



For Research Use Only, Not for use in diagnostic procedures

ELISA Kit for Measuring Human REG4

CircuLex Human REG4 ELISA Kit

Cat# CY-8213

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Intended Use

The MBL Research Product **CircuLex Human REG4 ELISA Kit** is used for the quantitative measurement of human REG4 in serum, plasma, 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

Regenerating islet-derived family, member 4 (REG4) was originally isolated from a cDNA library of inflammatory bowel disease (1). REG4 is a secreted calcium-independent lectin displaying mannose-binding specificity and able to maintain carbohydrate recognition activity in an acidic condition (2). REG4 consists of 158-amino acids with an N-terminal signal peptide of 22 amino acids and a predicted molecular mass of 18.2 kDa.

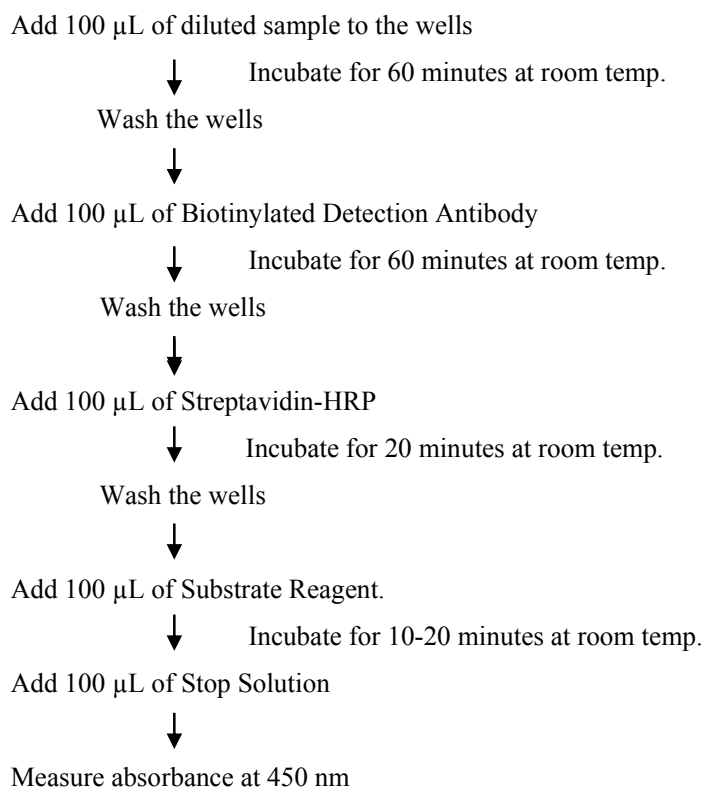
REG4 is widely expressed in the gastrointestinal tract including the duodenum, jejunum, ileum, ileocecum, appendix, descending colon, small intestine and pancreas, however poorly expressed in the large intestine of healthy individuals. The strong expression of REG4 have been especially observed in colon cancer. It was also reported that the mucosal damage caused by Crohn's disease and ulcerative colitis induce the expression of REG4 (3).

Further, REG4 is also secreted into the peripheral blood in a patient of stomach cancer, colon cancer, prostate cancer, pancreatic cancer, REG4 concentrations in these patients sera are significantly higher compared to those in healthy individuals (4-7). In particular, pancreatic and gastric cancer patients, REG4 concentrations was reported to be associated with susceptibility to chemotherapy (4,5). A recent study suggests that REG4 modulates proliferation, apoptosis, migration and invasion of ovarian cancer cells, and has an important role in early ovarian carcinogenesis (8).

Principle of the Assay

The MBL Research Product **CircuLex Human REG4 ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for human REG4 is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any human REG4 present. After washing away any unbound substances, a biotinylated monoclonal antibody specific for human REG4 is added to the wells. Following a wash to remove any unbound biotinylated antibody, Streptavidin-HRP is added to the wells. After washing away any unbound Streptavidin-HRP, HRP remaining on the well 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 REG4. A standard curve is constructed by plotting absorbance values versus human REG4 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 pre-coated with anti-human REG4 monoclonal antibody as a capture antibody.

