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Highly stable and robust protein S clotting assay validated for an easier laboratory practice

Amiral J.[1], Peyrafitte M.[2], Vissac A.M.[2]
[1]HYPHEN BioMed, Neuville sur Oise, [2]HYPHEN BioMed, Research, Neuville sur Oise,France

Introduction

Protein S (PS) is an anticoagulant factor, and its deficiency leads to increased thrombotic risk. Although PS clotting assays are well described and many commercial kits available, pitfalls are met in current laboratory practice, such as limited reagent stability (usually 4h), high variability (sometimes lot to lot for a same reagent) and possible interference from other plasma factors. This new clotting assay for the PS anticoagulant cofactor activity aims to overcome the usual inconveniences observed for current PS assays.

Aim

Development and validation of a new functional clotting assay (**HEMOCLOT Protein S**) for measuring the anticoagulant cofactor activity of PS, automatable onto the current laboratory coagulation instruments, stable and robust, and presenting a good reproducibility from lot to lot and run to run.

High specificity and accuracy in the clinical decision range.

Assay principle and protocol

The diluted tested plasma (1:10) is mixed with a PS deficient « substrate » plasma (R1). A clotting mixture containing optimized concentrations of Activated Protein C (APC), Factor IXa, and synthetic phospholipids (R2) is then added. Following a 3 min incubation step, clotting is initiated with 0.025M Ca++ ($CaCl_2$), and clotting time (CT) is recorded. There is a direct relationship between the PS concentration and the corresponding CT.

Water bath protocol:

Assay performed at 37°C

❶	❷	❸
50µl diluted test plasma (1:10) 50µl R1 (PS deficient plasma) → *1min at 37°C*	50µl R2 (APC/FIXa/PLPs) Preincubated at 37°C → *3min at 37°C*	100µl CaCl2 0.025M Preincubated at 37°C → *Record (CT, sec)*

Materials and Methods

Materials:

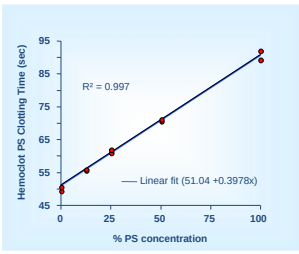
- PS deficient plasma: obtained by immunodepletion through anti-Protein S polyclonal antibodies, covalently linked to agarose beads.
- Factor IXa: Highly purified human Factor IX activated to Factor IXa at 37°C, through recycling onto activated contact phase covalently linked to agarose beads.
- Activated Protein C: Highly purified human PC activated by recycling through human thrombin covalently coupled onto agarose beads.
- Normal and pathological citrated plasmas; Instruments (START4, KC10, STAR)

Method evaluation:

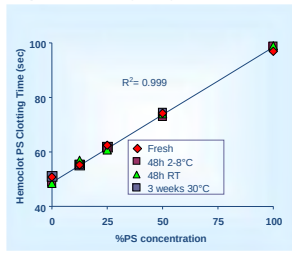
- Linearity of calibration curve over the dynamic range, and stability after reconstitution
- Specificity and interferences
- Precision
- Inter-lots homogeneity and comparison with other commercial assays (Staclot PS)
- Statistics performed with "Analyse-It" software.

Results

PS Calibration curve (START4 semi-automate



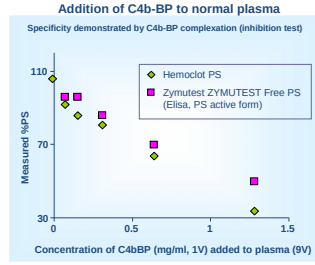
Stability after reconstitution and conservation (accelerated ageeing, 3 weeks at +30ºC)



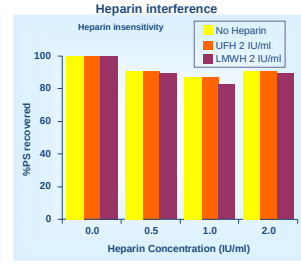
- A **linear dose response** relationship is obtained in the range 5-100% PS (1:10 dilution) (**r²>0.99**). The assay is robust and accurate in the clinical decision range, and PS deficiencies accurately detected.
  Results are well correlated when used on semi automate KC10 and STAR instrument
  (N=40 Range 39-151% Y=1.012X -7.286 r² =0.90).
- A **high stability of at least 48h at 2-8ºC or 12h at RT (and possibility to store the reagents frozen)** makes its use easy and consistent with current laboratory practice throughout the working day.
  Performances are maintained in accelerated ageing studies (overheating at 30ºC).

Specificity and heparin effect

Addition of C4b-BP to normal plasma



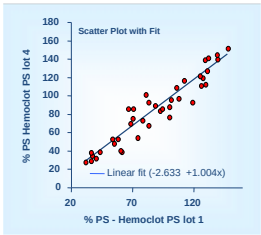
Heparin interference



- **The assay is specific**, as demonstrated by decreased PS concentrations with increased concentrations of C4bBP added to a normal plasma (complexation, leading to inactive PS).
- PS deficient plasma is measured <5%.
- When spiking heparin in normal plasma, the assay is insensitive to heparins (UFH or LMWH up to 2 IU/ml), so that **heparinized plasmas at usual therapeutic ranges can be used**.

