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biosensis® LR³IGF-I *Rapid*™ ELISA Kit: Human

Catalogue Number: BEK-2233-1P/2P

For the quantitative determination of LR³IGF-I in cell culture supernatants only if used as directed.

ASSAY SHOULD BE VALIDATED FOR SPECIFIC PURPOSE IN THE USER LABORATORY

For research use only, not for use in clinical and diagnostic procedures.

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1. Intended Use

This LR³IGF-I *Rapid*™ ELISA kit has been developed and validated to quantify LR³IGF-I protein in cell culture medium. It is primary use is to measure LR³IGF-I in media and during downstream processing of media following a production cycle and is not intended for other use.

For research use only. Not for diagnostic and clinical purposes.

2. Introduction

Human LR³ insulin-like Growth Factor-I (LR³IGF-I) is an 83 amino acid analogue of human IGF-I comprising the complete IGF-I sequence with the substitution of an Arginine for the Glutamine at position 3, plus a 13 amino acid extension peptide at the N-terminus.

Human LR³IGF-I is more potent than IGF-I *in vitro* and *in vivo*. This increased potency is due to reduced binding of LR³IGF-I to most of the IGF binding proteins that modify the biological actions of IGF-I. Human LR³IGF-I binds to the type I IGF receptor with similar affinity to wild type IGF-I.

LR³IGF-I was developed by GroPep Bioreagents specifically for supplementation of mammalian cell culture to support the survival and proliferation of cells. It is engineered to have a higher biological potency than native IGF-I or IGF-II and has several advantages over recombinant insulin. Supplementation of cell cultures with LR³IGF-I at a much lower concentration results in equivalent or better productivity than supplementation with standard concentrations of insulin. LR³IGF-I is better able to stimulate the type I IGF receptor and thus induce a higher level of activation of intracellular signalling molecules which are responsible for promoting cell survival by inhibition of apoptosis.

This LR³IGF-I *Rapid*™ ELISA kit combines GroPep Bioreagents' many years of expertise in the field of IGF research and Biosensis' established *Rapid*™ ELISA platform. This collaboration has resulted in the new LR³IGF-I *Rapid*™ ELISA kit which provides the sensitive, specific and reliable quantification of LR³IGF-I protein in less than 3 hours!

The ELISA kit consists of a pre-coated mouse monoclonal anti- LR³IGF-I capture antibody, a biotinylated anti- LR³IGF-I detection antibody and horseradish peroxidase (HRP)-conjugated streptavidin. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the concentration of LR3IGF-I present in samples and the supplied protein standard.

The provided Quality Control (QC) sample serves as a positive control with a defined concentration range of LR³IGF-I protein, formulated in a stabilized buffer solution and designed to assure assay performance.

NOTE: For research use only. Not for clinical and diagnostic purposes.

3. Protocol Summary

1. Prepare duplicate serial dilutions of standard and samples.
2. Add 100 uL standards, samples and blank to the pre-coated wells of the appropriate wells. Seal the plate and incubate on a shaker (140 rpm; 0.351 G*) for 90 minutes at room temperature.
3. Discard well contents and perform 5 washings with 1x wash buffer (200 uL per well).
4. Add 100 uL of the detection antibody (1x). Seal the plate and incubate on a shaker (140 rpm; 0.351 G*) for 30 minutes at room temperature.
5. Discard the solution inside the wells and wash as described above.
6. Add 100 µL of the 1x streptavidin-HRP conjugate into each well. Seal the plate and incubate on a shaker (140 rpm; 0.351 G*) for 30 minutes at room temperature.
7. Discard the solution inside the wells and wash as described above.
8. Add 100 uL of TMB to each well. Incubate the plate for 6-10 minutes at room temperature in the dark.
9. Add 100 uL of Stop Solution to each well. The color changes from blue to yellow. Read absorbance at 450 nm within 30 minutes of stopping the reaction.

* RCF= 1.12 x Orbit Radius x $(rpm/1000)^2$

4. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
LR³IGF-I antibody coated 96 well microplate	12 x 8 wells	24 x 8 wells
Assay diluent A (1x)	2 x 25 mL	4 x 25 mL
Recombinant LR³IGF-I standard	2 x 200 ng	4 x 200 ng
Quality Control (QC) Sample	2 Vials (see vial label for amount)	4 Vials (see vial label for amount)
LR³IGF-Idetection antibody (100x)	1 x 110 µL	2 x 110 µL
Streptavidin-HRP (100x)	1 x 110 µL	2 x 110 µL
Wash buffer (10x)	1 x 33 mL	2 x 110 µL
TMB substrate (1x)	1 x 11 mL	2 x 33 mL
TMB stop solution (1x)	1 x 11 mL	2 x 11 mL
Plate sealer	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard and QC sample	Use on same day; aliquot unused standard to prevent multiple freeze-thaw cycles and store at -20°C
Diluted detection antibody and HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer (1x)	2 weeks at 2-8°C

Note:

- Do not freeze the streptavidin-HRP conjugate.
- Do not use assay diluents from other ELISA kits.

