

# Quantikine<sup>®</sup> ELISA

## Human HE4/WFDC2 Immunoassay

Catalog Number DHE400

For the quantitative determination of Human Epididymal protein 4 (HE4) concentrations in cell culture supernates, serum, plasma, saliva, and urine.

This package insert must be read in its entirety before using this product.  
For research use only. Not for use in diagnostic procedures.

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## INTRODUCTION

Human Epididymal Protein 4 (HE4), also known as WAP Four-Disulfide Core domain protein 2 (WFDC2), is a secreted 25 kDa glycosylated member of the Whey Acidic Protein (WAP) family. WAP family proteins (*e.g.* SLPI, Trappin-2/Elafin, WFDC5, and WFDC12) share multiple conserved cysteines and exhibit protease inhibitory and anti-microbial activities (1, 2). Mature human HE4 consists of two tandem WAP domains, while mouse and rat HE4 have an additional 54 amino acid (aa) or 48 aa region between the WAP domains, respectively (3, 4). Mature human HE4 shares approximately 60% with comparable regions of mouse and rat HE4. Alternate splicing of human HE4 generates additional isoforms in which either the first or second WAP domain is deleted and may be replaced by substitutions of 10 aa, 22 aa, or 28 aa (3). HE4 is expressed in the normal epithelium lining the male and female genital tracts (4-6), upper respiratory tract (5-7), and ducts of the salivary glands and breast (5-7). It is also variably expressed in the renal distal convoluted tubule, colon, and endometrium (5, 6, 8).

HE4 is pathologically expressed in lung adenocarcinomas and cystic fibrosis lungs (7, 9, 10). Expression of the isoform which has 22 aa in place of the first WAP domain is associated with lung tumor size and invasiveness (9). Tissue and serum HE4 levels are elevated in type I and type II endometrial cancer, particularly of the endometrioid subtype (5, 8, 11, 12). HE4 elevation in the serum increases with endometrial tumor progression (8, 11). In breast cancer, tissue expression of HE4 correlates with lymph node involvement (13). Urinary HE4 levels are elevated in transitional cell cancer (14) as well as in early and late stage ovarian carcinoma (15). HE4 is not expressed in normal ovarian epithelium (5, 6), although the serum HE4 level rises during the ovulatory phase of the menstrual cycle (16). HE4 is strongly upregulated and secreted in ovarian cancer. Among ovarian cancer subtypes, serous and endometrioid tumors exhibit the most frequent and greatest magnitude of upregulation (5, 6, 17-19) with expression in clear cell and mucinous subtypes relatively sporadic and weak (5, 18). The serum elevation of HE4 is highest in advanced stages of ovarian cancer (17, 19, 20). Serum levels of the mucin glycoprotein CA125 are also elevated in ovarian cancer, and this marker exhibits comparable sensitivity but lower specificity than HE4 (19, 21, 22). The study of ovarian and endometrial cancer progression may be improved by the incorporation of both HE4 and CA125 values in the predictive computer model, risk of malignancy algorithm (ROMA) (8, 18-20, 23-25).

The Quantikine® Human HE4/WFDC2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human HE4 in cell culture supernates, serum, plasma, saliva, and urine. It contains NS0-expressed recombinant human HE4 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human HE4 showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human HE4.

## PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human HE4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any HE4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human HE4 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of HE4 bound in the initial step. The color development is stopped and the intensity of the color is measured.

## LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

## TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

## MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human HE4/WFDC2 Microplate	894187	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human HE4.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human HE4/WFDC2 Conjugate	894188	21 mL of a polyclonal antibody specific for human HE4 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human HE4/WFDC2 Standard	894189	Recombinant human HE4 in a buffered protein base with preservatives; lyophilized. <i>Refer to the vial label for reconstitution volume.</i>	
Assay Diluent RD1W	895117	11 mL of a buffer with preservatives. <i>For cell culture supernate, serum, plasma, and saliva samples.</i>	
Assay Diluent RD1-62	895330	11 mL of a buffered protein base with blue dye and preservatives. <i>For urine samples.</i>	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. <i>Use diluted 1:4 in this assay.</i>	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

\* Provided this is within the expiration date of the kit.

## OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 50 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human HE4 Controls (optional; R&D Systems®, Catalog # QC102).

## PRECAUTIONS

HE4 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

## SAMPLE COLLECTION & STORAGE

**The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.**

**Cell Culture Supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20$  °C. Avoid repeated freeze-thaw cycles.

**Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at  $\leq -20$  °C. Avoid repeated freeze-thaw cycles.

**Plasma** - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at  $\leq -20$  °C. Avoid repeated freeze-thaw cycles.

**Note:** *Citrate plasma has not been validated for use in this assay.*

**Saliva** - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at  $\leq -20$  °C. Avoid repeated freeze-thaw cycles.

**Urine** - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at  $\leq -20$  °C. Avoid repeated freeze-thaw cycles.

## SAMPLE PREPARATION

Serum and plasma samples require at least a 2-fold dilution. A suggested 2-fold dilution is 75  $\mu$ L of sample + 75  $\mu$ L of Calibrator Diluent RD5-26 (diluted 1:4)\*.

Saliva samples require at least a 500-fold dilution. A suggested 500-fold dilution is 10  $\mu$ L of sample + 490  $\mu$ L of Calibrator Diluent RD5-26 (diluted 1:4)\*. Complete the 500-fold dilution by adding 20  $\mu$ L of the diluted sample to 180  $\mu$ L Calibrator Diluent RD5-26 (diluted 1:4)\*.

Urine samples require at least a 200-fold dilution. A suggested 200-fold dilution is 10  $\mu$ L of sample + 190  $\mu$ L of Calibrator Diluent RD5-26 (diluted 1:4)\*. Complete the 200-fold dilution by adding 20  $\mu$ L of the diluted sample to 180  $\mu$ L Calibrator Diluent RD5-26 (diluted 1:4)\*.

\*See Reagent Preparation section.

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

**Note:** HE4 is detectable in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

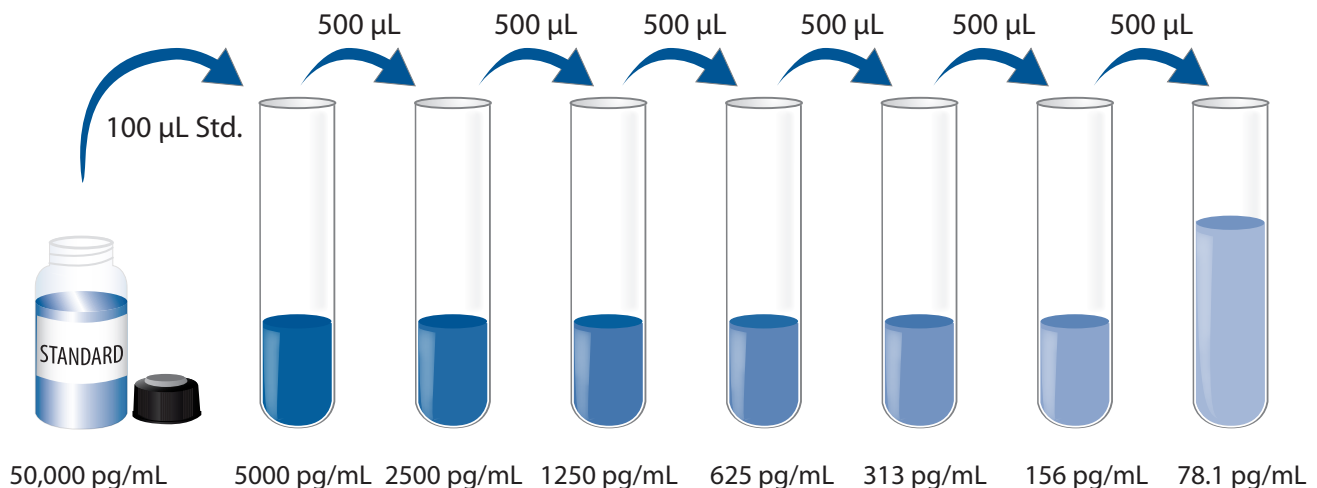
**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200  $\mu$ L of the resultant mixture is required per well.

