

## **Evaluation of chromogenic substrates : Hyphen BIOMED vs Chromogenix**

### **I/ Aim:**

Performances comparison of HYPHEN BioMed chromogenic substrates, with the corresponding substrates from Chromogenix, for specificity and reactivity.

### **Table of correspondences:**

Specificity	HYPHEN BioMed		Chromogenix	
	Name	Reference	Name	Reference
<b>Thrombin</b>	BIOPHEN CS-01(38)	A229001	S-2238	# 82 03 24
<b>Factor Xa</b>	BIOPHEN CS-11(32)	A229011	S-2732	-
	BIOPHEN CS-11(65)	A229014	S-2765	# 82 14 13
	BIOPHEN CS-11(22)	A229015	S-2222	# 82 03 16
<b>Activated Protein C</b>	BIOPHEN CS-21(66)	A229021	S-2366	# 82 10 90
<b>Kallikrein</b>	BIOPHEN CS-31(02)	A229031	S-2302	# 82 03 40
<b>Plasmin / Plasminogen-SK</b>	BIOPHEN CS-41(03)	A229041	S-2403	# 82 22 54
<b>Factor IXa</b>	BIOPHEN CS-51(09)	A229051	-	-
<b>Urokinase</b>	BIOPHEN Cs-61(44)	A229061	S-2444	# 82 03 57

### **II/ Protocol and reagents:**

- Reagents :**

- Chromogenic substrates : HBM: BIOPHEN CS-1132, CS11-65, CS1122, CS1138, CS2166, CS3102, CS4103, CS6144 vs the corresponding chromogenic substrates from Chromogenix (refer to the following protocols for the detailed lots).
- Lyophilised human FXa 10µg (HYPHEN BioMed, ref AEZ007A).
- Lyophilised bovine FXa (HYPHEN BioMed).
- Lyophilised human FIIa (Thrombin) 10 NIH (HYPHEN BioMed, ref AEZ006O).
- Lyophilised bovine FIIa (Thrombin) 21 NIH (HYPHEN BioMed, ref ABE102A).
- Lyophilised human activated Protein C (aPC) (HYPHEN BioMed, ref AEZ004B).
- Lyophilised human plasminogen 200µg (HYPHEN BioMed, ref APP005A).
- Lyophilised bovine plasminogen 5mg (HYPHEN BioMed, ref ABP105C).
- Streptokinase (HYPHEN BioMed, ref AEZ008B)
- Urokinase (HYPHEN BioMed, ref AEZ005A)
- Lyophilised prekallikrein pool (HYPHEN BioMed , ref APP501B).
- Lyophilised human FVIIa (HYPHEN BioMed, ref AEZ009Z).
- FIXa (HYPHEN BioMed)
- FXIa, FXIIa (ERL)
- TBSA buffer (Tris NaCl buffer with BSA, pH7.50, I=0.15, BSA1%) (HYPHEN BioMed, AAR005A/K).
- Tris (0.05M)-NaCl (0.30M) pH8.40 buffer (HYPHEN BioMed, ref AAR009A/K). (refer to the following protocols for the detailed lots).

- **FXa Substrate :**

Reconstitution: with 10 mL of sterile water, for a final working concentration of 2.5mg/mL.

Protocol:

400 µl Tris 0.05M, NaCl 0.30M pH : 8.40 buffer

100 µl human FXa (lot 050221A) or bovine FXa (lot BH6 « 61101 ») at 2.5 µg/mL, then further diluted to 1.25 & 0.625µg/mL or test of cross reactivity with other enzymes (prediluted in TBSA buffer) at 2.5µg/ml.

1 min at 37°C

100 µL FXa substrate at 2.5 mg/mL (rec by 10 ml, to obtain C=2.5mg/mL).

3 min at 37°C.

300 µL citric acid (stopping the reaction)

Read A405nm against the sample blank.

