ab136947 – SMN ELISA Kit

Instructions for Use

For quantitative detection of SMN (Survival Motor Neuron) in cell and tissue extracts of Human and mouse origin.

This product is for research use only and is not intended for diagnostic use.

Version 1 Last Updated 15 January 2014

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1. BACKGROUND

Abcam's SMN ELISA (Enzyme-Linked Immunosorbent Assay) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of SMN in Human and mouse cell and tissue extracts.

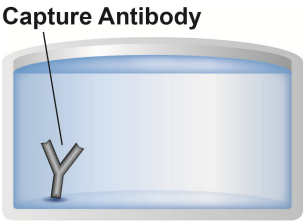
An anti-Human SMN antibody is precoated onto 96-well plates, standards or test samples are added to the wells and incubated at room temperature. The wells are washed and a polyclonal detector antibody specific to SMN is added, followed by incubation at room temperature. After further washing, a horseradish peroxidase (HRP) conjugated anti-species antibody is added to each well and incubated at room temperature. After incubation the excess reagents are washed away. TMB substrate is added to each well and after a short incubation the enzyme reaction is stopped and the yellow color generated is read at 450 nm. The intensity of the yellow coloration is directly proportional to the amount of SMN captured in the plate.

Survival Motor Neuron (SMN) is a ~38 kDa protein produced chiefly by the SMN1 gene, located on the telomeric portion of chromosome 5q1-4. A nearly identical centromeric copy of the gene (SMN2) also produces a small amount of full-length SMN protein, but due to a translationally silent C→T transition that results in alternative splicing of the pre-mRNA, most of the resulting SMN is truncated, causing reduced protein stability and lower overall SMN levels. Deletion or mutation of the SMN1 gene results in a reduced level of full-length SMN protein and manifests as a range of neuromuscular phenotypes in Humans as the disease spinal muscular atrophy (SMA). SMA is characterized by muscle weakness and atrophy, functional disability and is the most common lethal genetic disease of infants and toddlers. Approximately one in 35 adults is a carrier of the SMN1 mutation. The incidence of SMA is 1 in 6,000 to 1 in 10,000 live births.

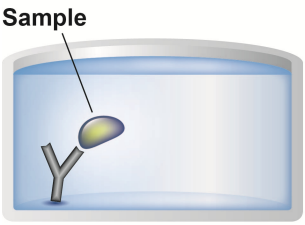
SMN protein is present in the cell cytoplasm, and also in the nucleus where it is concentrated in "gem" structures associated with Cajal

bodies. SMN protein is a constituent of Gemin-containing complexes, and is thought to participate in many aspects of RNA metabolism. SMN complexes have been shown to mediate the assembly of uridine-rich small nuclear ribonucleoproteins (snRNPs), which in turn act as critical components of spliceosomes.

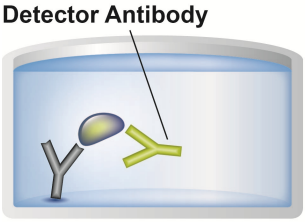
2. ASSAY SUMMARY



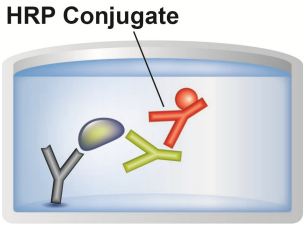
Prepare all reagents, samples and standards as instructed.



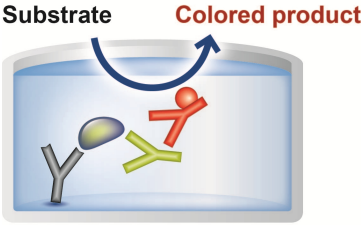
Add standard or sample to each well used. Incubate at room temperature.



Wash and add prepared detection antibody to each well. Incubate at room temperature.



Wash and add prepared antibody-HRP conjugate. Incubate at room temperature.



Add TMB Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- Stop Solution 2 is a 1 normal (1N) hydrochloric acid solution. This solution is caustic; care should be taken in use
- The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine
- We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results
- The SMN standard should be handled with care due to the unknown effects of the antigen

4. STORAGE AND STABILITY

All components should be kept at 4ºC except the standard which must be stored at -20ºC.Avoid multiple freeze-thaw cycles.

