures, enabling tissue imaging, and quantifying biomarker levels. The principles of MS as applied to cancer diagnostics are summarized here, together with recommendations for the use of this technique in clinical practice, based on currently published evidence and expert opinions. The main focus will be on matrix-assisted laser desorption/ionization (MALDI) and related MS techniques, such as surface-enhanced laser desorption/ionization (SELDI) for proteomic analysis.

PRINCIPLES OF DIAGNOSTIC MASS SPECTROMETRY

The typical mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge ratio (m/z) of the ionized analytes, and a detector that registers the number of ions at each m/z value (158). There are two approaches for biomarker discovery using MALDI/SELDI-TOF MS. One approach uses the differences between MS profiles of the disease and control specimens to generate a diagnostic model. A variation of this approach is to select several discriminate peaks and identify the nature of these protein/peptide peaks. Diagnostics are based on multiplex immuno-MS or ELISA. The other approach is to degrade enzymatically (usually with trypsin) the proteins to peptides, separate the peptides by techniques, such as high performance liquid chromatography (HPLC), and direct the eluted fractions into an ion source [electrospray ionization (ESI) or MALDI) where they are converted into ionized species that enter the mass spectrometer followed by identification of the protein fragments and parent proteins comprising the mass spectra by a variety of algorithmic approaches (159).

Mass spectrometric measurements are carried out in the gas phase of ionized species. Two commonly used techniques to volatize and ionize the proteins or peptides are ESI and MALDI (160–162). A variant of the latter is SELDI (Ciphergen, ProteinChip™) (163, 164). The mass analyzer separates ionic species according to their m/z ratios. Four basic types of mass analyzers are commonly used in proteomic research: the ion trap, time-of-flight (TOF), quadrupole, and Fourier transform ion cyclotron resonance (FT-ICR), with a potential fifth variant being the new Orbitrap mass spectrometer (Thermo Electron, Inc). These basic types may be variously combined in hybrid instruments.

Protein identification is achieved through either peptide mass fingerprinting or peptide sequencing. In the former, peptide masses are compared with mass spectra of proteins listed in databases using appropriate software (165, 166). Peptide sequencing is based on induction of random cleavage of peptide bonds between adjacent amino acid residues, using approaches such as collision-induced dissociation (CID). The resulting ion series is analyzed by software (167–171) to determine the amino acid sequence.

APPLICATION OF MASS SPECTROMETRY IN CANCER DIAGNOSTICS

MS has been applied to cancer in a variety of contexts, including

- Diagnosis, prognosis, and management
- Biomarker discovery
- Diagnostic tissue imaging
- Biological studies related to mechanism of disease.

MS is considered to be particularly well suited to serve as a diagnostic or biomarker discovery tool in cancer, given emerging evidence that during cancer development, cancer cells, and/or the surrounding microenvironment generate proteins and peptides of different type and in different concentrations than normal cells. These abnormal tissue distributions can be analyzed by imaging-based MS and the patterns compared with controls to identify cancer-specific changes that may prove to be clinically useful. Should leakage to the circulation occur from the tumor-host microenvironment, then a multiplex of cancer-specific analytes may be detectable in the blood as well, leading to even more widespread clinical utility and convenience of testing (172–175). This concept is graphically illustrated in Figure 1.

The identification of cancer-specific protein patterns in blood by MS was demonstrated by several investigators

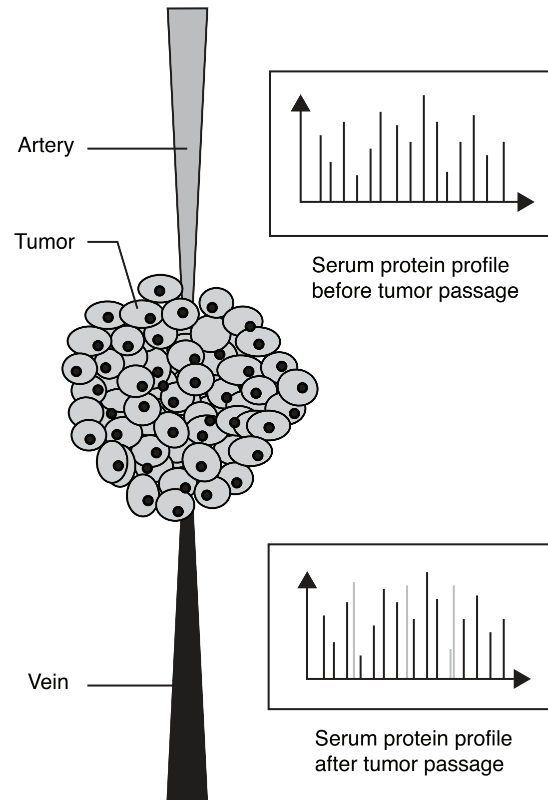

Figure 1. Secretion of specific biomarkers into the blood circulation by tumors. Tumor-specific proteins may be actively secreted by tumor cells or released into the circulatory system by necrosis and apoptosis of these cells. Either of these conditions leads to an alteration of the serum protein profile. When sera from normal and disease samples are compared, this alteration may result in differences detectable in relative and/or unique signal intensities. Reproduced from *Pharmacogenomics* 2003;4(4):463–76 (172) with the permission of Future Medicine Ltd.

