

ACSDKP ENZYME IMMUNOASSAY KIT

catalogue # A05881

96 wells

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U.S. patent # 50 47 330 European patent # 89 139 552

THE ACSDKP ENZYME IMMUNOASSAY HAS BEEN DEVELOPED AND VALIDATED BY SPI-BIO AND THE CEA.

For research laboratory use only. Not for human diagnostic use.



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ACSDKP ASSAY KIT

96-well - Storage: -20 ℃ Expiry date: stated on the package

This kit contains:

- R A covered 96 well plate, pre-coated with mouse anti-rabbit, ready to use after thawing
- One vial of AcSDKP tracer, lyophilised
- Two vials of AcSDKP standard, lyophilised
- One vial of AcSDKP antiserum, lyophilised
- I One vial of EIA buffer, lyophilised
- I One vial of concentrated Wash buffer, liquid
- 🖙 One vial of tween 20, liquid
- INT Two vials of Quality Control sample, lyophilised
- I One vial of Quality Control Stock Solution, liquid
- INT Two vials of Ellman's reagent, lyophilised
- Solution Solution Solution
- I One template sheet
- Real One well cover sheet

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 33 samples in duplicate.

PRECAUTIONS FOR USE

Users are recommended to read all instructions for use before starting work.

Each time a new pipet tip is used, aspirate a sample or reagent and dispense it back into the same vessel. Repeat this operation 2 or 3 times before distribution.

For research laboratory use only. Not for human diagnostic use. Do not pipet liquids by mouth. Do not use kit components beyond the expiration date. Do not eat, drink or smoke in area in which kit reagents are handled. Avoid splashing.

The total amount of reagents contains less than 100 μg of sodium azide. Flush the drains thoroughly to prevent the production of explosive metal azides.

MATERIALS AND EQUIPMENT REQUIRED

In addition to standard laboratory equipment, the following material is required:

- IS Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm)
- Microplate washer (or washbottles)
- Microplate shaker
- Distilled or deionized water
- Ref Polypropylene tubes



PRINCIPLE OF THE ASSAY

This Enzyme Immunoassay (EIA) is based on the competition between unlabelled AcSDKP and acetylcholinesterase (AChE) linked to AcSDKP (tracer) for limited specific rabbit anti-AcSDKP antiserum sites.

The complex rabbit antiserum-AcSDKP (free AcSDKP or tracer) binds to the mouse anti-rabbit antibody that is attached to the well.

The plate is then washed and Ellman's Reagent (enzymatic substrate for AChE and chromogen) is added to the wells.

The AChE tracer acts on the Ellman's Reagent to form a yellow compound.

The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of tracer bound to the well and is inversely proportional to the amount of free AcSDKP present in the well during the immunological incubation.

The principle of the assay is summarised on below:



SAMPLE COLLECTION & PREPARATION

This assay may be used to measure AcSDKP in plasma or serum and urine samples.

Urine sample may be assayed directly (without extraction) if diluted more than five fold in EIA buffer.

Plasma or serum samples should be measured after extraction. To do so, collect blood samples in tubes containing Heparine (do not use EDTA) and Captopril to 10^{-5} M final concentration (e.g.: $10 \ \mu$ I of 10^{-3} M Captopril solution for 1 ml of blood). Centrifuge the samples at 1 600 g for 20 minutes. Collect and keep plasma at-20 °C until assay.



EXTRACTION PROTOCOL

Thaw the plasma on ice water bath and keep the sample in the ice. Centrifuge 1 mL of plasma during 15 minutes at 4,500 rpm at 4° C.

Collect 0.15 mL of plasma and add 1 mL of methanol. Vortex the solution twice during 10 seconds and centrifuge it during 15 minutes at 4,500 rpm at 4 °C.

Decant the supernatant into a clean test tube and dry it by vacuum centrifugation. Reconstitute the sample with 0.45 mL of EIA buffer (dilution of initial plasma sample = three fold).

