

Instruction Manual for Herstatin Detection Kit (ELISA based)

Catalog # 17-354

Sandwich ELISA system for the colorimetric detection of herstatin using a monoclonal anti-herstatin as the capture antibody and an anti-herstatin biotin conjugate as the detection antibody.

Sufficient reagents for 96 assays per kit.

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DIAGNOSIS OF DISEASE IN HUMANS
DO NOT USE IN HUMANS**

I. STORAGE AND STABILITY

Storage: Upon receipt, see individual components for storage conditions. The High Binding 96-well Strip Plate (Catalog # 30-009) should be stored at room temperature. Herstatin Reporter Antibody, biotin conjugate (Catalog # 16-194) may be stored at 4°C for one month. For longer storage, aliquot and store at -20°C. Avoid multiple freeze-thaw cycles. Reconstituted Herstatin Standard (Catalog # 14-533) should be aliquoted and store at -70°C. All other components are stored at 4°C.

Stability: Components stable for 1 year from date of shipment if stored and handled correctly.

II. ASSAY OVERVIEW

Her2/*erbB*-2 (185kDa) is one of four members of the EGF receptor tyrosine kinase family capable of both homomeric and heteromeric interactions within the family. Her2 is frequently overexpressed in human tumors and is constitutively active as a dimer. An alternative transcript of the Her2 gene produces a 68kDa secreted protein, herstatin (1). Herstatin contains the first 340 amino acids of the extracellular domain of Her2 followed by a unique 79 amino acid C-terminal sequence, resulting from translation of a retained intron (1). Herstatin associates with p185Her2 with high affinity (1) and interrupts constitutive activation of Her2 (2), functioning as an autoinhibitor. Both the capture and reporter antibodies used in this assay have been generated to the unique region of herstatin, eliminating the cross reactivity with Her2/*erbB*-2.

The herstatin detection assay is a “sandwich” enzyme linked immunosorbent assay (ELISA) employing two mouse monoclonal antibodies specific for herstatin. Strip-wells are coated with the capture antibody and samples are incubated, allowing binding of herstatin to the capture antibody. Unbound material is removed in the wash steps and bound herstatin is detected using the biotinylated reporter antibody. Horseradish peroxidase (HRP)-conjugated streptavidin is then bound to the reporter antibody and Tetramethylbenzidine (TMB) is provided as the HRP substrate for colorimetric detection. Included in the kit is a herstatin standard that can be used as a positive control (if used in a single well) or to generate a standard curve (if a dilution series is prepared) to enable estimation of herstatin levels in test samples. The 8x12 strip well design allows the assay to be performed on more than one occasion and sufficient herstatin positive control is included to generate three standard curves (in duplicate).

III. SYSTEM COMPONENTS

A. Provided Kit Components

High Binding 96-well strip Plate

Catalog # 30-009

One high binding strip-well plate containing 12 strips of 8 wells each.

Herstatin Capture Antibody

Catalog # 05-662

One vial, containing **50µg** of protein G purified mouse monoclonal IgG in 50µl 0.1M Tris-Glycine pH 7.4, 0.15M NaCl, with 0.05% sodium azide.**Herstatin Reporter Antibody, biotin conjugate**

Catalog # 16-194

One vial containing **25µl** of protein G purified mouse monoclonal IgG, conjugated to biotin in PBS containing 0.05% Kathon[®] before the addition of glycerol to 30%.**Serum Diluent**

Catalog # 20-217

One vial containing **10ml** of serum diluent.**Herstatin Standard**

Catalog # 14-533

One vial containing **125ng** of recombinant herstatin protein lyophilized from a 5% sucrose solution.**20X TBS, 2% Tween[®]-20 (ELISA wash buffer)**

Catalog # 20-202

One vial containing **50ml** of 1M Tris, 3M NaCl, 2% Tween[®]-20, pH 7.4.**10% BSA in TBS, (ELISA blocking buffer)**

