ELISA Kit for Measuring Human Fibulin-3/EFEMP1

CircuLex Human Fibulin-3/EFEMP1 ELISA Kit

Cat# CY-8120

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Intended Use

The MBL Research Product **CircuLex Human Fibulin-3/EFEMP1 ELISA Kit** is used for the quantitative measurement of human Fibulin-3/EFEMP1 in serum and cell culture supernatant.

Individual users should determine appropriate conditions when using other types of samples.

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.
- Do not expose reagents to excessive light.

Introduction

Fibulin-3 is member of the fibulin family (1), also known as epidermal growth factor containing fibulin-like extracellular matrix protein 1 (EFEMP1), whose protein sequence contains a signal peptide, five tandem arrays of calcium binding EGF domains preceded by a modified EGF domain, and a C-terminal fibulin-type module (2).

It has been reported that Fibulin-3 was shown to act as tumor suppressors or activators in different cancers (3, 4). It has restricted expression in the body and is predominately localized in the extracellular matrix of elastic tissue (5). The levels of fibulin-3 expression have been found to be decreased in many cancer types due to promoter hypermethylation and have been correlated with poor survival of patients with lung cancer (6, 7), breast cancer (8), and hepatocellular carcinoma (9).

On the other hand, an increase in fibulin-3 was observed in malignant gliomas (10), cervical carcinomas (11), and pancreatic cancer (12). In addition, Fibulin-3 is suggested to be a new potential biomarker for malignant mesothelioma (13).

Principle of the Assay

The MBL Research Product **CircuLex Human Fibulin-3/EFEMP1 ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for human Fibulin-3/EFEMP1 is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any human Fibulin-3/EFEMP1 present. After washing away any unbound substances, an HRP conjugated antibody specific for human Fibulin-3/EFEMP1 is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H_2O_2-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 Fibulin-3/EFEMP1. A standard curve is constructed by plotting absorbance values versus human Fibulin-3/EFEMP1 concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure

Add 100 µL of diluted samples to the wells
↓ Incubate for 2 hours at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-human Fibulin-3/EFEMP1 antibody
↓ Incubate for 1 hour at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at room temp.
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

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 pre-coated with anti-human Fibulin-3/EFEMP1 monoclonal antibody as a capture antibody.

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

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for dilution of standards and samples. Ready to use.

Human Fibulin-3 Standard: One vial containing X* ng of lyophilized recombinant human Fibulin-3/EFEMP1.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-human Fibulin-3/EFEMP1 monoclonal antibody. 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 H_2SO_4. Ready to use.

Materials Required but not Provided

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/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.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**.
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- 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 residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. 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 Substrate Solution which contains hydrogen peroxide.
- **CAUTION: 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: Stop Solution is a strong acid. Wear disposable gloves and eye protection when handling the solution.**

Sample Collection and Storage

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.

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

Other biological samples: MBL has not tested.
(*e.g.* Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles. Individual users should determine appropriate conditions when using other types of samples.)

Detailed Protocol

The MBL Research Product **CircuLex Human Fibulin-3/EFEMP1 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 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 Fibulin-3 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 (**ddH_2O**). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute **Human Fibulin-3 Standard** with X* **µL** of **ddH_2O** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 150 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles. The concentration of the human Fibulin-3/EFEMP1 in vial should be **480 ng/mL**, which is referred to as the **Master Standard** of human Fibulin-3/EFEMP1.

***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

Prepare Standard Solutions as follows:

Use **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. **Std.1 (96 ng/mL)** serves as the highest standard. **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	120 µL of Master Standard (480 ng/mL)	480 µL	96 ng/mL
Std.2	300 µL of Std. 1 (96 ng/mL)	300 µL	48 ng/mL
Std.3	300 µL of Std. 2 (48 ng/mL)	300 µL	24 ng/mL
Std.4	300 µL of Std. 3 (24 ng/mL)	300 µL	12 ng/mL
Std.5	300 µL of Std. 4 (12 ng /mL)	300 µL	6 ng/mL
Std.6	300 µL of Std. 5 (6 ng/mL)	300 µL	3 ng/mL
Std.7	300 µL of Std. 6 (3 ng /mL)	300 µL	1.5 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. Discard any unused Standard Solutions after use.

Sample Preparation

Dilute samples with **Dilution Buffer**.

- Serum samples may require a 100 to 400-fold dilution.
- Cell culture supernatants require neat to appropriate dilution.

