m1A (N1-methyladenosine) Competitive ELISA kit

Catalog #: EIA-m1A

User Manual
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RayBiotech
Empowering your proteomics

ISO 13485 Certified

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Please read the entire manual carefully before starting your experiment

I. Introduction

RNA modifications affect RNA metabolism and are involved in many different biological processes. Mono-methylation of adenosine in the N1 position, N1-methyladensoine (m1A), is a reversible modification that was first discovered in tRNA in 1966. Decades later, m1A was found in tRNA, rRNA, mitochondrial RNA and mRNA. It has been verified that m1A dysregulation affects multiple cellular processes, including RNA structural stability, folding, interactions with proteins, cell viability, impaired self-renewal ability, cell proliferation, and cell death. They tend to be conserved and have been implicated in many critical biological processes including development, disease, circadian rhythm and embryonic stem cell fate transition, as well as age-related disorders such as Alzheimer's disease.

Today, it is recognized that the dynamic regulation of m1A in response to physiological stress and the dysregulated expression of m1A regulators are correlated with tumorigenesis and cancer recurrence. Urine samples of various cancers, rheumatoid arthritis, and AIDS patients have all demonstrated high levels of m1A, which supports its role as a functional detection biomarker. The urinary m1A correlates with m1A level in tissue from the same patients with esophageal cancer. Furthermore, elevated serum levels of m1A have been detected under stress conditions. Recent m1A research is uncovering new roles and mechanisms involving m1A. However, the complete role of 1-methyladenosine as a modified nucleoside has yet to be elucidated.

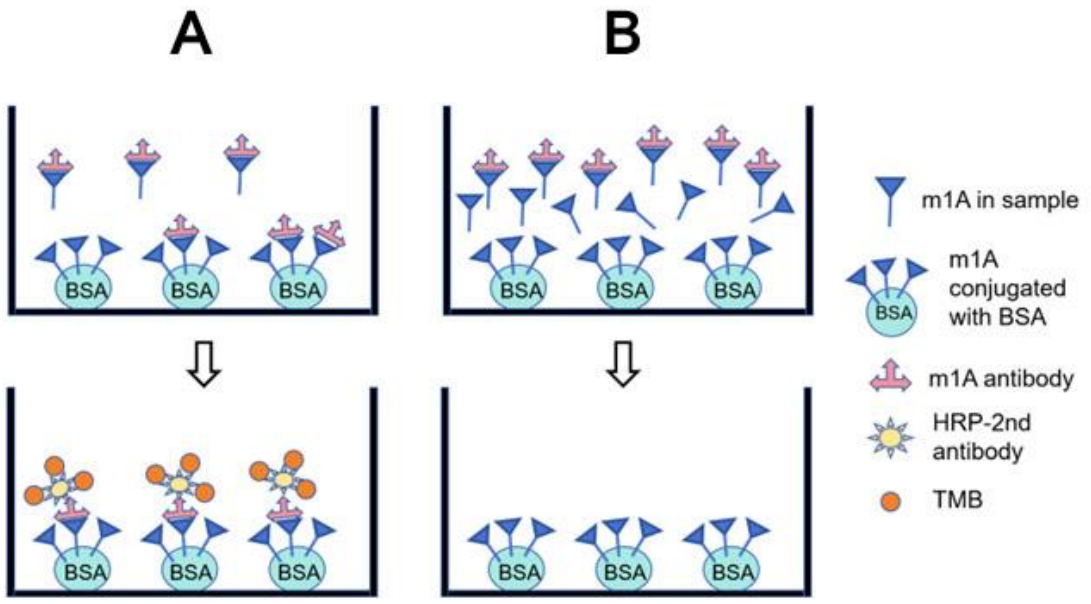
The RayBio® m1A ELISA Kit is a competitive enzyme immunoassay developed for rapid detection and quantitation of m1A in urine, serum, or plasma samples collected from human, rat or mouse. The quantity of m1A is determined by a known m1A standard curve. The m1A kit has a high sensitivity of approximately 2.544 ng/ml. The linearity, stability, precision and spiking & recovery were also confirmed and showed good performance. Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown samples.

