Quantikine® ELISA

Human EGFR Immunoassay

Catalog Number DEGFR0

For the quantitative determination of human Epidermal Growth Factor Receptor (EGFR) concentrations in cell culture supernates, serum, plasma, and human milk.

Note: The standard reconstitution method has changed. Read this package insert in its entirety before using this product.

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

The EGFR subfamily of receptor tyrosine kinases comprises four members: EGFR (also known as Her1, ErbB1, or ErbB), ErbB2 (Neu, Her2), ErbB3 (Her3), and ErbB4 (Her4). All family members are type I transmembrane glycoproteins with an extracellular domain (ECD) that contains two cysteine-rich regions separated by a spacer. The cytoplasmic domain contains a membrane-proximal tyrosine kinase domain followed by multiple serine, threonine, and tyrosine phosphorylation sites (1, 2). Mature human EGFR is a 170 kDa protein that is extensively and heterogeneously modified with N-linked glycosylation (3-5). Within the ECD, human EGFR shares 88% amino acid (aa) sequence identity with mouse and rat EGFR. It shares 43%-44% aa sequence identity with the ECDs of human ErbB2, ErbB3, and ErbB4. EGFR is widely expressed on epithelial cells, predominantly in the gastrointestinal tract and breast, and is required for epithelial cell development and proliferation (6-8).

EGFR binds the EGF family ligands EGF, Amphiregulin, TGF-α, Betacellulin, Epiregulin, HB-EGF, and Epigen. These molecules are expressed as transmembrane proteins but are shed from the cell surface by proteolytic cleavage (9). Ligand binding induces EGFR homodimerization as well as heterodimerization with ErbB2, ErbB3, or ErbB4. This results in activation of the kinase domains, tyrosine autophosphorylation, and internalization of the receptor-ligand complex (10, 11). EGFR signaling regulates multiple biological functions including cell proliferation, differentiation, motility, and apoptosis (7, 8, 12). Alterations in the structure, expression, and signaling of EGFR are involved in the development and metastasis of a wide variety of cancers, particularly those of epithelial origin (13, 14).

Soluble receptors consisting of the extracellular domain of EGFR are generated by alternate splicing in humans and mice (3, 15). The ECD can also be released by proteolytic cleavage (16, 17). The soluble forms retain ligand binding capability and may dimerize with membrane bound EGFR, resulting in inhibition of its tyrosine kinase activity (16, 18). Levels of the 110 kDa soluble EGFR are elevated in the serum of cervical and gastric carcinoma patients and in the urine of squamous cell carcinoma patients (19-21). Serum EGFR elevation is also associated with responsiveness to chemotherapy in advanced colorectal cancer (22). In contrast, serum EGFR is reduced in ovarian carcinoma, non-small cell lung cancer, and head and neck carcinoma (23, 24). A decrease of serum EGFR relative to soluble ErbB2 is associated with decreased life expectancy in patients with metastatic breast cancer (25).

The Quantikine® Human EGFR Immunoassay is a 4.5 hour solid phase ELISA designed to measure human EGFR in cell culture supernates, serum, plasma, and human milk. It contains NS0-expressed recombinant human EGFR and has been shown to accurately quantitate the recombinant factor. Results obtained using natural EGFR 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 EGFR.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for human EGFR has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any EGFR present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human EGFR 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 EGFR 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 the appropriate 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 EGFR Microplate	893730	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for human EGFR.	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 EGFR Conjugate	893731	21 mL of a polyclonal antibody specific for human EGFR conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human EGFR Standard	893732	Recombinant human EGFR in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-72	895367	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5K	895119	21 mL of a buffered protein base with preservatives. *For cell culture supernate/ human milk samples.*	
Calibrator Diluent RD5L	895028	21 mL of diluted animal serum in buffer with preservatives. *For serum/plasma samples.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated protein base of buffered surfactant with preservative. *May turn yellow over time.*	
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.
- 500 mL graduated cylinder.
- Test tubes for dilution of standards and samples.
- Human EGFR Controls (optional; R&D Systems®, Catalog # QC235).

PRECAUTIONS

This assay is temperature sensitive. Room temperature is defined as 18-23 °C. The high signal may be lowered if the assay is run below 18 °C.