5. Equipment Required but Not Supplied

- Single- and multi-channel pipettes capable of delivering 10-1000 µL volumes.
- Plastic and glassware for sample collection, sample preparation and buffer preparation.
- Plate shaker.
- Microplate reader capable of reading absorbance at 450 nm.

6. Before You Start….

- Read the entire protocol to familiarize yourself with the assay procedure; a tutorial about the use of *Rapid*™ ELISA kits is available online at: https://www.youtube.com/watch?v=7EOuc9qYL0E
- Bring the microplate and all reagents and solutions to room temperature before starting the assay
- Remove only the required amount of TMB substrate from the fridge to prevent multiple warm-ups and cool downs; keep the TMB substrate in the dark
- Remove the number of strips required and return unused strips to the pack and reseal
- Centrifuge all kit components to collect reagents to the bottom of tubes before use
- The stop solution provided with this kit is an acid; wear protective equipment when handling
- Please visit our Technical Notes section at www.biosensis.com for further information and helpful hints on ELISA-related topics

Careful experimental planning is required to determine the total number of samples that can be assayed per plate. Refer to Appendix A for help in determining the number of microplates required to conduct an ELISA assay experiment.

7. Sample Preparation

For unknown concentrations of LR³IGF-I in samples, it is important to perform several dilutions of the sample to allow the LR³IGF-I concentration to fall within the range of the LR³IGF-I standard curve (3.1 - 200 ng/mL).

Cell Culture Supernatants

- Remove particulates by centrifugation (10,000 x g for 5 minutes).
- Analyze immediately or store at -20°C in aliquots to prevent multiple freeze thaw cycles.
- If required, dilute samples with LR³IGF-I negative cell culture medium in order to measure LR³IGF-I concentrations. When testing in-house samples, Biosensis usually dilutes conditioned media with an equal quantity of fresh, serum-free medium (see table below: 'Recovery in Culture Media with 10%FCS').

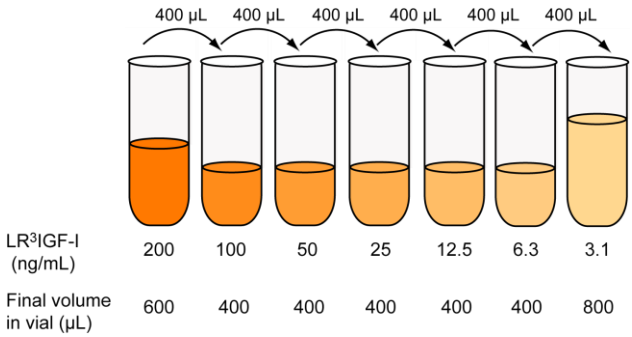
Note: Samples at extremes of pH should be buffered to neutral pH before adding to the assay wells. Since LR³IGF-I has a low affinity for insulin-like growth factor binding proteins, there is no requirement for an extraction step.

8. Preparation of LR³IGF-I Standard

- Reconstitute the lyophilized antigen standard with 1 mL of the **same diluent used for preparing sample dilutions.**
- Label the vial with the reconstituted LR³IGF-I standard as “200 ng/mL”; vortex and let stand for 15 minutes.
- **Note:** 200 ng/mL is the highest concentration of the LR³IGF-I standard curve.

In order to generate an LR³IGF-I standard curve, perform a 1:2 serial dilution down to 3.1 ng/mL. The volumes used for the dilution series depends on the number of repeats per LR³IGF-I concentration. For triplicate measurement (100 µL per well) of each LR³IGF-I standard concentration, we recommend this procedure:

1. Label 6 tubes with “100 ng/mL”, “50 ng/mL”, “25 ng/mL”, “12.5 ng/mL”, “6.3 ng/mL” and “3.1 ng/mL”, respectively.
2. Aliquot 400 µL of the sample diluent into each tube.
3. Take 400 µL from the “200 ng/mL” tube and transfer to the “100 ng/mL” tube.
4. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex.
5. Repeat steps 3 and 4 for each consecutive concentration until the last tube “3.1 ng/mL” is prepared and mixed well.