II. General Description

The RayBio® m1A Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting m1A based on the competitive enzyme immunoassay principle.

In this assay, the samples and standards are added to a microplate pre-coated with m1A. In the plate wells, the endogenous m1A or the standards compete with the pre-coated m1A for binding to the anti-m1A antibody. After a wash step, any bound m1A antibody then interacts with horseradish peroxidase (HRP)-secondary antibody, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of bound m1A antibody and inversely proportional to the amount of endogenous m1A in the standard or samples. A standard curve of known concentration of m1A can be established and the concentration of m1A in the samples can be calculated accordingly.

III. How It Works



A. Low to moderate level of endogenous m1A in sample. Anti-m1A binds to coated m1A due to low competition and is then detected by HRP-conjugated secondary antibody. **B.** High level of endogenous m1A in sample. Anti-m1A is bound by excess free m1A and washed off the plate.

IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store it at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

Component	**Size / Description**	**Storage / Stability <u>After Preparation</u>**
EIA-m1A Microplate	96 wells (12 strips x 8 wells) coated with m1A.	1 month at 4°C*
Assay Diluent B (5X)	15 ml concentrated buffer. Diluent for standards, samples, and antibodies.	1 month at 4°C
Standard m1A	2 vials. 1 vial is enough to run in two times.	1 week at 4°C
Positive Control	2 vials of Lyophilized Positive Control. 1 vial is enough to run in two times.	1 month at 4°C
Anti-m7G Antibody (1000X)	10 µl concentrated anti-m1A Antibody.	1 month at 20°C
HRP-conjugated secondary antibody (5000X)	5 µl concentrated antibody.	Do not store and reuse
Wash Buffer (20X)	25 ml of 20X concentrated solution.	1 month at 4°C
TMB One-Step Substrate Reagent	12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution.	1 month at 4°C
Stop Solution	12 ml of 0.2 M sulfuric acid.	12 months at 4°C

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Precision pipettes to deliver 2 µl to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1-liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. GraphPad Prism or SigmaPlot software (or other software which can perform four-parameter logistic regression models).
8. Tubes to prepare standard or sample dilutions.
9. Orbital shaker.
10. Aluminum foil.
11. Plastic wrap.

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

A. Preparation of Plate and Anti-m1A Antibody

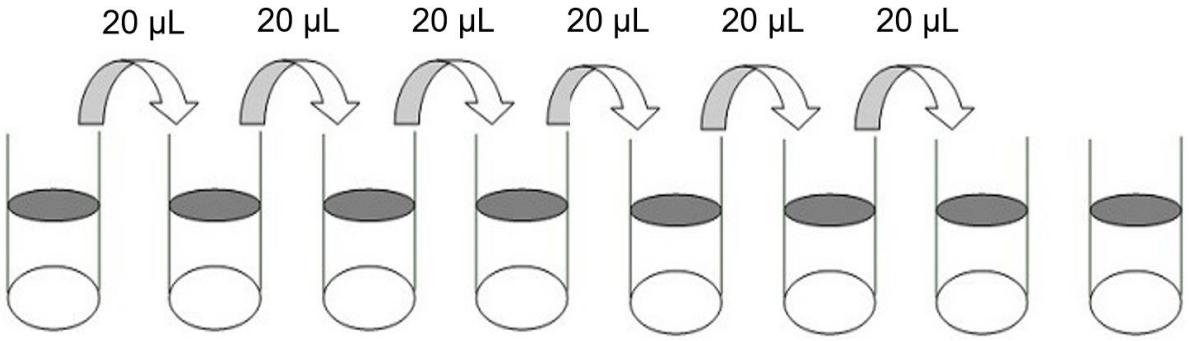
1. Equilibrate plate to room temperature before opening the sealed pouch.
2. Label removable 8-well strips as appropriate for your experiment.
3. 5X Assay Diluent B should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti-m1A antibody vial and dilute 1000-fold with 1X Assay Diluent B for working solution.

