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Mouse / Rat 25-OH Vitamin D ELISA Assay Kit

Catalog Number: VID21-K01 (1 x 96 wells)

For Research Use Only. Not for use in diagnostic procedures.

v. 6.0 (22 NOV 23)

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INTENDED USE

The Eagle Biosciences Mouse/Rat 25-OH Vitamin D ELISA Assay Kit is intended for use in the quantitative determination of total 25-OH Vitamin D (Vitamin D₂ and Vitamin D₃) in serum and EDTA plasma. This Mouse/Rat 25-OH Vitamin D ELISA Assay Kit is for Research Use Only.

ASSAY BACKGROUND

The group of compounds referred to as Vitamin D, are actually fat soluble steroidal prehormones. The main forms which occur in the body are Vitamin D₂ (ergocalciferol) and Vitamin D₃ (cholecalciferol). The active form of these molecules is Dihydroxyvitamin D₃ (1, 25(OH)₂ D₃). Vitamin D₃ is formed in the skin by photolysis of 7-dehydrocholesterol by ultraviolet radiation from sunlight. It is transported in blood circulation bound to proteins to the liver where it is hydroxylated. Further hydroxylation occurs in the kidneys to produce the most active form. Vitamin D levels are highest in newborns and decrease exponentially throughout life. Sufficient circulating levels of vitamin D are necessary for healthy bone maintenance and cell metabolism. Recent studies have shown that it may also lower incidents of certain cancers. Insufficient levels of Vitamin D can result in osteoporosis and bone fracture in the elderly, secondary hyperparathyroidism, abnormal cell metabolism and even increased incidents of cancer. Severe deficiency may lead to rickets in children and osteomalacia in adults. Disease associated with Vitamin D deficiency may also include: impaired immunity, increased autoimmunity, myopathy, diabetes mellitus, and an increased risk of colon, breast, and prostate cancers. Abnormally high levels (> 200 ng/ml) of Vitamin D leads to Vitamin D toxicity and may cause hypercalcaemia.

ASSAY PRINCIPLE

This Mouse/Rat 25-OH Vitamin D ELISA Assay Kit is designed developed and produced for the quantitative measurement of total 25-OH Vitamin D_{2/3} in serum utilizing the competitive immunoassay technique. This assay utilizes a monoclonal antibody that binds to both 25-OH Vitamin D₂ and 25-OH Vitamin D₃ equally. Assay calibrators, controls and test samples are added directly to wells of a microtiter plate that is coated with specific anti-25-OH Vitamin D₂, D₃ antibody. A buffer designed to release Vitamin D from binding proteins is then added to the wells. After the first incubation period, unbound material is washed away and biotinylated Vitamin D analogue is added to the wells and binds to remaining antibody sites. After the second incubation period, unbound biotin-D is washed away and horseradish peroxidase (HRP) conjugated streptavidin is added to each well. During the third incubation step, an immune complex of well coated "vitamin D antibody – vitamin D, biotin D and HRP conjugated streptavidin" is formed. The unbound matrix is removed in the subsequent washing steps. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction, which is terminated with an acidic reagent (ELISA stop solution). The absorbance is then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is inversely proportional to the amount of total 25-OH Vitamin D_{2/3} in the test sample. A calibration curve is generated by plotting the absorbance versus the respective Vitamin D concentration for each calibrator on a 4-parameter or point to point curve fitting. The concentration of total 25-OH Vitamin D_{2/3} in test samples is determined directly from this calibration curve.

REAGENTS: Preparation and Storage

This Mouse/Rat 25-OH Vitamin D ELISA Assay Kit must be stored at 2 – 8 °C upon receipt. Vitamin D is sensitive to heat and light. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Regents from different kit lot numbers should not be combined or interchanged.



1. Vitamin D Antibody Coated Microplate

One microplate with twelve by eight strips (96 wells total) coated with anti-Vitamin D2/D3 antibody. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

2. HRP - Streptavidin

One bottle containing **11.5 mL** ready to use HRP labeled streptavidin in a stabilized protein matrix. This reagent should be stored in 2-8°C and is stable until the expiration date on the kit box.

3. Biotinylated Vitamin D Analogue

One bottle containing **11.5 mL** of ready to use biotin-Vitamin D analogue in a stabilized buffer matrix with preservative. This reagent should be stored in 2-8°C and is stable until the expiration date on the kit box.

4. Vitamin D Assay Buffer

One bottle containing **15 ml** of ready-to-use buffered matrix. This buffer releases Vitamin D from its binding proteins. This reagent may be stored in room temperature and/or 2-8°C and is stable until the expiration date on the kit box.

