CAUTION: Use and interpretation of the 4Kscore Test is restricted to order of a physician

I. PROPRIETARY NAME

The 4Kscore® Test

II. INTENDED USE

The 4Kscore Test is an in vitro serum or plasma test that combines the results from four immunoassays (Roche Elecsys total PSA (prostate specific antigen), Roche Elecsys free PSA, intact PSA, and human kallikrein 2) into a single numerical score that also incorporates the following information: a patient's age, previous biopsy and digital rectal exam (DRE). The 4Kscore Test is indicated for use with other patient information as an aid in the decision for prostate biopsy in men 45 years of age and older who have an abnormal age-specific total PSA and/or abnormal DRE. The 4Kscore Test is intended to aid in detection of aggressive prostate cancer (Gleason Score ≥ 7/Gleason Grade Group ≥ 2) for whom a biopsy would be recommended by a urologist, based on current standards of care before consideration of the 4Kscore Test.

A 4Kscore < 5.0 is associated with decreased likelihood of a Gleason score ≥ 7 on biopsy. Prostate biopsy is required for the diagnosis of cancer. The test is not recommended more than once every 6 months.

The test is intended for professional use only, and is performed at a single-site BioReference Laboratories, Inc.

III. SUMMARY AND EXPLANATION

Prostate cancer is the most common non-skin cancer in men. Efforts toward early detection of prostate cancer are not without risk; prostate cancer early detection and diagnosis begins with an abnormal total PSA (tPSA) or digital rectal examination (DRE) and may proceed to prostate biopsy, which is an invasive, but diagnostically necessary procedure. Complications of prostate biopsy have been reported to include bleeding, genitourinary tract infection (6.1%), sepsis (0.5%) and may require hospitalization[1].

Prostate cancer is the second leading cause of cancer deaths in men, with 31,620 deaths projected in the US in 2019[2]. Prior standard of care for prostate cancer early detection was largely based on screening with total PSA (tPSA) testing and/or digital rectal examination (DRE), followed by referral for a prostate biopsy with an abnormal result. Since the implementation of widespread screening with PSA in the early 90's, there has been a 50% reduction in mortality due to the disease[2]. However, the low specificity of PSA has resulted in over 600,000 annual prostate biopsies performed in the US, of which approximately 75% find either no cancer or low grade, indolent (Gleason score ≤ 6) prostate cancer[3]. The over-diagnosis and overtreatment of indolent disease result in men needlessly exposed to the potential harms of biopsy (bleeding, infection, and hospitalization) and potential long term impaired urological function from unnecessary surgery[1, 4].

Men with a low PSA result, independent of, or crossed to DRE findings, which are also part of the standard of care screening, have a very low probability of high grade prostatic cancer and do not require a follow up test for at least 2 years[5, 6]. In the U.S., the primary care physician (PCP) is the first point of care for men's health and the most frequent prescriber of a PSA test. The decision to refer a patient to a urologist is usually made by a PCP based upon an abnormal,

elevated PSA result. Debate remains among physicians as to what level of PSA should trigger a referral. The National Comprehensive Cancer Network (NCCN) guidelines state that PSA levels of 1.0 ng/mL or lower are in the lower range of PSA values and are at a lower risk for potentially aggressive prostate cancer, and that a PSA level of 3.0 ng/mL or higher would be an indication for a prostate biopsy.

IV. PRINCIPLES OF THE PROCEDURE

Classic antigen-antibody sandwich immunoassays are used in the biomarker assays. Roche Elecsys total PSA and Elecsys free PSA are FDA approved tests performed on the Roche cobas platform[7, 8]. The intact PSA (iPSA) and human kallikrein 2 (hK2) tests are performed on the PerkinElmer AutoDELFIA platform. The iPSA test is a sandwich, non-competitive immunoassay that uses two mouse monoclonal antibodies. The hK2 test is a sandwich, non-competitive immunoassay that uses five mouse monoclonal antibodies.

V. PRODUCT INFORMATION

Roche Elecsys total PSA and Roche Elecsys free PSA are used to measure total PSA (tPSA) and free PSA (fPSA), respectively. Reagents used for both iPSA and hK2 tests are manufactured by OPKO Diagnostics, Woburn, MA. The 4Kscore Test is performed at BioReference Laboratories, Inc., Elmwood Park, NJ (BRL). Patients' serum or K_2EDTA plasma samples, if not frozen before shipping, should be received within 72 hours of blood draw. Shipping kits for sample transport are provided by BRL.

