# *Immunoassay Standardization: Is It Possible, Who Is Responsible, Who Is Capable?*

Standardization in clinical chemistry aims at improving trueness, i.e., lack of bias, but it does not directly affect assay imprecision. Standardization is important because it facilitates clinical interpretation and comparison of results from various studies *(1, 2)*. This is especially important with analytes used for screening, and this was one reason that cholesterol assays have been standardized in the US *(3)*. Standardization of assays for prostate-specific antigen (PSA) is important for the same reason, and also because these assays are important for monitoring of disease progression in prostate cancer. Large differences in assay calibration lead to misinterpretations of the clinical course when different assays are used *(4)*. Variation in assays for chorionic gonadotropin (CG) *(5)* cause similar problems when women with pregnancy-related disorders are monitored by determination of CG in serum *(6)*. It is a testimony of the clinical importance of standardization that the preparation of standards for PSA was initiated and carried out by a urologist, Thomas Stamey from Stanford *(7)*.

Standardization is a fairly new concept in clinical chemistry. The notion of "comprehensive measurement systems" and various levels of reference methods evolved in the 1970s *(1, 2)* and was formulated by Tietz in 1979 *(8)*. Pure standards, reference methods, and standard reference materials (SRMs) are now available for many of the most important analytes determined by conventional chemical methods, but immunoassay standardization is less well developed. Immunoassay standardization was extensively discussed at two meetings organized in 1990 and 1992 by the IFCC. At these, two standardization projects were initiated: the one for cortisol was intended to serve as a model for hapten immunoassays, the other project, for standardization of assays for CG, was intended to serve as a pilot study for protein immunoassays.

The problems associated with standardization of protein and hapten assays are different. Pure standards and reference methods based on mass spectrometry (MS) are available for many steroid hormone assays *(9)*. For peptide and protein immunoassay standardization there are no reference methods, value assignment of the standard is problematic, and the analyte is mostly heterogeneous and differs from the standard. Therefore, progress in standardization of protein immunoassays has been slow.

I have served as the chairman of the Working Group for Standardization of CG *(5)* and been involved in other projects aimed at immunoassay standardization *(10, 11)*. This experience has affected my thinking, but I am solely responsible for the views expressed here. Problems associated with standardization of hapten assays have been dealt with extensively in the recent literature *(9, 12)* and will only briefly be touched on here. The aim of this opinion is to stimulate discussion rather than to present a comprehensive and balanced review.

## Is Immunoassay Standardization Possible?

Roger Ekins, one of the inventors of immunoassays, claims that standardization of immunoassays for heterogeneous antigens is impossible *(13)*. It is therefore necessary to examine the basic problems before discussing possible solutions.

The prerequisites for standardization are availability of standards and reference methods *(8, 14)*. The primary standard should be homogeneous, pure, and identical to the corresponding substance in the sample that is measured. It is obvious that these requirements can be met only for some hapten immunoassays. Reference methods often are classified as primary and secondary *(8)*. The primary method should preferably be a "definitive method", e.g., isotope dilution MS. Such methods are available for steroids *(9)* and thyroid hormones. Recent developments in MS have made it possible to analyze peptides and proteins, and isotope dilution MS has been used to develop reference methods for two proteins *(15, 16)* and thyroid hormones *(17)*, but not for the free hormones, which have largely replaced assays for total thyroxine and triiodothyronine. However, MS is not useful for proteins and peptides occurring at low concentrations. In the absence of definitive methods, the best method available may be defined as the primary reference method, but this approach has not been applied in practice.

The problems hampering immunoassay standardization are different for different analytes. With steroid hormones, cross-reactions between closely related forms and complex formation with binding proteins in the sample are major problems. Previously used extraction methods are not compatible with automation, and the use of direct methods with short incubation times on automatic analyzers has actually aggravated the problem *(5)*. Although reference methods are available and standardization possible *(12)*, the accuracy and sensitivity of many automatic steroid assays are disappointing *(18)*. Immunoassay of urinary free cortisol is especially problematic because of the large excess of cross-reacting substances in urine. The use of immunoassay for this purpose is dubious, but urinary free cortisol can be accurately determined by HPLC *(19)*. However, this method is used by only 3 of 118 laboratories participating in the British quality assessment scheme (UKNEQAS) *(20)*. The clinicians in our hospital accept the higher costs of the HPLC assay because falsely increased immunoassay results often lead to much higher additional costs in the form of imaging examinations *(21)*. I believe that we should not give in to pressures to reduce laboratory costs when this leads to unacceptable compromises.