EGFR is detectable in saliva. Take precautionary measures to prevent contamination of the kit reagents while running the 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 ≤ -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 ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.
Note: *Citrate plasma has not been validated for use in this assay.*

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process twice more for a total of 3 times. Assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5L.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

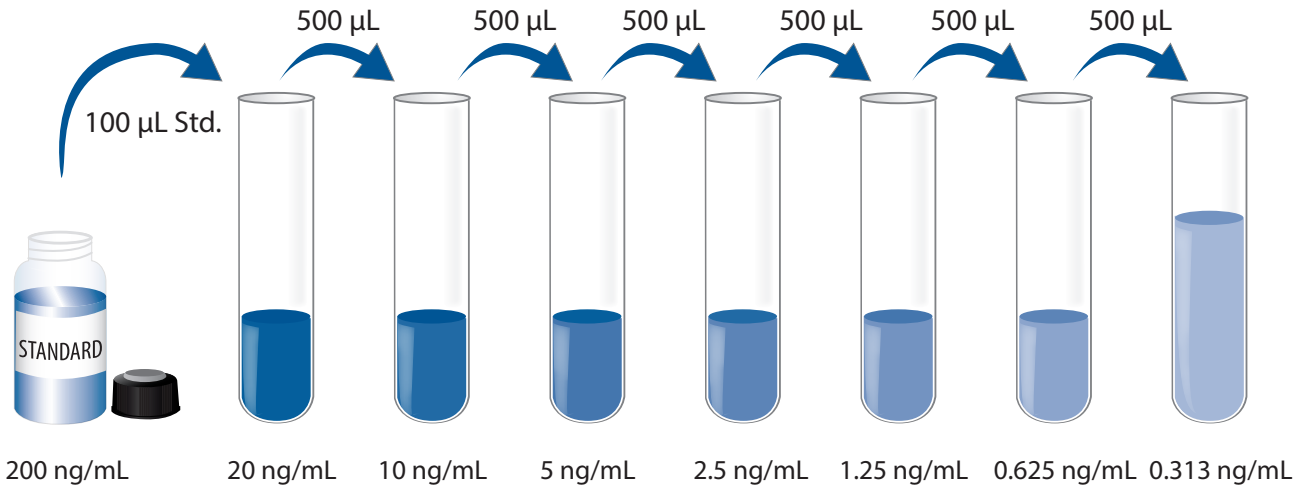
Note: *EGFR is detectable in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 µL of the resultant mixture is required per well.

Human EGFR Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human EGFR Standard with deionized or distilled water. This reconstitution produces a stock solution of 200 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5K (*for cell culture supernate/human milk samples*) or Calibrator Diluent RD5L (*for serum/plasma samples*) into the 20 ng/mL tube. Pipette 500 µL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20 ng/mL standard serves as the high standard. The appropriate calibrator diluent serves as the zero standard (0 ng/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: *EGFR 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 µL of Assay Diluent RD1-72 to each well.
4. Add 50 µL 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 µL) 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 µL of Human EGFR 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 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL 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 the 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.).

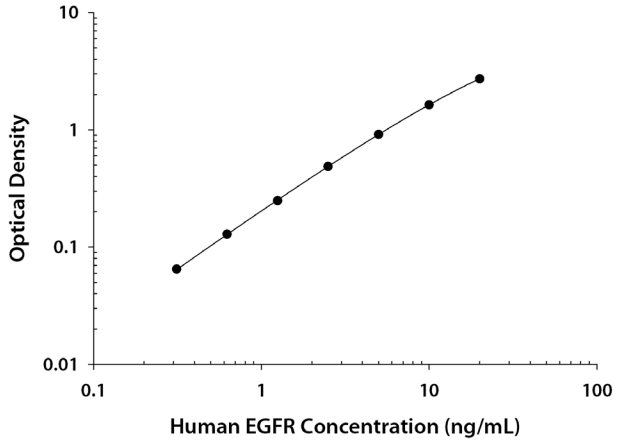
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human EGFR 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

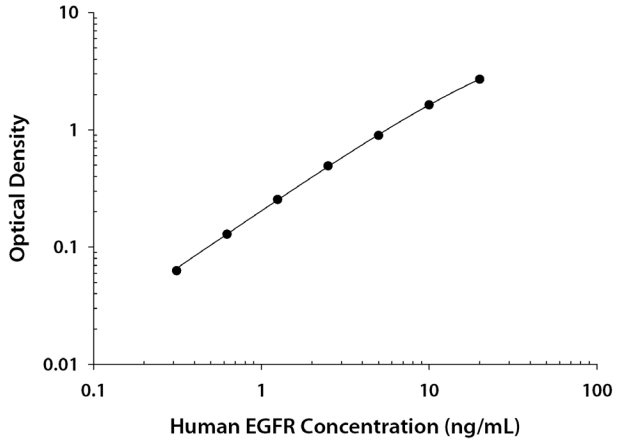
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/HUMAN MILK ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.007 0.007	0.007	—
0.313	0.069 0.075	0.072	0.065
0.625	0.135 0.137	0.136	0.129
1.25	0.250 0.261	0.256	0.249
2.5	0.490 0.497	0.494	0.487
5	0.919 0.925	0.922	0.915
10	1.614 1.667	1.641	1.634
20	2.687 2.772	2.730	2.723

