

*PRODUCT INFORMATION AND MANUAL*

***Human Active EGF-R  
ELISA***

***BMS270***

Enzyme-linked immunosorbent assay for quantitative  
detection of human active EGF-R.

For research use only.

Not for diagnostic or therapeutic procedures.

**Human Active EGF-R  
BMS270**



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## 1 Intended Use

The human EGF-R ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of active phosphorylated human EGF-R. **The human EGF-R ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

## 2 Summary

The epidermal growth factor receptor (EGF-R) is a glycoprotein that consists of 130 kDa protein (1186 amino acid residues) and 40 kDa sugar chain. EGF receptor molecule is made up of four domains; glycosylated extracellular domain that is the binding site of EGF and TGF $\alpha$ , a transmembrane domain, tyrosin kinase domain and auto-phosphorylation domain. The tyrosin kinase domain is an intracellular domain which is homologous with the oncogene v-erbB product. (1)

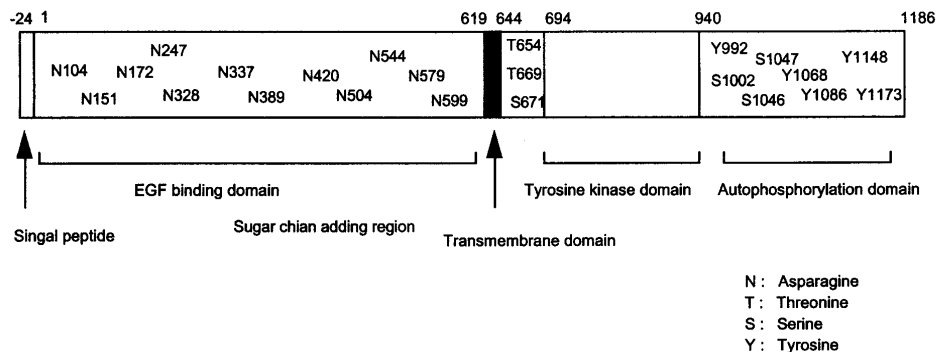
The binding of EGF to the receptor results in DNA reproduction and cell proliferation. During this step, ruffling of cell membrane, phosphorylation of EGF receptor, internalization, pH change, enzyme activation, re-organization of actin filament, and oncogene protein induction are occurred. Although the mechanism of the EGF signal transduction is not clear, it is assumed that EGF binding stimulation should initiate a series of signal transduction through tyrosine auto-phosphorylation and phosphorylation of interacting proteins. (2)

The half-life of EGF receptor on the cell surface is approximately 20 hours. When the receptor accepts the ligand, the degradation of the receptor is accelerated (degraded approximately in 5 hours). (3)

The human squamous epithelium carcinoma cell line, A431, possess many EGF receptors, 5-10 times of standard cell lines ( $2-3 \times 10^6$ /cell).

This kit enables to measure the quantity of phosphorylated EGF receptor by ELISA. It is useful for analysis of receptor phosphorylation and for in vitro screening of specific inhibitors to EGF receptor kinase.

## Structure of EGF-R and the position of its phosphorylation



Main phosphorylated amino acid residue (T:Threonin, S:Serin, Y:Tyrosin) positions are located in the auto-phosphorylation domain of EGF receptor. By accepting the EGF molecule, phosphorylation of EGF receptor is accelerated, and signals are transferred by phosphorylation. Using phosphorylated peptide mapping method, Shimizu and his co-workers have analyzed the position of phosphorylated amino acids in the receptor of NA cells which were highly expressing EGF receptor after stimulated with EGF or H<sub>2</sub>O<sub>2</sub>.(6)

According to their reports, in NA cells, T<sup>669</sup>, S<sup>1046</sup>, S<sup>1047</sup> are phosphorylated in normal condition, and S<sup>671</sup>, T<sup>669</sup>, S<sup>1046</sup>, S<sup>1047</sup>, Y<sup>1173</sup> are phosphorylated with H<sub>2</sub>O<sub>2</sub> stimulation and T<sup>654</sup>, T<sup>669</sup>, Y<sup>1173</sup> and other Y with EGF stimulation.

Thus, it is confirmed that tyrosin phosphorylation is remarkably triggered by EGF or H<sub>2</sub>O<sub>2</sub> exposure.

