

ELISA Kit for Measuring Human Lactoferrin

CircuLex Human Lactoferrin ELISA Kit

Cat# CY-8089

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Intended Use

The CycLex Research Product **CircuLex Human Lactoferrin ELISA Kit** is used for the quantitative measurement of human lactoferrin in serum, plasma and other biological media.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

Introduction

Lactoferrin is an iron-binding glycoprotein of the transferrin family that is expressed in most biologic fluids and is a major component of mammals' innate immune system (1). It is a glycoprotein consisting of a single polypeptide chain of about 80 kDa with two globular lobes each containing an iron-binding site. It is found in most exocrine secretions including milk, particularly the colostrums, and other secretions, such as tears, saliva, intestinal mucus and genital secretions, and in the specific granules of neutrophils.

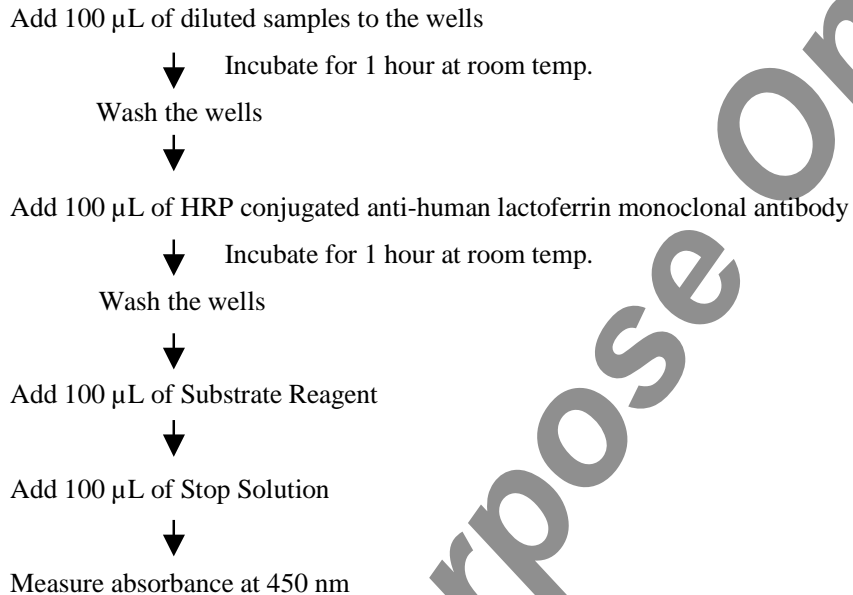
The physiological roles that have been proposed for lactoferrin include anti-inflammatory, immunomodulatory, antimicrobial, antiviral and antitumoral functions. For this reason, lactoferrin is regarded as a host-defense mediator. Specific lactoferrin receptors exist in a variety of cells, including monocytes, lymphocytes, adipocytes, hepatocytes, and endothelial cells (2). Therefore determination of the lactoferrin concentration in various body fluids can be a marker of inflammation (3-6). It was reported that fecal lactoferrin is useful as a sensitive and specific marker in identifying intestinal inflammation such as Crohn's disease and chronic inflammatory bowel disease (IBD) (7). Combination of several markers, such as S100A8/A9 complex, defensin, elastase, MPO and I-FABP may be useful for classifying IBD.

Lactoferrin also directly interacts with modified LDL to prevent its interaction with scavenger receptors (8, 9). A region rich in basic amino acid residues near the lactoferrin N-terminus is responsible for the interaction with acetylated or oxidized LDL.

Principle of the Assay

The CycLex Research Product **CircuLex Human Lactoferrin ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human lactoferrin is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any human lactoferrin present. After washing away any unbound substances, an HRP conjugated monoclonal antibody specific for human lactoferrin is added to the wells. Following a wash to remove any unbound monoclonal antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H₂O₂-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of human lactoferrin. A standard curve is constructed by plotting absorbance values versus human lactoferrin concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure



Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-human lactoferrin monoclonal antibody (AF-2D12) as a capture antibody.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing 2% Tween®-20

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for reconstitution of Human Lactoferrin Standard and sample dilution. Ready to use.