9. Other Reagents and Buffer Preparation

- Quality Control (QC) sample: Reconstitute the lyophilized sample with 1 mL of the same diluent used for preparing the LR³IGF-I standard curve. This will provide a QC sample within 70 – 130 ng/mL
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent A; **do not use** culture medium or other buffers and prepare enough volume to add 100 µL per well.
- Wash buffer (10x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly and dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

10. Assay Procedure

All steps are performed at room temperature (20-25°C, 70-75°F).

1. Add 100 µL of diluted LR³IGF-I standards, QC sample, samples and blank (sample diluent only) to the pre-coated microplate wells.
2. If available, include a negative and positive control sample in the assay procedure.
3. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 90 minutes.
4. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 µL per well). See the technical hints section for a detailed description of the washing procedure.
5. Add 100 µL of the detection antibody (1x) into each well.
6. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes.
7. Discard the solution inside the wells and wash as described in step 4.
8. Add 100 µL of the 1x streptavidin-HRP conjugate into each well.
9. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes.
10. Discard the solution inside the wells and wash as described in step 4.
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 6-15 minutes without shaking in the dark.
12. Stop the reaction by adding 100 µL of the stop solution into each well. Visible blue color will change to yellow. Read the absorbance at 450 nm on a plate reader. **Note:** Color will fade over time; hence, we recommend plate to be read within 5 minutes after adding the stop solution or no longer than 30 minutes after addition.

13. See Appendix B for a troubleshooting guide when unexpected difficulties are encountered.

* RCF= 1.12 x Orbit Radius x (rpm/1000)²

11. Technical Hints

1. Do not perform dilutions within the well.
2. At least duplicate measurements for each standard and sample dilution is recommended.
3. Dilute samples to an LR³IGF-I concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings.
4. Avoid touching the inside surface of the wells with the pipette tip.
5. Proper emptying and washing the plate is crucial for low background and non-specific binding. For manual plate washing, we recommend the following procedure:
    a. To remove liquid from the wells, place the plate on the palm of the hand and quickly invert the plate over the sink. Forcefully move the arm downwards and stop abruptly to force the liquid out of the wells. When done correctly, this technique should prevent liquid from getting onto the fingers or onto the outside of the microplate wells.
    b. Blot and forcefully tap the microplate against clean paper towels for 3-5 times.
    c. Wash the wells by pipetting 200 µL of wash buffer into each well and empty the wells as described in step a-b).
    d. Repeat this procedure for a total of 5 times.
6. Complete removal of liquid from the wells at each step is essential for reliable results. However, avoid letting the wells dry out at any time.
7. Add TMB and the stop solution to the wells in the same order.
8. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading.
9. Stopping the TMB reaction after 5 minutes is usually sufficient to obtain a very sensitive standard curve. However, TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm and perform all incubations at room temperature (20-25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.

12. Calculation of Results

1. Average the readings for each LR³IGF-I standard concentration, blank and sample.
2. Plot a standard curve with the LR³IGF-I standard concentration on the x-axis and the OD at 450 nm on the y-axis.
3. If values for the LR³IGF-I standards are adjusted for background absorbance, then subtract the blank value from the OD_{450} of the samples as well.
4. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression analysis
5. Perform a 4-PL regression analysis to calculate the concentration of LR³IGF-I in the QC sample. An observed concentration within the range of 70 –130 ng/mL indicates acceptable assay performance
6. Perform a regression analysis to calculate the concentration of LR³IGF-I in the samples. Multiply the result by the sample dilution factor.

Manual Plate Reading

The relative OD_{450} = (the OD_{450} of each well) – (the OD_{450} of Zero well).

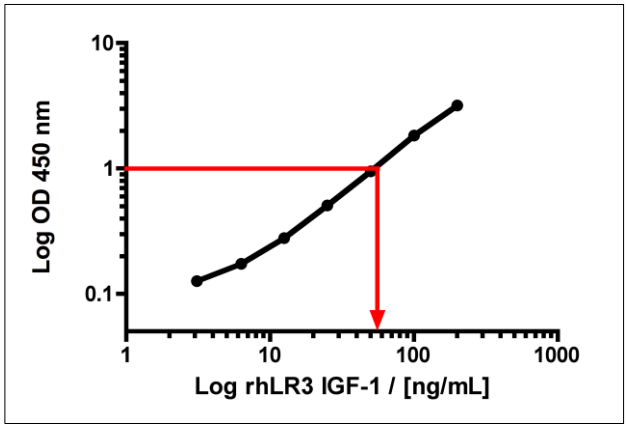
1. The **standard curve** can be plotted as the relative OD_{450} of each LR³IGF-I standard solution (Y-axis) vs. the respective known concentration of the LR³IGF-I standard solution (X-axis). The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding OD_{450}. The greater the concentration of target protein in the solution, the higher the OD_{450}
2. **Determine concentration of target protein in unknown sample.** The LR³IGF-I concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of LR³IGF-I in the unknown sample. In the exemplary standard curve on the right, a sample with OD_{450} = 1 reads as 58 ng/mL LR³IGF-I (red line). If the samples measured were diluted, multiply the concentrations from interpolation (see step 1) with

the dilution factor to obtain the actual LR^{3}IGF-I concentration in the sample.