Peptide and protein antigens will always represent a problem for immunoassay standardization because of the

heterogeneity of the antigens occurring in biological fluids *(13)*. In its strictest sense, standardization is possible only if standard and analyte are identical. This is the case with only a few small peptides, e.g., angiotensin-1 and -2. With larger peptides and proteins, the problems tend to become more complicated because biological samples often contain proforms, splice variants, fragments, and complexes of the analyte. Most hormones and tumor markers are glycoproteins. These are inherently heterogeneous, and it is virtually impossible to prepare a glycoprotein standard that is identical to the circulating form. There are no universal solutions to these problems, but if we identify them, accept some compromises, and set our ambitions at a realistic level, it is possible to improve the state of standardization of most immunoassays to a clinically adequate level. It is also necessary to identify analytes that cannot be appropriately standardized and to develop other solutions for these.

Many of the typical problems of immunoassay standardization have been encountered in the CG project, and the solutions that the CG working group has arrived at may be applicable to other similar assays. Intact CG is a heterodimer, but free subunits (CG$\alpha$ and CG$\beta$) and partially degraded (nicked) forms (CGn and CG$\beta$n) also occur in circulation and are measured to a variable extent by immunoassays *(5)*. Furthermore, a core fragment of CG$\beta$ (CG$\beta$cf) occurs in urine and is a potentially useful tumor marker. International standards are available for only part of these forms, and the CG standard is contaminated by nicked forms *(22)*.

### PRIMARY STANDARDS AND ASSIGNMENT OF VALUES FOR THESE

Many of the original hormone standards consisted of partially purified preparations, the potencies of which were determined by bioassay and expressed in arbitrary international units (IUs). IUs have also been assigned to some analytes without known biological activity (e.g., $\alpha$-fetoprotein, carcinoembryonic antigen, CG$\alpha$, and CG$\beta$). IUs based on bioactivity are important for characterization of therapeutic hormone preparations and standardization of biological assays. However, they are not necessarily well suited for immunoassays, and when new hormone standards are introduced, the ratio between biological activity and immunoreactivity is likely to change *(5)*. Furthermore, tracing of the values back to previously used impure standards loses its scientific basis when assay specificity is changed by the introduction of methods based on monoclonal antibodies.

The IFCC recommends the use of International System of Units (SI) units, and this is possible if the standard can be isolated in pure form. The primary structures of all clinically important hormones are known, and they can either be purified from natural sources or expressed by recombinant techniques. Therefore, their molar concentrations can be determined by amino acid analysis, and this should be the primary method for value assignment of new standards. The new CG standards will be assigned values by this method and expressed in substance concentrations, i.e., mol/L *(5)*. This is based on the following arguments:

- The molar concentration of a pure protein can be accurately determined by amino acid analysis.
- Antibodies primarily recognize peptide epitopes, which are related to the amino acid composition. Physiological variation in carbohydrate composition causes variation in bioactivity and weight of the preparation, but it rarely affects immunoreactivity.
- Immunoassays measure immunoreactivity rather than bioactivity.
- Molar concentrations are logical when comparing concentrations of related compounds with large differences in molecular weight, e.g., intact CG and subunits or free and complexed PSA.

The new CG standards are currently being evaluated by the National Institute of Biological Standards and Control (NIBSC; United Kingdom) for suitability as international standards. The biological activity will be determined for intact CG, and the standards will be cross-calibrated by immunoassay against the currently used standards. Thus, either IUs or molar concentrations can be used. When new standards need to be prepared, the ratio between molar concentration and immunoreactivity can be maintained, i.e., the determination of protein content by amino acid analysis will guarantee continuity of the value assignment. The molecular weight of the various CG preparations has been determined by MS, and the mass of protein in the standards can be calculated on the basis of the average molecular weight *(23)*.