Refer to list of materials supplied for storage conditions of individual components.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Microplate coated with anti-SMN monoclonal antibody (12 x 8 wells)	96 wells	4ºC
Rabbit polyclonal anti-Human SMN antibody	10 mL	4ºC
Assay Buffer 13	100 mL	4ºC
Anti-rabbit IgG-HRP conjugate	10 mL	4ºC
20X Wash Buffer Concentrate	100 mL	4ºC
Human SMN Standard	2 Vials	-20ºC
TMB Substrate	10 mL	4ºC
Stop solution 2	10 mL	4ºC
Extraction Reagent 4	100 mL	4ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Deionized or distilled water
- Precision pipets for volumes between 10 µL and 1,000 µL
- Disposable polypropylene test tubes for dilution of samples and standards
- Repeater pipettes for dispensing 100 µL
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- A microplate shaker
- Adsorbent paper for blotting
- Microplate reader capable of reading at 450 nm
- Hemocytometer for cell counts
- Cover slip for hemocytometer
- Trypan Blue 0.4%
- Mechanical homogenizer or manual dounce homogenizer
- Phosphate buffered saline
- Protease inhibitor cocktail (PIC)
- Phenylmethlysulphonyl fluoride (PMSF)

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Standards must be made up in polypropylene tubes
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results
- If inhibitors other than those recommended are used, the end user is responsible for assay validation. In some cases, some protease inhibitor cocktails may cause performance differences
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

9.1 1X Wash Buffer

Prepare the 1X Wash Buffer by diluting 50 mL of the supplied Wash Buffer Concentrate with 950 mL of distilled water. This can be stored at room temperature until the kit's expiration date, or for 3 months, whichever comes first.

9.2 Extraction Reagent 4 + Protease Inhibitor Cocktail

Add protease inhibitor cocktail to Extraction Reagent 4 prior to use. Add 0.5 µL of PIC per mL of Extraction Reagent 4 and add PMSF to a final concentration of 1 mM. Do not store Extraction Reagent 4 with protease inhibitors.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Diluted SMN standards should be used within 1 hour of preparation.

10.1 Allow the SMN standard to equilibrate to room temperature. Reconstitute one vial of SMN standard by adding 1 mL of Assay Buffer 13. Mix thoroughly and gently. Hold at room temperature for 5 minutes. This is the 3,200 pg/mL **Standard 1** Solution (see table below).

10.2 Label eight tubes with numbers 2 – 8**.**

10.3 Add 250 µL Assay Buffer 13 to each tube.

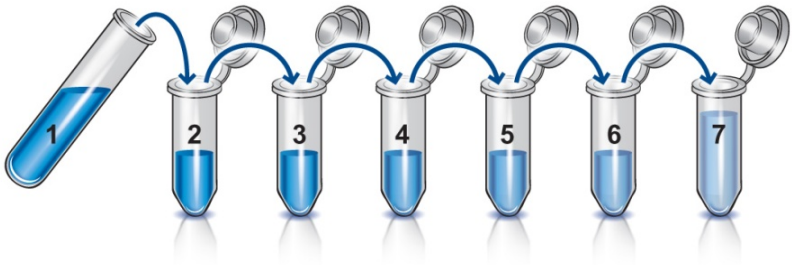
10.4 Prepare a 1,600 pg/mL **Standard 2** by transferring 250 µL from Standard 1 to tube 2. Mix thoroughly and gently.

10.5 Prepare **Standard 3** by transferring 250 µL from Standard 2 to tube 3. Mix thoroughly and gently.

10.6 Using the table below as a guide, repeat for tubes 4 through 7.

10.7 **Standard 8** contains no protein and is the Blank control.

Standard #	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	See Step 10.1				3,200
2	Standard 1	250	250	3,200	1,600
3	Standard 2	250	250	1,600	800
4	Standard 3	250	250	800	400
5	Standard 4	250	250	400	200
6	Standard 5	250	250	200	100
7	Standard 6	250	250	100	50
8	None	-	250	-	-



11. SAMPLE COLLECTION AND STORAGE

- This assay is compatible with Human and mouse SMN samples. Prior to assay, frozen samples should be brought slowly to 4oC (on ice) and centrifuged, if necessary, to isolate residual cell debris. Samples diluted sufficiently into the assay buffer can be read directly from a standard curve.
- A minimum 1:4 dilution is recommended for cell lysates and 1:8 dilution is recommended for tissue extracts. This is the minimum recommended dilution to remove matrix interference in the assay.

