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ab128573 –

GST 6XHis-tag ELISA Kit

Instructions for Use

For the quantitative measurement of 6XHis-tag protein expression

This product is for research use only and is not intended for diagnostic use.

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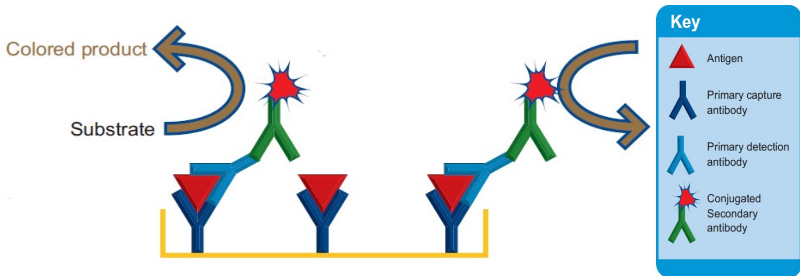
1. Introduction

Principle: ab128573 6xHis-tag ELISA Kit, is an in vitro enzyme-linked immunosorbent assay for the accurate measurement of 6xHistagged protein expression in cell lysates or after protein purification. The assay employs a mouse monoclonal antibody specific to the 6xHis-tag coated onto well plate strips. The immobilized antibody will bind 6xHis-tagged recombinant proteins in samples. Bound proteins can be detected using rabbit antibody specific to the 6XHistagged fusion protein of choice (not included). However this kit does include a detector antibody and standard protein for use with dual GST 6XHis-tagged proteins.

Standards and samples are pipetted into the wells and the 6XHis-tag present in the sample is bound to the wells by the immobilized antibody. The wells are washed and a rabbit analyte specific detector antibody is added. After washing away unbound detector antibody, an HRP-conjugated antibody specific for the rabbit antibody is pipetted to the wells. The wells are again washed, a HRP substrate solution is added to the wells and color develops in proportion to the amount of 6XHis-tag bound. The developing blue color is measured at 600 nm. Optionally the reaction can be stopped by adding hydrochloric acid which changes the color from blue to yellow and the intensity can be measured at 450 nm.



Background: The 6XHis-tag is a commonly used polyhistidine tag for the purification of recombinant proteins by immobilized metal ion affinity chromatography (IMAC) from *E. coli*, *Saccharomyces cerevisiae*, mammalian cells and baculovirus-infected *Spodoptera frugiperda* insect cells. It is typically used (1) as a single tag on proteins for purification, (2) as part of a dual tag for achieving proteins of higher purity (i.e. combined with GST fusion partner, albumin-binding protein or S-peptide) or (3) in tandem affinity purification of endogenous protein complexes.

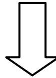
This 6XHis-tag detection assay kit is an enzyme immunoassay developed for the sensitive detection of 6XHis-tagged fusion proteins with a suitable detector antibody or for the quantification of GST6xHis double tagged proteins with the supplied detector antibody. The quantity of the GST 6XHis double tag can be determined in lysates or intermediate purification fractions by comparing their absorbance with that of a known recombinant GST 6XHis-tagged standard curve. The kit has a detection sensitivity limit of 0.5ng/mL of GST 6XHis-tagged protein. The kit is particularly suitable for screening in high-throughput conditions for maximum expression and optimal purification of 6XHis-tagged proteins as well

as for screening humoral immunity against His-tagged antigens. Each kit provides sufficient reagents to perform up to 96 assays including standard curve and 6XHis-tag fusion proteins.

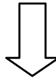
2. Assay Summary

Equilibrate all reagents to room temperature. Prepare all the reagents,

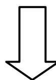
samples, and standards as instructed.



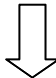
Add 50 µL standard or sample to each well used. Incubate 2 hours at room temperature.



Aspirate and wash each well three times. Add 50 µL prepared primary detector antibody to each well. Incubate 1 hour at room temperature.



Aspirate and wash each well three times. Add 50 µL prepared HRP labeled secondary detector antibody. Incubate 1 hour at room temperature.



Aspirate and wash each well three times. Add 100 µL HRP Development Solution to each well. Immediately begin recording the color development with elapsed time at 600 nm for 15 minutes. Alternatively add a Stop solution at a user-defined time and read at 450 nm.

