PG-ELISA

KAP1461

DIAsource ImmunoAssays S.A. - Rue du Bosquet, 2 - B - 1348 Louvain-la-Neuve – Belgium

History

Summary of change:

Previous Version: 121113-1	**Current Version: 200224-1**
LOT	Version :
EASIA	ELISA
No history	History added
PI number : ….	PI number removed
	Addition of the following sentence at the end of the English IFU: "Other translations of this Instruction for Use can be downloaded from our website: https://www.diasource-diagnostics.com/"

Read entire protocol before use.

PG-ELISA

For research use only. Not for use in diagnostic procedures.

An immunoenzymetric assay for the quantitative measurement of human aggrecan (PG)
in synovial fluid, serum and cell culture supernatant.

I GENERAL INFORMATION

A. Proprietary Name : DIAsource PG-ELISA kit

B. Catalogue Number : KAP1461 : 96 determinations

C. Manufactured by : DIAsource ImmunoAssays S.A.
Rue du Bosquet, 2 B-1348 Louvain-la-Neuve, Belgium.

For technical assistance or ordering information contact :
Telephone numbers : (Voice) +32/10/84.99.11 (Fax) +32/10/84.99.91

II APPLICATION AND INTENDED USE

Aggrecan (PG) is the predominant proteoglycan species in articular cartilage. It is composed of a core protein of 210 kDa to which over 100 chondroitin sulfate chains, about 20-50 keratan sulfate chains and O-linked as well as N-linked oligosaccharides are covently attached. The core protein contains three distinct globular domains (G1-G3). G1 is at the amino terminus, separated by a short extended segment from G2, while G3 is at the carboxy terminal end. The G1 amino terminal region can interact noncovalently with hyaluronic acid (HA) and has then be termed the hyaluronic acid binding region (HABR). A link protein interact with both the G1 region and the HA to stabilize this interaction. PG is produced by chondrocytes, and its production is regulated by cytokines and growth factors such as IL1β, TNFα, IGF1 or TGFβ. In extracellular matrix, as many as 200 aggrecan molecules can bind to one single HA molecule to form an aggregate (MW : $5\ 10^7$ to $5\ 10^8$).
An imbalance in the synthesis and degradation of the matrix components is a common feature of both osteoarthritis and rheumatoid arthritis. The loss of PG and other matrix components from the cartilage leads to destruction of the tissue, causing complete deterioration of the articular surface. Several recent publications suggest that the PG and PG fragments released in synovial fluid and serum during the degradation process might serve as markers of the metabolic changes in diseased cartilage. Cell culture is a commonly used procedure for the study of cartilage metabolism. The measurement of PG and other matrix components in culture supernatants and cellular contents can assist analysis of the effects of cytokines, growth factors, drugs and potential chondroprotective substances on the cartilage homeostasy. DIAsource has developped a ELISA for the measurement of human aggrecan to aid the study of this important cartilage constituent. The PG-ELISA is convenient, highly specific, and allow accurate measurement of PG in synovial fluid, serum and culture supernatant This assay is a new interesting tool for the exploration of the cartilage metabolism.

III. PRINCIPLES OF THE PG-ELISA ASSAY

The PG-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay (ELISA) performed on microtiter plate. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of PG are used. Antibody-producing cells are immortilized using the myeloma cell fusion method of Kohler and Milstein. A hybridoma cell is produced which secretes specific homogeneous antibodies. The use of a number of distinct MAbs avoids hyperspecificity and allows highly sensitive assays with extended standard range and short incubation time. Standards or samples containing PG react with capture monoclonal antibodies (MAbs 1) coated on the microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich : coated MAbs 1 - PG - MAb 2 - HRP, the microtiter plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme-labelled antibodies are measured through a chromogenic reaction. Chromogenic Solution ($TMB+H_2O_2$) is added and incubated. The reaction is stopped with the addition of Stop Solution (HCl) and the microtiter plate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance which is proportional to the PG concentration. A standard curve is plotted and PG concentrations in a sample is determined by interpolation from the standard curve. The use of the ELISA Reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in high sensitivity in the low range and in an extended standard range.