### ASSAY DESIGN

Most protein antigens are now determined by sandwich assays using two monoclonal antibodies or a combination of a monoclonal antibody and a polyclonal antiserum reacting with different epitopes. This facilitates tuning of assay specificity, e.g., CG assays can be designed to detect only heterodimeric CG, only CG$\beta$, or both components at the same time *(5)*. In many cases, the specificity obtained by sandwich assays improves clinical utility, e.g., determination of the proportion of free or complexed PSA provides better cancer specificity than total PSA *(24)*, and assays specific for CG$\beta$ provide better sensitivity for nontrophoblastic cancer than assays that detect both CG and CG$\beta$ *(25)*. Assays measuring two components together tend to underestimate either one *(26)*, but at least for PSA it has been possible to design assays that recognize free and complexed forms equally *(27)*. Thus, standardization may be possible even when the analyte occurs in quite different forms in serum.

Partial proteolytic degradation of the peptide chain or nicking is fairly common among hormones and tumor

markers, and this may cause variation in immunoreactivity. Some antibodies recognize intact and nicked forms equally, whereas others underestimate nicked forms of CG *(28)* and PSA *(29)*. It is therefore important to know the reactivity of the antibodies used in relation to the characteristics of analyte and standard.

### EPITOPE MAPPING

The specificity of an assay is dependent mainly on the epitope specificity of the antibody used. Detailed epitope maps are available for CG *(30, 31)*, and this was an important reason to choose it as a model compound for immunoassay standardization *(5)*. The International Society for Oncodevelopmental Biology and Medicine (ISOBM) has recently organized epitope mapping of several clinically important tumor markers in a series of workshops, in which the major immunoassay manufacturers have participated *(10, 32–34)*. These epitope maps form the basis for selection of antibodies for routine and reference methods.

### REFERENCE METHODS

Reference methods have been established for most clinically important steroid hormones *(9)* but only for two proteins, i.e., hemoglobin A1c *(16)* and apolipoprotein A-1. These methods are based on proteolytic digestion of the protein in the crude sample, which releases a peptide that is determined by MS. Because this approach is applicable only to proteins occurring at high concentrations, the working group for CG has planned to develop immunological reference methods. This is possible principally because standards *(23)* and antibodies with well-defined epitope specificity are available *(11)*. However, the companies supporting the CG project have not embraced this idea, and unless accepted by the industry, reference methods are of limited value. The main argument has been that an immunological reference method may be too dependent on a certain assay technology. Thus, even with the same antibodies and standards, different results could be obtained when shifting from a manual reference method to an automated routine assay. To be acceptable, a reference method would need to be insensitive to matrix effects, applicable to some generally available assays formats, and based on international standards and readily available monoclonal antibodies with known epitope specificities. It should be possible to either establish the reference method in any laboratory or to maintain a network of reference laboratories similar to that established for cholesterol *(3)*. However, the feasibility of immunological reference methods first needs to be demonstrated in pilot studies.

### SERUM-BASED REFERENCE MATERIALS

Panels of sera in which the content of one or several analytes has been determined by a definitive method have been prepared for many chemical and some immunochemical assays. These secondary standards are called SRMs by the NIST and Certified Reference Materials by the Institute for Reference Materials and Measurements (IRMM) *(12)*. Standardization of serum protein determinations has been accomplished by preparation of a serum-based standard in which values were assigned to the most commonly determined serum proteins *(35)*. The CG working group has also considered distributing calibrated serum samples *(23)*, but this is feasible only if reference methods are available. The use of serum-based secondary standards has the advantage that the matrix is similar to that in clinical samples. However, this does not eliminate the matrix problem, which varies from one sample to another. Furthermore, different immunoassays have different matrix problems.

### USING COMMERCIAL ASSAYS AS REFERENCE METHODS

Although assay manufacturers are skeptical about immunological reference methods, they often use a well-respected commercial assay or that of the market leader to calibrate their assays *(18)*. Would it be possible to utilize this principle for standardization? Could an expert group investigate available assays and declare the best one a reference method? This approach has several advantages: it is economical, it could be effectuated rapidly, and the reference method would be readily available. However, it also carries risks. The life span of the method might be limited because of changes in assay technology and company management. Furthermore, having the official reference method might be considered an unfair advantage by competitors. Because of this, it appears necessary to establish reference methods that are independent of commercial companies, but the use of commercial methods could be an interim solution in selected cases.