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

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

Human REG4 Standard: One vial containing X* ng of lyophilized recombinant human REG4.

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

Biotinylated Detection Antibody: One vial containing 12 mL of biotinylated anti-human REG4 monoclonal antibody. Ready to use.

100X Streptavidin-HRP: One vial containing 120 µL of 100X HRP (horseradish peroxidase) conjugated streptavidin.

Streptavidin-HRP Dilution Buffer: One bottle containing 12 mL of 1X buffer; use for dilution of 100X Streptavidin-HRP.

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 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 $1,000 \times g$. 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-2Na as the anticoagulant. If possible, collect the plasma into a mixture of EDTA-2Na and Futhan5 to stabilize the sample against spontaneous *in vitro* complement activation. Immediately centrifuge samples at 4°C for 15 minutes at $1,000 \times g$. 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: Heparin and Citrate plasma has not been validated for use in this assay.

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

Detailed Protocol

The MBL Research Product **CircuLex Human REG4 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**, **100X Streptavidin-HRP**, and **Human REG4 Standard**.

1. Prepare a working solution of **Wash Buffer** by adding **100 mL** of **10X Wash Buffer** to **900 mL** of deionized (distilled) water (**ddH₂O**). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.
2. Prepare **1X Streptavidin-HRP** by 100-fold diluting **100X Streptavidin-HRP** with **Streptavidin-HRP Dilution Buffer** at the time of use and discard any unused portion after use.
3. Reconstitute **Human REG4 Standard** with **X* µL** of **ddH₂O** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 100 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 human REG4 in vial should be **40 ng/mL**, which is referred to as the **Master Standard** of human REG4.

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

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. **Std.1 (4,000 pg/mL)** serves as the highest standard. **Sample Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Sample Dilution Buffer	Concentration
Std.1	60 µL of Master Standard (40 ng/mL)	540 µL	4,000 pg/mL
Std.2	300 µL of Std. 1 (4,000 pg/mL)	300 µL	2,000 pg/mL
Std.3	300 µL of Std. 2 (2,000 pg/mL)	300 µL	1,000 pg/mL
Std.4	300 µL of Std. 3 (1,000 pg/mL)	300 µL	500 pg/mL
Std.5	300 µL of Std. 4 (500 pg/mL)	300 µL	250 pg/mL
Std.6	300 µL of Std. 5 (250 pg/mL)	300 µL	125 pg/mL
Std.7	300 µL of Std. 6 (125 pg/mL)	300 µL	62.5 pg/mL
Blank	-	300 µL	0 pg/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Sample Dilution Buffer before dispensing.

Sample Preparation

Dilute samples with **Sample Dilution Buffer**.

- Serum and plasma samples may require a 50-fold dilution.
- Cell culture supernatants require appropriate dilutions.

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 **Sample 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 60 minutes, 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 **Biotinylated Detection Antibody** into each well.
7. Incubate the plate **at room temperature (ca.25°C) for 60 minutes, 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 **1X Streptavidin-HRP** into each well.
10. Incubate the plate **at room temperature (ca.25°C) for 20 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.**
11. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
12. 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
13. 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.
14. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.
15. 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 62.5 pg/mL to 4,000 pg/mL. Any sample reading higher than the highest standard should be diluted with Sample Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the human REG4 concentration.

Troubleshooting

1. All samples and standards should be assayed in duplicate, using the protocol described in **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 **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 REG4 ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

