

Standardization of Two Immunoassays for Human Glandular Kallikrein 2

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Background: Measurement of human kallikrein 2 (hK2) has improved early detection and staging of prostate cancer. However, reported concentrations of hK2 among currently used assays have not been standardized in any way. We compared two hK2 assays and five different recombinant hK2 variants (rhK2) and suggest a common calibrator as an important step and putative reference substance in hK2 assay standardization.

Methods: We measured 146 sera by two hK2 assays, using assay-specific calibrators to assess the difference between the two assays. Serial dilutions of five rhK2 preparations were measured repeatedly, with one preparation assigned as calibrator and the others as unknowns to define which variant provided the closest match between the two assays. This rhK2 variant was used to recalibrate both assays. We measured hK2 concentrations in the same 146 patients to evaluate the change in the difference.

Results: Use of assay-specific calibrators for comparison of the two assays yielded a Deming regression equation of: $y = 0.789$ (95% confidence interval, 0.674–0.922) $x + 0.014$ (0.004–0.025) $\mu\text{g/L}$; $R^2 = 0.667$. Analysis of five rhK2 variants revealed that the enterokinase (ek)-rhK2 form provided the best match between both assays. Using the ek-rhK2 as a common calibrator, we observed a change in the slope of the regression curve to: $y = 1.106$ (0.872–1.340) $x + 0.006$ (–0.002 to 0.016) $\mu\text{g/L}$; $R^2 = 0.648$,

suggesting an increase in the mean estimate of agreement between the two assays.

Conclusion: Calibration with a common calibrator substantially increased agreement between the assays. The ek-rhK2 variant provided the best performance of all tested rhK2 variants and should undergo mass spectrometry and amino acid analysis for exact mass determination and value assignment to evaluate its potential as a reference material for immunoassays for hK2.

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Human glandular kallikrein 2 (hK2)⁶ is a prostatic secretory protein (1) expressing 80% identity in amino acid (2) sequence to prostate-specific antigen (PSA). The physiologic link of hK2 to PSA may be its ability to cleave the propeptide of inactive pro-PSA, thus converting it into its mature active form (3, 4). Both hK2 and PSA are expressed under androgen control (5). Despite their similar origins, important differences exist between these proteins. Immunohistochemical studies demonstrated different tissue expression of hK2 and PSA. PSA is more intensively expressed in benign prostatic hyperplasia (BPH) than in prostate cancer (PCa). In PCa, expression is higher in low- compared with high-grade tumors. By contrast, the most intense staining of hK2 appears in high-grade PCa and lymph node metastases compared with low-grade PCa and is weaker still in BPH (6, 7).

Similar to PSA (8–10), hK2 forms complexes with antiproteases in seminal plasma (11) and blood (12), but hK2 concentrations are only as high as 1–2% of PSA concentrations in blood (13–16). Gel-filtration data show that 80–95% of detectable hK2 in blood occurs as free,

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⁶ Nonstandard abbreviations: hK2, human glandular kallikrein 2; PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; PCa, prostate cancer; rhK2, recombinant hK2; ek, enterokinase; Xa-rhK2, recombinant pro-hK2 that can be activated by factor Xa; TWT-rhK2, Turku wild-type recombinant hK2; MAAb, monoclonal antibody; thK2, total hK2; RLU, relative light unit(s); and CI, confidence interval.

noncomplexed 30-kDa forms (13, 16, 17), which may in part consist of catalytically inactive pro-hK2 (18). Five to 20% of hK2 is 90 kDa in size, which corresponds to the size predicted for hK2 complexed to antiproteases (12). The correlation coefficient of hK2 and PSA concentrations in serum is usually <0.6, suggesting that hK2 might provide clinical information additional to and independent of that provided by PSA. Because of the low concentration of hK2 and its homology to PSA, considerable effort has been necessary to develop immunoassays for specific and sensitive hK2 detection (13–20). Most of this work has been performed by groups from the US, Canada, and Scandinavia. Table 1 is a review of key studies on the development and clinical application of such assays.

Despite encouraging results for early detection and improved staging of clinically localized PCa, a major concern is the difference in reported serum concentrations of hK2. Klee et al. (19), who measured free hK2, and Black et al. (17) reported median hK2 concentrations of 0.026 vs 0.402 $\mu\text{g/L}$ in healthy males. Nam et al. (20), using the assay described by Black et al. (17), observed a median hK2 concentration of 1.180 $\mu\text{g/L}$ in clinically localized PCa, whereas the median value obtained Becker et al. (21) in the same scenario was only 0.079 $\mu\text{g/L}$. Finally, Partin et al. (22) and Kwiatkowski et al. (23) analyzed the PSA diagnostic gray zone (4–10 $\mu\text{g/L}$) to discriminate between PCa and BPH. Whereas Partin et al. (22) found identical median concentrations of hK2 (0.081 $\mu\text{g/L}$) for BPH vs PCa, Kwiatkowski et al. (23) found a highly significant difference of 0.090 vs 0.135 $\mu\text{g/L}$.

A major improvement in the utility of PSA was the establishment of a standard PSA preparation as a common calibrator. Substitution of manufacturer-specific by the common calibrator reduced differences between nine commercial immunoassays, and this preparation has been issued by the WHO as an official standard for PSA (24).

In contrast to the number of PSA assays at the time of standardization, we currently have only a limited number of hK2 assays. The time therefore seems favorable to standardize the two major hK2 immunoassays. A standardized hK2 assay will not only improve the comparability of different studies, but may also shed more light on the sometimes conflicting performance of hK2 as a PCa marker. Possibly hK2 can provide information that PSA fails to deliver, e.g., improved prediction of organ-confined cancer (25–27) or improved specificity in the early detection of PCa (21, 28).

In this study we assessed the differences in values measured by the two immunoassays for hK2 for identical patient samples and provide evidence for the usefulness of a reference reagent to serve as potential standard for detection of hK2 in serum.

Materials and Methods

PURIFIED RECOMBINANT hK2 VARIANTS

A total of five different recombinant (rhK2) variants were available. Harry G. Rittenhouse and Judith A. Finlay (Hybritech Inc, San Diego, CA) provided:

Table 1. Overview of studies describing development and clinical applications for immunoassays for hK2.