RECOVERY AND CALCULATION

To determine the recovery factor after extraction, use the QC Stock solution (500 nM) provided in this kit,

- ♦ Thaw 1 mL of plasma.
- Dilute 0.1 mL of QC Stock Solution (500 nM) with 0.9 mL of plasma, to prepare a plasma QC at the concentration of 50 nM.
- Scollect 0.15 mL of the plasma QC (50 nM) and add 1 mL of methanol. Vortex the solution twice during 10 seconds and centrifuge it during 15 minutes at 4,500 rpm at 4 ℃.
- Decant the supernatant into a clean test tube and dry it by vacuum centrifugation. Reconstitute the sample with 3 mL of EIA buffer (dilution of initial plasma sample = 1:20).

The recovery is determined by comparing the concentration of the QC determined by EIA multiplied by 20 (the dilution factor of the extraction) to the plasma concentration of the QC (50 nM).

REAGENT PREPARATION

The coated plates and reagents are provided ready to use.

Real EIA buffer

Reconstitute one vial with 50 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

Reality Control

Reconstitute one vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4 °C: 1 day.

Results AcSDKP standard

Short immunological reaction (**urine samples only**): Reconstitute the vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 25 nM. Prepare seven propylene tubes (for the seven other standards) and add 500 μ L of EIA buffer into each tube. Add 500 μ L of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes. Thus, standard concentrations are: 25 (S1), 12.5 (S2), 6.25 (S3), 3.12 (S4), 1.56 (S5), 0.78 (S6), 0.39 (S7) and 0.19 nM (S8), respectively. Stability at 4°C : 1 day.

For long immunological reaction (**plasma or urine**): Reconstitute the vial with 2 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 12.5 nM. Prepare seven propylene tubes (for the seven other standards) and add 500 μ L of EIA buffer into each tube. Add 500 μ L of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes. Thus, standard concentrations are: 12.5 (S1), 6.25 (S2), 3.12 (S3), 1.56 (S4), 0.78 (S5), 0.39 (S6), 0.19 (S7) and 0.09 nM (S8), respectively. Stability at 4°C: 1 day.

Rest AcSDKP-AChE tracer

Reconstitute the vial with 5 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4° C: 1 month.



Restance AcSDKP antiserum

Reconstitute the vial with **5 mL** of distilled or deionized water for **short immunological reaction** or **10 mL** of distilled or deionized water for **long immunological reaction**. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

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🖙 Wash buffer

Dilute 1 ml of the concentrated wash buffer to 400 mL with distilled or deionized water. Add 200 μ L of tween 20 (Use a magnetic stirrer to mix the contents). Stability at 4°C: 1 week.

Reagent Ellman's Reagent

Five minutes before use, reconstitute with 50 mL of distilled or deionized water. The tube contents should be tho-roughly mixed. Stability at 4 ℃ and in the dark: 4 days.

ASSAY PROCEDURE

It is recommended to perform the assays in duplicate and to follow the instructions hereafter.

PLATE PREPARATION

Prepare the wash buffer as indicated in the reagent preparation section. Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet (stored at 4 °C). Rinse each well five times with the wash buffer (300 μ L/well).

Just before distributing reagents and samples, remove the buffer from the wells by inverting the plate and shaking out the last drops.

DISTRIBUTION OF REAGENTS AND SAMPLES

A plate set-up is suggested on below. The contents of each well may be recorded on the sheet provided with the kit.



B : Blank

NSB : Non Specific Binding Bo : Maximum Binding S1-S8 : Standards 1-8 *: Samples or Quality Controls



PIPETTING THE REAGENTS

Note that the first column should be left empty for blanking Ellman's reagent. All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipet the buffer, standard, sample, tracer, antiserum and other reagents.

- EIA buffer: Dispense 100 μL to Non Specific Binding (NSB) wells and 50 μL to Maximum Binding (Bo) wells.
- AcSDKP standard: Dispense 50 μL of each of the eight standards (S1 to S8) in duplicate to appropriate wells. Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.
- Quality Control and samples: Dispense 50 μL in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA buffer.
- Solution Soluti Solution Solution Solution Solution Solution Solution S
- Solution Section 4. Solution (NSB) wells.

