

INSTRUCTIONS FOR USE

EGFr ELISA

Item No. 06489930

The logo for WILEX, featuring the word "WILEX" in a bold, black, sans-serif font. A small red triangle is positioned above the letter "X".

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OncogeneScience

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WILEX Inc.
Cambridge, MA 02140 USA

Intended Use

The EGFr ELISA is an enzyme-linked immunoassay used to quantitate human epidermal growth factor receptor (EGFr) in human serum or plasma.

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Method Principle	Solid Phase Sandwich ELISA
Analytical Range	0 ng/mL to 300 ng/mL
Specimen Types	Human serum or plasma
Sample Test Volume	10.0 microliters
Sensitivity	0.25 ng/mL
Purchase of this kit licenses its use under the following US patents: 5,344,760 and 5,674,753	

Table of Contents

Intended Use.....	1
Background	3
Principle of the Assay	6
Summary of Procedure.....	7
Materials Provided	8
Materials Required but Not Provided	9
Precautions and Recommendations	11
Sample Preparation	12
Detailed Protocol.....	12
Assay Procedure.....	14
2 Sample Standard Curve	18
Evaluation of Results	19
Sample Values	21
Troubleshooting.....	23
Reagent Stability and Storage	25
References	26
Technical Support.....	Back Cover

Background

The activation and overexpression of cellular oncogenes is an important factor in the development of human cancer. One important member of the oncogene family is the epidermal growth factor receptor (EGFr), which is structurally and functionally related to the oncogenic protein encoded by the *v-erb* B retrovirus. Retroviral oncogenes have been shown to convert normal cells to cancer cells [1]. The EGFr protein, also known as HER-1, is a transmembrane tyrosine kinase cell surface growth receptor that is expressed on normal epithelial cells and on a variety of malignant cell types. The full length EGFr oncoprotein has a molecular weight of 170,000 Daltons and is composed of three domains: the internal tyrosine kinase portion, responsible for signal transduction; a short transmembrane portion; and an external extracellular domain (ECD), which is the portion that binds growth factors such as EGF and which is shed from the cell surface [2].

The other members of the *v-erb* B family include HER-2/neu, HER-3, and HER-4, which are also cell surface receptors. Studies have shown that the ECDs of receptor tyrosine kinases interact with polypeptide ligands. Ligand binding to the ECD results in the formation of receptor homodimers or heterodimers, stimulates intrinsic kinase activity, and leads to phosphorylation of tyrosine residues in the intracellular domain. This, in turn, stimulates signal transduction [3].

Studies have shown that the EGFr ECD is shed into culture fluids of A431 cancer cells and has a molecular weight of 110,000 Daltons [4,5]. Circulating ECD is produced either by proteolytic cleavage [6] of the receptor, or by alternative transcription of primary RNAs [7]. Numerous immunohistochemistry

studies [8,9] have shown that EGFr is overexpressed in tumor cells of a variety of cancer types.

However, there are reports that the EGFr ECD can be either elevated or decreased in cancer patients when compared to a normal population. For instance, some immunoassay studies showed that soluble EGFr ECD levels are increased in the serum of patients with asbestosis-induced lung cancer [10] and in the urine of patients with squamous cell carcinomas of the head, neck, and lung [11]. In a 1999 report by Baron, et al., it was shown that serum EGFr ECD levels were significantly lower in epithelial ovarian cancer patients with stage III or IV disease compared to controls. The investigators also reported that serum EGFr ECD levels changed temporally during the course of a patient's disease [12]. In addition, a follow-up study by Baron, et al., in 2003 observed that soluble EGFr concentrations are useful in detecting stage I/II and stage III/IV epithelial ovarian cancer in young, premenopausal women [13]. Other investigators have concluded that the EGF/*ErbB* family of receptors may have clinical utility as serum biomarkers of disease activity and could be ideal for the development of novel therapeutics in the treatment of ovarian cancer patients [14].