13. Typical Data

Standard Curve

Standard curves are provided for demonstration only. A standard curve has to be generated for each LR³IGF-I ELISA assay.



In addition, we recommend measuring common samples when performing multiple assays across several days in order to normalize the standard curve numbers between the various runs.

In the above example graph, LR³IGF-I standards were run in triplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 6.5 minutes.

Typical optical densities and coefficient of variations for diluted standards are summarized in the following table:

	OD 450 nm			
LR³IGF-I/ [ng/mL]	**Mean**	**SD**	**SEM**	**CV**
200	3.174	0.058	0.034	1.89%
100	1.828	0.021	0.012	1.21%
50	0.950	0.019	0.011	2.00%
25	0.506	0.001	0.001	0.19%
12.5	0.278	0.009	0.005	3.03%
6.3	0.173	0.009	0.005	4.70%
3.1	0.126	0.002	0.001	1.50%
Blank	0.071	0.001	0.001	1.02%

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Limit of Detection

This LR³IGF-I ELISA kit detects a minimum of 1.0 ng/mL LR³IGF-I (defined as 150% of blank value).

Assay Precision

Two samples were assayed neat in triplicates on 3 different days.

		LR³IGF-I / [ng/mL]			Intra-Assay	Inter-Assay
		Mean	**SD**	**SEM**	**CV**	**CV**
1	Day 1	103	4.65	1.90	4.5%	4.3%
	Day 2	99	3.84	1.57	3.9%	
	Day 3	102	4.71	0.90	4.6%	
2	Day 1	102	3.03	1.24	3.0%	3.9%
	Day 2	98	5.07	2.07	5.2%	
	Day 3	100	3.60	1.37	3.6%	

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Recovery in Culture Media with 10%FCS

50 ng/mL of LR³IGF-I were spiked into DMEM/F12 with 10%FCS at different dilutions and recovery of LR³IGF-I.

Dilution	Recovery of LR³IGF-I
Undiluted	91%
1/2	101%
1/4	100%

Specificity

The assay is intended for quantification of LR³IGF-I. Cross-reaction with human IGF-I is 32%. Cross-reaction with human IGF-II is less than 0.01%

14. Other Information

For the latest IGF research reagents, LR³IGF-I (Catalogue # AM001, BU020) and its antibodies, please visit the GroPep Bioreagents website www.gropep.com

Biosensis offers a variety of *Rapid*™ ELISA kits for your research applications.

Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments

Standard curve, blank and controls:

- Standard (200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.3 ng/mL, 3.1 ng/mL): 7 wells
- Blank (0 pg/mL): 1 well
- Control vial (either provided in the ELISA kit or the researcher): 1 well

Gives a total of 9 wells used per Standard/Control set.

We recommend that the standard curve, blanks, and controls be run on each separate plate for the most accurate calculations. Note that if two (or more) independent assays are run on one plate, each assay requires its own Standard/Control set. We also recommend that ALL samples (i.e. standards, controls, blanks and test samples) be performed in duplicate at least.

Thus, for standards, blanks and controls, 9 x 2 = **18 standard wells are required per assay.** This leaves 96 wells – 18 wells = **78 sample wells per plate** for test samples for EACH of our standard 1P ELISA kits run with a standard curve and controls.

1P kit: 78 experimental wells per 96 well ELISA plate experiment
2P kit: 156 experimental wells per 192 well ELISA plate experiment

The 2-Plate Optional Single Control Set:
Some researchers using our 2P kits run a single, duplicate standard curve, blank and control sample set (18 wells) for the entire 2-plate experiment. This allows 78 test samples on the “standard curve plate” (the plate containing the standard curve, controls and blanks) and a full 96 test samples on the “test only plate” for a total available number of 174 tests per 2 plate kit. (78 wells +96 wells = 174).