3. Kit Contents

Item	Quantity
20X Buffer	20 mL
Extraction Buffer	15 mL
10X Blocking Buffer	6 mL
HRP Development Solution	12 mL
10X GST Protein Detector Antibody	700 µL
10X HRP Label	1 mL
GST 6xHis-tagged protein standard	2 µg
Anti 6XHis-tag Microplate (8 x 12 antibody coated strips)	96 Wells

4. Storage and Handling

Store all components at 4°C. This kit is stable for 6 months from receipt. After reconstitution the standard should be aliquoted and stored at -80°C. Unused microplate strips should be returned to the pouch containing the desiccant and resealed.

5. Additional Materials Required

- Rabbit antibody to 6XHis-tagged fusion protein when not using included anti-GST antibody.
- Microplate reader capable of measuring absorbance at 600 nm (or 450 nm after addition of Stop solution - not supplied.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water
- Multi and single channel pipettes
- PBS (1.4 mM KH_2PO4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3)
- Tubes for standard dilution
- Stop solution (optional) – 1N hydrochloric acid
- Optional plate shaker for all incubation steps

6. Preparation of Reagents

6.1 Equilibrate all reagents and samples to room temperature (18-25°C) before use.

6.2 Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water.

6.3 Prepare Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer. Excess unused Incubation Buffer may be stored at -20 °C for 6 months after performing the ELISA.

6.4 Prepare the GST 6XHis-tagged protein primary detector antibody by diluting 10-fold with Incubation Buffer immediately prior to use. If using a detector antibody specific to the tagged protein of interest, user optimization is required. A rabbit detector antibody (polyclonal or monoclonal) must be used with a dilution of 1:200 - 1:2000 in Incubation Buffer recommended. Prepare 0.5 mL for each 8 well strip used.

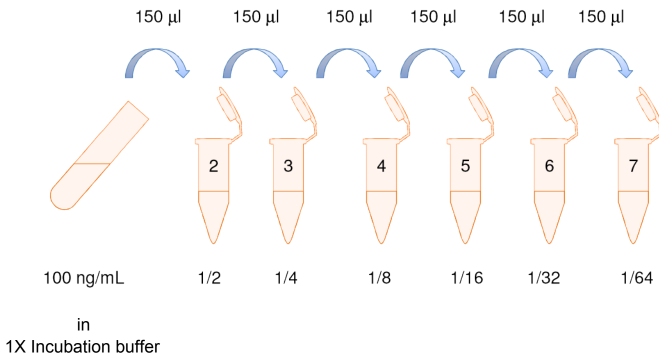
6.5 Prepare the anti-rabbit HRP labeled secondary antibody by diluting 10-fold with Incubation Buffer immediately before use. Prepare 0.5 mL for each 8 well strip used.

6.6 Reconstitute the 2 µg standard by adding 200 µL of nanopure water and vortex vigorously; the final concentration will be 10 µg/mL. Allow to sit for 10 minutes on ice. Aliquot and freeze spare tubes at -80C.

6.7 To prepare serially diluted standards, first label tubes #1-7. Add 150 µL of Incubation Buffer to each of tubes #2 through #7.

6.8 Prepare the standard curve sample (tube #1) at 100 ng/mL by diluting 10 µL of the 10 µg/mL reconstituted protein in 990 µL of Incubation Buffer. Transfer 150 µL from tube #1 to tube #2. Mix thoroughly. With a fresh pipette tip transfer 150 µL from #2 to #3. Mix thoroughly. Repeat for Tubes #4 through #7.

6.9 Use Incubation Buffer as the zero standard tube labeled #8. Use fresh standards for each assay.



7. Sample Preparation

Note: The extraction buffer provided with this kit, should only be used with mammalian expression systems. This buffer can be supplemented with phosphatase inhibitors, PMSF and protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions.

7.1 Mammalian Cell lysates.

7.1.1 Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 10 min at 4°C.

