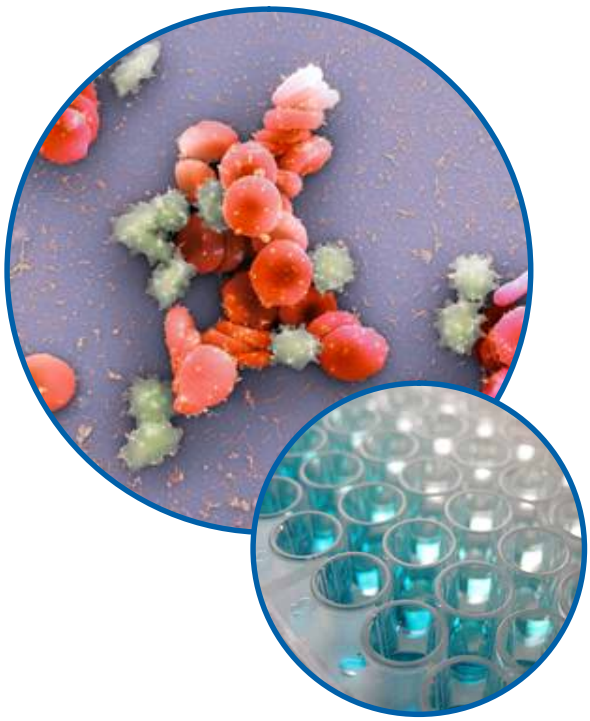

AdipoGen™

MANUAL

Nampt (Visfatin/PBEF) (mouse/rat) (IntraCellular) Dual ELISA Kit

For research use only. Not for diagnostic use.

Version 2 (14-March-2011)

Cat. No. AG-45A-0009EK-KI01

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1. Intended Use

The Nampt (Visfatin/PBEF) (mouse/rat) (IntraCellular) Dual ELISA Kit is to be used for the *in vitro* quantitative determination of mouse or rat Nampt in cell lysates or cell-based assays (screening). This ELISA Kit is for research use only.

2. Introduction

Nampt, nicotinamide phosphoribosyl-transferase, is the rate-limiting enzyme of the mammalian NAD, nicotinamide adenine dinucleotide, biosynthesis pathway from nicotinamide to NMN, nicotinamide mononucleotide (1), which is further converted to NAD by nicotinamide/nicotinic acid mononucleotide adenyltransferase abbreviated Nmnat. Nampt was originally identified in Haemophilus ducreyi (2), and was then found to have a significant homology to the mammalian pre-B cell colony-enhancing factor (PBEF) (3) or visfatin (4), which is also called extracellular Nampt (eNampt) (5) due to the fact that it is found as a circulating form in human serum and secreted from differentiated adipocytes. Although visfatin was originally reported as an insulin-mimicking hormone by its capability of binding to and activating the insulin receptor, subsequent studies do not support this observation (5, 6). However, Revollo et al. observed that haplodeficiency and chemical inhibition of Nampt caused defects in NAD biosynthesis and glucose-stimulated insulin secretion in pancreatic islets in vivo and in vitro and plasma visfatin and NMN levels were reduced in Nampt heterozygous females (5). This study proposed a model that NMN exists in high amounts in plasma, presumably derived from nicotinamide with help of eNampt. This circulating NMN and nicotinamide are uptaken by beta cells via unknown transport mechanism (s) are converted to NAD by Nmnat and intracellular Nampt (hereinafter abbreviated iNampt), respectively, concluding that Nampt-mediated systemic NAD biosynthesis is critical for insulin secretion presumably via a NAD-dependent histonedeacetylase, Sirt1. Since it has been shown that NAD(+) levels in mitochondria remain at physiological levels following genotoxic stress and can maintain cell viability even when nuclear and cytoplasmic pools of NAD(+) are depleted (7), the NAD(+) biosynthetic enzyme Nampt plays a critical role in enhancing life span and protecting against oxidative cell damage. Therefore, measurement of iNampt located at different cell compartments like cytoplasm, mitochondria, and nucleus may be able to give us some biological clue on the intracellular functions of Nampt.