Authors	MAb combination	Functional LDL, $\mu\text{g/L}$	Median hK2, $\mu\text{g/L}$				Total PSA, $\mu\text{g/L}$	Utility
			Healthy men	BPH	PCa loc ^a	PCa adv		
Piironen et al. (13)	Scavenger MAb 2H11 MAbs H50/H117	0.1					1–3400	NS
Kwiatkowski et al. (23)	Scavenger MAbs 2H11, 10,36/ MAbs 6H10/H50	0.05		0.09	0.135		4–10	PCa/BPH
Finlay et al. (14)	MAb G586/ MAb H449	0.120	0.330	0.886	6.770 ^b	+ ^b	0–6174	NS
Klee et al. (19)	MAb 449/599	0.004	0.026		0.072	0.116	7.4/9.3 ^c	NS
Black et al. (17)	MAb G586 MAb 8301 (hK2) MAb 8311 (PSA)	0.006	0.402				0.01–4.7	NS
Partin et al. (22)	MAbs 284/277			0.081	0.081		2–10	PCa/BPH
Nam et al. (20)	See Black et al. (17)	0.006		0.447	1.180		4–144	PCa/BPH
Becker et al. (21)	Scavenger MAbs 2H11, 10,36,2E9/ MAbs 6H10/H50	0.05		0.58	0.79		3–92	PCa/BPH
Haese et al. (26)	See Becker et al. (21)	0.05			0.09	0.30	3.5–68	oc/noc
Recker et al. (25)	See Becker et al. (21)	0.05			0.06	0.153	6.1/9.3 ^c	oc/noc
Finlay et al. (15)	MAb 284.4/277.4	0.008	0.013		0.085 ^d		2–10	NS
Haese et al. (27)	See Becker et al. (21)	0.050			0.08	0.120	<10	oc/noc

^a loc, clinically localized; adv, clinically advanced; NS, clinical application not specified; oc/noc, distinction of organ-confined vs non-organ-confined cancer.

^b Localized and advanced PCa.

^c Median tPSA concentration for both disease entities.

^d Clinical history of patients not known.

- (a) A wild-type rhK2 (HWT-rhK2) variant stock solution supplied at a concentration of 7399 $\mu\text{g/L}$. The preparation (29) and value assignment by mass spectrometry and amino acid analysis (15) have been described previously.
- (b) A mutated recombinant pro-hK2 variant of hK2 in which alanine at position 217 is replaced by valine (hence termed Ala-rhK2), described by Mikolajczyk et al. (29). The stock concentration was 8746 $\mu\text{g/L}$. As with HWT-rhK2, value assignment was done by mass spectrometry and amino acid analysis (15).

Other rhK2 variants were provided by Ville Vaisanen (University of Turku, Turku, Finland):

- (c) A recombinant proform of hK2 with a propeptide mutation in five of seven amino acids with increased stability and reduced activity that, because of the mutation, can be activated by enterokinase (ek-rhK2). The recombinant hK2 forms were generated using specific cell lines described by Lövgren et al. (30). The supernatants of the cell lines were purified and separated into stock solutions. The concentration of ek-rhK2 was determined by a PSA immunoassay that, to our knowledge, should fully cross-react with all known total, free, and pro-hK2 forms. The stock concentration was 10074 $\mu\text{g/L}$.
- (d) A recombinant pro-hK2 with a propeptide mutation at two of seven amino acids that can be activated by factor Xa (Xa-rhK2), in a concentration of 4306 $\mu\text{g/L}$ determined by immunoassay as described for the ek-rhK2 variant.
- (e) A recombinant, mature, one-chain wild-type hK2 variant (TWT-rhK2) with a concentration of 851.5 $\mu\text{g/L}$. Like the ek-rhK2 and Xa-rhK2, the concentration was determined by immunoassay as described.

HYBRITECH TOTAL hK2 ASSAY

Reagents were obtained from Beckman Coulter manufacturing and modified to run the assay similar to the microplate assay described earlier (15). The reagents were as follows: reagent packs containing mouse monoclonal anti-hK2 alkaline phosphatase conjugate, paramagnetic beads with streptavidin coating, a biotinylated tracer mouse anti-total hK2 (thK2) MAb, calibrators for a five-point calibration curve ranging from 0.00 to 3 $\mu\text{g/L}$, quality controls, substrate, wash buffer, and reaction vessels.

The Hybritech Access[®] thK2 assay is for research use only and not for use in diagnostic procedures. It is a two-site immunoenzymatic sandwich assay using a MAb/MAb antibody combination described earlier (15) and is performed on the automated Beckman Access system. The calibrator is the Hybritech wild-type rhK2 mentioned above. Initially, 50 μL of sample or control is pipetted into a reaction vessel with mouse monoclonal anti-thK2 alkaline phosphatase conjugate, paramagnetic particles coated with streptavidin, and a second, biotinyl-

ated mouse anti-thK2 MAb. The hK2 binds to the immobilized MAb in the solid phase; at the same time, the monoclonal anti-thK2 alkaline phosphatase conjugate reacts with a different epitope on the sample hK2. Subsequently, a magnetic field and washing remove unbound reactant. The chemiluminescent substrate is added, and the light generated, proportional to the concentration of thK2 in the sample, is measured by a luminometer. The result is provided as relative light units (RLU). In this study, we established the calibration curve and thK2 concentration in the samples with use of Biograph Software, VS 2.0 (Biotek Instruments). In accordance with the routine measurement of hK2 with the Access assay, we used a point-to-point calibration curve. The Hybritech assay has a lower detection limit of 0.008 $\mu\text{g/L}$, and the cross-reactivity to PSA is <0.001%. The assay detects several hK2-serpin complexes, e.g., protease inhibitor 6, protein C inhibitor, α_1 -antichymotrypsin, or plasminogen activator inhibitor type I, as reported by Finlay et al. (15). The reported mean CV for the assay when operated as a manual assay using pooled serum samples with concentrations between 0.032 and 0.064 $\mu\text{g/L}$ was 13% (15). With the automated device in this study, we analyzed two serum pools with mean hK2 concentrations of 0.088 (low) and 0.291 $\mu\text{g/L}$ (high). Using one lot of reagents, we assayed 24 samples over 6 days; the between-run precision analysis revealed a SD of 0.007 $\mu\text{g/L}$ and a CV of 8.5% for the low pool and a SD of 0.038 $\mu\text{g/L}$ and a CV of 12% for the high pool.

TIME-RESOLVED FLUOROMETRIC ASSAY

Ville Vaisanen (University of Turku, Turku, Finland) provided high-capacity streptavidin-coated microtiter wells, buffer, wash concentrate, enhancement solution, and the following MAb solutions: (a) a solution containing biotinylated capture MAb 6H10, which recognizes thK2, free hK2, and hK2-serpins; (b) a solution containing blocking MAbs 2E9, 5F7, and 5H6; and (c) a solution containing europium-labeled detection MAb 7G1 and calibrators for a seven-point calibration curve ranging from 0.00 to 4.6 $\mu\text{g/L}$. The TRF immunoassay for thK2 detection (13, 16) is a three-step manual assay using a combination of PSA-specific blocking and hK2-specific capture and detection MAbs. The calibrator is the Xa-rhK2 mutant mentioned above. Initially, 200 μL of a 300 $\mu\text{g/L}$ dilution of biotinylated capture MAb 6H10 is pipetted into a high-capacity streptavidin-coated microtiter well. After a 1-h incubation and washing, 125 μL of a solution containing 1000 ng of PSA-blocking MAbs 2E9 and 5F7 and 500 ng of PSA-blocking MAb 5H6, and 75 μL of calibrator, control, or sample are added and incubated for 2 h, followed by a second washing step. In the third step, 200 μL of a solution containing 100 ng of europium-labeled tracer MAb 7G1-Eu plus 500 ng of blocking MAb 5H6 is added and incubated for 30 min. A last washing step removes unbound reactant. After a 5-min incubation with 200 μL of enhancement solution, the resulting emission, propor-

