FluoBolt™-ASPORIN

METAL ENHANCED FLUORESCENCE IMMUNOASSAY for human ASPORIN

METAL ENHANCED FLUORESCENCE IMMUNOASSAY FOR
THE QUANTITATIVE DETERMINATION OF
ASPORIN IN HUMAN SERUM

CAT. NO. FIA-1702-F,-C3,-C5,-A6
96 Well Formate

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

rev.no. 191030

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FIANOSTICS
Fluorescence Immunoassays

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1) METAL / PLASMON ENHANCED FLUORESCENCE

Metal Enhanced Fluorescence (MEF) offers the possibility to increase the analytical sensitivity of systems based on fluorescence detection dramatically. MEF is based on the fact that excitation light interacts with the electrons of metal nano-structures thus generating very high electromagnetic fields (Localised Surface Plasmons, LSPs) Therefore, such structures are also called "plasmonic structures" and the combination of (e.g. polymeric) support and structure is known as "plasmonic substrate". These LSPs lead to an increase in emission output of fluorescent molecules (e.g. fluorescently labeled antibodies) when bound to surfaces with suitable nano-metal structures that enhances the signal dramatically. FIANOSTICS has developed a new plasmonic enhanced immunoassay platform in cooperation with Sony DADC BioSciences (now STRATEC Consumables since July 1st 2016), that allows up to 300 fold gains of sensitivity. This platform is fully compatible to standard laboratory methodology using 96 well microtiter plate format and assays based on this technology can be run on any standard fluorescence microplate reader. Its unique features enable fluorescence immunoassays with highest sensitivity and without washing steps.

2) ASPORIN

ASPORIN, also known as periodontal ligament-associated protein 1 (PLAP1) is a dimeric secreted extracellular matrix protein, which belongs to the small leucine-rich proteoglycan (SLRP) family. It consist out of 380 amino acids and has a highly conserved pro peptide sequence which contains a series of leucine rich repeats and are flanked by two cysteine residues in the C Terminal region. Further, it has four cysteine residues that form disulphide bonds as well as aspartic acid repeats in the N-Terminal region.

High levels can be found in aorta, uterus and osteoarthritic articular cartilage. Further, moderate levels of aspirin expression can be found in small intestine, heart liver, bladder, ovary, stomach, the adrenal-, thyroid-, and mammary gland. Lower levels of expression can be seen in the trachea, bone marrow and lung. ASPORIN is known to negatively regulate PDL differentiation and mineralisation as well as it inhibits BMP- dependent activation of SMAD proteins. Further, it directly binds to TGFβ-1 subsequently, binds to collagen by way of its LRR domain. Through its interaction with TGFβ-1, ASPORIN negatively regulates chondrogenesis in the articular cartilage by blocking the TGF-beta/receptor interaction on the cell surface and inhibiting canonical TGF-beta/Smad signalling. Moreover, it has the ability to bind calcium, giving it regulatory properties in osteoblast driven mineralisation and regulates FGF2 through direct and indirect interactions. Next to its regulatory properties in terms of cartilage and bone homeostasis, ASPORIN Researchers have investigated ASPORIN expression in cancer invasion and progression. However, its value as biomarker remains to be established yet.

3) CONTENT OF THE KIT

ID	KIT COMPONENT	QUANTITY
EM	Rabbit polyclonal anti-human ASPORIN antibody, pre-coated MEF-microtiter plate, packed in an aluminium bag with desiccant	1 x 96 well
WP	Wash buffer concentrate 20x, natural cap	1 x 25 ml
EAF, EA3 EA5, EAA	Anti-human ASPORIN antibody, black flask, labeled with FITC, Cy3, Cy5 or AlexaFluor680	1 x 5 ml
ES	Standards 1-6, (0, 12.5, 25, 50, 100, 200 pmol/l), white caps, lyophilized	6 vials, 0.25 ml
ECA/B	Control A and B, yellow cap, lyophilized (for concentrations see label)	2 vials, 0.25 ml
ED	Sample diluent, natural cap, ready to use	1 x 20 ml

