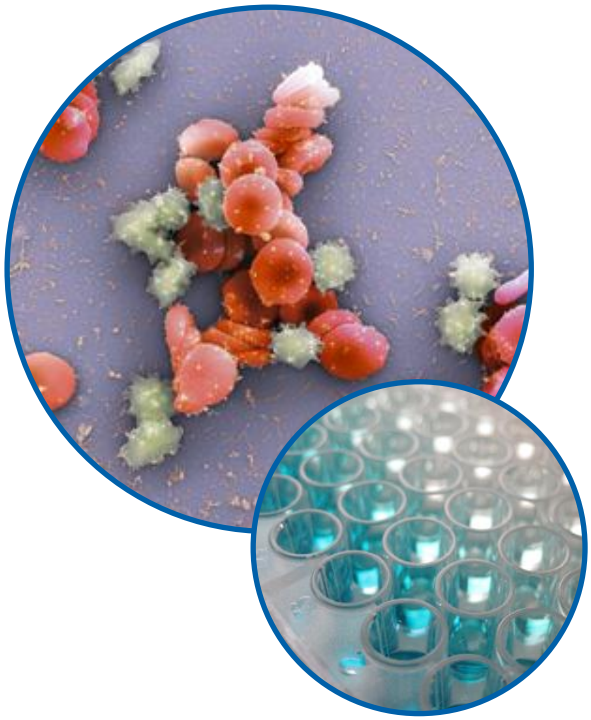


AdipoGen™

MANUAL

CTRP3 (human) Competitive ELISA Kit

For research use only. Not for diagnostic use.

Version 1 (28-July-2011)

Cat. No. AG-45A-0042EK-KI01

Table of Contents

1. Intended Use

The CTRP3 (human) Competitive ELISA Kit is to be used for the *in vitro* quantitative determination of human CTRP3 in serum, plasma and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

C1q- and tumor necrosis factor-related protein, CTRP or C1QTNF, is a rapidly-expanding cytokine family. There are 13 members of the CTRP family that have been identified. Each CTRP is comprised of the NH2 terminal half representing a proline-rich collagen domain preceded by a signal sequence and a hypervariable region, which can accelerate multimeric forms, and the COOH terminal half globular domain resembling TNF-α and RANKL in terms of 3D structure. Their overall structure is very similar to adiponectin. Therefore, CTRPs are the adiponectin paralogs (1). One interesting feature of the CTRP family is that their amino acids are quite conserved between species, implying their biological importance. CTRP3 or C1QTNF3 encodes a deduced 246-amino acid protein with a calculated molecular mass of 26 kDa. RT-PCR analysis detected expression in cDNAs from human osteosarcoma, chondroblastoma, and giant cell tumor, as well as in normal fibroblasts, white adipose tissue, and placenta. Circulating CTRP3 was observed in both mouse and human serum or plasma (2). CTRP3 was also known as Cors26 whose primary expression is in cartilage and overexpression of CTRP3 increased the growth of murine mesenchymal stem cells, suggesting that it plays a role in skeletal development (3). Circulating CTRP3 levels correlated inversely with circulating leptin levels in mice. CTRP3 expression decreased with diet-induced obesity in wild type mice with high leptin levels and increased with fasting in wild type mice and in obese leptin-deficient and insulin-resistant ob/ob mice. Administration of sufficient recombinant CTRP3 to increase its plasma levels 3-fold activated Akt signaling in liver, directly and independently of insulin suppressed hepatic glucose output via inhibition of gluconeogenesis, and lowered blood glucose levels in both normal and ob/ob mice (4). Visceral adipocytes or monocytes secrete CTRP3 that antagonizes lipopolysaccharide (LPS) via a physical interaction with the TLR4/MD-2 receptor complex (5). Due to these metabolically beneficial or anti-inflammatory feature(s) associated with CTRP3 the measurement of CTRP3 becomes intriguing.