VI. REAGENTS

Reagents required to obtain tPSA and fPSA are commercially available from Roche Diagnostics (Indianapolis, IN). Reagents required to obtain iPSA and hK2 are listed in the following table:

Table 1. iPSA and hK2 reagent list

Part number	Description	
43006	iPSA Master Lot (set of 200 plates' worth of reagents)	
Components of 43006 iPSA Master Lot:		
	Part number	Description
	33020	iPSA Capture 100x
	33021	iPSA Tracer 100x
	33055	Streptavidin-Coated plates
	43015	iPSA Standards
	43018	iPSA Assay Buffer
43007	hK2 Master Lot (set of 200 plates' worth of reagents)	
Components of 43007 hK2 Master Lot:		
	33022	hK2 Capture 100x
	33023	hK2 Tracer 100x
	33024	hK2 Blocker 50x
	33055	Streptavidin-Coated plates
	43016	hK2 Standards
	43010	hK2 Assay Buffer
43046	iPSA Assay Controls	
43047	hK2 Assay Controls	
33061	AutoDELFIA Vial Sleeves	

43106	iPSA and hK2 Assay Using AutoDELFIA – Patient Samples Procedure

VII. INSTRUMENT

Elecsys total PSA and Elecsys free PSA are performed on the cobas® e602 immunoanalyzer, Roche Diagnostics.

iPSA and hK2 are performed on the AutoDELFIA® immunoassay system, PerkinElmer.

VIII. SPECIMEN COLLECTION AND PREPARATION FOR ANALYSIS

Matrices Validated: Serum and K_2EDTA Plasma

Handling Conditions:

- Patient sample should be collected aseptically by an acceptable venipuncture technique into a blood collection tube.
- Specimen integrity can be maintained by following the handling processes of blood collection tube manufacturer's recommendation for centrifugation.
- Specimens should be collected in such a way as to avoid hemolysis

Specimen Handling and Processing:

Human plasma (K_2EDTA) or serum separator tubes (SST) can be used. Serum specimen should be allowed to clot. Centrifuge specimens and separate serum from the clot or plasma from the cells within one hour and ship with cold pack overnight to BioReference Lab. Specimen is received within 72 hours from the time of blood draw, and before 4PM Eastern time of a business day, at BioReference Lab, Elmwood Park, NJ, the specimen is processed the same day, if not, the specimen is stored at in -20^{o}C freezer until the next available run. No additives or preservatives are required to maintain integrity of the specimen.

Sample Processing Procedures

The 4Kscore Test is performed only at BioReference Laboratories, Elmwood Park, NJ.

Patient's file (sample type, prior biopsy status, DRE status and age) are uploaded to the Specimen Processing Module (SPM). Sample is tested in accordance with written procedures, EP-SOP-1150 version 17 for iPSA and hK2, EP-SOP- 0241 version 11 for tPSA and EP-SOP-0233 version 10 for fPSA. The results from the four assays are exported automatically to Lab Manager Application (B2 LIS) which automatically triggers the 4Kscore calculation when values of the four analytes are available. The 4Kscore test results are determined automatically by the validated laboratory information system algorithm calculation software.

IX. INTENDED USE POPULATION

The intended use population are:

- Men 45-54 years old and total PSA ≥ 2 ng/mL and/or abnormal DRE
- Men 55-75 years old and total PSA ≥ 3 ng/mL and/or abnormal DRE
- Men ≥76 years old and total PSA ≥ 4 ng/mL and/or abnormal DRE

X. CONTRAINDICATIONS

4Kscore Test is not indicated for use in men[3,9] with:

- A previous diagnosis of prostate cancer
- Digital rectal exam (DRE) performed within 96 hours before blood draw
- Use of 5-alpha reductase inhibitors within the previous 6 months
- Prostate procedures within the previous 6 months

XI. WARNINGS AND PRECAUTIONS

The warnings and precautions can be found in the 4Kscore Test Instructions for Use.

- For in vitro diagnostic use
- For Professional Use
- For Prescription Use only

XII. LIMITATIONS

1. The results of the 4Kscore Test should be used in conjunction with the patient's medical history, clinical examination, and other findings.
2. The performance of the 4Kscore Test is established only for men between the ages of 45 through 80 years old and for whom a biopsy would be recommended by urologist, based on current standards of care[3,9].
3. Biotin concentrations up to 25 ng/mL in serum demonstrate a less than or equal to 10% change in measurement of 4Kscore. Biotin concentrations greater than this may change 4Kscore results for patient samples.

