Citrullinated Histone H3 ELISA Kit

Item No. 501440

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
401350	Anti-Citrullinated Histone H3 HRP Conjugate	1 vial/1.5 ml
401352	Anti-Citrullinated Histone H3 ELISA Strip Plate	1 plate
401444	Citrullinated Histone ELISA Standard	2 vials
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml
400035	Polysorbate 20	1 vial/3 ml
400074	TMB Substrate Solution	1 vial/12 ml
10011355	HRP Stop Solution	1 vial/12 ml
400012	96-Well Cover Sheet	3 covers

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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman's Citrullinated Histone H3 ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified. The Stop Solution provided with this kit is an acid solution. Please wear appropriate personal protection equipment (*e.g.*, safety glasses, gloves, and lab-coat) when using this material.

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 as directed 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 with the ability to measure absorbance at 450 nm.
2. Adjustable pipettes and a repeating pipettor.
3. Materials used for **Sample Preparation** (see page 9).
4. A source of pure water; glass distilled water or HPLC-grade water is acceptable.

INTRODUCTION

About This Assay

Cayman's Citrullinated Histone H3 (CitH3) ELISA Kit is a sandwich (immunometric) assay which can be used to measure citrullinated histone from cell lysates, cell culture supernatant, plasma, and serum. Histone H3 is citrullinated by peptidylarginine deiminase 4 (PAD4) at residues R2, R8, and R17 in the processes of epigenetic chromatin remodeling and neutrophil extracellular trap (NET) formation.[1-4] CitH3 has been found in the plasma or serum of human patients or experimental animals in inflammatory disease conditions including septic shock.[5-8] *In vitro*, CitH3 is released from neutrophils that have been stimulated with calcium ionophore during the process of NETosis, and can be freed from NETs by treatment with nuclease. The measurement of CitH3, either in cell lysates or released during NETosis can be used as a functional readout of pharmacological PAD inhibition. The standard curve of the ELISA spans the range of 0.15-10 ng/ml, with a lower limit of quantification (LLOQ) of 0.15 ng/ml.

Description of Sandwich ELISAs

This immunometric assay is based on a double-antibody 'sandwich' technique. Each well of the microwell plate supplied in the kit has been coated with a monoclonal antibody specific for histone H3 (citrullinated at R2, R8, and R17). This antibody will bind any CitH3 introduced into the well. A second, polyclonal antibody also recognizing CitH3 (Antibody/HRP Conjugate) is added to the well. This allows the two antibodies to form a 'sandwich', each binding a different epitope on citrullinated H3. The 'sandwiches' are immobilized on the plate so the excess reagents may be washed away. The Antibody/HRP Conjugate is labeled with HRP, allowing quantitation of the CitH3. Addition of HRP Substrate TMB, followed by Stop Solution produces a yellow colored product which can be measured spectrophotometrically. The intensity of the color is directly proportional to the amount of bound Antibody/HRP Conjugate, which is proportional to the concentration of citrullinated histone H3.

$$\text{Absorbance} \propto [\text{Anti-CitH3/HRP}] \propto [\text{CitH3}]$$

A schematic of this process is shown in Figure 1, on page 7.

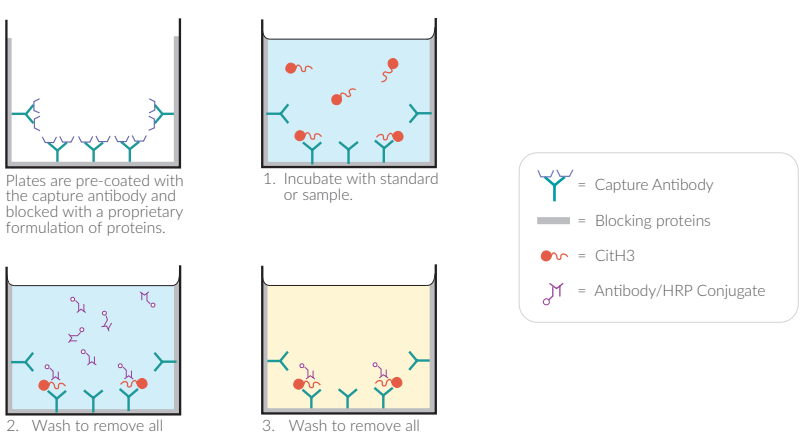


Figure 1. Schematic of the Sandwich ELISA

Definition of Key Terms

Lower Limit of Quantification (LLOQ): is defined as the lowest standard concentration in which absorbance (450 nm) - (1.64 x S.D.) is higher than the blank value of absorbance (450 nm) + (1.64 x S.D.).