Assay Procedure

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 samples 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 2 hours**, 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 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.

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 the 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 optical density of the average zero standard. Plot the optical density versus the concentration of standards and draw the best curve. Most microtiter plate readers perform automatic calculations of analyte concentration. The standard curve fits best to a sigmoidal four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a four-parameter logistic function.

A standard curve is also to be constructed by plotting the absorbance (Y) versus log of the known concentration (X) of standards, using a cubic function. Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of optical density (Y) is plotted versus log of the known concentration (X) of standards). To determine the concentration of each sample, first find the optical density 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 concentration.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Measurement Range

The measurement range is **1.5 ng/mL** to **96 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 Fibulin-3/EFEMP1 concentration.

Troubleshooting

1. All samples and standards should be assayed 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 MBL Research Product **CircuLex Human Fibulin-3/EFEMP1 ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

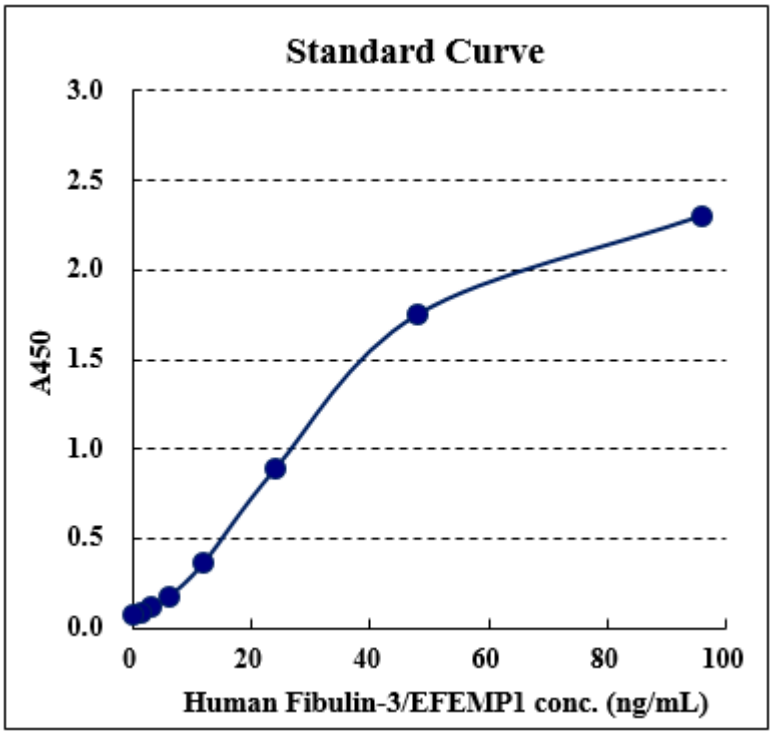
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of human Fibulin-3/EFEMP1 giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 0.626 ng/mL of sample.

* Dilution Buffer was pipetted into blank wells.

Typical Standard Curve



2. Precision

Intra-assay Precision (Precision within an assay)

Three samples* were tested sixteen times on one plate to assess intra-assay precision.

• Intra-assay (Within-Run, n=16) CV=1.9-3.4%

* Sample: Serum

Human Fibulin-3/EFEMP1 conc. (µg/mL)

	Sample 1	Sample 2	Sample 3
1	2.94	2.43	1.44
2	2.89	2.37	1.44
3	2.80	2.46	1.41
4	2.79	2.44	1.45
5	2.96	2.44	1.39
6	3.01	2.49	1.42
7	3.05	2.48	1.44
8	2.95	2.58	1.49
9	2.73	2.49	1.42
10	2.96	2.45	1.44
11	2.91	2.43	1.42
12	2.91	2.49	1.42
13	2.96	2.42	1.39
14	2.99	2.44	1.41
15	3.11	2.53	1.43
16	3.04	2.64	1.48
MAX.	**3.11**	**2.64**	**1.49**
MIN.	**2.73**	**2.37**	**1.39**
MEAN	**2.94**	**2.47**	**1.43**
S.D.	**0.10**	**0.06**	**0.03**
C.V.	**3.4%**	**2.6%**	**1.9%**

Inter-assay Precision (Precision between assays)

Three samples* were tested in five separate assays to assess inter-assay precision.