tional to the concentration of hK2 in the sample, is measured as cps using the Victor² (Wallac Oy) multilabel counter in the time-resolved immunofluorometric mode. Biograph Software, VS 2.0, was used to generate point-to-point calibration curves and to calculate the concentrations of samples. The TRF assay has a cross-reactivity with PSA of <0.01%. By diluting high-hK2 male sera into hK2-negative serum (10 replicates per dilution), we determined an analytical sensitivity of 0.0003 $\mu\text{g/L}$ and a functional sensitivity of 0.004 $\mu\text{g/L}$. Analytical sensitivity was defined as +2 SD of the zero calibrator, and functional sensitivity was defined as the lowest concentration at which the CV was <20%. Between-assay variation (CVs) at hK2 concentrations (18 replicates each) of 0.03, 0.1, and 0.5 $\mu\text{g/L}$ was 10%, 7%, and 11%, respectively. In this study, we analyzed two serum pools with mean hK2 concentrations of 0.069 (low) and 0.253 $\mu\text{g/L}$ (high). Using one lot of reagents, we assayed 24 samples over 6 days; between-run precision analyses revealed a SD of 0.01 $\mu\text{g/L}$ and a CV of 14% for the low pool and a SD of 0.024 $\mu\text{g/L}$ and a CV of 9.4% for the high pool.

SERUM SAMPLES

We obtained 237 serum samples stored at -70°C . Of these, 228 were from patients with clinically localized PCa scheduled for curative treatment, and 9 were from patients negative for PCa on prostate biopsy. All sera were obtained according to Institutional Review Board approval. Sera were drawn before prostatic manipulation. Patients undergoing antihormonal manipulation were not included. All samples were measured in triplicate.

CALIBRATOR CROSSOVER STUDY

We measured the seven-point calibrator of the TRF assay on the Hybritech assay and the five-point calibrator of the Hybritech assay on the TRF assay to assess observed vs expected concentrations of calibrator hK2 in either assay. All samples were measured in triplicate. Because results obtained at higher concentrations are likely to be more accurate, we excluded all concentrations $\leq 0.01 \mu\text{g/L}$ in the Hybritech assay and $\leq 0.0029 \mu\text{g/L}$ in the TRF assay to calculate the ratio.

MEASUREMENT OF THE FIVE DIFFERENT rhK2 VARIANT CALIBRATORS BY BOTH ASSAYS

For each of the recombinant hK2 variants (TWT-rhK2, Xa-rhK2, ek-rhK2, Ala-rhK2, and HWT-rhK2), we prepared serial dilutions (0, 0.01, 0.1, 1.0, 3.0, and 10.0 $\mu\text{g/L}$) using the stock solutions and assay buffer. The buffer consisted of 50 mmol/L Tris-HCl buffer (pH 7.75) containing 9 g/L NaCl, 0.5 g/L NaN_3 , 0.1 mL/L Tween 40, 0.5 g/L bovine γ -globulin, 20 $\mu\text{mol/L}$ diethylenetriamine pentaacetate, and 15 g/L bovine serum albumin. The dilutions were analyzed in both assays on 4 consecutive days in triplicate, yielding 12 measurements of each of the six concentrations of five different hK2 forms for each assay. Mean RLU and cps were calculated for the 12

measurements to represent calibrator or unknown samples. Subsequently, one hK2 variant (e.g., TWT-rhK2) was assigned as calibrator, and the others (Xa-rhK2, ek-rhK2, Ala-rhK2, HWT-rhK2) were assigned as unknowns. On the basis of their cps or RLU, we obtained the hK2 concentration of the unknown samples. The second variant (e.g., Xa-rhK2) was then used as calibrator and the variants TWT-rhK2, ek-rhK2, Ala-rhK2, and HWT-rhK2 were used as unknowns to obtain the hK2 concentrations when Xa-rhK2 was assigned to be calibrator. This procedure was repeated for all hK2 variants.

To identify the rhK2 variant that provided the smallest difference between the two assays, we calculated the ratio (as a percentage) between the hK2 concentrations obtained with both assays and performed Deming regression analysis. High and low (10.0, 0.00, and 0.01 $\mu\text{g/L}$) concentrations of the samples were excluded because the response of the samples in some cases was above or below that of the calibrators. Moreover, hK2 concentrations $\geq 10 \mu\text{g/L}$ seldom occur in clinical practice.

EVALUATION OF PATIENT SERA USING DIFFERENT CALIBRATORS

We measured the 237 described patient samples, using the assay-specific and common, ek-rhK2-based calibrator at concentrations of 0.00, 0.01, 0.1, 1.0, 3.0, and 10.0 $\mu\text{g/L}$. The ek-rhK2 calibrator was chosen to complement the standard calibrators because of the results of the previous evaluation of the five rhK2 variants and because it has a reported higher degree of stability than does the Xa-rhK2 variant (30). hK2 values $< 0.02 \mu\text{g/L}$ were excluded, yielding 146 evaluable patients. We compared the correlation of hK2 values measured by each assay with its specific calibrator vs those obtained with the ek-rhK2 calibrator. Again, Deming regression was applied.

SUBSET EVALUATION OF PATIENT SAMPLES

We aimed to describe the performance when the ek-rhK2 was used as a common calibrator compared with the performance of the assays when both the TRF and Hybritech assay were simultaneously calibrated with either the TRF calibrator (Xa-rhK2) or the Hybritech calibrator (HWT calibrator). For this purpose, a subset (148 of 237) patient samples were analyzed in parallel, with ek-rhK2 calibrator, the standard TRF, and Hybritech calibrators as unknowns to be used as calibrators in the subsequent evaluation of the patients. We calculated the hK2 concentration in the samples and controls based on the calibration curve obtained with the Hybritech calibrators, TRF calibrators, and ek-rhK2 calibrators when entered into Biograph software. This produced a total of three hK2 concentrations for each sample per assay. As previously, we excluded all patients with a hK2 concentration $< 0.02 \mu\text{g/L}$ for any of the six possible results, which produced a total of 80 evaluable patients. Deming regression analysis assessed the correlation of the 80 values for the following combinations:

- (a) TRF assay + TRF calibrator vs Hybritech assay + Hybritech calibrator
- (b) TRF assay + Hybritech calibrator vs Hybritech assay + Hybritech calibrator
- (c) TRF assay + TRF calibrator vs Hybritech assay + TRF calibrator
- (d) TRF assay + ek-rhK2 calibrator vs Hybritech assay + ek-rhK2 calibrator

Results

CALIBRATOR CROSSOVER STUDY

Overall, measurement of the Hybritech-specific calibrator on the TRF assay produced lower concentrations (60–90%) than anticipated. By contrast, when the TRF calibrators were measured in the Hybritech assay, much higher values (150–195%) were obtained. The complete results are shown in Table 2.