Lower Limit of Detection (LLOD): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a point two standard deviations away from the mean zero value.

Dynamic Range: the range in which the analyte is reliably quantifiable.

Standard Curve: a plot of the absorbance values *versus* concentration of a series of wells containing various known amounts of analyte.

PRE-ASSAY PREPARATION

Buffer Preparation

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

1. **ELISA Buffer Preparation**

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) (Item No. 400060) with 90 ml of 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. **Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (400X) (Item No. 400062): Dilute to a total volume of 2 L with water and add 1 ml of Polysorbate 20 (Item No. 400035). *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.*

Sample Preparation

Serum and plasma contain nuclease and other factors that can interfere with the functioning of assay. In general, serum or plasma (prepared using heparin or EDTA as the anticoagulant) can be used without a purification step in the assay if they are first diluted a minimum of 1:10 in ELISA Buffer. If experimental samples are collected in RPMI or PBS-based medium, we recommend diluting the sample 1:4 in ELISA Buffer before performing the assay. If cell culture supernatant samples are to be analyzed undiluted, we recommend adding a 1:10 volume of 500 mM EDTA to the sample prior to analysis.

Sample Matrix Properties

Linearity

Dilution	Concentration (ng/ml)	Dilution Linearity (%)
Neat	7.66	87
1:2	8.34	95
1:4	8.43	96
1:8	8.80	100
1:16	10.60	121
1:32	13.31	152

Table 1. Dilutional Linearity of culture supernatants from A-23187-stimulated and S7 nuclease-treated human neutrophils in the CitH3 ELISA Kit

Spike and Recovery

Cell culture medium (RPMI-1640 with 10% fetal bovine serum) and normal human plasma were spiked with CitH3 (human core histones citrullinated *in vitro* by PAD4) and analyzed using the CitH3 ELISA Kit. The results are shown below. Error bars represent the standard deviation obtained from multiple dilutions of each sample.

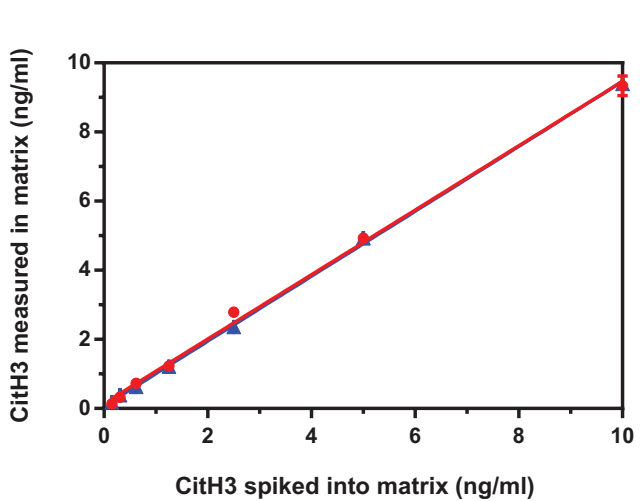


Figure 2. Spike and recovery in the CitH3 ELISA

Parallelism

To assess parallelism, culture supernatant and cell lysate from human neutrophils stimulated with A-23187 were checked at multiple dilutions and compared to the *in vitro*-citrullinated core histones kit standard and recombinant citrullinated histone H3 using the CitH3 ELISA. Absorbance (450 nm) values were plotted as a function of sample dilution. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow accurate determination of native CitH3 from biological samples from diverse sample matricies.