3. General References

(1) The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells: J.R. Revollo, et al.; J. Biol. Chem. 279, 50754 (2004)

(2) Identification of a plasmid-encoded gene from Haemophilus ducreyi which confers NAD independence: P.R. Martin, et al.; J. Bacteriol. 183, 1168 (2001)

(3) Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor: B. Samal, et al.; Mol. Cell. Biol. 14, 1431 (1994)

(4) Visfatin: a protein secreted by visceral fat that mimics the effects of insulin: A. Fukuhara, et al.; Science 307, 426 (2005)

(5) Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme: J.R. Revollo, et al.; Cell. Metab. 6, 363 (2007)

(6) Molecular characteristics of serum visfatin and differential detection by immunoassays: A. Körner, et al.; J. Clin. Endocrinol. Metab. 92, 4783 (2007)

(7) Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival: H. Yang, et al.; Cell 130, 1095 (2007)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of mouse or rat Nampt in cells. A monoclonal antibody specific for Nampt has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, Nampt is recognized by the addition of a purified polyclonal antibody specific for Nampt (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-IgG (Detector) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of Nampt in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use.
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

1 plate coated with Nampt Antibody	(12 x 8-well strips)
1 bottle Wash Buffer 10X	(50 ml)
1 bottle Diluent 5X	(50 ml)
1 bottle Lysis Buffer 10X	(12 ml)
1 bottle Detection Antibody	(12 ml)
1 vial Detector 100X (HRP Conjugated anti-IgG)	(150 µl)
1 vial mouse Nampt Standard (lyophilized)	(64 ng)
1 vial mouse Nampt QC sample (lyophilized)	
1 bottle TMB Substrate Solution	(12 ml)
1 bottle Stop Solution	(12 ml)
3 plate sealers (plastic film)	

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Calibrated precision single and multi-channel pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard
- Phenyl methylsulfonyl fluoride (PMSF)

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 50 ml Wash Buffer 10X + 450 ml water) to obtain Wash Buffer 1X.
- **Diluent 5X** has to be diluted with deionized water 1:5 before use (e.g. 50 ml Diluent 5X + 200 ml water) to obtain Diluent 1X.
- **Lysis Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 12 ml Lysis Buffer 10X + 108 ml water) to obtain Lysis Buffer 1X. Add 1 mM PMSF immediately before use.
- **Detector 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 120 µl in 12 ml of Diluent 1X (1:100).

  NOTE: *The diluted Detector is used within one hour of preparation.*

- **Mouse Nampt Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 64 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C*

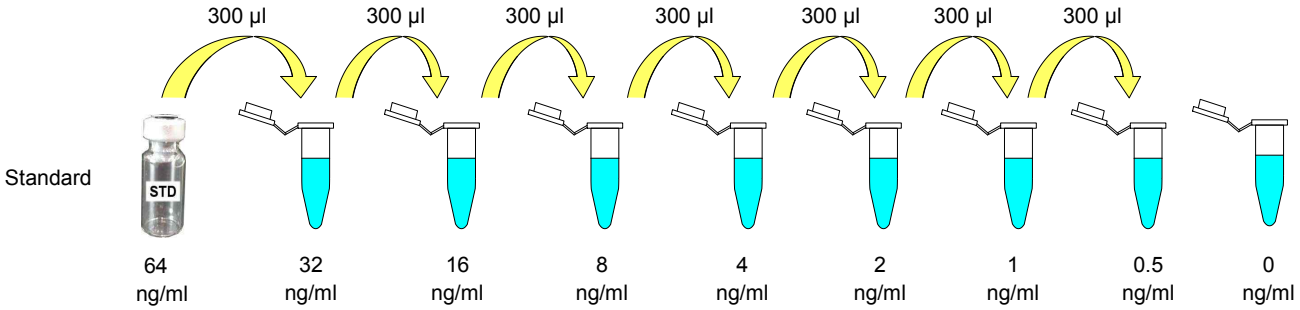
  - Dilute the standard protein concentrate (STD) (**64 ng/ml**) in Diluent 1X. A nine-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
  - Suggested standard points are:
    32 , 16 , 8 , 4 , 2 , 1 , 0.5 and 0 ng/ml.