Human Lactoferrin Standard: One vial containing 200 ng of lyophilized recombinant human lactoferrin.

HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-human lactoferrin monoclonal antibody (AF-1E4). Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H₂SO₄. Ready to use.

Materials Required but not Provided

- **Pipettors:** 2-20 μ L, 20-200 μ L and 200-1000 μ L precision pipettors with disposable tips
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation
- **Vortex mixer**
- **Microplate washer:** optional (Manual washing is possible but not preferable)
- **Plate reader:** capable of measuring absorbance in 96-well plates at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **Software package facilitating data generation and analysis :**optional
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residues from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain NaN_3 as preservatives. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Sample Collection, Storage and Preparation

Serum: Use a serum separator tube and allow samples to clot for 60 ± 30 minutes. Centrifuge the samples at 4°C for 10 minutes at 1,000 x g. Remove serum and assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of serum may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using EDTA-Na₂ as the anticoagulant. If possible, collect the plasma into a mixture of EDTA-Na₂ and Futhan5 to stabilize the sample against spontaneous *in vitro* complement activation. Immediately centrifuge samples at 4°C for 15 minutes at 1,000 x g. Assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of plasma may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Note: Citrate plasma has not been validated for use in this assay.

Other biological samples: Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles.

Sample Preparation

- Serum and plasma samples require a 100-fold dilution.
- Other biological samples require neat to appropriate dilution*.

*For example

Milk: 1.0×10^5 - to 1.0×10^6 -fold dilution

Tear: 1.0×10^5 - to 1.0×10^6 -fold dilution

Saliva: 1,000- to 5,000-fold dilution

Serum: 100- to 500-fold dilution

Urine: 1- to 10-fold dilution

Detailed Protocol

The CycLex Research Product **CircuLex Human Lactoferrin ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the Human Lactoferrin Standard within the kit should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer** and **Human Lactoferrin Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.
2. Reconstitute **Human Lactoferrin Standard** with **1.0 mL** of **Dilution Buffer**. The concentration of the human lactoferrin in vial should be **200 ng/mL**, which is referred as a **Master Standard** of human lactoferrin.

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20 ng/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	100 µL of Master Standard (200 ng/mL)	900 µL	20 ng/mL
Std.2	300 µL of Std. 1 (20 ng/mL)	300 µL	10 ng/mL
Std.3	300 µL of Std. 2 (10 ng/mL)	300 µL	5 ng/mL
Std.4	300 µL of Std. 3 (5 ng/mL)	300 µL	2.5 ng/mL
Std.5	300 µL of Std. 4 (2.5 ng/mL)	300 µL	1.25 ng/mL
Std.6	300 µL of Std. 5 (1.25 ng/mL)	300 µL	0.63 ng/mL
Std.7	300 µL of Std. 6 (0.625 ng/mL)	300 µL	0.31 ng/mL
Blank	-	300 µL	0 ng/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Master Standard should be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

Standard Assay Procedure for Human Lactoferrin

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.
2. Dilute serum sample with Dilution Buffer. (See "Sample Preparation" above.)
3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells.
4. Incubate the plate at room temperature (ca.25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.
7. Incubate the plate at room temperature (ca.25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
9. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed
10. Incubate the plate at room temperature (ca.25°C) for 10-20 minutes, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.
11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.
12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 0.25 units for the blank (zero concentration), or 3.0 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 30 minutes.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine human lactoferrin concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the human lactoferrin concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding human lactoferrin concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

1. The dose-response curve of this assay fits best to a sigmoidal 4-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.
2. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the 4-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).

Measurement Range

The measurement range is 0.31 ng/mL to 20 ng/mL. Any sample reading higher 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 human lactoferrin concentration.