MEASUREMENT OF THE FIVE DIFFERENT hK2 VARIANT CALIBRATORS IN BOTH ASSAYS

Although all hK2 variants were diluted to the same concentrations, considerable variation was observed in response. The ek-rhK2 variant delivered the strongest response on the Hybritech and TRF assays. By contrast, the weakest signal was seen for the Turku wild-type rhK2 (TWT). These differences in signal intensity translated into higher or lower concentrations than expected. With respect to the ratio of the Hybritech to the TRF concentration of hK2 when ek-rhK2 was used for calibration, the mean ratio was 96%. The Deming regression equation was (values in parentheses are the 95% confidence interval): $y = 0.949 (0.820-1.078)x - 0.001 (-0.064 \text{ to } 0.041) \mu\text{g/L}$ ($R^2 = 0.978$), where x is the hK2 concentration measured by the Hybritech assay, and y is that measured by the TRF assay.

When λ a-rhK2 was assigned as calibrator, we obtained a regression equation of $y = 1.042 (0.894-1.191)x - 0.041 (-0.126 \text{ to } 0.045) \mu\text{g/L}$ ($R^2 = 0.977$) and a mean ratio of 98%, which suggested a performance comparable to that of the ek-rhK2 calibrator. When TWT was used as calibrator, the mean ratio was 126%, as reflected in the regression equation of: $y = 1.350 (1.228-1.471)x - 0.097 (-0.221 \text{ to } 0.026) \mu\text{g/L}$; $R^2 = 0.988$. The Hybritech wild type and alanine-mutated variant as calibrators gave values between those extremes, both in terms of the mean Hybritech:TRF ratio and their regression slopes. In contrast

to the difference in the regression slope across the five calibrators, R^2 was similar in all cases. Complete data are shown in Table 3.

EVALUATION OF PATIENT SERA USING DIFFERENT CALIBRATORS

The means, medians, and ranges for the hK2 concentrations obtained with the different calibrators are given in Table 4. In the total ($n = 146$) and subset ($n = 80$) analyses, for both the TRF and Hybritech assays, the lowest hK2 concentrations were obtained when the ek-rhK2 calibrator was used. The TRF- and Hybritech-specific calibrators provided the second highest and the highest hK2 values, respectively. Panels A and B in Fig. 1 summarize the effect of the common ek-rhK2 calibrator on the combined dataset of 146 patients. The use of the assay-specific calibrators for comparison of the two assays yielded a regression equation of: $y = 0.789 (0.674-0.922)x + 0.014 (0.004-0.025) \mu\text{g/L}$; $R^2 = 0.667$. When we used the ek-rhK2, we observe a change in the slope of the regression curve to: $y = 1.106 (0.872-1.34)x + 0.0067 (-0.002 \text{ to } 0.016) \mu\text{g/L}$ ($R^2 = 0.648$), suggesting an increase in the mean estimate of agreement between the two assays.

EVALUATION OF PATIENT SAMPLE SUBSETS

Shown in Fig. 2 are the regression plots for 80 patients when each of the four possible assay-calibrator combinations was used. When each assay was calibrated with its own calibrator (Fig. 2A), we obtained a regression equation of: $y = 0.908 (0.656-1.161)x + 0.012 (-0.004 \text{ to } 0.029) \mu\text{g/L}$; $R^2 = 0.506$. When both assays were calibrated using either the Hybritech or TRF calibrator, the regression equations were: $y = 1.502 (1.086-1.918)x - 0.003 (-0.027 \text{ to } 0.021) \mu\text{g/L}$ ($R^2 = 0.504$; Fig. 2B) and $y = 1.981 (1.490-2.472); -0.001 (-0.037 \text{ to } 0.034) \mu\text{g/L}$ ($R^2 = 0.516$; Fig. 2C), respectively. The use of the ek-rhK2 (Fig. 2D) yielded a regression equation of: $y = 1.074 (0.746-1.401)x + 0.063 (-0.003 \text{ to } 0.016) \mu\text{g/L}$ ($R^2 = 0.544$), confirming the improvement in slope noted previously.

Discussion

Standardization of an analytical procedure should produce an improvement in agreement of reported concentrations obtained with different devices and be as close to the real value as possible. Clinical applicability and spread for a specific test are closely linked to the compa-

Table 2. Measurement of the Hybritech-specific calibrator in the TRF assay and vice versa.

TRF calibrators in Hybritech assay					
Value assigned, $\mu\text{g/L}$	0.0055	0.0155	0.043	0.43	4.6
Measured, $\mu\text{g/L}$	0.0107	0.0173	0.0725	0.6799	>3
Ratio, %	195	150	169	158	
Hybritech calibrators in TRF assay					
Value assigned, $\mu\text{g/L}$	0.1	1	3		
Measured, $\mu\text{g/L}$	0.060	0.900	2.620		
Ratio, %	60	90	87		

Table 3. Measurement of five different hK2 variants on both assays.^a

	ek calibrator ^b			Xa calibrator			TWT calibrator			HWT calibrator			ALA calibrator		
	TRF assay	Hybritech assay	Ratio, %	TRF assay	Hybritech assay	Ratio, %	TRF assay	Hybritech assay	Ratio, %	TRF assay	Hybritech assay	Ratio, %	TRF assay	Hybritech assay	Ratio, %
ek 0.1				0.107	0.111	96	0.180	0.150	120	0.108	0.125	86	0.148	0.134	110
ek 1.0				1.171	1.084	108	1.852	1.433	129	1.293	1.308	99	1.591	1.339	119
ek 3.0				3.580	3.307	108	5.956	4.326	138	3.995	4.130	97	4.813	4.223	114
Xa 0.1	0.094	0.089	106				0.170	0.135	126	0.100	0.111	90	0.139	0.120	115
Xa 1.0	0.868	0.917	95				1.579	1.321	120	1.109	1.203	92	1.363	1.233	110
Xa 3.0	2.606	2.720	96				5.105	3.935	130	3.349	3.727	90	4.149	3.808	109
TWT 0.1	0.055	0.064	86	0.056	0.072	79				0.049	0.079	62	0.075	0.086	87
TWT 1.0	0.589	0.677	87	0.675	0.739	91				0.740	0.897	83	0.892	0.923	97
TWT 3.0	1.633	2.050	80	1.892	2.254	84				2.068	2.769	75	2.555	2.825	90
HWT 0.1	0.095	0.081	117	0.100	0.090	111	0.170	0.123	139				0.139	0.109	127
HWT 1.0	0.790	0.754	105	0.909	0.823	110	1.417	1.103	128				1.227	1.027	119
HWT 3.0	2.393	2.216	108	2.758	2.438	113	4.645	3.231	144				3.790	3.061	124
ALA 0.1	0.071	0.074	96	0.073	0.082	89	0.128	0.113	113	0.069	0.091	76			
ALA 1.0	0.660	0.733	90	0.758	0.800	95	1.147	1.075	107	0.831	0.972	86			
ALA 3.0	1.925	2.174	89	2.225	2.392	93	3.632	3.173	114	2.427	2.942	82			
Mean ratio, %			96			98			126			85			110
Regression equation	$y = 0.949x - 0.001 \mu\text{g/L}$ ($R^2 = 0.978$)			$y = 1.042x - 0.041 \mu\text{g/L}$ ($R^2 = 0.977$)			$y = 1.350x - 0.097 \mu\text{g/L}$ ($R^2 = 0.988$)			$y = 0.905x - 0.039 \mu\text{g/L}$ ($R^2 = 0.984$)			$y = 1.122x - 0.026 \mu\text{g/L}$ ($R^2 = 0.982$)		

^a For the TRF and Hybritech assays, calculated hK2 concentration ($\mu\text{g/L}$) for each rhK2 variant is given when one variant serves as calibrator.