4) ADDITIONAL MATERIAL SUPPLIED WITH THE KIT

- 2 self adhesive plastic films
- Protocol sheet
- Instruction manual for use
- Desiccant bag

5) MATERIAL AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- Precision pipettes calibrated to deliver 10 µl, 20 µl, 50 µl, 200 µl, 500 µl and disposable tips
- Distilled or deionized water
- Plate washer, multichannel pipette or manifold dispenser for washing
- Refrigerator with 4°C (2-8°C)
- Fluorescence microplate reader
- Graph paper or software for calculation of results

6) REAGENTS AND SAMPLE PREPARATION

All reagents of the kit are stable at 4°C (2-8°C) until expiry date stated on the label of each reagent.

Sample preparation:
ASPORIN contains a calcium binding site. When using plasma samples, analytic recovery of the protein strongly varies depending on the amount and type of coagulant used. Therefore we recommend to only use serum samples for ASPORIN analysis. Collect venous blood samples by using standardized blood collection tubes for serum.

We recommend performing serum separation by centrifugation as soon as possible, e.g. 10 min at 2000 x g, preferably at 4°C (2-8°C). The acquired serum samples should be measured as soon as possible. For longer storage aliquot samples and store at -25°C or lower. Do not freeze-thaw samples more than 4 times. Lipemic or haemolysed samples may give erroneous results. Samples should be mixed well before assaying.

Reagent preparation:

Add 250 µl of distilled or deionized water to the lyophilized ES. Add 250 µl of distilled or deionized water to the lyophilized ES (Standards) and EC (Controls). Leave at room temperature (18-26°C) for 20 min. Reconstituted ES and EC are stable at -25°C or lower until expiry date stated on the label. Reconstituted ES and EC can undergo 4 freeze-thaw cycles.
Bring WP (Wash buffer) concentrate (20x) to room temperature. Make sure that the solution is clear and without any salt precipitates before further dilution. Dilute the WP to working strength by adding the appropriate amount of distilled or deionized water, e.g. 25 ml of WP + 475 ml water, prior to use in the assay. Undiluted WP is stable at 4°C (2-8°C) until expiry date on the label. Diluted WP is stable at 4°C (2-8°C) up to one month. Only use diluted WP in the assay.

7) ASSAY PROCEDURE

All reagents and samples must be at room temperature (18-26°C) before use in the assay.

Mark position for standards, controls and samples on the protocol sheet. We recommend to run samples and standards in duplicates. Take the plasmonic enhanced microtiter plate out of the aluminium bag.

Avoid touching the bottom of the plate with bare hands, because reading without washing is performed through the well bottom.

Seal all wells that will not be used in the following assay run with the accompanying adhesive film (cut to fit!).

In standard format, the kit is delivered with an AlexaFluor680 labeled detection antibody (EAA) because serum background fluorescence is minimal within this wavelength range. Therefore if your reader is equipped with monochromatic optics, please set Excitation/Emission to 679/702 nm or if you are using an optical filter based reader, select a suitable filter pair (e.g. 670/720 nm). On request the kit can also be delivered with FITC, Cy3 or Cy5 (Ex/Em = 495/518 nm, 550/570 nm or 650/670 nm) labeled detection antibody.

1) Add 50 µl of the selected fluorescence labeled detection antibody (EAF or EA3 or EA5 or EAA) to all wells required. Swirl gently.

2) Add 10 µl of standard, control or sample to the wells according to the marked positions on the protocol sheet, swirl gently, cover tightly with the delivered adhesive film and incubate over night at room temperature (18-26°C) in the dark.

3a) If your reader allows bottom reading, read the plate without any further processing at the Ex/Em wavelength fitting to the delivered detection antibody (495/518 nm for EAF, 550/570 nm for EA3, 650/670 nm for EA5, 679/702 nm for EAA). Gain should be set to achieve at least 10000 fluorescence units (F.U.) between the signal of the 0 pM and the 400 pM ASPORIN standard. Samples with signals exceeding the signal of the highest standard must be re-run with an appropriate dilution using sample diluent (ED).