IV. REAGENTS PROVIDED

Reagents	**96 tests Kit**	**Reconstitution**
Microtiter plate with 96 anti-PG coated wells	1 x 96 wells	**Ready** for use
Standards 0 to 5 in phosphate buffer with preservatives : see vial label for exact concentrations	6 vials lyophil.	**Add** 0.5 ml distilled water
Diluent buffer	2 vials 2 x 30 ml	**Ready** for use
Incubation buffer	1 vial 11 ml	**Ready** for use
Anti-PG-HRP Conjugate in a buffered solution with proteins and preservatives	1 vial 5.5 ml	**Dilute** with the conjugate buffer (see section 7)
Conjugate Buffer	2 vials 2 x 11 ml	**Ready** for use
Controls 1, 2 and 3 in phosphate buffer with preservatives	3 vials lyophil.	**Add** 0.5 ml distilled water
Washing Solution Concentrate (buffer with preservatives)	1 vial 10 ml	**Dilute** 2 ml in 400 ml distilled water or the vial contents in 2000 ml distilled water
Chromogenic Solution : TMB (Tetramethylbenzidine)	1 vial 25 ml	**Ready** for use
Stop Solution	1 vial 25 ml	**Ready** for use

Standard : Aggrecan purified from human articular cartilage (A1D1D1 fraction chromatographed on Sepharose CL 2B, material eluted at $0.1 < Kd < 0.7$).

V. PRECAUTIONS AND WARNINGS

1. The synovial fluid and serum components included in this kit (controls 1and 3) have been tested by European approved and USA FDA approved methods and found negative for HBsAg, anti-HCV and anti-HIV-1 and 2. No known method can offer complete assurance that synovial fluid and serum derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents or synovial fluid or serum specimens should be in accordance with local safety procedures.
2. Avoid any skin contact with Chromogenic Solution (TMB) and Stop Solution . In case of contact wash thoroughly with water.
3. Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
4. Do not pipet liquids by mouth.

VI. EQUIPMENT AND SUPPLIES REQUIRED BUT NOT PROVIDED

1. High quality distilled water.
2. Precision pipette : 50 µl, 100 µl, 200 µl, 1 ml and 10 ml.
3. Vortex mixer and magnetic stirrer.
4. Horizontal microtiter plate shaker capable of 700 rpm ± 100 rpm, microtiter plate reader capable of reading at 450 nm and 490 nm, microtiter plate washer.

VII. REAGENT PREPARATION

1. **Standards and Controls** : Reconstitute the lyophilized Standards and Controls to the volume specified on the vial label with distilled water (0.5 ml for Standards and Controls). Allow them to remain undisturbed until completely dissolved, then mix well by gentle inversion.
2. **Wash Solution**: Dilute 2 ml of Washing Solution Concentrate in 400 ml distilled water or all the contents of the Washing Solution Concentrate vial in 2000 ml distilled water (use a magnetic stirrer).
3. **Conjugate solution** : following the number of wells to be used, dilute the concentrated conjugate with the conjugate buffer in a clean glass. See below table the volume to pipet. Extemporaneous preparation is necessary.

TABLE OF CONJUGATE DILUTION (ml)

Number of wells	Concentrated conjugate	Conjugate diluent	Working volume
16	0.75	3	3.75
24	1.25	5	6.25
32	1.5	6	7.5
48	2.5	10	12.5
96	5	20	25

VIII. STORAGE AND SHELF LIFE OF REAGENTS

A. UNOPENED vials

Store the unopened vials at 2°C to 8°C. All kit components are stable until the expiry date printed on the labels.

