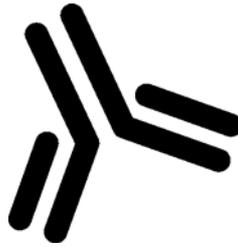


DRAFT
08/20/08



HE4 EIA

Prod. No. 404-10US

Instructions for use

Enzyme immunometric assay kit

DRAFT

For 96 determinations

 Use By	 In Vitro Diagnostic Medical Device
 Batch code	 Temperature limitation
 Date of manufacture	 Consult Instructions for Use
 Catalogue number	 Biological risks
 Manufacturer	 Contents of kit
 Contains sufficient for <96> tests	 From mouse
	 Human origin

INTENDED USE

The HE4 EIA is an enzyme immunometric assay for the quantitative determination of HE4 in human serum.

The HE4 EIA is to be used as an aid in monitoring recurrence or progressive disease in patients with epithelial ovarian cancer. Serial testing for patient HE4 assay values should be used in conjunction with other clinical methods used for monitoring ovarian cancer.

The HE4 EIA used in conjunction with the ARCHITECT CA 125 II assay creates a predictive probability of epithelial ovarian cancer using a mathematical function referred to as the Risk of Ovarian Malignancy Algorithm (ROMA™), for use in premenopausal and post-menopausal women presenting with an adnexal mass who have already been referred to an oncologic specialist and are scheduled for surgery. Subjects categorized as low risk for epithelial ovarian cancer using the ROMA value may have surgical intervention performed by a non-oncology specialist. The results must be interpreted in conjunction with other clinical findings in accordance with standard clinical management guidelines. The assay is not indicated as an aid in a decision to proceed to surgery.

SUMMARY AND EXPLANATION OF THE ASSAY

Human epididymis protein 4 (HE4) belongs to the family of whey acidic four-disulfide core (WFDC) proteins with suspected trypsin inhibitor properties. Other proteins in this family include SLPI, Elafin, and PS20 (WFDC1) (1, 2). The HE4 gene codes for a 13kD protein, although in its mature glycosylated form the protein is approximately 20-25 kD, and consists of a single peptide containing two WFDC domains (3). HE4 was first identified in the epithelium of the distal epididymis and originally predicted to be a protease inhibitor involved in sperm maturation (4, 5). HE4 has since been reported to be expressed in several normal tissues including epithelia of respiratory and reproductive tissues and also in ovarian cancer tissue (6-10). In addition to expression on a cellular level, secreted HE4 has been detected in high levels in the serum of ovarian cancer patients. In a case/control study comparing patients with ovarian cancer to healthy and benign conditions, Hellström *et al.* found that HE4 detected ovarian cancer with 67% sensitivity at a specificity level of 96% (11). In a subsequent study evaluating numerous known biomarkers for ovarian cancer, HE4 showed the highest sensitivity for the detection of ovarian cancer, particularly in early stage disease. In this study, the combination of HE4 and CA 125 was a more accurate predictor of malignancy than either marker alone, with a sensitivity of 76% and a specificity of 95% (12). It is estimated that 5 to 10 percent of women in the United States will undergo a surgical procedure for a suspected ovarian neoplasm during their lifetime, and 13 to 21 percent of these women will be found to have an ovarian malignancy (13). Since the majority of adnexal masses are benign, it is important to determine preoperatively whether a patient is at high risk for ovarian malignancy, in order to ensure proper management (14). The American College of Obstetricians and Gynecologists Practice Bulletin published in 2007 states the following (14):

“Women with ovarian cancer whose care is managed by physicians who have advanced training and expertise in the treatment of women with ovarian cancer, such as gynecologic oncologists, have improved overall survival rates compared with those treated without such collaboration.”

Since the initial report in 1988, clinical impression, serum CA125 and ultrasound along with CT scan, MRI and CT/PET have been the standards in the determination of whether an adnexal mass is suspicious for malignancy (15). Although the literature is replete with papers describing which modality is the more accurate, the combination of physical examination, CA 125 and imaging affords the highest positive predicative value (16-18). To improve the triage of patients presenting with adnexal mass, the HE4 EIA may be used in conjunction with the ARCHITECT CA125 II assay to create a predictive probability of epithelial ovarian cancer using a mathematical function referred to as the Risk of Ovarian Malignancy Algorithm (ROMA), for use in premenopausal and post-menopausal women presenting with an adnexal mass who have already been referred to an oncologic specialist and are scheduled for surgery. Subjects categorized as low risk for epithelial ovarian cancer using the ROMA value may have surgical intervention performed by a non-oncology

specialist. The results must be interpreted in conjunction with other clinical findings in accordance with standard clinical management guidelines. The assay is not indicated as an aid in a decision to proceed to surgery.

An additional use of the HE4 EIA is as an aid in monitoring recurrence or progressive disease in patients with epithelial ovarian cancer. Serial testing for patient HE4 assay values should be used in conjunction with other clinical methods used for monitoring ovarian cancer.

PRINCIPLE OF THE TEST

The HE4 EIA is a solid-phase, non-competitive immunoassay based upon the direct sandwich technique using two mouse monoclonal antibodies, 2H5 and 3D8, directed against two epitopes in the C-WFDC domain of HE4. Calibrators, controls and patient samples are incubated together with biotinylated Anti-HE4 monoclonal antibody (MAb) 2H5 in streptavidin coated microstrips. HE4 present in calibrators or samples is adsorbed to the streptavidin coated microstrips by the biotinylated Anti-HE4 MAb during the incubation. The strips are then washed and incubated with HRP labeled Anti-HE4 MAb 3D8. After washing, buffered Substrate/Chromogen reagent (hydrogen peroxide and 3, 3', 5, 5' tetra-methyl-benzidine) is added to each well and the enzyme reaction is allowed to proceed. During the enzyme reaction a blue color will develop if antigen is present. The intensity of the color is proportionate to the amount of HE4 present in the samples. The color intensity is determined in a microplate spectrophotometer at 620 nm (or optionally at 405 nm after addition of Stop Solution).

Calibration curves are constructed for each assay by plotting absorbance value versus the concentration for each calibrator. The HE4 concentrations of patient samples are then read from the calibration curve.

REAGENTS

- Each HE4 EIA kit contains reagents for 96 tests.
- The expiry date of the kit is stated on the label on the outside of the kit box.
- Do not use the kit beyond the expiry date.
- Do not mix reagents from different kit lots.
- Store the kit at 2–8°C. Do not freeze.
- Opened reagents are stable according to the table below provided they are not contaminated, stored in resealed original containers and handled as prescribed. Return to 2-8°C immediately after use.

Component	Quantity	Storage and stability after first use
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MICROPLA

Streptavidin Microplate	1 Plate	2—8°C until expiry date stated on the plate
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12 x 8 breakable wells coated with streptavidin. After opening, immediately return unused strips to the aluminium pouch, containing desiccant. Reseal carefully to keep dry.

