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ONE METHOD – TWO INSTRUMENTS. ANALYTICAL PERFORMANCE OF ELECTROCHEMILUMINESCENT ASSAY FOR HE4 IN SERUM

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Summary

Human epididymis protein 4 (HE4) is a tumor marker approved by authority organizations in clinical chemistry for the detection and monitoring recurrence of ovarian cancer. Before the assay becomes part of standard laboratory practice, every laboratory has to confirm the analytical performance of the assay, declared by its manufacturers. The verification of the analytical performance of the electrochemiluminescent assay was performed on 2 analytical platforms produced by the same manufacturer. Within-run (reapeatability) and within-laboratory precision were determined in accordance with the CLSI EP15 protocol on both analyzers. The linearity study was determined in accordance with the CLSI EP6 protocol. The Roche HE4 assays showed excellent within-run and within-laboratory precision (CV< 4%). Within-run precision at 2 concentration levels is the same on both analyzers (2.1% CV and 1.2% CV). Within-laboratory precision was CV3.3 %, and 2.8% on Elecsys, respectively, 2.8% and 3.4 % on Cobas e411. The HE4 assay on Elecsys showed good linearity (r^2=0,99), within the range of 53 to 982 pmol/L. Completely automated analytical systems for determining HE4 in serum, Elecsys and Cobase411 that were verified in this research have shown remarkable analytical characteristics and they meet all the analytical goals necessary for their introduction to laboratory practice.

KEY WORDS: *HE4, analytical performance, verification, precision, linearity*

JEDNA METODA – 2 ANALITIČKA SUSTAVA.
ANALITIČKE ZNAČAJKE ELEKTROKEMILUMINISCENTNE METODE ZA ODREĐIVANJE HE4 U SERUMU

Sažetak

Humani epididimis protein 4 (HE4) je tumorski biljeg za otkrivanje i praćenje bolesnica s karcinomom jajnika preporučen od međunarodnih organizacija i autoriteta za kliničku kemiju. Prije uvođenja testa u standardni laboratorijski rad, svaki laboratorij mora potvrditi analitičke karakteristike testa navedene od strane proizvođača. Verifikacija analitičkih značajki elektrokemiluminiscentne metode za određivanje HE4 u serumu provedena je na 2 analitička sustava istog proizvođača. Preciznost u seriji (ponovljivost) i unutar laboratorijska preciznost na oba analitička sustava provedene su prema CLSI smjernicama, protokl EP15. Linearnost je ispitana prema CLSI smjernicama, protokol EP6. Test HE4 proizvođača Roche Diagnostics GmbH pokazao je izvrsnu preciznost u seriji i unutar laboratorijsku preciznost (CV < 4%). Preciznost u seriji na 2 koncentracijske razine bila je jednaka na oba analizatora (CV2,1% i 1,2%). Unutarlaboratorijska preciznost imala je na Elecsys analizatoru CV3,3 %, i 2,8 %, a na Cobas e411 2,8% i 3,4 % . Test HE4 pokazao je izvrsnu linernost (r^2=0,99) u rasponu vrijednosti od 53 do 982 pmol/L na Elecsys analizatoru. Potpuno automatizirani analitički sustavi za određivanje HE4 u serumu ,Elecsys i Cobase411 pokazali su izvrsne analitičke karakteristike i zadovoljavaju sve analitičke zahtjeve za uvođenje u svakodnevni laboratorijski rad.

KLJUČNE RIJEČI: *HE4, analitičke značajke testa, verifikacija, preciznost, linearnost*

INTRODUCTION

There is a long distance between discovering a tumor marker molecule and its introduction to clinical practice. Along that road, a tumor marker has to meet some strict requirements of analytical and clinical acceptability. A lot of effort has been put into tumor markers, so as to diagnose malignant tumors on time, to provide adequate care for patients suffering from tumor, as well as to detect cancer recurrence. As a result, today we have a growing number of tumor markers. However, some of them have shown questionable clinical applicability in different scientific publications over a period of time. In order to put a tumor marker to medical practice and prove its clinical usability, it has to pass the following steps in the cancer biomarker validation, listed below (1):

1. Sample collection and processing-evaluation of preanalytical factors
2. Analytical validation of biomarker assays
3. Clinical validation of biomarker tests
4. Demonstration of clinical value
5. Regulatory approval
6. Postmarketing evaluation

The analytical validation confirms that this method for determining a tumor marker has been accurate, precise, specific, robust, and stable over time (1-5). For quantitative methods, linearity with the sample dilution, parallelism, recovery following the analyte addition and functional sensitivity need additionally to be tested. A diagnostic company should give the results of every analytical validation of a tumor marker to its end user – a clinical laboratory. The laboratory needs to confirm the manufacturer's test performances in its own work conditions and surroundings before the introduction of the marker to everyday practice. The Clinical and Laboratory Standard Institute (CLSI) has issued a number of guidelines in order to ensure that the validation process remains synchronised everywhere in the world.