   Patients taking supplements containing biotin should discontinue usage for at least 3 days prior to 4Kscore testing.
4. 4Kscore clinical performance may be different in patients taking ciprofloxacin.
5. **The 4Kscore Test is not intended for use for[3,9]:**
   - A patient with a previous diagnosis of prostate cancer.
   - A patient that has received a DRE in the previous 96 hours (4 days) before phlebotomy. A DRE performed after the phlebotomy is acceptable.
   - A patient that has undergone, within the previous 6 months, any procedure or therapy to treat symptomatic benign prostatic hyperplasia (BPH) or any invasive, urologic procedure that may be associated with a secondary PSA elevation prior to phlebotomy. Such therapies or procedures include, but are not limited to: prostate biopsy, thermotherapy, microwave therapy, laser therapy, Transurethral Resection of the Prostate (TURP), urethral catheterization and lower genitourinary tract endoscopy.
   - A patient has received within the previous 6 months 5-alpha reductase inhibitor (5-ARI) therapy such as Avodart (dutasteride) or Proscar (finasteride).
6. False negative: a patient with a low 4Kscore result (<5.0) has in average likelihood of GS≥7 of 4.1% with 95%CI: (2.1%; 7.9%) and a clinically significant Gleason 7 (Grade Group 2 or 3) cancer would not be detected.
7. False positive: the patient may be biopsied to confirm presence of prostate cancer, resulting in no cancer found or indolent cancer detected.

XIII. REFERENCE INTERVAL

Expected values in normal healthy males

At least 120 samples from apparently healthy male age 45-54, 55-64, and ≥65 groups, totaling 411 samples, were collected for the determination of the 4Kscore Test reference interval (normal range).

The demographic information of these subjects are shown below:

Table 2. Summary of demographic information of the sample cohort used in the 4Kscore Reference Range Study

Category	Subject (N=411)
Ethnicity	
Hispanic or Latino	131 (31.9%)
Non-Hispanic / Non-Latino	208(50.6%)
Not indicated	72 (17.5%)
Race	
Caucasian / White	202 (49.1%)
Black or African American	31 (7.4%)
Asian or Asian American	24 (5.8%)
American Indian or Alaska Native	4 (1.0%)
Other*	112 (27.3%)
Not indicated	38 (9.2%)

* 81.3% of subjects who indicated "Other" for race had identified as Hispanic or Latino Ethnicity.

Table 3. Mean, Median and 95% Percentile of the 4Kscore in Healthy Male and by Age Group

Age Group	N	4Kscore		
		95th Percentile	Median	Mean
All subjects	411	13.2	4.32	5.7
Age 45 to <54	113	4.1	2.4	2.4
Age 55 to <65	160	9.4	4.28	5.3
Age 65 to 80	138	15.6	7.5	8.7

XIV. CLINICAL RESULTS OF THE 4KSCORE TEST IN THE INTENDED USE POPULATION

A. The 4Kscore Test for the Detection of Aggressive Prostate Cancer in Clinical Studies

The 4Kscore Test was evaluated[10] for its clinical validity to aid in detecting aggressive high grade prostate cancer (Gleason score 7, GG2 or higher) and to assist the decision to biopsy in men with

an abnormal total PSA and/or abnormal DRE, for whom biopsy would be recommended by a urologist based on current standards of care.

Two prospective studies were carried out in contemporary subjects in the United States in 2013 to 2017. The Intended Use population includes: men age 45-54 with total PSA ≥2.0 ng/mL, age 55-74 with total PSA ≥3.0 ng/mL, and age 75-80 with total PSA ≥4.0 ng/mL or men age 45 and above and/or abnormal DRE. Based on these criteria, total qualified subjects from the two prospective studies[3,9] contributing 574 and 363, respectively, were combined for a total of 937 subjects. The performance of the 4Kscore test compares to tPSA alone are shown by Receiver Operating Characteristic (ROC) plot and the Area Under the Curve of the ROC statistics in the Figures and Table below. The values and performance characteristics of the 4Kscore Test by age groups, race and 4Kscore ranges for the N=937 intended use population are included in this section.

Figure 1. ROC Plot (4Kscore vs. tPSA)

