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Manufactured By: HYPHEN BioMed

Summary of validation criteria for chromogenic substrates (especially CS-11(65) and CS-01(38)) for human Antithrombin, human Thrombin, bovine FXa.

1. CS-11(65) and CS-01(38) substrates:

METHOD FOR ANALYTICAL DATA	SPECIFICATIONS CS-11(65) #A229014 (25mg)	SPECIFICATIONS CS-11(65) #A229114 (100mg)	SPECIFICATIONS CS-01(38) #A229001/A229001C	SPECIFICATIONS CS-01(38) #A229101 (100mg)
1. **Content** From manufacturing process	≥ 22 mg	≥ 90 mg	≥ 22 mg	≥ 90 mg
2. **HPLC** analysis (Purity grade): (From supplier CoA)	≥ 95 %			
3. **Solubility in water** (From supplier CoA and/or testing)	≥ 5 mg/mL			
4. **Free pNA content** (From supplier CoA)	< 0.05%			
5. **Experimental Molecular weight** (From supplier CoA, basic structure)	641.7 ± 5		553 ± 5	

Internal Quality control specifications for substrates:

- Free pNA content (A405 for substrate at 2.5mg/ml): ≤0.30
- pH in compliance.
- A405 level measured in a chromogenic assay in optimized conditions: in compliance.
- Stability after reconstitution is verified for each lot (free pNA and A405 stability in a chromogenic assay), for sufficient lot size.

2. Human Antithrombin:

METHOD FOR ANALYTICAL DATA	SPECIFICATIONS
1. Protein Content (per vial, by Lowry method)	> 120 µg (#APP004A/K 150µg); > 1.35 mg (#APP004B/L 1.5mg) 10 ± 0.5 mg (#APP004C 10mg) > 3.50 mg (#APP004D 3.75mg) >95 mg (#APP004Z 100mg)
2. Purity: SDS-PAGE	1 major band of about 58,000 daltons
3. Specific AT activity (anti-Xa method): Determined by chromogenic anti-Xa assay in presence of heparin (Biophen AT). Expressed in International Units (IU) of AT, standardized against WHO/NIBSC for AT, human, plasma.	AT concentration > 6 IU/mg
4. Absence of heparin : Verified by toluidine blue test, and verified by measurement in a specific heparin anti-Xa assay.	Absence
5. Batch homogeneity: Verified on different vials, on intra assay, on A405 measured in chromogenic anti-Xa assay in presence of heparin (Biophen AT)	N≥5 vials tested CV (A405) ≤ 2% (except APP004 C and Z, or depending on lot size)

Stability after reconstitution and in overheating studies (3 week at 30°C for the lyophilized product) is verified for each lot (on AT concentration, using Biophen AT assay) (for sufficient lot size).

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3. Human Thrombin:

METHOD FOR ANALYTICAL DATA	SPECIFICATIONS
1 Protein Content (per vial, by Lowry method)	≥ 30 µg (# AEZ006A/L, 100 NIH) ≥ 300 µg (# AEZ006B, 1000 NIH) ≥ 3 µg (# AEZ006 0/K, 10NIH)
2 Purity: SDS-PAGE (4-12% acrylamide)	1 major band of about:≈ 35, 000 daltons
3 Clotting time: When tested at 10 NIH/ml, on purified human Fibrinogen at 5mg/ml:	CT = 5 ± 1 sec.
4 Clotting activity Determined in a clotting assay, on purified human Fibrinogen at 4mg/ml, and expressed in NIH (or IU) respectively to the harmonized WHO/NIBSC standard for human thrombin	≥ 90 NIH (or IU) (# AEZ006A/L, 100 NIH) ≥ 900 NIH (or IU) (# AEZ006B, 1000 NIH) ≥ 8 NIH (or IU) (# AEZ006 0/K, 10NIH)
5 Chromogenic activity: On IIa substrate CS-01(38), expressed as A405/min.NIH.ml	A405 ≥ 0.75 / min.NIH.ml
6 Specific activity ▪ NIH (or IU) /mg (clotting activity) ▪ nkats/µg (determined in a chromogenic assay at 37°C, in optimized conditions in a Tris 0.05M, NaCl 0.30M PH 8.40 buffer, using CS-01(38) at 2.5 mg/ml) ▪ nkats/NIH (or nkats/IU)	≥ 1,500 NIH (or IU)/mg* ≥ 2 nkats/µg ≥ 1 nkat/NIH
7 Batch homogeneity : Verified on different vials, on intra assay reproducibility on CT measured on human Fibrinogen (see 3).	N≥5 vials tested; CV ≤ 5% (except AEZ006B, or depending on lot size)

Stability after reconstitution and in overheating studies (3 week at 30°C for the lyophilized product) is verified for each lot (clotting and chromogenic assay),(for sufficient lot size).
*NIH and IU (clotting units for thrombin) have now been harmonized and are identical.

