His-Tag Detection ELISA Kit

Item No. 10012445

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
400242	His ELISA Monoclonal Antibody	1 vial/100 dtn
400240	His-AP Tracer	1 vial/100 dtn
400247	His-Protein ELISA Standard	1 vial
400084	TBS Assay Buffer Concentrate (10X)	2 vials/10 ml
411007	AP Wash Buffer Concentrate (150X)	1 vial/5 ml
400008/400009	Goat Anti-Mouse IgG Coated Plate	1 plate
400012	96-Well Cover Sheet	1 cover
400089	*p*NPP Substrate Solution	2 vials/12 ml

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of 'UltraPure' water. Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Buffer Preparation** and **Sample Preparation** (see pages 10 and 11, respectively).

INTRODUCTION

Background

Recombinant protein expression is a valuable tool in the production of large amounts of protein for both functional and structural studies. To facilitate purification and detection, recombinant proteins are often labeled with affinity tags, such as hexahistidine (6X-His), GST, and FLAG. 6X-His tags are often the tag of choice due to their small size, less potential to interfere in protein folding, weak immunogenicity, and high affinity for Ni^{2+} ions. His-tagged proteins expressed in bacteria or baculovirus/insect cells can be easily purified by Ni^{2+}-resin chromatography.[1] Cell lysates and samples at different stages of purification are generally analyzed by SDS-PAGE to determine the expression of His-proteins, which can add several days to the purification and analysis protocol. A semi-quantitative screening assay would provide the ability to rapidly screen for His-tagged proteins at each stage of expression and purification, permitting the user to determine if there is sufficient protein expression to continue with purification and to monitor loss or enrichment at each stage.

About This Assay

Cayman's His-Tag Detection ELISA Kit is a competitive assay designed for the rapid, semi-quantitative screening of cell lysates and affinity column fractions for His-tagged proteins. It can be used as a substitute for SDS-PAGE to expedite the screening of affinity column fractions within a few hours.

Principle of the Assay

This assay is based on the competition between free His-protein and a 6X-His Tracer (6X-His linked to alkaline phosphatase (AP)) for a limited number of His-specific monoclonal antibody binding sites. The concentration of the 6X-His Tracer is held constant while the concentration of free His-protein (standard or sample) varies. Thus, the amount of Tracer that is able to bind to the monoclonal antibody will be inversely proportional to the concentration of free His-protein in the well. This monoclonal antibody-His (either free or tracer) complex binds to the goat anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then *p*NPP is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Tracer bound to the well, which is inversely proportional to the amount of free His-protein present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound His Tracer}] \propto 1/[\text{His-protein}]$$

A schematic of this process is shown in Figure 1 on page 8.

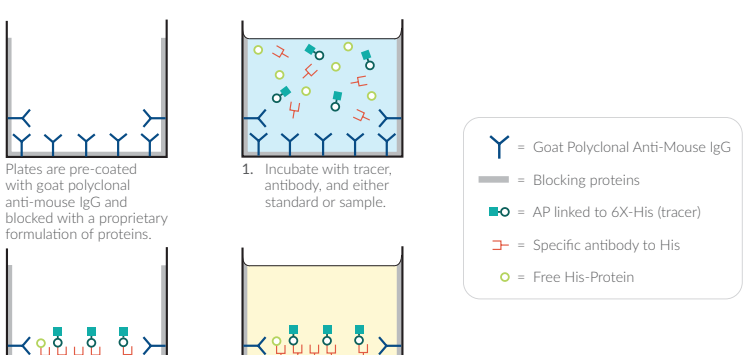


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blank: background absorbance caused by *p*NPP Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including NSB wells.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding. Do not forget to subtract the Blank absorbance values.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. UltraPure water may be purchased from Cayman (Item No. 400000).

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for about two months.

1. **TBS Assay Buffer Preparation**

   Dilute the contents of one vial of TBS Assay Buffer Concentrate (10X) (Item No. 400084) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **AP Wash Buffer Preparation**

   Dilute the contents of the vial of AP Wash Buffer Concentrate (150X) (Item No. 411007) to a total volume of 750 ml with UltraPure water. Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:150.

Sample Preparation

Crude cell lysates contain materials that interfere in the assay and give false positives or erroneously high levels of His-tagged protein. It is recommended that cell lysates be cleared by high speed centrifugation before assaying.