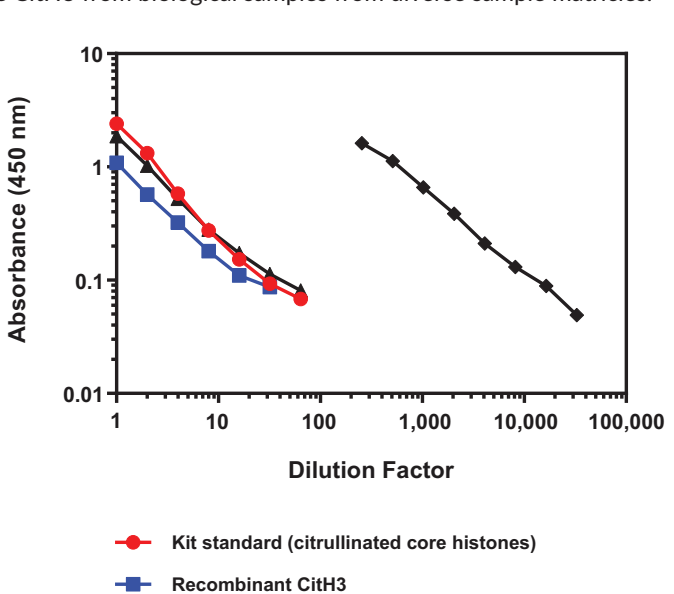


Figure 3. Parallelism of sample matrices in the CitH3 ELISA

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Citrullinated Histone H3 ELISA Standard

Reconstitute the lyophilized CitH3 ELISA Standard (Item No. 401444) with 2 ml of ELISA Buffer. Mix gently. The concentration of this solution (the bulk standard) is 500 ng/ml. The reconstituted standard is relatively unstable at 4°C and should be used within three hours of reconstitution. A second lyophilized standard has been supplied should the assay need to be repeated.

To prepare the standard for use in the ELISA: Obtain eight clean test tubes and label them, #1 through #8. Aliquot 4.9 ml of ELISA Buffer into tube #1. Aliquot 500 µl of ELISA Buffer into tubes #2-8. Transfer 0.1 ml of freshly prepared stock standard (500 ng/ml) to tube #1. Serially dilute the standard by removing 500 µl from tube #1 and placing into tube #2. Mix gently. Next, remove 500 µl from tube #2 and place into tube #3; mix gently. Repeat this process for tubes #4-7. Do not add any CitH3 ELISA Standard to tube #8. This tube is the zero-point vial, the lowest point on the standard curve.

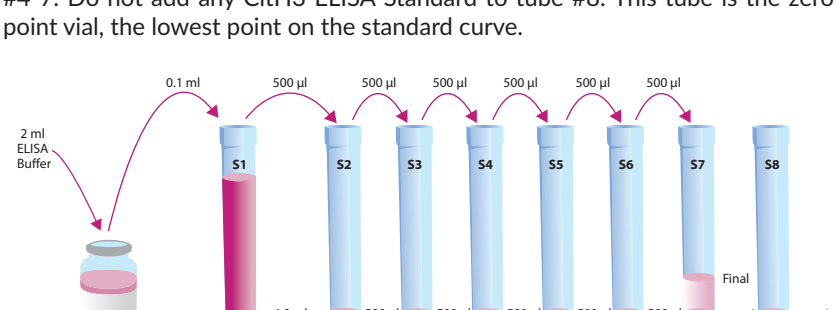


Figure 4. Preparation of the CitH3 standards

Anti-Citrullinated Histone H3 HRP Conjugate

This reagent is supplied as a concentrated (10X) stock solution of a rabbit anti-CitH3 polyclonal antibody conjugated to HRP. On the day of the assay, thaw the reagent (Item No. 401350) at room temperature.

For a full plate, dilute 1.2 ml of HRP Conjugate into 10.8 ml of 1X ELISA Buffer (Item No. 600040); for a half plate, dilute 0.6 ml of HRP Conjugate into 5.4 ml of 1X ELISA Buffer. Do not prepare diluted HRP Conjugate until immediately before use. Store unused Anti-CitH3 HRP Conjugate at 4°C.

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 of the strips at once, place the unused strips back in the plate packet and store according to the plate insert at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain an eight 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 a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 3, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 18, for more details). We suggest you record the contents of each well on the template sheet provided (see page 25).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	1	1	1	9	9	9	17	17	17	25
B	S2	S2	2	2	2	10	10	10	18	18	18	25
C	S3	S3	3	3	3	11	11	11	19	19	19	25
D	S4	S4	4	4	4	12	12	12	20	20	20	26
E	S5	S5	5	5	5	13	13	13	21	21	21	26
F	S6	S6	6	6	6	14	14	14	22	22	22	26
G	S7	S7	7	7	7	15	15	15	23	23	23	27
H	S8	S8	8	8	8	16	16	16	24	24	24	27

S1-S8 - Standards 1-8
1-27 - Samples

Figure 5. Sample plate format

Performing the Assay

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(s).