Factor Xa Substrate lots:

HBM (CS-1165) : 71902-1 exp 2009-11

HBM (CS-1122) : 64702-1 exp : 2009-05

HBM (CS-1132) : 70301-1 exp : 2009-07

Chromogenix (S2765) : N0454925 exp : 2008-04

Chromogenix (S2222) : N0261184 exp : 2009-06

- \* **Factor IIa (Thrombin) substrate :**

Reconstitution: with 10 mL of sterile water, for a final working concentration of 2.5mg/mL.

Protocol:

400 µl Tris buffer 0.05M, NaCl 0.30M pH 8.40

100 µl human IIa (070323A) or bovine IIa (070426F) at 3 NIH/mL (**3NIH=1.5µg/mL**), then further diluted to 1.5 & 0.75 NIH/mL or test of cross reactivity with other enzymes (prediluted in TBSA buffer) tested at the same concentration of 1.5µg/mL. .

1 min at 37°C

100 µL IIa substrate at 2.5 mg/mL (rec by 10 ml, to obtain C=2.5mg/mL).

3 min at 37°C.

300 µL citric acid (stopping the reaction)

Read A405nm against the sample blank.

Factor IIa (Thrombin) Substrate lots:

HBM (CS-0138) : 70602-1 exp : 2009-08

Chromogenix (S2238) : N0862392 exp : 2009-08

- \* **Activated Protein c (aPC) substrate :**

Reconstitution: with 10 mL of sterile water, for a final working concentration of 2.5mg/mL.

Protocol:

400 µl Tris 0.05M, CsCL 0.26M, CaCl<sub>2</sub> : 0.004M pH : 8.30 buffer

100 µl human APC (lot 061012A) at 2.5µg/mL, then further diluted to 1.25 & 0.625µg/mL or test of cross reactivity with other enzymes (prediluted in TBSA buffer) at the same final concentration of 2.5µg/mL.

1 min at 37°C

100 µL APC substrate at 2.5 mg/mL (rec by 10 ml, to obtain C=2.5mg/mL).

5 min at 37°C.

300 µL citric acid (stopping the reaction)

Read A405nm against the sample blank.

APC Substrate lots:

HBM (CS-2166) : 54702-1 exp : 2008-05

Chromogenix (S2366) : N0152994 exp : 2008-01

### **\* Plasmin / Plasminogen-SK Substrate :**

Reconstitution: with 10 mL of sterile water, for a final working concentration of 2.5mg/mL.

Protocol:

200 µl human plasminogen (lot 060614A) or bovine plasminogen (lot 060330C) at 10µg/mL then further diluted to 5 & 2.5 µg/mL or test of cross reactivity with other enzymes (prediluted in TBSA buffer) tested at the same concentration of 10µg/mL. .

200µl streptokinase at 10000 IU/ml (for human plasminogen) or urokinase à 1000IU/mL (for bovine plasminogène)

3 min at 37°C

200 µL plasmin substrate at 2.5 mg/mL (rec by 10 ml, to obtain C=2.5mg/mL).

3 min at 37°C.

300 µL citric acid (stopping the reaction)

Read A405nm against the sample blank.

#### Plasmin/Plasminogen-SK Substrate lots:

HBM (CS-4103) : 54703-1 exp : 2008-05

Chromogenix (S2403) : N1263082 exp : 2008-09

### **\* Kallikrein substrate :**

Reconstitution: with 10 mL of sterile water, for a final working concentration of 2.5mg/mL.

Protocol: (protocol 1)

200 µl prekallikrein pool (lot 070413E) pure,

200µl FXIIa (lot 2072AL) at 1 µg/mL then further diluted to 0.5 & 0.25µg/mL, or test of cross reactivity with other enzymes at C=2µg/mL

5 min at 37°C

200 µl Tris 0.05M, NaCl 0.15M pH 8.00 buffer

2 min at 37°C

200 µL kallikrein substrate at 2.5 mg/mL (rec by 10 ml to obtain C=2.5mg/mL).

2 min at 37°C.