B. OPENED vials

1. The Conjugate vial must be stored at 2° to 8°C.
2. The reconstituted Standards and Controls are stable for 1 day at 2°C to 8°C. Aliquots held for longer periods of time should be frozen, a maximum of two times, at -20°C (maximum 2 months) or at -70°C for longer storage (until expiration date).
3. Store the unused strips at 2°C to 8°C in the sealed bag containing the dessiccant until expiration date.
4. The Wash Solution Concentrate is stable at room temperature until expiration date. In order to avoid washerhead obstructions, it is recommended to prepare a fresh diluted Wash Solution each day.

IX. SPECIMEN COLLECTION, PREPARATION, STORAGE AND DILUTION

A. Specimen Collection and preparation

The PG-ELISA kit may be used to measure PG in human synovial fluids, sera and culture supernatants.

Synovial fluids can be collected into sterile tubes with EDTA or without additives, and must be centrifuged (at 1800g for 20 minutes) to remove cells.

Culture supernatants can be collected into plastic tubes and must be centrifuged (at 1800g for 20 minutes) to remove cells.

At the present stage of our studies, the following culture media have been used in human chondrocytes cultures.

- DMEM supplemented with 1% ULTROSER G (GIBCO)
- DMEM supplemented with transferin (6.25µg/ml), selenium (6.25 ng/ml), BSA (1.25 mg/ml) and linoleic acid (5.35 µg/ml).
- DMEM supplemented with 0% to 10% fetal calf serum.

No interferences were detected in the PG-ELISA with these culture media. Nevertheless we recommend that each laboratory check the absence of interferences in its culture media.

B. Storage

Synovial fluid samples, sera and culture media must be kept at -20°C for maximum 2 months, and for longer storage (maximum one year) at - 70°C.

C. Sample Dilution

At the present stage of our studies, only preliminary results can be provided and we thus recommend that each laboratory establishes its own dilution range. For guidance, see the following informations.

1. **Synovial fluid**
The PG levels measured in 300 synovial fluids ranged between 2 and 800 µg/ml and the mean concentration was 80 µg/ml. Therefore we recommend to dilute the synovial fluids 1/500 with the dilution buffer, according to the following procedure.
1/5 dilution : Pipet 100 µl dilution buffer and 25 µl SF in a 3ml plastic tube. Vortex the tube.
1/50 dilution : Pipet 225 µl dilution buffer and 25 µl 1/5 dilution in a 3 ml plastic tube. Vortex the tube.
1/500 dilution : Pipet 225 µl dilution buffer and 25 µl 1/50 dilution in a 3ml plastic tube. Vortex the tube.
If the sample generates values out of the standard curve, adapt the sample dilution and repeat the assay.

2. **Serum**
The PG levels measured in adult sera (age : 18-66 years) ranged between 1 and 4.4 µg/ml and the mean concentration was 2.8 µg/ml. PG levels in children sera (age : 2-16 years) ranged between 4 and 15 µg/ml and the mean concentration was 10.4 µg/ml. Therefore, we recommand to dilute adult sera 1/20 and children sera 1/100 according to the following procedure.
1/20 dilution : Pipet 380 µl dilution buffer and 20 µl serum in a 3 ml plastic tube. Vortex the tube.
1/100 dilution : Pipet 100 µl dilution buffer and 25 µl 1/20 dilution in a 3 ml plastic tube. Vortex the tube.

3. **Cell culture supernatant**
PG levels measured in 5 different human chondrocyte culture supernatants ranged between 1 and 10 µg/ml. We thus suggest to dilute the culture supernatant 1/50 with the dilution buffer.
Pipet 490 µl dilution buffer and 10 µl culture supernatant in a 3 ml plastic tube. Vortex the tube.

X. PG-ELISA PROCEDURE

The instructions of the assay procedure must be followed to obtain reliable results.