3. General References

(1) Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-gamma agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions: G.W. Wong, et al.; Biochem. J. 416, 161 (2008)

(2) Genomic organization, promoter, amino acid sequence, chromosomal localization, and expression of the human gene for CORS-26 (collagenous repeat-containing sequence of 26-kDa protein): A. Schaffler, et al.; Biochem. Biophys. Acta. 1630, 123 (2003)

(3) Molecular cloning and characterization of a novel gene, CORS26, encoding a putative secretory protein and its possible involvement in skeletal development: T. Maeda, et al.; J. Biol. Chem. 276, 3628 (2001)

(4) C1q/TNF-related protein-3 (CTRP3), a novel adipokine that regulates hepatic glucose output: J.M. Peterson, et al.; J. Biol. Chem. 285, 39691 (2010)

(5) C1q/TNF-related protein-3 represents a novel and endogenous lipopolysaccharide antagonist of the adipose tissue: A. Kopp, et al.; Endocrinology 151, 5267 (2010)

4. Assay Principle

This assay is a competitive Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human CTRP3 in biological fluids. A polyclonal antibody recognizing native human CTRP3 reacts with a series of predetermined recombinant human CTRP3 standard proteins or samples under competition in the human CTRP3-coated plate. Their relative reactivity is plotted with that of the standard proteins.

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 human CTRP3 Recombinant Protein	(12 x 8-well strips)
1 bottle Wash Buffer 10X	(50 ml)
1 bottle Diluent 5X	(50 ml)
1 bottle Detection Antibody	(12 ml)
1 vial Detector 100X (HRP Conjugated anti-IgG)	(150 µl)
1 vial human CTRP3 Standard (lyophilized)	(1 µg)
1 vial human CTRP3 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

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.
- **Detector 100X (HRP Conjugated anti-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.*

- **Human CTRP3 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 1 µg/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) (**1 µg/ml**) in Diluent 1X. A seven-point standard curve in Diluent 1X is recommended.
  - Suggested standard points are:

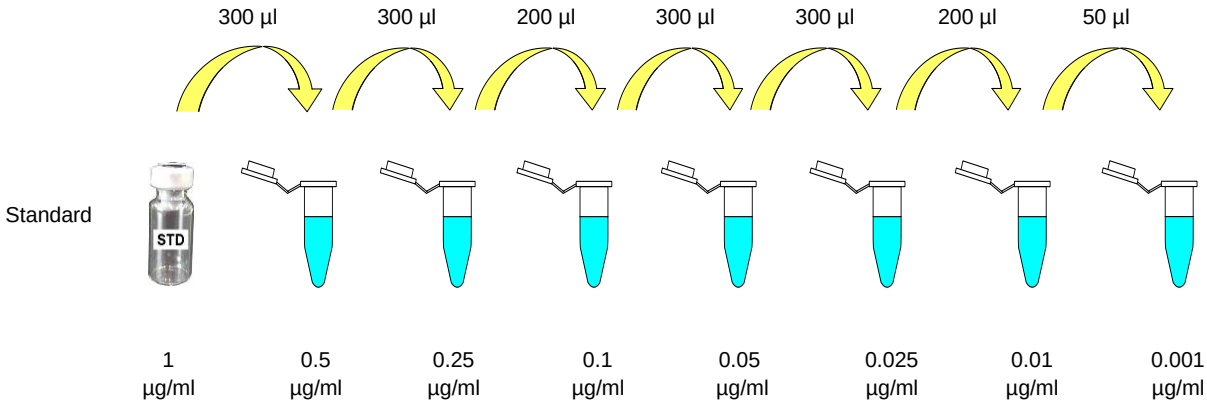
    1 , 0.5 , 0.25 , 0.1 , 0.05 , 0.025 , 0.01 and 0.001 µg/ml.

- **Human CTRP3 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
1 µg/ml	-	-
0.5 µg/ml	300 µl of CTRP3 (1 µg/ml)	300 µl of Diluent 1X
0.25 µg/ml	300 µl of CTRP3 (0.5 µg/ml)	300 µl of Diluent 1X
0.1 µg/ml	200 µl of CTRP3 (0.25 µg/ml)	300 µl of Diluent 1X
0.05 µg/ml	300 µl of CTRP3 (0.1 µg/ml)	300 µl of Diluent 1X
0.025 µg/ml	300 µl of CTRP3 (0.05 µg/ml)	300 µl of Diluent 1X
0.01 µg/ml	200 µl of CTRP3 (0.025 µg/ml)	300 µl of Diluent 1X
0.001 µg/ml	50 µl of CTRP3 (0.01 µg/ml)	450 µl of Diluent 1X



8.2. Sample Collection, Storage and Dilution

Serum : Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Plasma : Collect plasma using heparin, EDTA, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -20°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma or **Cell Culture Supernatant** have to be diluted in Diluent 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/4 dilution of serum or plasma is recommended! If samples fall the outside range of assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