• Inter-assay (Run-to-Run, n=5) CV=2.3-7.1%

* Sample: Serum

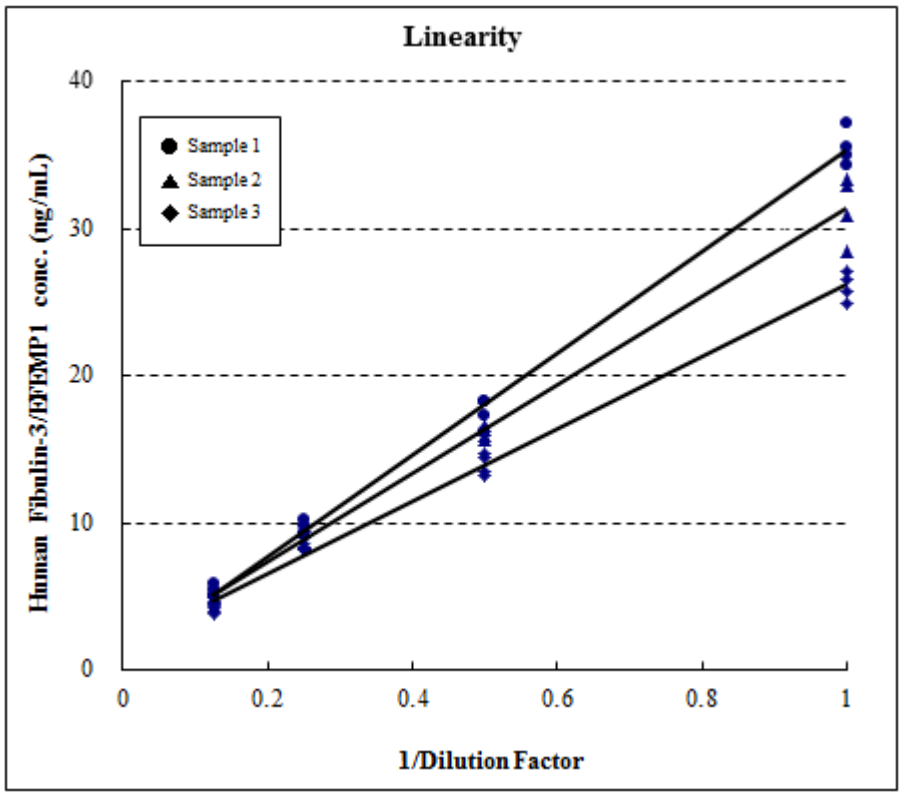
Human Fibulin-3/EFEMP1 conc. (µg/mL)

	Serum 1	Serum 2	Serum 3
1	4.07	3.44	2.12
2	4.04	3.59	2.20
3	3.97	3.62	2.09
4	3.84	3.08	2.14
5	3.74	3.20	2.07
MAX.	**4.07**	**3.62**	**2.20**
MIN.	**3.74**	**3.08**	**2.07**
MEAN	**3.93**	**3.39**	**2.12**
S.D.	**0.140**	**0.239**	**0.049**
C.V.	**3.6%**	**7.1%**	**2.3%**

3. Linearity

To assess the linearity of the assay, three samples* were 10^2-fold diluted with Dilution Buffer and assayed.

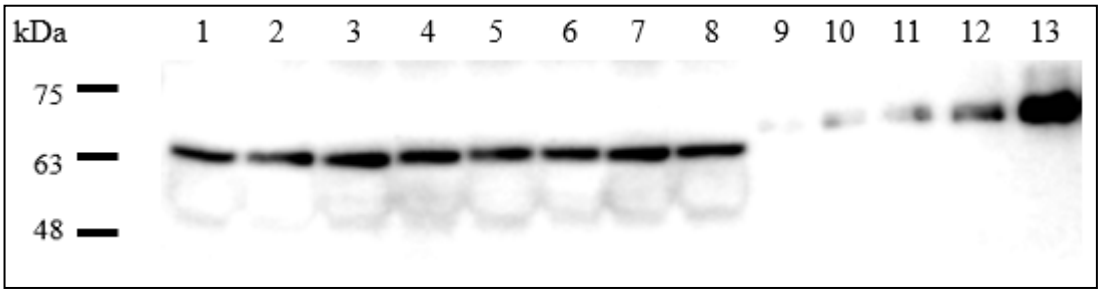
* Sample: Serum



4. The concentration of human Fibulin-3/EFEMP1 in serum

To assess the measurement range of the assay and the concentration of human Fibulin-3/EFEMP1 in sera, human sera were measured with densitometry following western blotting.