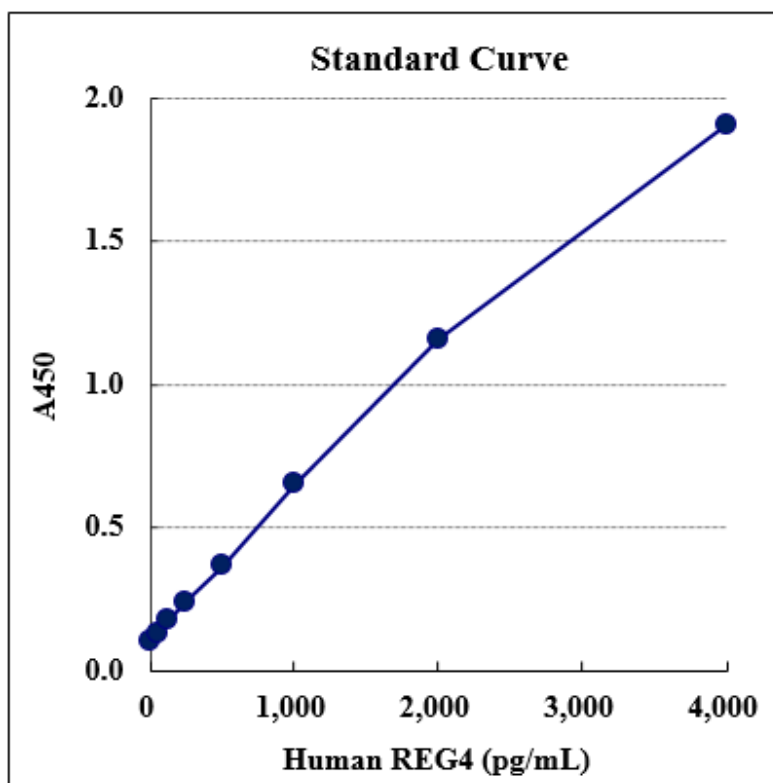
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of human REG4 giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 25 pg/mL of sample.

* Dilution Buffer was pipetted into blank wells.

Typical standard curve



2. Precision

Intra-assay Precision (Precision within an assay)

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

- Intra-assay (Within-Run, n=16) CV=3.5, 3.1, 6.5 %

* Sample: Cell culture supernatant

Human REG4 conc. (ng/mL)			
	Sample 1	Sample 2	Sample 3
1	0.63	1.36	3.64
2	0.62	1.38	4.10
3	0.64	1.42	3.59
4	0.63	1.46	3.76
5	0.66	1.44	4.04
6	0.64	1.38	3.49
7	0.64	1.34	3.53
8	0.67	1.45	3.57
9	0.59	1.44	3.76
10	0.62	1.43	3.60
11	0.63	1.44	3.62
12	0.60	1.43	3.60
13	0.63	1.46	4.08
14	0.63	1.50	4.22
15	0.63	1.45	3.98
16	0.64	1.46	3.83
MAX.	6.53	1.50	4.22
MIN.	0.59	1.34	3.49
MEAN	0.63	1.42	3.76
S.D.	0.02	0.04	0.24
C.V.	3.5%	3.1%	6.5%

Inter-assay Precision (Precision between assays)

Three samples* of known concentration were tested in four separate assays to assess inter-assay precision.