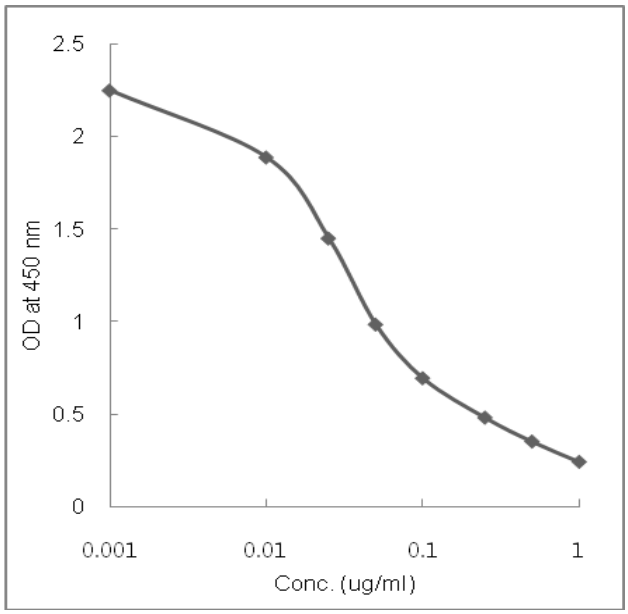
☐	1. Determine the number of 8-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 8-well strips coated with CTRP3 protein when opened can be stored at 4°C for up to 1 month*.
☐	2. Add 50 µl of the different standards and reconstituted QC sample into the appropriate wells in duplicate! At the same time, add 50 µl of diluted serum, plasma or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples).**
☐	3. Add 50 µl to each well of the Detection Antibody and tap gently on the side of the plate to mix.
☐	4. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	5. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multi-channel 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.
☐	6. Add 100 µl to each well of the diluted Detector **(see 8.1. Preparation and Storage of Reagents)**.
☐	7. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	8. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multi-channel 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.
☐	9. Add 100 µl to each well of TMB Substrate Solution.
☐	10. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 20 minutes**.
☐	11. 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!
☐	12. 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.
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs the corresponding CTRP3 concentration (µg/ml) on the horizontal (X) axis (see **10.** TYPICAL DATA).
- Calculate the CTRP3 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a 4-parameter logistic equation.
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of human CTRP3 in the samples.

10. Typical Data

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



Standard hCTRP3 (µg/ml)	Optical Density (mean)
1	0.2426
0.5	0.3522
0.25	0.4811
0.1	0.6939
0.05	0.9835
0.025	1.4482
0.01	1.8852
0.001	2.2461

Figure: Standard curve

11. Performance Characteristics

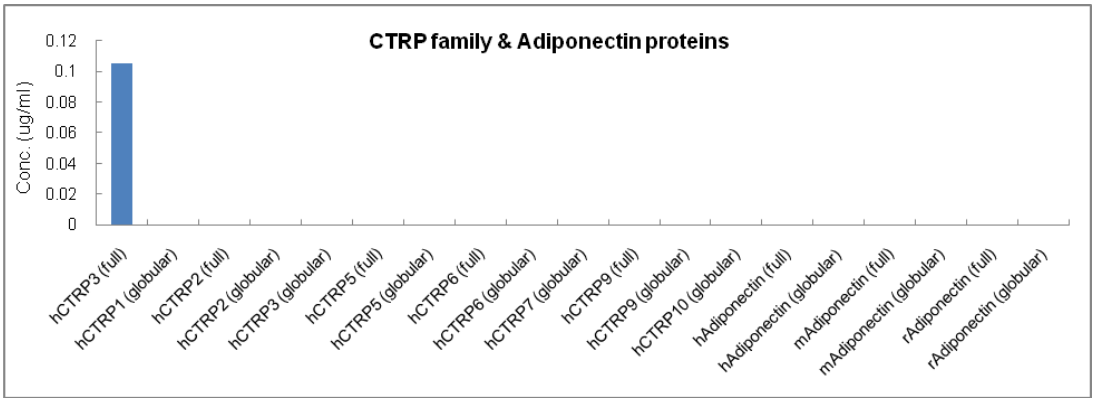
A. Sensitivity (Limit of detection):