5. ELISA Wash Concentrate

One bottle containing **30 mL** of 30-fold concentrate. Before use, the contents must be diluted with **870 mL** of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non-mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the kit box.

6. ELISA HRP Substrate

One bottle containing **12 mL** of ready to use tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

7. ELISA Stop Solution

One bottle containing **12 mL** of ready to use stop solution. This reagent may be stored at 2-8°C or room temperature and is stable until the expiration date on the kit box.

8. Vitamin D Calibrators 0 to 5

Six vials contain **0.5 mL** each of ready to use liquid 25-OH Vitamin D3 in a bovine serum albumin-based matrix with a non-azide preservative. **Refer to the vial for exact concentration.** This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

9. Vitamin D Controls

Two vials each contain **0.5 mL** of ready to use liquid Vitamin D3 in a human serum-based matrix with a non-azide preservative. **Refer to vials for concentration range for each control.** This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.



SAFETY PRECAUTIONS

The reagents of the Mouse/Rat 25-OH Vitamin D ELISA Assay Kit must be used in professional laboratory. Source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they are potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 25 μ l, 100 μ l, 500 μ L, etc.
2. Disposable pipette tips suitable for above volume dispensing.
3. Aluminum foil.
4. Plastic microtiter well cover or polyethylene film.
5. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
6. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.
7. ELISA plate shaker.

SPECIMEN COLLECTION

Serum, EDTA-plasma and citrate plasma samples were validated with this Mouse/Rat 25-OH Vitamin D ELISA Assay Kit. Only 50 μ L total (25 μ L each) of human EDTA-plasma or serum is required for the 25-OH Vitamin D measurement in duplicate. No special preparation of individual is necessary prior to specimen collection. Collect whole blood with Vacutainer and separate the serum or plasma from cells according to manufacturer's instruction. Serum and plasma samples can be stored at room temperature for 3 days. For longer term storage, sample can be kept at -15°C . Avoid more than three freeze-thaw cycles of specimen.

Animal serum Total 25-OH Vitamin D from bovine/calf, goat, horse, chicken, mouse, and equine can be detected using this kit.

ASSAY PREPARATION

1. Reagent Preparation

- (1) Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
- (2) ELISA Wash Concentrate must be diluted to working solution prior to use. Please see REAGENTS section for details.



2. Assay Procedure

Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	Cal 0	Cal 4	SAMPLE 1
B	Cal 0	Cal 4	SAMPLE 1
C	Cal 1	Cal 5	SAMPLE 2
D	Cal 1	Cal 5	SAMPLE 2
E	Cal 2	Control 1	SAMPLE 3
F	Cal 2	Control 1	SAMPLE 3
G	Cal 3	Control 2	SAMPLE 4
H	Cal 3	Control 2	SAMPLE 4

- (1) Add **25 µl** of calibrators, controls and test samples into the designated microwells.
- (2) Add **100 µl Vitamin D Assay Buffer** to each well.
- (3) Seal the plate securely, cover with aluminum foil and place on an ELISA plate shaker (170 rpm or 450 rpm). Incubate the plate at **room temperature (20°C – 25°C) for 60 minutes.**
- (4) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
- (5) Add **100 µl of Biotinylated Vitamin D Analogue** to each well.
- (6) Seal the plate securely, cover with aluminum foil and place on an ELISA plate shaker (170 rpm or 450 rpm). Incubate the plate **at room temperature (20 °C - 25 °C) for 30 minutes.**
- (7) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
- (8) Add **100 µL of Streptavidin-HRP** into each of the wells.
- (9) Seal the plate securely, cover with aluminum foil and place on an ELISA plate shaker (170 rpm or 450 rpm). Incubate the plate at **room temperature (20 °C - 25 °C) for 20 minutes.**
- (10) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
- (11) Add **100 µl TMB** reagent to each of the wells.
- (12) Cover plate with aluminum foil, and incubate at **room temperature (20 °C - 25 °C) for 20 minutes, static.**
- (13) Immediately add **100 µL of ELISA Stop Solution** into each of the wells. Mix gently.
- (14) Read the absorbance at 450 nm. 4-parameter curve is recommended.