NOTE: Due to the interference of denaturing reagents, this assay is not suitable for proteins that require denaturing to expose the His-tag.

Buffers and reagents that have been tested and demonstrated to be compatible with the assay:

Reagents	
Compatible Buffers and Reagents	PBS (phosphate buffered saline)
	PBS (1.0% NP-40)
	TBS (Tris buffered saline)
	50 mM potassium phosphate, 500 mM sodium chloride, pH 7.2
	50 mM potassium phosphate, 500 mM sodium chloride (1.0% NP-40)
	B-PER Protein extraction reagent
	Imidazole (<300 mM)
Incompatible Reagents	Urea (>0.5 M)
	Guanidine-HCl (>0.5 M)
	Imidazole (>300 mM)

Preparation of Assay-Specific Reagents

His-Protein ELISA Standard

This kit contains one His-Protein ELISA Standard (Item No. 400247). As this is a rapid screening assay, and the immunoreactivity of different His-tagged proteins is highly variable,[2] the standard is not designed to be used as a quantitative tool, but provide a means of monitoring protein gain or loss during the various purification steps.

NOTE: A standard curve is NOT recommended for screening column fractions eluted in imidazole (see page 20).

Reconstitute the His-Protein Standard with 400 µl TBS Assay Buffer. The concentration of this solution (the bulk standard) will be 50 µg/ml. Store this solution at 4°C; it will be stable for at least two weeks. For longer storage, we recommend the bulk standard be aliquoted and stored at -20°C or lower. Avoid repeated freeze/thaw cycles.

To prepare the standard for use in ELISA: Obtain six clean test tubes and number them #1 through #6. Aliquot 940 µl TBS Assay Buffer to tube #1 and 500 µl TBS Assay Buffer to tubes #2-6. Transfer 60 µl of the standard to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-6.

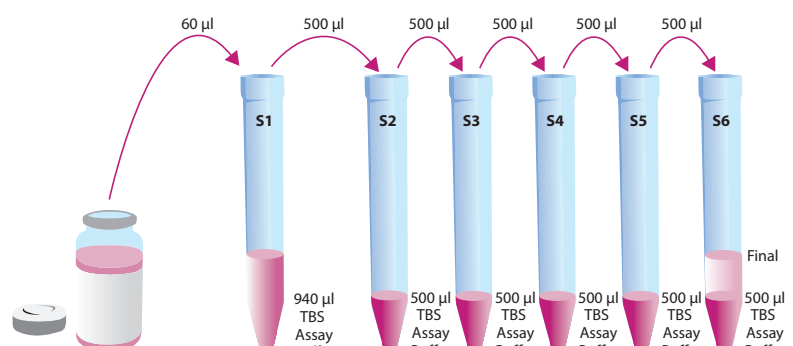


Figure 2. Preparation of the His-Protein standard

His-AP (Alkaline Phosphatase) Tracer

Reconstitute the His-AP Tracer (Item No. 400240) with 6 ml TBS Assay Buffer. Store the reconstituted His-AP Tracer at 4°C *(do not freeze!)* and use within two weeks. A 20% surplus of tracer has been included to account for any incidental losses.

His ELISA Monoclonal Antibody

Reconstitute the His ELISA Monoclonal Antibody (Item No. 400242) with 6 ml TBS Assay Buffer. Store the reconstituted His ELISA Monoclonal Antibody at 4°C. It will be stable for at least four weeks. A 20% surplus of antibody has been included to account for any incidental losses.

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

There is no specific pattern for using the wells on the plate. A suggested plate format is shown in Figure 3, below. Each plate or set of strips should contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), and a six point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate. We suggest you record the contents of each well on the template sheet provided (see page 30).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	B_0	B_0	8	8	16	16	24	24	32	32
B	S2	S2	1	1	9	9	17	17	25	25	33	33
C	S3	S3	2	2	10	10	18	18	26	26	34	34
D	S4	S4	3	3	11	11	19	19	27	27	35	35
E	S5	S5	4	4	12	12	20	20	28	28	36	36
F	S6	S6	5	5	13	13	21	21	29	29	37	37
G	NSB	NSB	6	6	14	14	22	22	30	30	38	38
H	Blk	Blk	7	7	15	15	23	23	31	31	39	39

Blk - Blank
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S6 - Standards 1-6
1-39 - Samples

Figure 3. Sample plate format

Performing the Assay (Non-Imidazole)

Instructions for Imidazole fractions (<300 mM imidazole) can be found on pages 23-27.