CAL HE4 A

HE4 Calibrator A	1 x 8 mL	2—8°C until expiry date stated on the vial
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Phosphate buffered salt solution containing bovine serum albumin, an inert yellow dye, and a non-azide antimicrobial preservative. Ready for use. Should also be used for dilution of samples.

HE4 Calibrators B-F	5 vials, lyophilized	Stability after reconstitution 4 weeks at 2-8°C 4 months at -20°C or below
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CAL HE4 B

1 x 1 mL

CAL HE4 C

1 x 1 mL

CAL HE4 D

1 x 1 mL

CAL HE4 E

1 x 1 mL

CAL HE4 F

1 x 1 mL

The lyophilized calibrators contain HE4 antigen in a phosphate buffered salt solution containing bovine serum albumin, an inert yellow dye, and a non-azide antimicrobial preservative. To be reconstituted with distilled or deionized water before use.

NOTE: The exact HE4 concentration is lot specific and is indicated on the label of each vial.

Component	Quantity	Storage and stability after first use
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HE4 Controls	2 vials, lyophilized	Stability after reconstitution 4 weeks at 2-8°C 4 months at -20°C or below
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CONTROL	HE4	1	1 x 1 mL
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CONTROL	HE4	2	1 x 1 mL
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The lyophilized controls contain HE4 antigen in a human serum matrix and a non-azide antimicrobial preservative. To be reconstituted with distilled or deionized water before use.

BIOTIN	Anti-HE4
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Biotin Anti-HE4	1 x 15 mL	2–8°C until expiry date stated on the vial
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Biotin Anti-HE4 monoclonal antibody from mouse, approximately 1 µg/mL. Contains phosphate buffered saline (pH 7.2), bovine serum albumin, blocking agents, detergent, an inert red dye, and a non-azide antimicrobial preservative. Ready for use.

CONJ	Anti-HE4
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Tracer, HRP Anti-HE4	1 x 0.75 mL	2–8°C until expiry date stated on the vial
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Stock Solution of HRP Anti-HE4 monoclonal antibody from mouse, approximately 40 µg/mL. Contains non-azide antimicrobial preservatives. To be diluted with Tracer Diluent prior to use.

DIL	CONJ
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Tracer Diluent	1 x 15 mL	2–8°C until expiry date stated on the vial
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Phosphate buffered saline (pH 7.2) with bovine serum albumin, blocking agents, detergents, an inert blue dye, and a non-azide antimicrobial preservative. Ready for use.

SUBS	TMB
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TMB HRP-Substrate	1 x 12 mL	2–8°C until expiry date stated on the vial
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Contains buffered hydrogen peroxide and 3, 3', 5, 5' tetra-methylbenzidine (TMB). Ready for use.

Component	Quantity	Storage and stability after first use
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STOP

STOP Solution	1 x 15 mL	2—8°C until expiry date stated on the vial
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Contains 0.12 M hydrochloric acid. Ready for use.

WASHBUF 25X

Wash Concentrate	1 x 50 mL	2—8°C until expiry date stated on the bottle
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A Tris-HCl buffered salt solution with Tween 20. Contains Germall II as preservative.

To be diluted with distilled or deionized water 25 times before use.

Indications of instability

The TMB HRP-Substrate should be colorless or slightly bluish. A blue color indicates that the reagent has been contaminated and should be discarded.

WARNINGS AND PRECAUTIONS

For In Vitro Diagnostic Use:

- For professional use only
- Follow the instructions in the Package insert. Reliability of assay results cannot be guaranteed if there are any deviations from the instructions in this package insert.
- Handle all patient specimens as potentially infectious. It is recommended that human source reagent and human specimens be handled in accordance with the OSHA Standard on Bloodborne pathogens (19). Biosafety level 2 (20) or other appropriate biosafety practices should be used for material that contain or are suspected of containing infectious agents.
- Follow local guidelines for disposal of all waste material.

Caution

Material used in the preparation of human source reagent has been tested and found to be Non-Reactive for HIV 1 and 2 Antibody, HCV Antibody and Hepatitis B Surface Antigen (HBsAg). Since no method can completely rule out the presence of blood borne diseases, the handling and disposal of human source reagents from this product should be made as if they were potentially infectious.

SPECIMEN COLLECTION AND HANDLING

The HE4 EIA is intended for use with serum (including serum collected in separator tubes (SST)). Plasma and other body fluids have not been validated for use with the HE4 EIA.

Collect blood by venipuncture and follow the tube manufacturer's processing instructions for collection tubes. When serial specimens are being evaluated, the same type of specimen should be used throughout the study.

Serum can be stored at 2–8°C for 3 days before being tested. For longer periods store samples at -40°C or colder.

Bring frozen samples to room temperature and mix THOROUGHLY by gently inverting multiple times before analysis. Samples that contain gross particulates should be centrifuged at 10,000 x g for 10 minutes prior to use to eliminate any particulate matter that may have developed from the thawing process.

PROCEDURE

Materials required but not supplied with the kit

1. Microplate shaker

Shaking should be medium to vigorous, approximately 700-1100 oscillations/min.

2. Microplate washer

Automatic plate washer capable of performing 1, 3 and 6 washing cycles, and with a minimal fill volume of 350 µL/well/washcycle.

An 8-channel pipette with disposable plastic tips for delivery of 350 µL is recommended if an automatic microplate washer is not used.

3. Microplate spectrophotometer

With a wavelength of 620 nm and/or 405 nm, and an absorbance range of 0 to 3.0.

4. Precision pipettes

With disposable plastic tips for dispensing microlitre volumes. An 8-channel pipette or dispenser pipette with disposable plastic tips for delivery of 100 µL is recommended but not required. Pipettes for dispensing millilitre volumes.

5. Distilled or deionized water

For reconstitution of HE4 Calibrators, HE4 Controls and for preparation of diluted Wash Solution.

Procedural notes

1. A thorough understanding of this package insert is necessary to ensure proper use of the HE4 EIA kit. The reagents supplied with the kit are intended for use as an integral unit. Do not mix identical reagents from kits having different lot numbers. Do not use the kit reagents after the expiry date printed on the outside of the kit box.
2. Reagents should be allowed to reach room temperature (20–25°C) prior to use. Frozen specimens must be gently but thoroughly mixed after thawing. **The assay should only be performed at temperatures between 20–25°C to obtain accurate results.**
3. Before starting to pipette calibrators and patient specimens it is advisable to mark the strips to be able to clearly identify the samples during and after the assay.