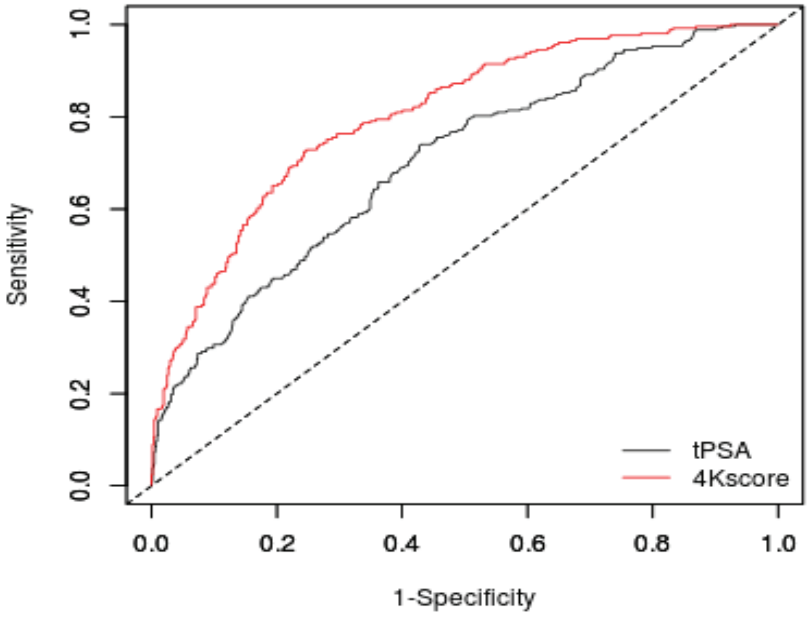

Table 4. ROC Statistics

	N	AUC	95% CI
tPSA	937	0.7021	0.6651; 0.7391
4Kscore	937	0.8039	0.7734; 0.8344

B. The 4Kscore Test Performance at 5.0 Cut Point in N=937 Intended Use Population

Table 5. Performance Characteristics of the 4Kscore Test at 5.0 Cut Point by Age Group

Age Group	N	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	FPR (1-Specificity) (95% CI)	FNR (1-Seensitivity) (95% CI)
All Subjects	937	96.9% (94.0%, 98.4%)	27.4% (24.2%, 30.9%)	33.7% (30.3%, 37.1%)	95.9% (92.1%, 97.9%)	72.6% (69.1%, 75.8%)	3.1% (1.6%, 6.0%)
Age 45 to 54	106	94.1% (73.0%, 99.0%)	42.7% (32.9%, 53.1%)	23.9% (15.3%, 35.3%)	97.4% (86.8%, 99.5%)	57.3% (46.9%, 67.1%)	5.9% (0.5%, 13.2%)
Age 55 to 75	785	96.9% (93.7%, 98.5%)	25.3% (21.9%, 29.0%)	33.9% (30.3%, 37.7%)		74.7% (71.0%, 78.1%)	3.1% (1.5%, 6.3%)
Age 76 to 80	46	100.0% (82.4%, 100%)	21.4% (10.2%, 39.5%)	45.0% (30.3%, 60.2%)	100.0% (61.0%, 100.0%	78.6% (60.5%, 89.8%)	0.0% (0.0%, 17.6%)

C. Probability of Having Aggressive Prostate Cancer in All Intended Use Population and Subgroups of African American and Non-African American by 4Kscore Ranges

Table 6. All Subjects (N=937)

4Kscore Range	Total Number of Subjects	Number of Subjects with Gleason Score ≥ 7	% Probability	95% CI
<5	194	8	4.1%	2.1%, 7.9%
5 to <10	146	14	9.6%	5.8%, 15.5%
10 to <20	198	39	19.7%	14.8%, 25.8%
≥20	399	197	49.4%	44.5%, 54.3%
All	937	258	27.5%	24.8%, 30.5%

Table 7. Non-African American (N=676)

4Kscore Range	Total Number of Subjects	Number of Subjects with Gleason Score ≥ 7	% Probability	95% CI
<5	150	6	4.0%	1.8%, 8.5%
5 to <10	118	10	8.5%	4.7%, 14.9%
10 to <20	144	24	16.7%	11.5%, 23.6%
≥20	264	114	43.2%	37.3%, 49.2%
All	676	154	22.8%	19.8%, 26.1%

Table 8. African American (N=254)

4Kscore Range	Total Number of Subjects	Number of Subjects with Gleason Score ≥ 7	% Probability	95% CI
<5	42	2	4.8%	1.3%, 15.8%
5 to <10	26	4	15.4%	6.2%, 33.5%
10 to <20	53	14	26.4%	16.4%, 39.6%
≥20	133	81	60.9%	52.4%, 68.8%
	254	101	39.8%	33.9%, 45.9%

Note: 7 subjects with unknown race were excluded

The statistics from the US National Cancer Institute indicate that African American men are 1.7 times more likely to be diagnosed with prostate cancer compared to white men[11]. Separate analyses of the African American and non-African American subsets in the N=937 intended use population would be valuable. The median, mean, 95th percentile and the 4Kscore Test performance shown in the Table below is at the 4Kscore cut point of 5.0. It is noted that African American have higher median and mean scores as compared to the non-African American men however the performance of the 4Kscore Test in AA population does not yield to high false negative rate when compared to non-AA sub-group.