PROCEDURAL NOTES

1. Vitamin D is sensitive to heat and light. It is important to avoid direct exposure to these conditions.
2. It is recommended that all calibrators and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results. It is recommended to add external controls to each assay.
3. Keep light sensitive reagents in the original amber bottles.
4. Store any unused antibody coated strips in the foil zipper bag with desiccant to protect from moisture.
5. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the Mouse/Rat 25-OH Vitamin D ELISA Assay test.
6. Incubation times or temperatures other than those stated in this insert may affect the results.
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
8. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
9. It is important to seal the plate properly to avoid evaporation.

INTERPRETATION OF RESULTS

It is recommended to use a 4-parameter calibration curve fitting.

1. Calculate the average absorbance for each pair of duplicate test results.
2. The calibration curve is generated by the corrected absorbance of all calibration levels on the ordinate against the calibrator concentration. Appropriate computer assisted data reduction programs should be used for the calculation of results.

The total 25 OH vitamin D concentrations for the test samples are read directly from the calibration curve using their respective average absorbance.

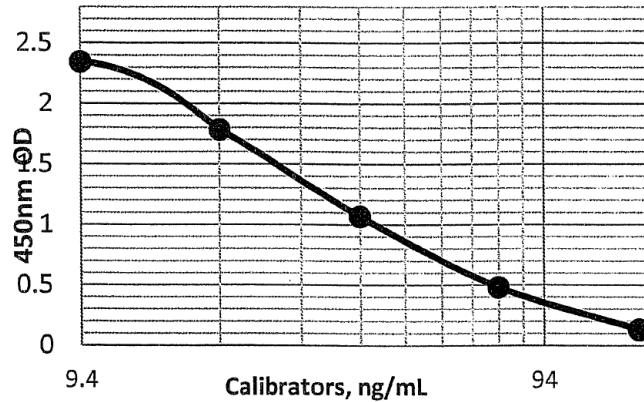
EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting calibration curve from this Mouse/Rat 25OH Vitamin D ELISA is represented. **This curve should not be used in lieu of calibration curve generated with each assay.**

Well I.D	OD 450 nm Absorbance		B/B0
	Readings	Average	
Cal-0: 0 ng/mL	3.123	3.159	100%
	3.195		
Cal-1: 9.4 ng/mL	2.729	2.723	86%
	2.717		
Cal-2: 18.8 ng/mL	2.181	2.152	68%
	2.123		
Cal-3: 37.5 ng/mL	1.219	1.258	40%
	1.297		
Cal-4: 75 ng/mL	0.468	0.454	14%
	0.440		
Cal-5: 150 ng/mL	0.159	0.158	5%
	0.156		



25-OH Vitamin D Calibrator Curve



EXPECTED VALUES

Dietary intake, race, season and age are known to affect the normal levels of Total 25-OH Vitamin D. The following data is provided for guidance only. It is important for each laboratory to establish its own reference ranges, which may better represent its typical population and region.

Recent literature has suggested the following ranges for the classification of Total 25-OH Vitamin D status:

Level	ng/mL
Severe Deficiency	<10
Insufficient	10-24
Optimal	25-100
Potential Toxicity	>100

The Endocrine Society Clinical Practice Guideline (2011) has suggested a higher target level of at least 30 ng/ml (see ref. #6):

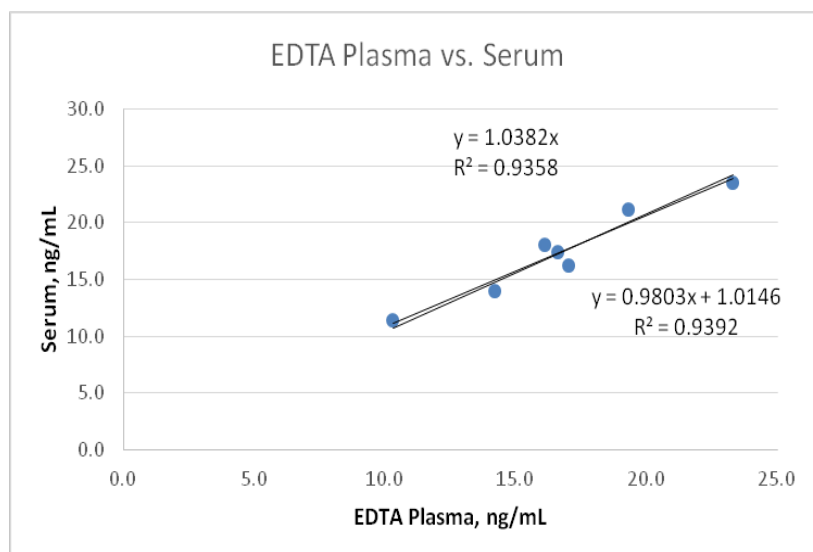
Level	ng/mL
Deficiency	<20
Insufficient	20-29
Sufficiency	30-100

We have validated the above reference range with 56 apparently healthy individuals. Donors that were not taking Vitamin D supplements from which samples were collected were tested. Patient EDTA plasma and serum were used to obtain the summarized data below.

	ng/mL
Mean	32.4
Highest	74.6
Lowest	12.6



Donor serum and EDTA plasma paired samples were correlated using this kit. The result yielded an excellent slope and correlation.