4. The requirement for efficient and thorough washing for separation of bound and unbound antigen and reagents from the solid-phase bound antibody-antigen complexes is one of the most important steps in an EIA. **In order to ensure efficient washing make sure that all wells are completely filled to the top edge with wash solution during each wash cycle, that wash solution is dispensed at a good flow rate, that the aspiration of the wells between and after the wash cycles is complete and that the wells are empty. If there is liquid left, invert the plate and tap it carefully against absorbent paper.**
 - Automatic strip washer: Follow the manufacturer's instructions for cleaning and maintenance diligently and wash the required number of wash cycles prior to and after each incubation step. The aspiration/wash device should not be left standing with the Wash Solution for long periods, as the needles may get clogged resulting in poor liquid delivery and aspiration.
5. The TMB HRP-Substrate is very sensitive for contamination. For optimal stability of the TMB HRP-Substrate, pour the required amount from the vial into a carefully cleaned reservoir or preferably a disposable plastic tray to avoid contamination of the reagent. Be sure to use clean disposable plastic pipette tips (or dispenser pipette tip).
6. Be sure to use clean disposable plastic pipette tips and a proper precision pipetting technique when handling samples and reagents. Do not allow the pipette tip to touch the surface of the liquid in order to avoid carry-over. A diligent pipetting technique is of particular importance when handling the samples and the TMB HRP-Substrate solution.

Preparation of reagents	Stability of prepared reagent
HE4 Calibrators B-F	4 weeks at 2–8°C 4 months at -20°C or below
Add exactly 1.0 mL of distilled or deionized water to each vial. Allow to stand for at least 15 minutes to reconstitute and mix gently before use. NOTE: The concentration of the calibrators is stated on the labels and should be used for calculation of results.	
HE4 Controls 1 and 2	4 weeks at 2–8°C 4 months at -20°C or below
Add exactly 1.0 mL of distilled or deionized water to each vial and mix gently. Allow to stand for at least 15 minutes to reconstitute and mix gently before use. NOTE: The ranges of the controls are stated on the labels.	
Wash Solution	2 weeks at 2–25°C in a sealed container
Pour the 50 mL Wash Concentrate into a clean container and dilute 25-fold by adding 1200 mL of distilled or deionized water to give a buffered Wash Solution.	

Preparation of reagents	Stability of prepared reagent
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Tracer Working Solution

4 weeks at 2–8 °C in a sealed container

Prepare the required quantity of Tracer working solution by mixing 50 µL of Tracer, HRP Anti-HE4 with

1 mL of Tracer Diluent per strip (see table below):

No. of Strips	Tracer, HRP Anti-HE4 (µL)	Tracer Diluent (mL)
1	50	1
2	100	2
3	150	3
4	200	4
5	250	5
6	300	6
7	350	7
8	400	8
9	450	9
10	500	10
11	550	11
12	600	12

Be sure to use a clean plastic or glass tube for preparation of Tracer working solution.

Alternative: Pour the contents of the Tracer, HRP Anti-HE4 into the vial of Tracer Diluent and mix gently. Make sure that the entire content of the Tracer, HRP Anti-HE4 vial is transferred to the vial of Tracer Diluent.

NOTE: The Tracer working solution is stable for 4 weeks at 2–8°C. Do not prepare more Tracer working solution than will be used within this period and make sure that it is stored properly.

ASSAY PROCEDURE

Perform each determination in duplicate for both calibrators, controls and unknown specimens. A calibration curve should be run with each assay. All reagents and specimens must be brought to room temperature (20–25°C) before use.

1. Start preparing Calibrators B-F, Controls 1 and 2, Wash Solution and Tracer working solution. It is important to use clean containers. Follow the instructions carefully.
2. Transfer the required number of microplate strips to a strip frame. (Immediately return the remaining strips to the aluminium pouch containing desiccant and reseal carefully). Wash each strip once with the Wash Solution. Do not wash more strips than can be handled within 30 min.
3. Pipette 25 µL of each of the HE4 Calibrators (CAL A, B, C, D, E and F), HE4 Controls (C1, C2)

and unknown specimens (Unk) into the strip wells according to the following scheme:

	1	2	3	4	5	6	7 etc
A	Cal A	Cal E	1 st Unk				
B	Cal A	Cal E	1 st Unk				
C	Cal B	Cal F	2nd Unk				
D	Cal B	Cal F	2nd Unk				
E	Cal C	C1					
F	Cal C	C1					
G	Cal D	C2					
H	Cal D	C2					

4. Add 100 µL of Biotin Anti-HE4 to each well using a 100 µL precision pipette (or an 8-channel 100 µL precision pipette). Do not allow the pipette tip to touch the surface of the liquid in order to avoid carry-over.
5. Incubate the plate for 1 hour (± 10 min) at room temperature (20-25°C), constantly shaking the plate using a microplate shaker.
6. After the first incubation aspirate and wash each strip 3 times using the wash procedure described in Procedural notes, item 4.
7. Add 100 µL of Tracer working solution to each well. Use the same pipetting procedure as in item 4 above.
8. Incubate the frame for 1 hour (± 5 min) at room temperature (20–25°C) with constant shaking.
9. After the second incubation aspirate and wash each strip 6 times, using the wash procedure described in Procedural notes, item 4.
10. Add 100 µL of TMB HRP-Substrate to each well using the same pipetting technique as described in item 4 above.

The TMB HRP-Substrate should be added to the wells as quickly as possible and the time between addition to the first and last well should not exceed 5 min.

11. Incubate for 30 min (\pm 5 min) at room temperature (20–25°C) with constant shaking. Avoid exposure to direct sunlight.

12. Immediately read the absorbance at 620 nm in a microplate spectrophotometer.

Option

If the laboratory does not have access to a microplate reader capable of reading at 620 nm, the absorbance can be determined as described in the alternative item 12 below:

Alt. 12. Add 100 μ L of Stop Solution, mix and read the absorbance at 405 nm in a microplate spectrophotometer within 15 min after addition of Stop Solution.

Measurement range

The HE4 EIA measures concentrations between 15 and 900 pM. If HE4 concentrations above the measuring range are expected, it is recommended that samples be diluted with HE4 Calibrator A prior to analysis (see “Calculation of results with diluted samples”).

Quality control

HE4 Control 1 and 2 should be used for validation of each assay series. Ranges of expected results are indicated on the vial labels.

The HE4 assay results should be considered valid if:

- The mean values of control duplicates are within the specified ranges.
- The duplicate replicates of calibrators B-F and controls do not exceed a CV of 15%.
- The duplicate replicates of calibrator A (zero) are not more than 0.06 OD units different from each other.

If an assay results in invalid calibrator or control results, a complete check of reagents, accuracy of pipettes, plate washer and reader performance should be made and the analysis repeated. Each laboratory may also prepare its own serum pools at different levels, which can be used as internal controls in order to assure the precision of the assay.