### 3 Principles of the Test

An anti-human EGF-R coating antibody is adsorbed onto microwells.

Human EGF-R present in the sample or standard binds to antibodies adsorbed to the microwells.

Following incubation unbound biological components are removed during a wash step and a HRP-conjugated anti-phosphotyrosine antibody is added and binds to active phosphorylated human EGF-R captured by the first antibody.

Following incubation unbound HRP-conjugated anti-phosphotyrosine antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 1

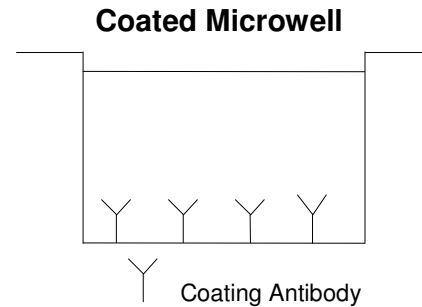


Figure 2

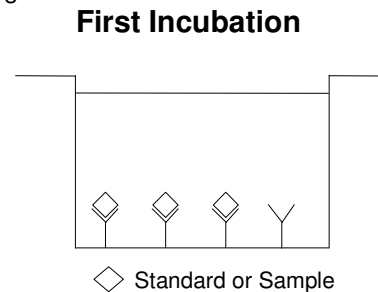


Figure 3

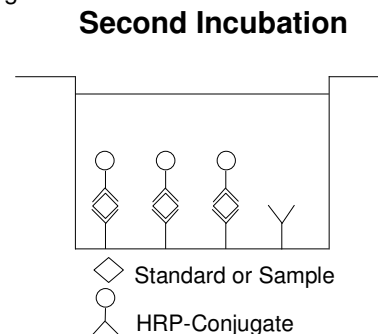
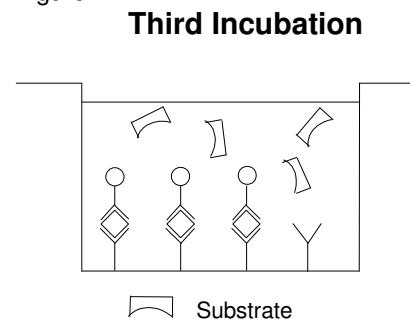
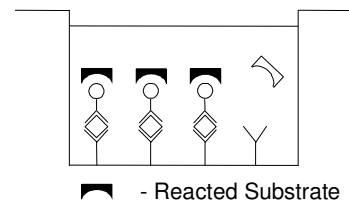


Figure 4



A coloured product is formed in proportion to the amount of active phosphorylated human EGF-R present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human EGF-R standard dilutions and human EGF-R concentration determined.

Figure 5



#### 4 Reagents Provided

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human EGF-R
- 1 vial **HRP-Conjugate** anti-phosphotyrosine monoclonal antibody, lyophilized
- 1 vial human active phosphorylated EGF-R **Standard**, lyophilized
- 2 vials (11 ml) **Sample Diluent**
- 1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)
- 1 vial (11 ml) **Receptor Extraction Buffer**
- 1 vial (12 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (12 ml) **Stop Solution** (1N sulfuric acid)
- 2 **Adhesive Films**

## 5 Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C, store reconstituted standard at -80°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

## 6 Specimen Collection and Storage Instructions

Cell lysates and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human EGF-R. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

### 6.1 Sample Preparation – Cell Lysate Protocol

#### For adhesion cells:

- Culture cells on a  $\phi$ 90mm dish up to confluent (average cell number  $10^7$  cells).
- Remove the supernatant (no washing). Add 1 ml of Receptor Extraction Buffer and recover the cell solution from the dish with a cell scraper. Transfer the solution to a 1.5 ml microcentrifuge tube.
- Spin at 4°C for 5 min. at 10000 g and collect the supernatant as a sample.

#### For suspended cells:

- Transfer cells to a microcentrifuge tube, and spin at 300 g.

- Remove the supernatant (no washing). Add 1 ml of Receptor Extraction Buffer and suspend the cells by pipeting.
- Spin at 4°C for 5 min. at 10000 g and use the supernatant as a sample.

NOTE:

- Cell lysate samples should be prepared freshly for the assay. If necessary store cell lysate samples frozen at -80°C.
- Cell lysate samples must be diluted 10 - 50 times depending on the cell line used.
- Human serum and plasma samples are assayed with no dilution or 2-fold dilution.