Moreover, due to the guidelines set by the CLSI, the diagnostic company has to validate every method for determining tumor markers, while the end user – the clinical laboratory – has to verify that same method, i.e. to test if the given method in the laboratory surroundings also meets the specifications obtained by manufacturer's validation. The analytical verification performed by the clinical laboratory does not have to contain all the elements of the analytical validation performed by the assay manufacturer. The following parameters are usually part of the analytical verification process:

a) precision
b) accuracy
c) linearity
d) method comparison

After analytical verification has been conducted, the clinical laboratory has to assess if the method meets the requirements set in advance, so as to make a decision whether or not it can be put to routine medical practice. The acceptance criteria are defined in scientific literature (Ricos et al. for the majority of biochemical parameters and some tumor markers)(6), by renowned institutions, assay manufacturers or external quality assessment (EQA) organizers. For example, according to the National Academy of Clinical Biochemistry, clinically used serum-based immunoassays should have interassay CV of 10% and within-assay variability of 5% at clinical decision concentrations (7).

More than 20 years have passed from the discovery of HE4 to its introduction to clinical practice. HE4 was first identified and described by Kirchhoff et al. in 1991, as a transcript exclusively expressed in distal epididymis (8). In 1999, Schummer and his colleagues demonstrated that the HE4 gene was primarily overexpressed in patients with ovarian carcinomas (9). In 2003, Hellström et al. performed the first measurement of HE4 in the serum of patients with ovarian carcinoma (10). Finally, the human epididymis protein 4 was approved as a tumor biomarker by the Food and Drug Administration (FDA) for monitoring recurrence of ovarian cancer in 2009.

In 2014, a group of medical biochemistry specialists and gynaecologists launched an initiative to perform analytical verification of the H4 tumor marker first on Elecsys and then on the Cobase411 analyser at the Department of Medical Biochemistry in Oncology. The analytical verification was performed prior to clinical/diagnostic validation (diagnostic sensitivity, diagnostic specificity, positive and negative predictive values), so HE4 was introduced to the routine procedure of treating patients suffering from ovarian cancer in the University Hospital for Tumors at the University Hospital Center Sestre milosrdnice.

MATERIALS AND METHODS

Study design

The evaluation of the analytical performance of electrochemiluminiscent assay was conducted from December 2014 to July 2015 on 2 platforms of the same manufacturer: Elecsys and Cobas e411 (Roche Diagnostics GmbH, Germany). Within-run and within-laboratory precision were determined according to the CLSI EP15 protocol on both analyzers (11), using one assay reagent lot. Furthermore, linearity testing was determined according to CLSI EP6 (12).

Before the evaluation began, the HE4 assay (Roche Diagnostics GmbH, Germany, catalogue number: 05950929 190, lot number: 184436) was calibrated on Elecsys® and Cobas e411, using HE4 CalSet (Roche Diagnostics GmbH, Germany, catalogue number: 05950945 190, lot number: 179364). The method has been standardized against the HE4 EIA method from Fujirebio Diagnostics, Inc. The success of calibration was verified using 2 levels of PreciControl HE4 (Roche Diagnostics GmbH, Germany, catalogue number: 05950953 190, lot number: 180300). After reconstitution in 1 mL of deionised water, the liquid quality control material was dissolved in smaller volumes and kept frozen at -20°C for 5 days. The quality control material listed above was used in the evaluation process of within-run and within-laboratory precision. The patient samples used in the linearity study were collected in Vacuette® 5 mL Z Serum Sep Clot Activator tubes (Greiner Bio-One GmbH, Austria, catalogue number: 456071). After being left at room temperature for 30 minutes, the samples were centrifuged (Rotofix 32A, HettichZentrifugen, Germany) at 3500 rpm for 10 minutes. If the samples were not used on the same day, the serum was separated from the separation gel into secondary tubes and frozen at -20°C.