SERUM/PLASMA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.007 0.007	0.007	—
0.313	0.069 0.071	0.070	0.063
0.625	0.135 0.137	0.136	0.129
1.25	0.261 0.263	0.262	0.255
2.5	0.495 0.504	0.500	0.493
5	0.891 0.918	0.905	0.898
10	1.622 1.660	1.641	1.634
20	2.691 2.720	2.706	2.699

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 forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE/HUMAN MILK ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	1.65	5.08	10.6	1.63	5.00	10.0
Standard deviation	0.094	0.170	0.507	0.144	0.416	0.816
CV (%)	5.7	3.3	4.8	8.8	8.3	8.2

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	1.69	5.33	11.2	1.70	5.21	10.4
Standard deviation	0.072	0.199	0.616	0.161	0.522	1.03
CV (%)	4.3	3.7	5.5	9.4	10.0	10.0

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	98	86-106%
Serum* (n=4)	101	89-117%
EDTA plasma* (n=4)	99	91-108%
Heparin plasma* (n=4)	101	92-111%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human EGFR were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	104	93	92	95
	Range (%)	100-107	93-94	89-96	90-102
1:4	Average % of Expected	104	93	91	97
	Range (%)	102-106	90-96	84-95	92-100
1:8	Average % of Expected	104	97	96	102
	Range (%)	100-106	93-100	88-99	98-106
1:16	Average % of Expected	103	101	99	104
	Range (%)	101-105	97-104	93-105	98-110

*Sample were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Eighty-four assays were evaluated and the minimum detectable dose (MDD) of human EGFR ranged from 0.005-0.036 ng/mL. The mean MDD was 0.014 ng/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 recombinant human EGFR produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human EGFR in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=34)	65.2	51.3-77.8	7.18
EDTA plasma (n=34)	64.7	52.7-77.7	7.23
Heparin plasma (n=34)	64.7	50.7-84.0	8.22
Human milk (n=6)	0.84	0.35-1.84	0.56

Cell Culture Supernates:
Human peripheral blood leukocytes were cultured in DMEM supplemented with 5% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human EGFR. No detectable levels were observed.

A431 human epithelial carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and non-essential amino acids. An aliquot of the cell culture supernate was removed, assayed for human EGFR, and measured 188 ng/mL.

HT-29 human colon adenocarcinoma cells were cultured in McCoy's media 5A supplemented with 10% fetal bovine serum until confluent. An aliquot of the cell culture supernate was removed, assayed for human EGFR, and measured 0.41 ng/mL.

MDA-MB-231 human breast cancer cells were cultured in Leibovitz L-15 media supplemented with 10% fetal bovine serum and 2 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for human EGFR, and measured 0.92 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human EGFR.

The factors listed below were prepared at 200 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range recombinant human EGFR control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Amphiregulin
Betacellulin
EGF
Epiregulin
ErbB2
ErbB3
ErbB4
HB-EGF
TGF-α
TGF-β1
TGF-β2
TGF-β3

Recombinant mouse:

EGF

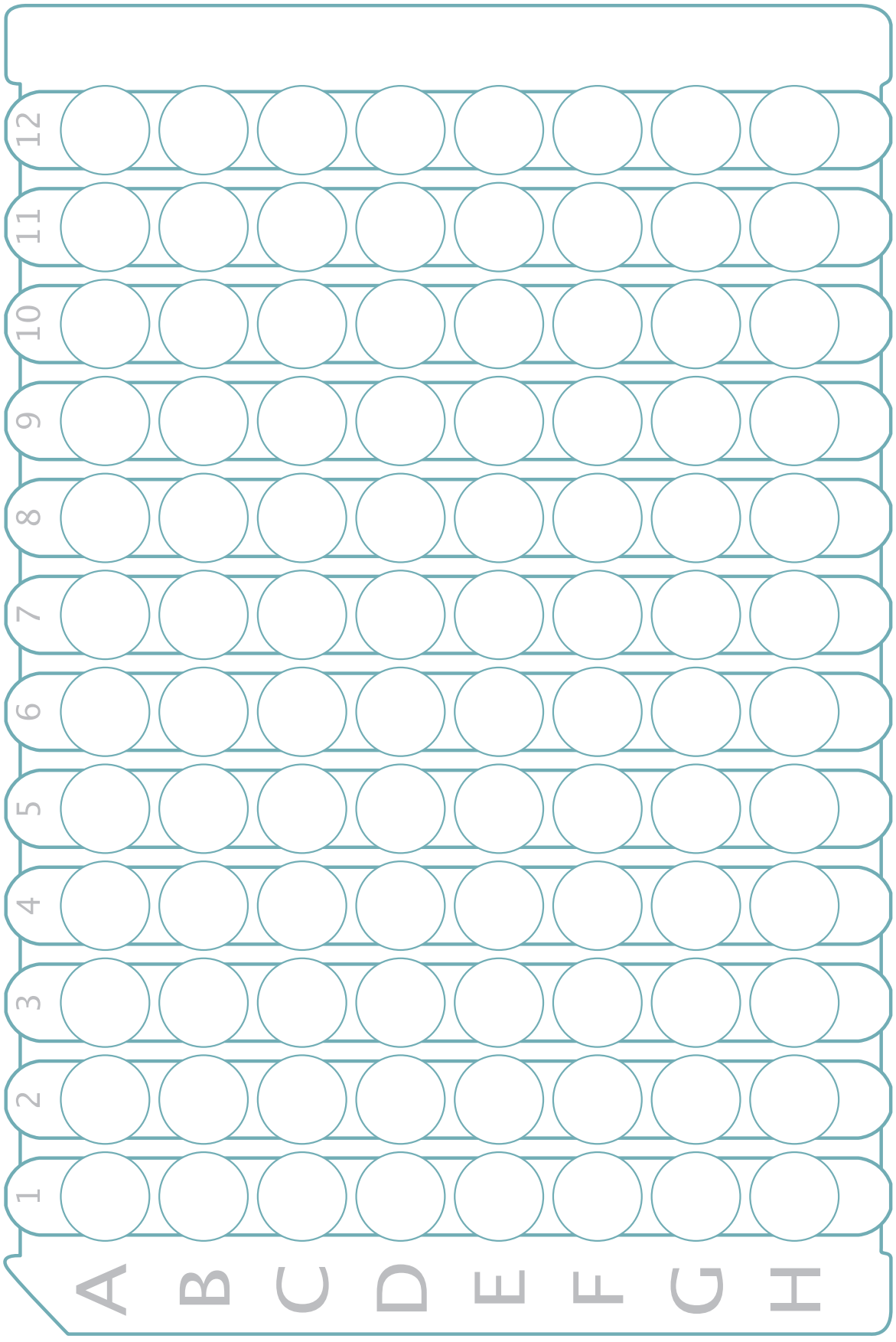
Recombinant mouse EGFR cross-reacts approximately 6% in this assay.

REFERENCES

1. Singh, A.B. and R.C. Harris (2005) Cell. Signal. **17**:1183.
2. Burgess, A.W. (2008) Growth Factors **26**:263.
3. Lin, C. *et al.* (1984) Science **224**:843.
4. Ullrich, A. *et al.* (1984) Nature **309**:418.
5. Stroop, C.J. *et al.* (2000) Glycobiology **10**:901.
6. Real, F.X. *et al.* (1986) Cancer Res. **46**:4726.
7. Sibilia, M. and E.F. Wagner (1995) Science **269**:234.
8. Miettinen, P.J. *et al.* (1995) Nature **376**:337.
9. Higashiyama, S. *et al.* (2008) Cancer Sci. **99**:214.
10. Yarden, Y. *et al.* (1987) Biochemistry **26**:1434.
11. Lemmon, M.A. *et al.* (1997) EMBO J. **16**:281.
12. Lui, V.W. and J.R. Grandis (2002) Anticancer Res. **22**:1.
13. Rowinsky, E.K. (2004) Annu. Rev. Med. **55**:433.
14. Citri, A. and Y. Yarden (2006) Nat. Rev. Mol. Cell Biol. **7**:505.
15. Reiter J.L. *et al.* (2001) Genomics **71**:1.
16. Sanderson, M.P. *et al.* (2008) J. Cell. Biochem. **103**:1783.
17. Perez-Torres, M. *et al.* (2008) Exp. Cell Res. **314**:2907.
18. Basu, A. *et al.* (1989) Mol. Cell. Biol. **9**:671.
19. Oh, M.J. *et al.* (2000) Clin. Cancer Res. **6**:4760.
20. Choi, J.H. *et al.* (1997) Cancer **79**:1879.
21. Witters, L.M. *et al.* (1995) Clin. Cancer Res. **1**:551.
22. Zampino, M.G. *et al.* (2008) Cancer Chemother. Pharmacol. **63**:139.
23. Baron, A.T. *et al.* (2003) Cancer Epidemiol. Biomarkers Prev. **12**:103.
24. Lemos-Gonzalez, Y. *et al.* (2007) Br. J. Cancer **96**:1569.
25. Souder, C. *et al.* (2006) Cancer **107**:2337.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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