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ProductInformation

c-Met ELISA

Product Number CS0560
Storage Temperature 2-8 °C

Technical Bulletin

Product Description
c-Met ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative determination of c-Met protein in cell lysates. A monoclonal antibody specific for c-Met (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. c-Met standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the c-Met antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and an Anti-c-Met antibody specific for total c-Met, is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized c-Met. After removal of excess detection antibody, horseradish peroxidase-labeled (HRP) anti-rabbit IgG is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess anti-rabbit IgG-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of c-Met present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of c-Met.

c-Met ELISA is designed to detect and quantify the level of c-Met protein, independent of its phosphorylation state. Reactivity of this ELISA kit with other species is not assured. This ELISA kit detects full length c-Met protein but not soluble c-Met. This kit can be used to normalize the c-Met content of the samples when examining quantities of phosphorylated sites on Phospho-c-Met [pTyr^{1230}/pTYr ^{1234}/pTyr ^{1235}] (Prod. No. CS0590).

c-Met, a member of the tyrosine kinase superfamily, is the receptor for hepatocyte growth factor, also known as scatter factor (HGF/SF). The mature c-Met protein is a disulfide-linked heterodimer with MW 190 kDa composed of a heavily glycosylated α subunit that is completely extracellular in localization, and a β subunit comprised of an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain. c-Met is transcribed from a single open reading frame and translated into a protein precursor that is proteolytically cleaved, yielding the heterodimeric mature protein. Alternative splicing yields several c-Met isoforms, including proteins that remain in the uncleaved, monomeric state or that lack various portions of the c-Met cytoplasmic domain. Cells expressing c-Met include epithelial cells, endothelial cells, blood cells of various types, and glomerular mesenchymal cells.

The ligand for c-Met, HGF/SF, is a member of the plasminogen-related growth factor family, which is synthesized as an inactive pro-form. HGF/ SF activation requires cleavage with either urokinase plasminogen activator (uPA), HGF activator, or Coagulation Factor Xa. Sources of HGF/SF include mesenchymal cells, mesanglial cells, endothelial cells, macrophages, and tumor cells.

HGF/SF binding to c-Met stimulates receptor dimerization and the phosphorylation of numerous residues within the receptor's cytoplasmic domain, including tyrosines 1230, 1234, and 1235 within the Tyr-X-X-X Tyr-Tyr motif of c-Met's activation loop. This motif is conserved among the activation loops of several receptor tyrosine kinases including insulin receptor, insulin like growth factor 1 receptor, nerve growth factor receptor/Trks, and RON. Phosphorylation of tyrosines 1234 and 1235 of c-Met is required for activation of the receptor's tyrosine kinase activity. c-Met phosphorylation also generates docking sites for numerous signaling molecules and stimulates receptor internalization via clathrin-coated vesicles. Signaling proteins that are phosphorylated and/ or localized in response to c-Met phosphorylation include: Grb2, Shc, Cbl, Crk, cortactin, paxillin, GAB1, PI-3 K, FAK, Src, Ras, ERK1 and 2, JNK, PLC-γ, AKT, and STAT3.

HGF/SF stimulation of c-Met expressing cells enhances proliferation, migration, morphogenesis, and protease synthesis, characteristics that are associated with invasive cell phenotype. Indeed, many types of cancer

exhibit sustained c-Met stimulation, overexpression, or mutation, including carcinomas of the colon, breast, ovary, lung, liver, prostate, thyroid, kidney, as well as melanomas and sarcomas. In addition to cancer studies, other research areas in which c-Met is under investigation include organogenesis, organ regeneration, angiogenesis, and surgical wound healing.

Reagents

- **c-Met Standard, Lyophilized, 2 vials, Product No. C 9365 –** Cell extract from GTL 16 cells. Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 3943,** contains sodium azide as preservative.
- **Monoclonal-Anti-c-Met-Coated 96 well plate, Product No. C 9490 -** A plate using break-apart strips coated with monoclonal antibody specific for full-length c-Met (regardless of phosphorylation state).
- **Anti-c-Met Detection Antibody, 11 mL, Product No. C 9615**
  A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.
- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. R 7778 -** contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 5788 -** contains 3.3 mM thymol. Ready to use.
- **Wash Buffer concentrate, 25X, 100 mL, Product No. W 2639 -** *See Reagent Preparation for handling, dilution and storage instructions*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.

Cell Extraction Buffer

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
1% Triton® X-100
10% glycerol
0.1% SDS
0.5% deoxycholate

1 mM PMSF (stock is 0.3 M in DMSO) *PMSF is very unstable and must be added prior to use, even if added previously*.
Protease inhibitor cocktail (Product No. P 2714). Add 250 µl of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 °C.

