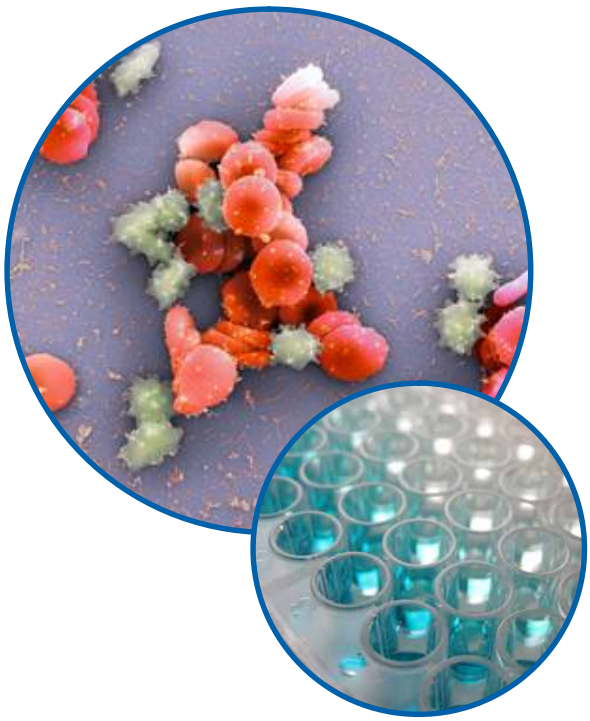


AdipoGen™

MANUAL

IL-33 (mouse) ELISA Kit

For research use only. Not for diagnostic use

Version 2 (18-March-2011)

Cat. No. AG-45A-0038EK-KI01

Table of Contents

1. Intended Use

The IL-33 (mouse) ELISA Kit is to be used for the *in vitro* quantitative determination of mouse IL-33 in serum, plasma and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

IL-33 is a multifunctional proinflammatory cytokine. By searching databases for IL-1β-related proteins, two cDNAs encoding mouse and human IL-33 were isolated (1). The predicted 270-amino acid human protein has a calculated molecular mass of 30 kD. It was converted by activated caspase 1 *in vitro* to a mature 18-kD protein with a predicted cleavage site after residue 111, which is analogous to the cleavage of pro-IL-1β or IL-18. IL-33 was also discovered as a nuclear factor for the N-terminus contains a bipartite nuclear localization signal and a homeodomain-like helix-turn-helix domain with homology to Drosophila 'engrailed' and POU transcription factors (2). This protein compartmentalization and inflammatory feature resemble HMGB1 (3). TNF-α- and IL-1β-induced expression of IL-33 was detected in primary lung, dermal, or synovial fibroblasts and keratinocytes with low levels in dendritic cells and macrophages (1, 4). The IL-33 receptor complex is comprised of ST2 (IL1RL1) and IL1R accessory protein (IL-18Rap). Engagement of the receptor complex with IL-33 recruited Myd88, IRAK, IRAK4, and TRAF6, followed by phosphorylation of ERK1/ERK2, p38, and JNK. Addition of IL-33 to polarized mouse Th2 lymphocytes expressing ST2 resulted in increased production of Il5 and Il13, thereby stimulating allergy reaction (1) whereas IL-33 reduced the level of IFN-γ produced by polarized mouse Th1 cells. It has been demonstrated that synovial membranes from rheumatoid arthritis (RA) patients expressed both IL-33 and ST2 in the lining layer and the interstitial sublining layers (5). Two murine RA models showed that interaction between IL-33 and ST2 plays a key role in initiation and/or worsening the disease severity via mast cells expressing ST2 (4, 6). While none of healthy individuals showed detectable IL-33 levels in serum the patients group undergoing atopic anaphylaxis exhibited a remarkable IL-33 levels (7). IL-33 and ST2 are expressed in cardiomyocytes. It has been shown that their interaction blunts the apoptosis of cardiomyocytes (8). These multiple cellular functions of IL-33 may provide some novel therapeutic interventions for several inflammatory diseases.