A. Procedural notes

1. Allow the samples and reagents to equilibrate to room temperature (18°C to 25°C) before commencing the assay. Thoroughly mix the reagents and samples before use by gentle agitation or swirling.
2. Do not use kit components beyond the expiration date.
3. Do not mix materials from different kit lots.
4. Do not mix strips from different plates.
5. Perform Standards,Controls and Unknowns in duplicate. Vertical alignment is recommended.
6. A standard curve should be run with each assay run or each plate run.
7. To avoid drift, the time between pipetting of the first standard and the last sample must be no longer than 30 minutes. Otherwise, results will be affected.
8. Use a clean disposable plastic pipette for each reagent, standard, control or specimen addition in order to avoid cross contamination .
9. For the dispensing of the Chromogenic Solution and Stop Solution avoid pipettes with metal parts.
10. Use a clean plastic container to prepare the Wash Solution.
11. During incubation with Chromogenic Solution, avoid direct sunlight on the microtiter plate.
12. Respect the incubation times described in the assay procedure.

B. Assay Procedure

1. **Select the required number of strips for the run.** The unused strips should be resealed in the bag with desiccant and stored at 2-8°C.
2. **Secure** the strips into the holding frame.
3. **Pipette 50 µl of each Standard, Control, or Diluted Sample** into the appropriate wells.
4. **Pipette 100 µl of incubation buffer** into the appropriate wells foreseen for the Standards, Controls and Samples.
Incubate for **2 hours** at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
6. **Aspirate** the liquid from each well ;
7. **Wash** the plate three times by :
a) dispensing of 0.4 ml of DIAsource Wash Solution into each well ;
b) aspirating the content of each well.
8. **Pipette 200 µl of anti-PG conjugate** into all the wells.
9. **Incubate** for **1 hour** at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
10. **Aspirate** the liquid from each well ;
11. **Wash** the plate three times by :
a) dispensing of 0.4 ml of DIAsource Wash Solution into each well ;
b) aspirating the content of each well.
12. **Pipette 100 µl of Chromogenic Solution** into each well within 15 min. following the washing step.
13. **<u>Exactly incubate</u>** the plate for **<u>15 min.</u>** at room temperature on an horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight.
14. **Pipette 200 µl of Stop Solution** into each well.
15. **Read** absorbances at 450 nm and 490 nm (reference filter : 630 or 650 nm) within 3 hours and calculate the results as described in section XI.

XI. CALCULATION OF ANALYTICAL RESULTS

A. Reading the plate with the ELISA Reader

. Read the plate according to the instructions of the ELISA Reader and ELISA$^{AID™}$ Software.

B. Reading the plate with other equipment

. Read the microtiter plate at 450 nm (reference filter : 630 or 650 nm).
. Construct a standard curve using all standard points for which absorbances are below the limit of linearity of reader used.
. Plot the OD on the ordinate against the standard concentrations on the abscissa using either linear or semi-log graph paper and draw the curve by connecting the plotted points with straight lines.
. Determine PG concentrations of Samples or Controls for which absorbance is no greater than those of the last standard plotted at 450 nm.
. If any Control or Sample has an absorbance greater than the absorbance of the last standard read at 450 nm, a second reading at 490 nm (reference filter : 630 or 650 nm) is needed. Proceed as described above to construct a second standard curve at 490 nm using all the standard points. The segment of the curve drawn between the last standard read at 450 nm and the most concentrate standard will be considered at 490 nm. The concentration of Samples and Controls for which absorbance is included in this segment, is read at 490 nm. So, the first reading gives the high sensitivity of the assay and the second reading allows an extended standard range.

Note : The readings at 490 nm are only for off-scale values at 450 nm (above the limit of reader linearity)and should not replace the reading at 450 nm for values below the limit of reader linearity.

C. Example of a typical reference curve

The following data are for demonstration purpose only and can not be used in place of data generated at the time of assay. These data are provided by using the ELISA reader and the ELISA^{AID} software.

PG-ELISA		Polychromatic model (OD units)
Standard	0 ng/ml	0.044
	10 ng/ml	0.202
	25 ng/ml	0.441
	50 ng/ml	0.660
	100 ng/ml	1.466
	250 ng/ml	3.339

XII. QUALITY CONTROL

- The **three Controls** provided in the kit can be used as internal laboratory controls.