## **7 Materials Required But Not Provided**

- 5 ml and 10 ml graduated pipettes
- 5  $\mu$ l to 1000  $\mu$ l adjustable single channel micropipettes with disposable tips
- 50  $\mu$ l to 300  $\mu$ l adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

## 8 Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

## 9 Preparation of Reagents

**Buffer Concentrates** should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

### 9.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25 °C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

### 9.2 HRP-Conjugate

**Please note that the HRP-Conjugate should be used within 30 minutes after dilution.**

Reconstitute the lyophilized **HRP-Conjugate** by addition of 11 ml distilled water. Swirl or mix gently to insure complete and homogeneous solubilisation. Proceed with 10 min of occasional mixing, avoid foaming.

### 9.3 Human active phosphorylated EGF-R Standard

Reconstitute **human EGF-R standard** by addition of 1 ml distilled water. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard (fmol/ml) is stated on the vial). Proceed with 10 min of occasional mixing, avoid foaming.

The reconstituted standard lyophilisate is stable for 2 weeks at -80 °C.

**Standard dilutions** can be prepared directly on the microwell plate (see 10.d) or alternatively in tubes (see 9.3.1).

#### 9.3.1 External Standard Dilution

Label 7 tubes, one for each standard point.

S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the standard curve as follows:

Pipette 225 µl of Sample Diluent into tubes S2 – S7.

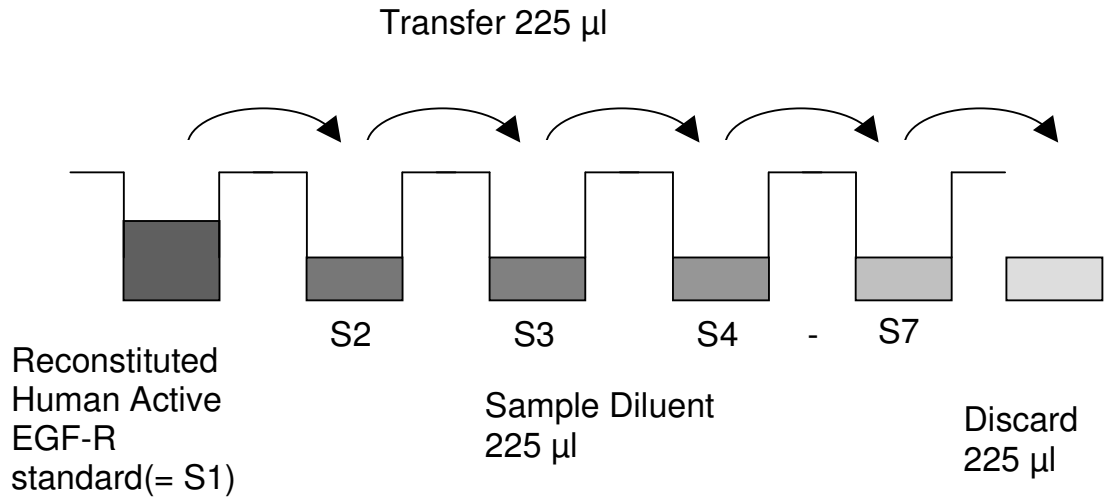
Pipette 225 µl of reconstituted standard (serves as the highest standard S1) into the first tube, labelled S2, and mix.

Pipette 225 µl of this dilution into the second tube, labelled S3, and mix thoroughly before the next transfer.

Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 6).

Sample Diluent serves as blank.