3b) If your reader has no bottom read option or if you want to store the plate for documentation purposes, discard or aspirate the content of the wells and wash 3x with diluted wash buffer. Use a minimum of 200 µl wash buffer per well. After the final wash, remove remaining fluid by strongly tapping the plate against a paper towel. Read the plate in top configuration without any further processing at the Ex/Em wavelength fitting to the chosen detection antibody (495/518 nm for EAF, 550/570 nm for EA3, 650/670 nm for EA5, 679/702 nm for EAA).

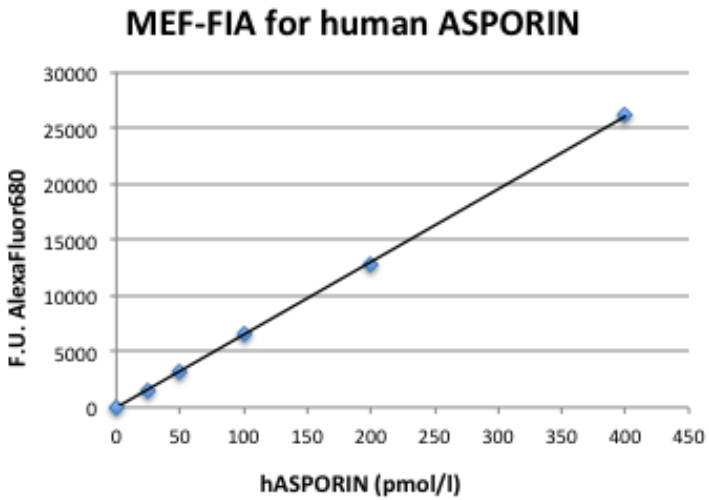
Hint: Quality of bottom reading (3a) may vary between microplate readers. For first time users we suggest to perform the washing step and follow protocol 3b.

Gain should be set to achieve at least 10000 fluorescence units (F.U.) between the signals of the 0 pM and the 400 pM ASPORIN standard. Samples with signals exceeding the signal of the highest standard must be re-run with appropriate dilution using sample diluent (ED).
4) Store the plate with desiccant at 4°C (2-8°C) in the aluminium bag. Unused wells are stable until expiry date stated on the label. Fluorescence signals of standards, controls and samples remain detectable for at least two month at the plate surface, depending on signal intensity achieved.

8) CALCULATION OF RESULTS

Subtract the fluorescence intensity of the 0 pM standard from all other standards, samples and controls. Construct a calibration curve from the fluorescence units (F.U.) of the standards using commercially available software or graph paper. Read sample and control concentrations from this standard curve. The assay was evaluated with 4PL algorithm. Different curve fitting methods need to be evaluated by the user.

Example of a typical calibration curve



The quality control (QC) protocol supplied with the kit shows the results of the final release QC for each kit lot at production date.

Fluorescence intensity obtained by customers may differ due to various influences and/or due to the normal decrease of signal intensity during shelf life.

However, this does not affect validity of results as long as the supplied kit controls read according to specifications (target ranges see labels).

9) ASSAY CHARACTERISTICS

Method	Metal Enhanced Direct Sandwich Fluorescence Immunoassay in 96-well plate format
Sample type	Serum
Standard range	0 to 400 pmol/l (6 standards and 2 controls in a serum based matrix)
Conversion factor	1 ng/ml = 11,25 pmol/l (MW: 43,417 kD / Monomer)
Sample volume	10 µl (undiluted sample) / well
Incubation time / temperatu	over night / RT
Sensitivity	LOD (0 pmol/l + 3 SD): 10 pmol/l; LLOQ: 25 pmol/l
Specificity	This assay detects human ASPORIN
Cross-reactivity	Human ASPORIN shares around 99% aa sequence identity with higher apes (e.g. gorilla or chimpanzee) but only 90% with rat/mouse and 87% with bovine/ equine ASPORIN. Cross reactivity of this assay with other species than human has not been tested.