For information, data extracted from internal evaluation:

HUMAN THROMBIN	nkat/µg (CS-0138)	nkat/µg (CS-0181)	nkats/NIH (CS01-38)	nkats/NIH (CS01-81)
(h)IIa lot: 081215A	2.42	3.08	1.1	1.4
(h)IIa lot: 090213B	2.86	3.55	1.3	1.6
(h)IIa lot: 090407A	2.85	3.58	1.3	1.6
(h)IIa lot: 091106A	3.28	4.08	1.5	1.8
(h)IIa lot: 091208A	2.15	2.69	1.0	1.2
(h)IIa lot: 091217B	3.07	3.80	1.4	1.7
(h)IIa lot: 090918A	3.61	4.42	1.6	2.0
Mean :	**2.89**	**3.60**	**1.3**	**1.6**

BOVINE THROMBIN	*nkat/µg (CS-0138)*	*nkat/µg (CS-0181)*	nkats/NIH (CS01-38)	nkats/NIH (CS01-81)
(b)IIa lot: 071115B	4.47	4.62	2.0	2.1
(b)IIa lot: 090921A	5.01	5.12	2.3	2.3
(b)IIa lot: 071115C	4.63	4.68	2.1	2.1
(b)IIa lot: 070426F	3.83	4.06	1.7	1.8
Mean :	**4.48**	**4.62**	**2.0**	**2.1**

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4. Bovine FXa:

METHOD FOR ANALYTICAL DATA	SPECIFICATIONS
1. **Protein Content** Per vial, measured by Lowry method and based on FX zymogen	> 12 µg (# ABE101C 15µg) > 25 µg (# ABE101D/L 30µg) > 45 µg (# ABE1010/K 50µg) > 450 µg (# ABE101B 500µg)
2. **Purity:** SDS-PAGE (4-12% acrylamide)	FX zymogen: 1 major band of about 55,000 daltons
3. **Chromogenic activity on FXa substrate (10 µg/ml)** A405 is measured in a purified system with or without RVV, full activation of FX zymogen into FXa is verified by:	Difference with/without RVV: Δ A405 < 10%
4. **Enzymatic activity (on substrate CS-11 (22)):** (chromogenic assay at 37°C, in optimized conditions in a Tris 0.05M, NaCl 0.30M PH 8.40 buffer, using CS-11(22) at 2.5 mg/ml)	≥ 30nkats (# ABE101C 15µg) ≥ 60nkats (# ABE101D/L 30µg) ≥ 100nkats (# ABE1010/K 50µg) ≥ 1000nkats (# ABE101B 500µg)
5. **Specific activity (**nkats/µg) (deduced from previous chromogenic test on CS-11(22), and protein content based on FX zymogen)	≥ 1.75 nkats/µg
6. **Batch homogeneity** Verified on different vials, on intra assay reproducibility on A405 measured in chromogenic assay (see 4) for FXa at 2.5 µg/ml.	N≥5 vials tested CV ≤ 3% (for ABE101C and ABE101D/L, or depending on lot size)

Stability after reconstitution and in overheating studies (3 week at 30°C for the lyophilized product) is verified for each lot (chromogenic assay),(for sufficient lot size).

For information, data extracted from internal evaluation:

Using chromogenic substrate:	**CS-11(22)**	**CS-11(32)**	**CS-11(65)**
BOVINE FXa lot:	**Mean nkat/µg**	**Mean nkat/µg**	**Mean nkat/µg**
100125B (raw material 090629F)	2.56	4.14	7.50
090323A (raw material 080523J)	2.30	3.77	6.90
080616A (raw material 071009C)	2.05	3.38	6.15
061117B (raw material 060920)	2.18	3.52	6.32
Mean:	**2.27**	**3.70**	**6.72**

Using chromogenic substrate:	**CS-11(22)**	**CS-11(32)**	**CS-11(65)**
HUMAN FXa lot:	**Mean nkat/µg**	**Mean nkat/µg**	**Mean nkat/µg**
090213C (raw material 070125B)	0.91	2.08	4.18
090825C (raw material 090217A)	1.17	2.59	5.21
Mean:	**1.04**	**2.34**	**4.69**

Note: It is considered that 1 unit FX in plasma is about 10µg/ml, and that all FX zymogen is converted to FXa.

HYPHEN reagents are fully controlled for each manufacturing lot.
Exact values are indicated for each lot on specific Certificate of Analysis.

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