Addition of Standards and Samples and First Incubation

1. Add 100 µl of the standards or diluted sample to the appropriate wells on the plate. Each sample should be assayed in duplicate, triplicate recommended.
2. Cover the plate with 96-Well Cover Sheet (Item No. 400012). Incubate for two hours at room temperature on an orbital shaker.

Addition of HRP Conjugate and Second Incubation

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 100 µl of the HRP Conjugate working solution to each well of the plate.
3. Cover the plate with plastic film and incubate for one hour 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 100 µl of TMB Substrate Solution (Item No. 400074) to each well of the plate.
3. Cover the plate with plastic film and incubate for 30 minutes at room temperature in the dark on an orbital shaker.
4. DO NOT WASH THE PLATE. Add 100 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. *NOTE: The Stop Solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

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 of 450 nm.

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used.

Calculations

Plotting the Standard Curve and Determining the Sample Concentration

Using computer reduction software, plot absorbance (linear y-axis) *versus* concentration (linear x-axis) for standards (S1-S7) and fit the data with a four-parameter logistic equation, or alternatively a linear curve fit.

Performance Characteristics

Representative Data

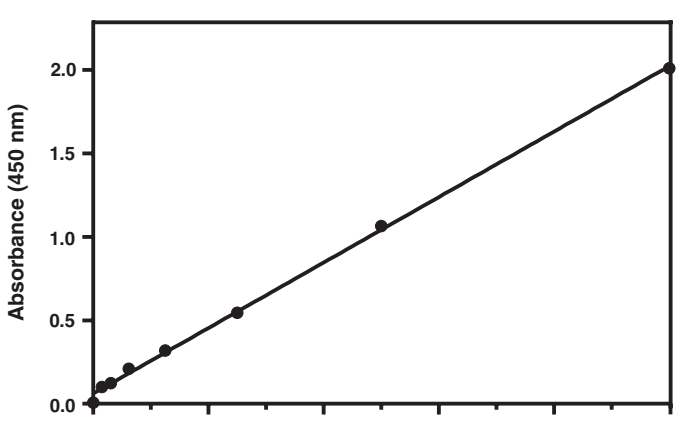
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples.

Absorbance (450 nm) at 30 min

CitH3 Standards (ng/ml)	Raw Absorbance	%CV Intra-Assay Precision	%CV Inter-Assay Precision
10	2.009	2.49	0.21
5	1.065	5.06	1.26
2.5	0.545	14.20	4.64
1.25	0.320	19.12	14.76
0.62	0.210	11.78	16.16
0.31	0.123	30.10*	13.12
0.15	0.101	21.84*	36.04
0	0.071	--	--

Table 2. Typical results

*Data in this range of the standard curve should be evaluated cautiously.



Assay Range = 0.15-10 ng/ml
Dynamic Range = 0.62-10 ng/ml
Sensitivity (defined as LLOQ) = 0.3 ng/ml
Lower Limit of Detection = 0.1 ng/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with ELISA Buffer.

Figure 6. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (RPMI media spiked with analyte) in a single assay.

Matrix Control (ng/ml)	%CV
6.1	6.1
0.9	6.8
0.5	20.2

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three matrix controls (RPMI media spiked with analyte) in separate assays spanning across several days.