^b ek, ek-rhK2; Xa, Xa-rhK2; TWT, TWT-rhK2; HWT, hWT-rhK2; ALA, Ala-rhK2.

rability of reported results. The application of a common calibrator for PSA consisting of 90% α_1 -antichymotrypsin-PSA and 10% free PSA is an example of a common calibrator for a tumor marker in the urologic specialty (24).

hK2 has been described in several reports to complement PSA detection for improvement in specificity and identification of high-risk groups within certain PSA ranges for early detection of PCa (21–23, 28). Other studies reported on improved differentiation of pathologically non-organ-confined from organ-confined cancer (26, 27) or aggressive grade 3 from less aggressive grade 1 and 2 cancers (25). It is important to note that a consistent improvement in the research assays used for clinical

studies has taken place, including assay precision, lower detection limits, and cross-reactivity to PSA. In addition, these studies were analyzing samples with clinically relevant PSA concentrations of 3 $\mu\text{g/L}$ and above. Both early detection and staging of PCa lack predictive accuracy if only PSA is used. In these studies (21–23, 25–27), it was shown that hK2 can be detected reliably. In the present study, the most recent hK2 assays were used. Both assays had good reproducibility (CV <15%) at hK2 concentrations as low as 0.03 $\mu\text{g/L}$. Even if the assays could have reliably measured lower hK2 concentrations, values <0.02 $\mu\text{g/L}$ were removed from analysis to eliminate any variation caused by sensitivity problems.

One major concern is the reported differences in hK2

Table 4. Descriptive statistics on total PSA and hK2 concentrations as measured by two assays using three different calibrators each.

Patient group	hK2, $\mu\text{g/L}$						PSA range, $\mu\text{g/L}$
	TRF assay			Hybritech assay			
	TRF calibrator	ek-rhK2 calibrator	Hybritech calibrator ^a	Hybritech calibrator	ek-rhK2 calibrator	TRF calibrator ^a	
All patients (n = 146)							2.05–39.47
Mean	0.105	0.061		0.108	0.050		
Median	0.087	0.042		0.091	0.037		
Range	0.035–0.332	0.02–0.259		0.042–0.396	0.02–0.225		
Subset (n = 80)							
Mean	0.090	0.060	0.153	0.078	0.043	0.063	
Median	0.085	0.047	0.143	0.071	0.034	0.056	
Range	0.035–0.200	0.02–0.167	0.072–0.322	0.042–0.173	0.02–0.135	0.033–0.140	2.15–39.47

^a Measured only on a subset of 80 patients.

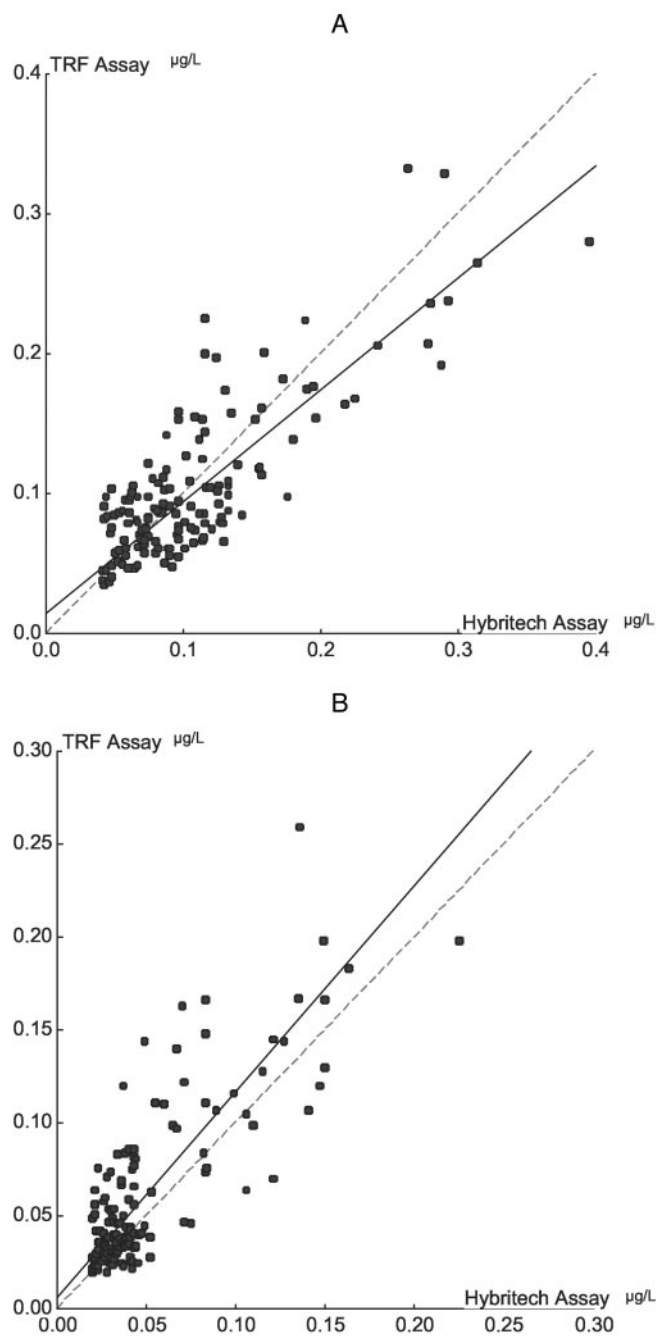


Fig. 1. Comparison of Hybritech assay + Hybritech calibrator vs TRF assay + TRF calibrator (A) and Hybritech assay + ek-rhK2 calibrator vs TRF assay + ek-rhK2 calibrator (B).