B. Preparation of Standards

5. Label 8 microtubes with the following concentrations: S1 (4000 ng/ml), S2 (400 ng/ml), S3 (40 ng/ml), S4 (4 ng/ml), S5 (0.4 ng/ml), S6 (0.04 ng/ml), S7 (0.004 ng/ml) and S8 (0 ng/ml). Pipette 180 µl of 1x Assay Diluent B into S2-S8.
6. Briefly centrifuge the vial of m1A Standard. Reconstitute with 200 µl of 1x Assay Diluent B. Mix thoroughly. This solution serves as S1.

7. To make the S2 (400 ng/ml) standard, pipette 20 µl of the S1 standard into the tube labeled S2 containing 180 µl of 1x Assay Diluent B. Mix thoroughly.

8. Repeat this step with successive concentration, preparing a dilution series as shown in the illustration below. Each time, add 20 µl of the prior concentration until the S7 is reached. Mix each tube thoroughly before the next transfer. 1x Assay Diluent B serves as the blank (S8, 0 ng/ml).



	S1	S2	S3	S4	S5	S6	S7	S8
Diluent Volume	200 µl	180 µl	180 µl	180 µl	180 µl	180 µl	180 µl	180 µl
Conc.	4000 ng/ml	400 ng/ml	40 ng/ml	4 ng/ml	0.4 ng/ml	0.04 ng/ml	0.004 ng/ml	0 ng/ml

C. Positive Control Preparation

9. Briefly centrifuge the Positive Control vial and reconstitute with 200 µl of 1X Assay Diluent B.

The positive Control is a mouse serum sample that serves to verify that the kit components are functioning. The resulting OD is not used to calculate final concentrations of samples. If no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired.

D. Sample Preparation

10. Urine, serum, and plasma samples from humans, rats, and mice can be detected. Use immediately or aliquot and store at -20 ºC until use. Avoid repeated freeze-thaws. Dilute your sample with the 1x Assay Diluent B.

I. Urine: Urine should be aseptically collected from the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge samples at 5000 g for 10 minutes, or filter through 0.45 µm filter, prior to use in the assay.

II. Serum: Avoid hemolyzed and lipemic blood samples. Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 5000 x g for 10 minutes at room temperature. Remove the yellow serum supernatant without disturbing the white buffy layer.

III. Plasma: Avoid hemolyzed and lipemic blood samples. Collect blood with EDTA, heparin or citrate and centrifuge at 5000 g for 10 minutes at room temperature. Remove the plasma layer and avoid disturbing the white buffy layer.

IV. Cell or Tissue RNA Samples:

1. Dissolve total RNA, mRNA or small RNA from cell or tissue samples in nuclease free water at 0.1-2 µg/µl. The input RNA/DNA should be at 0.5-5 µg/well. Ensure the same amount of RNA/DNA loading across all reactions.
2. Remove any RNA secondary structure by incubating the sample at 95ºC for 5 minutes and rapidly chilling on ice.
3. Centrifuge the reaction mixture for 5 minutes at 6000 x g at 4 ºC and collect the supernatant (0.5-5 µg of RNA sample) for EIA assay.

If you have any questions regarding the recommended dilutions, you may contact technical support at 888-494-8555 or techsupport@raybiotech.com.

E. Preparation of Wash Buffer and HRP

11. If the Wash Buffer contains visible crystals, warm them to room temperature and mix gently until dissolved.

12. Dilute 20 ml of Wash Buffer (20X) Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.

13. Briefly centrifuge the HRP-Secondary Antibody vial before use. Dilute the HRP-Secondary Antibody concentrate 5000-fold with 1X Assay Diluent B.

VIII. Assay Procedure

1. Remove the appropriate number of microtiter wells of m1A coated microplate from foil pouch and place them into the well holder. Return any unused wells to the foil pouch with desiccant pack, reseal along entire edge, and store at -20 ºC.

2. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.

3. Add 50 µl of each standard (S1-S8), Positive Control and sample to appropriate wells. Incubate at room temperature for 10 minutes on an orbital shaker.

4. Add 50 µL of the diluted Anti-m1A Antibody to each well. Cover wells and incubate at room temperature for 1 hour on an orbital shaker.