INCUBATING THE PLATES

or

Cover the plate with a plastic film and incubate for :

- ⇔ 3 hours (short immunological reaction) at room temperature (22 °C), for **urine samples only**,
- ♥ 18 hours (long immunological reaction) at +4 °C for plasma or urine samples.

DEVELOPING AND READING THE PLATE

Reconstitute the wash buffer and Ellman's Reagent as indicated in reagent preparation section. Empty the plate by turning over and shaking. Then, wash each well five times with the wash buffer ($300 \mu L$ /well). Dispense 200 μL of Ellman's Reagent to the 96 wells. Incubate in the dark (plate covered with an aluminium sheet) at room temperature. Optimal development is obtained using an orbital shaker. The plate should be read between 405 and 414 nm (yellow colour) when the Maximum Binding (Bo) wells reach an absorbance of 0.2-0.8 unit.

		Enzyme Immunoassay	Protocol (Volumes are in µl)		
	Blank	Non specific binding	Maximum binding	Standard	Sample
Buffer		100	50		
Standard	-		•	50	
Sample	•		•		50
Tracer	•	50	50	50	50
Antiserum	•		50	50	50
	Cover	the plate, incubate 3 hours +4 °C	at room temperature (for urine sample: ; (for plasma or urine samples)	s only), or 18	hours at
			Wash the plate 5 times		
Ellman's reagent	200	200	200	200	200
		Incubate th	e plate with an orbital shaker in the		
		c	lark at room temperature		
		Read th	e plate between 405 and 414 nm		



DATA ANALYSIS

Make sure that your plate reader has subtracted the absorbance readings of the blank well (absorbance of Ellman's reagent) from the absorbance readings of the rest of the plate. If not, do it now.

- ♦ Calculate the average absorbance for each NSB, Bo, standards and samples.
- Calculate the B/Bo (%) for each standard and sample: (average absorbance of standards or sample average absorbance of NSB) divided by (average absorbance of Bo average absorbance of NSB) & multiplied by 100.
- Using a semi-log graph paper, plot the B/Bo (%) for each standard point (y axis) versus the concentration (x axis). Draw a best-fit line through the points.
- To determine the concentration of your samples, find the B/Bo (%) value on the y axis. Read the corresponding value on the x axis which is the concentration of your unknown sample. Samples with a concentration greater than 12.5 nM should be re-assayed after dilution in EIA buffer.
- Solution with the solution of the solution of

TYPICAL DATA

EXAMPLE DATA

The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 1.5 hour developing at 20 °C, reading at 414 nm. A 4-parameter curve fitting was used to determine the concentrations.

	Short immunological reaction		Long immunological reaction	
Standard	MAU	B/Bo (%)	mAU	B/Bo (%)
NSB	0	0.0	2	0.0
Во	292	100.0	347	100.0
25.0 nM	28	9.6		
12.5 nM	45	15.4	23	6.6
6.25 nM	76	25.9	36	10.4
3.12 nM	119	40.7	66	19.0
1.56 nM	163	55.9	97	28.0
0.78 nM	197	67.6	154	44.4
0.39 nM	232	79.8	223	64.3
0.19 nM	258	88.5	283	81.6
0.09 nM			296	85.3
QC	140	48	93	26.8

ACCEPTABLE RANGE

- [™] Bo absorbance: > 200 mAU in the conditions indicated above.
- Ratio NSB absorbance / Bo absorbance: < 0.1.
- I® 50% B/Bo%:
- 2.0 nM, after short immunological reaction
- 0.5 nM, after long immunological reaction
- Real QC sample: see the label on the vial.





ACSDKP STANDARD CURVE



ASSAY VALIDATION & CHARACTERISTICS

IMMUNOCHROMATOGRAMS (PLASMA SAMPLE)



Fig. 1. Chromatograms obtained from plasma samples with selected ion monitoring. Plasma free of AcSDKP (A), plasma free of AcSDKP (b), plasma free of AcSDKP (b), plasma free of AcSDKP (b), pooled plasma from AcSDKP (b), pooled plasma from AcSDKP-treated volunteers (D) and pooled plasma from AcSDKP-treated volunteers (E). The internal standard peak is not shown.