Precision:

Intra-assay:
5 samples of known concentrations were tested 3 times within 1 assay run.
CVs ranged from 3-7%

Inter-assay:
2 samples of known concentrations were tested in duplicates within 3 different assay runs:
CVs ranged from 8-11%

Spike/Recovery:
The recovery of ASPORIN was evaluated by adding known amounts of human recombinant ASPORIN to 4 different human serum samples.
Mean recovery was 69%

Linearity:

4 human serum samples were spiked with recombinant ASPORIN and diluted 1+2 and 1+9 with the sample diluent (ED) supplied with the kit. Mean linearity was 78%.

Specificity:

Analyte Specificity:
This assay detects human ASPORIN. Addition of recombinant TGF-β1 to the standards supplied with this kit did not reduce signal intensity.

Species Specificity:
Human ASPORIN shares around 99% aa sequence identity with higher apes (e.g. gorilla or chimpanzee) but only 90% with rat/ mouse and 87% with bovine/ equine ASPORIN. Cross reactivity of this assay with other species than human has not been tested. However, spike/recovery experiments in mouse and rat serum worked well, indicating no matrix interferences caused by this sample type. Using this assay with samples from other species must be evaluated by the user. FIANOSTICS does not take responsibility for functionality of the assay in non-human samples.

10) TECHNICAL HINTS

- Do not mix or substitute reagents with those from other lots or sources.
- Do not mix stoppers and caps from different reagents or use reagents between lots.
- Do not use reagents beyond expiration date.
- Protect reagents from direct sunlight.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.

11) PRECAUTIONS

- All test components of human source were tested against HIV-Ab and HBsAg and were found negative. Nevertheless, they should be handled and disposed as if they were infectious.
- Liquid reagents contain ≤0.1% Proclin 300 as preservative. Proclin 300 is not toxic in concentrations used in this kit. It may cause allergic skin reactions – avoid contact with skin, eyes or mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply cosmetics where reagents are used.
- Wear gloves, protective glasses and lab jacket while performing this assay.
- For Research Use Only. Not for Diagnostic Purposes.

12) LITERATURE

- **Metal-enhanced fluorescence: an emerging tool in biotechnology.** Aslan K, et al. Curr Opin Biotechnol, Feb 2005; 16(1):55-62.

- **Asporin and Osteoarthrities** Xu et al., Osteoarthritis and Cartilage. 2015 Jun; 23(6): 933-939. Volume 23, Issue 6, June 2015, Pages 933-939

- **Replication of association of the D-repeat polymorphism in asporin with osteoarthristis** Ikegawa et al. , Arthritis Res Ther. 2006; 8(4): 403.

- **Cartilage Intermediate Layer Protein and Asporin Polymorphisms Are Independent Risk Factors of Lumbar Disc Degeneration in Male Collegiate Athletes** Seok-KiMin et al., Cartilage. 2014 Jan; 5(1): 37-42.

- **Asporin, a susceptibility gene in osteoarthritis, is expressed at higher levels in the more degenerate human intervertebral disc** Gruber et al., Arthritis Res Ther. 2009; 11(2): R47.

- **Asporin Is a Fibroblast-Derived TGF-β1 Inhibitor and a Tumor Suppressor Associated with Good Prognosis in Breast Cancer.** Maris et al., PLoS Med. 2015 Sep 1;12(9)

- **Asporin Is a Fibroblast-Derived TGF-β1 Inhibitor and a Tumor Suppressor Associated with Good Prognosis in Breast Cancer.** Maris P, et al. PLoS Med. 2015 Sep 1;12(9):e1001871.

- **The dual role of asporin in breast cancer progression.** Simkova et al. Oncotarget. 2016 Aug 9; 7(32): 52045–52060.

- **Asporin is a stromally expressed marker associated with prostate cancer progression**. Rochette et al. Br J Cancer. 2017 Mar 14;116(6):775-784.

13) NOTES

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