Troubleshooting

1. The Human Lactoferrin Standard should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.
2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.
3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the CycLex Research Product **CircuLex Human Lactoferrin ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the reconstituted Lactoferrin Standard must be stored at below -70°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

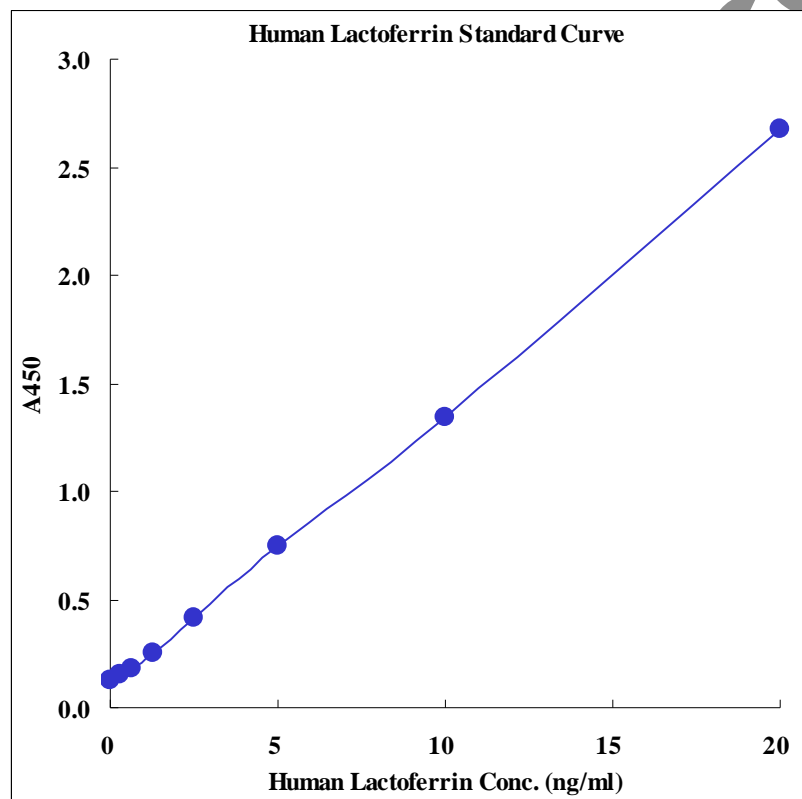
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of human lactoferrin giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 259 pg/ml of sample.

* Dilution Buffer is pipetted into blank wells.

Typical Standard Curve



2. Precision

Intra-assay Precision (Precision within an assay)

Five samples of known concentration were tested seven times on one plate to assess intra-assay precision.

- Intra-assay (Within-Run, n=7) CV=4.2-7.3 %

Human lactoferrin concentration

	Sample 1 (ug/ml)	Sample 2 (ug/ml)	Sample 3 (ng/ml)	Sample 4 (ng/ml)	Sample 5 (ug/g)
1	1454.6	1375.8	39.9	328.7	9.0
2	1483.9	1447.7	39.1	338.4	7.6
3	1597.5	1477.5	38.2	330.0	8.4
4	1701.3	1308.3	38.9	343.1	8.9
5	1423.6	1333.9	35.5	330.0	7.5
6	1464.2	1271.1	36.4	340.0	8.1
7	1509.7	1356.1	37.1	383.4	8.4
max.	1701.3	1477.5	39.9	383.4	9.0
min.	1423.6	1271.1	35.5	328.7	7.5
mean	1519.3	1367.2	37.9	341.9	8.3
SD	97.4	73.8	1.6	19.2	0.6
CV(%)	6.4	5.4	4.2	5.6	7.3

Sample 1: Milk
 Sample 2: Tear
 Sample 3: Urine
 Sample 4: Serum
 Sample 5: Stool

Inter-assay Precision (Precision between assays)

Five samples of known concentration were tested in three separate assays to assess inter-assay precision.