Reference material

Since no common reference material is available for HE4 antigen, HE4 EIA Calibrator values are assigned against a set of in-house reference standards.

CALCULATION OF RESULTS

If a microplate spectrophotometer with built-in data calculation program is used, refer to the manual for the spectrophotometer and create a program using the concentration stated on the label of each of the HE4 Calibrators.

For automatic calculation of HE4 results it is recommended to use either of the following methods:

- Cubic spline curve fit method. Calibrator A should be included in the curve with the value 0 pM.

- Interpolation with point-to-point evaluation. Calibrator A should be included in the curve with the value 0 pM.
- Quadratic curve fit method. Calibrator A should be included in the curve with the value 0 pM.

NOTE: 4-parametric or Linear regression evaluation methods should not be used.

For manual evaluation, a calibration curve is constructed by plotting the absorbance (A) values obtained for each HE4 Calibrator against the corresponding HE4 concentration (in pM).

The unknown HE4 concentrations can then be read from the calibration curve using the mean absorbance value of each patient specimen.

Calculation of results with diluted samples

If samples in an initial analysis give HE4 levels higher than 900 pM the samples should be diluted 1/10 and 1/100 with HE4 Calibrator A to obtain the accurate HE4 concentration of the samples.

- 1/10 dilution = 50 µL of specimen + 450 µL of HE4 Calibrator A
- 1/100 dilution = 50 µL of 1/10 dilution + 450 µL of HE4 Calibrator A

The HE4 concentration of the undiluted sample is then calculated as:

- Dilution 1/10: 10 x measured value
- Dilution 1/100: 100 x measured value

Risk of Ovarian Malignancy Algorithm (ROMA) for estimating the risk of epithelial ovarian cancer in premenopausal and postmenopausal women presenting with adnexal mass

Calculation of Predictive Index (PI)

The Predictive Index (PI) is calculated for premenopausal and postmenopausal women separately using the equations (1) and (2) below. To calculate the PI, the assay values obtained from the HE4 EIA and ARCHITECT CA125 II assays, respectively, are inserted into the applicable equation of the algorithm below, depending on menopausal status of the woman.

(1) Premenopausal woman

$$\text{Predictive Index (PI)} = -12.0 + 2.38 \cdot \text{LN}[\text{HE4}] + 0.0626 \cdot \text{LN}[\text{CA125}]$$

(2) Postmenopausal woman

$$\text{Predictive Index (PI)} = -8.09 + 1.04 \cdot \text{LN}[\text{HE4}] + 0.732 \cdot \text{LN}[\text{CA125}]$$

Calculation of ROMA value (i.e. Predictive Probability)

To calculate the ROMA value, insert the calculated value for Predictive Index into equation (3):

$$(3) \text{ ROMA value \%} = \exp(\text{PI}) / [1 + \exp(\text{PI})] \cdot 100$$

The examples below can be used in order to validate calculations of PI and PP:

Menopausal status	HE4 (pM)	CA125 (U/mL)	PI calculation	PI	ROMA (%)
Premenopausal	37.5	74.9	-12.0+(2.38*3.624)+(0.0626*4.316)	-3.10388	4.29%
Premenopausal	386.6	21.8	-12.0+(2.38*5.957)+(0.0626*3.082)	2.371517	91.5%
Postmenopausal	66.7	11.3	-8.09+(1.04*4.200)+(0.732*2.425)	-1.94683	12.5%
Postmenopausal	383.1	22.7	-8.09+(1.04*5.948)+(0.732*3.122)	0.381799	59.4%

LIMITATIONS OF THE PROCEDURE

Patients with confirmed ovarian cancer may have HE4 assay values in the same range as healthy women. Certain histological types of ovarian cancer e.g. mucinous or germ cell tumors, rarely express HE4, therefore HE4 is not recommended for monitoring of patients with known mucinous or germ cell ovarian cancer (7). Conversely, elevated levels of HE4 antigen may be present in individuals with non-malignant disease. Therefore, the level of HE4 cannot be used as absolute evidence for the presence or absence of malignant disease and the HE4 test should not be used in cancer screening. The results of the test should be interpreted only in conjunction with other investigations and procedures in the diagnosis of disease and the management of patients, and the HE4 test should not replace any established clinical examination.

The algorithm for estimating risk of epithelial ovarian cancer has not been validated for the following patient groups: patients previously treated for malignancy, patients currently being treated with chemotherapy and patients < 18 years of age.

The form of the mathematical function referred to as the Risk of Ovarian Malignancy Algorithm (ROMA), using the combination of HE4 and ARCHITECT CA125 II assay values, only depends on the premenopausal or postmenopausal status of a woman. The premenopausal or postmenopausal status must be based on ovarian function determined with information available from clinical evaluation and medical history.

The ROMA value does not include age, family history, clinical findings, or imaging results and should be interpreted in conjunction with these parameters.

Failure of the HE4 EIA and/or the ARCHITECT CA125 II assay to perform as indicated, or error in the calculation of results could lead to inaccurate risk assessment and improper management of the patient. Specifically, a falsely low result of the assay(s) could result in a determination that the patient is at lower risk of having epithelial ovarian cancer, which could triage the patient to a less specialized level of care. Use of the assay results without consideration of the other laboratory findings, imaging studies, and clinical assessment could therefore pose a risk.

Anti-reagent antibodies (human anti-mouse antibody (HAMA) or heterophilic antibodies) in the patient sample may occasionally interfere with the assay, even though specific blocking agents are included in the buffers.

The assay must be performed in a temperature controlled environment since incubation at temperatures above the recommended temperature range 20 - 25°C may give false low results.

EXPECTED VALUES

The distribution of HE4 levels determined in 1147 specimens is shown in the table below:

Distribution of HE4 Assay Values					
	Number of subjects	0 - 150 pM	150.1 - 300 pM	300.1 - 500 pM	> 500 pM
APPARENTLY HEALTHY					
Females (Premenopausal)	76	72	3	0	1
Females (Postmenopausal)	103	97	5	0	1
BENIGN CONDITIONS					
Pregnancy	22	21	1	0	0
Benign Gynecological Disease	347	324	18	1	4
Other Benign Disease	108	82	8	7	11
Hypertension/Cong. Heart Failure	96	75	16	2	3
CANCER					
Ovarian Cancer	127	27	18	21	61
Breast Cancer	46	40	4	2	0
Lung Cancer	50	29	15	6	0
Endometrial Cancer	116	86	15	4	11
Gastrointestinal Cancer	56	47	8	0	1

In this study 94.4% of the healthy female subjects had a HE4 assay value at or below 150 pM. It is recommended that each laboratory establish its own reference value for the population of interest.

