

Supporting Information

Differentiation and Identification of Recombinant Human Erythropoietin and Darbepoetin Alfa in Equine Plasma for Doping Control by LC-MS/MS

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Included in this online Supporting Information are figures and tables listed below.

S-Figure 1. LC-MS chromatograms of rhEPO and DPO (50 µg/mL each) following digestion by trypsin alone, indicating identical tryptic peptide profiles and lack of differentiation between DPO and rhEPO without deglycosylation of the associated glycopeptides.

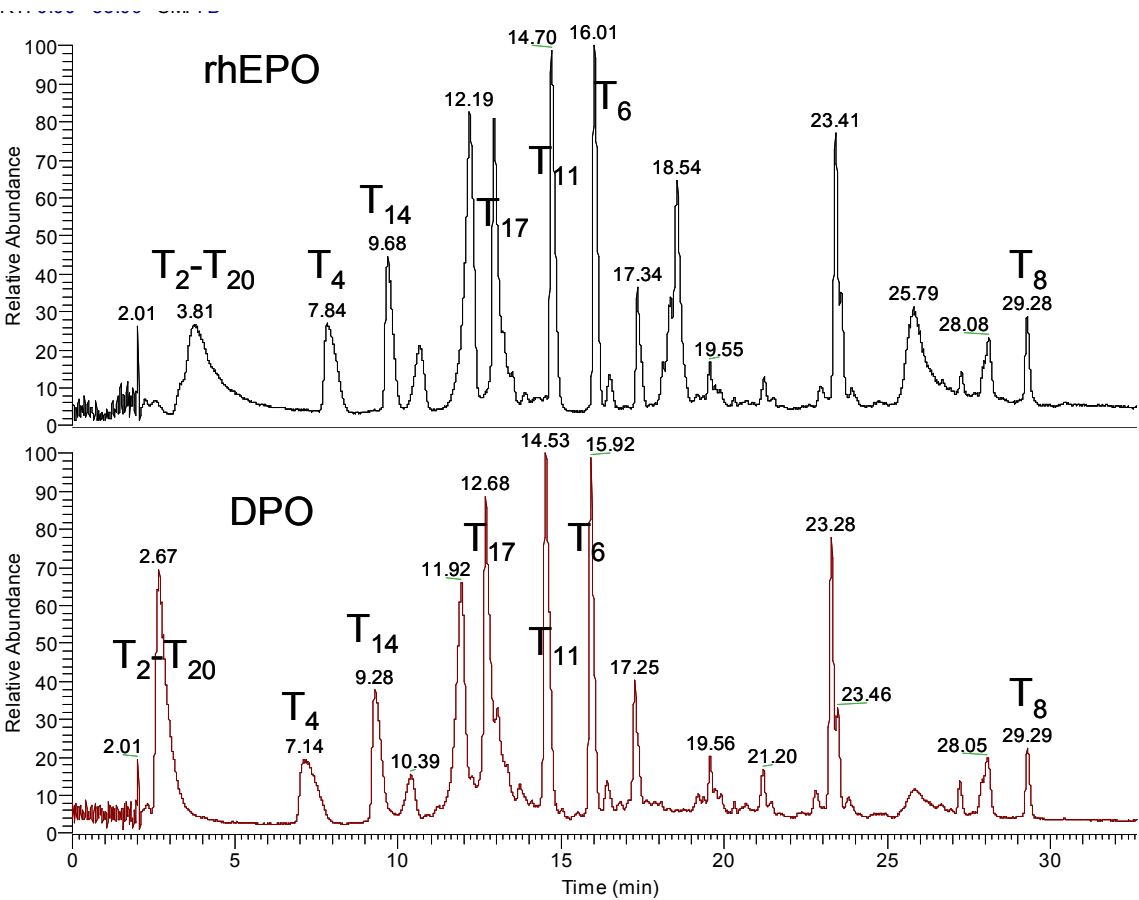
S-Figure 2. LC-MS/MS chromatograms and product ion spectra indicating that 0.2 ng/mL of rhEPO and 0.1 ng/mL of DPO spiked to equine plasma were identified.

S-Figure 3. Calibration curves for quantification of rhEPO and DPO in equine plasma by external calibration.