- Inter-assay (Run-to-Run, n=4) CV=5.5, 7.4, 4.3 %

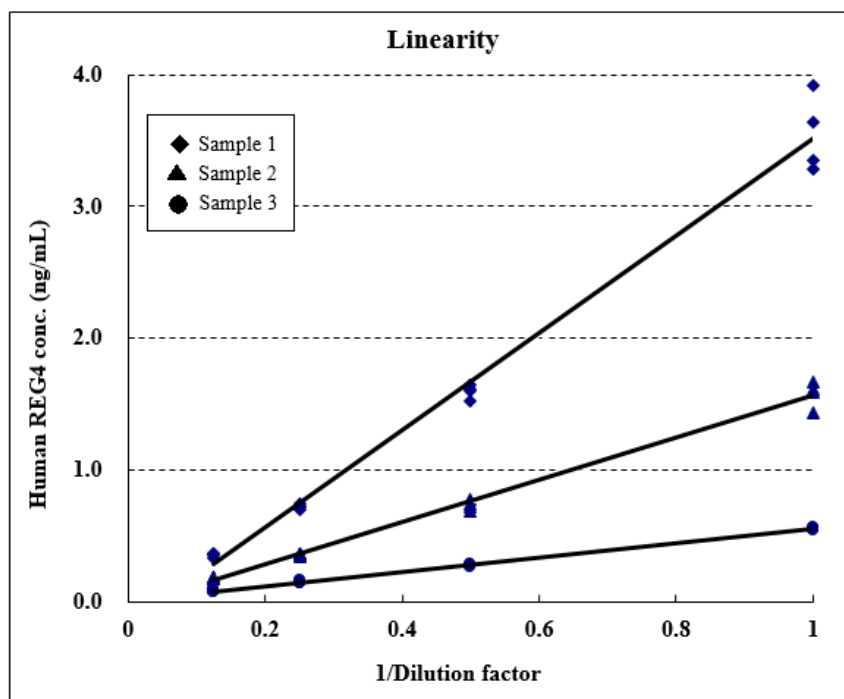
* Sample: Cell culture supernatant

Human REG4 conc. (ng/mL)			
	Sample 1	Sample 2	Sample 3
1	0.62	1.48	3.47
2	0.63	1.41	3.77
3	0.61	1.27	3.54
4	0.56	1.50	3.77
MAX.	0.63	1.50	3.77
MIN.	0.56	1.27	3.47
MEAN	0.60	1.41	3.64
S.D.	0.033	0.104	0.158
C.V.	5.5%	7.4%	4.3%

3. Linearity

To assess the linearity of the assay, three samples* were serially diluted with Sample Dilution Buffer to produce samples with values within the dynamic range of the assay.

* Sample: Cell culture supernatant



Example of Test Results

Fig.1 Human REG4 concentration in cell culture supernatants

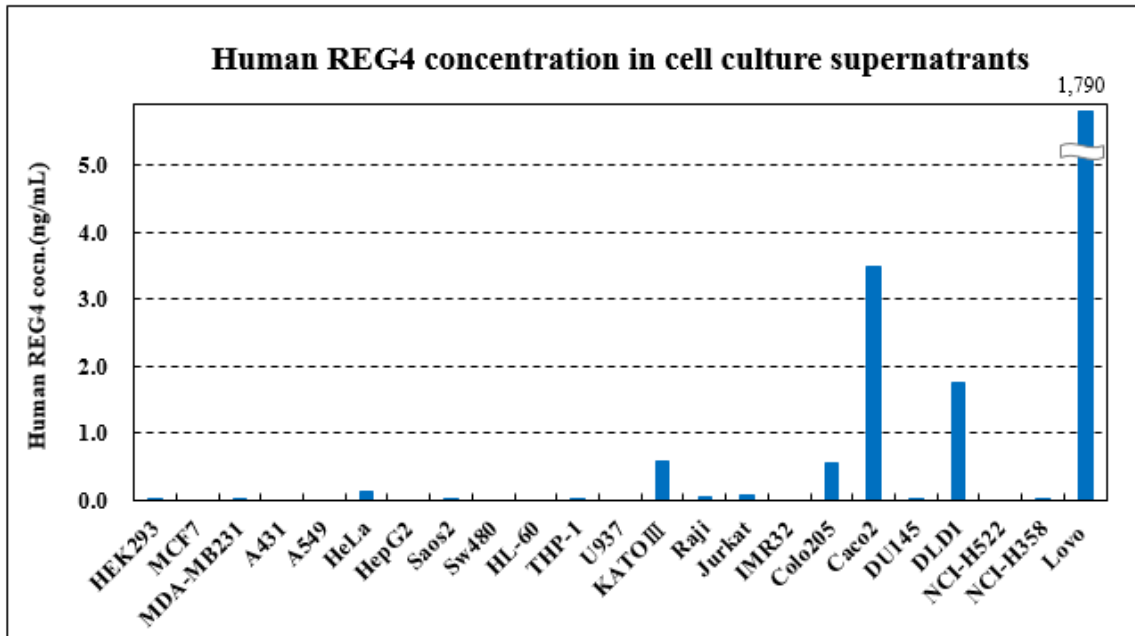
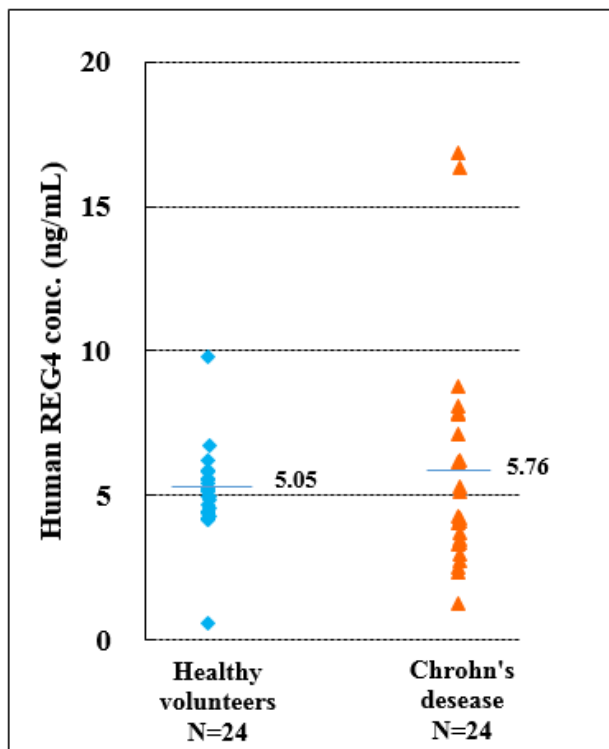