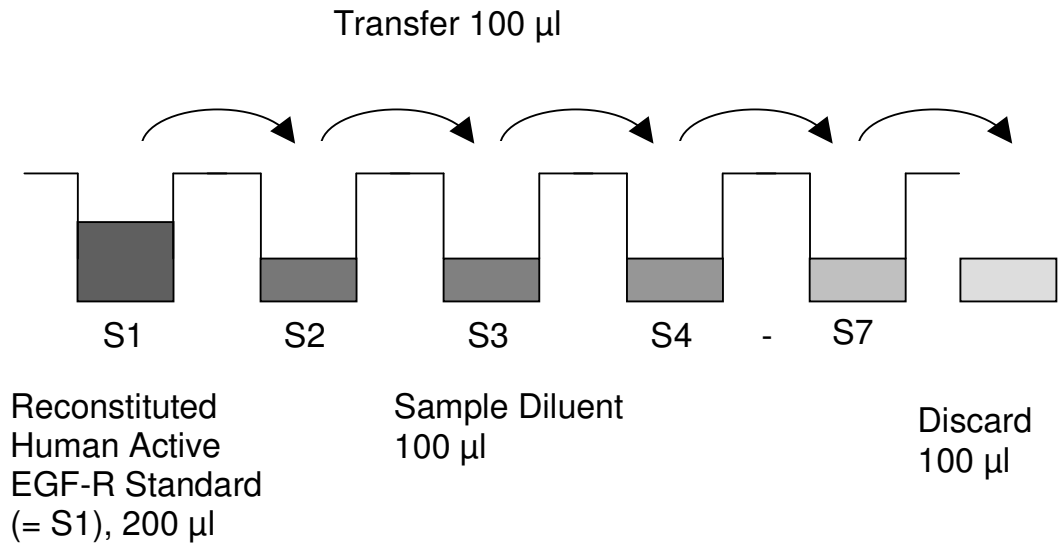
Figure 6



## 10 Test Protocol

- a. For cell lysis follow the cell lysate protocol (see 6.1):
- b. Predilute your samples before starting with the test procedure. Dilute cell lysate samples 1:10 to 1:50 with Sample Diluent. Dilute serum and plasma samples 1:2 with Sample Diluent if necessary.
- c. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- d. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.3.1):  
Add 100 µl of Sample Diluent in duplicate to **standard wells** B1/2 - G1/2, leaving A1/A2 empty. Pipette 200 µl of prepared **standard** (see Preparation of Standard 9.3) in duplicate into well A1 and A2 (see Table 1). Transfer 100 µl to wells B1 and B2. Mix the contents of wells B1 and B2 by repeated aspiration and ejection, and transfer 100 µl to wells C1 and C2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human EGF-R standard dilutions. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 7



In case of an **external standard dilution** (see 9.3.1), pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Standard 1	Standard 1	Sample 1	Sample 1
<b>B</b>	Standard 2	Standard 2	Sample 2	Sample 2
<b>C</b>	Standard 3	Standard 3	Sample 3	Sample 3
<b>D</b>	Standard 4	Standard 4	Sample 4	Sample 4
<b>E</b>	Standard 5	Standard 5	Sample 5	Sample 5
<b>F</b>	Standard 6	Standard 6	Sample 6	Sample 6
<b>G</b>	Standard 7	Standard 7	Sample 7	Sample 7
<b>H</b>	Blank	Blank	Sample 8	Sample 8

- e. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
- f. Add 100 µl of each prediluted **sample** in duplicate to the **sample wells**.
- g. Cover with an adhesive film and incubate at 37°C for 1 hour, if available on a microplate shaker set at 100 rpm.
- h. Prepare **HRP-Conjugate** (see Preparation of HRP-Conjugate 9.2).
- i. Remove adhesive film and empty wells. **Wash** microwell strips 3 times with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on

absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**

- j. Add 100 µl of **HRP-Conjugate** to all wells.
- k. Cover with an adhesive film and incubate at 37°C for 1 hour, if available on a microplate shaker set at 100 rpm.
- l. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point i. of the test protocol. Proceed immediately to the next step.
- m. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- n. Incubate the microwell strips at room temperature (18° to 25°C) for about 15 min if available on a microplate shaker set at 100 rpm. Avoid direct exposure to intense light.

**The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.**

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.6 – 0.65.

- o. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- p. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the

blank wells. Determine the absorbance of both the samples and the standards.

**Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.**

## 11 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human EGF-R concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human EGF-R for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human EGF-R concentration.
- **If instructions in this protocol have been followed cell lysate samples have been diluted 1:10 – 1:50, the concentration read from the standard curve must be multiplied by the dilution factor (x 10 – 50).  
If serum or plasma samples have been diluted 1:2 the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect human EGF-R levels. Such samples require further external predilution according to expected human EGF-R values with Sample Diluent in order to precisely quantitate the actual human EGF-R level.**
- It is suggested that each testing facility establishes a control sample of known human EGF-R concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 8

Representative standard curve for human EGF-R ELISA. Human EGF-R was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