(A), Hybritech assay–Hybritech calibrator combination is on the x axis; the TRF assay–TRF calibrator combination is on the y axis ($n = 146$ cases). The regression equation, represented by the solid line, is: $y = 0.789 (0.674–0.922)x + 0.014 (0.004–0.025) \mu\text{g/L}$; $R^2 = 0.667$. (B), Hybritech assay–ek-rhK2 calibrator combination is on the x axis; the TRF assay–ek-rhK2 calibrator combination is on the y axis ($n = 146$ cases). The regression equation, represented by the solid line, is: $y = 1.106 (0.872–1.340)x + 0.006 (–0.002 \text{ to } 0.016) \mu\text{g/L}$; $R^2 = 0.648$.

concentrations (Table 1). As a consequence, the Diamandis group in Toronto has modified the calibration of their hK2 assay to provide results comparable to the assay

reported (15), the Hybritech Access thK2 assay of our study, which is for research use and not for use in diagnostic procedures. Several explanations for the reported differences in hK2 concentrations may be assumed. Differences in epitope recognition and epitope specificity by various antibodies could contribute, as could different assay designs, e.g., a combination of two hK2-specific MAbs as reported by Klee et al. (19) and Finlay and coworkers (14, 15) compared with a scavenger hK2 assay with excessive blocking of all PSA forms to prevent their further reaction, as reported by Piironen et al. (13) and Becker et al. (16). Likewise, all assays use recombinant hK2, which might differ from the hK2 in plasma or serum. Finally, various free hK2 forms potentially exist (analogous to PSA pro variants, cleaved and noncleaved variants), which are likely to be detected differently by different antibodies. Moreover they might also occur in different concentrations in benign vs malignant prostatic disease.

In this study, we were able to analyze the two most cited hK2 assays for the first time on an identical set of patients. In our study, evaluation using Deming regression analysis of patient samples revealed a difference of 21% (Fig. 1A) and therefore does provide sufficient rationale for standardization efforts. Deming regression analysis of the results obtained for the same samples with the ek-rhK2 calibrator reveals an improved agreement in the obtained values as observed by an increase in the regression slope. Because of the higher signal intensity of the ek-rhK2 calibrator, however, we observed a decrease in the obtained values compared with the values obtained with the assay-specific calibrators. Because of the lower signal intensity of the Hybritech wild-type rhK2 variant, the usual calibrator in the Hybritech assay, compared with the Xa-rhK2 variant, which is the usual calibrator in the TRF assay, the effect of decreased concentrations measured with use of the common ek-rhK2 calibrator is more profound with the Hybritech than with the TRF assay results.

Subset analysis of 80 evaluable patient samples, using three different calibrator substances as common standards reveals an important result: Use of the three common calibrators (the ek-rhK2 calibrator; the Xa-rhK2 calibrator, which is the usual calibrator in the TRF assay; or the Hybritech wild-type rhK2, which is the usual calibrator in the Hybritech assay) leads to an increase in the slope of the regression curve in all cases. However, most notably the Hybritech wild-type variant produces an overestimation of the obtained values. The regression slope obtained with the Xa-rhK2 calibrator is closer to the optimum. However, the closest match was seen with the ek-rhK2 calibrator. This observation in the clinical sample material coincides with the almost equally good performance of both the ek-rhK2 and Xa-rhK2 variants over the other variants noted in the analysis of the five available rhK2 variants shown in Table 3. One explanation for the differences in response of different hK2 variants could be

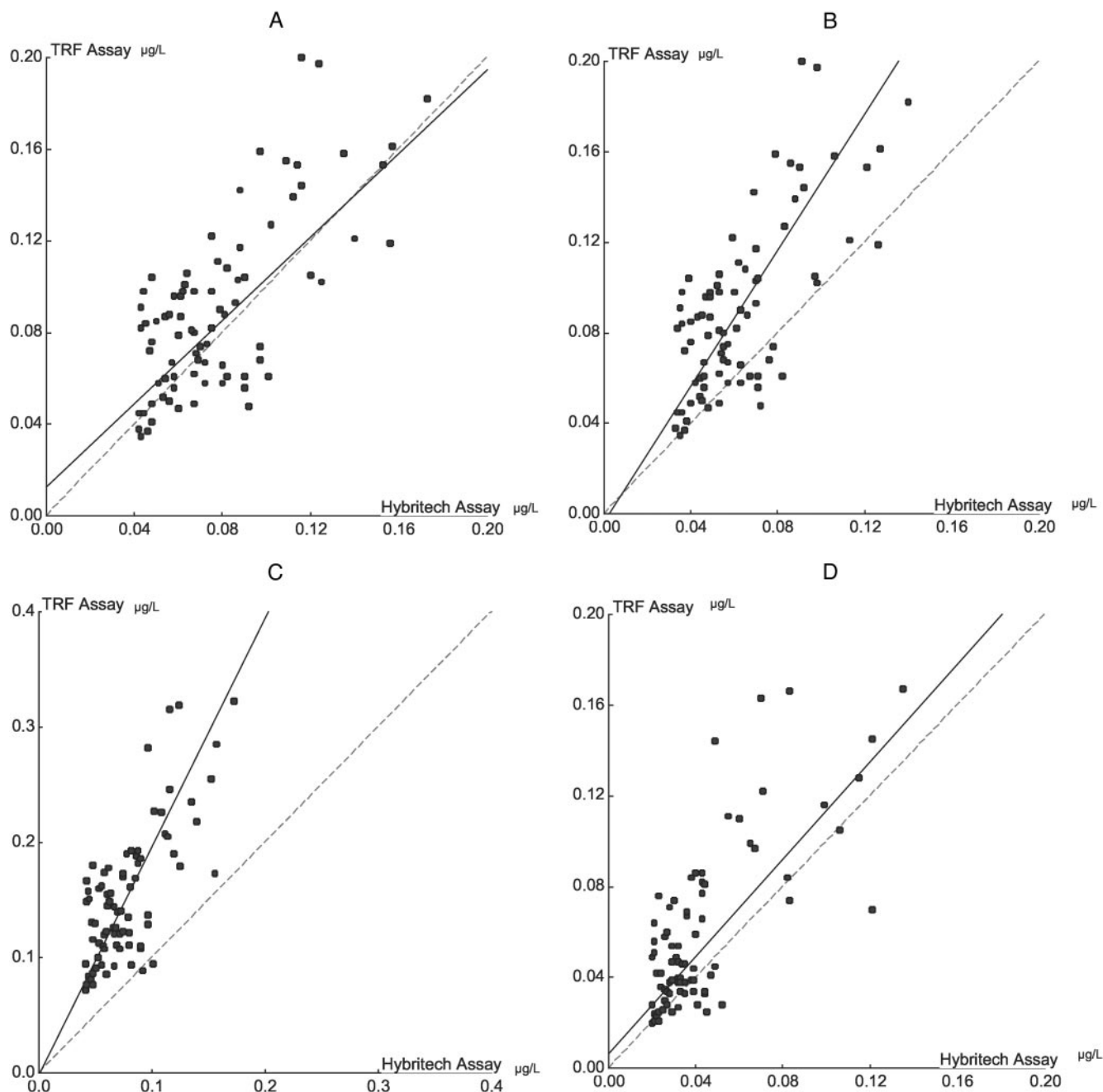


Fig. 2. Deming regression plots for the four assay-calibrator combinations outlined in the section on subset evaluation of patient samples.

