



**Instruction Manual**

**Pentosan Polysulfate (PPS) ELISA Kit**

Catalog No K1126

Immunoassay for quantitative determination of the content of Pentosan Polysulfate (PPS) in human plasma

*Please read this instruction manual carefully before using the kit.*

*(Version 2.8, Last updated on 17 April 2012)*

**For Research Use only (RUO)**

**Not for use in Diagnostic Procedures**

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## 1. Intended Use and Background Information

Pentosan Polysulfate (PPS) is a heparin-like product obtained after sulfation of xylan. Sold under brand name Elmiron® [1], it is the only oral medication approved by FDA for the relief of bladder pain or discomfort associated with interstitial cystitis [2]. Further, a number of clinical and non-clinical studies suggested roles of PPS in tumor growth inhibition [3,4], as an anti-HIV agent [5], as inhibitor of atherosclerosis[6] and for the treatment of osteoarthritis [7].

This ELISA kit uses the principle of an immunoassay for detection of PPS in clinical samples. Use of an immunoassay for detection of PPS was reported in a few earlier studies [4,8,9]. However, none of these methods was available for usage by others.

This kit can be used for detection of PPS in plasma samples of subjects dosed with PPS. It can also be used (with a few modifications in the protocol) for detection of PPS in human urine and in aqueous solution.

Recommended therapeutic dose for oral PPS for Interstitial Cystitis patients is 300 mg/day (100 mg thrice daily), whereas for certain trials with cancer patients [4] a much higher dose of 400 mg/m<sup>2</sup> ( $\cong$  700 – 800 mg/day) has been used. Most studies show that >90% of PPS is excreted intact through urine and feces and the bioavailability of oral PPS in blood is estimated to be around 3-4% of the dose administered.

In pharmacokinetic study with healthy subjects [1] who were dosed with 300 or 450 mg of single dose of PPS, the observed steady state level of PPS in plasma was 20 – 50 ng/ml, whereas in an efficacy study with cancer patients [4] dosed with 2-3 times the therapeutic dose, the steady state level of PPS in human plasma on day 1 of the treatment was  $68.7 \pm 6.8$  ng/ml.

Sensitivity of this ELISA kit is 2.6 ng/ml in human plasma, which is ~ 10% of the lowest observed steady state level with 300 mg of single dose. For a single-dose pharmacokinetic study, a higher dose (450 mg) could be used in view of the poor bioavailability of the molecule. With such a dose, the sensitivity of the assay would be < 5% of the observed steady-state level of PPS in plasma.

## 2. Principle of the Assay

This assay method uses an indirect ELISA format in which the primary reagent is the rabbit polyclonal antibody against PPS developed by Abexome Biosciences. In this assay, matrix containing the analyte i.e. PPS is coated on microtiter plates pre-coated with Poly-L Lysine. The bound PPS is then detected by adding Rabbit PPS polyclonal antibody followed by detection with Goat anti-Rabbit IgG peroxidase conjugate/TMB substrate. The signal produced is measured at 450 nm in a micro plate reader.

Key steps of the assay and approximate time taken for each step are shown in the table below:

Sl No	Step	Time Taken
1	Analyte Coating and Incubation	2.5 hr
2	Blocking	1 hr
3	Primary Antibody Binding	1.5 hr
4	Detection Antibody Binding	1.5 hr
5	Substrate Addition and Measurement	45 min
6	Calculation of Results	15 min

The assay has been validated in human plasma according to regulatory guidelines and industry-recommended practices (see § 8 below for details). Key performance characteristics of the assay are:

Parameter	Value (in human plasma)
Sensitivity / Lower Limit of Quantification	2.6 ng/ml
Upper Limit of Quantification	166.5 ng/ml
Range of Quantification	2.6 – 166.5 ng/ml

### 3. Materials

#### 3.1 Materials Supplied with the Kit

Sl No	Description	Quantity	Strength	Recommended Storage on Receipt
1	PPS standard	2.5 µg	NA	-20°C
2	Anti-PPS Antibody	67 µl	150 X	-20°C
3	Anti-PPS Detection Antibody	2 µl	10,000 X	-20°C
4	Blocking Reagent	200 mg	NA	25°C
5	TMB Substrate	12 ml	1 X	4°C
6	Pre-coated microtiter plate	1 x 96-well	NA	-80°C

All kit components are stable for minimum 4-5 months if stored at recommended storage temperature.