Table 9. 4Kscore Performance in the Intended Use Population by African American and Non-African American at 4Kscore cut point of 5.0

Race	N	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	FPR (95% CI)	FNR (95% CI)
All Subjects	937	96.9% (94.0%, 98.7%)	27.4% (24.1%, 30.9%)	33.7% (32.5%, 34.8%)	95.9% (92.1%, 97.9%)	72.6% (69.1%, 75.9%)	3.1% (1.4%, 6.0%)
Non-African American	676	96.1% (91.8%, 98.2%)	27.6% (23.9%, 31.6%)	28.1% (24.5%, 3.1%)	96.0% (91.5%, 98.2%)	72.4% (68.4%, 76.1%)	3.9% (1.8%, 8.2%)
African American	254	98.0% (93.1%, 99.5%)	26.1% (19.8%, 33.6%)	46.7% (44.1%, 53.4%)	95.2% (83.2%, 98.7%)	73.9% (66.4%, 80.2%)	2.0% (0.5%, 6.9%)

Note: 7 subjects with unknown race excluded

The reported 4Kscore result is expressed on a unitless scale from 0.1 through 100.0.

D. Performance data in specific population

1. Performance of the 4Kscore Test in Subjects with Comorbidity

The 4Kscore Test performance in the intended use populations, who have comorbidities commonly found in men of the same age groups were evaluated. Data for the subset of subjects who have known comorbidities are presented in the Table below. The false positive rate and false negative rate are similar for the entire intended use population and those with known specific comorbidities.

Table 10. 4Kscore Test Performance Characteristics by Comorbidity at 4Kscore 5.0 cut point

Comorbidity	N	Sensitivity	Specificity	PPV	NPV	FPR = (1-Specificity)	FNR (1-Sensitivity)
All Subjects	937	96.9%	27.4%	33.7%	95.9%	72.6%	3.1%
Allergies	47	100.0%	31.6%	25.7%	100.0%	68.4%	0.0%
Arthritis	113	96.9%	25.9%	34.1%	95.5%	74.1%	3.1%
Asthma	36	90.9%	28.0%	35.7%	87.5%	72.0%	9.1%
Atrial Fibrillation	17	100.0%	7.1%	18.8%	100.0%	92.9%	0.0%
Benign GI Disease	188	97.9%	29.8%	31.7%	97.7%	70.2%	2.1%
BPH	232	96.2%	33.3%	29.4%	96.8%	66.7%	3.9%
Cardiovascular, Hypertension	535	97.0%	23.9%	36.7%	94.6%	76.1%	3.0%
COPD	46	90.9%	31.4%	29.4%	91.7%	68.6%	9.1%
Diabetes	162	94.4%	23.2%	38.1%	89.3%	76.9%	5.6%
Hyper-Lipidemia	388	97.6%	30.3%	40.5%	96.3%	69.7%	2.4%
Hypogonadism	30	83.3%	25.0%	21.7%	85.7%	75.0%	16.7%
Kidney Disease	32	100.0%	13.6%	34.5%	100.0%	86.4%	0.0%
Skin Cancer	33	100.0%	11.1%	20.0%	100.0%	88.9%	0.0%
Vitamin D Deficiency	12	100.0%	25.0%	40.0%	100.0%	75.0%	0.0%
All Cancer*	51	100.0%	17.5%	25.0%	100.0%	82.5%	0.0%

* All Cancer includes 16 categories: adrenal gland (2), back muscle (1), bladder (1), bone (1), breast (0), colorectal (3), GB, gastric, pancreatic (1), kidney (3), leukemia (4), lung, liver (2), lymphoma (1), penile (2), pituitary gland (0), skin (33), testicular (1), and thyroid (0), and the population consists of 51 unique subjects.

2. Performance of The 4Kscore Test in Subjects with Concomitant Medications

Table 11. 4Kscore Test Performance Characteristics at 4Kscore 5.0 Cut Point by Concomitant Medication