(A), Hybritech assay-Hybritech calibrator combination is on the *x* axis; TRF assay-TRF calibrator combination is on the *y* axis (*n* = 80 cases). The regression equation, represented by the *solid line*, is: $y = 0.908 (0.656-1.161)x + 0.012 (-0.004 \text{ to } 0.029) \mu\text{g/L}$; $R^2 = 0.506$. (B), Hybritech assay-TRF calibrator combination is on the *x* axis; TRF assay-TRF calibrator combination is on the *y* axis (*n* = 80 cases). The regression equation, represented by the *solid line*, is: $y = 1.502 (1.086-1.918)x - 0.003 (-0.027 \text{ to } 0.020) \mu\text{g/L}$; $R^2 = 0.504$. (C), Hybritech assay-Hybritech calibrator combination is on the *x* axis; TRF assay + Hybritech calibrator combination is on the *y* axis (*n* = 80 cases). The regression equation, represented by the *solid line*, is: $y = 1.981 (1.490-2.472)x - 0.001 (-0.037 \text{ to } 0.034) \mu\text{g/L}$; $R^2 = 0.516$. (D), Hybritech assay-ek-rhK2 calibrator combination is on the *x* axis; TRF assay-ek-rhK2 combination is on the *y* axis (*n* = 80 cases). The regression equation, represented by the *solid line*, is: $y = 1.074 (0.746-1.401)x + 0.063 (-0.003 \text{ to } 0.016) \mu\text{g/L}$; $R^2 = 0.544$.

the hK2 concentration assigned to each calibrator. If the original concentration of a calibrator is overestimated, samples will show a falsely high concentration, whereas if the calibrator concentration is underestimated, samples will present falsely low concentrations. Although the methods used to determine the calibrator concentrations

are not as accurate as possible (especially immunoassay determination for ek-rhK2, Xa-rhK2, and TWT-rhK2), it is highly unlikely that value assignment would have been so unsuccessful as to produce a >50% difference in signal intensity between two hK2 variants that were value-assigned simultaneously. Moreover, when the same cali-

brator is used for both assays, value assignment becomes insignificant. Although the determined concentration may not be accurate, the crucial difference between assays is not altered.

Although the common calibrator does consistently improve the slope of the regression curve, which reflects the mean estimate of agreement, we did not observe a comparable increase in the dispersion of single values around such an estimate. We believe that a major reason for this observation is the variety of potentially different hK2 variants in patient serum: Deducting from the nature of free PSA isoforms in serum (31–34), such free hK2 forms may include pro-hK2 variants, potentially internally cleaved hK2 variants, and hK2 variants with an intact amino acid chain. Because in contrast to PSA, the majority of hK2 in serum, ~80–95%, occurs in its free form, a much larger proportion of potentially different hK2 variants may cause the consistent degree of dispersion of single values despite an increase in the mean estimate of agreement. This is in line with the results obtained from the measurement of the five different hK2 variants (Table 3): If there is only a single hK2 variant in the sample, both the slope and R^2 of the regression analysis are significantly better than in patient serum. This suggests that a degree of hK2 variability in serum contributes to the scatter of values.

Preanalytical conditions, including sample handling, storage, and rapid processing, have been shown to affect the free fraction of PSA in serum. With the much lower concentrations of hK2, which as stated is a dominantly free serum protein, these considerations may be even more important.

Potentially, imprecision of hK2 detection, specifically in the low range, might contribute to the dispersion of single values around the regression line. The results of our precision analysis as well as our exclusion of values that for either assay-calibrator combination read $<0.02 \mu\text{g/L}$, however, should decrease the likelihood that imprecision alone is responsible for the observed scatter of values. Additionally, assay imprecision would be more pronounced with low hK2 concentrations, but the dispersion of values seen in our material was equal in all concentration ranges.

Two major points with regard to suggesting a reference material need to be addressed. The first is that the standardization of PSA uses a mixture of free and complexed PSA. With hK2, this would be the best approach as well, but at present, too little is known about the free hK2 variants in serum, their composition, and possibly their variability according to disease status. In addition, PSA occurs in a quite steady free/complex ratio, whereas hK2 molecular forms might demonstrate a much larger variation. Therefore, at this point we cannot suggest a mixture of different hK2 variants to serve as a common calibrator. With emerging knowledge, however, this may be a potential aim for future improvement.

The second point is that a more complete characteriza-

tion, in addition to the initial report (30), of the ek-rhK2 variant by mass spectroscopy and/or amino acid analysis needs to be done. This way, the calibrator values would be based on substance concentrations (moles). The exact mass units of the calibrator substance can be calculated on the basis of the molar content and molecular weight determined by mass spectrometry. Serial dilutions of this calibrator may subsequently be used to test the performance of the common ek-rhK2 based calibrator in a multi-institutional trial.

In conclusion, differences in the detection of hK2 in identical patient sera are significant and warrant standardization of the assays. Of the five recombinant hK2 variants tested, including the assay-specific Xa-rhK2 and Hybritech wild-type hK2 variants, the ek-rhK2 variant provided the closest match between the two assays. Calibration of the two assays by use of a common, ek-rhK2-based calibrator revealed a substantial gain in agreement between the assays. These results suggest that this variant should undergo further characterization by mass spectroscopy and amino acid analysis for exact mass determination and value assignment to evaluate its potential as a reference material for immunoassays for hK2.

