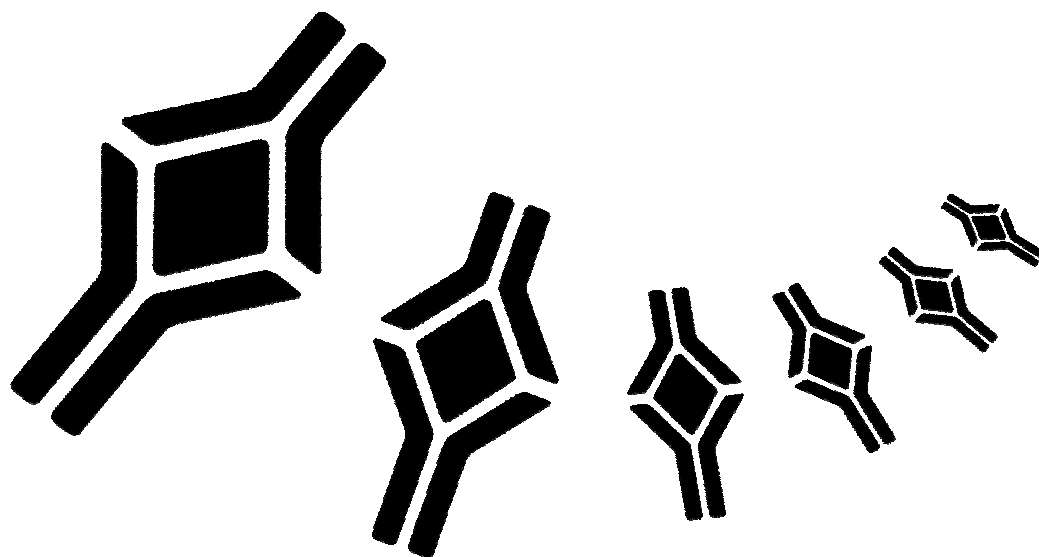


**BioVendor**

Research  
and Diagnostic Products



# Human Active EGF receptor ELISA

Product Data Sheet

Cat. No.: RBMS270R

For Research Use Only

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**»» This kit is manufactured by:  
BioVendor – Laboratorní medicína, a.s.**

**»» Use only the current version of Product Data Sheet enclosed with the kit!**

## 1 INTENDED USE

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The Active EGF-R ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of Active epidermal growth factor (EGF) receptor levels in cell lysates, human serum, and other biological fluids. **The EGF-R ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

## 2 SUMMARY

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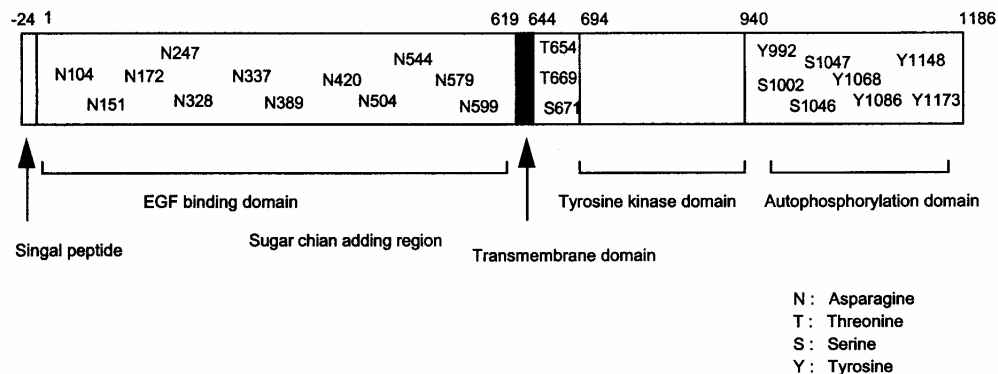
The epidermal growth factor (EGF) receptor is a glycoprotein that consists of 130 kDa protein (1186 amino acid residues) and 40 kDa sugar chain. EGF receptor molecule is made up of four domains; glycosylated extracellular domain that is the binding site of EGF and TGF $\alpha$ , a transmembrane domain, 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 receptor and its position of phosphorylation



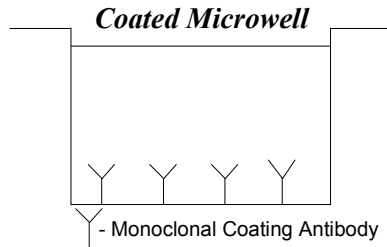
Main phosphorylated amino acid residue (T:Threonin, S:Serin, Y:Tyrosin) position are located in auto-phosphorylation domain of EGF receptor. By accepting the EGF molecule, phosphorylation of EGF receptor is accelerated, and signals are transferred by phosphorylation. Using phosphorylated peptide mapping 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.