5. Discard the solution and wash wells 5 times with 1X Wash Solution Buffer (200-300 µl each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µl of prepared HRP-Secondary antibody solution (see Reagent Preparation step 13) to each well. Incubate for 1 hour at room temperature with gentle shaking.

7. Discard the solution and wash 5 times as directed in Step 5.

8. Add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate for 2-10 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).

9. Add 100 µl of Stop Solution to each well. Read at 450 nm immediately. Results should be read immediately (color will fade over time).

Note: Reliable standard curves are obtained when OD values do not exceed 2.5 units for the blanks (S8).

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.

2. Add 50 µl standards or samples to appropriate well. Incubate for 10 minutes at room temperature.

3. Add 50 µl anti-m1A to appropriate well. Incubate 1 hour at room temperature. And wash 5 times.

4. Add 100 µl prepared HRP-Secondary antibody solution. Incubate 1 hour at room temperature. And wash 5 times.

5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 2-10 minutes at room temperature.

6. Add 100 µl Stop Solution to each well. Read at 450 nm immediately.

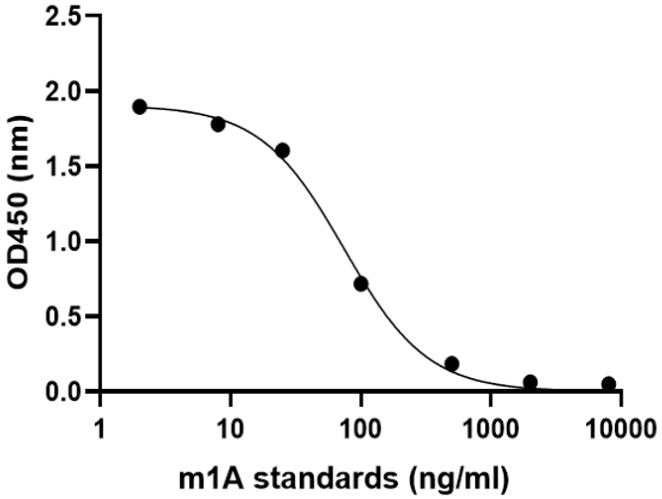
X. Calculation of Results

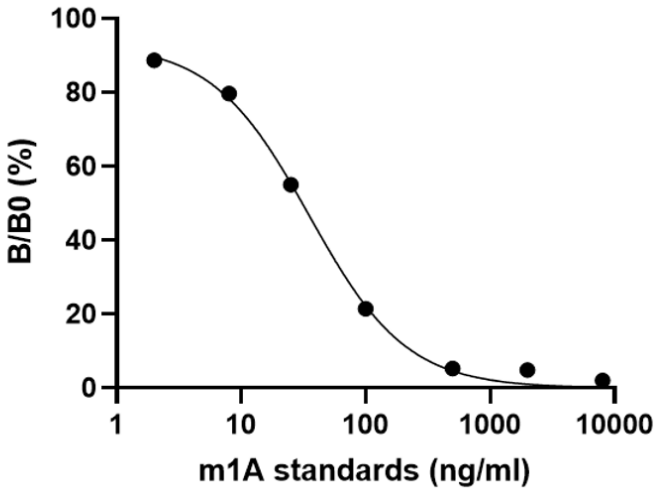
Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using GraphPad Prism or Sigma Plot software (or other software which can perform four-parameter logistic regression models). If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

The measurement range is 2.544 – 8000 ng/ml. Any sample reading lower than the highest standard should be diluted with dilution buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the m1A concentration.

Percentage absorbance = B/B0 X 100%, where
B = OD of sample or standard
B0 = OD of Blank (S8, or zero standard)