12. SAMPLE PREPARATION

10 **Peripheral Blood Mononuclear Cell (PMBC) Collection**

12.1.1 Collect blood samples using standard venipuncture into tubes. Invert tubes 8 to 10 times to mix anticoagulant additive with blood. Blood samples should be centrifuged within two hours of blood collection. Centrifuge tube/blood samples at room temperature (18–25ºC) for 20 minutes at 1,500 to 1,800 RCF.

12.1.2 After centrifugation, mononuclear cells and platelets will be in a whitish layer just under the plasma layer. Immediately process the PBMCs, by aspirating approximately half of the plasma without disturbing the cell layer. Collect the cell layer and transfer to a 15 mL conical centrifuge tube with cap.

12.1.3 Add PBS to the PBMCs to bring the volume to 15 mL. Cap tube and invert to mix cells.

12.1.4 Centrifuge tube for 15 minutes at 300 RCF, 4ºC. Aspirate supernatant without disturbing the cell pellet.

12.1.5 Resuspend cell pellet in residual PBS by gently vortexing or tapping tube with index finger.

12.1.6 Add PBS to resuspended pellet to bring volume to 10 mL. Cap tube and invert to mix cells.

12.1.7 Centrifuge tube for 15 minutes at 300 RCF, 4ºC. Aspirate supernatant without disturbing the cell pellet.

12.1.8 Repeat washing steps 8 and 9 for a total of 3 washes.

12.1.9 Assay immediately or freeze down in freezing media and store in liquid nitrogen.

12.2 **PBMC Thawing**

12 Remove vials containing frozen cells from liquid nitrogen and place in a water bath set at 37ºC.

12.1.2 Remove vials form water bath when no ice crystals remain

12.1.3 Transfer cell solution to 15 mL conical centrifuge tube with cap.

12.1.4 Add PBS to the PBMCs to bring the volume to 15 mL. Cap tube and invert to mix cells.

12.1.5 Centrifuge tube for 15 minutes at 300 RCF, 4ºC. Aspirate supernatant without disturbing the cell pellet.

12.1.6 Resuspend cell pellet in 2 mL PBS for performing cell counts.

12.3 **Cell Counts with Hemocytometer**

12.3.1 Transfer 50 µL of cell suspension to a solution containing 75 µL PBS and 125 µL

12.3.2 Trypan blue. Vortex the trypan-blue cell solution.

12.3.3 With the cover slip in place, transfer a small amount of trypan blue-cell suspension to a chamber on the hemocytometer. Ensure that the entire area under the cover slip contains the staining solution before removing any excess staining solution from the edge of the cover slip.

12.3.4 Both chambers of the hemocytometer must contain staining solution before performing cell counts.

12.3.5 Place hemocytometer on the microscope and count the number of trypan-blue excluding (viable) cells in the 4 outer squares. If there are less than 10 cells or more than 100 cells per square, repeat the procedure adjusting to an appropriate dilution factor.

12.3.6 Calculate the cell concentration as follows: Cell concentration per milliliter = Total cell count in 4

squares x 2,500 x 5 (dilution factor) Total cell count = Cell concentration per milliliter x 2.0 mL (cell suspension)

12.3.7 Centrifuge cell suspension for 10 minutes at 300 RCF, 4ºC. Aspirate supernatant without disturbing cell pellet.

12.3.8 Proceed to cell lysis immediately.

12.4 Cell Lysis

12.4.1 Resuspend cell pellet in Extraction Reagent 4, containing protease inhibitors.

12.4.2 Add 1 mL of extraction reagent per 108 cells. See Reagent Preparation Section for addition of protease inhibitors to Extraction Reagent 4.

12.4.3 Incubate cell suspension on ice for 30 minutes for complete lysis.

12.4.4 Transfer cell lysis to 1.5 mL centrifuge tube. Centrifuge cell lysates for 10 minutes at 14,000 RCF, 4ºC.

12.4.5 Clarified lysates may be assayed immediately, or aliquoted and stored at -70ºC.

12.4.6 No degradation of SMN in cell lysate was observed after 2 freeze-thaw cycles.

12.5 Cell Lysate Sample Handling

12.5.1 If cell lysates were frozen prior to assay, the frozen lysate samples should be brought slowly to 4ºC (on ice) and, if residual precipitate is present, centrifuge to isolate residual cell debris. Samples diluted sufficiently into the assay buffer can be read directly from a standard curve. A minimum 1:4 dilution is recommended for cell lysates to remove matrix interference in the assay.