300 µL citric acid (stopping the reaction)

Read A405nm against the sample blank.

#### Kallikrein Substrate lots:

HBM (CS-3102) : 70302-1 exp : 2009-07

Chromogenix (S2302) : N0448702 exp : 2007-08

### **\* Urokinase substrate :**

Reconstitution: with 10 mL of sterile water (ie at 2.5mg/ml), then diluted for a working concentration of 1mg/mL.

Protocol:

100 µl uPA substrate (at 1mg/ml)

600 µl Tris 0.05M , NaCl 0.05M pH 8.80 buffer

2 min at 37°C

100 µl uPA at 5000 U/ml

3 min at 37°C

400 µl citric acid (stopping the reaction)

Read A405nm against the sample blank.

#### uPA substrate lots:

HBM (6144) : 80701-1

Chromogenix (S-2444) : N0532838

### **III/ Results for the respective substrates reactivities :**

- **Factor Xa substrates:**

	Factor Xa Substrate						
		Xa (human)			Xa (bovine)		
concentration ( $\mu\text{g/mL}$ )	2.5	1.25	0.625	2.5	1.25	0.625	
<b>HBM 11(65)</b>	OD (405nm)	2.092	1.116	0.570	2.534	1.47	0.73
<b>Chromogenic (2765)</b>	OD (405nm)	2.104	1.131	0.592	2.507	1.503	0.747

	Factor Xa Substrate						
		Xa (human)			Xa (bovine)		
concentration ( $\mu\text{g/mL}$ )	2.5	1.25	0.625	2.5	1.25	0.625	
<b>HBM 11(22)</b>	OD (405nm)	0.486	0.255	0.123	0.978	0.518	0.256
<b>Chromogenic (2222)</b>	OD (405nm)	0.552	0.282	0.146	1.141	0.595	0.281

	Factor Xa Substrate						
		Xa (human)			Xa (bovine)		
concentration ( $\mu\text{g/mL}$ )	2.5	1.25	0.625	2.5	1.25	0.625	
<b>HBM 11(32)</b>	OD (405nm)	1.032	0.554	0.285	1.525	0.809	0.404

- **Thrombin substrates:**

	Factor IIa Substrate						
		Thrombin (human)			Thrombin (bovine)		
3NIH=1,5 $\mu\text{g/mL}$	concentration (NIH/mL)	3	1.5	0.75	3	1.5	0.75
<b>HBM 01(38)</b>	OD (405nm)	0.83	0.394	0.216	2.142	1.136	0.514
<b>Chromogenic (2238)</b>	OD (405nm)	0.868	0.413	0.224	2.246	1.182	0.54

- **Activated Protein (aPC) substrates:**

	APC Substrate			
		APC (human)		
concentration ( $\mu\text{g/mL}$ )	2.5	1.25	0.625	
<b>HBM 21(66)</b>	OD (405nm)	1.5	0.778	0.368
<b>Chromogenic (2366)</b>	OD (405nm)	1.642	0.84	0.476

- **Plasmin / Plasminogen-SK substrates:**

	Plasmin Substrate		
		Plasminogen (human)	
<b>with streptokinase (10000U/mL)</b>	concentration ( $\mu\text{g/mL}$ )	10	5
<b>HBM 41(03)</b>	OD (405nm)	1.619	0.707
<b>Chromogenic (2403)</b>	OD (405nm)	2.5	0.332
		1.714	0.768
		0.358	

	Plasmin Substrate		
		Plasminogen (bovine)	
<b>with urokinase (1000U/mL)</b>	concentration ( $\mu\text{g/mL}$ )	10	5
<b>HBM 41(03)</b>	OD (405nm)	2.708	1.407
<b>Chromogenic (2403)</b>	OD (405nm)	2.5	0.749
		2.796	1.459
		0.764	