## Who Is Responsible?

Several organizations are involved in various aspects of immunoassay standardization, but the responsibility of each of these has not been defined. At the top of the standardization hierarchy is the International Standardization Organization (ISO), to which national and regional standardization organizations that have established standards and reference materials for substances of importance in clinical chemistry, e.g., NIST in the United States and the Community Bureau of Reference (BCR) in Europe, report. The WHO issues standards for most biologically important hormones and several other clinically important antigens determined by immunoassay. These standards are validated by the NIBSC in the United Kingdom, and if found acceptable, are issued as International Standards by the Expert Committee on Biological Standards (ECBS) and distributed by NIBSC. To date, WHO deals only with standards but not with assays and reference methods. Other important organizations include the NIH, which provides hormone preparations for research purposes, and the CDC. In addition to these official bodies, several professional organizations have

projects concerning immunoassay standardization, e.g., IFCC, the International Union for Pure and Applied Chemistry (IUPAC), ISOBM, the College of American Pathologists (CAP), NCCLS, and the American and Canadian societies for clinical chemistry, AACC and CFCC.

Although quite a few organizations deal with standardization, it is not clear who is responsible for what. Because standardization is an international rather than a national or regional problem, it is desirable that one international organization should be responsible for the coordination of various standardization projects.

## Who Is Capable?

The requirements imposed by various bodies controlling the use of immunoassays vary from one country to another. The need to obtain certification for new assays by the Food and Drug Administration (FDA) in the US has led to considerable restrictions in assay availability, but this does guarantee that the assays are properly standardized. The European Community (EC) has recently issued the In Vitro-Diagnostics Directive (IVDD; directive 98/79/EC), which should be implemented by 2003. It states that "the traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order". The European Committee for Standardization (CEN or CENELEC) has authorized its technical committees to develop standards. Those for in vitro medical devices are handled by CEN/TC 140, which works in close collaboration with the ISO technical committee ISO/TC 212. NCCLS has the Secretariat responsibility for ISO/TC 212 and is the home of the National Reference System for the Clinical Laboratory (NRSCL). However, the agency or authority responsible for authorization of reference systems has not yet been determined. Methods conforming to the IVDD may be labeled "CE", but this label is not required for accreditation of a method in Europe. These requirements are determined by national authorities. I believe that proper standardization should be required for registration and licensing once the rules have been defined and the tools, i.e., standards and reference methods, have been made available. However, it is obvious that reference systems for protein immunoassays will not be available by 2003, when the IVDD should be implemented.

The actual work on development and validation of standards and reference methods is performed by experts in working groups. A potential problem is that many of the experts either have a vested interest in antibodies and assay principles or they have close collaboration with, or are employed by, companies. If we exclude these, the number of capable experts will be substantially reduced. Utilization of all expertise available, including that in the diagnostic industry, appears both necessary and desirable.

At least for the CG project, the working group has also raised most of the funding from assay manufacturers. This is not a viable long-term solution, and it may be hard to engage capable experts unless funding is guaranteed. Although many aspects of standardization involve pure science, it is not easy to compete for research money with standardization projects. Therefore, these should be funded from other sources. Most laboratories spend considerable resources on quality assurance, which like standardization serves the aim of improving the quality of laboratory results. I think that laboratorians would be willing to pay for better standardization if the mechanisms were available and progress could be guaranteed.

## Alternative Approaches

In addition to being theoretically problematic and practically complicated, standardization is also time-consuming and expensive. The preparation of PSA standards was first discussed at two meetings in Stanford organized by Thomas Stamey in 1992 and 1994, and the final report on these standards just appeared in this journal *(36)*. The CG project was initiated at the same time, and the standards will probably be available this year. This time scale is typical; therefore, the problems of immunoassay standardization will not be solved within this or even the next decade even if funding was made available. We therefore need to consider the following interim solutions.