IMMUNOCHROMATOGRAMS (URINE SAMPLE)



measurements in immunoextracted samples. Pooled plasma from untreated volunteers (one sample) (\bullet) , pooled plasma from captopril-treated volunteers (two samples) (\blacksquare) , pooled plasma from AcSDKP-treated volunteers (one samples) (\bullet), pooled urine from untreated volunteers (two samples), (O) and pooled urine from captopril-treated volunteers (two samples) (\Box) .

ASSAY TROUBLE SHOOTING

- IS Bo value is too low: incubation in wrong conditions (time or temperature) or reading time too short or AcSDKP-AChE tracer, AcSDKP antiserum or Ellman's reagent have not been dispensed.
- INSB value too high: contamination of NSB wells with AcSDKP antiserum or inefficient washing.
- Bigh dispersion of duplicates: poor pipetting technique or irregular plate washing.
- IS IC₅₀ or QC concentrations not within the expected range: wrong preparation of standards.

CORRELATION



Analyses of two dilutions of a biological sample do not agree: Interfering substances are present. Sample must be purified prior to EIA analysis (excepting plasma samples).

These are a few examples of trouble shooting that may occur. If you need further explanation, SPI-BIO will be happy to answer any question or information about this assay. Please feel free to contact our technical support staff by letter, phone (33 (0)1 39 30 62 60), fax (33 (0)1 39 30 62 99) or E-mail (contact@spibio.com), and be sure to indicate the lot number of the kit (see outside the box).

SPI-BIO proposes a training workshop in EIA practice & theory. This workshop is given twice a year. For further information, please contact our Customer Relation Representative (33 (0)1 69 53 14 00).

BIBLIOGRAPHY

Struthers A.D., McFayden R., Fraser R., Robson C., Morton J.J., Junot C., Ezan E. Non adherence with ACE inhibitor therapy: a comparison of different ways of measuring it with observations on its frequency in heart failure. *JACC*, 1999, 34, 2072-2077.

- Azizi M., Ezan E., Reny J.L., Wdzieczak-Bakala J., Gerineau V., Menard J. Renal and metabolic clearance of N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) during angiotensinconverting enzyme inhibition in human. *Hypertension. 1999 Mar, 33(3), 879-86.*
- Azizi M., Ezan E., Nicolet L., Grognet J.-M., Menard J. High plasma level of N-acetyl-seryl-aspartyl-lysyl-proline: a new marker of chronic angiotensin-converting enzyme inhibition. *Hypertension. 1997 Nov, 30(5),1015-9.*
- Azizi M., Rousseau A., Ezan E., Guyene T.T., Michelet S., Grognet J.-M., Lenfant M., Corvol P., Menard J.

Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator N-acetyl-seryl-aspartyl-lysyl-proline. *J Clin Invest.* 1996 Feb 1, 97(3), 839-44.

🖙 Grassi J. & Pradelles Ph.

Compounds labelled by the acetylcholinesterase of *Electrophorus Electricus*. Its preparation process and its use as a tracer or marquer in enzymo-immunological determinations. *United States patent*, $N \circ 1,047,330$. September 10, 1991.

🖙 Pradelles Ph., Frobert Y., Créminon C., Ivonine H., Frindel E.

Distribution of a negative regulator of haematopoietic stem cell proliferation (AcSDKP) and thymosin B4 in mouse tissues.

FEBS 10125, 1991, 289(2), 171-175.

Real Pradelles Ph., Frobert Y., Créminon C., Liozon E., Massé A., Frindel E.

Negative regulator of pluripotent hematopoiectic stem cell proliferation in human white blood cells and plasma as analysed by enzyme immunoassay.

Bioch. Bioph. Res. Com., 1990, August 16, 170-3, 986-993.