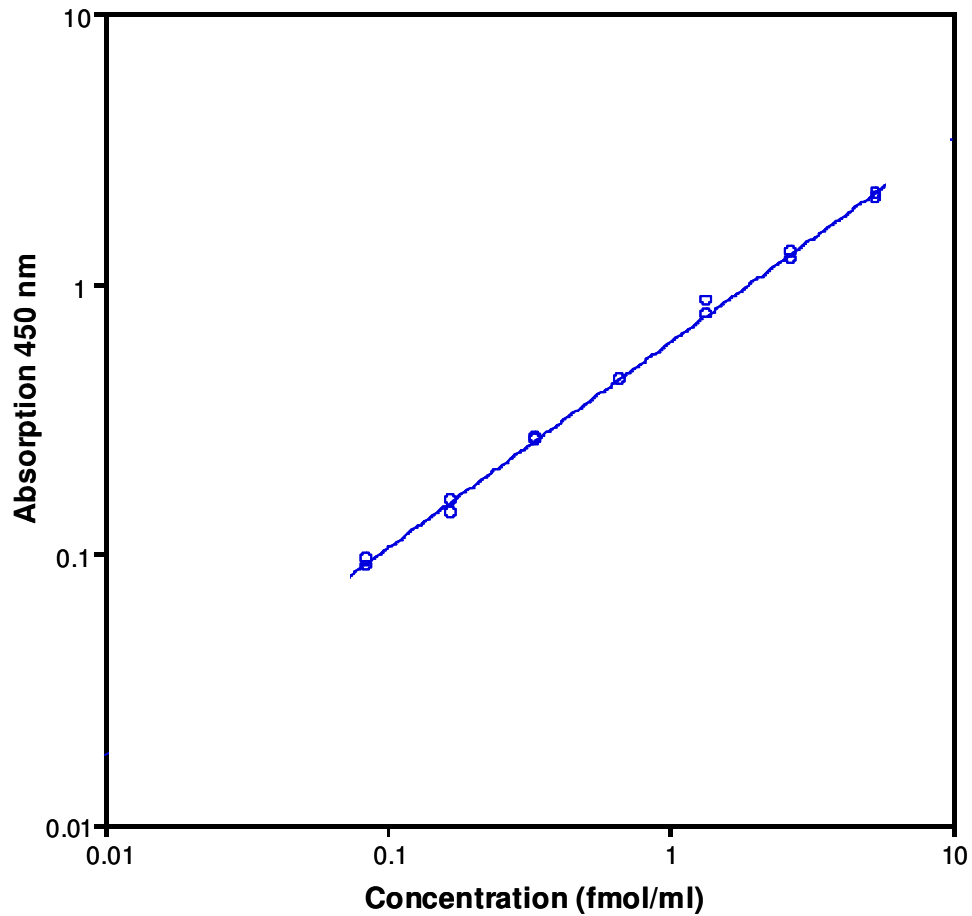


Table 2

Typical data using the human EGF-R ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human EGF-R Concentration (fmol/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	5.400	2.156	2.114	2.0
	5.400	2.072		
2	2.700	1.313	1.266	3.7
	2.700	1.218		
3	1.350	0.863	0.814	6.0
	1.350	0.765		
4	0.675	0.439	0.438	0.3
	0.675	0.436		
5	0.338	0.264	0.266	0.5
	0.338	0.267		
6	0.169	0.141	0.149	5.5
	0.169	0.157		
7	0.084	0.090	0.093	3.7
	0.084	0.096		
Blank	0	0.021	0.020	
	0	0.019		

**NOTE:** The concentration of the standard curve may vary. Please refer to the concentration stated on the standard vial for concentration of S1.

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

## 12 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

## 13 Performance Characteristics

### 13.1 Sensitivity

The limit of detection of human EGF-R defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.051 fmol/ml (mean of 10 independent assays).

### 13.2 Reproducibility

#### 13.2.1 Intra-assay

Reproducibility within the assay was evaluated with 16 replicates of 3 cell lysate samples (A431 cell extract) containing different concentrations of human EGF-R. Data below show the mean human active EGF-R concentration, the standard deviation and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 5.4%.