### leave the problem to the industry

Currently, most of the standardization problems are taken care of by assay manufacturers, and this situation will not change soon. Appropriate standardization is an important sales argument, and much expertise on this subject is found within companies. In spite of this the results are unsatisfactory, which can be blamed on both the lack of reference systems and technical difficulties. These problems have actually become worse because of the inflexibility of automated analyzers with respect to assay design. Therefore, acceptable standardization may not be possible for all assays on all instruments. Although this problem is understandable, it is also a reason not to leave solving the problem completely to the industry.

### harmonization

If standardization is not possible, harmonization can be used to calibrate various assays to give the same results. However, perfectly harmonized assays may all be biased. Harmonization would solve some of the clinical problems, but from a principal point of view it is dubious, and maintenance of calibration is problematic in the absence of reference methods. Therefore, standardization should be the goal whenever possible. For assays of antigens that have not been defined, e.g., CA 125, only harmonization is possible at present.

### method-specific reference values

With the present state of standardization, reference values need to be determined separately for each assay. The

laboratorian is ultimately responsible for the reference values used, but I believe that assay manufacturers should be obliged to prepare these according the recommendations of the IFCC. At present, few companies do this. Establishment of reference values can be quite demanding, especially for hormones, the concentrations of which are dependent on age and sex. Therefore, collaboration among laboratorians, clinicians, and assay manufacturers is necessary. Ideally, panels of samples from well-characterized reference populations should be established and made available to assay manufacturers. This could be accomplished in multicenter collaborative studies, and it would probably be economically feasible because of the value of such panels. Method-specific reference values are necessary because of the unsatisfactory standardization, but they do not replace standardization.

### RISK CALCULATION

The interpretation of laboratory results can be significantly improved by providing the results not only as concentrations but also as an estimate of the probability that the result indicates a certain diagnosis. This approach is especially useful for interpretation of results of multiple determinations as used in screening for Down syndrome *(37)*. Recently, risk calculation on the basis of free and total PSA in combination with clinical data has been shown to substantially improve the diagnostic accuracy for prostate cancer *(38)*. Estimation of the risk of cardiovascular disease can be substantially improved by combing the results for total and HDL cholesterol and C-reactive protein *(39)*. The results are not dependent on standardization and reference values, but the algorithms are valid only for the assays used to establish them unless the assays are identically standardized. Development of risk calculation algorithms is demanding because it requires both reference groups of subjects who do not develop the disease and large groups of patients with accurately established diagnoses. Therefore, this process is more demanding than establishment of reference values, and use of risk calculation algorithms will increase rather than decrease the need for standardization.

### EXTERNAL QUALITY ASSESSMENT

The results of quality assessment programs provide valuable information about the comparability of various methods, but they do not tell whether an assay is properly standardized. More information is obtained if the quality-control samples have been supplemented with pure standards or analyzed by a reference method *(40)*. The results for such samples reflect the state of standardization, but assay-specific matrix effects and commutability problems make it impossible to judge the calibration of individual assays on the basis of the results of a few quality-control samples *(41)*.

## Conclusions

I do not make any claims to have provided adequate answers to the questions posed in the title of this article, but I would like to present a list of tasks and problems that need to be addressed:

- *Definition of goals*. Because standardization of many immunoassays requires compromises, we need to define the quality required for various assays considering clinical needs and theoretical and practical limitations.
- *Setting priorities*. Because resources are limited and standardization is time-consuming and expensive, we need to determine in which order assays should be standardized.
- *Evaluation of the applicability of immunological reference methods*. Can we specify reference methods accurately enough that they can be used anywhere, or should we establish a network of reference laboratories or prepare serum-based secondary standards? These alternatives need to be tested.
- *Collaboration and agreement on responsibilities*. A common organ for organizations working on immunoassay standardization should be established and the responsibilities of the various organizations determined.
- *Funding*. Without additional funding, improvement is not possible. Organizations responsible for accreditation, licensing, and quality assessment of assays should have an interest in this. The funding problems would be solved if a small percentage of the money spent on these activities could be diverted to standardization.