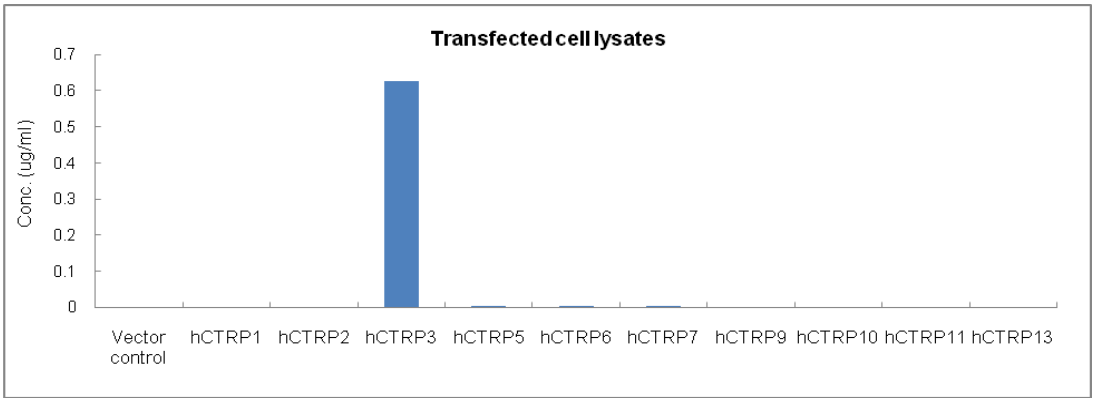
The lowest level of CTRP3 that can be detected by this assay is 1 ng/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.001 µg/ml – 1 µg/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human CTRP3.





D. Intra-assay precision:

Four samples of known concentrations of human CTRP3 were assayed in replicates 8 times to test precision within an assay.

Samples	Means (µg/ml)	SD	CV (%)	n
1	0.275	0.017	6.16	8
2	0.289	0.020	6.93	8
3	0.341	0.028	8.15	8
4	0.298	0.025	8.35	8

E. Inter-assay precision:

Four samples of known concentrations of human CTRP3 were assayed in 4 separate assays to test precision between assays.

Samples	Means (µg/ml)	SD	CV (%)	n
1	0.247	0.006	2.43	4
2	0.349	0.017	5.01	4
3	0.285	0.024	8.25	4
4	0.282	0.021	7.57	4

F. Recovery:

When samples (serum) are spiked with known concentrations of human CTRP3, the recovery averages 104% (range from 85% to 115%).

Samples	Average recovery (%)	Range (%)
1	102.60	85-110
2	110.05	85-115
3	100.42	85-110
4	102.09	85-110

G. Linearity:

Different human serum samples containing CTRP3 were diluted several fold (1 to 1/4) and the measured recoveries ranged from 95% to 107%.

Samples	Sample Dilution	Expected (ug/ml)	Observed (ug/ml)	% of Expected
1	1	0.300	0.300	100
	1 : 2	0.150	0.156	104.00
	1 : 4	0.080	0.079	105.33
2	1	0.362	0.362	100
	1 : 2	0.181	0.179	98.90
	1 : 4	0.090	0.089	98.34
3	1	0.300	0.300	100
	1 : 2	0.150	0.146	97.33
	1 : 4	0.080	0.072	96.00

H. Expected values:

CTRP3 levels range in plasma and serum from **0.1 to 1 ug/ml**.