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **TBS Assay Buffer**

   Add 100 µl TBS Assay Buffer to NSB wells. Add 50 µl TBS Assay Buffer to B_0 wells.

2. **His-Protein ELISA Standard**

   Add 50 µl from tube #6 to both of the lowest standard wells (S6). Add 50 µl from tube #5 to each of the next two standard wells (S5). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **His-AP Tracer**

   Add 50 µl to each well *except* the Blk wells.

5. **His ELISA Monoclonal Antibody**

   Add 50 µl to each well *except* the NSB and the Blk wells.

Well	TBS Assay Buffer	Standard/Sample	AP Tracer	His ELISA Monoclonal Antibody
Blk	-	-	-	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate for 90 minutes at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse four times with Wash Buffer. Each well should be completely filled with Wash Buffer during each wash. Invert the plate between wash steps to empty the fluid from the wells. After the last wash, gently tap the inverted plate on absorbent paper to remove the residual Wash Buffer.
2. Add 200 µl of *p*NPP Substrate Solution to each well of the plate.
3. Cover the plate with plastic film and incubate for approximately 60 minutes at room temperature. The assay typically develops in 30-60 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells in the range of 0.3-1.5 A.U. (blank subtracted).

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as either $\%B/B_0$ *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit.

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain $\%B/B_0$ for a logistic four-parameter fit, multiply these values by 100.)

Plot the Standard Curve

Plot %B/B_0 for standards S1-S6 *versus* His-Protein concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot - The data can also be lineraized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation.*

$$\text{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample by using the equation obtained from the standard curve plot. *NOTE: Remember to account for any dilution or concentration of the sample prior to the addition to the well.* Samples with %B/B_0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference in the assay.

Performance Characteristics

Due to variability in immunodetection of the His-tag on His-tagged proteins, the sensitivity of this assay will vary depending on the protein being analyzed. The purified His-tagged standard provided typically show a sensitivity of 100 ng/ml. If the tertiary structure of the experimental protein partially occludes the His-tag, the sensitivity of the assay may be reduced.