## References


1. Cali JP. An idea whose time has come. Clin Chem 1973;19:291–3.
2. Rodgerson DO, Tietz NW. Selection of "recommended methods" for use in a clinical laboratory—some urgent considerations and a suggested approach [Editorial]. Clin Chem 1975;21:1057–8.
3. Myers GL, Kimberly MM, Waymack PP, Smith SJ, Cooper GR, Sampson EJ. A reference method laboratory network for cholesterol: a model for standardization and improvement of clinical laboratory measurements. Clin Chem 2000;46:1762–72.
4. Semjonow A, Brandt B, Oberpenning F, Roth S, Hertle L. Discordance of assay methods creates pitfalls for the interpretation of prostate-specific antigen values. Prostate Suppl 1996;7:3–16.
5. Stenman UH, Bidart JM, Birken S, Mann K, Nisula B, O'Connor J. Standardization of protein immunoprocedures. Choriogonadotropin (CG). Scand J Clin Lab Invest Suppl 1993;216:42–78.
6. Cacciatore B, Stenman U-H, Ylöstalo P. Early screening for ectopic pregnancy in high-risk symptom-free women. Lancet 1994;343:517–8.
7. Stamey TA, Chen Z, Prestigiacomo AF. Reference material for PSA: the IFCC standardization study. International Federation of Clinical Chemistry. Clin Biochem 1998;31:475–81.
8. Tietz NW. A model for a comprehensive measurement system in clinical chemistry. Clin Chem 1979;25:833–9.
9. Stöckl D, Franzini C, Kratochvila J, Middle J, Ricos C, Siekmann L, Thienpont LM. Analytical specifications of reference methods compilation and critical discussion (from the members of the European EQA-Organizers Working Group B). Eur J Clin Chem Clin Biochem 1996;34:319–37.
10. Stenman UH, Paus E, Allard WJ, Andersson I, Andres C, Barnett TR, et al. Summary report of the TD-3 workshop: characterization of 83 antibodies against prostate-specific antigen. Tumour Biol 1999;20:1–12.
11. Gerth R, Schleret S, Bidart J-M, Sturgeon C, Birken S, Stenman U-H, Berger P. Three dimensional molecular epitope assignment of monoclonal antibodies (mAb) against hCG and derivatives [Abstract]. Tumour Biol 2000;21S1: 86.
12. Thienpont LM. Standardization of steroid immunoassays—in theory an easy task. Clin Chem Lab Med 1998;36:349–52.
13. Ekins R. Immunoassay standardization. Scand J Clin Lab Invest Suppl 1991;205:33–46.