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References

1. Chapdelaine P, Paradis G, Tremblay RR, Dube JY. High level of expression in the prostate of a human glandular kallikrein mRNA related to prostate-specific antigen. *FEBS Lett* 1988;236:205–8.
2. Schedlich LJ, Bennetts BH, Morris BJ. Primary structure of a human glandular kallikrein gene. *DNA* 1987;6:429–37.
3. Lovgren J, Airas K, Lilja H. Enzymatic action of human glandular kallikrein 2 (hK2). Substrate specificity and regulation by Zn^{2+} and extracellular protease inhibitors. *Eur J Biochem* 1999;262:781–9.
4. Lovgren J, Rajakoski K, Karp M, Lundwall A, Lilja H. Activation of the zymogen form of prostate-specific antigen by human glandular kallikrein 2. *Biochem Biophys Res Commun* 1997;238:549–55.
5. Young CY, Andrews PE, Montgomery BT, Tindall DJ. Tissue-specific and hormonal regulation of human prostate-specific glandular kallikrein. *Biochemistry* 1992;31:818–24.
6. Darson MF, Pacelli A, Roche P, Rittenhouse HG, Wolfert RL, Young CY, et al. Human glandular kallikrein 2 (hK2) expression in prostatic intraepithelial neoplasia and adenocarcinoma: a novel prostate cancer marker. *Urology* 1997;49:857–62.
7. Darson MF, Pacelli A, Roche P, Rittenhouse HG, Wolfert RL, Saeid MS, et al. Human glandular kallikrein 2 expression in prostate adenocarcinoma and lymph node metastases. *Urology* 1999;53:939–44.
8. Stenman UH, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, Alfthan O. A complex between prostate-specific antigen and α_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.
9. Lilja H, Christensson A, Dahlen U, Matikainen MT, Nilsson O, Pettersson K, et al. Prostate-specific antigen in serum occurs predominantly in complex with α_1 -antichymotrypsin. *Clin Chem* 1991;37:1618–25.
 10. Christensson A, Bjork T, Nilsson O, Dahlen U, Matikainen MT, Cockett AT, et al. Serum prostate specific antigen complexed to α_1 -antichymotrypsin as an indicator of prostate cancer. *J Urol* 1993;150:100–5.
 11. Deperthes D, Chapdelaine P, Tremblay RR, Brunet C, Berton J, Hebert J, et al. Isolation of prostatic kallikrein hK2, also known as hGK-1, in human seminal plasma. *Biochim Biophys Acta* 1995;1245:311–6.
 12. Grauer LS, Finlay JA, Mikolajczyk SD, Pusateri KD, Wolfert RL. Detection of human glandular kallikrein, hK2, as its precursor form and in complex with protease inhibitors in prostate cancer serum. *J Androl* 1998;19:407–11.
 13. Piironen T, Lovgren J, Karp M, Eerola R, Lundwall A, Dowell B, et al. Immunofluorometric assay for sensitive and specific measurement of human prostatic glandular kallikrein (hK2) in serum. *Clin Chem* 1996;42:1034–41.
 14. Finlay JA, Evans CL, Day JR, Payne JK, Mikolajczyk SD, Millar LS, et al. Development of monoclonal antibodies specific for human glandular kallikrein (hK2): development of a dual antibody immunoassay for hK2 with negligible prostate-specific antigen cross-reactivity. *Urology* 1998;51:804–9.
 15. Finlay JA, Day JR, Evans CL, Carlson R, Kuus-Reichel K, Millar LS, et al. Development of a dual monoclonal antibody immunoassay for total human kallikrein 2. *Clin Chem* 2001;47:1218–24.
 16. Becker C, Piironen T, Kiviniemi J, Lilja H, Pettersson K. Sensitive and specific immunodetection of human glandular kallikrein 2 in serum. *Clin Chem* 2000;46:198–206.
 17. Black M, Magklara A, Obiezu C, Melegos DN, Diamandis EP. Development of an ultrasensitive immunoassay for human glandular kallikrein with no cross-reactivity from prostate-specific antigen. *Clin Chem* 1999;45:790–9.
 18. Saedi MS, Hill TM, Kuus-Reichel K, Kumar A, Payne J, Mikolajczyk SD, et al. The precursor form of the human kallikrein 2, a kallikrein homologous to prostate-specific antigen, is present in human sera and is increased in prostate cancer and benign prostatic hyperplasia. *Clin Chem* 1998;44:2115–9.
 19. Klee GG, Goodmanson MK, Jacobsen SJ, Young CY, Finlay JA, Rittenhouse HG, et al. Highly sensitive automated chemiluminometric assay for measuring free human glandular kallikrein-2. *Clin Chem* 1999;45:800–6.
 20. Nam RK, Diamandis EP, Toi A, Trachtenberg J, Magklara A, Scorilas A, et al. Serum human glandular kallikrein-2 protease levels predict the presence of prostate cancer among men with elevated prostate-specific antigen. *J Clin Oncol* 2000;18:1036–42.
 21. Becker C, Piironen T, Pettersson K, Bjork T, Wojno KJ, Oesterling JE, et al. Discrimination of men with prostate cancer from those with benign disease by measurements of human glandular kallikrein 2 (hK2) in serum. *J Urol* 2000;163:311–6.
 22. Partin AW, Catalona WJ, Finlay JA, Darte C, Tindall DJ, Young CY, et al. Use of human glandular kallikrein 2 for the detection of prostate cancer: preliminary analysis. *Urology* 1999;54:839–45.
 23. Kwiatkowski M, Recker F, Piironen T, Pettersson K, Otto T, Wernli M, et al. In prostatic patients the ratio of human glandular kallikrein to free PSA improves the discrimination between prostate cancer and benign hyperplasia within the diagnostic “gray zone” of total PSA 4 to 10 ng/mL. *Urology* 1998;52:360–5.
 24. Rafferty B, Rigsby P, Rose M, Stamey T, Gaines DR. 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.
 25. Recker F, Kwiatkowski MK, Piironen T, Pettersson K, Huber A, Lummen G, et al. Human glandular kallikrein as a tool to improve discrimination of poorly differentiated and non-organ-confined prostate cancer compared with prostate-specific antigen. *Urology* 2000;55:481–5.
 26. Haese A, Becker C, Noldus J, Graefen M, Huland E, Huland H, et al. Human glandular kallikrein 2: a potential serum marker for predicting the organ confined versus non-organ confined growth of prostate cancer. *J Urol* 2000;163:1491–7.
 27. Haese A, Graefen M, Steuber T, Becker C, Pettersson K, Piironen T, et al. Human glandular kallikrein 2 levels in serum for discrimination of pathologically organ-confined from locally-advanced prostate cancer in total PSA-levels below 10 ng/ml. *Prostate* 2001;49:101–9.
 28. Becker C, Piironen T, Pettersson K, Hugosson J, Lilja H. Clinical value of human glandular kallikrein 2 and free and total prostate-specific antigen in serum from a population of men with prostate-specific antigen levels 3.0 ng/mL or greater. *Urology* 2000;55:694–9.
 29. Mikolajczyk SD, Millar LS, Kumar A, Saedi MS. Human glandular kallikrein, hK2, shows arginine-restricted specificity and forms complexes with plasma protease inhibitors. *Prostate* 1998;34:44–50.
 30. Lovgren J, Tian S, Lundwall A, Karp M, Lilja H. Production and activation of recombinant hK2 with propeptide mutations resulting in high expression levels. *Eur J Biochem* 1999;266:1050–5.
 31. Mikolajczyk SD, Grauer LS, Millar LS, Hill TM, Kumar A, Rittenhouse HG, et al. A precursor form of PSA (pPSA) is a component of free PSA in prostate cancer serum. *Urology* 1997;50:710–4.
 32. Mikolajczyk SD, Marker KM, Millar LS, Kumar A, Saedi MS, Payne JK, et al. A truncated precursor form of prostate-specific antigen is a more specific serum marker of prostate cancer. *Cancer Res* 2001;61:6958–63.
 33. Mikolajczyk SD, Marks LS, Partin AW, Rittenhouse HG. Free PSA in serum is becoming more complex. *Urology* 2002;59:797–802.
 34. Nurmikko P, Vaisanen V, Piironen T, Lindgren S, Lilja H, Pettersson K. Production and characterization of novel anti-prostate-specific antigen (PSA) monoclonal antibodies that do not detect internally cleaved Lys¹⁴⁵-Lys¹⁴⁶ inactive PSA. *Clin Chem* 2000;46:1610–8.