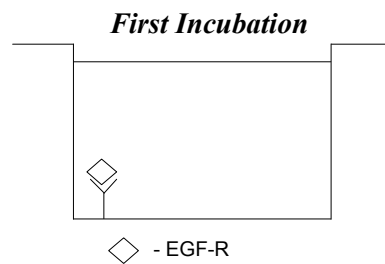
Thus, it is confirmed that tyrosin phosphorylation is remarkably triggered by EGF or H<sub>2</sub>O<sub>2</sub> exposure.

### 3 PRINCIPLES OF THE TEST

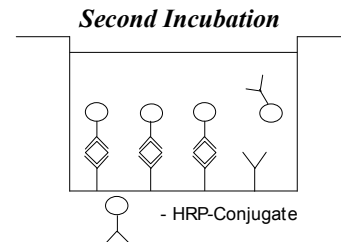
An anti-EGF receptor monoclonal coating antibody is adsorbed onto microwells.



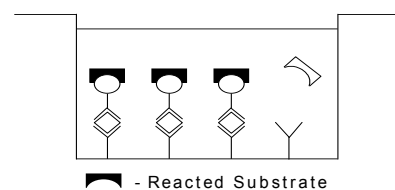
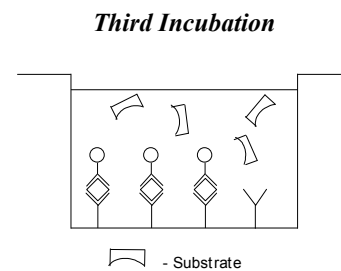
EGF-R present in the sample or standard binds to antibodies adsorbed to the microwells; After incubation, a HRP-conjugated monoclonal anti-phosphotyrosine antibody is added and binds to activated EGF receptor captured by the first antibody.



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



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



## 4 REAGENTS PROVIDED

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- 1 aluminium pouch with a **Antibody Coated Microtiter Strips** with Monoclonal Antibody (murine) to human EGF receptor
- 1 vial **HRP-Conjugate** anti-Phosphotyrosine monoclonal (murine) antibody, lyophilized
- 1 vial **active EGF-R Standard** (concentration (fmol/ml) on vial label), lyophilized
- 1 bottle (50 ml) **Wash Buffer Concentrate** 20x (phosphate-buffered saline with 1% Tween 20)
- 2 bottles (11 ml) **Sample diluent** (buffered protein matrix)
- 1 vial (12 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (11 ml) **Receptor Extraction Buffer** (to prepare cell lysate)
- 1 vial (12 ml) **Stop Solution** (1N Sulfuric acid)
- 2 adhesive **Plate Covers**

### **Reagent Labels**

## 5 STORAGE INSTRUCTIONS

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Store kit reagents between 2° and 8°C. Immediately after use reagents should be returned to cold storage (2° to 8°C) except the reconstituted standard lyophilisate. The reconstituted standard lyophilisate is stable for 2 weeks at -80°C. Expiry of the kit and reagents is stated on labels. The 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, the reagent is not contaminated by the first handling.

## 6 SPECIMEN COLLECTION

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Cell lysates, human serum, and other biological fluids are suitable for use in the assay. Remove the serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

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

Samples must be stored frozen at -20°C to avoid loss of bioactive 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, frozen sera or plasma should be brought to room temperature slowly and mixed gently.

### **Method of preparing adhesion cell lysate:**

- 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 Cell Scraper. Transfer the solution to a 1.5 ml microcentrifuge tube.
- Spin at 4°C for 5 min. at 10000g and collect the supernatant as a sample.
- Cell lysate sample may be diluted up 10-50 times because of high productivity of EGF receptor depending on the cell line used.

### **Method of preparing suspension cell lysate:**

- Transfer cells to a microcentrifuge tubes, and spin at 300g.
- Remove the supernatant (no washing). Add 1 ml of Receptor extraction buffer and suspend the cell by pipeting.
- Spin at 4°C for 5 min. at 10000g and use the supernatant as a sample.

### **NOTE:**

- Samples should be prepared each time of assay. In case of storing a sample, it should be frozen at  $-80^{\circ}\text{C}$ .
- Human serum and plasma are used for assay with no dilution or 2-fold dilution.