Note : Other controls which contain azide will interfere with the enzymatic reaction and cannot be used.

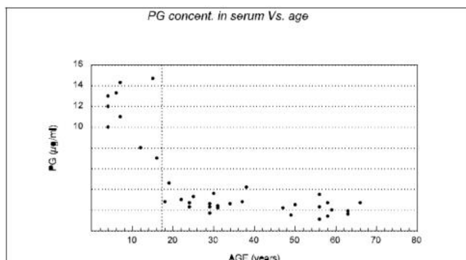
- Diluted synovial fluids, diluted sera as well as diluted cell culture supernatants can be collected and frozen immediately in aliquot to serve as controls. Repeated freezing and thawing are not recommended.
- **Record keeping** : it is good laboratory practice to record the kit lot numbers and date of reconstitution for the reagents in use.
- **Controls** : it is recommended that Controls be routinely assayed as unknown samples to measure assay variability. It is recommended that quality controls charts be maintained to monitor the performance of the kits. Control ranges are indicated on vial labels. Out of range control results indicate the assay must be repeated. Repeat patient samples may also be used to measure interassay precision.
- **Sample handling** : strictly adhere to the instruction for handling and storage of samples. Standards, Controls, and Unknowns should be run in duplicate. A clean disposable tip should always be used to avoid carryover contamination.
- **Data reduction** : it is good practice to construct a standard curve for each run to check visually the curve fit selected by the computer program.

XIII. EXPECTED RANGE

In a preliminary study, PG concentrations were measured in culture supernatants of human chondrocytes cultivated in the presence or not of IL1β (10^{-10} M) and IGF- I (10^{-8} M), as well as in 97 synovial fluids of patients with meniscus (Men), osteoarthritis (OA), reactive arthritis (ReA), gout (Gout), and rheumatoid arthritis (RA). The results are tabled below.

Culture Supernatants	**PG concentrations in (ng/µg DNA) (m ± SD)**	**Synovial Fluids**	**PG concentrations (µg/ml) (m± SEM)**
Control (4)	306 ±47	Men(16)	226 ± 36
IL1β (4)	162 ± 12	Gout (6)	291 ± 35
IGFI (4)	527 ± 35	ReA(15)	185 ± 61
		OA(14)	71 ± 14
		RA(46)	61 ± 11

The PG production measured in culture supernatants was inhibited by IL1β and increased by IGF1 as already reported by other using different immunoassay systems for the PG detection. Similar PG concentrations were measured in the meniscus and gout synovial fluids. As compared to these groups, the PG levels were significantly lower in the ReA ($p<0.05$), OA ($p<0.001$) and RA ($p<0.001$) synovial fluids.



The figure shows the PG concentrations in serum of normal subjects aged from 3 to 66 years.
The PG levels measured in adult sera (age : 16-66 years) ranged between 1 and 4.4 µg/ml and the mean concentration was 2.8 µg/ml. PG levels measured in children sera (age : 3-16 years) ranged between 7 and 15 µg/ml. The mean PG concentration was 10.4 µg/ml.

XIV. PERFORMANCE CHARACTERISTICS

1. Minimum Detectable Concentration (MDC).
   The MDC is estimated to be 0.9 ng/ml and is defined as the PG concentration corresponding to the average OD of 20 replicates of the zero standard + 2 standard deviations.

2. Precision

INTRA-ASSAY			
Sample (Dilution)	**n**	**<X> ± SD (ng/ml)**	**CV %**
Syn. fluide 1 (dil:1/100)	20	127.6 ± 2.3	1.8
2 (dil:1/70)	20	75 ± 1.15	1.53
Serum 1 (dil:1/20)	20	78 ± 1.48	1.9
2 (dil:1/40)	20	56.3 ± 1.5	2.6
Cult. sup. (dil:1/30)	20	119 ± 3.2	2.7

INTER-ASSAY (day-to-day)			
Sample	**n**	**<X> ± SD (ng/ml)**	**CV %**
Syn. fluid 1 (dil:1/250)	20	157.9 ± 6.9	4.3
2 (dil:1/250)	20	47.3 ± 2.6	5.5
Serum 1 (dil:1/20)	20	76 ± 4.6	6.05
2 (dil:1/40)	20	55 ± 3.6	6.5
Cult. sup. 1 (dil:1/30)	20	145 ± 5.8	4
2 (dil:1/100)	20	48.8 ± 3.5	7

3. Specificity
Cross reactivity was determined by addition of differents analytes to 0, 23 or 159 ng/ml of PG and the apparent PG concentration was measured.