7.1.2 Rinse cells twice with PBS.

7.1.3 Solubilize cell pellet at $2x10^7$/mL in Extraction Buffer.

7.1.4 Incubate on ice for 20 minutes. Centrifuge at 16,000 x g, 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

7.2 Bacterial and Yeast cell lysates.

7.2.1 Disrupt cells by french pressing, sonication, freeze thaw or bead-vortexing according to standard protocols.

7.2.2 Clarify lysates by centrifuging at 16,000 x g, 4°C for 20 minutes.

7.2.3 If fusion protein is insoluble, use either 6 M Guanidine hydrochloride *(CH_5N_3HCL)*, 0.1 M NaH_2PO_4; 0.01 M Tris-HCl; pH 8.0 or 7 M Urea *($CO(NH_2)_2$)*; 0.1 M NaH_2PO_4; 0.01 M Tris-HCl; pH 8.0.

7.2.4 Assay samples immediately or aliquot and store at 80°C. The sample protein concentration in the extract may be quantified using a protein assay.

8. Assay Procedure

Equilibrate all reagents to room temperature before use. It is recommended all samples and standards be assayed in duplicate. Note - The GST 6XHis-tagged standard and detector can be used to accurately quantify GST 6XHis-tagged or as a positive control alongside 6XHis-tagged fusion proteins.

8.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

8.2 Remove unused microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack and reseal.

8.3 Add 50 µL of each serially diluted GST 6XHis-tagged protein standard or test sample per well. Also include a Incubation Buffer as a zero standard.

8.4 The samples should be diluted to within the working range of the assay in Incubation Buffer. As a guide, typical ranges of sample concentration and assay tolerance for commonly used sample buffers are shown below in Data Analysis.

8.5 Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

8.6 Aspirate each well and wash, repeat this twice more for a total of **three** washes. Wash by aspirating or decanting from wells then dispensing 300 µL 1X Wash buffer into each well as described above. Complete removal of liquid

at each step is essential to good performance. After the last wash, remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.

8.7 Immediately prior to use prepare sufficient (0.5 mL/strip used) 1X Primary Detector Antibody (step 6.4) in Incubation Buffer. Add 50 µL 1X Primary Detector antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

8.8 Repeat the aspirate/wash procedure above.

8.9 Immediately before use, prepare sufficient (0.5 mL/strip used) 1X HRP labeled secondary detector antibody (step 6.5) in Incubation Buffer. Add 50 µL 1X HRP labeled secondary detector antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

8.10 Repeat the aspirate/wash procedure above.

8.11 Add 100 µL HRP Development Solution to each empty well and immediately record the blue color development with time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nM
Time:	up to 15 min.
Interval:	20 sec. - 1 min.
Shaking:	Shake between readings

Alternative– In place of a kinetic reading, at a ***user defined*** time record the endpoint OD data at (i) 600 nm or (ii) stop the reaction by adding 100 µL stop solution (1N HCl) to each well and record the OD at 450 nm. Analyze the data as described below.

9. Data Analysis

Average the duplicate standard readings and plot against their concentrations after subtracting the zero standard reading. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Read protein concentrations of GST 6XHis double-tagged proteins for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in Incubation Buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

TYPICAL STANDARD CURVE - *For demonstration only.*

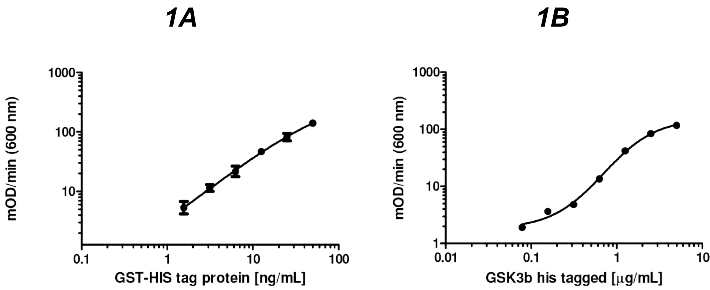


Figure 1: A) Example GST 6XHis tagged standard curve. *GST-His tagged protein (provided with the kit) was diluted from 50 ng/mL to 0.7 ng/mL. Background was subtracted from data points prior to log transformation and curve fitting (log-agonist vs. response – variable slope). Sensitivity was calculated mathematically to be as low as 0.5 ng/mL.*