- Inter-assay (Run-to-Run, n=3) CV=2.1-9.5 %

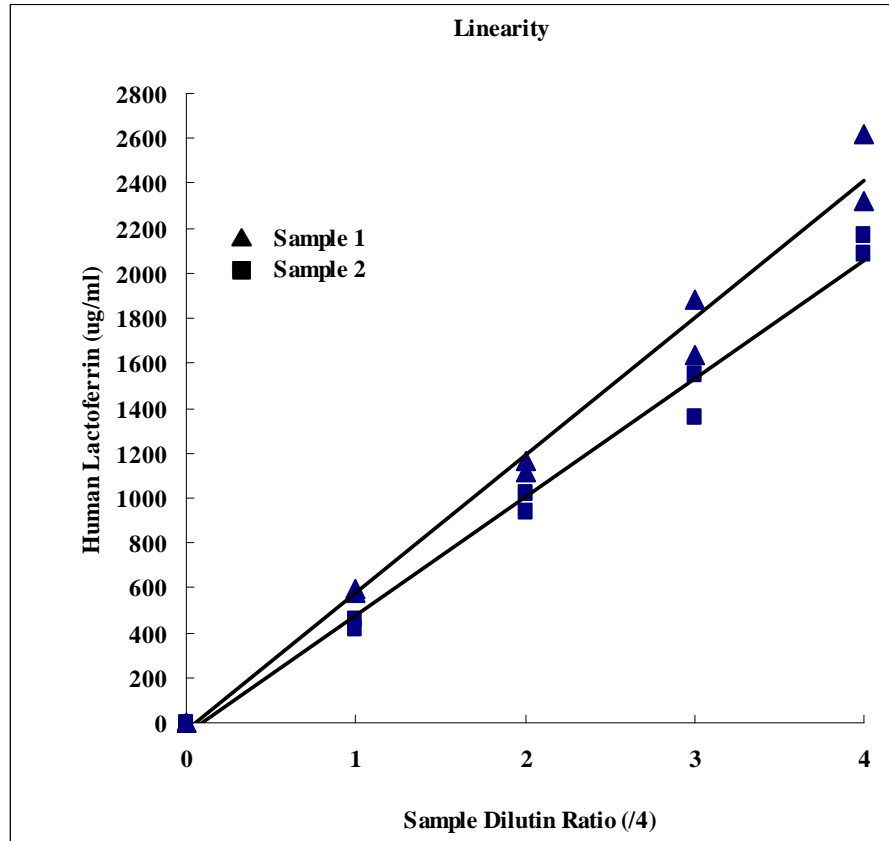
Human lactoferrin concentration

Assay	Sample 1 (ug/ml)	Sample 2 (ug/ml)	Sample 3 (ng/ml)	Sample 4 (ng/ml)	Sample 5 (ug/g)
1	1514.5	1420.1	37.8	324.8	8.0
2	1574.0	1413.6	40.6	377.0	8.9
3	1725.3	1366.9	36.7	323.5	9.7
max.	1725.3	1420.1	40.6	377.0	9.7
min.	1514.5	1366.9	36.7	323.5	8.0
mean	1604.6	1400.2	38.4	341.8	8.9
SD	108.7	29.0	2.0	30.5	0.8
CV(%)	6.8	2.1	5.2	8.9	9.5

Sample 1: Milk
 Sample 2: Tear
 Sample 3: Urine
 Sample 4: Serum
 Sample 5: Stool

3. Linearity

Two biological samples* were diluted with Dilution Buffer and assayed after dilution. The neat sample is set to 1 (4/4). Please note all samples including the neat sample are 200,000-fold diluted as stated in the Assay Procedure. The results are summarized in the figure below.



*Sample 1: Milk
Sample 2: Tear

Example of Test Results

Fig.1. Lactoferrin levels in many biological samples

a) In milk of healthy volunteers

sample	hLF conc. (mg/ml)
1	2.4
2	2.2
3	6.2
4	2.7

b) In tear of healthy volunteers

sample	hLF conc. (mg/ml)
1	5.3
2	1.8
3	5.3
4	4.7
5	1.3

c) In urine of healthy volunteers

sample	hLF conc. (ng/ml)
1	1.5
2	0.3
3	28.8
4	9.9
5	4.5

d) In saliva of healthy volunteers

sample	hLF conc. (ug/ml)
1	3.0
2	4.8
3	20.6
4	5.0
5	9.9
6	3.7

e) In serum of healthy volunteers

sample	hLF conc. (ng/ml)
1	474.5
2	260.7
3	296.8

f) In stool of healthy baby volunteers

sample	hLF conc. (ug/g)
1	776.4
2	90.4
3	33.4
4	55.2
5	18.6

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Related Products

* CircuLex S100A12/EN-RAGE ELISA Kit: Cat# CY-8058

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