- **Mouse Nampt QC sample** has to be reconstituted with 1 ml of deionized water.

  - Refer to the Certificate of Analysis for current QC sample concentration. Mix the QC sample to ensure complete reconstitution and allow the QC sample to sit for a minimum of 15 minutes. The reconstituted QC sample is ready to use, do not dilute it.

Dilute further for the standard curve:

To obtain	Add	Into
32 ng/ml	300 µl of Nampt (64 ng/ml)	300 µl of Diluent 1X
16 ng/ml	300 µl of Nampt (32 ng/ml)	300 µl of Diluent 1X
8 ng/ml	300 µl of Nampt (16 ng/ml)	300 µl of Diluent 1X
4 ng/ml	300 µl of Nampt (8 ng/ml)	300 µl of Diluent 1X
2 ng/ml	300 µl of Nampt (4 ng/ml)	300 µl of Diluent 1X
1 ng/ml	300 µl of Nampt (2 ng/ml)	300 µl of Diluent 1X
0.5 ng/ml	300 µl of Nampt (1 ng/ml)	300 µl of Diluent 1X
0 ng/ml	300 µl of Diluent 1X	Empty tube



8.2. Sample Collection, Storage and Dilution

Cell Lysates : Grow cell until 90% confluency. Scrap cells off the plate and transfer to an appropriate tube. Keep on ice and microcentrifuge at 1,200 rpm for 5 minutes at 4°C. Remove supernatant, rinse cells once with ice-cold PBS. Remove PBS and add 200 µl ice-cold 1x lysis buffer supplemented with 1 mM phenyl methylsulfonyl fluoride (PMSF) to ten million cells of interest and incubate on ice for 30 minutes. Microcentrifuge at 12,000 rpm for 5 minutes at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Use freshly prepared cell lysate samples.

Cell Lysates have to be diluted in Diluent 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/10 to 1/1,000 dilutions of cell lysates are recommended! If samples fall the outside range of assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 8-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 8-well strips coated with Nampt antibody when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted cell lysates samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples).**
☐	3. Cover the plate with plate sealer and incubate for **overnight at 4°C.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the Detection Antibody.
☐	6. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted Detector **(see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB Substrate Solution.
☐	12. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 10 minutes**.
☐	13. Stop the reaction by adding 100 µl of Stop Solution. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
	! CAUTION: CORROSIVE SOLUTION!
☐	14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

9. Calculation of Results

- Average the duplicate readings for each standard, QC and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding Nampt concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the Nampt concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of mouse or rat Nampt in the samples.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