Catalog # 20-191B

One vial containing **25ml** of 10% BSA in TBS, pH 7.4, containing 0.05% Kathon[®].**TMB (tetramethylbenzidine) Substrate Reagent A**

Catalog # 20-182a

One vial containing **7.5ml** of 0.04% (w/v) TMB solution.**TMB (tetramethylbenzidine) Substrate Reagent B**

Catalog # 20-182b

One vial containing **7.5ml** of 0.02% hydrogen peroxide in citric acid buffer.**Streptavidin HRP**

Catalog # 18-152a

One vial containing **25µg** of active conjugate in **25µl** of 0.05M NaHCO₃, pH 8.3, containing 0.05% thimerosal as a preservative.

B. Required Materials Not Provided

- 0.05M sodium bicarbonate buffer
- 2M Sulfuric Acid (for Stop Solution)
- Ice bucket
- Timer
- Variable volume (5-200µl) pipet + tips
- Variable volume (5-200µl) multichannel pipet + tips
- Reagent troughs for multichannel pipettes
- Microtiter plate washer (optional), shaker or platform vortex
- Shaking incubator
- Wash bottle or multichannel dispenser for washing
- 96-well spectrophotometer capable of measuring absorbance at 450nm and 570nm.

C. Stock Solutions

Prepare the following solutions in advance of performing the Assay:

Wash Buffer (1X TBS/T): Dilute 50ml 20X TBS, 2% Tween[®]-20 (Catalog # 20-202) with 950ml of sterile water to create a working solution of 1X TBS/T. Store at room temperature.

ELISA Blocking Buffer (3% BSA in TBS/T): Add 58ml of 1X TBS/T to 25ml 10% BSA in TBS (Catalog # 20-191b) to make a working solution of 3% BSA in TBS/T.

Stop Solution: Prepare a solution of 2M (4N) Sulfuric Acid. (NOT PROVIDED)

Herstatin Standard: Add 125 μ l of sterile water to the lyophilized positive control. Place on ice for 30 minutes to one hour with periodic gentle swirling to dissolve. Aliquot and store at -70°C.

0.05M Sodium Bicarbonate, pH ~9.0: Dissolve 0.42g sodium bicarbonate (NaHCO₃ F.W. 84.01) in 100ml sterile water. This solution must be prepared fresh. Discard unused portion following assay completion. (NOT PROVIDED)

IV. HERSTATIN ASSAY PROCEDURE

Safety Warnings and Precautions: The Herstatin Detection Assay Kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

A. General Notes

1. The microtiter plate (Catalog # 30-009) is provided as strip wells enabling the assay to be performed on multiple occasions. If the entire plate is not required for the assay remove the appropriate number of microtiter strip wells from the well holder and store at 4°C for future use.
2. Human sera or plasma samples should be diluted 1:20 – 1:40 in sample diluent.
3. If the serum diluent is cloudy, filter using a 0.2 μ m syringe filter.
4. When performing washes manually, avoid introducing bubbles when dispensing liquids into the wells, and ensure each well is filled with buffer, but not overflowing to avoid cross-contamination between wells. Empty wells with a wrist-flick motion over an appropriate receptacle, and while still inverted, blot any remaining moisture on clean paper towels. If an automated plate washer is used, follow manufacturer's recommendation for operation.
5. Agitation of the wells during the ELISA wash steps is recommended to reduce non-specific background. If microtiter plate agitator is not available, a platform vortex at a low setting can be used (e.g. level 1 of Fisher's Genie II platform vortex). If background problems occur, simply increase the number and/or duration of washes.
6. A brief water rinse is recommended prior to the addition of the TMB substrate mixture to remove traces of Tween[®]-20 which can interfere with HRP activity. TBS can be used in place of water. All traces of moisture must be removed before the addition of the TMB substrate, since volume fluctuations between wells affect OD measurement accuracy.
7. The incubation period for the HRP reaction with the TMB substrate may vary based on the experimental conditions. Stop the reaction at the first trace of color in negative control wells (no sample or 0ng herstatin standard wells). This is a very important step to help keep background levels low.
8. Well to well variations of the plastic (background) can be determined by reading the plate at 570nm. The 570nm values should then be subtracted from the 450nm values.