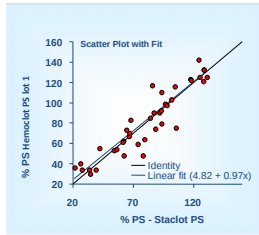
Inter-lots consistency and method comparison on patients plasmas (STAR):

Inter-lots :



N	40
Range	31 - 148%
r²	0.90

Method comparison:



N	40
Range	31 - 148%
r²	0.87

Inter lots consistency was verified on normal and pathological plasmas (deficiencies of PS or other factors, thrombosis risk check up, dicumarol treatment, cirrhosis, FV-L…) , and **HEMOCLOT Protein S** results globally well correlated with another commercial assay (**Staclot PS**).

Normal and clinical values:

	PS clotting assay		ZYMUTEST Free PS (Elisa, active form)	Staclot PS
	Mean %PS (Range)	SD	Mean %PS (Range)	Mean %PS (Range)
Normals: Males (N=12)	118 (91-143)	17	98 (70-125)	112 (86-130)
Females (N=6)	88 (63-123)	22	80 (52-105)	83 (61-117)
Dicumarol treated (N=5)	41 (35-56)	9	37 (33- 43)	30 (21-42)

Precision

Using one lot of Hemoclot PS reagent on STAR analyzer, 5 levels ranging from 37 to 120% PS were analyzed for a total of **n=20** (tests in duplicate, twice a day with new calibration curve in each, over 5 days) :

			Within run Repeatability		Total Precision	
Sample	n	Mean%PS	SD	CV%	SD	CV%
High	20	120.2	5.5	4.6	11.1	9.2
Mid	20	72.3	3.0	4.2	4.3	6.2
Low	20	46.1	3.9	8.5	3.4	7.4
Normal Control	20	76.0	3.5	4.6	4.5	5.9
Abnormal Control	20	37.2	2.8	7.6	3.2	8.6

Within run repeatability SD was in the range 2.8 - 5.5 (CV 4.2-8.5%) and Total precision SD in the range 3.2 to 11.1 (CV 5.9 to 9.2%), to be precised with additional series.

Conclusions

- **Designed with only 2 reagents, this assay is simple, very stable, and fully automatable on the main coagulation analyzers.**
- **The assay is reliable and accurate, and highly performing at the clinical decision range, for a safe identification of Protein S deficiencies.**
- **This improved PS clotting assay is proposed as a new efficient and reliable tool for current PS testing, and could introduce easier and safer clinical laboratory practice.**

ANIARA
7768 Service Center Drive • West Chester OH 45069
Phone: 513.770.1991 **Toll Free:** 866.783.3797 **Fax:** 513.573.9241 **Email:** info@aniara.com
www.aniara.com

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Aim: Protein S (PS) is an anticoagulant factor, and its deficiency leads to increased thrombotic risk . Although PS clotting assays are well described and many commercial kits available, pitfalls are met in current laboratory practice, such as limited reagent stability (usually 4h), high variability (sometimes lot to lot for a same reagent) and possible interference from other plasma factors. A new PS clotting assay was developed to overcome those limits and facilitate current use.

Method: The diluted tested plasma (1:20) is mixed with a PS deficient « substrate » plasma. A clotting mixture containing optimized concentrations of Activated Protein C (APC), FIXa with trace amounts of F Xa, and phospholipids (R2) is then added. Following a 2 min incubation step, clotting is initiated with 0.025M Ca++, and clotting time (CT) recorded.

Results: A linear dose response is obtained in the range 10-200% PS (r^2>0.995). PS deficiencies and high PS concentrations are directly and accurately measured. Designed with only 2 reagents, this assay is simple and fully automatable on the main coagulation analyzers. A high stability of at least 48h at 2-8°C, 24h at RT (and possibility to freeze) makes its use easy and consistent with current laboratory practice throughout the working day. Intra and inter assay variability evaluated on STAR were of 4-8% and 7-10% on the range 40 to 130 %. Inter lots consistency was verified (r^2>0.98) on N=50 normal and pathological plasmas (deficiencies of PS or other factors, thrombosis risk check up, dicumarol treatment, cirrhosis, Factor V-L…) ranging from 0 to 150%, and results well correlated with another commercial assay (r>0.94). The assay is insensitive to heparin (UFH or LMWH up to 2 IU/ml). Heparinized plasmas at usual therapeutic ranges can be tested.

Conclusions: This improved PS clotting assay is proposed as a new efficient and reliable tool for current PS testing, and could introduce an easier and safer clinical laboratory practice.