including Vlahou et al (176) for bladder cancer, by Li et al (177) for breast cancer, by Petricoin et al (174) and Rai et al (178) for ovarian cancer, and Adam et al (179) for prostate cancer. Subsequently, many other investigators have used similar approaches to identify multiple markers and informative profiles for many other types of cancer (Table 9). Blood and urine, the most accessible and diagnostically useful body fluids, have been most studied, although other fluids such as nipple aspirate fluid and conditioned media have value as sources for biomarker discovery.

In almost every published article, the profiles generated by MALDI-TOF MS have been shown to yield better diagnostic sensitivities and specificities than the established cancer biomarkers in current use. Because of this, the MALDI-TOF MS approaches have received extensive publicity since they promise to revolutionize early cancer detection, sub-classification, prognosis, prediction of therapeutic response. However, the initial enthusiasm has been tempered somewhat by parallel reports that have identified potential problems with this approach and its clinical reliability (128, 180–197). These issues are not unlike those facing the gene transcript profiling community (198). Future validation studies will determine how ready this technology is for clinical application.

CURRENT ADVANCES AND EXISTING LIMITATIONS OF MALDI-TOF MASS SPECTROMETRY–BASED PROFILING FOR CANCER DETECTION

If MALDI-TOF profiling is to be successful in the transition from a research technique to a clinical diagnostic tool, then an extensive understanding of pre-analytical, analytical, and post-analytical sources of variation must be realized and controlled (128, 180–197). For example, the effect of sample storage and processing, sample type, patient selection, and demographic variables (sex, age) on test outcome must be clearly established (199). Analytical performance must improve to the point where sensitivity, specificity, and the dynamic range become comparable to those of established techniques, such as ELISA. The reproducibility of protein patterns across different batches of chips (when SELDI-TOF is employed), different analysts, different sites, and different instrumentation is still

Table 9. Mass Spectrometry for Cancer Diagnosis and Imaging

Application	Cancer Type	Reference
Cancer Diagnosis		
	Nasopharyngeal	(213)
	Ovarian	(174, 178, 210)
	Breast	(177, 211, 214–219)
	Prostate	(179, 220–224)
	Bladder	(176, 225, 226)
	Pancreatic	(227–230)
	Head and Neck	(231)
	Lung	(232)
	Colon	(233)
	Melanoma	(234, 235)
	Hepatocellular	(236–238)
	Leukemia	(181)
Tissue imaging		
	Gliomas	(239)
	Breast	(240–242)
	Lung	(243)

under investigation. Robustness of the methodology, in general, is of concern, as are issues related to bioinformatic artifacts, data over-fitting, and bias arising from experimental design. However, a large number of these issues relate to inappropriate analysis of publicly available mass spectral data sets that were not meant to be compared. Recently, a large consortium of investigators has shown success at reproducibly obtaining mass spectral signatures, including diagnostically important ones, at multiple sites across time and instruments. This finding establishes a very positive result for those attempting to employ MALDI-TOF type approaches for protein fingerprinting based diagnostics (193).

The current limitations and promises of MALDI-TOF, particularly as applied to clinical practice and cancer diagnostics, are addressed more fully in several recently published reviews (128, 173, 175, 180, 182, 194–197).