#### 3.2 Materials and Equipments to be Supplied by End-user

Sl No	Item	Recommended Make (Catalog No) if any
1	1X Phosphate Saline Buffer(PBS), pH 7.4	PBS tablet, Sigma Aldrich (P4417)
2	1X Phosphate Saline Buffer with 0.05% Tween-20(PBST)	-
3	2N Sulphuric Acid (H <sub>2</sub> SO <sub>4</sub> )	-
4	Tween-20 (AR grade)	-
5	Powder free gloves	-
6	Sterile Distilled Water	-
7	Microtiter plate orbital shaker	-
8	Microtiter plate reader with 450 nm filter	-
9	Human Plasma (to be used as matrix)	-

### 4. Precautions

- Avoid freeze/thaw cycles for all reagents.
- Handle all reagents wearing gloves and other protective gears. Avoid direct contact with eyes and skin. Do not pipette any reagents by mouth.
- Wear gloves which would not interfere with proper grip of fingers.
- Use well calibrated pipettes.
- TMB Substrate and Detection Antibody are light sensitive, hence should not be exposed to light.

## 5. Reagent Preparation



- ✓ Use 0.2% K-EDTA as anti-coagulant for plasma separation



- ✓ Vortex well to mix during each step
- ✓ Use 1.5 or 2 ml Eppendorf tube only
- ✓ Use appropriate pipette range and do not change pipette in between the assay procedure

### 5.1 Matrix Selection and Handling

- Blood samples from healthy subjects, not dosed with Pentosan Polysulfate, should be used for preparing the plasma matrix.
- Plasma samples should be clear, non-haemolyzed and non-turbid. No further processing is required for plasma samples.

### 5.2 Standards

- Reconstitute the PPS standard into 62.5  $\mu$ l plasma matrix to get main stock of 40  $\mu$ g/ml concentration (to be made fresh on the day of experiment for single time use).
- Prepare a sub-stock of 4000 ng/ml concentration by diluting 40  $\mu$ l of main stock to 400  $\mu$ l with plasma matrix (to be made fresh on the day of the experiment).
- Prepare standards S1-S12 and blank in plasma matrix as explained in the Table below. Seven of these points (S4-S10) act as standard calibrators and the remaining five are used to facilitate curve-fitting by 4-parameter logistic (4PL) method.

Sub stock of PPS (ng/ml)	Volume from sub stock ( $\mu$ l)	Plasma Volume ( $\mu$ l)	Final concentration (ng/ml)	Standard ID
4,000	333.4	666.6	1332.00	S1
1332.00	400	400	666.00	S2
666.00	400	400	333.00	S3
333.00	400	400	166.50	S4
166.50	400	400	83.25	S5
83.25	400	400	41.63	S6
41.63	400	400	20.81	S7
20.81	400	400	10.41	S8
10.41	400	400	5.20	S9
5.20	400	400	2.6	S10
2.6	400	400	1.3	S11
1.3	400	400	0.65	S12
	0	400	0	Blank



- ✓ Vortex well to mix during each step
- ✓ Use 1.5 or 2 ml Eppendorf tube only
- ✓ Use appropriate pipette range and do not change pipette in between the assay procedure

### 5.3 QC Samples preparation

- Prepare at least three QC samples – HQC: 125 ng/ml, MQC: 30 ng/ml and LQC: 7.8 ng/ml by spiking plasma matrix from 4,000 ng/ml sub stock of PPS.
- To be made fresh on the day of experiment for one time use.



- ✓ *Make sure not to allow the contents of the blocking buffer to boil during the microwave step.*
- ✓ *Since the Tween-20 is highly viscous reagent, take the accurate volume and add to the solution properly.*
- ✓ *Mix the solution on magnetic stirrer at least for 10 min.*
- ✓ **Do not** store the warm solution immediately at 4°C

#### 5.4 Blocking Buffer

- Take 40 ml of 1X PBS pH 7.4 into a glass conical flask/reagent bottle.
- Transfer the blocking reagent into the solution.
- Dissolve the reagent by slightly warming in microwave oven until the solution becomes clear and keep it stirring on magnetic stirrer for 1 hour at 25°C.
- This solution can be stored at 4°C for 4-5 days.
- Add 0.5% (v/v) Tween-20 on the day of the experiment to make the working Blocking Buffer.

#### 5.5 Primary Antibody

- Spin the tube to bring content to the bottom.
- Transfer entire content to a larger container and make up the volume to 10.5 ml with 1X PBST to prepare ready-to-use solution
- To be made fresh on the day of experiment for single time use.