In a study presented at American Association for Cancer Research (AACR) in 2002, it was reported that, compared to the normal EGFr ECD range (45–78 ng/mL), patients with cancer had decreased levels of serum EGFr ECD. In fact, it was reported that 42% of the patients with lung cancer, 44% of patients with late stage prostate cancer, 48% of patients with ovarian cancer, 62%

of patients with colon cancer, 44% of women with stage III breast cancer, and 32% of women with metastatic breast cancer had significantly decreased serum EGFr ECD levels compared to normal subjects [15].

In studies reported at American Society of Clinical Oncology (ASCO) in 2002, it was shown that pretreatment serum EGFr levels were significantly decreased in metastatic breast cancer patients compared to healthy controls. The breast cancer patients who had decreased serum EGFr levels had reduced clinical benefit, shortened time to progression, and a shortened overall survival compared to patients with normal serum EGFr levels [16].

Recent work has shown that tumors that overexpress EGFr may be amenable to treatment with a variety of therapies which target EGFr. Among these are small molecule inhibitors of the kinase activity or antibody-based therapies that are directed to the surface EGFr proteins. In addition, these novel anti-EGFr therapies are being combined with traditional therapies to increase therapeutic efficacy [17–20].

The EGFr ELISA is designed to provide the investigator with a convenient, accurate, and reproducible method to determine EGFr levels in human serum or plasma. Comparison of results between investigators, using a common method such as the EGFr ELISA, will contribute to consistency in observations and will aid in more clearly defining the role of EGFr in the development and progression of cancer. In addition, the EGFr ELISA may be used in conjunction with other immunoassays.

Principle of the Assay

The EGFr ELISA is a sandwich type immunoassay that uses a mouse monoclonal Capture Antibody and an alkaline phosphatase-labeled mouse monoclonal antibody as detector. Both capture and detector reagents specifically recognize the extracellular domain of EGFr. The Capture Antibody recognizes a protein domain on the extracellular portion of EGFr, does not inhibit EGF binding, and does not crossreact with *erbB-2* oncoprotein or human blood group A antigen. The Capture Antibody has been immobilized on the interior surface of the microtiter plate wells. To perform the test, an appropriate volume of specimen is incubated in the wells to allow binding of the antigen by the Capture Antibody. The immobilized antigen is then exposed to the alkaline phosphatase-labeled Detector Antibody. Addition of Substrate to the wells allows the catalysis of a chromogen into a colored product, the intensity of which is proportional to the amount of EGFr that is bound to the plate.

6

Standards are provided in the kit that allow accurate, quantitative determinations of EGFr in suitable samples. Using a microtiter plate reader, one can measure simultaneously the absorbance of the colored product in the Standards and sample wells. Correlating the absorbance values of samples with the Standards allows the investigator to determine the levels of EGFr in a sample. Samples may be assigned a quantitative value of EGFr in nanograms per mL (ng/mL) of serum or plasma.

For instructions, see the Detailed Protocol and Evaluation of Results sections of this booklet.

Summary of Procedure

Steps	Incubations
1. Add samples and Standards to wells	1.5 hours, 37°C
2. Wash	
3. Add Conjugate to wells	0.5 hour, RT*
4. Wash	
5. Add Substrate to wells	1.0 hour, RT*
6. Add Stop Solution to wells	
7. Read plate at 650 nm	

*Room temperature (20–27°C)

Materials Provided

The following components are supplied:

Microtiter plate—One (1) precoated microtiter plate supplied ready to use, with 96 wells (12 strips of eight) in a zip-lock bag with a desiccant pack. Wells are coated with an anti-human epidermal growth factor receptor monoclonal antibody.

EGFr Standards—Six (6) separate vials containing EGFr (p110) obtained from A431 cell supernatant. Standards are calibrated using several independent quantitative amino acid analyses of immunoaffinity purified EGFr and are labeled with values that are 50-fold greater than the actual vial dose. Assigning these label values to a standard curve obviates the need to correct the reported dose for a 1:50 diluted sample (2% serum in buffer). See Evaluation of Results. Standards contain 0.09% sodium azide.