Test principle

The electrochemiluminescenceimunoassay (ECLIA) is intended to be used on Elecsys and Cobas e411 immunoassay analyzers. The electrochemiluminescent (ECL) reaction is a process in which highly reactive species are generated from a stable precursor at the surface of an electrode. This highly reactive species react with another, producing light.

The HE4 assay is based on preferences of a sandwich electrochemiluminescence imumunoassay. In the first step, the patient's sample is combined with a reagent containing a biotinylated monoclonal HE4 specific antibody and a monoclonal HE4 specific antibody, labelled with a ruthenium complex. During the 9-minute incubation, the antibodies capture the HE4 present in the sample to form a sandwich complex. After adding streptavidin –coated paramagnetic microparticlein the second step, during another 9-minute incubation, the biotinylated antibody attaches to the streptavidin-coated surface of the microparticles. The reaction mixture is transported into the measuring cell where the microparticles are magnetically captured on the surface of electrode. The unbound reagent and sample are washed away by ProCell. The application of voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier. The amount of the chemiluminescent signal produced is directly proportional to the amount of HE4 in the sample. The results are calculated using a calibration curve which is analyzer-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Repeatability (within-run precision)

Repeatability is a quantitative value indicating the disagreement among a set of replicate measurements when all the measurements are made under identical conditions (or within a single run of a procedure)(11). Repeatability (within-run precision) was assessed according to CLSI EP15 over 5 days in the triplicate measurement of 2 levels of the quality control material. The mean, the standard deviation (SD) and the coefficient of variation (CV,%) were calculated from these data.

Within-laboratory precision

Within-laboratory precision is a quantitative value indicating the disagreement among replicate measurements over a longer time when all known, major sources of measurement error within the laboratory (except for major maintenance, recalibration, or reagent lot changes) are accounted for. Within-laboratory precision reflects the accumulation of various error sources, including repeatability (11).

Within-laboratory precision was assessed in accordance with CLSI EP15 over 5 days in the triplicate measurement of 2 levels of the quality control material. The mean, SD and CV were calculated from these data (12).

Linearity

A quantitative analytical method is linear when there exists a mathematically verified straightline relationship between the observed values and the true concentrations or activities of the analyte (13). The linearity study was assessed in accordance with the CLSI EP6 procedures, to verify the linear measuring interval of a measurement for a measuring system. The laboratory staff collected native serum samples in order to find extremely high and low concentrations of HE4. Three dilutions were prepared by mixing the high concentration sample and low concentration sample in the following ratio: 1:3, 1:1 and 3:1. Each dilution and high and low concentration samples were analyzed in duplicate. The observed values were plotted against the expected values.

Statistical analysis

All the statistical analysis were performed using Microsoft Excel 2007. The mean, SD and CV were calculated from the data obtained from the within-run precision and within-laboratory precision studies. The equation and R-squared value were obtained for the linearity study.

RESULTS

Repeatability (within-run precision)

Within-run precision, expressed as CV, for the quality control material at the HE4 concentration of 46.9 pmol/L was 2.1% on both, Elecsys and eCobas analyzers. For the quality control material at the HE4 concentration of 352 pmol/L within-run CV was also the same (1.2%CV) on both analyzers (Table 1).

Table 1.

WITHIN-RUN PRECISION AND WITHIN-LABORATORY PRECISION ON THE ELECSYS AND ECOBAS ANALYZERS IN ACCORDANCE WITH CLSI EP15. ANALYTICAL GOALS FROM MANUFACTURER AND DERIVED FROM BIOLOGICAL VARIABILITY - BRAGA ET AL.

	Within-run precision (CV)		Within-laboratory precision (CV)	
PreciControl HE4	Elecsys	eCobas	Elecsys	eCobas
L1 – 46,9 pmol/L	2.1 %	2.1 %	3.3 %	2.8 %
L2 – 352,0 pmol/L	1.2 %	1.2 %	2.8 %	3.4 %
Manufacturer precision:				
L1 – 45,7 pmol/L	1.4 %		4.2 %	
L2 – 345,0 pmol/L	1.4 %		3.4 %	
Braga et al. analytical goals for precision (14)				
	optimal	desirable	minimum	
HE4 PreM	3.0 %	6.0 %	9.1 %	
HE4 PostM	1.6 %	3.2 %	4.6 %	

Table 2

LINEARITY DATA OF EXPECTED AND OBSERVED CONCENTRATION ON ELECSYS.