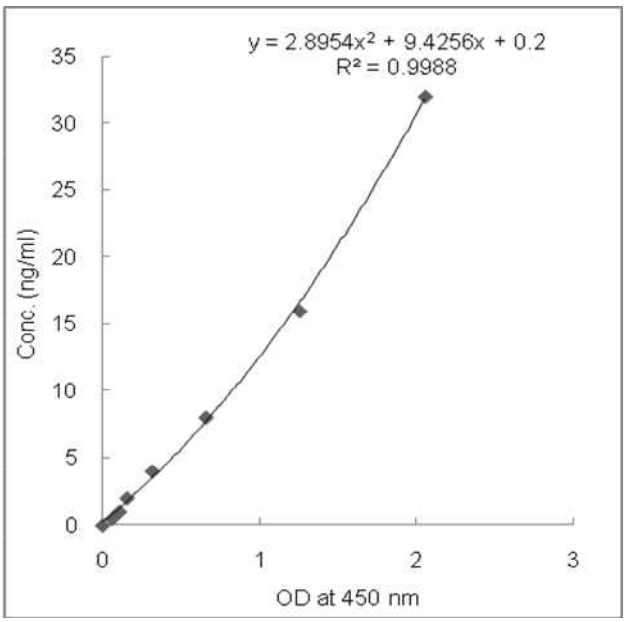


Figure: Standard curve

Standard mNampt (ng/ml)	Optical Density (mean)
32	2.0563
16	1.2556
8	0.6584
4	0.3157
2	0.1551
1	0.1060
0.5	0.0612
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of Nampt that can be detected by this assay is 50 pg/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.5 ng/ml – 32 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant mouse and rat Nampt. It does not cross-react with human Nampt, human adiponectin, mouse adiponectin, mouse resistin, mouse vaspin, mouse RBP4, mouse GPX3, mouse progranulin, mouse IL-33, mouse clusterin, mouse ANGPTL3, mouse ANGPTL4, mouse ANGPTL6, mouse leptin, mouse TNF-α.

D. Intra-assay precision:

Two samples of known concentrations of mouse and rat Nampt were assayed in replicates 10 times to test precision within an assay.

Samples	Means (µg/ml)	SD	CV (%)	n
Mouse 1	1.04	0.04	3.50	10
Rat 1	5.89	0.13	2.25	10

E. Inter-assay precision:

Two samples of known concentrations of mouse and rat Nampt were assayed in 3 separate assays to test precision between assays.

Samples	Means (µg/ml)	SD	CV (%)	n
Mouse 1	0.99	0.07	6.80	3
Rat 1	3.72	0.29	5.12	3

NOTE: *The mouse cell lysates sample was prepared from mouse 3T3L1 cells (1:200 dilutions). The rat cell lysates sample was prepared from rat hepatoma cells (1:400 dilutions).*

F. Recovery:

When samples (cell lysates) are spiked with known concentrations of mouse and rat Nampt, the recovery averages 102% (range from 95% to 105%).

Samples	Average recovery (%)	Range (%)
Mouse 1	100.81	95-105
Rat 1	104.25	95-105

G. Linearity:

Different mouse and rat cell lysates samples containing Nampt were diluted several fold (1/200 to 1/1,600) and the measured recoveries ranged from 95% to 110%.

Samples	Sample Dilution	Expected (µg/ml)	Observed (µg/ml)	% of Expected
Mouse 1	1 : 200	1.50	1.50	100
	1 : 400	0.75	0.78	104.05
	1 : 800	0.37	0.41	109.54
Rat 1	1 : 400	0.61	0.61	100
	1 : 800	0.31	0.32	103.23
	1 : 1,600	0.15	0.17	108.72