A. Typical Data
These standard curves are for demonstration only. A standard curve must be run with each assay.

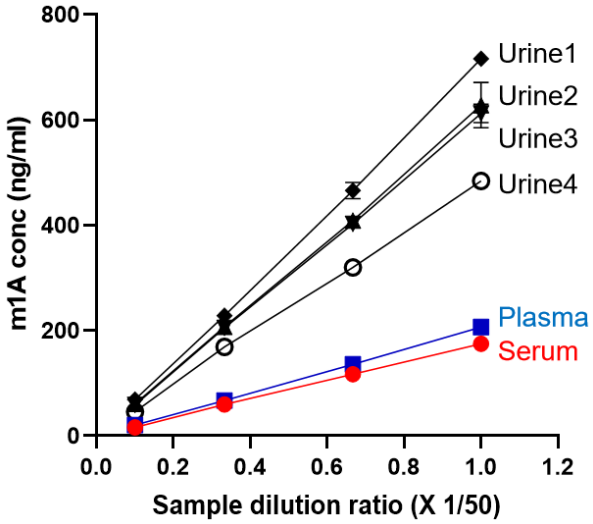




B. Sensitivity
The limit of detection (LOT, Blank-3SD) is 2.544 ng/ml.

C. Linearity

Human serum, plasma and urine samples were serially diluted from 50-500-fold (n=3). The recovery ranges from 94.55 to 104.64.



Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
Serum	1/50	174.946		
	1/75	117.199	116.631	100.487
	1/150	59.762	58.315	102.480
	1/500	16.197	17.495	92.583
Plasma	1/50	206.764		
	1/75	135.757	137.843	98.487
	1/150	67.196	68.921	97.497
	1/500	20.388	20.676	98.606
Urine 1	1/50	628.097		
	1/75	408.897	418.731	97.651
	1/150	207.197	209.366	98.964
	1/500	60.446	62.810	96.237
Urine 2	1/50	612.113		
	1/75	402.699	408.075	98.683
	1/150	204.860	204.038	100.403
	1/500	58.721	61.211	95.931
Urine 3	1/50	715.903		
	1/75	465.870	477.269	97.612
	1/150	228.412	238.634	95.716
	1/500	68.789	71.590	96.087
Urine 4	1/50	484.180		
	1/75	320.092	322.787	99.165
	1/150	168.884	161.393	104.641
	1/500	45.781	48.418	94.554

C. Precision

Intra-assay (Within-Run, n=5) CV%=2.17-11.371. 5 cases of human urine samples were tested in 8 separate assays.

Repeat time	Urine 1 (ng/ml)	Urine 2 (ng/ml)	Urine 3 (ng/ml)	Urine 4 (ng/ml)	Urine 5 (ng/ml)
1	1603.810	75.087	8695.778	9577.803	12387.618
2	1633.138	75.087	8381.984	9152.439	12643.756
3	1638.139	78.006	8814.189	9577.803	12695.993
4	1842.378	82.411	8754.688	9749.363	12591.860
5	1936.437	85.364	8725.159	9819.291	12591.860
6	1880.793	75.087	8996.337	9377.873	12188.575
7	1681.861	76.545	8965.593	9443.813	12962.408
8	1837.623	56.448	8844.165	9345.162	12854.782
Mean	1756.772	75.504	8772.237	9505.444	12614.607
SD	130.873	8.585	190.941	220.014	245.003
CV%	**7.450**	**11.371**	**2.177**	**2.315**	**1.942**

Inter-assay (Run-to-Run, n=4) CV%=3.751-10.148. 6 cases of human urine samples were tested in 4 separate assays.

Repeat time	Urine 1 (ng/ml)	Urine 2 (ng/ml)	Urine 3 (ng/ml)	Urine 4 (ng/ml)	Urine 5 (ng/ml)	Urine 6 (ng/ml)
1	6326.030	15934.945	14606.109	18816.803	17840.353	27551.106
2	5724.446	19697.172	14288.123	18641.347	18153.810	27961.674
3	7057.465	16863.948	15200.052	19392.831	17938.125	27863.230
4	6963.973	18008.083	16907.025	22948.011	19326.111	32670.554
Mean	6517.978	17626.037	15250.327	19949.748	18314.600	29011.641
SD	620.885	1620.302	1167.337	2024.452	686.938	2445.546
CV%	**9.526**	**9.193**	**7.655**	**10.148**	**3.751**	**8.430**

D. Spiking & Recovery

Human urine sample average recovery range (n=3): 87.768-125.7511%.