#### 5.6 Detection Antibody

- Spin the tube to bring content to the bottom.
- Add 100 µl 1X PBST, mix by vortexing and spin to bring content to the bottom of the tube.
- Transfer entire content to a larger container and make up the volume to 20 ml with 1X PBST to prepare ready-to-use solution
- To be made fresh on the day of experiment for single time use

### 6. Assay Procedure

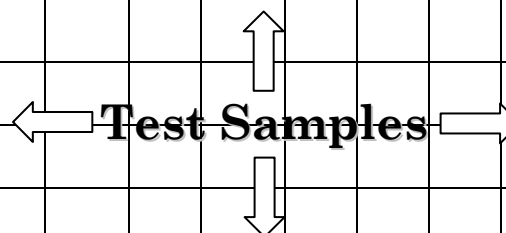
#### 6.1 Analyte Coating

1. Bring the PLL-coated plate to room temperature, wash once with 1X PBS.
2. Prepare standards, QC samples and test samples.
3. Add standards, QC samples and test samples at 100 µl/well to the PLL-coated plate. Suggested plate map for sample analysis is given below.



*Keep plate map next to you to avoid mistakes*

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
<b>B</b>	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12
<b>C</b>	HQC	MQC	LQC	PB								
<b>D</b>	HQC	MQC	LQC	PB								
<b>E</b>												
<b>F</b>												
<b>G</b>												
<b>H</b>												



*PB- Plasma Blank*

- Seal plates with adhesive plate sealer and close the plate lid.
- Incubate the plate at 4°C for 2 hours on plate orbital shaker at low (~ 100) rpm.

### 6.2 Blocking

- Bring the plates to ~ 25°C by keeping it on the bench for ~15 minutes.
- Aspirate out the contents of each well so that there is no cross contamination between the wells.
- Wash the plate twice with 1X PBS for ~30 seconds each.
- Blot plate on absorbent paper to remove any residual reagent from the well.
- Block the plates by adding 300 µl of freshly prepared Blocking Buffer.
- Seal plates with fresh adhesive plate sealer and close the plate lid.
- Incubate at 25°C for 1 hr on plate orbital shaker at low (~ 100) rpm.

### 6.3 Primary Antibody Binding

- Discard the contents of each well.
- Wash the plate twice with 1X PBS for ~30 seconds each.
- Blot plate on absorbent paper to remove any residual reagent from the well.
- Add 1X Anti-PPS Antibody 100 µl/well to the plate.
- Seal plates with fresh adhesive plate sealer and close the plate lid.
- Incubate the plate on orbital shaker at low (~ 100) rpm at 25°C for 1 hr.



Make sure that the Blocking Buffer is ready (section 5.5)



- ✓ Use multichannel pipette
- ✓ Make sure all the tips have same volume of liquid while taking from the reagent boat
- ✓ Make sure there are no air bubbles in the tips



- ✓ Take care not to add antibody in the blank or control wells

#### **6.4 Detection Antibody Binding**

1. Discard the contents of each well and wash 3 times with 1X PBST followed by 3 times with 1X PBS allowing ~3 minutes for soaking during each wash.
2. Blot plate on absorbent paper to remove any residual reagent from the well.
3. Add Anti-PPS Detection Antibody solution 100 µl/well to the plate.
4. Seal plates with adhesive plate sealer and close the plate lid.
5. Incubate the plate on orbital shaker at low (~ 100) rpm at 25°C for 45 minutes.

#### **6.5 Substrate Addition and Measurement**

1. Wash the plate and blot subsequently as mentioned in steps 1 and 2 of section 6.5 above.
2. Add 100µl of TMB substrate per well. Incubate the plate undisturbed in dark at 25°C for 25 minutes.
3. Stop the reaction by adding 50µl/well stop solution (2N H<sub>2</sub>SO<sub>4</sub>).
4. Record the optical absorbance of the wells at 450nm using the microtiter plate reader.

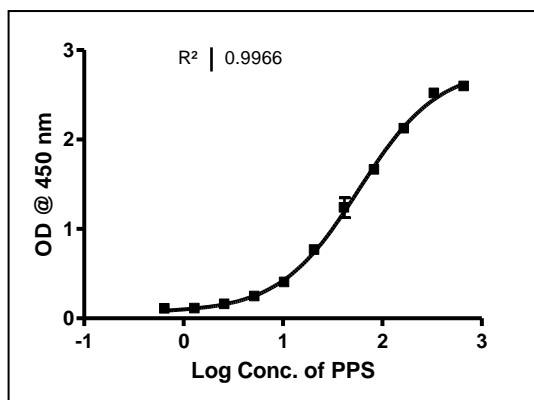
#### **6.6 Calculation of Results**

With the standards, construct a standard curve and determine the linear range. It is recommended to use a software program to plot the standard curve and to determine the concentration of the samples. The data analysis given in the subsequent section was done using Graphpad Prism® version 4. Absorbance of the test and the QC samples are interpolated by using 4PL method.

## 7. Example Data

The following results are provided for demonstration only and are not be used for any calculations. End-user must run a standard curve for each assay.