12.6 Mouse Tissue Homogenization

12.6.1 Prepare Extraction Reagent 4 with protease inhibitors. Recommended protease inhibitors are 0.5 µL of PIC8340 per mL of reagent and PMSF to a final concentration of 1 mM.

12.6.2 Transfer tissue sample to appropriate sized tube for homogenization with 1mL of prepared Extraction Reagent 4.

12.6.3 For mechanical homogenizer, disrupt the tissue with three pulses of 3-4 seconds each. For manual dounce homogenizer, complete a minimum of 5 passes of the pestle past the buffer/tissue volume, or until tissue appears completely homogenized. Keep samples on ice while completing all preparations.

12.6.4 Pellet out tissue/cellular debris via centrifugation at 14,000g for 10 minutes at 4°C and transfer supernatant to a clean tube.

12.6.5 Measure the protein content of the supernatant using the protein assay.

12.6.6 Prepare tissue homogenates for use in the SMN assay by diluting the extracted samples in assay buffer. Samples must be diluted at least 1:8. Dilute brain, muscle, and spinal cord tissue samples to final assay protein concentrations of 25 µg/mL, 50 µg/mL and 100 µg/mL, respectively.

13. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section

14. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **It is recommended to assay all standards, controls and samples in duplicate**

13 Prepare all reagents, working standards, and samples as directed in the previous sections.

14.2 Add 100 µL of each Standard into the appropriate wells.

14.3 Add 100 µL of the Samples into the appropriate wells.

14.4 Seal the plate and incubate for 1 hour on a plate shaker at 500 rpm and at room temperature.

14.5 Empty the contents of the wells and wash by adding 300 µL of 1X Wash Buffer to every well. Repeat the wash 3 more times for a total of 4 Washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

14.6 Add 100 µL of the anti-SMN monoclonal detection antibody to every well.

14.7 Seal the plate and incubate for 1 hour on a plate shaker at 500 rpm and at room temperature.

14.8 Wash as described in step 14.5.

14.9 Add 100 µL of the anti-rabbit IgG – HRP conjugate to all wells.

14.10 Seal the plate and incubate for 30 minutes on a plate shaker (~500) at room temperature.

14.11 Wash as described in step 14.5.

14.12 Add 100 µL TMB substrate solution to each well.

14.13 Seal the plate and incubate for 30 minutes on a plate shaker at 500 rpm and at room temperature.

14.14 Add 100 µL Stop Solution to each well.

14.15 Read the O.D. absorbance at 450 nm, preferably with correction between 570 and 590 nm.

15. CALCULATIONS

A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

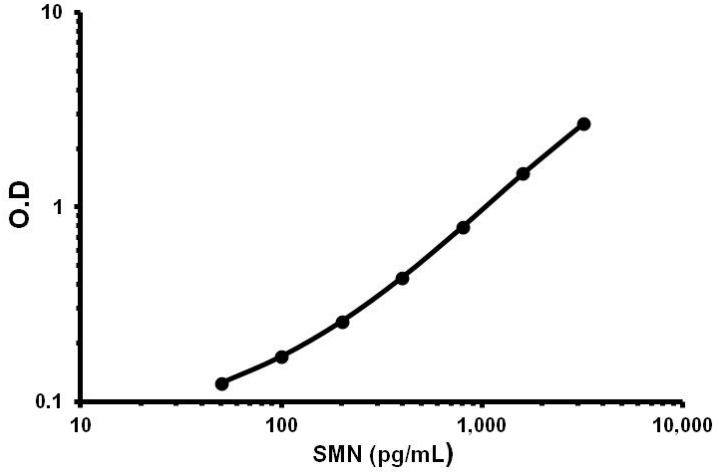
- Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD bound:

  Average Net OD = Average Bound OD - Average Blank OD

- Plot the average Net OD for each standard versus SMN concentration in each standard. Sample concentrations may be calculated off of Net OD values using the desired curve fitting

16. TYPICAL DATA

Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Sample	SMN Conc. (pg/mL)	Net OD
Sample 8	0	0.088
Sample 7	50	0.124
Sample 6	100	0.17
Sample 5	200	0.258
Sample 4	400	0.434
Sample 3	800	0.789
Sample 2	1,600	1.482
Sample 1	3,200	2.679

17. TYPICAL SAMPLE VALUES

SENSITIVITY –

The sensitivity of the assay, defined as the concentration of SMN measured at 2 standard deviations from the mean of 20 replicates of zero standard along the standard curve, was determined to be 50 pg/mL.