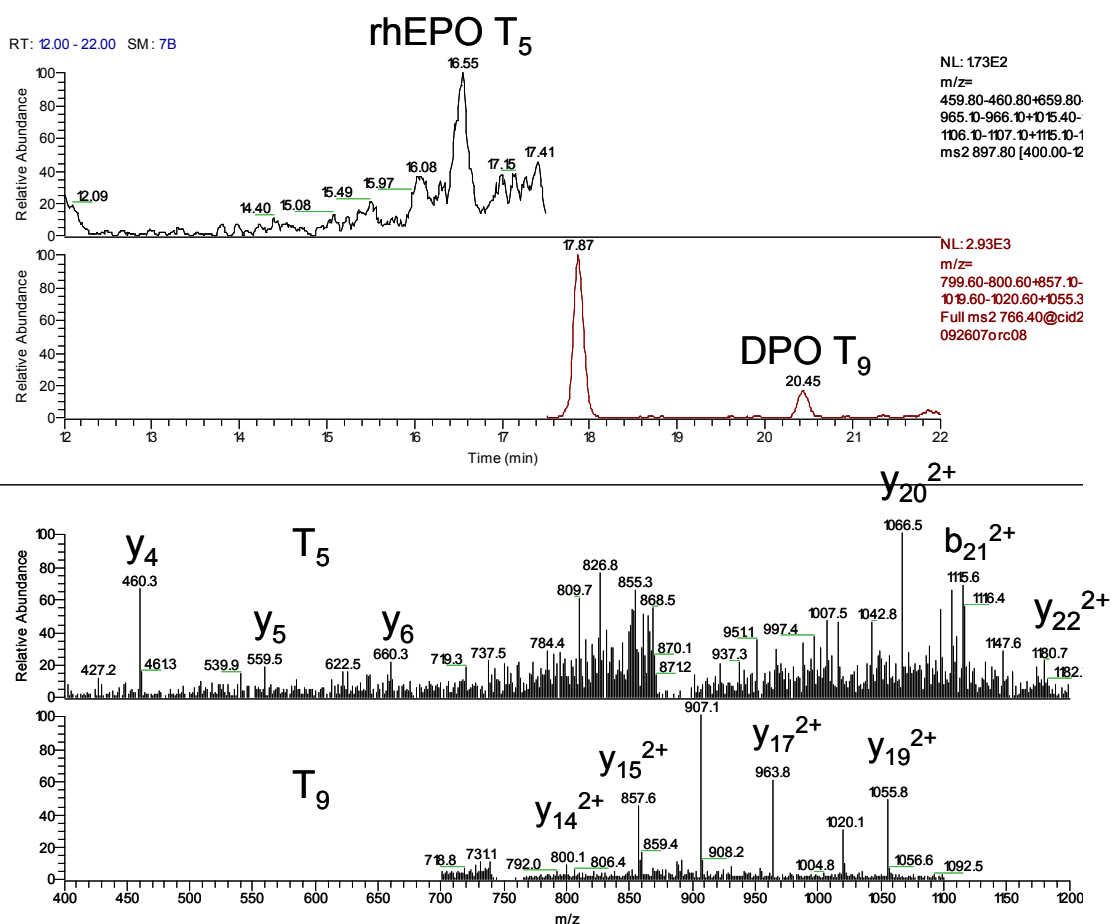
S-Table 1. Accurate mass measurements and those predicted for product ions of the deglycosylated T₅ of rhEPO and T₉ of rhEPO and DPO.

S-Table 2. BLAST search result for the unique tryptic peptides, T₅ of rhEPO and T₉ of DPO, indicating their inherent specificity for identification of rhEPO and DPO in equine plasma.