Monitoring of Disease status in Patients Diagnosed with Ovarian Cancer

The effectiveness of the HE4 EIA as an aid in monitoring of disease status in ovarian cancer patients was determined by assessing changes in HE4 levels in serial serum samples from 80 patients compared to changes in disease status. A study involving a total of 354 pairs of observations was undertaken with an average number of 4.4 observations per patient. A positive change in HE4 was defined as an increase in the value that was at least 25% greater than the previous value of the test. This level of change takes into account the variability of the assay and the biological variability. Sixty percent (60%) or 76/126 of the patient samples with a positive change correlated with the disease progression while seventy-five percent (75%) or 171/228 of the patient serial samples with no significant change in HE4 value correlated with no progression. The total concordance was seventy percent (70% or 247/354). The following table presents the data in a 2 x 2 format.

Change in Disease State per Sequential Pair			
Increase in HE-4 concentration	Progression	No Progression	Total
>25%	76	57	133
≤25%	50	171	221
Total	126	228	354

The following table shows the resulting sensitivities and specificities of the HE4 EIA compared to the disease status at various changes in HE4 EIA concentrations.

Sensitivity is represented as a concordance of the HE4 EIA to progression of disease.

Specificity is represented as a concordance of the HE4 EIA to no progression of disease.

Percent Change in HE4 Concentration (%)	Sensitivity (%)	Specificity (%)
10	71	62
25	60	75
50	43	88
75	38	92
100	31	95

Risk estimation in patients presenting with pelvic mass

The effectiveness of HE4 EIA in combination with ARCHITECT CA125 II for risk estimation for epithelial ovarian cancer of patients presenting with adnexal mass based on their HE4 and CA125 values was determined in a prospective, multi-center, double blind clinical trial. An algorithm (ROMA) was developed for estimation of the risk of epithelial ovarian cancer. The algorithm takes into account the HE4 and CA125 values determined with the HE4 EIA and ARCHITECT CA125 II assays as well as the menopausal status of the patient. The algorithm calculates a predictive probability of finding epithelial ovarian cancer on surgery. In the prospective study a total of 502 patients were included and the predictive probability for ovarian cancer as well as the ability for separation into a low and a high risk group based on ROMA values was determined.

The cumulative frequency distribution of the ROMA values for benign and epithelial ovarian cancer cases including tumors of low malignant potential (LMP) respectively using the algorithm is shown in Figures 1 and 2. The frequency distribution graphs illustrate the distribution of patients with benign disease and epithelial ovarian cancer (including tumors of Low Malignant Potential) at different ROMA value cut-points.

Fig. 1 Cumulative frequency distribution of ROMA values for **premenopausal women**

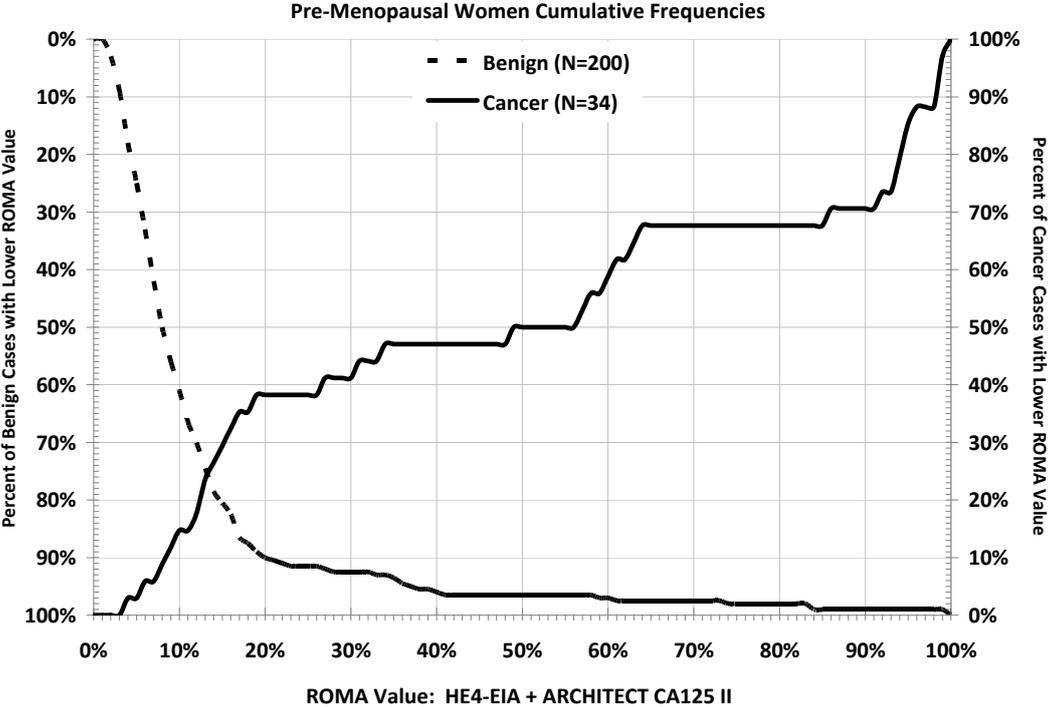
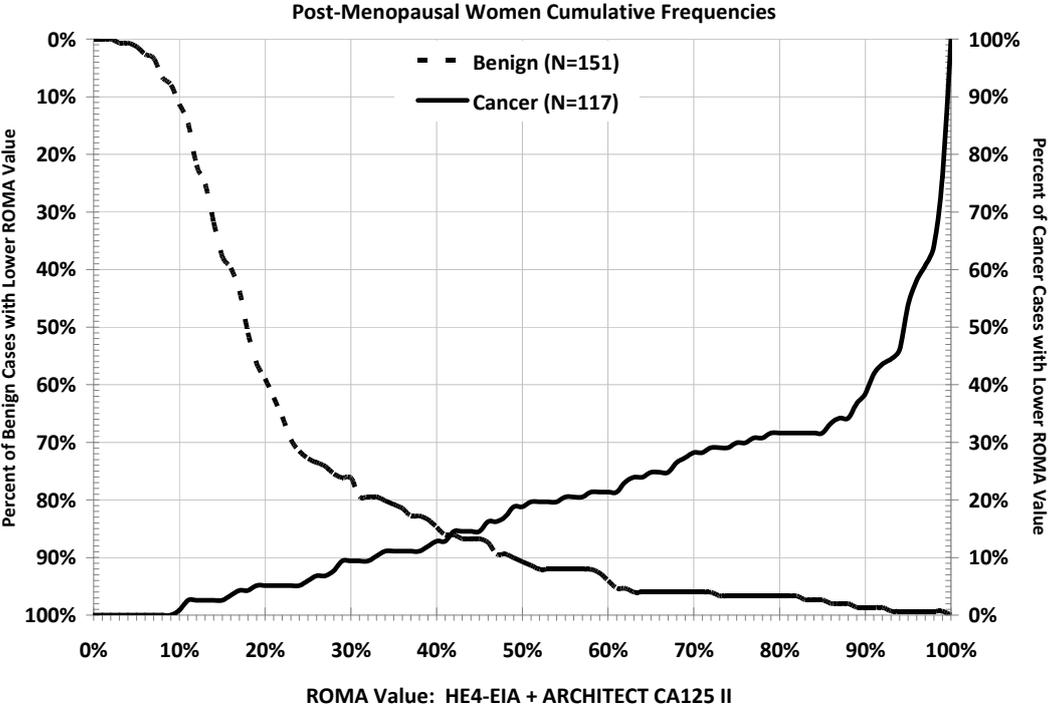