Thaw on ice. *Add the protease inhibitors just before use.*

Procedure for Extraction of Proteins from Cells
This protocol has been successfully applied to several cell lines of human origin. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –70 °C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals.
5. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of c-Met. For example, 10^8 HeLa cells grown in RPMI plus 10% FBS can be extracted in 1 mL of Extraction Buffer. Under these conditions, use of 1-10 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer is sufficient for the detection of c-Met.
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 °C.
7. Aliquot the clear lysate to clean microcentrifuge tubes

Before assay: extracted cell lysate samples containing protein should be diluted with Standard Diluent Buffer at least 1:10. This dilution is necessary to reduce the matrix effect of the cell extraction buffer.

Reagent Preparation

Standard
Note: This c-Met standard (lyophilized cell extract from GTL 16 cells) was calibrated against the mass of a highly purified, truncated, recombinant human c-Met protein expressed in a mouse myeloma cell line, N50.

1. Reconstitute c-Met Standard with Standard Diluent Buffer. Refer to standard vial label for instructions.
2. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 50 ng/mL c-Met. Use standard within 1 hour of reconstitution.
3. Prepare serial standard dilutions as follows

Tube#	Standard Buffer	Standard from tube #:	Final ng/mL
1	Reconstitute according to label instructions		50 ng/mL
2	0.25 mL	0.25 mL (1)	25 ng/mL
3	0.25 mL	0.25 mL (2)	12.5 ng/mL
4	0.25 mL	0.25 mL (3)	6.25 ng/mL
5	0.25 mL	0.25 mL (4)	3.12 ng/mL
6	0.25 mL	0.25 mL (5)	1.6 ng/mL
7	0.25 mL	0.25 mL (6)	0. 8 ng/mL
8	0.25 mL	-	0 ng/mL

4. Remaining reconstituted standard should be discarded or frozen at –70 °C. for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Anti-rabbit IgG Horseradish Peroxidase (HRP)
Note: The *Anti-rabbit IgG-HRP* 100x concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution:
1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Within 1 hour of use, dilute 10 µL of this 100x concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-rabbit IgG-HRP Working Solution.
4. Return the unused concentrate to the refrigerator
5. For more strips use the following amounts:

# of 8 well strips	IgG-HRP Concentrate µL	Diluent mL
2	20	2
4	40	4
6	60	6
8	80	8
10	100	10
12	120	12

Wash Buffer
1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as *Working Wash Buffer.*
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability
All components of this kit are stable at 2 - 8 °C. Any unused reconstituted standard should be discarded or frozen at -70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www.sigma-aldrich.com

Procedure
Precautions
- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2 – 8 °C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions
- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

c-Met ELISA Assay Summary

1) **100 µL of c-Met Standards or Samples (samples diluted 1:10 or higher in Standard Diluent Buffer)**
   Incubate 2 hours at RT
   aspirate and wash 4x

2) **Add 100 µL c-Met Detection Antibody**
   Incubate 1 hour at RT.
   aspirate and wash 4x

3) **Add 100 µL Anti-Rabbit IgG-HRP**
   Incubate 30 min at RT.
   aspirate and wash 4x

4) **Add 100 µL Stabilized Chromogen**
   Incubate 30 minutes at RT
   ***(in the dark)*.**

5) **Add 100 µL of Stop Solution**
   Read at 450nm.

Total Assay Time - 4 hours

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilution wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch

1st incubation
a Add 100 µL Standard Diluent Buffer to zero wells.
b Add 100 µL c-Met standards, samples or controls to the appropriate wells.
c Samples prepared in cell extraction buffer or brain homogenate buffer must be diluted 1:10 or greater in Standard Diluent Buffer (for example, 10 µL sample plus 90 µL buffer). *The dilutions should be optimized for each assay.*

d Cell culture supernatants or buffered solutions; dilute 1:2 in *Standard Diluent Buffer* (50 µL buffer + 50 µL sample).
e Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature.
f Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 4 times following washing instructions.

2nd incubation
a Add 100 µL Anti-c-Met-Detection antibody to all wells (except chromogen blanks).
b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation
a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation
a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: *If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

Stop reaction
a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading
a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Blank the plate reader against the Chromogen Blank wells (containing Chromogen and Stop Solution).

Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution

Results
1. The results may be calculated using any immunoassay software package.
2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of c-Met may be calculated manually.
4. Calculate the Average Net OD (average reading of 2 wells) for each standard dilution and samples as follows:
5. Average Net OD = Average Bound OD – Average Chromogen Blank OD
6. On graph paper plot the Average Net OD of standard dilutions against the standard concentration (ng/mL) of c-Met. Draw the best curve through these points to construct the standard curve.
7. The c-Met concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
8. Multiply the values obtained for the samples by dilution factor of each sample.
9. Samples producing signals higher than the 50 ng/mL standard should be further diluted and assayed again.

