Proteomics and
Protein Markers

Comparison of Two Assays for Human Kallikrein 2

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Background: We compared two recently developed research assays for the measurement of human kallikrein 2 (hK2) in serum: one fully automated assay (Beckman Coulter Access® immunoanalyzer) and one manual assay based on the DELFIA® technology.

Methods: We used two subsets of clinical specimens consisting of 48 samples from prostate cancer patients and 210 samples from participants in an ongoing screening study (ERSPC). Both subsets were measured in the Rotterdam laboratory, and the prostate cancer samples were used for analytical comparison with the originating sites for the assays: Beckman Coulter Research Department (San Diego, CA) and Turku University (Turku, Finland).

Results: Both the Beckman Coulter and the Turku assays performed very similarly between the Rotterdam laboratory and the originating sites: the R^2 value for both comparisons was 0.99, and the slope difference between sites was <20%. Deming regression analysis of the DELFIA (y) and Access (x) assays yielded the following: for the prostate cancer group, $y = 1.17x - 0.01$ ($R^2 = 0.88$; n = 48); and for the ERSPC group, $y = 0.62x - 0.01$ ($R^2 = 0.77$). Breakdown of the latter group into subgroups (nondiseased, benign prostatic hyperplasia, and prostate cancer samples) gave only minor differences. The Access calibrators were underrecovered by 13% in the DELFIA assay, whereas the DELFIA calibrators were overrecovered by 45% in the Access assay.

Conclusion: The DELFIA and Access assays for hK2, which have similar analytical features, show differences that cannot be explained by calibration.
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An important tumor marker in use in clinical oncology is prostate-specific antigen (PSA).[5] Because of its sensitivity and its organ specificity, this marker is applied in population screening and diagnosis and monitoring of patients with prostate cancer (PCa) *(1)*. However, the discriminatory power of total PSA between the prostate disorders benign prostate hyperplasia (BPH) and PCa is limited *(2)*.

To increase both the sensitivity and the specificity of PSA, many efforts have been made: use of different molecular forms of PSA (free PSA, complexed PSA, total PSA), combination of markers with clinical information (PSA density, PSA velocity, age-specific reference values for PSA), and more recently, the search for new molecular markers *(3–5)*. Promising data have appeared in the literature on the application of human kallikrein 2 (hK2) as a prostate disease marker *(6)*, and several assays for hK2 have been described. We realized that for hK2 to be successfully used, current assays would need to be compared and eventually standardized as was done for PSA.

Our three institutions, University Hospital Rotterdam, the University of Turku, and Hybritech, a subsidiary of Beckman Coulter Inc. (HBC), have collaborated to start the standardization process on two assays that have been the subject of several reports *(7–13)*. The current report describes the first comparison between these two hK2 assays.

Materials and Methods

SAMPLES
The well-characterized samples (serum) belonging to the assay comparison study were part of an informed con-

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[5] Nonstandard abbreviations: PSA, prostate-specific antigen; PCa, prostate cancer; BPH, benign prostate hyperplasia; hK2, human kallikrein 2; HBC, Hybritech-Beckman Coulter; ERSPC, European Randomized Study of Screening for Prostate Cancer; MDC, minimum detectable concentration; and ACT, α_1-antichymotrypsin.

sent-based side study of the Rotterdam section of the European Randomized Study of Screening for Prostate Cancer (ERSPC) (14). Samples were stored for up to 4 years at −80 °C and were thawed and frozen twice during this study. All 210 samples came from individuals from whom we had obtained prostate biopsy results. On the basis of biopsy results, transrectal ultrasound prostate volume, and serum PSA, the individuals were divided into three groups: group 1, healthy individuals (biopsy negative for cancer; prostate volume <40 mL, total PSA, 1.0–7.0 µg/L; n = 72); group 2, BPH patients (biopsy negative for cancer; prostate volume >40 mL; total PSA, 2.8–9.6 µg/L; n = 60); and group 3, PCa patients (biopsy positive for cancer; total PSA, 1.4–10.2 µg/L; n = 78).

An additional panel of 48 less-characterized samples was obtained from our clinical serum bank (samples also stored at −80 °C) for offsite vs onsite assay comparisons. These were nontraceable excess clinical serum samples from PCa patients with PSA values between 1.1 and 28.8 µg/L that were selected at random. For the sake of clarity, we will use the term "uncharacterized" for these samples.

AUTOMATED hK2 Access® ASSAY

The automated hK2 assay, developed for research purposes, is an adaptation of the microtiter version described previously (15) to be performed on the Beckman Coulter Access immunoanalyzer (Beckman Coulter Inc.). The automated version of the hK2 assay is very similar in assay performance to the manual microplate version. The minimum detectable concentration (MDC) for this assay is 0.008 µg/L, and its cross-reactivity to PSA is <0.001%. The calibrator used in this assay was recombinant mature hK2 (15).