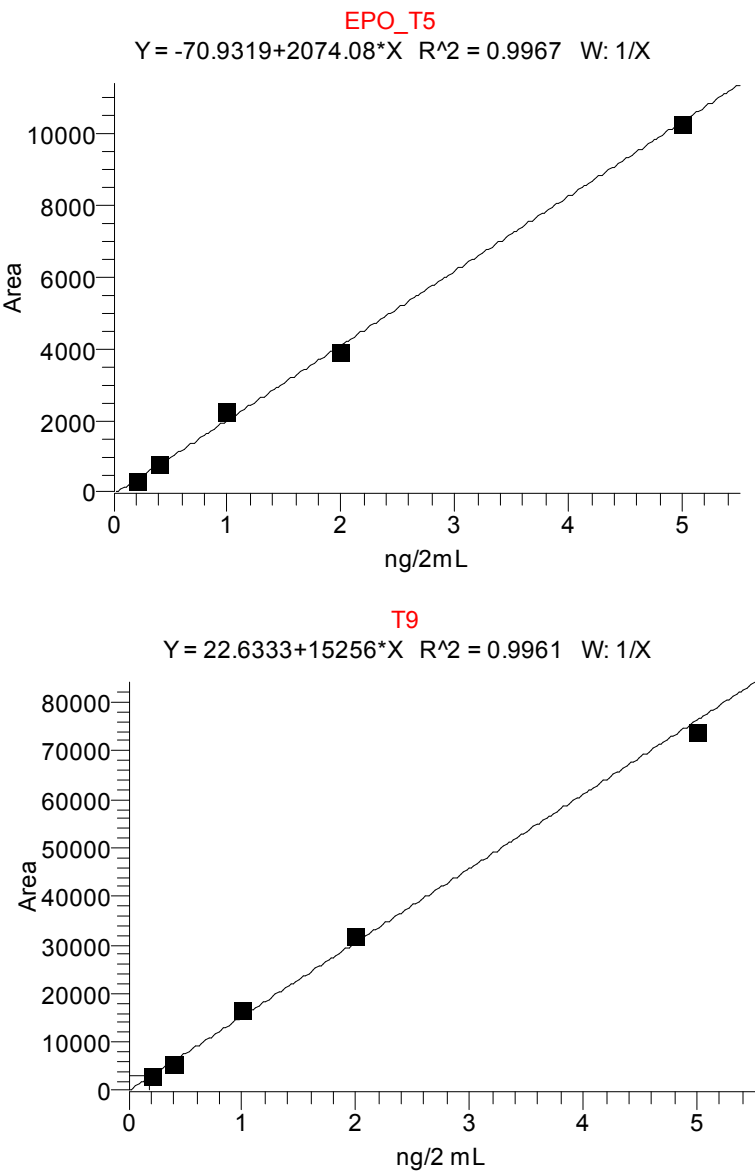
S-Table 3. SEQUEST search results of the product ion spectra of the deglycosylated T₅ of rhEPO, deglycosylated T₉ of DPO, T₆ and T₁₇ of rhEPO and DPO indicating the specificity of the T₅ and the T₉ for identification of rhEPO and DPO.



S-Figure 1. LC-MS chromatograms of rhEPO (top panel) and DPO (bottom panel) (50 µg/mL each) following digestion by trypsin alone, indicating identical tryptic peptide profiles and lack of differentiation between DPO and rhEPO without deglycosylation of the associated glycopeptides.



S-Figure 2. LC-MS/MS chromatograms and product ion spectra indicating that 0.2 ng/mL of rhEPO and 0.1 ng/mL of DPO spiked to equine plasma were identified using the triply charged T₅ at m/z 897.8 and the T₉ at m/z 766.4.



S-Figure 3. Calibration curves for quantification of rhEPO (top panel) and DPO (bottom panel) in equine plasma by external calibration.

S-Table 1. Accurate mass measurements and exact masses predicted for product ions of the deglycosylated T₅ of rhEPO and T₉ of rhEPO and DPO

Peptide	Measured monoisotopic <i>m/z</i> of product ion ^a	Product ion name	Predicted <i>m/z</i> ^b	Δ <i>m/z</i> (a.m.u.) ^c	Δ <i>m/z</i> (ppm) ^d
T ₅ of rhEPO	1115.4623	b ₂₁ ²⁺	1115.4674	-0.0051	-4.5
	1106.463	(b ₂₁ - H ₂ O) ²⁺	1106.4621	0.0009	0.8
	1097.4534	(b ₂₁ - 2H ₂ O) ²⁺	1097.4569	-0.0035	-3.2
	1066.4657	y ₂₀ ²⁺	1066.4672	-0.0015	-1.4
	1015.9449	y ₁₉ ²⁺	1015.9434	0.0015	1.5
	965.4146	y ₁₈ ²⁺	965.4195	-0.0049	-5.0
	460.2389	y ₄	460.2402	-0.0013	-2.8
T ₉ of rhEPO	1088.0728	y ₁₉ ²⁺	1088.0759	-0.0031	-2.8
	1052.5538	y ₁₈ ²⁺	1052.5573	-0.0035	-3.3
	996.0127	y ₁₇ ²⁺	996.0153	-0.0026	-2.6
	939.4706	y ₁₆ ²⁺	939.4732	-0.0026	-2.8
	889.9364	y ₁₅ ²⁺	889.939	-0.0026	-2.9
	832.4221	y ₁₄ ²⁺	832.4256	-0.0035	-4.2
	681.3622	y ₁₁ ²⁺	681.3642	-0.002	-2.9
T ₉ of DPO	1055.5515	y ₁₉ ²⁺	1055.555	-0.0035	-3.3
	1020.0323	y ₁₈ ²⁺	1020.0364	-0.0041	-4.0
	963.4911	y ₁₇ ²⁺	963.4944	-0.0033	-3.4
	906.9505	y ₁₆ ²⁺	906.9523	-0.0018	-2.0
	857.416	y ₁₅ ²⁺	857.4181	-0.0021	-2.4
	799.9018	y ₁₄ ²⁺	799.9047	-0.0029	-3.6

^a Product ions were generated in the LTQ linear ion trap with CID parameters described in Materials and Methods, and their *m/z* values were measured using the Orbitrap at a resolving power of 30,000 (at *m/z* 400).