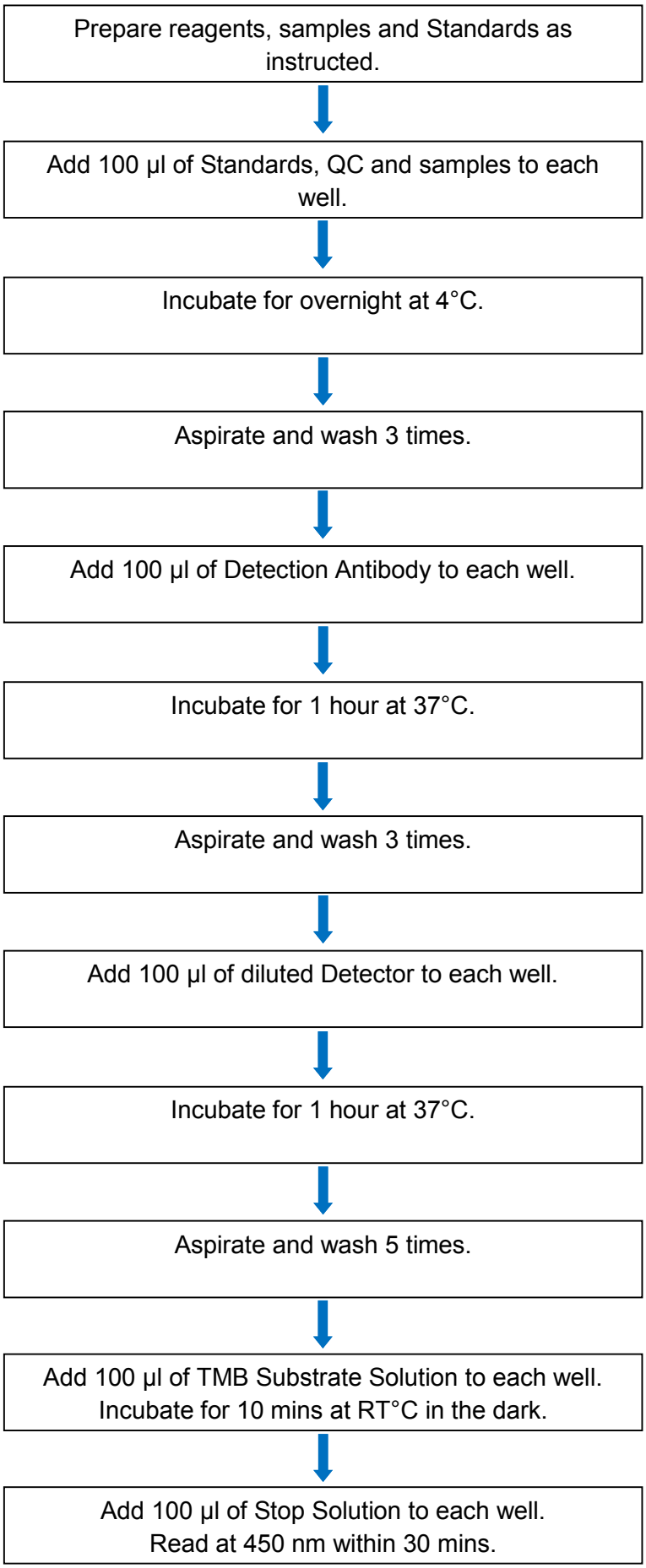
12. Technical Hints and Limitations

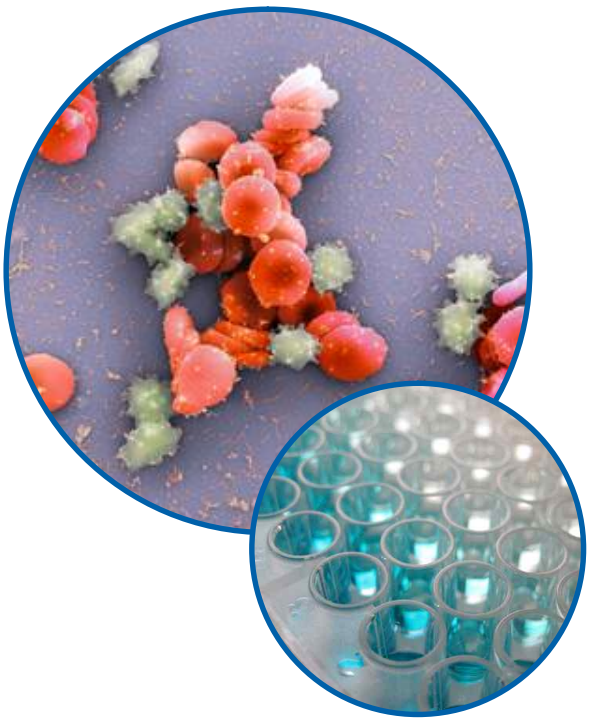
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 8-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution protected from light.
- The Stop Solution consists of phosphoric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of detector too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Assay Flow Chart





Product Specific References:

For more References please visit www.adipogen.com!

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