Comorbidity	N	Sensitivity	Specificity	PPV	NPV	FPR = (1-Specificity)	FNR (1-Sensitivity)
All Subjects	937	96.9%	27.4%	33.7%	95.9%	72.6%	3.1%
Acetaminophen	58	100.0%	19.4%	43.1%	100.0%	80.6%	0.0%
Acetylsalicylic Acid	169	95.9%	25.0%	34.3%	93.8%	75.0%	4.1%
Simvastatin	128	97.5%	25.0%	37.1%	95.7%	75.0%	2.5%
Allopurinol	53	100.0%	12.1%	40.8%	100.0%	87.9%	0.0%
Amlodipine	162	100.0%	25.7%	44.9%	100.0%	74.3%	0.0%
Atorvastatin	168	100.0%	21.2%	35.0%	100.0%	78.8%	0.0%
Biotin	5	100.0%	0.0%	60.0%	-	100.0%	0.0%
Ciprofloxacin	200	91.1%**	30.3%	27.5%	92.2%	69.7%	8.9%
Doxazosin	12	80.0%	28.6%	44.4%	66.7%	71.4%	20.0%
Fish Oil	49	100.0%	20.5%	24.4%	100.0%	79.5%	0.0%
Hydro-chlorothiazide	132	97.7%	22.5%	37.8%	95.2%	77.5%	2.3%
Ibuprofen	49	100.0%	26.5%	37.5%	100.0%	73.5%	0.0%
Levofloxacin	94	95.7%	26.8%	29.7%	95.0%	73.2%	4.4%
Levothyroxine	46	100.0%	12.1%	31.0%	100.0%	87.9%	0.0%
Lisinopril	223	97.0%	22.9%	34.6%	94.7%	77.1%	3.0%
Losartan	47	100.0%	13.8%	41.9%	100.0%	86.2%	0.0%
Metformin	118	92.6%	20.9%	25.8%	90.5%	79.1%	7.4%
Metoprolol	103	97.4%	27.7%	44.1%	94.7%	72.3%	2.6%
Multivitamin	130	100.0%	27.6%	31.1%	100.0%	72.5%	0.0%
Omeprazole	133	100.0%	22.6%	35.7%	100.0%	77.4%	0.0%
Pravastatin	64	94.1%	34.0%	34.0%	94.1%	66.0%	5.9%
Sildenafil	112	100.0%	28.4%	48.4%	100.0%	71.6%	0.0%
Tamsulosin	119	94.1%	29.4%	18.2%	96.8%	70.6%	5.9%
Trimethoprim	14	100.0%	10.0%	30.8%	100.0%	90.0%	0.0%
Vitamin D	88	100.0%	22.4%	40.0%	100.0%	77.6%	0.0%

** 4Kscore clinical performance may be different in patients taking ciprofloxacin:

It is noted that the sensitivity appears to be lower for the 200 subjects who reported taking ciprofloxacin as a prophylaxic prophylactic antibiotic treatment prior to the prostate biopsy procedure.

Table 12. Likelihood of Gleason scores 7 or higher, by 4Kscore values, stratified by ciprofloxacin use

	4Kscore	N		Likelihood of Gleason ≥7	
		Total	Gleason ≥7	Point est.	(95% CI)
cipro(+)	<5.0	51	4	7.8%	(3.1%; 18.5%)
	5.0–<10.0	41	7	17.1%	(8.5%; 31.3%)
	10.0–<20.0	34	5	14.7%	(6.4%; 30.1%)
	≥20.0	74	29	39.2%	(28.9%; 50.6%)
	total:	200	45	22.5%	
cipro(−)	<5.0	143	4	2.8%	(1.1%; 7.0%)
	5.0–<10.0	105	7	6.7%	(3.3%; 13.1%)
	10.0–<20.0	164	34	20.7%	(15.2%; 27.6%)
	≥20.0	325	168	51.7%	(46.3%; 57.1%)
	total:	737	213	28.9	

XV. ANALYTICAL PERFORMANCE CHARACTERISTICS

1. Matrix comparison study

To demonstrated that a conversion factor applied to serum measurement of component analytes provides 4Kscore values calculated from serum comparable to those calculated from K_2 EDTA plasma. The conversion factors were established for the four analytes individually then validated in clinical samples from 349 subjects with paired serum and K_2 EDTA plasma samples collected. The study follows the CLSI EP35-A for the demonstration of similarity of serum and K_2 EDTA plasma samples for the measurement of 4Kscore. The Passing-Bablok Regression analyses of the 349 paired serum and K_2 EDTA plasma samples with the 4Kscore values range from 0.6 to 99.5. The results of the Passing-Bablok regression (X-axis is serum and Y-axis is K_2 EDTA plasma) were slope=0.989 with 95% CI: (0.980; 0.996) and intercept = -0.001 with 95% CI: (-0.000; 0.004).

2. Serum and K_2EDTA Plasma Stability of Patients Samples

Stability of samples used for the 4Kscore Test is based on the stability of samples used with each individual assay, i.e., tPSA, fPSA, iPSA and hK2. Stability studies for all four constituent measurement procedures (i.e., tPSA, fPSA, iPSA, hK2) were performed in accordance with CLSI EP25-A, "Evaluation of Stability of In Vitro Diagnostic Reagents". A panel of 20 samples from 20 donors were collected and prepared for serum (on-gel) and plasma. Stability of serum samples were evaluated by keeping the serum samples on-gel at room temperature for (24, 48, and 72 hours) and then transferred and stored at 2–8ºC for additional 24, 48, 72, and 96 hours. Stability of K_2EDTA plasma samples were evaluated at 2–8ºC for 24, 48, 72, 96, 120, and 144 hours. At

each time point, samples were tested in four replicates for each of the tPSA, fPSA, iPSA and hK2 assays. 4Kscore value was also evaluated by comparing value obtained at 96 hours to the value from the initial day. The results support the following initial stability for four assays and 4Kscore when no more than 10% deviation of the value of samples was observed:

- K2EDTA plasma samples are stable up to 120 hours (5 days) at 2–8ºC
- Serum samples are stable on-gel up to 72 hours at room temperature followed with up to 72 hours stored at 2–8ºC.