Table 3

Sample	Mean Human Active EGF-R Concentration (fmol/ml)	Standard Deviation (fmol/ml)	Coefficient of Variation (%)
1	1.940	0.115	5.9
2	0.541	0.026	4.8
3	0.167	0.009	5.4

#### 13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments over 3 days. Data below show the mean human active EGF-R concentration, the standard deviation and the coefficient of variation for each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 4.4%.

Table 4

Sample	Mean Human Active EGF-R Concentration (fmol/ml)	Standard Deviation (fmol/ml)	Coefficient of Variation (%)
1	1.940	0.095	4.9
2	0.522	0.026	5.0
3	0.162	0.006	3.4

### 13.3 Recovery Studies

The recovery was evaluated by combining 2 samples out of 10 different human active EGF-R samples with known concentration. The measured value of 20 combined samples was compared with the calculated value and recovery determined (see Table 5).

The recovery ranged from 94.5% to 109.1% with an overall mean recovery of 99.3%.

Table 5

	Sample A Human EGF-R Conc. (fmol/ml)	Sample B Human EGF-R Conc. (fmol/ml)	Sample A+B Observed Human EGF-R Conc. (fmol/ml)	Sample A+B Expected Human EGF-R Conc. (fmol/ml)	Recovery (%)
1	2.640	0.000	1.440	1.320	109.1
2	2.640	2.640	2.520	2.640	95.5
3	2.640	1.360	1.890	2.000	94.5
4	2.640	0.636	1.590	1.640	97.1
5	2.640	0.288	1.420	1.460	97.0
6	2.640	0.142	1.340	1.390	96.3
7	1.360	0.000	0.675	0.680	99.3
8	1.360	1.360	1.330	1.360	97.8
9	1.360	0.636	0.946	0.998	94.8
10	1.360	0.288	0.790	0.824	95.9
11	1.360	0.142	0.720	0.751	95.9
12	0.636	0.000	0.317	0.318	99.7
13	0.636	0.636	0.634	0.636	99.7
14	0.636	0.288	0.471	0.462	101.9
15	0.636	0.142	0.390	0.389	100.3
16	0.288	0.000	0.151	0.144	104.9
17	0.288	0.288	0.299	0.288	103.8
18	0.288	0.142	0.218	0.215	101.4
19	0.142	0.000	0.072	0.071	101.4
20	0.142	0.142	0.141	0.142	99.3

### 13.4 Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human active EGF-R positive cell lysate. There was no crossreactivity detected.

## 14 Bibliography

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For literature update refer to **[www.bendermedsystems.com](http://www.bendermedsystems.com)**

## 15 Ordering Information

### + Europe

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[www.ebioscience.com](http://www.ebioscience.com)

## 16 Reagent Preparation Summary

### 16.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20x (50 ml) to 950 ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

### 16.2 HRP-Conjugate

Reconstitute lyophilized **HRP-Conjugate** with 11 ml distilled water.

### 16.3 Human Active EGF-R Standard

Reconstitute lyophilized **human active EGF-R standard** with 1 ml distilled water.

## 17 Test Protocol Summary

1. Prepare cell lysates according to protocol (see 6.1).
2. Predilute cell lysate samples with Sample Diluent 1:10 – 1:50. If necessary predilute serum and plasma samples with Sample Diluent 1:2.
3. Determine the number of microwell strips required.
4. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells leaving the first wells empty. Pipette 200 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.  
Alternatively external standard dilution in tubes (see 9.3.1): Pipette 100 µl of these standard dilutions in the microwell strips.
5. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
6. Add 100 µl prediluted sample in duplicate, to designated sample wells.
7. Cover microwell strips and incubate 1 hour at 37°C.
8. Prepare HRP-Conjugate.
9. Empty and wash microwell strips 3 times with Wash Buffer.
10. Add 100 µl HRP-Conjugate to all wells.
11. Cover microwell strips and incubate 1 hour at 37°C.
12. Empty and wash microwell strips 4 times with Wash Buffer.
13. Add 100 µl of TMB Substrate Solution to all wells.
14. Incubate the microwell strips for about 15 minutes at room temperature (18° to 25°C).
15. Add 100 µl Stop Solution to all wells.
16. Blank microwell reader and measure colour intensity at 450 nm.

**Note: If instructions in this protocol have been followed cell lysate samples have been diluted 1:10 – 1:50, the concentration read from the standard curve must be multiplied by the dilution factor (x 10 – 50).**

**If serum or plasma samples have been diluted 1:2, the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**