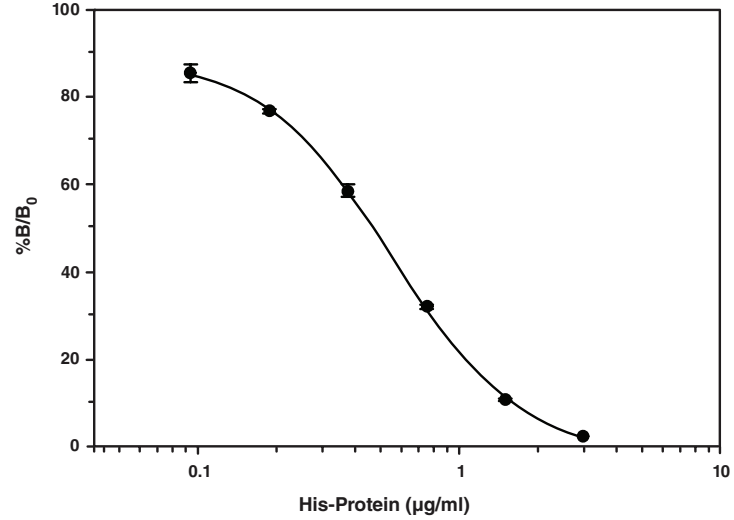


Figure 4. Typical standard curve

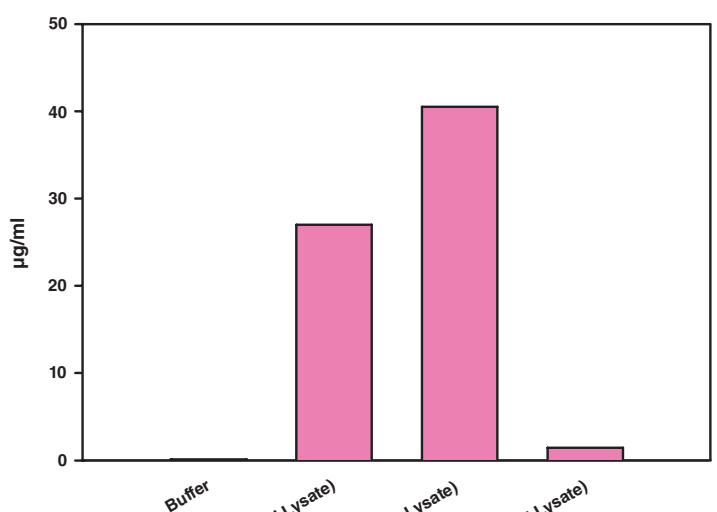


Figure 5. His-Protein determination in baculovirus/insect cell lysates expressing recombinant His-tagged or GST-tagged protein

Appendix

Performing the Assay - Imidazole fractions (<300 mM Imidazole)

Plate Set Up

Due to varying degrees of interference of imidazole in the assay, control wells containing imidazole buffer alone MUST be included. For example, if proteins are eluted with 100 mM, 200 mM, and 300 mM imidazole from a column, control wells containing equivalent concentrations of imidazole must also be included. A standard curve is NOT recommended for screening column fractions. Although there is no specific pattern for using wells on the plate, a suggested plate format is shown in Figure 6 below. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.* Each plate or set of strips should contain a minimum of two Blk wells, two NSB wells, two B_0 wells, duplicate samples, duplicate buffer control wells for each imidazole concentration, and duplicate positive controls prepared in imidazole.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Blk	P2	P2	C5	C5	S8	S8	P10	P10	C13	C13
B	NSB	NSB	S3	S3	P5	P5	C8	C8	S11	S11	P13	P13
C	B_0	B_0	C3	C3	S6	S6	P8	P8	C11	C11	S14	S14
D	S1	S1	P3	P3	C6	C6	S9	S9	P11	P11	C14	C14
E	C1	C1	S4	S4	P6	P6	C9	C9	S12	S12	P14	P14
F	P1	P1	C4	C4	S7	S7	P9	P9	C12	C12	S15	S15
G	S2	S2	P4	P4	C7	C7	S10	S10	P12	P12	C15	C15
H	C2	C2	S5	S5	P7	P7	C10	C10	S13	S13	P15	P15

Blk - Blank
NSB - Non-Specific Binding
B_0 - Maximum Binding
S - Sample
C - Imidazole Buffer Control
P - Positive Control in Imidazole Buffer

Figure 6. Sample plate format

Positive Control Preparation

Reconstitute the His-Protein ELISA Standard (Item No. 400247) with 400 µl TBS Assay Buffer. The concentration of this solution will be 50 µg/ml. Store this solution at 4°C; it will be stable for at least two weeks. For longer storage, we recommend the bulk standard be aliquoted and stored at -20°C or lower. Avoid repeated freeze/thaw cycles.

Prepare a separate positive control for every concentration of imidazole. For example, if samples were eluted in 100 mM, 200 mM, and 300 mM imidazole, prepare a separate positive control in each of these imidazole concentrations. To prepare the positive control, transfer 10 µl of standard to tubes containing 1 ml of the varying imidazole buffer concentrations. These tubes are the positive controls.

Addition of the Reagents

1. **TBS Assay Buffer**

   Add 100 µl TBS Assay Buffer to NSB wells. Add 50 µl TBS Assay Buffer to B_0 wells.

2. **Positive Controls**

   Add 50 µl per well.

3. **Negative Control (Imidazole Buffer)**

   Add 50 µl per well.

4. **Samples**

   Add 50 µl of sample per well.

5. **His-AP Tracer**

   Add 50 µl to each well *except* the Blk wells.

6. **His ELISA Monoclonal Antibody**

   Add 50 µl to each well *except* the NSB and the Blk wells.

Well	TBS Assay Buffer	Positive Controls	Negative Control (Imidazole Buffer)	Sample	His-AP Tracer	His ELISA Monoclonal Antibody
Blk	-	-	-	-	-	-
NSB	100 µl	-	-	-	50 µl	-
B_0	50 µl	-	-	-	50 µl	50 µl
Sample	-	-	-	50 µl	50 µl	50 µl
C	-	-	50 µl	-	50 µl	50 µl
P	-	50 µl	-	-	50 µl	50 µl

Table 2. Pipetting summary

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate for 90 minutes at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse four times with Wash Buffer. Each well should be completely filled with Wash Buffer during each wash. Invert the plate between wash steps to empty the fluid from the wells. After the last wash, gently tap the inverted plate on absorbent paper to remove the residual Wash Buffer.
2. Add 200 µl of *p*NPP Substrate Solution to each well of the plate.
3. Cover the plate with plastic film and incubate for approximately 30 minutes at room temperature. The assay typically develops in 30-60 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells in the range of 0.3-1.5 A.U. (blank subtracted).