LIMITATION OF THE PROCEDURE

1. This Mouse/Rat 25-OH Vitamin D ELISA Assay Kit requires serum or plasma sample for testing.
2. Serum or plasma samples from different species may show different matrix background.
3. For sample values greater than 150 ng/mL, it is recommended to re-assay samples with dilution (i.e. 1:10 or 1:100). The best dilution matrix is vitamin D free human serum.
4. Cell culture or tissue culture samples should be validated with total binding and other performance specifications before being used.
5. Severely hemolyzed samples, icteric or lipaemic sample should not be used
6. If Spike Recovery is desired, use controls to spike into the samples.

QUALITY CONTROL

The performance of the Mouse/Rat 25-OH Vitamin D ELISA Assay Kit was determined a correlation study test using an FDA approved kit 25-OH Vitamin D ELISA test. To assure the validity of the results each assay should include adequate controls with known Vitamin D levels. We recommend that all assays include the laboratory's own Vitamin D controls in addition to those provided with this kit.

PERFORMANCE CHARACTERISTICS

Sensitivity

The Limit of Blank (LoB) was calculated by measuring the Calibrator zero in 16 replicates and calculating the 95th percentile of the distribution of the test values. The LoB was calculated to be 1.000ng/mL

The Limit of Detection (LoD) was calculated by measuring the Calibrator 0, 1, and a low sample and calculating the 95th percentile of the distribution of the test values. The LoD was calculated to be 4.781ng/mL

The Limit of Quantitation (LoQ) was calculated to be 8.558ng/mL.



Specificity

Cross reactivity of this Mouse/Rat 25-OH Vitamin D ELISA kit was determined by testing sera with spiked and unspiked cross reactants. The results are summarized in the following table:

Compound and Concentration	% Cross reaction
25-OH Vitamin D ₃ at 10ng/mL	100
25-OH Vitamin D ₂ at 10ng/mL	100
1,25(OH) ₂ Vitamin D ₃ at 200 ng/mL	20
1,25(OH) ₂ Vitamin D ₂ at 690 ng/mL	1.9
Vitamin D ₃ at 200ng/mL	2.9
Vitamin D ₂ at 200ng/mL	1.3
24,25(OH) ₂ Vitamin D ₃ at 20 ng/mL	>100
24,26(OH) ₂ Vitamin D ₃ at 4 ng/mL	>100
3-epi 25OH Vitamin D ₃ at 20 ng/mL	0.1

Interference

Interference was tested by spiking (95%) serum and plasma samples with (5%) concentrations of hemoglobin, lipid, and bilirubin. The results are provided below:

	Results	Bias	Amount (mg/mL)
Test Control	13.263	-	-
Bilirubin	13.014	-2%	10
	12.877	-3%	2
	13.443	1%	0.4
Test Control	18.699	-	-
Hemoglobin	20.389	9%	10
	18.409	-2%	2
	17.738	-5%	0.4
Lipids	18.541	-1%	200
	18.197	-3%	40
	15.786	-16%	8



	Results	Bias	Amount (mg/mL)
Test Control	38.645	-	-
Bilirubin	41.13	6%	10
	39.837	3%	2
	37.574	-3%	0.4
Test Control	63.075	-	-
Hemoglobin	64.491	2%	10
	67.079	6%	2
	69.287	10%	0.4
Lipids	64.176	2%	200
	59.401	-6%	40
	52.97	-16%	8

Precision

The intra-assay precision was validated by measuring three samples in sixteen (16) replicate determinations. The inter-assay precision was validated by measuring three samples in twelve (12) different assays in duplicate. The results indicate below:

Intra-Assay Precision			
	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	94.6	50.2	26.2
Std Dev	1.4	2.8	2.4
%CV	1.4%	5.5%	9.0%

Inter-Assay Precision			
	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	22.17	60.51	48.60
Std Dev	1.672142	4.581008	2.53009
%CV	7.5%	7.6%	5.2%

Linearity

Three (3) calibrators were diluted with standard matrix and tested. The results of dilution recovery value are summarized as follows:



Samples	Expected	Observed %	Recovery
Calibrator A	135.089	-	-
80%	108.0712	117.862	109%
60%	81.0534	94.227	116%
40%	54.0356	57.989	107%
20%	27.0178	28.486	105%
Calibrator B	120.639	-	-
80%	96.5112	102.441	106%
60%	72.3834	73.992	102%
40%	48.2556	46.322	96%
20%	24.1278	24.973	104%
Calibrator C	87.229	-	-
80%	69.7832	68.734	98%
60%	52.3374	49.002	94%
40%	34.8916	34.0894	98%
20%	17.4458	17.861	102%

Three (3) samples were diluted with Vitamin D-free human serum and tested. The results of dilution recovery value are summarized as follows:

Samples	Expected	Observed %	Recovery
Serum 1	146.749	-	-
80%	117.3992	124.74	106%
60%	88.0494	98.388	112%
40%	58.6996	60.741	103%
20%	29.3498	27.408	93%
Serum 2	129.63	-	-
80%	103.704	109.594	106%
60%	77.778	77.106	99%
40%	51.852	47.916	92%
20%	25.926	22.001	85%
Serum 3	91.502	-	-
80%	73.2016	74.395	102%
60%	54.9012	50.319	92%
40%	36.6008	30.751	84%
20%	18.3004	16.534	90%



Spike Recovery

Calibrators were spiked each other in equal volume and assayed. The results indicate below:

Samples	Expected	Observed	% Recovery
Cal 1: 9.4 ng/mL	-	9.4	-
+ Cal 3: 37.5 ng/mL	23.45	26.448	113%
+ Cal 4: 75.0 ng/mL	42.2	41.662	99%
+ Cal 5: 150.0 ng/mL	79.7	85.259	107%
Cal 2: 18.8 ng/mL	-	18.8	-
+ Cal 3: 37.5 ng/mL	28.15	29.836	106%
+ Cal 4: 75.0 ng/mL	46.9	48.15	103%
+ Cal 5: 150.0 ng/mL	84.4	93.95	111%
Cal 3: 37.5 ng/mL	-	37.5	-
+ Cal 4: 75.0 ng/mL	56.25	54.931	98%
+ Cal 5: 150.0 ng/mL	93.75	106.491	114%
Cal 4: 75.0 ng/mL	-	9.4	-
+ Cal 5: 150.0 ng/mL	112.5	128.548	114%

Six (6) serum/plasma samples were spiked with each other in equal volume and tested. The results indicate below.

Samples	Expected	Observed	% Recovery
Sample 1:	-	65.472	-
+ Sample A: 67.774	66.623	66.715	100%
+ Sample B: 68.757	66.6145	76.127	114%
+ Sample C: 20.025	42.7485	44.484	104%
Sample 2:	-	43.743	-
+ Sample A: 67.774	55.7585	58.859	106%
+ Sample B: 67.757	55.75	54.864	98%
+ Sample C: 20.025	31.884	30.081	94%
Sample 3:	-	19.137	-
+ Sample A: 67.774	43.4555	39.619	91%
+ Sample B: 68.757	43.447	39.444	91%
+ Sample C: 20.025	19.581	19.621	100%

Hook Effect

The hook effect was validated using a high concentrated 25-OH-D3 stock. The assay showed no hook effect at a concentration of 19.65µg/mL (19,650 ng/mL).

Assay Delay

The assay delay was tested using real human samples. The samples were added after the calibrators in different times.



Samples	Concentration	%Bias
Sample 1	18.516	-
After 5 min	20.414	10%
After 15 min	18.094	-2%
After 25 min	19.481	5%
Sample 2	65.839	-
After 5 min	68.271	4%
After 15 min	57.089	-13%
After 25 min	62.023	-6%

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Mouse/Rat 25 OH Vitamin D ELISA: Condensed Assay Protocol

25 μ L calibrators, controls, and unknown samples
+ 100 μ L Incubation Buffer



*Incubate @Room Temperature for 60 min, shaking
Wash 5x*

100 μ L Working Biotin-Vitamin D



*Incubate @Room Temperature for 30 min, shaking
Wash 5x*

100 μ L Working Conjugate Streptavidin



*Incubate @Room Temperature for 20 min, shaking
Wash 5x*

100 μ L HRP Substrate



Incubate @Room Temperature for 20 min, static

100 μ L Stop Solution



Immediately

Read absorbance at 450 nm (4 – parameter)

Within 10 minutes



Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

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For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.