Fig. 2 Cumulative frequency distribution of ROMA values for **postmenopausal women**



Stratification into low risk and high risk groups

The risk of ovarian malignancy algorithm was used to stratify women into risk groups for finding epithelial ovarian cancer. The following cut-points were used in order to provide a specificity level of 75%:

Premenopausal women

ROMA value $\geq 13.1\%$ = High risk of finding epithelial ovarian cancer

ROMA value $< 13.1\%$ = Low risk of finding epithelial ovarian cancer

Postmenopausal women

ROMA value $\geq 27.7\%$ = High risk of finding epithelial ovarian cancer

ROMA value $< 27.7\%$ = Low risk of finding epithelial ovarian cancer

The risk stratification into high risk of harboring epithelial ovarian cancer of all patients presenting with adnexal mass using the ROMA values at 75% specificity level is shown in Table 1 including the risk stratification obtained for the separate premenopausal and postmenopausal patient groups respectively. The sensitivity for stratifying patients with epithelial ovarian cancer stage I-IV into the high risk group was 94% and the specificity was 75%, such that 75% of women with benign pelvic mass were classified into the low risk group. The positive and negative predictive values were 58 % and 97% respectively.

Table 1: Risk stratification into high risk of harboring Epithelial Ovarian Cancer (EOC) in patients presenting with adnexal mass using the ROMA value.

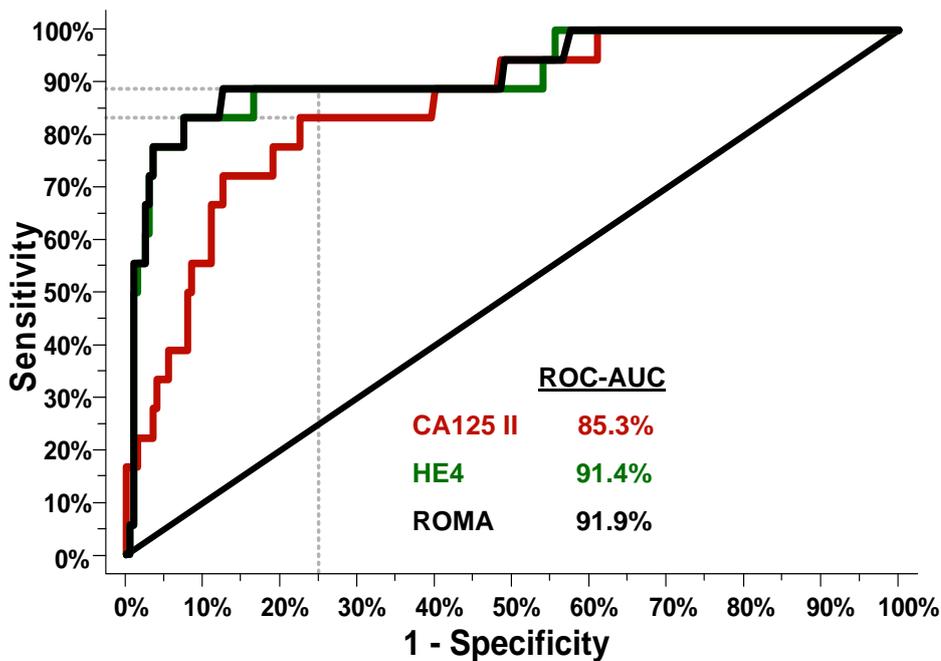
Premenopausal cut-point for stratification into high risk group at 75% specificity level $\geq 13.1\%$, postmenopausal cut-point for stratification into high risk group at 75% specificity level $\geq 27.7\%$.

	Premenopausal Women n = 234	Postmenopausal Women n = 268	Pre- & Postmenopausal Women Combined n = 502
Low Malignant Potential	10/16 (63%)	3/6 (50%)	13/22 (59%)
Stage I-II EOC	6/7 (86%)	24/28 (86%)	30/35 (86%)
Stage I – IIIC^a EOC	7/8 (88%)	35/39 (90%)	42/47 (89%)
Stage I – IV EOC	16/18 (89%)	105/111 (95%)	121/129 (94%)

^aStage I – IIb & Stage IIIC (Omentum negative, lymphnode positive) Epithelial Ovarian Cancer

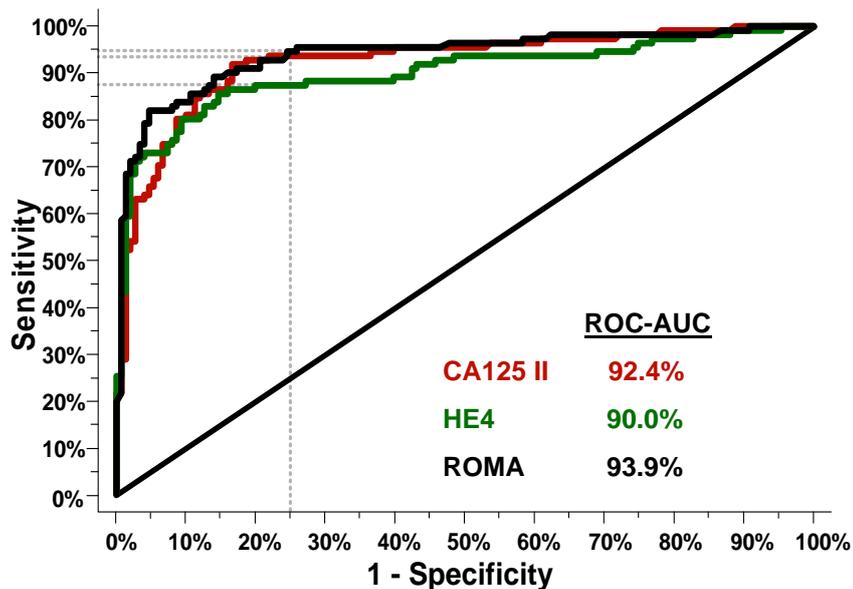
Receiver Operating Characteristic (ROC) curves for HE4 EIA, ARCHITECT CA125 II and ROMA in Epithelial Ovarian Cancer Stage I – IV in premenopausal patients and for HE4 EIA, ARCHITECT CA125 II and ROMA in Epithelial Ovarian Cancer Stages I – IV in post menopausal patients are shown in Figures 3 and 4, respectively.

