

FOR INFORMATION ONLY.  
WHEN PERFORMING  
THE ASSAY ALWAYS REFER  
TO PACKAGE INSERT  
SUPPLIED  
WITH THE KIT



# HE4 EIA

## Prod. No. 404-10US

Instructions for use. 2011-07

Enzyme immunometric assay kit

For 96 determinations



Use By

IVD

In Vitro Diagnostic Medical Device



Batch code



Temperature limitation



Date of manufacture



Consult Instructions for Use



Catalogue number



Biological risks



Manufacturer

CONT

Contents of kit

ORIG MOU

From mouse



Contains sufficient for <96> tests

ORIG HUM

Human origin

**WARNING:** HE4 assay values obtained with different assay methods cannot be used interchangeably due to differences in assay methods and reagent specificity. The results reported by the laboratory to the physician must include the identity of the HE4 assay used. If, in the course of monitoring a patient, the assay method used for determining serial HE4 levels is changed, additional sequential testing should be carried out. Prior to changing assays, the laboratory **MUST** confirm baseline values for patients being serially monitored. **The HE4 EIA should not be used as a cancer screening test.**

## **INTENDED USE**

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

The assay 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.

## **SUMMARY AND EXPLANATION OF THE ASSAY**

The HE4 gene, also known as WFDC2, encodes a protein with a WAP-type four disulphide core (WFDC) domain. HE4 is one of 14 homologous genes on chromosome 20q12-13.1 which encode proteins with WAP-type four disulphide core (WFDC) domains (1). HE4 belongs to the family of whey acidic four-disulfide core (WFDC) proteins with suspected trypsin inhibitor properties but no biological function has so far been identified (2). Other proteins in this family include SLPI, Elafin, and PS20 (WFDC1) (2). Members of the WFDC family are characterized by an approximately 50 amino acid sequence with eight highly conserved cysteine residues that form four disulphide bridges (3). 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 and two WFDC domains (4).

Human epididymis protein 4 (HE4) was first identified in the epithelium of the distal epididymis and originally predicted to be a protease inhibitor involved in sperm maturation (5,6). HE4 was reported to be expressed in a number of normal tissues including epithelia of respiratory and reproductive tissues (4,7). Elevated levels were found in several tumor cell lines including ovarian, lung, colon and breast cancer. Most interestingly, HE4 is overexpressed 93% of serous, 100% of endometrioid epithelial ovarian cancers and 50% of clear cell but not in mucinous ovarian carcinomas (8). Although it is not tissue-specific, a number of independent microarray studies identified HE4 as one of the most useful biomarkers for ovarian cancer (7,9,10,11).

In addition to expression on a cellular level, secreted HE4 was detected in high levels in the serum of ovarian cancer patients (12). Hellström et al. found elevated HE4 levels in cancer patients compared to healthy and to benign conditions in a case/control study. Furthermore, the area under the ROC curve was comparable to that of established biomarker CA125. At the chosen cut-off, HE4 achieved 96% specificity and 80% sensitivity comparing healthy subjects and cancer patients. Similar results were obtained in other studies using either the research ELISA system or a bead-based array system (13).

## 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 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

<b>Streptavidin Microplate</b>	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.

Component	Quantity	Storage and stability after first use
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CAL	HE4	A
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<b>HE4 Calibrator A</b>	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.

<b>HE4 Calibrators B-F</b>	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
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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.

<b>BIOTIN</b>	<b>Anti-HE4</b>
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<b>Biotin Anti-HE4</b>	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.

Component	Quantity	Storage and stability after first use
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CONJ	Anti-HE4
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<b>Tracer, HRP Anti-HE4</b>	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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<b>Tracer Diluent</b>	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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<b>TMB HRP-Substrate</b>	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.

STOP
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<b>Stop Solution</b>	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
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<b>Wash Concentrate</b>	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:

- 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 (14). Biosafety level 2 (15) 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.

Serum can be stored at 2–8°C for 3 days before being tested. For longer periods store samples at -40°C or colder. Sample storage has been validated for up to 3 years at ≤ -40°C. 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  $\mu\text{L}$ /well/washcycle.

An 8-channel pipette with disposable plastic tips for delivery of 350  $\mu\text{L}$  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 microliter volumes. An 8-channel pipette or dispenser pipette with disposable plastic tips for delivery of 100  $\mu\text{L}$  is recommended but not required. Pipettes for dispensing millilitre volumes.

5. **Distilled or deionized water**

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

6. **Fujirebio Diagnostics Tumor Marker Control (sold separately, REF 108-20)**

### 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, controls 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.