3. General References

(1) IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines: J. Schmitz, et al.; Immunity 23, 479 (2005)

(2) Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules: E.S. Baekkevold, et al.; Am. J. Path. 163, 69 (2003)

(3) The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'?: C. Moussion, et al.; PLoS ONE 3, e3331 (2008)

(4) IL-33 exacerbates antigen-induced arthritis by activating mast cells: D. Xu, et al.; Proc. Nat. Acad. Sci. 105, 10913 (2008)

(5) IL-33 mediates antigen-induced cutaneous and articular hypernociception in mice: W.A. Verri, Jr., et al.; Proc. Nat. Acad. Sci. 105, 2723 (2008)

(6) IL-33 exacerbates autoantibody-induced arthritis: D. Xu, et al.; J. Immunol. 184, 2620 (2010)

(7) The cytokine interleukin-33 mediates anaphylactic shock: P.N. Pushparaj, et al.; Proc. Nat. Acad. Sci. 106, 9773 (2009)

(8) IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system: S. Sanada, et al.; J. Clin. Invest. 117, 1538 (2007)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of mouse IL-33 in biological fluids. A monoclonal antibody specific for IL-33 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, IL-33 is recognized by the addition of a purified polyclonal antibody specific for IL-33 (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-rabbit 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 IL-33 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 mouse IL-33 Antibody	(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-rabbit IgG)	(150 µl)
1 vial mouse IL-33 Standard (lyophilized)	(2 ng)
1 vial mouse IL-33 QC sample (lyophilized)	
1 bottle Substrate Solution I (TMB)	(6 ml)
1 bottle Substrate Solution II (Peroxidase)	(6 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-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.*

- **Substrate Solution I and II** have to be mixed together in equal volumes within 15 minutes of use.

  NOTE: *Freshly prepare just before use the Substrate Solution and protect from light!*

- **Mouse IL-33 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 2 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) (**2 ng/ml**) in Diluent 1X. A seven-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
  - Suggested standard points are:

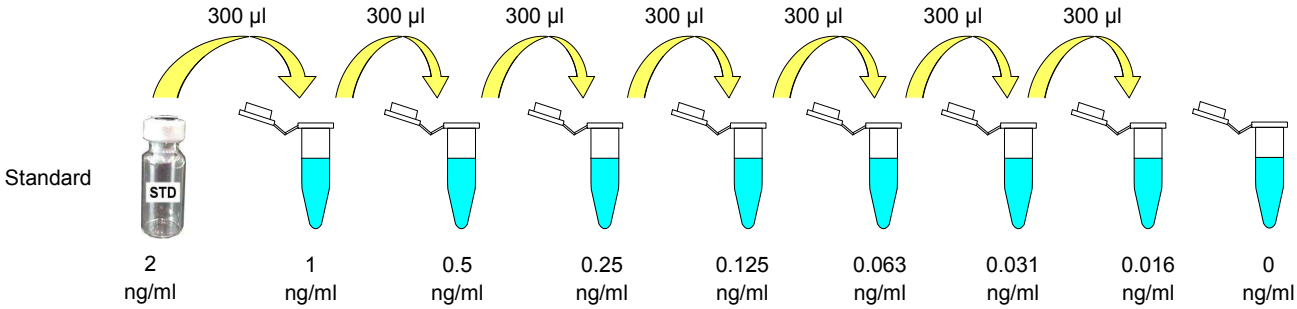
    1 , 0.5 , 0.25 , 0.125 , 0.063 , 0.031 , 0.016 and 0 ng/ml.

- **Mouse IL-33 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 ng/ml	300 µl of IL-33 (2 ng/ml)	300 µl of Diluent 1X
0.5 ng/ml	300 µl of IL-33 (1 ng/ml)	300 µl of Diluent 1X
0.25 ng/ml	300 µl of IL-33 (0.5 ng/ml)	300 µl of Diluent 1X
0.125 ng/ml	300 µl of IL-33 (0.25 ng/ml)	300 µl of Diluent 1X
0.063 ng/ml	300 µl of IL-33 (0.125 ng/ml)	300 µl of Diluent 1X
0.031 ng/ml	300 µl of IL-33 (0.063 ng/ml)	300 µl of Diluent 1X
0.016 ng/ml	300 µl of IL-33 (0.031 ng/ml)	300 µl of Diluent 1X
0 ng/ml	300 µl of Diluent 1X	Empty tube