8

Standard#	Concentration	Volume/Vial
6	300.0 ng/mL	1 mL
5	200.0 ng/mL	1 mL
4	125.0 ng/mL	1 mL
3	60.0 ng/mL	1 mL
2	12.5 ng/mL	1 mL
1	0.0 ng/mL	1 mL

Sample Diluent—One (1) bottle containing 100 mL of BSA, mouse IgG, and 0.09% sodium azide.

50X Conjugate—One (1) vial containing 0.4 mL of 50X alkaline phosphatase-labeled anti-EGFr antibody. Must be diluted to 1X with Conjugate Diluent. See **Table 1**, p. 17.

Conjugate Diluent—One (1) bottle containing 12 mL of buffered solution (pH 7.0) with BSA and 0.09% sodium azide.

Substrate—Two (2) vials, A and B, containing 6 mL each of BluePhos Substrate. Mix equal parts of A and B before use.

Stop Solution—One (1) bottle containing 12 mL of EDTA.

Platwash Concentrate—One (1) 100-mL bottle. Dilute one (1) part concentrate in 19 parts high-quality deionized water prior to use.

Materials Required but Not Provided

- Pipettors: 2–20 μL , 20–200 μL , and 200–1000 μL precision pipettors with disposable tips
- Precision manual or automated pipettor for 5- and 10-mL pipets
- Disposable 5- and 10-mL pipets
- Wash bottle, multichannel dispenser, or automated 96-well microtiter plate washer for plate washing
- Microcentrifuge and tubes for sample preparation

- 500- or 1000-mL graduated cylinder
- High-quality deionized water
- 12 x 75 mm culture tubes for sample preparation
- Vortex mixer
- Reagent reservoirs
- Dry heat incubator capable of maintaining 37°C
- Plastic wrap or adhesive plate sealers
- Water bath
- Microtiter plate reader capable of measuring absorbance at a wavelength of 650 nm
- Liquid household bleach for inactivating clinical specimens and decontamination of plate washer
- Disposable paper towels

EGFr ELISA Controls—Controls have been developed to provide customers with control material for quality monitoring of day-to-day assay performance. EGFr ELISA Controls consisting of EGFr p110 in buffer are sold separately. Refer to EGFr ELISA Controls, Item No. 06489949, when ordering. Volumes are 0.5 mL each. Store undiluted EGFr ELISA Controls at 2–8°C. Protect from light. Controls that have been diluted in Sample Diluent may be stored at 4°C, protected from light, and used for up to 2 weeks.

Precautions and Recommendations

- Store components at 2–8°C, except for Platewash Concentrate. Do not expose reagents to excessive light. Do not freeze any of the kit components.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain sodium azide as a preservative. Care should be taken to avoid direct contact with this reagent.
- Do not mouth pipet or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Wear protective gloves and dispose of biological samples properly.

Sample Preparation

Suitable samples for analysis by the EGFr ELISA include human serum or plasma samples. Due to possible interfering factors, special care must be taken in the preparation and assay of human serum or plasma. Predilute serum and plasma at 1:50 as described below.

SERUM OR PLASMA

The initial concentration of the serum or plasma specimen to be examined should not exceed a concentration of 2% (a 1:50 dilution of specimen in Sample Diluent). For example, 0.020 mL of sample may be diluted into 0.980 mL of Sample Diluent and 100 μ L added to the microtiter plate wells.

Detailed Protocol

RECOMMENDED PROCEDURES

12

1. Addition of reagents must be in the order specified.
2. All six Standards and the test specimens should be run in duplicate. For greater accuracy, test each sample at more than one concentration. Change tips during this process. Avoid carry-over of one Standard into another.
3. Before addition of the Conjugate or Substrate, equilibrate all reagents to room temperature (20–27°C) for at least 10 minutes prior to use. However, keep any unused Substrate at 2–8°C.