Product Profile

Typical Results
The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Standard	Optical Density
ng/mL	450 nm
50	2.76
25	1.87
12.5	1.25
6.25	0.95
3.13	0.72
1.25	0.49
0.78	0.39
0	0.17

Limitations
- Do not extrapolate the standard curve beyond the 50 ng/mL standard point.

- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of cell extract and brain homogenate samples have not been thoroughly investigated.
- The rate of degradation of native c-Met in various matrices has not been investigated.

Performance characteristics

Sensitivity

The analytical sensitivity of this assay is <0.4 ng/mL of c-Met. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

The sensitivity of this ELISA was compared to immunoblotting using known quantities of c-Met. The data presented in Figure 1 show that the sensitivity of c-Met ELISA is 2X greater than immunoblotting. The bands shown in the immunoblotting were developed using rabbit anti-cMet, an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

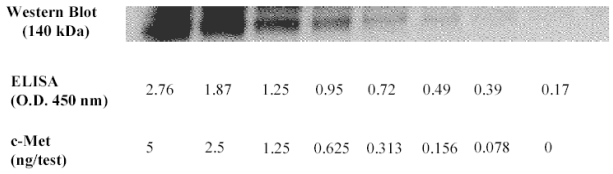

Figure 1 Detection of c-Met by ELISA vs immunoblot

Precision

1. Intra-Assay Precision

Samples of known concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	37.8	16.3	8.4
Standard Deviation (SD)	3.4	1.4	0.7
% Coefficient of Variation	9.1	8.4	8.4

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	37.1	16.6	8.0
Standard Deviation (SD)	3.9	1.8	0.9
Coefficient of Variation %	10.4	10.6	11.4

Recovery

To evaluate recovery, extraction buffer was diluted 1:10 with *Standard Diluent Buffer* to bring the SDS concentration to <0.01%. c-Met standard was spiked into the cell extraction buffer. The average recovery was 99.9%.

Parallelism

Natural c-Met from a GTL 16 and HeLa cell lysates was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the c-Met standard curve. Parallelism was demonstrated by Figure 2 and indicates that the standard accurately reflects c-Met content in samples.

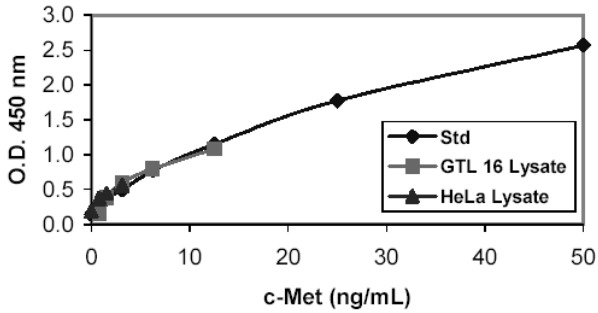

Figure 2 c-Met ELISA - Parallelism

Linearity of Dilution

GTL 16 cells were grown in cell culture medium containing 10% FCS and lysed with Cell Extraction Buffer. This lysate was diluted with *Standard Diluent Buffer* over the range of the assay and measured for c-Met content. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.98.

Dilution	Cell lysate		
	Measured ng/mL	Expected ng/mL	% Expected
Neat	50.0	50.0	100
1:2	29.6	25.0	118
1:4	15.8	12.5	126

Specificity

- The c-Met ELISA is specific for measurement of total c-Met protein regardless of phosphorylation status.
- To determine the specificity of this ELISA, cell extracts from different cell lines, each at the concentration of 200 µg/mL total protein, were analyzed.
- The data presented in Figure 3 show that the ELISA detects c-Met protein in lysates from human GTL 16, HeLa, Colo 201, HEPG2, Jurkat and K562 cells.
  The levels of c-Met detected with this ELISA are consistent with the results obtained by immunoblot (insert)

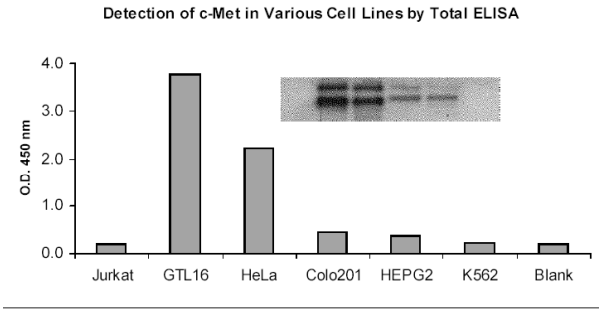

Figure 3

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