Sample	Ratio	1st replicate	2nd replicate	Expected concentration	Observed concentration
H (ID 0042)	1	983.70	980.60	982.15	982.15
H:L	3:1	747.10	739.50	749.85	743.30
H:L	1:1	524.60	519.30	517.56	521.95
H:L	1:3	273.40	282.00	285.26	277.70
L (ID 0004)	1	52.81	53.11	52.96	52.96

H – sample with high HE4 concentration; L – sample with low HE4 concentration

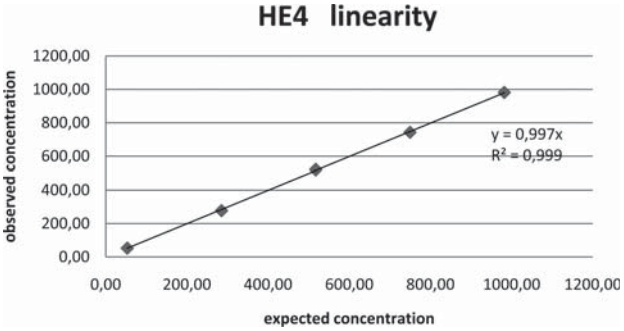

Figure 1. Observed HE4 values plotted against expected values in linearity study.

Within-run precision reached desirable analytical goals derived from biological variability (14).

Within-laboratory precision

Within-laboratory CV on the Elecsys analyzer for PreciControl HE4 Level1 was higher (3.3%) than within-laboratory CV on the eCobas analyzer (2.8%). On the contrary, within-laboratory CV on Elecsys analyzer for PreciControl HE4 Level2 was lower (2.8%) than within-laboratory CV on the eCobas analyzer (3.4%).

Within-laboratory CV fully met the manufacturer criteria for within-laboratory precision (Table 1.)

Linearity

The linearity data for the Elecsys analyzer are shown in Table 2 and Figure 1. The manufacturer has declared that the linearity range of HE4 assay is15 – 15000 pmol/L. In accordance with CLSI EP6, linearity was demonstrated throughout the measuring range, from53 to 982 pmol/L, with R^2 of 0,9998.

DISCUSSION

This research has demonstrated some of the HE4 immunoassay analytical performances. Although the verification process is one of the aspects of the ISO 15189:2012 standard in the process of medical laboratory accreditation, its true value becomes clear when a new test is being introduced into a laboratory. We had such a situation when we were the first laboratory in Croatia to introduce a completely new parameter, HE4, to routine laboratory practice.

When introducing assay into laboratory practice, it is necessary to confirm the technical performances received from the manufacturer, as well as to compare them with relevant findings in scientific literature. Taking into consideration the fact that HE4, as a new test, has been available to the laboratories for only a few years, it is not such a strange fact that the biological variability and analytical requirements were defined only by Braga F. et al. in 2014 (14). They conducted their research on an analyzer produced by the same manufacturer and they also used the same method, but on different analytical system. The precision study of the HE4 method on the Roche analyzers obtained in this research cannot be compared to any other similar research studies in scientific literature, since there are almost no other works dealing with that topic. In study of the referent intervals for HE4 and CA125 for Asian population, Park et al. described the precision of the HE4 assay, but conducted it on the Abbott Architect i2000SR analyzer (Abbott Laboratories, Abbott Park, IL), through testing based on a completely different method: chemiluminescentmicroparticle immunoassay (15).

Within-run precision in this research is the same on both analyzers for normal (2.1%) and high (1.2%) analyte concentration, which meets the desirable specification for precision (HE4PreM ≤6.0% ; HE4PostM ≤ 3.2%), determined by Braga F. et al. (14) Within-run precision is in accordance with the optimal specification, but only for HE4PreM≤ 3.0%(14). Although these are two different methods, we can only state that the repeatability by Park et al.(15) on the Abbott analytical system was 2.8% CV for low and high concentrations, in comparison with our 2.1 and 1.2% CV. Within-run precision for normal concentrationon both analyzers does not meet the specifications set by the manufacturer. The reason for that might be the fact that the manufacturer used the CLSI EP5 protocol to test the precision: 2 runs per day in a duplicate, each for 21 days, which is different from our CLSI EP15: a triplicate measurement during a 5-day period.