4. CAUTION: When inverting the microtiter plate to decant or blot, press the side tabs of the frame inward to prevent the strips from falling out. Fill the open portion of the frame with uncoated or used strips when 96-port mechanical washers are used.
5. Preparation of Platewash
 - a. If the Platewash Concentrate is cold, allow it to reach room temperature (20–27°C) before use (about 45 minutes). Make sure all crystals are dissolved. If necessary, warm at 37°C and stir.
 - b. Dilute one (1) volume of Platewash Concentrate with 19 volumes of distilled or deionized water. Mix well. This solution is Platewash. The total volume required will depend on the washing method/instrument used. Approximately 1 L of this solution is required to prime an automated washer and run one microtiter plate; about 700 mL is required for each microtiter plate when manual washing is performed.
 - c. Platewash must be freshly prepared each day. Do not store Platewash.
6. Microtiter plate washing may be automated, semi-automated, or manual, but must be carried out with care to ensure optimal performance of the assay. Plate washing equipment must be properly adjusted, cleaned, and maintained. Whichever method is used, the solution used to wash plates is Platewash.

- a. Automatic Microtiter Plate Washer—Set the fill volume to 300 μL /well. Prime the instrument with Platewash. Use two 3-cycle washes. After the initial 3-cycle wash, rotate the plate 180° and repeat.
- b. Manual Microtiter Plate Washer—Wash six times, using 300 μL per well per wash. Fill the entire plate, then aspirate in the same order.
- c. Hand-Held Syringe—Wash six times, using 300 μL per well per wash. Blot the plate upside-down between washes.

After the final wash, invert the microtiter plate and tap it on an absorbent surface to remove excess liquid. Wells should not be completely dry. Residual liquid protects the bound reagents from desiccation. This is particularly important for maintaining enzyme activity.

7. The transfer of samples and Standards from tubes to the microtiter wells can be greatly simplified by using semi-automation. The hand-held expandable and programmable 8-place pipet, Impact EXP® (Matrix Technologies Corp., Hudson, NH), is ideal for this purpose.

Assay Procedure

The EGF α ELISA is provided with removable strips of wells so the assay can be carried out on multiple occasions. A standard curve must be included in each separate assay. Both the Standards and samples should be assayed in duplicate. Disposable pipet tips and clean reagent troughs should be used for all transfers to avoid cross-contamination of reagents and samples.

1. Cut the foil pouch between the notches at the zip-lock end, break the zip-lock seal, and remove the plate from the foil pouch. Select the number of 8-well strips needed. Remove unused strips from the frame and return to the pouch. Seal the zip lock and store at 2–8°C. (Save the frame for future assays.)
2. Prepare a working solution (1X) of Platewash. Add one (1) part Platewash Concentrate to 19 parts of deionized water. Mix well. The total volume required will depend on the washing method used. Approximately 1 L of this buffer is required to prime an automated washer and run one microtiter plate. Platewash must be prepared fresh each day.
IMPORTANT: Warm all samples, Standards, and other kit reagents to room temperature (20–27°C) before addition to assay plate wells.
3. Add diluted samples and each of the six EGFr Standards (0 to 300 ng/mL) in duplicate by pipetting 100 µL into appropriate wells using clean pipet tips for each sample and Standard. (Do NOT dilute Standards.) Add Standard Level 1 to one additional well to be used for determination of **Substrate blank**. When sample dilution is necessary, use the Sample Diluent supplied. Return unused Standards to 2–8°C.
4. Cover wells with clean plastic wrap or plate sealer. **Incubate microtiter plate for 1.5 hours at 37°C.**
5. Prepare Working Conjugate by diluting an appropriate volume of Conjugate Concentrate into Conjugate Diluent. Refer to **Table 1**, p. 17, for volumes appropriate for the number of strips used.