## 7 MATERIALS REQUIRED BUT NOT PROVIDED

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- 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 linear regression analysis

## 8 PRECAUTIONS FOR USE

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- 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 statements(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 reagents.
- Exposure to acids will inactivate 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 if 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

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### A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 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 is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

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

### B. Preparation of HRP-Conjugate

The **HRP-Conjugate** must be solubilized with 11 ml of distilled water. Mix gently followed by 10 min occasional mixing, avoiding foam formation.

### C. Preparation of Standard

Rehydrate **Standard** with 1 ml distilled water. Slowly roll for approximately 10 min or let vial stand and sporadically mix gently. The concentration (fmol/ml) is shown on the vial label. The reconstituted standard lyophilisate is stable for 2 weeks at -80°C.

## 10 TEST PROTOCOL

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- a. Mix all reagents thoroughly without foaming before use.
- b. Remove **Microwell Plate coated with Monoclonal Antibody** (murine) to human EGF-R from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- c. Add 100 µl of **Sample Diluent**, in duplicate, to the standard wells, leaving the first wells empty. Prepare standard dilutions by pipetting 200 µl of **EGF-R Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Transfer 100µl from well A1 and A2 to well B1 and B2 respectively. Mix the contents of well B1 and B2 and transfer 100 µl to well C1 and C2 respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of EGF-R standard dilutions. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of EGF-R standard dilutions:

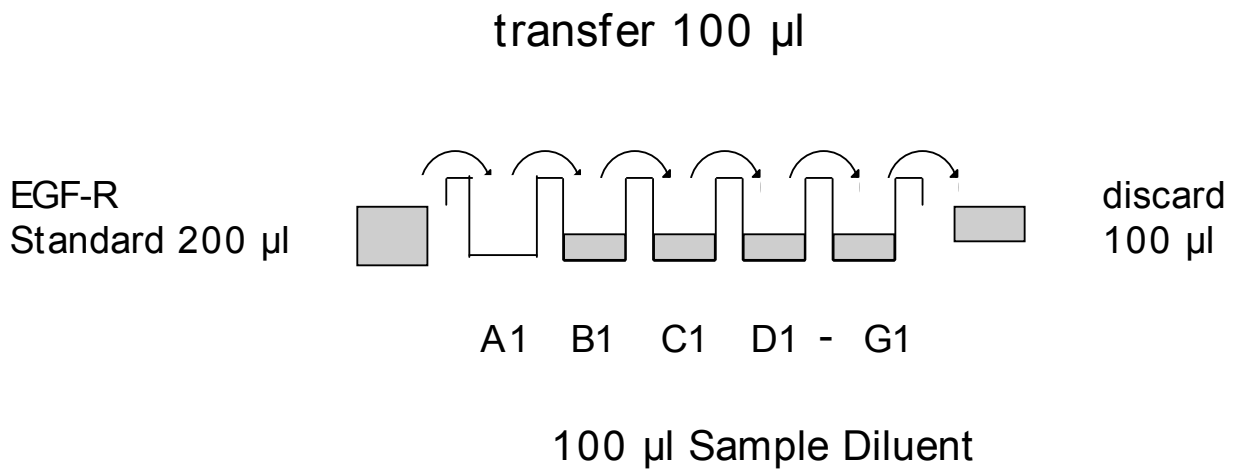


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	<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

- d. Add 100 µl of **Sample Diluent**, in duplicate, to the blank wells.
- e. Add 100 µl of each **Sample**, in duplicate, to the designated wells.
- f. Cover with a **Plate Cover** and incubate at 37°C for 1 hour, if available on a rotator set at 100 rpm.
- g. Remove Plate Cover. Wash the microwell strips three times with approximately 300 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

After the last wash, tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

- h. Add 100 µl of **HRP-conjugate** to all wells.
- i. Cover with a Plate Cover and incubate for 1 hour at 37°C.
- j. Remove **Plate Cover** and empty wells. Wash microwell strips 4 times according to point g. of the test protocol. Proceed immediately to the next step.
- k. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.
- l. Incubate the microwell strips at room temperature (18° to 25°C) for about 15 minutes, if available on a rotator 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 point m. of this protocol) before positive wells are no longer properly recordable.**

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 an OD of 0.6 – 0.65 is reached.