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, 1/5 dilution of serum or plasma is recommended!*

8.3. Assay Procedure (Checklist)

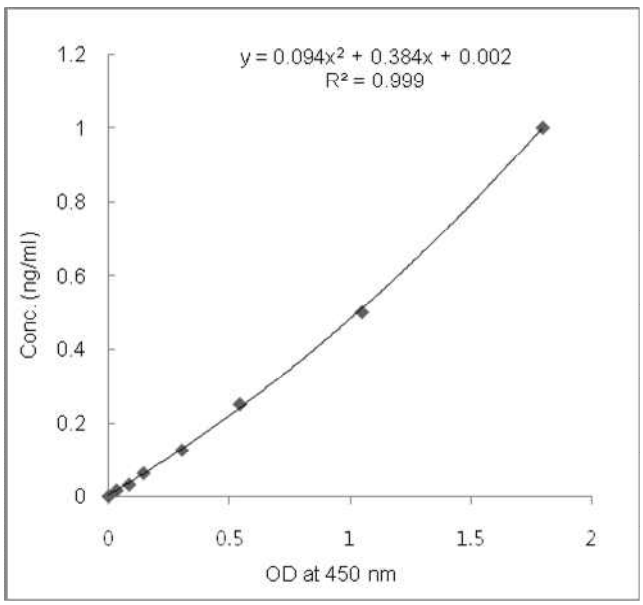
☐	1. Determine the number of 8-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 8-well strips coated with IL-33 antibody when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted 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. 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 mixed substrate solution.
☐	12. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 20 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 IL-33 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the IL-33 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 IL-33 in the samples.

10. Typical Data

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



Standard mIL-33 (ng/ml)	Optical Density (mean)
1	1.796
0.5	1.048
0.25	0.543
0.125	0.303
0.063	0.144
0.031	0.085
0.016	0.033
0	0

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of IL-33 that can be detected by this assay is 10 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: 0.016 ng/ml – 1 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant mouse IL-33. It does not cross-react with human IL-33, human IL-6, human IL-24, human IL-23, human IL-17A, human ST2, mouse adiponectin, rat adiponectin, mouse RBP4, mouse resistin, mouse vaspin, mouse Nampt, mouse clusterin.

D. Intra-assay precision:

One sample from collagen induced arthritis (CIA) mouse of known concentration of mouse IL-33 was assayed in replicates 2 times to test precision within an assay.

Sample	Means (ng/ml)	SD	CV (%)	n
1	0.194	0.01	4.18	2

E. Inter-assay precision:

One sample from CIA mouse of known concentration of mouse IL-33 was assayed in 3 separate assays to test precision between assays.

Sample	Means (ng/ml)	SD	CV (%)	n
1	0.181	0.02	9.56	3

F. Recovery:

When samples (serum or plasma) are spiked with known concentrations of mouse IL-33, the recovery averages 93% (range from 85% to 100%).

Samples	Average recovery (%)	Range (%)
1	94.11	90-100
2	91.80	85-100

G. Linearity:

One mouse serum sample containing IL-33 was diluted several fold (1/5 to 1/10) and the measured recoveries ranged from 94% to 105%.

Sample	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1 : 5	0.200	0.200	100
	1 : 10	0.100	0.942	94.26