B. Protocol

1. Dilute the capture antibody (Catalog # 05-662) to 5ng/ μ l (1:200) in 0.05M sodium bicarbonate (pH 9.4). Prepare adequate solution to cover all wells to be used; 50 μ l in 10ml of 0.05M sodium bicarbonate, (pH 9.4) is sufficient for the entire plate.
2. Coat the wells with 100 μ l per well of the diluted capture antibody. Cover the plate and incubate for either 2 hours at room temperature or overnight at 4°C.
3. Remove the capture antibody solution by inverting the plate over a sink or aspirating the liquid. Rinse wells once with 1X TBS/T (Wash Buffer) ensuring the wells are completely filled. Tap the plate on absorbent paper to remove excess liquid.
4. Add 150 μ l per well of ELISA Blocking Buffer and incubate at 37°C for 1 hour.
5. Remove the Blocking Buffer by inverting the plate over a sink or aspirating the liquid. Rinse wells once with Wash Buffer, ensuring the wells are completely filled. Tap the plate on absorbent paper to remove excess liquid.
6. Dilute human serum samples (at least 1:20) with serum diluent (Catalog # 20-217). The positive control herstatin (Catalog # 14-533) should also be prepared in the serum diluent. Prepare a standard curve using serial dilutions of the herstatin standard. 10ng/well is recommended as the high standard and serum diluent is the zero standard (see appendix B).
7. Add 100 μ l of diluted human serum samples or controls per well. Incubate the plate for 1 hour at room temperature.
8. Remove the samples and controls by inverting the plate over a sink or aspirating the liquid.
9. Wash the plate with 150 μ l of Wash Buffer per well and soak for 2 to 3 minutes (gentle agitation may reduce background). Invert the plate over a sink or aspirate and repeat the wash twice more. Tap the plate on absorbent paper to remove excess liquid.
10. Dilute sufficient Herstatin Reporter Antibody, biotin conjugate (Catalog # 16-194) 1:500 in 1X Blocking Buffer.
11. Add 100 μ l of diluted Herstatin Reporter Antibody per well. Cover the plate and incubate at room temperature for 45 to 60 minutes.
12. Remove the Herstatin Reporter Antibody and wash the plate three times, following steps 8 and 9.
13. Dilute the Streptavidin HRP Conjugate (Catalog # 18-152a) 1:5000 in 1X Blocking Buffer.
14. Add 100 μ l per well of the diluted Streptavidin HRP, cover the plate and incubate at 37°C for 30 to 45 minutes.
15. Remove the Streptavidin HRP solution and wash the plate, following steps 8 and 9. Following the washes, rinse wells twice with 1X TBS (NOT PROVIDED) or ultrapure water ensuring the wells are completely filled. Tap the plate on absorbent paper to remove excess liquid.
16. Prepare sufficient TMB substrate by mixing equal volumes of TMB substrate A and TMB substrate B.
17. Add 75 μ l per well of the TMB substrate and incubate for 10 to 30 minutes at room temperature.
18. Add 75 μ l per well of Stop Solution in the same order the substrate was added.
19. Determine the absorbance at 450nm and 570nm within 30 minutes of adding Stop Solution.