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#### Preparation of reagents

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#### 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.

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#### Tumor Marker Control 1-2

Please consult the Instructions for Use for the Fujirebio Diagnostics Tumor Marker Control (sold separately, REF 108-20).



Preparation of reagents	Stability of prepared reagent
<b>Wash Solution</b>	2 weeks at 2–25°C in a sealed container
<p>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.</p>	
<b>Tracer Working Solution</b>	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):

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, Tumor Marker Control 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 aluminum 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  $\mu\text{L}$  of each of the HE4 Calibrators (CAL A, B, C, D, E and F), Tumor Marker Control (TMC 1 and 2) 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	TMC 1					
F	Cal C	TMC 1					
G	Cal D	TMC 2					
H	Cal D	TMC 2					

4. Add 100  $\mu\text{L}$  of Biotin Anti-HE4 to each well using a 100  $\mu\text{L}$  precision pipette (or an 8-channel 100  $\mu\text{L}$  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 ( $\pm$  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  $\mu\text{L}$  of Tracer working solution to each well. Use the same pipetting procedure as in item 4 above.

8. Incubate the frame for 1 hour ( $\pm 5$  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  $\mu\text{L}$  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  $\mu\text{L}$  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**

Fujirebio Diagnostics Tumor Marker Control (sold separately, REF 108-20) should be used for validation of the assay series. Ranges of expected results are shown in the lot specific Assigned Values sheet provided with the Tumor Marker Control.

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 1 and 2 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.

# 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	<table border="1"><tr><td>CAL</td><td>HE4</td></tr></table> <p>B, C, D, E, F</p>	CAL	HE4	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.																												
CAL	HE4																															
Prepare Tumor Marker Controls	Tumor Marker Control 1, 2	Please consult the Instructions for use for the Fujirebio Diagnostics Tumor Marker Control, REF 108-20.																														
Prepare Wash Solution	<table border="1"><tr><td>WASHBUF</td><td>25X</td></tr></table>	WASHBUF	25X	Dilute 50 mL of Wash Concentrate with 1200 mL of distilled or deionised water.																												
WASHBUF	25X																															
Prepare Tracer working solution	<table border="1"><tr><td>CONJ</td><td>Anti-HE4</td></tr><tr><td>DIL</td><td>CONJ</td></tr></table>	CONJ	Anti-HE4	DIL	CONJ	Mix 50 µL of Tracer, HRP Anti-HE4 with 1mL of Tracer Diluent per strip:																										
CONJ	Anti-HE4																															
DIL	CONJ																															
	<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></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	
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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	25 $\mu$ L in each well		
4. Add Biotin Anti-HE4		BIOTIN Anti-HE4	100 $\mu$ L 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 $\mu$ L 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 $\mu$ L 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 $\mu$ L 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		

## 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  $\mu$ L of specimen + 450  $\mu$ L of HE4 Calibrator A
- 1/100 dilution = 50  $\mu$ L of 1/10 dilution + 450  $\mu$ L 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

## 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.

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:

<b>Distribution of HE4 Assay Values</b>					
	<b>Number of subjects</b>	<b>0 - 150 pM</b>	<b>150.1 - 300 pM</b>	<b>300.1 - 500 pM</b>	<b>&gt; 500 pM</b>
<b>APPARENTLY HEALTHY</b>					
Females (Premenopausal)	76	72	3	0	1
Females (Postmenopausal)	103	97	5	0	1
<b>BENIGN CONDITIONS</b>					
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
<b>CANCER</b>					
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.

<b>Change in Disease State per Sequential Pair</b>			
<b>Increase in HE4 concentration</b>	<b>Progression</b>	<b>No Progression</b>	<b>Total</b>
>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.

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



## 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 (16). 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  $\leq 15$  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 (17) 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  $\leq 25$  pM. The functional sensitivity is expressed as the concentration of an analyte at which the CV is 20%. The NCCLS guideline EP5-A2 (16) 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=Observed HE4 Concentration (pM)/Endogenous HE4 Conc. (pM) + 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 (18). 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 = 98%

\*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 (19) was used to design the interference experiments. The following substances and concentrations were tested and found not to interfere with the test.

<b>Endogenous serum interferences</b>	<b>Test Concentration</b>
Triglycerides	30 mg/mL
Billirubin	0.2 mg/mL
Hemoglobin	10 mg/mL
Total Protein	120 mg/mL

<b>Chemotherapeutic drug interferences</b>	<b>Test Concentration</b>
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 µg/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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