Within-laboratory precision on both analyzers completely meets the specifications set by the manufacturer on both concentration levels. It is interesting that the within-laboratory CV is lower on the Elecsys analyzer at a high concentration level (3.3% vs. 2.8%), while the within-laboratory

CV is lower on the eCobas analyzer at a normal concentration level (2.8% vs. 3.4%). The total precision described in the works of Park et al. ranges from 2.9 to 6.5%CV, depending on the concentration level (15).

It is difficult to make an analogy between the analytical goals for precision set by Braga F. et al. and the results of our research, since in their research they used the serum of patients that were divided into two groups: premenopausal women and postmenopausal women. In this research, instead of patients' serum we tested precision by using commercially available material - PreciControl HE4 that contains 2 concentration levels, which is in accordance with CLSI EP15.

Within-run and within-laboratory precision completely met the criteria set by the National Academy of Clinical Biochemistry: the interassay CV of 10% and within-assay variability of 5% (7), so we can conclude that the HE4 immunoassay, set on both tested analytical systems, is an assay of distinguished precision.

The test linearity in this research was also outstanding. We did not cover the whole linearity interval declared by the manufacturer. The linearity at lower levels of HE4 concentration in serum was not tested, because it does not have any clinical significance. We used patient's serum for linearity testing, so it is difficult to find human serum with a concentration level on the upmost borderline of the declared linearity (1500 pmol/L). An extremely pathologically high concentration level of 982 pmol/L was best we could reach to test linearity.

Verification requests for quantitative method is also method comparison and accuracy. The accuracy study was not conducted, because the certified referential material (CRM) was not available at the time when the verification was done, when the assay still had not been introduced to laboratory practice. When it comes to the relevance of HE4, it is enough to say that until a year ago none of the leading European institutions for the external quality assessment of medical-biochemistry laboratories had HE4 as one of their standard parameters. The RfB DGKL external quality assessment organizer from Germany still have not offered HE4 in their panel of tumor markers, while Labquality (Finland) introduced it only last year.

The accuracy study comparison with the already existent referential system was also impossible, since HE4 determining was yet to be introduced to laboratory practice. It was not possible to make a comparison with other laboratories in Croatia, because we were the first laboratory in our country to introduce HE4 to our laboratory practice. The comparison results between Elecsys and Cobas e411 would certainly be interesting, but also inapplicable, because one analytical system was replaced by another one in laboratories throughout the world (RocheDiagnostics GmbH recalled the Elecsys analyzers, having it replaced by the eCobas systems).

The introduction of in-serum HE4 to routine laboratory practice and the use of that tumor marker for treating patients suffering from ovarian cancer (16) inevitably lead to a question whether or not the results of determining HE4 in serum from different laboratories are comparable (17). Will changing the laboratory in which the tumor marker is determined cause bias in concentration that is not a sign of recovery or a recurring disease, but simply an indicator that the analytical method has been changed?

This is why specialist in laboratory medicine has to write a information about the manufacturer, the method and the analyzer on which the tumor marker was determined below the results of a tumor marker. Medical specialists are completely aware that different immunochemical methods are mutually incomparable, which is why they always send the same patients to the same laboratory. That way they ensure that the bias in the test results is caused only by a patient's changed condition and not by variations of the method, the analyzer manufacturer or the reagent. Also, specialist in laboratory medicine should to inform doctors who diagnose, operate and monitor patients about a properly performed process of analytical verification and, eventually, about its results. It is one of the ways to build trust in the doctor – biochemist relationship. The final decision on the applicability and use of a tumor marker is always made by the doctor anyway. As it seems now, the future of HE4 in the University Hospital for Tumors is unquestionable.

CONCLUSION

The completely automated analytical systems for determining HE4 in serum –Elecsys and

Cobas e411 – verified in this research have shown some remarkable analytical characteristics and they meet all the analytical goals for setting the assay to laboratory practice. We can say for sure that bias in the concentration of HE4 in serum during the treatments of patients suffering from gynaecological cancer does not lie in the analytical method, but in the real change of the concentration of tumor markers in the bodies of the patients. Availability of a precise, linear and robust immunoassay for determining HE4 is the main precondition for an early diagnosis and proper treatment of patients suffering from ovarian cancer in Croatia.

