Version 10-11/24

User's Manual

Human Chemerin (Retinoic Acid Receptor Responder Protein 2) ELISA Kit

REF **DEIA-NB24-04**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The Chemerin ELISA Kit is intended to be used for quantitative measurement of human Chemerin in human serum and plasma samples.

General Description

Chemerin, also known as tazarotene-induced gene 2 (TIG2) or retinoic acid receptor responder 2 (RARRES2), is synthesized as precursor protein of 163 amino acids including a N-terminal signal peptide of 20 amino acids, which is chipped of enduring secretion. The inactive, circulation proform of Chemerin contains six cystein residues and thus three intramolecular disulfide bridges are suggested.

Prochemerin expression has been demonstrated for liver, lung, pituitary, lymph node, stomach and adipose tissue. It has been detected in blood, ascitic fluids from ovary and liver cancer and synovial fluids from arthritic patients. Different receptors have been found in spleen, lymph node, small intestine, lung tissue as well as in macrophages and immature dendritic cells.

Prochemerin is converted into its biologically active form by serine or cystein proteases, resulting in pro- or anti-inflammatory actions of the active protein, respectively. Proteolytic cleavage of Chemerin has been described for following serine proteases tryptase, plasmin, elastase, cathepsin G as well as for the cystein proteases cathepsin S and calpains.

The active protein is involved in innate and adaptive immune responses and for instance acts as a strong chemoattractant for immature dendritic cells and macrophages. It influences intracellular signaling by binding to its specific receptors. The C-terminal domain of Chemerin allows binding to the receptor ChemR23/CMKLR1 and elicits a pro-inflammatory stimulus by inducing Ca^{2+} influx and ERK1/2 activation. Further, the N-terminal domain of Chemerin binds to the CCRL2 receptor and allows presentation of the C-terminal domain to neighbor cells. In case of Prochemerin cleavage by cystein proteases the resulting peptides act inhibitory on the ChemR23/CMKLR1 receptors and thus have anti-inflammatory effects.

Recently, the relevance of Chemerin in adipogenesis and adipocyte metabolism has been discovered. Goralski et al. demonstrated in mice that Chemerin as well as receptor (ChemR23) expression is present in adipocytes of visceral and subcutaneous adipose tissue. The expression and secretion increases enduring adipocyte differentiation. The active Chemerin of 16 kDa has been found in the conditioned media of adipocytes. Intracellular signaling networks in adipocytes are also influenced by Chemerin e.g. stimulation of MAPK p42/44 (ERK1/2) phosphorylation was demonstrated. Further, the authors also show a post-differentiational effect of Chemerin on gene expression in adipocytes. Based on these results an influence of Chemerin on metabolic syndrome and insulin resistance was purposed. This studies reveal that Chemerin is more expressed by adipose tissue of obese patients and is able to impair insulin sensitivity of muscle cells and insulin mediated lipolysis and lipogenesis in adipocytes. It has been shown that Chemerin is associated with markers of inflammation and components of the metabolic syndrome as well as with renal function.

For the further investigation of Chemerin functions and its quality as biomarker in metabolic diseases reliable measurement of Chemerin in different body fluids is a prerequisite. Creative Diagnostics offers a sensitive and reproducible test system for the quantitative measurement of Chemerin. Based on highly specific antibodies total Chemerin is measured.

Principles of Testing

The Chemerin ELISA Kit is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. The Chemerin in the sample binds to the immobilized first antibody on the microtiter plate. In the following step, the biotinylated and Streptavidin-Peroxidase conjugated second specific anti-Chemerin-Antibody binds in turn to the immobilised Chemerin. In the closing substrate reaction the turn of the colour will be high specific catalysed, quantitatively depending on the Chemerin level of the samples.