- **Kallikrein substrates:**

	Kallikreine Substrate		
		XIIa (human)	
<b>protocol 1</b>	concentration ( $\mu\text{g/mL}$ )	1	0.5
<b>HBM 31(02)</b>	OD (405nm)	>3	0.25
<b>Chromogenic (2302)</b>	OD (405nm)	>3	0.913
		2.128	1.332

- **Urokinase substrates:**

	UPA Substrate 25 mg		
		urokinase	
<b>with urokinase</b>	concentration (U/mL)	5000	2500
<b>HBM 61(44)</b>	OD (405nm)	1,085	0,511
<b>Chromogenic (2444)</b>	OD (405nm)	1250	0,239
		0,729	0,366
		0,178	

## **IV/ Results for the respective substrates specificities (and cross reactivities with other enzymes in the standard working conditions):**

- **Factor Xa substrates:**

enzyme	concentration of use	<b>Factor Xa Substrate (CS11-65)</b>	
		<b>HBM (CS-1165)</b>	<b>Chromogenix 2765</b>
OD 405 nm			
IIa human	5 NIH/ml	0.020	0.033
IIa Bovine	5 NIH/ml	0.052	0.065
Xa human	2.5 µg/mL	2.09	2.10
Xa bovine	2.5 µg/mL	2.53	2.51
IXa	2.5 µg/mL	0.004	0.006
XIa ERL (HFXIa 2291)	2.5 µg/mL	0.106	0.115
XIIa (ERL)	2.5 µg/mL	0.058	0.064
aPC	2.5 µg/mL	0.010	0.018
VIIa (060622B)	2.5 µg/mL	0.008	0.002
Plasminogen SK	2.5 µg/mL	0.005	0.005
Plasminogen UK	2.5 µg/mL	0.100	0.108

enzyme	concentration of use	<b>Factor Xa Substrate (CS11-22)</b>	
		<b>HBM (1122)</b>	<b>Chromogenix 2222</b>
OD 405 nm			
IIa human	5 NIH/ml	0.018	0.018
IIa Bovine	5 NIH/ml	0.029	0.032
Xa human	2.5 µg/mL	0.49	0.55
Xa bovine	2.5 µg/mL	0.98	1.14
IXa	2.5 µg/mL	0	0.001
XIa ERL	2.5 µg/mL	0.039	0.046
XIIa ERL	2.5 µg/mL	0.064	0.062
APC	2.5 µg/mL	0	0
VIIa	2.5 µg/mL	0	0.001
Plasminogen SK	2.5 µg/mL	0.005	0.003
Plasminogen UK	2.5 µg/mL	0.033	0.041

enzyme	<b>Factor Xa Substrate (CS-1132)</b>	
	concentration of use	<b>HBM (1132)</b>
		OD 405 nm
IIa human	5 NIH/ml	0.058
IIa Bovine	5 NIH/ml	0.149
Xa human	2.5 µg/mL	1.03
Xa bovine	2.5 µg/mL	1.53
IXa	2.5 µg/mL	0.005
XIa ERL	2.5 µg/mL	0.033
XIIa ERL	2.5 µg/mL	0.062
APC	2.5 µg/mL	0.001
VIIa	2.5 µg/mL	0.003
Plasminogen SK	2.5 µg/mL	0.004
Plasminogen UK	2.5 µg/mL	0.04

- **Thrombin substrates:**

enzyme	<b>Factor IIa Substrate (CS0138)</b>		
	concentration of use	<b>HBM (0138)</b>	<b>Chromogenix 2238</b>
		OD 405 nm	
IIa human	3 NIH/ml	0.83	0.86
IIa Bovine	3 NIH/ml	2.10	2.20
Xa human	1.5 µg/mL	0.036	0.033
Xa bovine	1.5 µg/mL	0.008	0.008
IXa	1.5 µg/mL	0.001	0
XIa ERL	1.5 µg/mL	0.117	0.100
XIIa	1.5 µg/mL	0.004	0.004
APC	1.5 µg/mL	0.103	0.099
VIIa	1.5 µg/mL	0.001	0.001
Plasminogen SK	1.5 µg/mL	0.053	0.053
Plasminogen UK	1.5 µg/mL	0.098	0.096