Western blotting



Lane 1-8: Human serum 1-8 from healthy volunteers (1.0 µL/lane, albumin-depleted)
Lane 9-13: Recombinant human Fibulin-3 (0.25, 0.5, 0.75, 1.0, 2.0 ng/lane)
Detection: Anti-human Fibulin-3/EFEMP1 rabbit polyclonal antibody

Densitometric analysis of western blotting

	Human Fibulin-3/EFEMP1 conc.
Serum 1	1.21
Serum 2	1.21
Serum 3	1.59
Serum 4	1.50
Serum 5	1.37
Serum 6	1.33
Serum 7	1.56
Serum 8	1.41
	(µg/mL)

The concentrations measured were somewhat lower than ones done by ELISA*, but showed approximately the same range of values. Some of the proteins may be lost by the albumin depletion procedure.

* See Fig.1 in the section "Example of Test Results" below.

Example of Test Results

Fig.1 Human Fibulin-3/EFEMP1 concentration in sera from healthy volunteers (n=16)

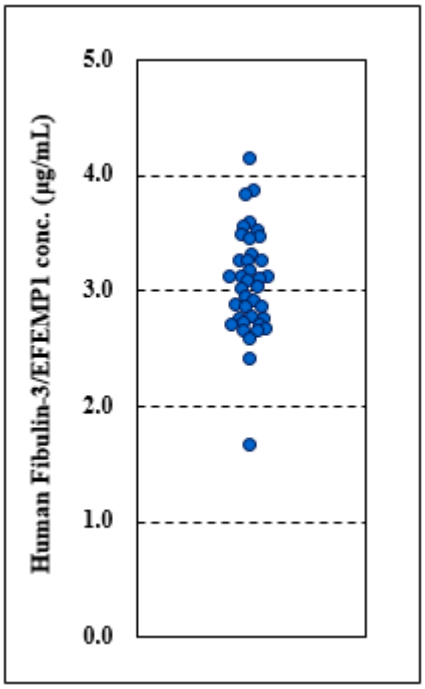
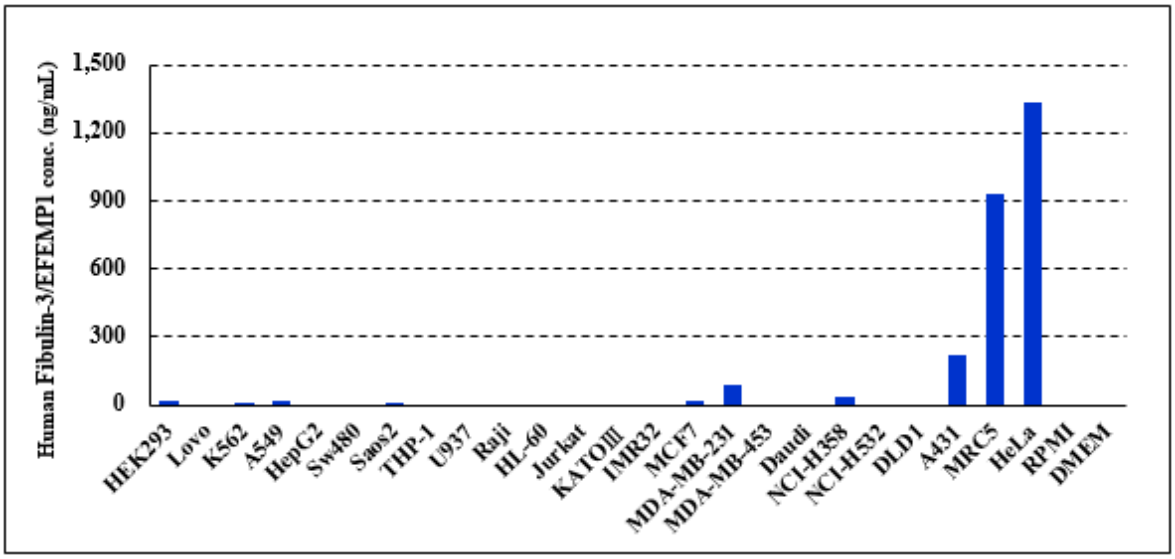


Fig.2 Human Fibulin-3/EFEMP1 concentration in cell culture supernatants.



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