Fig. 3. ROC curves of HE4 EIA, ARCHITECT CA125 II and ROMA in **premenopausal** patients for differentiation between Benign (N=200) and Epithelial Ovarian Cancers^a (N=18)



^a Tumors of Low Malignant Potential (LMP) not included

Fig. 4. ROC curves of HE4 EIA, ARCHITECT CA125 II and ROMA in **postmenopausal** patients for differentiation between Benign (N=151) and Epithelial Ovarian Cancers^a (N=111)



^a Tumors of Low Malignant Potential (LMP) not included

The ROC curves demonstrated that the ROMA algorithm provided higher AUC as compared to HE4 or CA125 alone in both pre-and postmenopausal women.

The False Negative Rates and percentage of epithelial ovarian cancer stratified into low risk of harboring epithelial ovarian cancer in patients presenting with adnexal mass using the ROMA value at 75 % specificity level is shown in Table 2. Stratification into low and high risk group of harboring epithelial ovarian cancer using the ROMA algorithm at 75 % specificity level resulted in an overall False Negative Rate of 6.2%. Three (3) percent of all cases stratified into the low risk group represented epithelial ovarian cancer.

Table 2. False Negative Rate (FNR) and percentage of Epithelial Ovarian Cancers for all cases stratified into low risk group in patients presenting with adnexal mass using the ROMA value. Premenopausal cut-point for stratification into low risk group at 75% specificity level < 13.1%, postmenopausal cut-point for stratification into low risk group at 75% specificity level < 27.7%.

Epithelial Ovarian Cancer ^a	False Negative Rate (FNR)			Percentage of cancers in Low Risk Group		
	False Negative Cancers	Total Cancers	FNR ^b	False Negative Cancers	True Positive Benign	(%) ^c
Premenopausal	2	18	11.1%	2	149	1.3%
Postmenopausal	6	111	5.4%	6	113	5.0%
All patients	8	129	6.2%	8	262	3.0%

^a Tumors of Low Malignant Potential (LMP) not included; ^b FNR = False Negative/(True Positive + False Negative); ^c False Negative/(True Negative + False Negative)

PERFORMANCE CHARACTERISTICS

Precision

The HE4 assay precision is $\leq 15\%$ total CV. A study was performed as described per the National Committee for Clinical Laboratory Standards NCCLS (CLSI) guideline EP5-A2 (21). A panel of four serum samples was assayed, using two lots of reagents, in replicates of two, at two separate times per day for 20 days. Data from this study is summarized below.*

Sample	Reagent lot	n	Mean conc. (pM)	Within-run SD (pM)	Within-run CV %	Total SD (pM)	Total CV %
1	1	80	50.3	0.81	1.6	2.34	4.7
	2	80	48.0	0.69	1.4	2.17	4.5
2	1	80	75.3	1.81	2.4	2.96	3.9
	2	80	72.4	1.73	2.4	4.70	6.5
3	1	80	255	5.68	2.2	12.0	4.7
	2	80	242	5.21	2.2	12.8	5.3
4	1	80	407	6.22	1.5	14.5	3.6
	2	80	385	8.71	2.3	21.6	5.6

*Representative data; results in individual laboratories may vary from these data.

Detection limit

The limit of detection of the HE4 EIA assay is ≤ 15 pM. The limit of detection (LoD) corresponds to the upper limit of the 95% confidence interval and represents the lowest concentration of HE4 antigen that can be distinguished from zero. The NCCLS guideline EP17-A (22) was used to design the LoD experiments. A study was conducted where HE4 Calibrator A (zero) and 4 samples from healthy subjects diluted to 5 pM with Sample Diluent was tested in replicates of 24 per run in 4 runs on two separate days. The LoD was calculated as follows:

$$\text{LoD (pM)} = 5.0 \text{ pM} \times (1.65 \times \text{SD}_0 + 1.65 \times \text{SD}_5) / (\text{OD}_5 - \text{OD}_0)$$

The Limit of Detection of the HE4 EIA Kit was calculated to be < 2.5 pM.

Functional sensitivity

The functional sensitivity of the HE4 EIA assay is ≤ 25 pM. The functional sensitivity is expressed as the concentration of an analyte at which the CV is 20%. The NCCLS guideline EP5-A2 (21) was used to design the experiments for determination of functional sensitivity. A study was conducted where a five member sensitivity panel was tested in replicates of 4 in 2 runs on twenty separate days with two lots of reagents. The functional sensitivity determined for the HE4 EIA was found to be < 5 pM.

Recovery

The HE4 EIA assay mean recovery is $100 \pm 15\%$. A study was performed where dilutions of a patient sample with known concentrations of HE4 were added to normal human serum samples. The concentration of HE4 was determined using the HE4 EIA assay and the resulting percent recovery was calculated. Representative data from this study is summarized in the table below*.

Sample	Endogenous Assay Value (pM)	HE4 Antigen Added (pM)	Observed HE4 Assay Value (pM)	Percent Recovery** %
1	44.6	15	60.6	102
		75	96.0	89
		350	397	96
		650	686	96
2	41.1	15	55.7	99
		75	95.2	91
		350	400	98
		650	657	93
3	40.6	15	54.0	97
		75	95.1	91
		350	403	99
		650	680	96
4	46.6	15	63.3	103
		75	106	97
		350	410	99
		650	645	90
5	40.2	15	56.5	102
		75	102	98
		350	402	99
		650	676	96

The average recovery across the four separate spiked concentrations shown above was found to be 97%.

*Representative data; results in individual laboratories may vary from these data.

**% Recovery = $\frac{\text{Observed HE4 Concentration (pM)}}{\text{Endogenous HE4 Conc. (pM)} + \text{HE4 Added (pM)}}$

High Dose Hook

High dose hook is a phenomenon whereby very high level specimens may read within the dynamic range of the assay. For the HE4 EIA, no high dose hook effect was observed for samples containing up to 300 000 pM HE4 native antigen.

Dilution Linearity

The HE4 EIA assay mean dilution linearity is $100 \pm 15\%$. A study was conducted for the HE4 EIA modeled after the NCCLS (CLSI) guideline EP6-A (23). Serum samples with elevated HE4 values were diluted with HE4 Calibrator A (zero). The HE4 concentration was determined for each dilution and the percent (%) recovery was calculated. Representative data from this study is summarized in the table below*.