Samples	Average (ng/ml)				**Recovery rate (%)**		
	0	+500ng/ml	+100ng/ml	+25ng/ml	**+500ng/ml**	**+100ng/ml**	**+25ng/ml**
U1	35.1354	539.0456	158.4644	57.2881	**100.7820**	**123.3290**	**88.6105**
U2	1.5101	520.1914	111.9871	27.8786	**103.7363**	**110.4770**	**105.4740**
U3	190.1089	636.8586	315.6331	212.0509	**89.3499**	**125.5243**	**87.7680**
U4	252.2921	738.8891	352.6780	283.7299	**97.3194**	**100.3858**	**125.7511**

E. Stability

The whole kit including coated plates, 1st antibody, 2nd antibody, standards were saved at -20℃ and 4℃ for one week. The standards and samples were measured.

m1A (ng/ml)	OD 450				CV %
	m1A-20	m1A-20	m1A-4	m1A-4	
8000	0.055	0.049	0.049	0.048	6.37%
2000	0.077	0.07	0.065	0.067	7.53%
500	0.147	0.135	0.12	0.12	10.02%
100	0.653	0.592	0.5	0.517	12.50%
25	1.829	1.909	1.714	1.753	4.79%
8	2.85	2.89	2.675	2.656	4.32%
2	3.201	3.009	2.797	2.767	6.88%
0	3.245	3.123	2.905	2.905	5.54%

Samples	Human plasma (ng/ml)		Rat plasma (ng/ml)		Mouse plasma (ng/ml)		Human Urine (ng/ml)	
Saved at	-20℃	4℃	-20℃	4℃	-20℃	4℃	-20℃	4℃
1	34.508	31.707	75.830	74.290	56.871	51.843	2959.600	2637.598
2	35.328	34.247	83.716	79.943	56.992	50.364	5860.807	5489.751
3	45.974	43.458	106.421	110.342	65.420	67.967	8051.951	8018.040
4	92.241	89.032	62.353	56.113	91.964	92.243	7729.802	7948.054
Mean	52.010	49.610	82.080	80.170	67.810	65.600	6150.000	6022.500
Std. Deviation	27.320	26.760	18.470	22.540	16.590	19.470	2336.250	2545.000
Std. Error	13.660	13.380	9.235	11.270	8.296	9.733	46.730	50.900

F. Example of Test Results.

The concentration of m1A in serum and plasma from human, rat and mouse, as well as in urine from human.

	Human serum	Human plasma	Rat serum	Rat plasma	Mouse serum	Mouse plasma	Human urine
Numbers	4	3	2	3	2	3	10
Minimum (ng/ml)	16.65	48.81	83.46	60.06	47.65	48.23	2224
Maximum (ng/ml)	115	121.5	85.19	117.7	56.79	126.4	10386
Range (ng/ml)	98.32	72.65	1.736	57.65	9.142	78.16	8162
Mean (ng/ml)	**43.85**	**93.54**	**84.33**	**93.7**	**52.22**	**94.99**	**6366**
Std. Deviation	47.48	39.13	1.228	30.01	6.464	41.28	2691
Std. Error	23.74	22.59	0.8681	17.32	4.571	23.83	850.9

XIV. Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	○ Inaccurate pipetting ○ Improper standard dilution	○ Check pipettes ○ Briefly centrifuge and dissolve the powder thoroughly by gently mixing
Low signal	○ Improper preparation of standard and/or biotinylated antibody ○ Too brief incubation times Inadequate reagent volumes or improper dilution	○ Briefly spin down vials before opening. Dissolve the powder thoroughly. ○ Ensure sufficient incubation time; assay procedure step 2 may be done overnight. ○ Check pipettes and ensure correct preparation.
Large CV	○ Inaccurate pipetting Air bubbles in wells	○ Check pipettes. ○ Remove bubbles in wells
High background	○ Plate is insufficiently washed. ○ Contaminated wash buffer	○ Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. ○ Make fresh wash buffer
Low sensitivity	○ Improper storage of the ELISA kit ○ Stop solution	○ Follow storage recommendations in sections IV and V. Keep substrate solution protected from light. ○ Add stop solution to each well before reading plate

RayBio® ELISA Kits

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