Matrix Control (ng/ml)	%CV
6.5	7.4
1.1	15.7
0.5	24.7

Table 4. Inter-assay precision

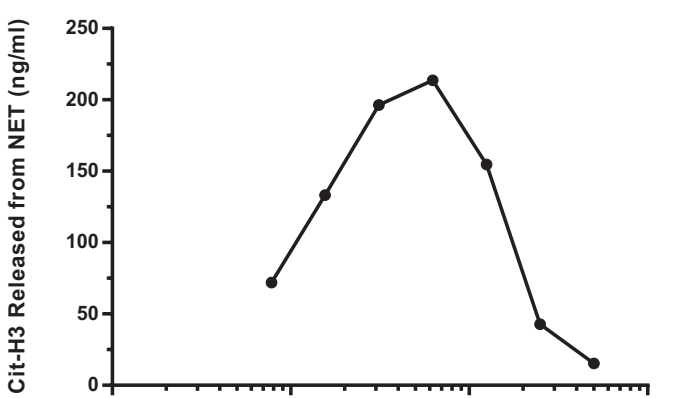


Figure 7. Measurement of CitH3 from primary human neutrophils stimulated to form extracellular traps. Human peripheral blood neutrophils were stimulated for three hours at 37°C with 25 µM A-23187 to induce NET formation. Various concentrations of micrococcal nuclease were added to the wells for an additional hour of incubation to release CitH3 from the trap. The supernatants were centrifuged to remove cellular debris and then tested in the ELISA. Increasing the concentration of nuclease to 50 U/ml increases the amount of available NET-free CitH3 in the supernatant. At concentrations of nuclease above 100 U/ml, the signal is reduced in a dose-dependent manner. This likely represents the disruption of the citrullinated multimeric core histone within the extracellular trap.

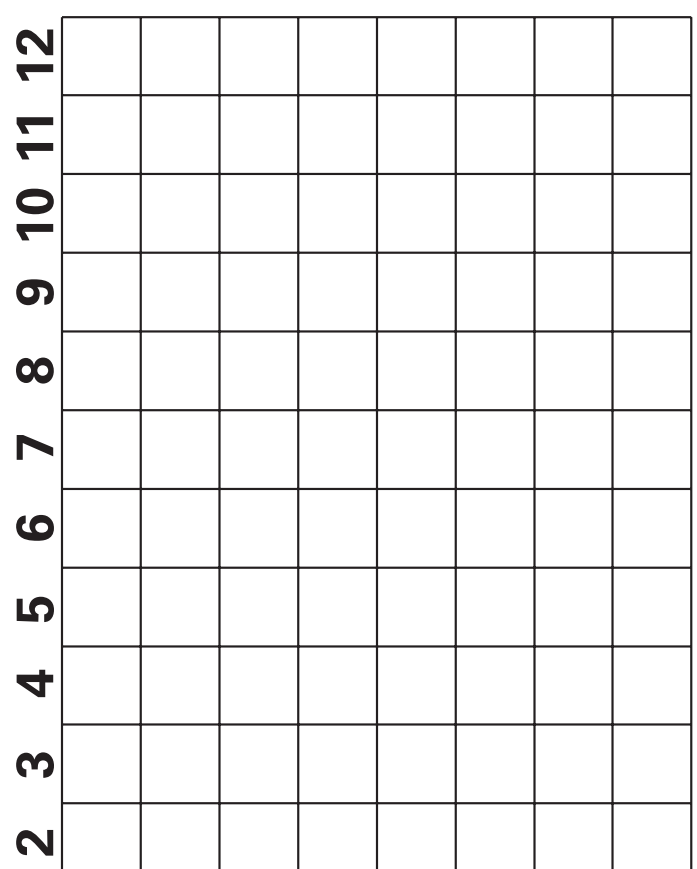
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Poor development of test sample	CitH3 is a part of a complex extracellular trap and unavailable for binding the capture antibody	Treat the sample with DNAse to disrupt the extracellular trap, thereby releasing the free CitH3
Poor development (low signal) of standard curve	A. Plate required more development time B. Standard was diluted incorrectly C. The standard is degraded	

Procedure	Standards/Samples (µl)
Mix all reagents gently	--
Add standards/samples to plate	100
Seal the plate and tap gently to mix	
Incubate plate for 2 hours at RT, shaking	
Aspirate wells and wash 4 x well volume (~400 µl) with Wash Buffer (1X)	
Apply 1X HRP-Conjugate Solution	100
Incubate for 1 hour at RT, shaking	
Aspirate wells and wash 4 x well volume (~400 µl) with Wash Buffer (1X)	
Apply TMB Substrate Solution	100
Incubate 30 min at RT, shaking, sealed, and *protected from light*	
Do Not Wash, apply HRP Stop Solution	100
Read absorbance at 450 nm	

Table 6. CitH3 Assay Summary



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NOTES

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