Sample	Final Dilution Factor	Obtained Value (pM)	Expected Value (pM)	Percent Recovery** %
1	Undiluted	889.6	889.6	100
	1:1.25	720.0	711.7	101
	1:1.7	543.1	533.8	101
	1:2	450.6	444.8	101
	1:2.5	345.9	355.8	97.2
	1:5	183.6	177.9	103
	1:10	97.6	89.0	109
	1:20	49.1	44.5	110
	1:40	25.9	22.2	116
2	Undiluted	697.0	697.0	100
	1:1.25	544.9	557.6	97.7
	1:1.7	429.8	418.2	103
	1:2	361.1	348.5	104
	1:2.5	275.9	278.8	99.0
	1:5	134.5	139.4	96.5
	1:10	74.4	69.7	107
	1:20	39.1	34.9	112
	1:40	21.0	17.4	120
3	Undiluted	680.2	680.2	100
	1:1.25	499.7	544.2	91.8
	1:1.7	354.4	408.1	86.8
	1:2	296.7	340.1	87.2
	1:2.5	247.2	272.1	90.9
	1:5	124.9	136.0	91.8
	1:10	61.7	68.0	90.7
	1:20	34.6	34.0	102
	1:40	18.4	17.0	109

Average recovery across the three diluted samples shown above = 101%

*Representative data; results in individual laboratories may vary from these data.

**% Recovery= HE4 Concentration obtained x Dilution factor / Undiluted HE4 Concentration.

Analytical Specificity

The HE4 EIA assay mean assay specificity is $100 \pm 15\%$. Recovery studies were performed to compare sera containing the following compounds at the indicated concentrations with control sera. The NCCLS guideline EP7-A (24) was used to design the interference experiments. The following substances and concentrations were tested and found not to interfere with the test.

Endogenous serum interferences	Test Concentration
Lipids	30 mg/mL
Billirubin	0.2 mg/mL
Hemoglobin	10 mg/mL
Total Protein	120 mg/mL
Chemotherapeutic drug interferences	Test Concentration
Carboplatin	500 µg/mL
Cisplatin	165 µg/mL
Clotrimazole	0.3 µg/mL
Cyclophosphamide	500 µg/mL
Dexamethasone	10 µg/mL
Doxorubicin	1.16 µg/mL
Leucovorin	2.68 µg/mL
Melphalan	2.8 µg/mL
Methotrexate	45 µg/mL
Paclitaxel	3.5 ng/mL

Potentially interfering clinical conditions

The HE4 EIA assay was evaluated using specimens with HAMA and Rheumatoid Factor (RF) to further assess the assay specificity. Five specimens positive for HAMA and five specimens positive for RF were evaluated for % recovery with HE4 antigen spiked into each specimen at approximately 50 and 450 pM. Mean recovery results are summarized in the following table.*

Clinical condition	Number of specimens	Mean % recovery
HAMA	5	101
RF	5	95

*Representative data; results in individual laboratories may vary from these data.

WARRANTY

The performance data presented here were obtained using the assay procedure indicated. Any change or modification of the procedure not recommended by Fujirebio Diagnostics may affect the results, in which event Fujirebio Diagnostics disclaims all warranties expressed, implied or statutory including the implied warranty of merchantability and fitness for use.

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Protocol Sheet

HE4 EIA REF 404-10US

Prepare the components directly before use. Use wash and incubation conditions according to the Instructions.

Note. The assay should only be performed at temperatures between 20–25°C to obtain accurate results.

Step	Vial/Plate	Procedure																																							
1. Prepare HE4 Calibrators	CAL HE4 B, C, D, E, F	Add 1 mL of distilled or deionised water to each vial and mix gently. Allow to stand for at least 15 minutes. NOTE: The exact concentration of each calibrator is stated on the label. Reconstituted stability: 4 weeks at 2-8°C.																																							
Prepare HE4 Controls	CONTROL HE4 1, 2																																								
Prepare Wash Solution	WASHBUF 25X	Dilute 50 mL of Wash Concentrate with 1200 mL of distilled or deionised water.																																							
Prepare Tracer working solution	CONJ Anti-HE4 DIL CONJ	Mix 50 µL of Tracer, HRP Anti-HE4 with 1 mL of Tracer Diluent per strip:																																							
		<table border="1"> <thead> <tr> <th>No. of Strips</th> <th>Tracer, HRP Anti-HE4 (µL)</th> <th>Tracer Diluent (mL)</th> </tr> </thead> <tbody> <tr><td>1</td><td>50</td><td>1</td></tr> <tr><td>2</td><td>100</td><td>2</td></tr> <tr><td>3</td><td>150</td><td>3</td></tr> <tr><td>4</td><td>200</td><td>4</td></tr> <tr><td>5</td><td>250</td><td>5</td></tr> <tr><td>6</td><td>300</td><td>6</td></tr> <tr><td>7</td><td>350</td><td>7</td></tr> <tr><td>8</td><td>400</td><td>8</td></tr> <tr><td>9</td><td>450</td><td>9</td></tr> <tr><td>10</td><td>500</td><td>10</td></tr> <tr><td>11</td><td>550</td><td>11</td></tr> <tr><td>12</td><td>600</td><td>12</td></tr> </tbody> </table>	No. of Strips	Tracer, HRP Anti-HE4 (µL)	Tracer Diluent (mL)	1	50	1	2	100	2	3	150	3	4	200	4	5	250	5	6	300	6	7	350	7	8	400	8	9	450	9	10	500	10	11	550	11	12	600	12
No. of Strips	Tracer, HRP Anti-HE4 (µL)	Tracer Diluent (mL)																																							
1	50	1																																							
2	100	2																																							
3	150	3																																							
4	200	4																																							
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8	400	8																																							
9	450	9																																							
10	500	10																																							
11	550	11																																							
12	600	12																																							
2. Wash	MICROPLA	Wash each well once with Wash Solution. Use manual or automatic washer.																																							
3. Add calibrators, controls and samples	CAL HE4 A, B, C, D, E, F CONTROL HE4 1, 2	25 µL in each well																																							
4. Add Biotin Anti-HE4	BIOTIN Anti-HE4	100 µL in each well																																							
5. Incubate	MICROPLA	1 hour shaking at 20–25°C																																							
6. Wash	MICROPLA	Wash each well three times with Wash Solution. Use manual or automatic washer.																																							
7. Add Tracer working solution	TRACER WORKING SOLUTION	100 µL in each well																																							
8. Incubate	MICROPLA	1 hour shaking at 20–25°C																																							
9. Wash	MICROPLA	Wash each well six times with Wash Solution. Use manual or automatic washer.																																							
10. Add TMB HRP-Substrate	SUBS TMB	100 µL in each well																																							
11. Incubate	MICROPLA	30 min shaking at 20–25°C																																							
12. Read absorbance	MICROPLA	620 nm																																							
Alt.12 Add Stop Solution	STOP	100 µL in each well																																							
Alt.13 Mix	MICROPLA	Allow to mix at 20–25°C																																							
Alt.14 Read absorbance	MICROPLA	Read at 405 nm within 15 min																																							



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