V. APPENDICES

A. Abbreviated Herstatin Assay Procedure for Experienced Users




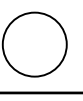
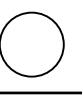

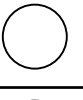
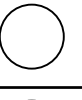

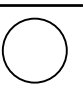
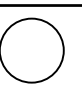

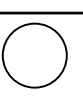
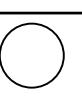

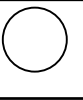
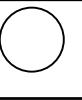

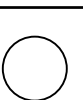

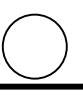
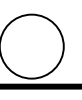
Note: This abbreviated procedure is provided as a convenience to outline and facilitate completion of the assay. It is recommended that a photocopy of the page be used. As a step is completed, it may be checked off in the appropriate box. After completion of the assay, the page can then be incorporated into your lab notebook if desired.

- 1. Dilute the capture antibody to 5ng/ μ l in 0.05M sodium bicarbonate (100 μ l/well).
- 2. Coat each well with 100 μ l of diluted capture antibody. Incubate two hours at room temperature (RT) or overnight at 4°C.
- 3. Add 150 μ l per well of 1X Blocking Buffer. Incubate one hour at 37°C.
- 4. Dilute human serum samples at least 1:20 in serum diluent (100 μ l/well). Dilute positive control herstatin in serum diluent.
- 5. Add 100 μ l of samples or controls per well. Incubate 1 hour at RT.
- 6. Wash with 150 μ l of Wash Buffer per well and soak 2-3 minutes (agitation may reduce background). Remove liquid and repeat wash twice more.
- 7. Dilute Herstatin Reporter Antibody 1:500 in 1X Blocking Buffer (100 μ l/well).
- 8. Add 100 μ l of diluted antibody per well. Cover plate and incubate at RT for 45 to 60 minutes.
- 9. Remove the Herstatin Reporter Antibody. Wash 3 times.
- 10. Dilute the Streptavidin HRP Conjugate 1:5,000 in 1X Blocking Buffer.
- 11. Add 100 μ l per well of the diluted Streptavidin HRP, cover and incubate at 37°C for 30 to 45 minutes.
- 12. Remove the solution and wash the plate three times. Rinse twice with water or TBS.
- 13. Mix equal volumes of TMB substrates A and B (75 μ l/well).
- 14. Add 75 μ l per well of the TMB solution. Incubate 10-30 minutes at RT.
- 15. Add 75 μ l per well of Stop Solution in the same order the substrate was added.
- 16. Within 30 minutes of adding Stop Solution, determine the absorbance at 450nm.

B. Standard Curve

Generate a standard curve (0-10ng herstatin/well) using the following procedure. We recommend performing the standard curve in duplicate.

1. Pipet 100 μ l of Serum Diluent (Catalog # 20-217) in all wells required for the standard curve except the first wells (*i.e.*, add 100 μ l to all wells except A1 and A2).
2. Dilute the Herstatin Standard (Catalog # 14-533) to 0.1ng/ μ l by adding 42 μ l of reconstituted Herstatin Standard to 378 μ l of Serum Diluent. Pipet 200 μ l of the diluted Herstatin Standard to each of two empty wells (A1 and A2).
3. Transfer 100 μ l of the 0.1ng/ μ l Herstatin Standard to the next well containing 100 μ l of Serum Diluent, mixing thoroughly before the next transfer. Repeat this process to make successive dilutions, with the initial wells (A1 and A2) containing 10ng Herstatin Standard per well. Remove 100 μ l from final dilution (G1 and G2), leaving 100 μ l in all standard curve wells. Use 100 μ l of Serum Diluent (see step 1) in the last wells (H1 and H2) for the zero standard.
4. Follow steps 8-18 pf the Assay Protocol from page 5 section B.
5. Record the absorbance at 450nm.

		1	2	Herstatin Standard per well (ng)
				10
100 μ l				5
100 μ l				2.5
100 μ l				1.25
100 μ l				0.625
100 μ l				0.3125
100 μ l				0.1563
				0

C. References

1. Doherty, J.K., *et al.*, Proc. Natl. Acad. Sci. USA **96**:10869-10874, 1999.
2. Azios, N.G., *et al.*, Oncogene **20**:5199-5209, 2001.