**Calibrator Diluent RD5-26 (diluted 1:4)** - Add 12.5 mL of Calibrator Diluent RD5-26 Concentrate to 37.5 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5-26 (diluted 1:4).

**Human HE4/WFDC2 Standard - Refer to the vial label for reconstitution volume.**

Reconstitute the Human HE4/WFDC2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 900  $\mu$ L of Calibrator Diluent RD5-26 (diluted 1:4) into the 5000 pg/mL tube. Pipette 500  $\mu$ L into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 5000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) serves as the zero standard (0 pg/mL).



## ASSAY PROCEDURE

**Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.**

**Note:** HE4 is detectable in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100  $\mu$ L of Assay Diluent RD1W (*for cell culture supernate, serum, plasma, and saliva samples*) or Assay Diluent RD1-62 (*for urine samples*) to each well.
4. Add 50  $\mu$ L of standard, control, or sample\* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400  $\mu$ L) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200  $\mu$ L of Human HE4/WFDC2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200  $\mu$ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. **Protect from light.**
9. Add 50  $\mu$ L of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

\*Samples may require dilution. See Sample Preparation section.



## CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

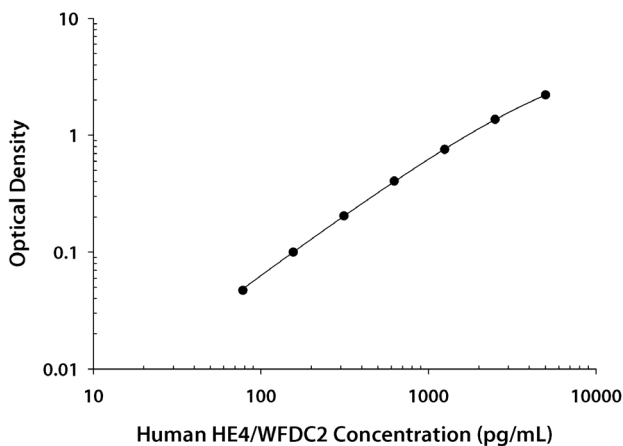
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human HE4 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

## TYPICAL DATA

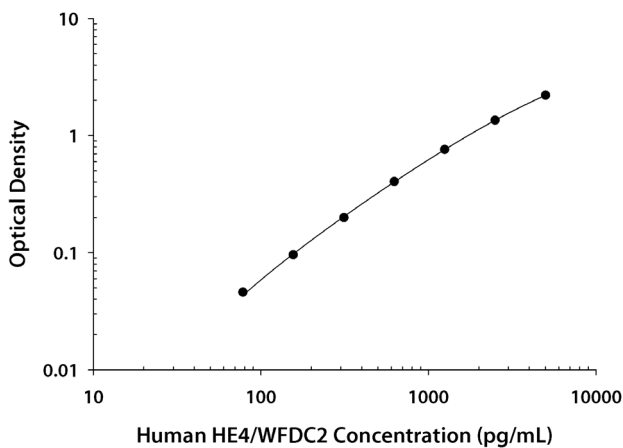
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

### CELL CULTURE SUPERNATE/SERUM/PLASMA/SALIVA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.021 0.026	0.024	—
78.1	0.069 0.073	0.071	0.047
156	0.124 0.124	0.124	0.100
313	0.226 0.229	0.228	0.204
625	0.424 0.433	0.429	0.405
1250	0.766 0.792	0.779	0.755
2500	1.378 1.395	1.387	1.363
5000	2.207 2.252	2.230	2.206