In addition, long-term sample stability was evaluated real-time for iPSA and hK2 using six serum and six plasma sample stored at -80ºC (±10ºC). The data support sample stability up to one year for these two assays. The sample storage stability are summarized in **Table 13**:

Table 13. Sample Stability for the 4Kscore Test and individual assays

Assay	Sample Type	Ambient (RT)	Refrigerated	Frozen
		20–25°C	**2–8°C**	**-20°C, unless specified**
tPSA (P990056)	Serum and plasma (K_2EDTA, Li-Heparin)	24 hours	5 days (120 hours)	6 months
fPSA (P000027)	Serum and plasma (K_2EDTA, Li-Heparin)	8 hours	5 days (120 hours)	3 months
iPSA	Serum	72 hours	3 days (72 hours)	12 months (-90 – -70°C)
	Plasma (K_2EDTA)	3 day (72 hours)	5 days (120 hours)	12 months (-90 – -70°C)
hK2	Serum	72 hours	3 days (72 hours)	12 months (-90 – -70°C)
	Plasma (K_2EDTA)	3 day (72 hours)	5 days (120 hours)	12 months (-90 – -70°C)
4Kscore	Serum	72 hours	3 days (72 hours)	3 months
	Plasma (K_2EDTA)	not tested	5 days (120 hours)	

Patient samples are shipped overnight to the testing lab, shipping studies follows ISTA 7D 2007 shipping standard (24-hour domestic freight transport) in North America for the summer and winter simulation studies. Additional temperature cycle period are added to the recommended summer and winter simulation profiles (Modified Summer Cycles, and Modified Winter Cycles), which serves to evaluate the potential effect of possible delays during shipment. When compared to control samples, which were kept refrigerated for a maximum of 2 days during the temperature cycling for each test group, demonstrates that all samples are within 10% of the control sample results.

3. Precision studies

a. The within laboratory precision of 4Kscore Test were evaluated with single donor serum samples at 5 clinically relevant 4Kscore values range from below 5.0 to above 40.0. CLSI EP05-A3 guideline procedure of 80 replicates were determined for each sample. The study yielded the following results:

Table 14. 4Kscore Within-Laboratory Precision

Sample	Replicates, N	4Kscore Mean	Within-Run (Repeatability)		Between-Day/Run		Total	
			SD	CV%	SD	CV%	SD	CV%
6879	75	2.8	0.31	11.0%	0.29	10.4%	0.42	15.3%
6886	80	15.0	1.00	6.7%	1.59	10.6%	1.88	12.6%
6887	78	6.6	0.57	8.6%	0.58	8.8%	0.81	12.3%
6889	80	15.0	1.57	10.5%	2.73	18.2%	3.15	21.0%
6892	76	48.2	1.59	3.3%	2.00	4.1%	2.55	5.3%

b. Another 6-day study was conducted to evaluate the precision of the 4Kscore incorporating sources of variability as different operator and different instrument. Five serum samples were tested in four replicates per run, one run per day for six days on three instruments (three operators, one operator per instrument) using one reagent lot, for a total of 72 measurements per sample. Operators were cycled through instruments over the course of this 6-day study. The results are summarized in **Table 15**:

Table 15. 6-day 4Kscore Within-Laboratory Precision

ID	Mean	N	Repeatability (Within-Run)		Between-Day		Between-Instrument		Between-Operator		Within-Lab	
			SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
1	3.00	71	0.0	0.0%	0.0	0.0%	0.0	0.0%	0.0	0.0%	0.0	0.0%
2	7.63	71	0.45	5.9%	0.05	0.7%	0.21	2.7%	0.00	0.0%	0.50	6.5%
3	11.38	72	1.55	13.6%	1.03	9.1%	0.19	1.7%	0.30	2.7%	1.90	16.7%
4	17.03	70	0.77	4.5%	0.30	1.8%	0.30	1.8%	0.09	0.5%	0.89	5.2%
5	82.00	72	1.08	1.3%	0.50	0.6%	0.81	1.0%	0.14	0.2%	1.44	1.8%

c. Reagent lot-to-lot imprecision of 4Kscore was evaluated using five serum samples and three reagent lots at clinically relevant 4Kscore values of less than 5.0 to above 60.0. Each sample was tested in five replicates per run, one run per day, for five days, for a total of 75 measurements per sample. For each of the five samples, the mean, repeatability, between-day/run between-lot component of variance were calculated. The %CV for the between-lot imprecision was ≤5.4% for all five samples.