Fig.2 Human REG4 concentration in sera from healthy volunteers and Crohn's disease patients



References

1. Hartupée JC *et al.* Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV. *Biochim Biophys Acta.* 2001 Apr 16;1518(3):287-93.
2. Ho MR *et al.* Human RegIV protein adopts a typical C-type lectin fold but binds mannan with two calcium-independent sites. *J Mol Biol.* 2010 Oct 1;402(4):682-95.
3. Kämäräinen M *et al.* RELP, a novel human REG-like protein with up-regulated expression in inflammatory and metaplastic gastrointestinal mucosa. *Am J Pathol.* 2003 Jul;163(1):11-20.
4. Mitani Y *et al.* Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy. *Oncogene.* 2007 Jun 28;26(30):4383-93.
5. Eguchi H *et al.* Serum REG4 level is a predictive biomarker for the response to preoperative chemoradiotherapy in patients with pancreatic cancer. *Pancreas.* 2009 Oct;38(7):791-8.
6. Hayashi T *et al.* Immunohistochemical analysis of Reg IV in urogenital organs: Frequent expression of Reg IV in prostate cancer and potential utility as serum tumor marker. *Oncol Rep.* 2009 Jan;21(1):95-100.
7. Numata M *et al.* Relationship between RegIV gene expression to outcomes in colorectal cancer. *J Surg Oncol.* 2011 Aug 1;104(2):205-9.
8. Chen S *et al.* The role of the REG4 gene and its encoding product in ovarian epithelial carcinoma. *BMC Cancer.* 2015 Jun 16;15:471.

Related Products

- * CircuLex Human FAM3D ELISA Kit: Cat# CY-8201
- * CircuLex Human MICA ELISA Kit: Cat# CY-8206
- * CircuLex Human MICB ELISA Kit: Cat# CY-8207
- * CircuLex Human LYPD6B ELISA Kit: Cat# CY-8212
- * CircuLex Human REG4 ELISA Kit: Cat# CY-8213
- * CircuLex Human Elafin/SKALP ELISA Kit: Cat# CY-8214
- * CircuLex Human soluble Nectin-4 ELISA Kit: Cat# CY-8217
- * CircuLex Human soluble Tie-1 ELISA Kit: Cat# CY-8218
- * CircuLex Human Amphiregulin/AREG ELISA Kit: Cat# CY-8220
- * CircuLex Human TGF α ELISA Kit: Cat# CY-8221
- * CircuLex Human soluble IL-6 Receptor ELISA Kit: Cat# CY-8222

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