- aPC substrates:

		<b>APC Substrate (CS2166)</b>	
enzyme	concentration of use	<b>HBM (2166)</b>	<b>Chromogenix 2366</b>
OD 405 nm			
IIa human	5 NIH/ml	2.234	2.479
IIa Bovine	5 NIH/ml	>3	>3
Xa human	2.5 µg/mL	0.119	0.142
Xa bovine	2.5 µg/mL	0.104	0.128
IXa	2.5 µg/mL	0.095	0.015
XIa ERL	2.5 µg/mL	1.034	1.191
XIIa ERL	2.5 µg/mL	0.086	0.102
APC	2.5 µg/mL	1.50	1.64
VIIa	2.5 µg/mL	0.017	0.019
Plasminogen SK	2.5 µg/mL	0.276	0.401
Plasminogen UK	2.5 µg/mL	0.458	0.582

- Plasmin/Plasminogen-SK substrates:

		<b>Plasmine – Plasminogen/SK Substrate (CS4103)</b>	
enzyme	concentration of use	<b>HBM (4103)</b>	<b>Chromogenix 2403</b>
OD 405 nm			
IIa human	20 NIH	0.017	0.013
IIa Bovine	20 NIH	0.036	0.039
Xa human	10µg/mL	0.194	0.137
Xa bovine	10µg/mL	0.086	0.099
IXa	10µg/mL	0.009	0.003
XIa ERL	10µg/mL	0.063	0.057
XIIa ERL	10µg/mL	0.001	0.001
APC	10µg/mL	0.022	0.004
VIIa	10µg/mL	0.004	0.002
Plasminogen SK	10µg/mL	1.62	1.71
Plasminogen UK	10µg/mL	2.71	2.79

- **Kallikrein substrates:**

		<b>Kallikrein Substrate (CS3102)</b>	
enzyme	concentration of use	<b>HBM (3102)</b>	<b>Chromogenix 2302</b>
OD 405 nm			
Illa human	4 NIH/ml	0.169	0.155
Illa Bovine	4 NIH/ml	0.289	0.284
Xa human	2µg/mL	0.805	0.778
Xa bovine	2µg/mL	0.688	0.675
IXa	2µg/mL	0.046	0.026
XIa ERL	2µg/mL	0.06	0.041
XIIa ERL	1µg/mL	>3	>3
	0.5µg/ml	1.50	2.12
APC	2µg/mL	0.089	0.056
VIIa	2µg/mL	0.043	0.028
Plasminogen SK	2µg/mL	0.241	0.255
Plasminogen UK	2µg/mL	0.133	0.142

- **Urokinase substrates:**

enzym	concentration of using	UPA Substrate 25 mg	
		<b>HBM (6144)</b>	<b>Chromogenic (2444)</b>
OD 405 nm			
Illa human	6 NIH	0,008	0,08
Illa Bovin	6 NIH	0,031	0,024
Xa human	5 µg/mL	0,017	0,013
Xa bovin	5 µg/mL	0,063	0,042
XIIa	5 µg/mL	0,017	0,019
APC	5 µg/mL	0,003	0,004
Plasminogen SK	5 µg/mL	0,008	0,005
Plasminogen UK	5 µg/mL	0,216	0,143

(note: the reactivity obtained in the protocol using “urokinase activated” plasminogen is due to the added uPA).

## **V/ CONCLUSIONS :**

All the substrates exhibit the strong expected reactivity to their specific enzyme. In most of the cases, measured cross reactivities are very low (<2%) excepted for (but identical for HBM and Chromogenix):

-APC substrate (CS2166 / S2366) with Thrombin, FXIa and plasmin.

- Kallikrein substrate (CS3102 / S2302) with FXa.

The assay conditions must be chosen in order to make the measurement specific for the targeted enzyme.