MANUAL DELFIA® hK2 ASSAY

The DELFIA-based assay for hK2 is a modification of a previously described time-resolved fluorescence method developed for research (16). The whole procedure is performed manually. Its cross-reactivity to PSA is <0.01%. The calibrator used in this assay is an activated form of mutated recombinant hK2 (17).

MDC, PRECISION, AND RECOGNITION OF hK2 AND hK2–α_1-ANTICHYMOTRYPSIN

The MDC was assessed by assaying 10 replicates of the zero calibrators and 3 replicates of the lowest non-zero calibrator. The assays were performed once each on 2 consecutive days (15). The between-run precision was measured with use of one serum pool (hK2 concentration, 0.056 µg/L; Access hK2 assay). We analyzed two samples consisting of free hK2 and hK2 complexed with α_1-antichymotrypsin (ACT), respectively, to directly compare the recognition of both molecular forms. These samples were, by definition, matched on a molar basis (15). The calibrators for the DELFIA hK2 assay were run in the Access hK2 assay and vice versa so that the calibration for the two assays could be compared.

CORRELATION OF ASSAYS AMONG THE THREE SITES

We performed a comparison study to check the correlation of the two hK2 assays being run at University Hospital Rotterdam laboratory with the originating laboratories for each of the two hK2 assays. For this purpose, we tested 48 uncharacterized clinical samples in the Rotterdam laboratory and then shipped aliquots of those same samples to both HBC (San Diego) for comparison with the hK2 Access assay and to the Department of Biotechnology of the University of Turku (Finland) for comparison with the AutoDELFIA® hK2 assay. In both cases the chemistries were identical and the reagent lots were the same between our site and the originating laboratory.

COMPARISON OF THE TWO ASSAYS ON PATIENT SAMPLES

Both hK2 assays in Rotterdam were used to assay the uncharacterized and the characterized panels.

STATISTICS

All statistical calculations, including Deming regression analysis, were performed with the statistical program Analyze-It (Analyze-It Software Ltd.).

Results

In Rotterdam, the imprecision (as CV) at a hK2 concentration of 0.056 µg/L was: for the Access, a CV of 3.0% over 6 days; and for the DELFIA, a CV of 8.9% over 11 days. The lower limits of detection were 0.008 µg/L for the Access assay and 0.007 µg/L for the DELFIA assay.

The activities of both assays toward free and uncomplexed hK2 were compared directly by measuring matched calibrators for these analytes at Rotterdam and at their original sites. The hK2/hK2-ACT ratio was similar for both assays, with the DELFIA assay being slightly more equimolar than the Access assay: DELFIA, 1.15 and 1.10; Access, 1.28 and 1.29.

The calibrators for the DELFIA assay were measured with the Access assay and vice versa to compare calibrator recovery between the two assays. Regression analysis yielded the following equations: for the Access calibrators, y (Rotterdam, DELFIA) = $0.79x - 0.01$ µg/L and y (Turku, DELFIA) = $0.95x$; and for the DELFIA calibrators, y (Rotterdam, Access) = $1.31x - 0.01$ µg/L and y (San Diego, Access) = $1.59x + 0.01$ µg/L.

Both the Turku and the HBC assays performed very similarly between the Rotterdam site and their respective originating sites (Fig. 1A and B). The regression line for Fig. 1A is: $y = 0.82x + 0.02$ µg/L ($R^2 = 0.99$), and that for Fig. 1B is: $y = 0.87x - 0.01$ µg/L ($R^2 = 0.99$).