Standard	Conc (ng/ml)	Mean Abs
S1	1332.00	2.602
S2	666.00	2.594
S3	333.00	2.517
S4	166.50	2.122
S5	83.25	1.662
S6	41.63	1.237
S7	20.81	0.765
S8	10.41	0.402
S9	5.20	0.244
S10	2.6	0.155
S11	1.3	0.109
S12	0.65	0.106
	Blank	0.096



**Mean absorbance values  
 in plasma matrix**

## 8. Assay Characteristics

### 8.1 Validation summary

Abexome Biosciences have validated this assay in plasma matrix according to FDA Bioanalytical Method Validation Guidelines [10] and industry-recommended practices for ligand-binding assays [11,12,13]. However, such validation is generic in nature and it is intended to only supplement but not substitute specific validation as required by regulations or otherwise in each case. The end-user is expected to validate the assay parameters as appropriate.

## **8.2 Standard Curve and Regression Model**

The calibration standards were generated by spiking PPS in plasma. The standard curve consisted of twelve non-zero standards including seven calibrators and five anchor points. A 4-PL regression model was used to fit the curve. Anchor points were excluded from acceptance criteria and used to facilitate curve fitting. The regression model was accepted as the %AR was within 80-120% for each calibrator. Cumulative mean precision (%CV) of all calibrators was less than 20%.

## **8.3 Accuracy and Precision**

Intra-assay precision (%CV) between replicates of the QC samples was  $\leq 20\%$ , while accuracy (%AR) of the QC samples was between 80-120%

## **8.4 Robustness**

Robustness of the assay was tested by repeating the experiment with two analysts, at two laboratory locations using two different plate readers and during two different days. Inter-assay variation and pooled intra-assay variations (%CV) of the QC samples was  $\leq 20\%$  indicating robustness of the assay.

## **8.5 Selectivity**

Five different human plasma matrices were tested for selectivity in a single batch experiment by recovery studies at two QC samples. 4 out of 5 different individual plasma samples passed the acceptance criteria of %AR to be within 80-120% for both QC samples.

## **8.6 Sensitivity**

Across six batch runs LLOQ-QC at 2.6 ng/ml showed %AR within 75-125% range confirming the sensitivity of the assay to be around 2.6 ng/ml.

## **8.7 Drift**

The drift parameter was evaluated by placing LQC and HQC samples at two opposite edges of the 96-well microtiter plate, Difference in %AR of each QC sample between the two edges was  $\leq 20\%$  indicating no appreciable drift across the plate.

## **8.8 Cross-Reactivity**

Cross-reactivity of the assay was assessed with Heparin and Chondroitin sulphate. For each molecule, recovery studies were done

at three QC points and the %AR values were calculated. The %AR for all three QCs (HQC and LQC) showed recovery outside 80-120% indicating no cross-reactivity of the kit with Heparin and Chondroitin sulfate.

### 8.9 Dilution Linearity

From the sub-stock a 1000x-ULOQ sample was made which were further diluted into multiple QC samples, which were tested for recovery. All samples within range of quantification – between ULOQ (166.5 ng/ml) and LLOQ (2.6 ng/ml) showed acceptable recovery within 80-120% range. As expected, QC samples well above ULOQ did not pass the criteria due to possible Hook Effect. Samples below LLOQ also failed to recover,

## 9. References

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### 10. Troubleshooting Guide

Problem	Probable Causes	Solution
Sample readings are out of range	Samples contain no or below detectable levels of analyte. Matrix interference observed for the sample that may be due to haemolyzed matrix.	Spike the standards in non- haemolyzed matrix and check for recovery. If the recovery is 80-120%, then analyte concentration in sample may be below the detection limit of the kit
	Samples contain analyte concentrations greater than highest standard point.	Samples may require dilution and reanalysis.
High variation in samples and/or standards	Pipetting errors	Use calibrated pipettes
		Make sure pipette tips are tightly secured.
		Ensure that no bubbles are formed in the wells after the addition of reagents.
	Plate washing was not adequate or uniform	Confirm complete removal of residual wash buffer.
	Non-homogenous samples	Thoroughly mix samples before pipetting.
	Edge effect	Use the plate sealer and maintain the uniform temperature during the assay
	Samples may have high particulate matter	Remove the particulate matter by centrifugation.
	Insufficient plate mixing	Plate should be mixed thoroughly by agitation during all incubation steps using an ELISA plate orbital shaker.
Low or poor signal for the standard curve	Standard was incompletely reconstituted or was inappropriately stored.	Do not reuse plate sealers
		Avoid touching the plate with pipette tips.
	Reagents added to wells with incorrect concentrations.	Reconstitute standard according to protocol. Check for calculation and pipetting errors.