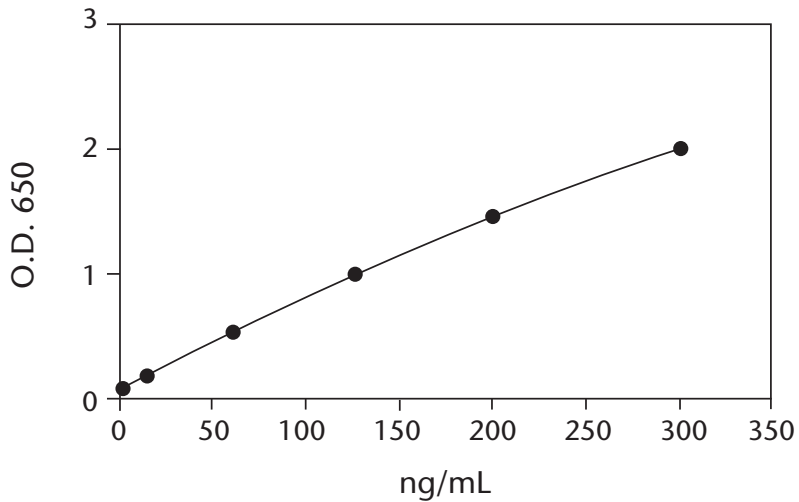
6. Carefully remove the plastic wrap or plate sealer. Wash wells using 300 μL per well with six cycles of Platewash buffer. (Wash for three cycles, rotate the plate 180°, and wash for three more cycles.)
7. Pipet 100 μL of Working Conjugate into all wells **except the Substrate blank well**, which is left empty. Cover the wells with a fresh piece of plastic wrap. **Incubate the microtiter plate at room temperature (20–27°C) for 30 minutes.**
8. Prepare Working Substrate by combining equal parts of Solution A and Solution B. Six mL of each Substrate solution will provide 12 mL of Working Substrate, sufficient to develop one microtiter plate. Mix well.
9. Dispense Working Substrate into a clean reagent trough and allow to come to room temperature. Do not dispense more than needed. Discard unused Working Substrate.
10. Wash wells as in Step 6. CAUTION: Do not allow plates to dry out. Proceed immediately to Step 11.
11. Pipet 100 μL of Working Substrate into all wells and cover the plate with plastic wrap or plate sealer. **Incubate the microtiter plate at room temperature (20–27°C) for 60 minutes.**
12. Pipet 100 μL of Stop Solution into all wells.
13. Measure absorbance in each well using a spectrophotometric plate reader at a wavelength of 650 nm. Wells should be read within 30 minutes of adding the Stop Solution.

TABLE 1. EGFr ELISA—PREPARATION OF WORKING CONJUGATE

# Strips Used	Conj. Concentrate	Conj. Diluent
1	20 μ L	0.98 mL
2	40 μ L	1.96 mL
3	60 μ L	2.94 mL
4	80 μ L	3.92 mL
5	100 μ L	4.90 mL
6	120 μ L	5.88 mL
7	140 μ L	6.86 mL
8	160 μ L	7.84 mL
9	180 μ L	8.82 mL
10	200 μ L	9.80 mL
11	220 μ L	10.78 mL
12	240 μ L	11.76 mL

Figure 1

Sample Standard Curve



Evaluation of Results

The antibodies used in the EGFr ELISA recognize the extracellular, ligand-binding domain of the EGF receptor. This form of EGFr has been identified with a molecular weight of approximately 110 kDa. The Standards in the kit are calibrated in nanograms, which take into account this molecular weight form found in serum and are prepared from a naturally occurring form of the 110 kDa EGFr.

1. Average the absorbance values for each Standard and all sample dilutions to obtain the mean absorbances.
2. Determine the concentration of unknowns by interpolation from the standard curve. There are a variety of microtiter plate reader software packages available for analysis of microtiter plate data (SoftMax Pro™, Molecular Devices Corporation, Sunnyvale, CA; KC4™, Bio-Tek Instruments, Inc., Winooski, VT) that simplify this process. Use a quadratic curve fitting algorithm (second order polynomial).

NOTE: Do not assign “blank” wells using software. This will subtract the average blank readings from all other wells. It is useful for quality control and troubleshooting purposes to be able to inspect the absorbance values reported for all wells without adjustments applied to the raw data.