- m. Stop the enzyme reaction by quickly pipetting 100  $\mu$ l of **Stop Solution** into each well, including the blank wells. 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.
- n. 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 EGF-R 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

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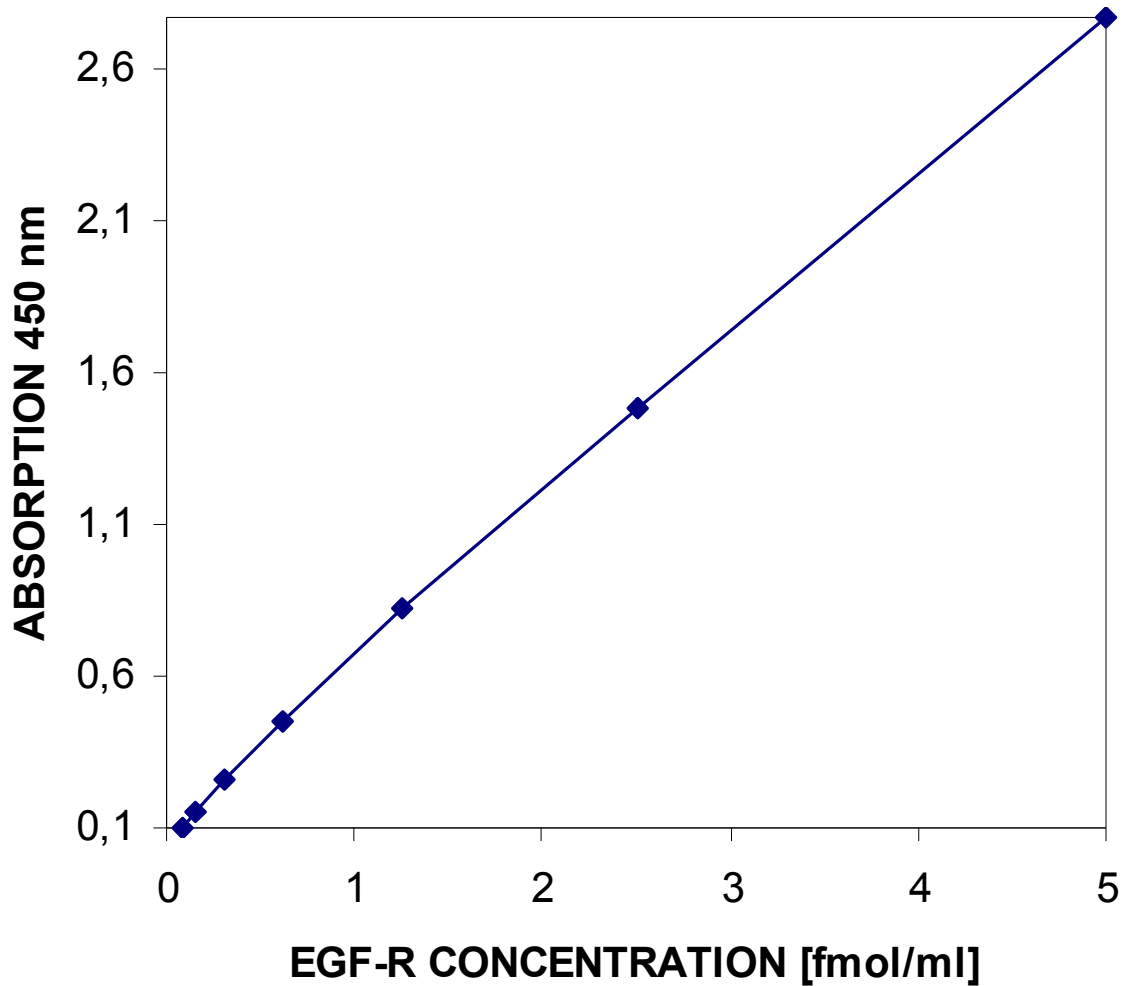
- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the EGF-R concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating 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 EGF-R concentration.

**Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low EGF-R levels. Such samples require further dilution with Sample Diluent in order to precisely quantitate the actual EGF-R level.**

- It is suggested that each testing facility establishes a control sample of known 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 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for EGF-R ELISA.