REFERENCES

1. Duffy MJ, Sturgeon CM, Sölétormos G,Barak V, Molina R, Hayes DF, Diamandis EP, Bossuyt PM. Validation of new cancer biomarkers: a position statement from the European group on tumor markers. Clin Chem. 2015;61(6):809-20.
2. Horvath AR, Lord SJ, StJohn A, Sandberg S, Cobbaert CM, Lorenz S, Monaghan PJ, Verhagen-Kamerbeek WD, Ebert C, Bossuyt PM.TestEvaluation Working Group of the European Federation of Clinical Chemistry Laboratory Medicine. From biomarkers to medical tests: the changing landscape of test evaluation. Clin Chim Acta. 2014;427:49-57.
3. Khleif SN, Doroshow JH, Hait WN.AACR-FDA-NCI Cancer Biomarkers Collaborative. AACR-FDA-NCI Cancer Biomarkers Collaborative consensus report: advancing the use of biomarkers in cancer drug development. Clin Cancer Res. 2010;16(13):3299-318.
4. Jennings L, Van Deerlin VM, Gulley ML. College of American Pathologists Molecular Pathology Resource Committee. Recommended principles and practices for validating clinical molecular pathology tests. Arch Pathol Lab Med. 2009;133:743–55.
5. Sturgeon C, Hill R, Hortin GL, Thompson D. Taking a new biomarker into routine use-a perspective from the routine clinical biochemistry laboratory. Proteomics Clin Appl. 2010;4(12):892-903.
6. Ricós C, Alvarez V, Cava F, García-Lario JV, Hernández A, Jiménez CV, Minchinela J, Perich C, Simón M. Current databases on biological variation: pros, cons and progress. Scand J Clin Lab Invest. 1999;59(7): 491-500.
7. Sturgeon CM, Hoffman BR, Chan DW, Ch'ng SL, Hammond E, Hayes DF, Liotta LA, Petricoin EF, Schmitt M, Semmes OJ, Söletormos G,van der Merwe E, Diamandis E P.National Academy of Clinical Biochemistry. National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines for use of tumor markers in clinical practice: quality requirements. Clin Chem. 2008;54(8):e1-e10.
8. Kirchhoff C, Habben I, Ivell R, Krull N. A major human epididymis-specific cDNAencodes a protein with sequence homology to extracellular proteinase inhibitors. Biol Reprod. 1991;45:350–357.
9. Schummer M, Ng WV, Bumgarner RE, Nelson PS, Schummer B, Bednarski DW, Hassell L, Baldwin RL, Karlan BY, Hood L. Comparative hybridization of an array of 21,500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas. Gene. 1999;238(2):375-85.
10. Hellström I, Raycraft J, Hayden-Ledbetter M, Ledbetter JA, Schummer M, McIntosh M, Drescher C, Urban N, Hellström KE. The HE4 (WFDC2) protein is abiomarker for ovarian carcinoma. Cancer Res. 2003;63(13): 3695-700.
11. CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS). User verification of precision and estimation of bias: approved guideline, second edition. NCCLS 2005; document EP15-A2.
12. Westgard JO. Useful measures and models for analytical quality management in medical laboratories. Clin Chem Lab Med. 2016;54(2):223–33.
13. CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS). Evaluation of the linearity of quantitative measurement procedures: a statistical approach; approved guideline NCCLS 2003; document EP6.
14. Braga F, Ferraro S, Mozzi R, Panteghini M. The importance of individual biology in the clinical use of serum biomarkers for ovarian cancer. Clin Chem Lab Med. 2014;52(11):1625-31.
15. Park Y, Kim Y, Lee EY, Lee JH, Kim HS. Reference ranges for HE4 and CA125 in a large Asian population by automated assays and diagnostic performances for ovarian cancer. Int J Cancer. 2012;130(5):1136-44.
16. Sölétormos G, Duffy MJ, Othman Abu Hassan S,Verheijen RH,Tholander B, Bast RC Jr, Gaarenstroom KN, Sturgeon CM, Bonfrer JM, Petersen PH, Troonen H, CarloTorre G, Kanty Kulpa J, Tuxen MK, Molina R. Clinical Use of Cancer Biomarkers in Epithelial Ovarian Cancer: Updated Guidelines From the European Group on Tumor Markers. Int J Gynecol Cancer. 2016;26(1):43-51.
17. Ferraro S, Panteghini M. Is serum human epididymis protein 4 ready for prime time? Ann Clin Biochem. 2014;51(2):128-36.

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