3. Results for samples and Controls are expressed in ng/mL by reading directly from the standard curve concentrations as designated on the vials and in the Materials Provided section of this booklet. For convenience, no mathematical dilution correction is needed for 1:50 diluted samples since the actual concentration in the Standard preparations is at 2% of the labeled dosage (i.e., they have been prediluted at 1:50).
4. For samples that give absorbance (OD) values exceeding the range of the standard curve, subsequent assay at greater dilutions will be necessary. Any such sample result will require correcting the value obtained from the assay for any dilution beyond 1:50.

Example:

Sample Dilution	Dilution Correction Factor (multiply reported result by)
1:100	2
1:200	4
1:400	8

Sample Values

The levels listed should be used as a guideline only. The determination of normal ranges should be carried out by each laboratory using appropriate samples.

MATCHED SERA AND EDTA PLASMA SAMPLES (N=20)

Sample Type	Mean Value (ng/mL)	Range (ng/mL)
Serum	61	48–72
EDTA Plasma	62	52–75

Assay Characteristics

SENSITIVITY

The EGFr ELISA will detect 0.25 ng/mL of EGFr analyte in a Sample Diluent matrix. The signal of the 0.25 ng/mL Standard is approximately two times the zero signal.

SPECIFICITY

HER-2/neu p105 is not detected by the EGFr ELISA when tested at levels ten-fold higher than the Level 6 Standard.

PRECISION

1. Intra-Assay

Normal human plasma spiked with EGFr Standards at three levels were tested in one assay with 12 replicates/test point.

Sample	High	Medium	Low
n	12	12	12
Mean	4.96	3.91	1.17
% C.V.	3.43	6.58	5.73

2. Inter-Assay

Normal human plasma spiked with EGFr Standards at three levels were tested in seven assays with 12 replicates/test point.

Sample	High	Medium	Low
n	84	84	84
Mean	4.88	3.71	1.17
% C.V.	5.43	8.83	8.16

RECOVERY

1. Human Serum

A measured amount of purified p110 was spiked into a human sera pool and into four individual human sera samples. A replicate amount of p110 was spiked into Sample Diluent. After an initial 1:50 dilution, all samples were tested in the EGFr ELISA. After subtracting the amount of p110 found in the sera samples, and using the Sample Diluent preparation as a basis, the mean recovery of p110 was determined to be 96%.

Troubleshooting

A. ASSAY DOES NOT DEVELOP COLOR OR ODs ARE LOW

- Plate allowed to dry out after Conjugate step.
- Step(s) omitted or in wrong sequence.
- Room temperature during incubations fell below 20°C.
- Substrate was not warmed to room temperature (20–27°C) prior to addition to wells.

B. HIGH BACKGROUND SIGNAL

- Insufficient washing between steps.

- The Substrate blank well should read ≤ 0.07 absorbance units. Zero Standard should read ≤ 0.10 absorbance units. Higher readings indicate deterioration of Substrate or exposure of Substrate to light before or during the incubation step.
- Be certain the plate is read at the correct wavelength.

C. POOR DUPLICATES

- Insufficient washing, especially when accompanied by high background. Take special care when washing plates by hand, or have automatic washer serviced.
- Sporadic high signals may indicate contamination of Substrate by Conjugate. Be sure to use a fresh piece of plastic wrap or adhesive plate sealer for this step. Residual droplets of Conjugate on re-used plastic wrap or adhesive plate sealer may lead to false positive signals.
- Generating bubbles in wells on addition of reagents. Use care in pipetting.
- Splashing of reagents between wells will lead to erroneous results. Avoid jarring the plate.

Reagent Stability and Storage

All of the reagents included with the EGFr ELISA have been tested for stability. Reagents should not be used beyond the stated expiration date. Kit reagents should be stored at 2–8°C with the exception of Platewash Concentrate, which may be stored at room temperature (20–27°C). Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

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Notes:



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OncogeneScience

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