Recombinant soluble EGF-R was diluted in serial two-fold steps in Sample Diluent; symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



## Typical data using the EGF-R ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	EGF-R Concentration (fmol/ml)	O.D. Mean
1	5.00 5.00	2.727
2	2.50 2.50	1.443
3	1.25 1.25	0.779
4	0.625 0.625	0.404
5	0.312 0.312	0.215
6	0.156 0.156	0.106
7	0.078 0.078	0.054

## 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 detergent before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

## 13 PERFORMANCE CHARACTERISTICS

### A. Sensitivity

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

### B. Reproducibility

#### a. Intra-assay (n=16)

Assay was carried out with 16 replicates of 3 samples containing different concentration of active EGR-receptor. (Sample: A431 cell extract)

	Ave. (fmol/ml)	S.D. (fmol/ml)	CV (%)
Sample A	1.940	0.115	5.9
Sample B	0.541	0.026	4.8
Sample C	0.167	0.009	5.4

#### b. Inter-assay (performance 3 times)

Assay to assay precision with one laboratory was evaluated in three independent experiments over 3 days.

	Ave. (fmol/ml)	S.D. (fmol/ml)	CV (%)
Sample A	1.940	0.095	4.9
Sample B	0.522	0.026	5.0
Sample C	0.162	0.006	3.4

## C. Recovery Studies

The recovery of active EGF receptor was tested by adding two samples out of ten different level in various matrices.

Sample A	Sample B	A+B Measured	A+B Calculated	Recovery (%)
2.640	0.000	1.440	1.320	109.1
2.640	2.640	2.520	2.640	95.5
2.640	1.360	1.890	2.000	94.5
2.640	0.636	1.590	1.640	97.1
2.640	0.288	1.420	1.460	97.0
2.640	0.142	1.340	1.390	96.3
1.360	0.000	0.675	0.680	99.3
1.360	1.360	1.330	1.360	97.8
1.360	0.636	0.946	0.998	94.8
1.360	0.288	0.790	0.824	95.9
1.360	0.142	0.720	0.751	95.9
0.636	0.000	0.317	0.318	99.7
0.636	0.636	0.634	0.636	99.7
0.636	0.288	0.471	0.462	101.9
0.636	0.142	0.390	0.389	100.3
0.288	0.000	0.151	0.144	104.9
0.288	0.288	0.299	0.288	103.8
0.288	0.142	0.218	0.215	101.4
0.142	0.000	0.072	0.071	101.4
0.142	0.142	0.141	0.142	99.3

## D. Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a EGF-R positive serum. There was no detectable cross reactivity with any of the tested proteins.

## 14 REFERENCES

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## 15 REAGENT PREPARATION SUMMARY

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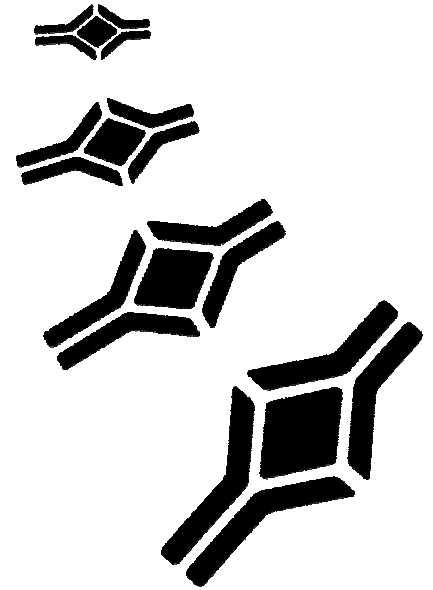
- A. Wash Buffer**            Add **Wash Buffer Concentrate** 20 x (50 ml) in to 950 ml distilled water.
- B. HRP-Conjugate**        Add 11 ml of distilled water to HRP-Conjugate.
- C. Standard**                Add 1 ml of distilled water to lyophilized Standard.

## 16 TEST PROTOCOL SUMMARY

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- Add 100 µl **Sample Diluent**, in duplicate, to standard wells except the first wells
- Pipette 200 µl **EGF-R Standard** into the first standard wells and create standard dilutions by transferring 100 µl from well to well; Discard 100µl from the last wells
- Add 100 µl **Sample Diluent**, in duplicate, to the blank wells
- Add 100 µl **Sample** to designated wells
- Cover microwell strips and incubate 1 hour at 37°C
- Empty and wash microwell strips 3 times with Wash Buffer
- Add 100 µl **HRP-Conjugate** to all wells
- Cover microwell strips and incubate 1 hour at 37°C
- Empty and wash microwell strips 4 times with **Wash Buffer**
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 15 minutes at room temperature (18° to 25°C)
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

**Note:** For samples which have been diluted according to the instructions given in this manual the concentration read from the standard curve must be multiplied by the dilution factor. Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low EGF-R levels. Such samples require further dilution with Sample Diluent in order to precisely quantitate the actual EGF-R level.



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