KEY POINTS: MASS SPECTROMETRY PROFILING IN CANCER DIAGNOSTICS

Despite numerous publications describing impressive results of MALDI-TOF MS as a diagnostic tool (Table 9), the level of published evidence, as described by Hayes et al (37), is level IV–V (evidence from either retrospective or small pilot studies that estimate distribution of marker levels in sample population). According to the criteria of Pepe et al (200), the stage of development of this technology as a biomarker tool is phase I (preclinical exploratory studies). Based on this information, the recommendations presented in Table 10 have been formulated. There is little question that MALDI-TOF MS approaches are promising for biomarker discovery and validation. As for direct profiling of patient specimens for diagnostic use, the issues discussed in this document would need to be resolved. The advantages of proteomic profiling include analysis without the need for a labeling molecule, potentially high specificity, multiparametric analysis, high-throughput, very low sample volume requirements, and direct interface with computer algorithms. The major limitations of the MALDI-TOF technology for MS profiling type work are, at present, the cross-platform reliability of the signatures generated; dramatic effects on final spectral composition from subtle changes in sample handling and processing; and analytical sensitivity, especially when the analyte is present in minute amounts in a highly complex mixture that includes high abundance molecules. However, there are inherent advantages of certain MALDI-TOF approaches. For instance, combining immune isolation prior to MALDI-TOF analysis allows for elimination of secondary antibodies and detection of multiple derivative analytes such as protein isoforms (201, 202). In addition, exciting new research has indicated that many low abundance proteins and low molecular weight analytes exist in a bound state in the serum, and are effectively amplified by carrier protein based sequestration (203–208). These low molecular weight analytes appear to have underpinned many past spectral fingerprints, thus indicating that many of these ions may be generated from low abundant analytes. A list of these low molecular weight carrier protein bound analytes has recently been provided for early-stage ovarian cancer patients (206, 209), and the concept verified in an independent study with Alzheimer's disease detection (207). In that study, high-resolution MALDI-TOF serum proteomic profiling of Alzheimer's disease samples reveals disease-specific, carrier-protein-bound mass signatures. These recent findings, together with other recent publications (181, 210–212) describing truncated or fragments of proteins ("fragmentome") of the circulatory proteome, indicate that MALDI-TOF based approaches may be measuring

Table 10. NACB Recommendations for Use of MALDI-TOF MS in Cancer Diagnostics

1. MALDI-TOF MS profiling in the realm of cancer diagnostics should be considered an investigational and research tool that at this time, like all unvalidated methods, is insufficiently reliable to be the basis of clinical decisions.
2. For MALDI-TOF MS profiling to become a clinically reliable tool, it must undergo validation according to principles such as those described by Pepe et al (68) and avoid biases, as described by Ransohoff (128).
3. For MALDI-TOF MS profiling testing, validation of discriminatory peaks in the mass spectra should include statistically powered independent testing and validation sets that include large numbers of inflammatory controls and samples from patients with benign disorders and from healthy controls, as well as samples from patients with other cancers. The degree of statistical powering of the validation studies should be carried out under methods such as those described by Pepe et al (200) along with taking into consideration the intended clinical use of the test itself.
4. Stability of bioinformatic algorithms should be evaluated using large numbers of samples, preferably from several institutions and countries.
5. Standardized protocols should be developed for sample collection, handling and processing.
6. Quality control and reference materials for MALDI-TOF MS must be developed and used more widely to monitor and improve method reliability.
7. Protein/peptide sequence identification or specific immune recognition of the analytes facilitates reproducibility, robustness, and overall biomarker validation.

analytes that are disease specific, of lower abundance than previously thought, and novel.

As for all technologies that directly impact patient health, until extensive validation studies are performed, MALDI-TOF MS fingerprinting approaches should not be used as diagnostic tests for cancer in clinical practice. Investigators should perform thorough validation experiments following CAP/CLIA-based codes of good laboratory practice and should provide data in a transparent form for full evaluation by the scientific community. In experiments in which MS fingerprinting is being employed, appropriate independent validation sets should also be employed using inflammatory and benign controls along with high numbers of unaffected controls, since specificity will be an important determining factor of success in the clinic, especially for screening indications. Despite recent difficulties in extending research observations for genomic and proteomic profiling, the field is now evolving with a better understanding of potential sources of bias and instrument variances, as well greater appreciation of the stringent requirements for developing good laboratory practices and standard operating procedures that may make clinical adoption and validation achievable in the foreseeable future.

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Appendix

NACB Quality Requirements Subcommittee Members: Catharine M Sturgeon, Chair; Soo-Ling Ch'ng; Elizabeth Hammond; Daniel F. Hayes; and Györg Sölétormos. Dr Jean-Pierre Basayau and Professors Per Hyltoft Petersen, Mathias Müller, and Hans Schneider were invited expert reviewers.

NACB Microarray Subcommittee Members: Eleftherios P. Diamandis, Chair; Manfred Schmitt; and Elene van der Merwe. Professor Roger Ekins was an invited expert reviewer.

NACB Mass Spectrometry Subcommittee Members: Daniel W. Chan, Chair; O. John Semmes; Emmanuel F. Petricoin; Lance A. Liotta; Elene van der Merwe; and Eleftherios P. Diamandis. Professors William T. Morgan and Roz Banks were invited expert reviewers.