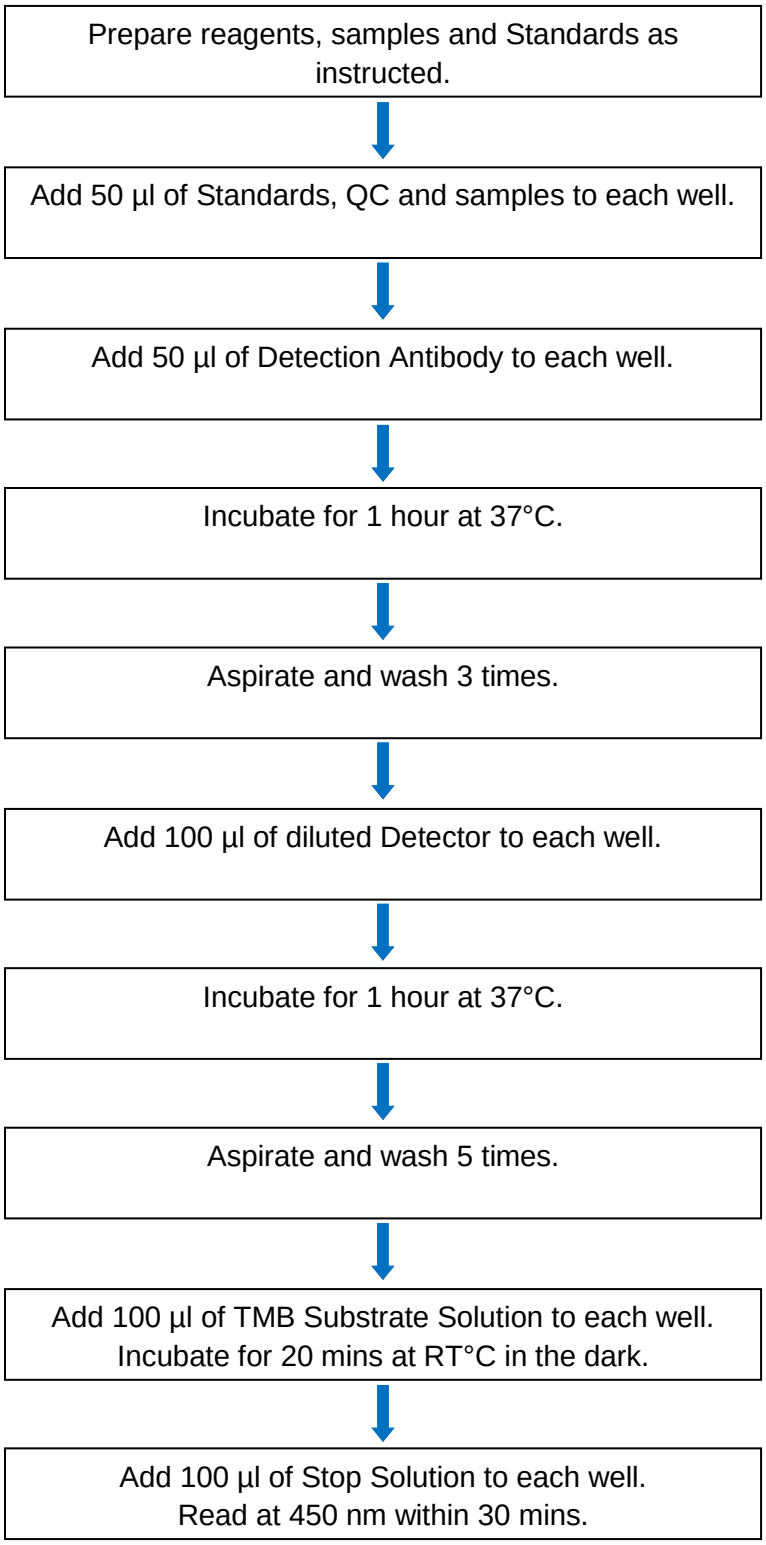
12. Technical Hints and Limitations

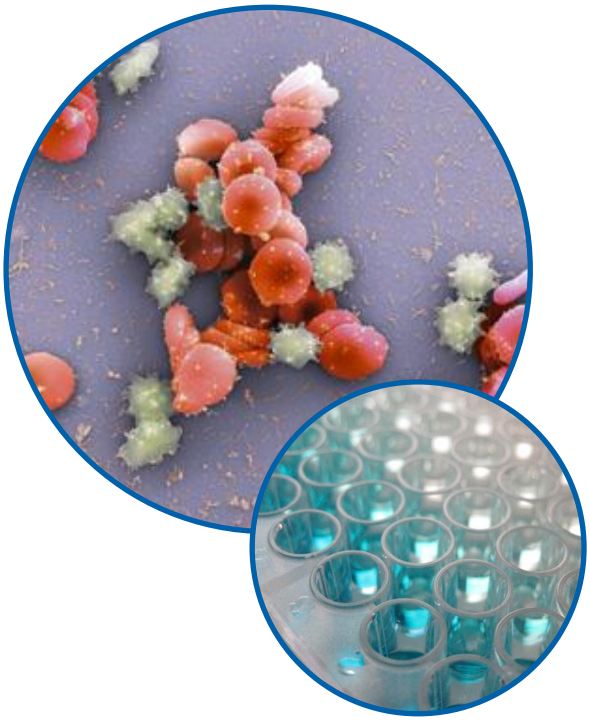
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last 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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