14. Jeffcoate SL. Role of reference materials in immunoassay standardization. Scand J Clin Lab Invest Suppl 1991;205:131–3.
15. Barr JR, Maggio VL, Patterson DG Jr, Cooper GR, Henderson LO, Turner WE, et al. Isotope dilution-mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. Clin Chem 1996;42:1676–82.
16. Kobold U, Jeppsson JO, Dulffer T, Finke A, Hoelzel W, Miedema K. Candidate reference methods for hemoglobin A1c based on peptide mapping. Clin Chem 1997;43:1944–51.
17. Thienpont LM, Fierens C, De Leenheer AP, Przywara L. Isotope dilution-gas chromatography/mass spectrometry and liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of triiodo-L-thyronine in serum. Rapid Commun Mass Spectrom 1999;13:1924–31.
18. Stenman U-H. Immunoassay standardisation. In: Price CP, Newman DJ, eds. Principles and practices of immunoassay. London: Macmillan, 1997:245–68.
19. Murphy BE. How much "UFC" is really cortisol? [Editorial; Comment]. Clin Chem 2000;46:793–4.
20. Middle JG. Urinary free cortisol. In: Annual review for the UK NEQAS for Clinical Chemistry Specialist Advisory Group for Immunoassay, November 1999. Birmingham, UK: UK NEQAS, 2000:19–23.
21. Turpeinen U, Markkanen H, Valimaki M, Stenman UH. Determination of urinary free cortisol by HPLC. Clin Chem 1997;43:1386–91.
22. Birken S, Chen Y, Gawinowicz MA, Lustbader JW, Pollak S, Agosto G, et al. Separation of nicked human chorionic gonadotropin (hCG), intact hCG, and hCG$\beta$ fragment from standard reference preparations and raw urine samples. Endocrinology 1993;133:1390–7.
23. Sturgeon C, Stenman U-H, Bidart J-M, Birken S, Berger P, Lequin R, et al. IFCC Working Group for Standardisation of hCG Measurements: progress report. Proc UK NEQAS Meeting 1998;3:94–103.
24. Stenman U-H, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, Alfthan O. A complex between prostate-specific antigen and $\alpha$1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: assay of the complex improves clinical sensitivity for cancer. Cancer Res 1991;51:222–6.
25. Alfthan H, Haglund C, Roberts P, Stenman U-H. Elevation of free $\beta$ subunit of human choriogonadotropin and core $\beta$ fragment of human choriogonadotropin in serum and urine of patients with malignant pancreatic and biliary disease. Cancer Res 1992;52:4628–33.
26. Graves HC. Issues on standardization of immunoassays for prostate-specific antigen: a review. Clin Invest Med 1993;16:415–24.
27. Mitrunen K, Pettersson K, Piironen T, Bjork T, Lövgren T. Dual-label one-step immunoassay for simultaneous measurement of free and total prostate-specific antigen concentrations and ratios in serum. Clin Chem 1995;41:1115–20.
28. Cole LA, Kardana A. Discordant results in human chorionic gonadotropin assays. Clin Chem 1992;38:263–70.
29. Leinonen J, Zhang W, Paus E, Stenman U-H. Reactivity of 77 antibodies to prostate-specific antigen with isoenzymes and complexes of prostate-specific antigen. Tumour Biol 1999;20(Suppl 1):28–34.
30. Bidart JM, Birken S, Berger P, Krichevsky A. Immunochemical mapping of hCG and hCG-related molecules. Scand J Clin Lab Invest Suppl 1993;216:118–36.
31. Berger P, Bidart JM, Delves PS, Dirnhofer S, Hoermann R, Isaacs N, et al. Immunochemical mapping of gonadotropins. Mol Cell Endocrinol 1996;125:33–43.
32. Nustad K, Bast RC Jr, Brien TJ, Nilsson O, Seguin P, Suresh MR, et al. Specificity and affinity of 26 monoclonal antibodies against the CA 125 antigen: first report from the ISOBM TD-1 workshop. International Society for Oncodevelopmental Biology and Medicine. Tumour Biol 1996;17:196–219.
33. Price MR, Rye PD, Petrakou E, Murray A, Brady K, Imai S, et al. Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin, San Diego, CA, November 17–23, 1996. Tumour Biol 1998;19:1–20.
34. Stigbrand T, Andres C, Bellanger L, Bishr Omary M, Bodenmuller H, Bonfrer H, et al. Epitope specificity of 30 monoclonal antibodies against cytokeratin antigens: the ISOBM TD5–1 Workshop. Tumour Biol 1998;19:132–52.
35. Whicher JT, Ritchie RF, Johnson AM, Baudner S, Bienvenu J, Blirup-Jensen S, et al. New international reference preparation for proteins in human serum (RPPHS). Clin Chem 1994;40:934–8.
36. Rafferty B, Rigsby P, Rose M, Stamey T, Gaines Das R. Reference reagents for prostate-specific antigen (PSA): establishment of the first international standards for free PSA and PSA (90:10). Clin Chem 2000;46:1310–7.
37. Wald NJ, Cuckle HS, Densem JW, Nanchahal K, Royston P, Chard T, et al. Maternal serum screening for Down's syndrome in early pregnancy. BMJ 1988;297:883–7.
38. Virtanen A, Gomari M, Kranse R, Stenman U-H. Estimation of prostate cancer probability by logistic regression: free and total prostate-specific antigen, digital rectal examination, and heredity are significant variables. Clin Chem 1999;45:987–94.
39. Rifai N, Ridker P. Proposed cardiovascular risk assessment algorithm using high-sensitivity C-reactive protein and lipid screening. Clin Chem 2001;47:28–30.
40. Middle J. Standardization of steroid hormone assays. Ann Clin Biochem 1998;35:354–63.
41. Thienpont LM, Stockl D. EQA with accuracy-based kits: bringing together consensus/reference method targets and method dependent targets. Proc UK NEQAS Meeting 1994;1:44–7.



**Ulf-Håkan Stenman**

*Department of Clinical Chemistry*
*Helsinki University Central Hospital*
*POB 140*
*FIN-00029 Helsinki, Finland*
*Fax 358-9-47174804*
*E-mail ulf-hakan.stenman@hus.fi*