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with human Chemerin antibody. Wells are separately breakable. (8x12) wells.
2. **Dilution Buffer (VP):** ready for use. Please use this for the reconstitution of Standards and Control Sera and for the dilution of Control Sera and Samples. 1 x 125 mL
3. **Standards (A-E):** lyophilized (recombinant Chemerin), Standard values are between 0.025 - 0.6 ng/ml (25, 100, 250, 400 und 600 ng/ml). 6 x 1 mL

   Attention: If the standards are required for more than one assay process, we recommend to store the reconstituted Standards frozen at -20°C. Standards should be thawed only once – where required please store aliquoted in adequate volumes.
4. **Control Serum KS1&KS2:** lyophilized, (human serum). The dilution of the Control Sera KS 1&2 should be according to the dilution of the respective samples, the target values and the respective ranges are given on the certificate. 2 x 250 µL
5. **Antibody-HRP-Conjugate (AK):** ready for use, contains a mixture of biotinylated anti-human Chemerin Antibody and HRP (Horseradish Peroxidase)-labelled Streptavidin. 1 x 12 mL
6. **Washing Buffer (WP):** 20-fold concentrated solution, dilute 1:20 in A.dest. or in deionized Water. 1 x 50 mL

   Attention: After dilution, the Washing Buffer is only 4 weeks stable, dilute only according to requirements.
7. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised Tetramethylbencidine. 1 x 12 mL
8. **Stopping Solution (SL):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
9. **Sealing Tape:** for covering the microtiter plate. 2

Materials Required But Not Supplied

1. Precision pipettes and multichannel pipettes with disposable plastic tips
2. Distilled or deionized water for dilution of the Washing Buffer (WP)
3. Vortex-mixer
4. Microtiterplate shaker (350 rpm)
5. Microtiterplate washer (recommended)
6. Microplate reader ("ELISA-Reader") with filter for 450 and≥590 nm
7. Polyethylene PE/Polypropylene PP tubes for dilution of samples

Storage

All reagents are stable until the indicated expiry, if stored unopened and protected from sunlight at 2 – 8°C. The shelf life of the components after initial opening is limited to 4 weeks, if stored appropriately.

Specimen Collection And Preparation

1. Sample type:

Serum samples are suitable. Further neither EDTA (5.4 mmol/l), Sodium Citrate (10.6 mmol/l) nor Heparin (30 IU/ml) did interfere with Chemerin measurement.

2. Storage of the samples

Storage at RT max. 3 days

Storage at 4°C max. 6 days

Storage at -20°C max. 2 years

in tightly closable plastic tubes.

The measured values of serum and plasma samples did not show significant deviations up to 10 thaw/freezing cycles, values within the range of 92 to 104 % of the target value were found.

3. Sample Preparation

Samples have to be diluted in Dilution Buffer (VP). In most determinations (serum or plasma samples, and no extreme values expected) a dilution from 1:505 with Dilution Buffer VP should be suitable. According to expected Chemerin levels the dilution with VP can be higher or lower. The excellent linearity of this test system allows sample dilution of 1:250 to 1:1000 (see Linearity).

Chemerin concentrations may be completely different in body fluids of human origin other than serum or cell culture supernatants.

4. Sample dilution

Pipette 1 ml Dilution Buffer VP in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add 10 µl Serum- or Plasma (dilution 1:101). Dilute in the second step 50 µl of the pre diluted sample with 200 µl Dilution Buffer VP (dilution 1:505). After mixing use 100 µl per determination of this dilution in the assay.

Reagent Preparation

Reagents with different lot numbers cannot be mixed. All reagents are stable until the indicated expiry, if stored unopened and protected from sunlight at 2 – 8°C. The shelf life of the components after initial opening is limited to 4 weeks, if stored appropriately. Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming.

1. The Standards A – E is reconstituted with 1mL Dilution Buffer VP. After resuspension, the standard is diluted according to a gradient - A (25 pg/mL), B (100 pg/mL), C (250 pg/mL), D (400 pg/mL) and E (600 pg/mL), which are prepared for immediate use.
2. The Control Sera KS1 and KS2 are reconstituted with 250 µL Dilution Buffer VP. After reconstitution, dilute the Controls KS1 and KS2 with the Dilution Buffer VP in the same ratio (1:505) as the sample and use within

max. 60 min.

Note: It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer. The reconstituted standard and controls can be stored for 4 weeks at –20°C. Repeated freeze/thaw cycles have to be avoided.

3. The required volume of Washing Buffer WP is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest. The diluted Washing Buffer is stable for 4 weeks at 2-8°C. It has to be at room temperature for usage!