2P kit, single set of controls (in duplicate): 174 experimental wells per 192 well ELISA plate experiment

Example: 60 test samples, duplicate measurement at one single dilution
How many wells and plates do I need for sixty test samples at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 samples, x 2 draws each (e.g. before and after) = 120 stock samples
2. Factor in the number of repeat tests desired, in order to calculate the number of wells required. Biosensis recommends that ALL samples be run in duplicate at least.
3. Multiply the number of stock samples by the number of dilutions per sample. In this example, we will be testing EACH stock sample at a single dilution (i.e. 1:100) in duplicate, so 120 stock samples x 2 wells = 240 microplate wells required for sixty test samples. In summary: 60 samples, 4 tests per sample, equals 240 total number of wells required.
4. Decide on the number of standards, blanks and controls run on each plate. We recommend standard, control and blank duplicates for each 96-well plate. Alternatively, one standard, blank and control duplicate set can be run on every 2 plates (2-Plate Optional Single Control Set option discussed above).

Running the Test:
Running the 60 test sample experiment with a standard curve, blanks and controls on *each* test plate, in duplicate (our recommended option):

Single Dilution per Test Sample:

(240 wells required) / (78 wells per plate available) = 3.077 plates required, or just over 3 plates, thus researcher will need to order **4 x 1-plate kits or 2 x 2-plate kits** (there will be unused wells) to ensure enough wells for the entire sixty test samples, tested in duplicate (two draws per sample, 1 dilution, 4 wells per test, total of 240 wells). The unused 8-well strips can be used for other assays later.

Two Dilutions per Test Sample:

If testing at two dilutions, (e.g. 1:50 & 1:100 per sample) and doing duplicates, then the number of wells/tests is doubled to 480 (e.g. 120 x 2 @ 1:50 = 240, PLUS 120 x 2 @1:100 = 480). Then the number of plates is determined by (480 wells required) / (78 wells per plate available) = 6.15 plates required, or just over 6 plates, thus the researcher will need to order **3 x 2-plate kits and 1 x 1-plate kit** to ensure the minimum number of wells for sixty test samples tested in duplicate at two dilutions (two draws per test, 2 dilutions, 8 tests/wells per test, total of 480 tests/wells).

2-Plate Optional Single Control Set Option:

If running 60 test samples with a single dilution, and a single duplicate standard curve, blank, and control sample for every two plates, then one has **174 available test wells per 2P kit**.

For the single dilution, sixty test samples, 2 draws per sample experiment (240 tests), one would need (240/174) = 1.38 2-plate kits, or **2 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty test samples.

For the two dilutions per sample, sixty test samples, 2 draws per sample experiment (480 tests), one would need (480/174) = 2.76 2-plate kits, or 3 x 2-plate kits would need to be ordered to ensure enough wells for all sixty test samples.

Appendix B: Troubleshooting Guide

This LR³IGF-I ELISA kit has been developed to deliver reproducible results when following the provided assay protocol. Please refer to the troubleshooting guide below when unexpected difficulties are encountered. If you require further assistance, talk to a scientist at Biosensis (biospeak@biosensis.com).

Problem	Cause	Solution
High background (blank OD > 0.30)	Insufficient washing	Make sure to perform 5 washes after each incubation step with sample, detection antibody and streptavidin-HRP conjugate. Follow the recommended manual washing procedure for optimal washing performance
	Excessive concentration of detection antibody and/or HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume.
	Increased incubation time and temperature	Please follow incubation times as stated in the protocol and perform incubations at room temperature
	Contamination	Ensure that all plastic- and glassware used is clean. Do not use the same pipette tip to transfer samples and blank solution into the wells
Low absorbance readings	Concentration of LR³IGF-I in the sample is lower than the detection limit of this assay	Confirm that the assay protocol was carried out correctly by either spiking a known amount of LR³IGF-I into your sample and/or check that the QC sample value falls within the expected LR³IGF-I concentration range
	Insufficient antibody or insufficient HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume
	Reagents expired	Ensure correct storage of the kit and do not use the kit beyond its expiry date
Low absorbance readings	Decreased incubation times and temperature	Please follow incubation times as stated in the protocol and warm reagents to room temperature before use
	Microplate wells dried out	Do not leave microplate wells without solution for an extended time
	Wash buffer not diluted	Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution
	Stop solution not added	Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm
Standard OD values above plate reader limit	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
Sample OD values above standard curve range	LR³IGF-I concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
High coefficient of variations (CV)	TMB and stopping solution not added consistently	Add TMB and stopping solution to the wells in the same order and speed
	Inconsistent pipetting	Ensure pipettes are working correctly and are calibrated; review your pipetting technique; add standards and samples to the wells using a single-channel rather than a multi-channel pipette
	Insufficient mixing of reagents	Briefly vortex to mix solutions before pipetting into the wells
	Bottom of the plate is dirty	Clean the bottom of the plate before reading the plate

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