Once the assays were compared site to site, the characterized set of 210 samples from the ERSPC was tested in both assays in Rotterdam (Fig. 1C). We found the following equation for the regression line: $y = 0.62x - 0.01$ µg/L ($R^2 = 0.77$). The slopes and R^2 values for the three subgroups differed only slightly from the values obtained
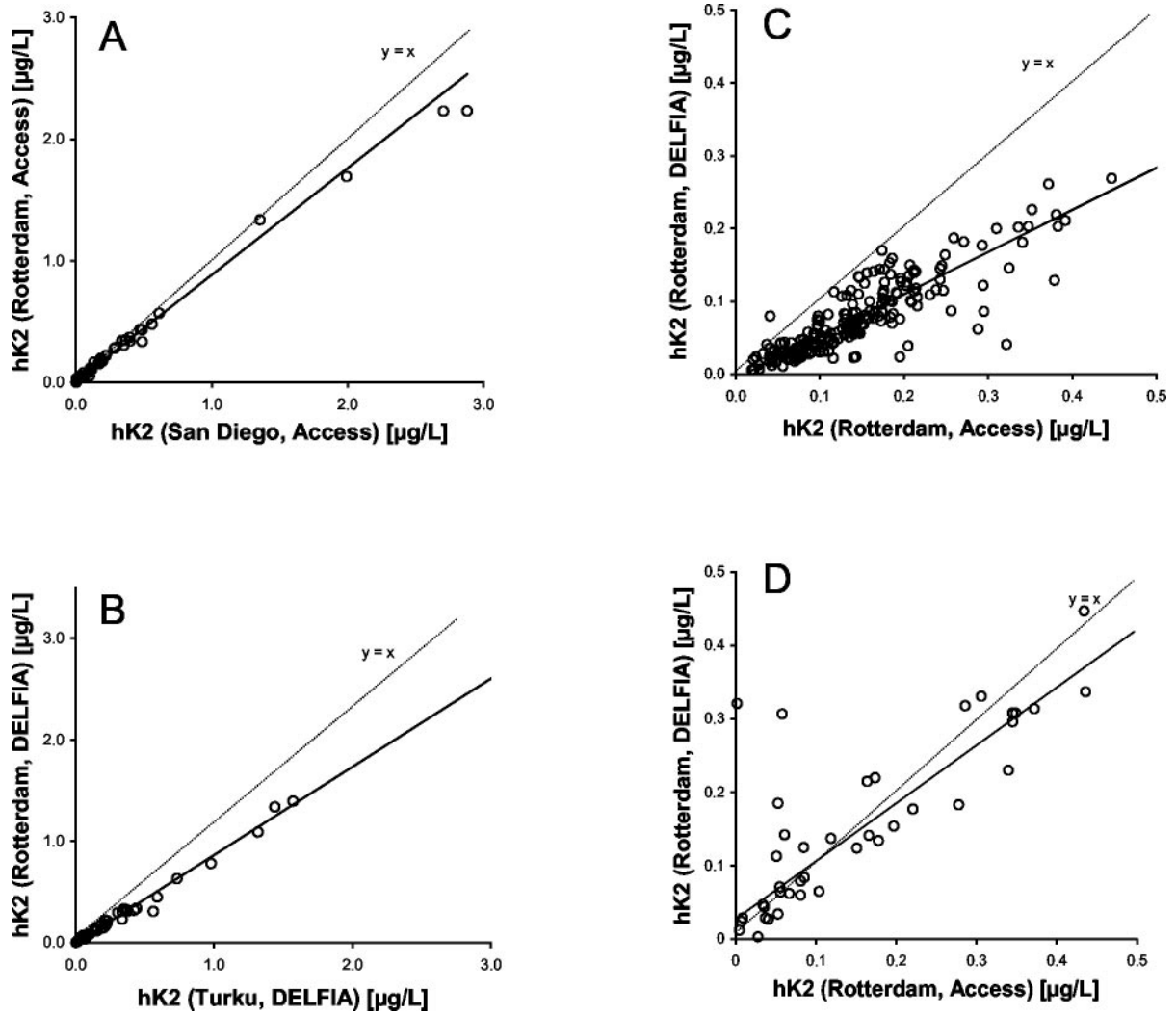
Fig. 1. Comparisons of the hK2 assay between different laboratories.

(*A* and *B*), comparison of the Access assay between San Diego and Rotterdam (*A*) and the DELFIA assay between Turku and Rotterdam (*B*), both with clinical samples. (*C* and *D*), comparison of the DELFIA and Access assays using ERSPC samples (*C*) and clinical samples (limited range; *D*). The regression equations for the lines are given in the text.

for the entire group (data not given). Fig. 1D is a graph comparing the results obtained for the uncharacterized specimens as measured in the Rotterdam laboratory using the same scale as in Fig. 1C. The equation for the regression line is: $y = 0.87x + 0.03\ 0.01\ \mu g/L$ ($R^2 = 0.64$; n = 40). Table 1 contains statistical information on both markers, hK2 and PSA, for both sample sets.

Finally, when we applied the Bland–Altman procedure

Table 1. Marker comparison between ERSPC and clinical samples.[a]

			hK2, $\mu g/L$	
	n	PSA, $\mu g/L$	DELFIA	Access
ERSPC samples				
Total	210	3.9 (1.0–10.2)	0.06 (0.01–0.41)	0.13 (0.02–0.51)
Healthy individuals	72	1.9 (1.0–7.0)	0.04 (0.01–0.20)	0.08 (0.02–0.38)
BPH	60	4.7 (2.8–9.6)	0.10 (0.01–0.41)	0.17 (0.02–0.51)
PCa	78	4.4 (1.4–10.2)	0.08 (0.01–0.23)	0.15 (0.03–0.38)
Clinical samples				
PCa	48	12.1 (1.1–28.8)	0.15 (0.00–2.91)	0.14 (0.00–2.23)

[a] Results are given as the median (range).