Assay Procedure

NOTES: All determinations (Standards, Control Sera and Samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended. When performing the assay, the Standards, Control Sera and the samples should be pipette as fast as possible (e.g. < 15 minutes). To avoid distortions due to differences in incubation times, the Antibody POD-Conjugate AK, the Substrate Solution S as well as the Stop Solution SL should be added to the plate in the same order and in the same time interval each, respectively.

1. Add 100 µl Dilution Buffer VP in wells A1/A2 (blank).
2. Pipette in positions B1/2 100 µl of the Standard A (25 pg/ml),

   pipette in positions C1/2 100 µl of the Standard B (100 pg/ml),

   pipette in positions D1/2 100 µl of the Standard C (250 pg/ml),

   pipette in positions E1/2 100 µl of the Standard D (400 pg/ml),

   pipette in positions F1/2 100 µl of the Standard E (600 pg/ml).
3. To control the correct accomplishment 100 µl of the 1:505 (or in respective dilution rate of the sample) in Dilution Buffer VP diluted Control Sera KS1 or KS2 can be pipetted in positions G1/2 and H1/2.
4. Pipette 100 µl each of the diluted sample (e.g. dilute 1:505 with Dilution Buffer VP) in the rest of the wells, according to requirements.
5. Cover the wells with sealing tape and incubate the plate for 1 hour at room temperature (shake at 350 rpm). After incubation aspirate the contents of the wells and wash the wells 5 times with 300 µl Washing Buffer WP / well.
6. Following the last washing step pipette 100 µl of the Antibody-POD-Conjugate AK in each well.
7. Cover the wells with the sealing tape and incubate 1 hour at room temperature (shake at 350 rpm).
8. After incubation wash the wells 5 times with Washing Buffer WP as described in step 5.
9. Pipette 100 µl of the TMB-substrate solution S in each well.
10. Incubate the plate for 30 minutes in the dark at room temperature.
11. Stop the reaction by adding 100 µl of Stopping Solution SL to all wells.
12. Measure the absorbance within 30 minutes at 450 nm (reference filter: ≥ 590 nm).

Quality Control

For the evaluation of the assay, it is preconditioned, that the absorbance values of the blank should be below

0.3, these of standard E should exceed 0.8. Samples, which yield higher absorbance values than Standard E are beyond the standard curve, for reliable determinations these samples should be tested anew with a higher dilution.

Calculation

1. Calculate the mean absorbance value for the blank from the duplicated determination (well A1/A2).
2. Subtract the mean absorbance of the blank from the mean absorbances of all other values.
3. Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.
4. Recommendation: Calculation of the standard curve should be done by using a computer program because the curve is in general (without respective transformation) not ideally described by linear regression. A higher-grade polynomial, or four parametric logistic (4 PL) curve fit or non-linear regression are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).
5. The Chemerin concentration of the diluted sample or the diluted control sera in pg/ml (or ng/ml according the chosen unit for the standards) is calculated in this way, the Chemerin concentrations of the undiluted samples and of control sera are calculated by multiplication with the respective dilution factor.

Exemplary calculation of the Chemerin concentration of a 1:505 diluted sample

Measured extinction of your sample.....0.56

Measured extinction of the blank.....0.02

Your measurement program will calculate the Chemerin concentration of the diluted sample automatically by using the difference of sample (0.02) and blank for the calculation. You only have to determine the most suitable curve fit (here: polynomial 3 degree).

In this exemplary case, the following equation is solved by the program to calculate the Chemerin concentration in the sample:

$$0.54 = 0.0039285+0.0042173x+2.6356 \times 10^{-6}x^2-3.5684 \times 10^{-9}x^3$$

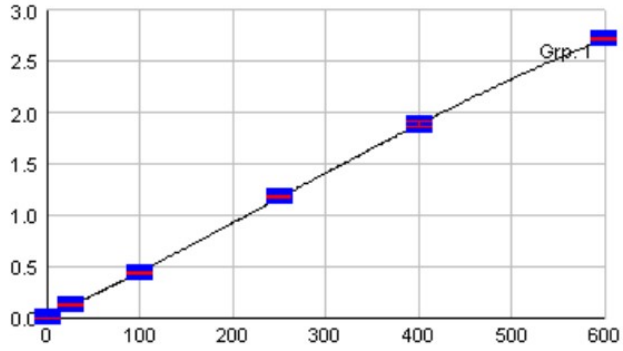
$$0.12 = x$$

If the dilution factor 1:505 is taken into account, the Chemerin concentration of the undiluted sample is

$$0.12 \times 505 = 60.65 \text{ ng/ml}.$$

Typical Standard Curve

The exemplary shown standard curve in Figure below cannot be used for calculation of your test results. You have to establish a standard curve for each test you conduct!



