

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
Thrombin	BIOPHEN CS-01(38)	A229001	S-2238	# 82 03 24
Factor Xa	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
Activated Protein C	BIOPHEN CS-21(66)	A229021	S-2366	# 82 10 90
Kallicrein	BIOPHEN CS-31(02)	A229031	S-2302	# 82 03 40
Plasmin / Plasminogen-SK	BIOPHEN CS-41(03)	A229041	S-2403	# 82 22 54
Factor IXa	BIOPHEN CS-51(09)	A229051	-	-
Urokinase	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, CaCl₂ : 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

*** Kallicrein substrate :**

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

Protocol: (protocol 1)

200 µl prekallicrein 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 kallicrein 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.

Kallicrein 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
HBM 11(65)	OD (405nm)	2.092	1.116	0.570	2.534	1.47	0.73
Chromogenic (2765)	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
HBM 11(22)	OD (405nm)	0.486	0.255	0.123	0.978	0.518	0.256
Chromogenic (2222)	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
HBM 11(32)	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
HBM 01(38)	OD (405nm)	0.83	0.394	0.216	2.142	1.136	0.514
Chromogenic (2238)	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
HBM 21(66)	OD (405nm)	1.5	0.778	0.368
Chromogenic (2366)	OD (405nm)	1.642	0.84	0.476

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

	Plasmin Substrate			
		Plasminogen (human)		
with streptokinase (10000U/mL)	concentration (µg/mL)	10	5	2.5
HBM 41(03)	OD (405nm)	1.619	0.707	0.332
Chromogenic (2403)	OD (405nm)	1.714	0.768	0.358

	Plasmin Substrate			
		Plasminogen (bovine)		
with urokinase (1000U/mL)	concentration (µg/mL)	10	5	2.5
HBM 41(03)	OD (405nm)	2.708	1.407	0.749
Chromogenic (2403)	OD (405nm)	2.796	1.459	0.764

- **Kallicrein substrates:**

	Kallicreine Substrate			
		XIIa (human)		
protocol 1	concentration (µg/mL)	1	0.5	0.25
HBM 31(02)	OD (405nm)	>3	1.496	0.913
Chromogenic (2302)	OD (405nm)	>3	2.128	1.332

- **Urokinase substrates:**

	UPA Substrate 25 mg			
		urokinase		
with urokinase	concentration (U/mL)	5000	2500	1250
HBM 61(44)	OD (405nm)	1,085	0,511	0,239
Chromogenic (2444)	OD (405nm)	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	Factor Xa Substrate (CS11-65)		
	concentration of use	HBM (CS-1165)	Chromogenix 2765
		OD 405 nm	
Ila human	5 NIH/ml	0.020	0.033
Ila 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	Factor Xa Substrate (CS11-22)		
	concentration of use	HBM (1122)	Chromogenix 2222
		OD 405 nm	
Ila human	5 NIH/ml	0.018	0.018
Ila 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

Factor Xa Substrate (CS-1132)		
enzyme	concentration of use	HBM (1132)
		OD 405 nm
Ila human	5 NIH/ml	0.058
Ila 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:**

Factor IIa Substrate (CS0138)			
enzyme	concentration of use	HBM (0138)	Chromogenix 2238
		OD 405 nm	
Ila human	3 NIH/ml	0.83	0.86
Ila 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:**

	APC Substrate (CS2166)		
enzyme	concentration of use	HBM (2166)	Chromogenix 2366
		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:**

	Plasmin – Plasminogen/SK Substrate (CS4103)		
enzyme	concentration of use	HBM (4103)	Chromogenix 2403
		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

- **Kallicrein substrates:**

Kallicrein Substrate (CS3102)			
enzyme	concentration of use	HBM (3102)	Chromogenix 2302
OD 405 nm			
Ila human	4 NIH/ml	0.169	0.155
Ila 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:**

UPA Substrate 25 mg			
enzym	concentration of using	HBM (6144)	Chromogenic (2444)
OD 405 nm			
Ila human	6 NIH	0,008	0,08
Ila 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.

- Kallicrein substrate (CS3102 / S2302) with FXa.

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