### URINE



(pg/mL)	O.D.	Average	Corrected
0	0.022 0.023	0.023	—
78.1	0.066 0.071	0.069	0.046
156	0.117 0.121	0.119	0.096
313	0.219 0.224	0.222	0.199
625	0.427 0.428	0.428	0.405
1250	0.751 0.814	0.783	0.760
2500	1.352 1.391	1.372	1.349
5000	2.213 2.246	2.230	2.207

## PRECISION

### Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

### Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

## CELL CULTURE SUPERNATE/SERUM/PLASMA/SALIVA ASSAY

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	470	1278	2448	476	1408	2731
Standard deviation	11.5	35.3	86.1	26.0	79.1	162
CV (%)	2.4	2.8	3.5	5.5	5.6	5.9

## URINE ASSAY

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	451	1337	2629	452	1409	2761
Standard deviation	14.0	39.8	85.7	25.0	78.0	202
CV (%)	3.1	3.0	3.3	5.5	5.5	7.3

## RECOVERY

The recovery of human HE4 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=8)	103	95-114%
Serum (n=4)	104	92-115%
EDTA plasma (n=4)	108	94-115%
Heparin plasma (n=4)	107	96-115%
Saliva (n=4)	100	98-107%
Urine (n=4)	98	85-111%

## LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human HE4 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to this assay.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva (n=4)	Urine (n=4)
1:2	Average % of Expected	100	103	103	106	104	102
	Range (%)	97-104	95-107	102-106	102-109	102-104	100-106
1:4	Average % of Expected	101	103	104	110	105	100
	Range (%)	96-106	94-108	96-107	107-114	101-107	94-104
1:8	Average % of Expected	101	104	106	110	106	101
	Range (%)	98-106	96-108	100-110	102-115	103-108	96-105
1:16	Average % of Expected	99	103	105	107	109	103
	Range (%)	95-105	98-108	97-110	102-115	104-112	96-109

## SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of human HE4 ranged from 2.44-32.2 pg/mL. The mean MDD was 10.6 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

## CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed full length mature recombinant human HE4 produced at R&D Systems® (aa28-124 Accession Number Q14508).

## SAMPLE VALUES

**Serum/Plasma/Saliva/Urine** - Samples from apparently healthy volunteers were evaluated for the presence of human HE4 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=35)	4283	3143-6430	792
EDTA plasma (n=35)	4068	3043-5973	724
Heparin plasma (n=35)	3889	2760-5707	717

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Saliva (n=10)	1032	233-1835	565
Urine (n=12)	502	229-890	247

### Cell Culture Supernates:

ZR-75 human breast cancer cells were cultured in DMEM and supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum until confluent. An aliquot of the cell culture supernate was removed, assayed for human HE4, and measured 1610 pg/mL.

OVCAR-3 human ovarian carcinoma cells were cultured in RPMI and supplemented with 20% fetal bovine serum, 10 µg/mL bovine insulin, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate until confluent. An aliquot of the cell culture supernate was removed, assayed for human HE4, and measured 133 ng/mL.

MCF-7 human breast cancer cells were cultured in DMEM and supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. An aliquot of the cell culture supernate was removed, assayed for human HE4, and measured 256 pg/mL.

## SPECIFICITY

This assay recognizes natural and recombinant human HE4.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human HE4 control were assayed for interference. No significant cross-reactivity or interference was observed.

### Recombinant human:

CA125/MUC16

Mesothelin

SLPI

Trappin-2/Elafin

WFDC1

WFDC6

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## PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

The diagram shows a microplate layout with 12 rows and 8 columns. The rows are numbered 1 through 12 on the left side, and the columns are labeled A through H at the bottom. Each well is represented by a circle. The layout is as follows:

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								

**NOTES**

**NOTES**

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