Precision

The inter and intra assay coefficients of variability are below 5.16 and 2.17 %, respectively.

Intra-Assay-Variation

	Determinations [n]	Mean value	SD	CV [%]
Sample 1	20	84.33	1.71	2.02
Sample 2	20	142.33	3.93	2.76

Inter-Assay Variation

	Sample 1	Sample 2	Sample 3
Mean	70.91	123.88	147.96
SD	3.42	6.80	6.06
CV [%]	5.16	5.87	4.38

Sensitivity

The analytical sensitivity of the assay yields 0.005 pg/ml (5 pg/ml; as 2 x SD of zero standard in 19-fold determination).

Specificity

Commercially available sera from bovine, cat, chicken, dog, donkey, goat, guinea pig, horse, mouse, pig, rabbit, rat and sheep were diluted (1:10) and used as samples in this assay system and the signal intensity was measured. No cross reactivity was detected.

Linearity

The Chemerin ELISA Kit is over a very wide range dilution authentic, the linearity of serum dilutions is over a dilution range of 1:250 to 1:1000 excellent (see table below).

Dilution	Sample 1 [ng/ml]	Sample 2 [ng/ml]	Sample 3 [ng/ml]
1:250	57.03	113.35	129.71
1:375	58.63	110.44	129.99
1:500	58.07	112.49	131.99
1:625	54.88	111.31	134.34
1:750	54.29	107.36	133.61
1:875	53.26	103.61	129.31
1:1000	53.51	101.59	126.43
AV / SD / CV%	**55.67 / 2.21 / 3.97**	**108.59 / 4.55 / 4.19**	**130.77 / 2.74 / 2.09**

AV= Average Value; SD = Standard Deviation, VC = Coefficient of Variation

Recovery

The recovery of recombinant Chemerin in serum and plasma samples varied from 81 to 103%.

Matrix effects: % Recovery of recombinant Chemerin in different body fluids.

Matrix effects						
Dilution [1:x]	**2**	**5**	**10**	**100**	**500**	**1000**
Saliva	35	52	70	-	-	-
Urine	92	92	91	-	-	-
Breast milk	90	88	86	-	-	-
Cell culture media	91	100	95	104	-	-
Cerebrospinal fluid	> max.	> max.	> max.	96	92	94

Interferences

Interference of physiological appearing substance with the Chemerin measurement was investigated. Serum samples have been enriched with different concentrations of possibly interfering substances and the amount of Chemerin was measured and compared with the Chemerin concentration in the same sample without any enrichment. In table below the relative results are shown. None of the tested substances interfered significantly with Chemerin measurement.

	Triglycerides [100 mg/ml]	**Bilirubin** [200 µg/ml]	**Haemoglobin** [1 mg/ml]
%	107.6	98.6	108.5

Effects of coagulation inhibitors were investigating by adding indicated amounts of inhibitors to VP or PBS enriched with 125 pg/ml Chemerin. Relative amounts of Chemerin determined in inhibitor containing samples in comparison to inhibitor free samples are shown in table below. None of the tested substances interfered significantly with Chemerin measurements.

		% Recovery
[3.8 g/l]	Citrate	103.4
[5.4 mmol/l]	EDTA	100.4
[30 IE/ml]	Heparin	103.0

Precautions

1. For research and professional use only. The Creative Diagnostics kit is suitable only for in vitro use and not for internal use in humans and animals.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
3. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
4. This kit contains material of human and/or animal origin. Disposal of containers and unused contents should be done in accordance with federal and local regulatory requirements.
5. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step. Use separate pipette tips for each sample, control and reagent to avoid cross contamination. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
6. Following components contain human serum: Control Sera KS1 / KS2, Standards A-E. Source human serum for the control sera provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.