(differential plots) and calculated the standard error of the mean difference, we found no evidence for systematic bias for the comparisons shown in panels A and D of Fig. 1 vs those shown in panels B and C.

Discussion

It has been shown that the hK2 concentration in serum corresponds to ∼1–2% of the total PSA concentration (6, 11, 15). The exact physiologic role of hK2 in relation to the biochemistry of PSA is not sufficiently known, although several aspects have been described (11). From the recent reports on hK2 in the literature, it appears that hK2 concentrations in serum may be increased in malignant prostate disease. Preliminary data show that hK2 measurements may aid in the detection of PCa (7) or may help predict the stage and grade of PCa (9–11, 13). These data indicate that hK2 may have value as a pretreatment marker in PCa evaluation.

To date, no commercial hK2 assays are available. The assays we have compared are (modifications of) the assays that were used in the majority of the clinical publications. To our knowledge, this is the first report of an analytical comparison study on hK2 assays. It is therefore difficult to compare the current clinical literature for the usefulness of hK2 without knowing how the assays compare directly in the laboratory. The results shown in Fig. 1 indicate that the performance of the two assays in the Rotterdam laboratory was very similar to their performance in the originating laboratories. Therefore, the comparison of the assays at the Rotterdam site is indicative of and confirms the reliability of the data produced by these assays in the previous clinical studies.

We next compared the assay data from both the characterized (ERSPC) samples and the uncharacterized samples. The slope of the correlation curve between the two assays for all 210 of the ERSPC samples indicated that in the Access hK2 assay, on average, recovery of hK2 was ∼38% higher than in the DELFIA assay. There was considerable scatter between the assays.

Differences in calibration can explain part of slope differences between the assays. We noticed that the Access calibrators were underrecovered by 13% (averaged values of both Access sites) in the DELFIA assay, whereas the DELFIA calibrators were overrecovered by 45% (averaged values of both DELFIA sites) in the Access assay. However, we emphasize that calibration cannot be the only source of the assay differences because in addition to the differences in calibrator recovery, there was substantial scatter in the correlation curves (Fig. 1C).

There are several possibilities for these additional differences between the assays. Simple analyte stability should not be an issue in this comparison because the same samples were mostly run under the same conditions on the same day. If degradation (e.g., proteolytic degradation of the serum hK2, which would completely eliminate the signal in both assays) had occurred, it should have occurred equivalently in all samples. Nevertheless, a more complex type of degradation (e.g., partial proteolytic degradation of specific epitopes within the serum hK2) that would partially eliminate the signal in one assay but not the other may be possible. The Access assay recovers less hK2 signal in samples containing hK2 that has been proteolytically degraded (15). In addition, we have observed that the DELFIA assay does not recognize degraded forms of hK2 (unpublished results). Nothing is known about the reaction pattern of possible glycosylated hK2 forms.

PSA assays have actually exploited differences in proteolysis of the antigen to aid in discriminating benign disease from PCa (18, 19). Therefore, differences in proteolytically degraded hK2 forms could also explain some of the differences found between the different clinical groups (20).

Some of the differences between the correlations in the different groups (Table 1) might be attributable to the hK2 range that is being measured. The hK2 concentration range in samples from healthy individuals is obviously different from that in the cancer samples. This difference is even clearer if we consider the uncharacterized sample group, which consisted of only cancer specimens. We found several high hK2 values in this group. However, the sample concentration range does not completely explain the observed differences (Fig. 1).

Another possibility for differences between the two assays could be their ability to measure complexed vs free forms of hK2. The assays showed very similar ratios of reactivity on hK2 vs hK2-ACT, so it is unlikely those differences in hK2/hK2-ACT ratios in the patient samples would contribute much to the difference between assay values. Furthermore, it has already been shown that the vast majority of hK2 in serum appears to be free (16, 21). Recognition of complexed forms of hK2 may be not be as important for hK2 as it is for PSA.

In conclusion, we have shown that the Access and DELFIA for hK2 actually are quite different. Even if both assays are calibrated with the same hK2 standard, we predict that there will still be some differences in patient correlations between these two assays. Nevertheless, use of a common calibrator for both assays could eliminate one significant cause of error, and other factors influencing the result could then be identified more easily. This should eliminate much confusion and should be much easier to implement at this point in the clinical use of hK2 than it was to standardize PSA (22, 23) because PSA was already being used routinely in clinical laboratories.

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