Analysis

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings of the duplicate (or triplicate) negative control wells (C) and samples wells (S).
2. To correct for interference from the imidazole buffer, subtract the Sample (S) wells from the corresponding negative Control (C) wells (*i.e.*, C1 minus S1, C2 minus S2, etc). This value is the corrected O.D. for each fraction. The corrected O.D. of fractions containing His-protein should be greater than zero.

Positive control (P) wells are not used in the analysis but serve as a diagnostic tool. The O.D. for the positive control wells MUST be less than the O.D. for corresponding imidazole buffer control wells.

Sample Data

Shown below is an example of data for varying imidazole concentrations containing 0.5 µg/ml His-Protein.

Imidazole Concentration (mM)	Negative Control (C) Wells (O.D.)	Sample (S) Wells (O.D.)	Corrected O.D. (C minus S)
300	0.098	0.064	0.034
200	0.174	0.121	0.053
100	0.337	0.259	0.078

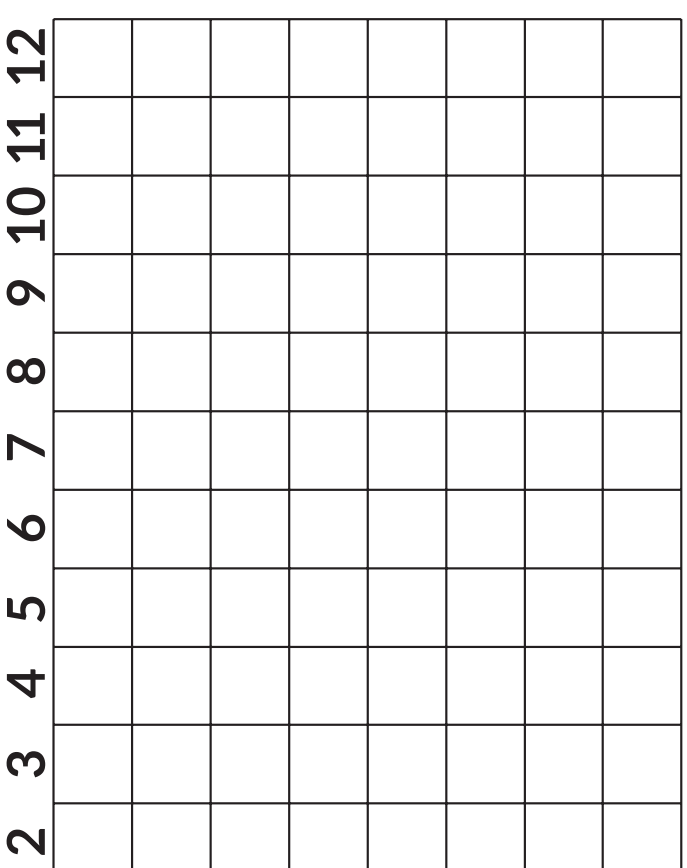
Table 3. Typical results

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure water
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody	A. Re-wash plate and redevelop
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time C. Dilution error in preparing reagents	A. Replace activated carbon filter or change source of UltraPure water B. Return plate to shaker and re-read later
Low sensitivity (shift in dose response curve)	Standard is degraded	Replace standard
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by ELISA[3]

References

1. Bornhorst, J.A. and Falke, J.J. Purification of proteins using polyhistidine affinity tags. *Methods Enzymol.* **326**, 245-254 (2000).
2. Debeljak, N., Feldman, L., Davis, K.L., *et al.* Variability in the immunodetection of His-tagged recombinant proteins. *Anal. Biochem* **359(2)**, 216-223 (2006).
3. Maxey, K.M., Maddipati, K.R., and Birkmeier, J. Interference in enzyme immunoassays. *J. Clin. Immunoassay* **15**, 116-120 (1992).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.