B) Example GSK3B-His tagged standard curve. *GSK3B-His tagged protein (not provided with the kit) was diluted from 5ug/mL to 0.07ug/mL and loaded on to the His plate. GSK3b was detected with a rabbit monoclonal antibody and labeled with the provided HRP label. Background was subtracted from data points prior to log transformation and curve fitting (logagonist vs. response – variable slope).*

TYPICAL SAMPLE RANGE

Typical working ranges	
Sample Type	Range
GST His tagged	1 – 50 ng/mL
GSK3b His tagged	70 – 5000 ng/mL

SENSITIVITY

Calculated minimum detectable dose of GST 6XHis-tagged protein = 0.5 ng/mL (zero dose n=8 + 2 standard deviations)

LINEARITY OF DILUTION

GST-His tagged recombinant protein on a cell extract background (ng/mL)	% Expected Value
50	100%
25	97.8%
12.5	85.5%
6.25	98.2%

ASSAY TOLERANCE

Tolerance of this assay was tested with buffers typically used in recombinant protein chemistry sample preparation:

Buffer Name	Composition	Maximum concentration tolerated by assay	Effect at higher concentrations
Guanidine buffer	6 M GuHCl; 0.1 M NaH_2PO_4; 0.01 M Tris-HCl pH 8.0	0.1%	Inhibition
Urea buffer	7 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-HCl pH 8.0	10%	Inhibition
Imidazole buffer	250mM Imidazole; 300mM NaCl; 50mM NaH_2PO_4 pH8.0	2.5%	Agonist
Mammalian detergent	Provided with the kit	50%	Inhibition
Yeast detergents	Commercially available	2%	Agonist

RECOVERY

Sample Type	Average Recovery (%)	Range (%)
50% extraction buffer (supplied with the kit)	94.15%	66%- 112%
50% Lauryl Maltoside	86%	71% - 97%
10% Serum	75%	66% - 90%
50% Cultured Media	90.12%	78% - 106%
0.1% Guanidine Buffer	65.8%	45% - 81%
10% Urea buffer	84.8%	25% - 114%
2.5% Imidazole buffer	129%	73% - 155%
2% Yeast extraction buffer	113%	77% - 136%

If measuring 6XHis-tagged proteins solubilized with guanidine buffer, dilute standard curve (or reference sample) in the same final concentration that the test samples are loaded onto the plate (dilution factor should be 1:1000 or greater). Note that 10% Urea can inhibit the ELISA assay at low concentration of the tagged protein (<10ng/mL). If measuring serum samples to screen for antibodies against the His-tagged protein of interest, dilute IgG standards (not provided in the kit) in an equal concentration of serum.

REPRODUCIBILITY

Parameter	CV%
Intra (n= 8)	6.4%
Inter (n=3 days)	10%

10. Specificity

This kit is reactive with any 6XHis-tagged recombinant protein. Note that it is possible that lack of exposure of the 6XHis-tag on the surface of the protein may prevent the anti-6XHis antibody from binding in this kit.

The capture antibody provided in this kit was evaluated for specificity on western blot.

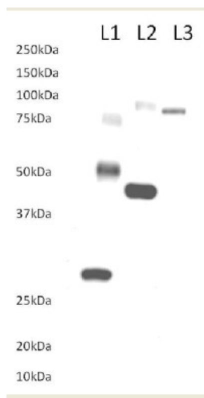


***Figure 2: Validation by Western blot**. Capture antibody was tested by western blot against three His tagged proteins: GST recombinant protein loaded at 50ng/well (lane 1), GSK3b recombinant protein loaded at 50ng/well (lane 2) and Hexokinase 1 recombinant protein loaded at 250ng/well (lane 3). Blots were blocked with 5% Milk in TBST buffer, incubated overnight in anti 6XHis-tag primary antibody and labeled with GAM HRP.*

11. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipets
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Fusion protein detector antibody does not bind	Change detector antibody and optimize
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper sorage of the ELISA kit	Store the reconstituted protein at -80°C, all other assay components 4°C. Keep substrate solution protected from light.
Very high background	Fusion protein detector antibody binds not specifically	Change detector antibody and optimize

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