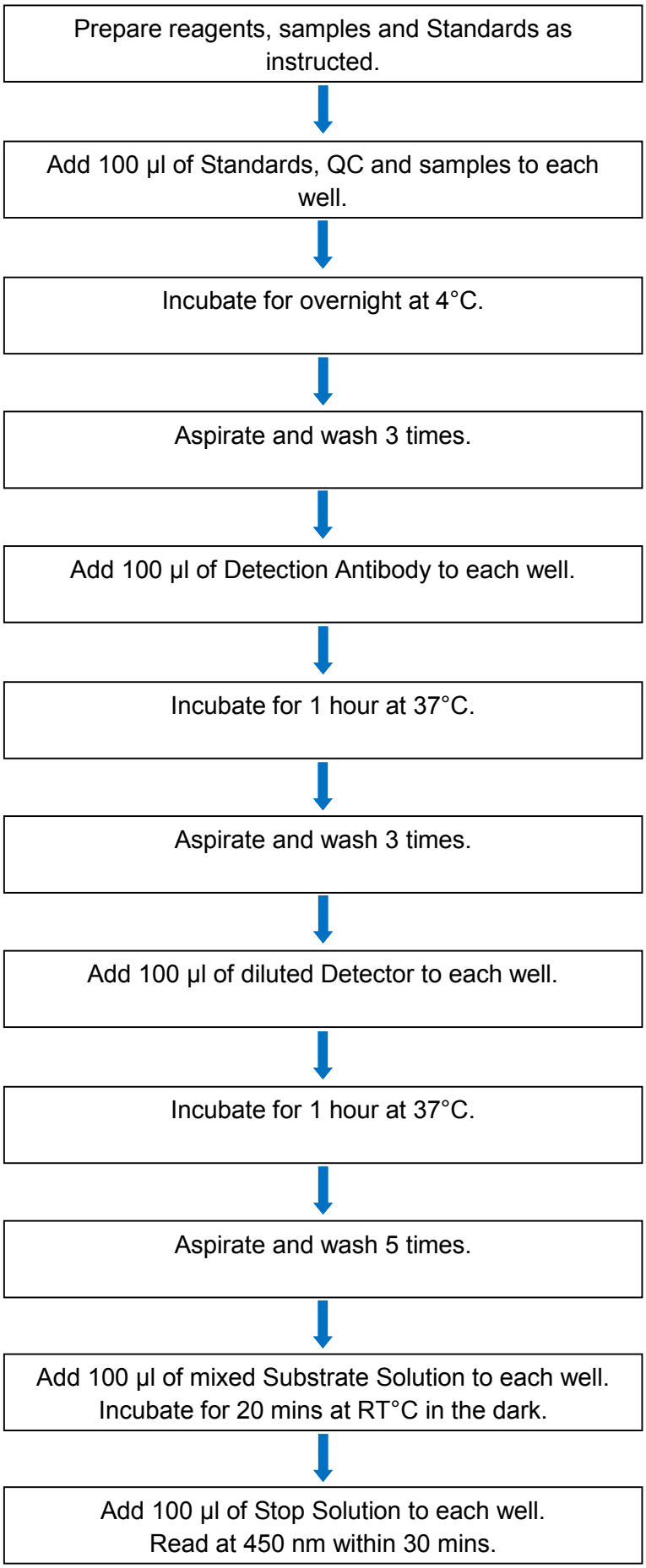
12. Technical Hints and Limitations

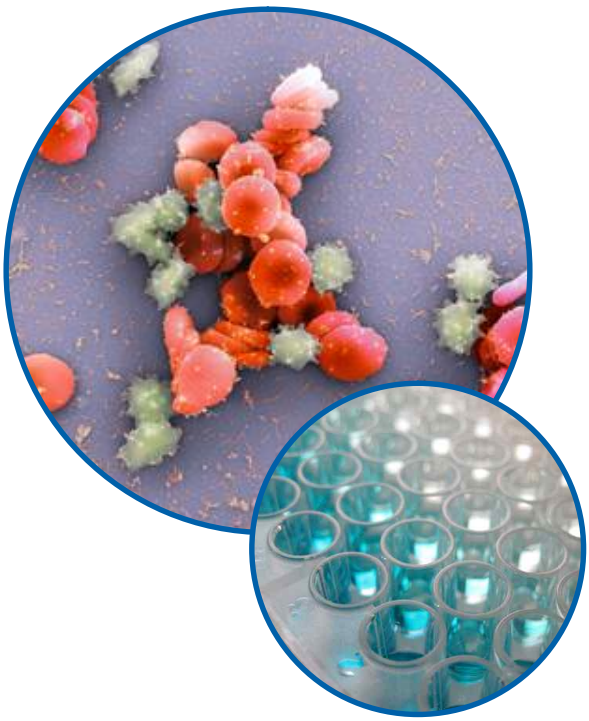
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last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