LINEARITY OF DILUTION –

The minimum required dilution for several common samples was determined by serially diluting samples into the assay buffer and identifying the dilution at which linearity is observed.

Dilution	Human cell lysate (%)	Mouse Brain Tissue (%)	Mouse Muscle Tissue (%)	Mouse Spinal Cord Tissue (%)
Neat	-	-	-	-
1:2	-	>LOD	74	65
1:4	98	>LOD	84	82
1:8	102	>LOD	90	88
1:16	105	>LOD	99	89
1:32	100	94	100	97
1:64	-	100	<LOD	106

RECOVERY –

After diluting each sample matrix to its minimum required dilution, recombinant Human SMN was spiked at high, medium, and low concentrations. The recovery of the standard in spiked samples was compared to the recovery of identical spikes in the assay buffer. The mean and range of percent recovery at the three concentrations are indicated below for each matrix.

Mean Spike and Recovery Results			
Sample Matrix (# of samples)	**Minimum Required Dilution**	**Spike Concentration (pg/mL)**	**Average % Recovery (range)**
Human PBMC lysate (n=5)	1:4	1667	100 (88 - 116)
		667	100 (88 - 116)
		267	99 (79 - 134)
Mouse Brain Extract (n=2)	≥1.8[a]	1250	84 (83-84)
		250	86 (85-87)
		50	104 (96-112)
Mouse muscle extract (n=2)	≥1.8[b]	1250	79 (76-81)
		250	88 (85-90)
		50	125 (103-146)
Mouse spinal cord extract (n=2)	≥1.8[c]	1250	68 (66-69)
		250	68 (67-68)
		50	44 (39-48)

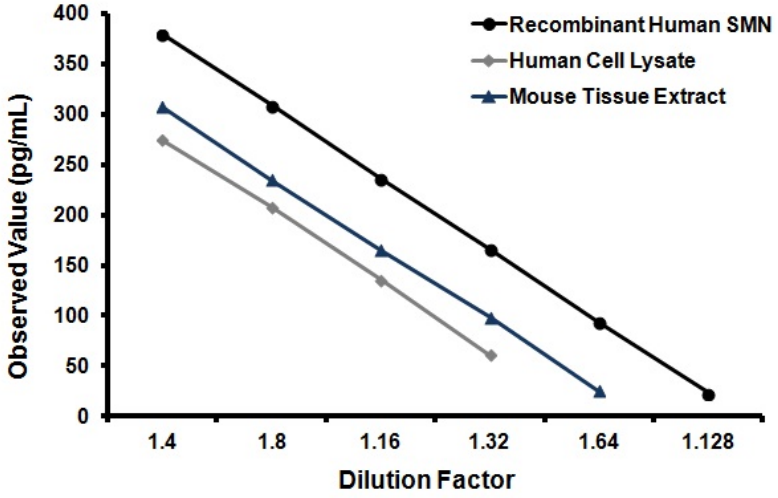
[a] = Dilute mouse brain tissue extract such that the final protein concentration in the assay sample is 25µg/mL with a minimum dilution of 1:8.

[b] = Dilute mouse muscle tissue extract such that the final protein concentration in the assay sample is 50µg/mL with a minimum dilution of 1:8.

[c] = Dilute mouse spinal cord tissue extract such that the final protein concentration in the assay sample is 100µg/mL with a minimum dilution of 1:8.

PARALLELISM –

A parallelism experiment was carried out to determine if the recombinant Human SMN standard accurately determines SMN concentrations in biological matrices. To assess parallelism, values for Human PBMC lysate and mouse tissue extract was obtained from a standard curve using four parameter logistic curve fitting. The observed concentration was plotted against the dilution factor. Parallelism of the curves demonstrates that the antibody binding characteristics are similar enough to allow the accurate determination of analyte levels in diluted samples.



PRECISION –

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing SMN in a single assay.

SMN (pg/mL)	% CV
928	0.8
322	1.0
122	3.2

Inter-assay precision was determined by measuring buffer controls (n=12) of varying SMN concentrations in multiple assays over several days.

SMN (pg/mL)	% CV
983	7.1
378	8.9
134	11.4

18. ASSAY SPECIFICITY

This kit detects SMN protein of both Human and mouse origin. Other species have not been tested.

19. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

20. NOTES

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