4. The 4Kscore Test Precision Simulation

The precision of 4Kscore was evaluated at five different 4Kscore value levels. For a multivariate index assay, the precision performance can be different at the same score value when the underlying combinations for tPSA, fPSA iPSA, hK2, age, previous biopsy, and DRE result are different. In order to evaluate the precision characteristics of 4Kscore numerical values under different combinations of underlying variables, precision was computationally simulated from empirically obtained precision of the component immunoassays for iPSA and hK2, and tPSA, fPSA from manufacturer's labeling. Random measurement error was considered normally

distributed and a generator of random normally distributed numbers was used. Mean values of the analytes were considered as values of the corresponding analyte of 937 subjects from the clinical validation study. The standard deviation (SD) for each individual subject for each of the four individual analytes was calculated based on the precision profile of the analyte by linear interpolation. Each subject random measurement error was simulated with 1,000 iterations. There were simulated repeatability of the 4Kscore based on repeatability of the individual analytes and within-laboratory precision of the 4Kscore based on the within-laboratory precision of the individual analytes. There were considered six ranges of the 4Kscore values and for each range, there were calculated: mean of 4Kscore, maximum of SD and maximum %CV.

Table 16. Simulated Precision for 4Kscore

4Kscore	N	Repeatability (Within-Run)			Within-Laboratory Precision		
		Mean	SD_{max}	$\%CV_{max}$	Mean	SD_{max}	$\%CV_{max}$
<5	194	2.66	0.42	11.0%	2.67	0.72	17.9%
5–10	146	7.46	0.95	10.3%	7.48	1.67	16.8%
11–20	198	14.48	1.33	9.0%	14.51	2.19	14.8%
21–40	188	28.34	2.48	8.1%	28.36	3.63	13.8%
41–60	95	49.17	3.36	7.9%	49.17	5.31	12.4%
>60	116	80.52	6.90	8.4%	80.47	8.03	9.9%

%CV of the repeatability of the 4Kscore values was ≤11% and the %CV of the within-laboratory precision of the 4Kscore values was ≤18%.

5. Analytical Specificity of the 4Kscore Test

Nine endogenous and 20 exogenous substances at concentrations at least 3 folds higher blood concentration than the normal prescribed doses, were tested for interference according to CLSI EP07-A3. The 9 endogenous substances are bilirubin, hemoglobin, human serum albumin, human IgG, triglycerides, rheumatoid factor, human anti-mouse antibody, prostatic acid phosphatase, and alpha1 antichymotrypsin. The 20 exogenous substances are tamsulosin, silodosin, doxazosin mesylate, ciprofloxacin, nitrofurantoin, sulfamethoxazole, trimethoprim, sildenafil, acetaminophen, acetylsalicylic acid, ibuprofen, Lisinopril, atorvastatin, amlodipine, hydrochlorothiazide, metformin, omeprazole, gadobutrol, and biotin. With the exception of biotin, no evidence of interference was observed at the doses tested.

Biotin interference:

Biotin concentrations up to 25 ng/mL in serum demonstrate a less than or equal to 10% change in measurement of 4Kscore. Biotin concentrations greater than this may change 4Kscore results for patient samples.

The recommended daily intake for biotin is 0.03 mg and normal serum concentrations of biotin

are stated to range from below 0.1 to 0.8 ng/mL[12]. High doses of biotin (containing up to 100 mg of biotin, with recommendations to take multiple pills per day) may be taken as a dietary supplement promoted for hair, nail, or skin benefits. Some pharmacokinetic studies have shown that in subjects taking daily doses of 5 mg, 10 mg, and 20 mg of biotin serum concentrations of biotin can reach up to 73 ng/mL, 141 ng/mL, and 355 ng/mL, respectively, or plasma concentrations up to 1160 ng/mL for subjects taking doses of biotin up to 300 mg/day[13]. These studies were performed in a small number of apparently healthy, white subjects. Clearance of biotin could be different in other patient populations, such as in patients with impaired renal function, which could lead to higher concentrations of biotin in serum or plasma.

ADDITIONAL INFORMATION

The 4Kscore® Test is a registered trademark of OPKO Diagnostic, LLC, a subsidiary of OPKO Health, Inc.
The manufacturer of the 4Kscore Test is:
BioReference Laboratory, 481 Edward H. Ross Drive, Elmwood Park, NJ, 07407, USA

Patent: https://www.opko.com/what-we-do/our-research/patents

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