Added analyte to PG PG samples		**Observ. values for 0 ng/ml of PG**	**Observ. values for 23 ng/ml of PG**	**Observ. values for 159 ng/ml of PG**
Hyaluronic acid	1000 ng/ml	0	22.7	152.5
Link-protein (1)	10 ng/ml	0	23	153.3
Hyaluronic acid 1000ng/ml + Link-protein	10 ng/ml	0	21.1	141.8
Chondroitine sulfate (2)	1500 ng/ml	0	26.8	154.8
Keratan sulfate (3)	1500 ng/ml	0	23	147

(1) Purified in our laboratory
(2) From porcine rib cartilage (SIGMA n° C0914)
(3) From bovine cornea (SIGMA n° K3001)

No ELISA reactive material was detected in extracts of human- bone - liver - kidney -heart-artery - vein or in culture supernatants of human - Osteoblasts - Synovial fibroblasts - Skin fibroblasts - Hepatocytes - Endothelial cells.

4. Accuracy

RECOVERY			
Sample	Added PG (ng/ml)	Reco-vered PG (ng/ml)	Reco-very (%)
Syn. fluid 1 (dil:1/580)	125	128	102
	42	43	102
	16	18	112
Syn. fluid 2 (dil:1/70)	125	124	99
	42	48	114
	16	19	118
Serum 1 (dil:1/30)	21.4	24	112
	40	36	90
	81	83	102
Serum 2 (dil:1/90)	21.4	23	107
	40	42	105
	81	88	109
Cult. sup. 1 (dil:1/150)	22	22.5	102
	57	57.5	100
	172	181	105
Cult. sup. 2 (dil:1/60)	22	20.2	92
	57	52	91
	172	177	102

DILUTION TEST			
Sample	Dilu-tion	Theor. conc (ng/ml)	Meas. conc. (ng/ml)
Syn. fluid	1/70	275	275
	1/140	137.5	139
	1/280	68.7	71
	1/560	34.3	37.6
	1/1120	17.2	19.3
Serum	1/16	163	163
	1/32	81.5	88.7
	1/64	40.7	47
	1/128	20.3	20.6
Cult. sup.	1/60	195	195
	1/120	97.5	97.5
	1/240	48.7	45.3
	1/480	24.4	22.2
	1/960	12.2	12

XV. LITERATURE REFERENCES

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XVII. SUMMARY OF ASSAY PROCEDURE

	Standards (µl)	Diluted synovial fluid samples (µl)	Diluted Sera (µl)	Diluted culture supernat. (µl)
Standards (0-5), controls	50	-	-	-
Diluted Synovial fluid samples	-	50	-	-
Diluted sera	-	-	50	-
Diluted Culture supernatants	-	-	-	50
Incubation buffer	100	100	100	100
Incubate for 2 hour at R.T. with continuous shaking (700 RPM) **Aspirate** the contents of each well **Wash** 3 times with 0.4 ml of Wash Solution and aspirate				
Anti-PG-HRP Conjugate	200	200	200	200
Incubate for 1 hour at R.T. with continuous shaking (700 RPM) **Aspirate** the contents of each well **Wash** 3 times with 0.4 ml of Wash Solution and aspirate				
Chromogenic Solution	100	100	100	100
Exactly incubate 15 min. at R.T. with continuous shaking				
Stop Solution	200	200	200	200
Read on a microtiter plate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm) and 490 nm (versus 630 or 650 nm).				

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