^b Predicted *m/z* values for the product ions were obtained using ProteinProspector software developed by Dr. Alma Burlingame and his group at the University of California, San Francisco, CA. Predicted *m/z* values for y₁₈²⁺, y₁₉²⁺, y₂₀²⁺, (b₂₁ - 2H₂O)²⁺, b₂₁²⁺, and y₂₂²⁺ of the

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- 1 T₅ of rhEPO were the *m/z* values predicted from amino acid sequence of the T₅ by
- 2 ProteinProspector minus 1.00783 (mass of hydrogen atom), by taking into account the disulfide
- 3 bond between ²⁹Cys and ³³Cys.
- 4 ^c Calculated as *m/z* measured minus *m/z* predicted.
- 5 ^d Δ *m/z* (a.m.u.) divided by *m/z* predicted.

S-Table 2. BLAST search result for the tryptic peptide T₅ of rhEPO and T₉ of DPO indicating their inherent specificity for identification of rhEPO and DPO in equine plasma ^a

T ₅ of rhEPO, ²¹ EAENITTGCAEHCSLNENITVPDTK ⁴⁵			
gi number	Protein	Species	Match to a sequence section
62240997	Erythropoietin	Homo sapiens (human)	100%
114615073	Erythropoietin	Pan troglodytes (chimpanzee)	100%
23379782	Erythropoietin	Gorilla gorilla (gorilla)	100%
23379784	Erythropoietin	Pongo pygmaeus (orangutan)	88%
23379788	Erythropoietin	Saguinus oedipus	88%
112293295	Erythropoietin	Macaca mulatta	84%
27806897	Erythropoietin	Bos taurus	84%
126352399	Erythropoietin	Equus caballus (horse)	76%

Deaminated form of the T ₅ , ²¹ EAEDITTGCAEHCSLNEDITVPDTK ⁴⁵			
62240997	Erythropoietin	Homo sapiens	92%
114615073	Erythropoietin	Pan troglodytes	92%
23379782	Erythropoietin	Gorilla gorilla	92%
23379784	Erythropoietin	Pongo pygmaeus	80%
23379788	Erythropoietin	Saguinus oedipus	80%
112293295	Erythropoietin	Macaca mulatta	76%
27806897	Erythropoietin	Bos taurus	76%
126352399	Erythropoietin	Equus caballus (horse)	68%

T ₉ of DPO, ⁷⁷ GQALLVNSSQVNETLQLHVDK ⁹⁷			
6573159	Erythropoietin	Homo sapiens (human)	85%
57163805	Erythropoietin	Felis catus (domestic cat)	85%
23379780	Erythropoietin	Pan troglodytes (chimpanzee)	85%
23379782	Erythropoietin	Gorilla gorilla (gorilla)	85%
23379784	Erythropoietin	Pongo pygmaeus (orangutan)	85%
126352399	Erythropoietin	Equus caballus (horse)	80%
55742715	Erythropoietin	Sus scrofa (pig)	80%
126722603	Erythropoietin	Oryctolagus cuniculus	80%
21389309	Erythropoietin	Mus musculus	80%

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Deaminated form of the T₉, ⁷⁷GQALLV**D**SSQV**D**ETLQLHVDK⁹⁷

62240997	Erythropoietin	Homo sapiens (human)	80%
57163805	Erythropoietin	Felis catus (domestic cat)	80%
23379780	Erythropoietin	Pan troglodytes (chimpanzee)	80%
23379782	Erythropoietin	Gorilla gorillar (gorilla)	80%
23379784	Erythropoietin	Pongo pygmaeus (orangutan)	80%
126352399	Erythropoietin	Equus caballus (horse)	76%
55742715	Erythropoietin	Sus scrofa (pig)	76%

^a The result shows that only a few exact matches (100%) in amino acid sequence to the T₅ of rhEPO but neither to its deaminated form nor to the T₉ of DPO or the deaminated T₉ was found in the database of the All Non-Redundant GenBank CDS (translations + PDB + SwissProt + PIR + PRF excluding environmental samples) of December 3, 2007 from the National Center for Biotechnology Information (NCBI). The search program used was BLASTP 2.2.17 (Au-26-2007) from NCBI.

S-Table 3. SEQUEST search results of the product ion spectra of the deglycosylated T₅ of rhEPO, deglycosylated T₉ of DPO, T₆ and of T₁₇ of rhEPO and DPO indicating the specificity of the T₅ and the T₉ for identification of rhEPO and DPO ^a

Peptide	Database	Ranking No.	Protein found	P (pro)	Sf	Score	Accession #	Note	Candidate peptide
T5 of rhEPO	Human	1	lines homolog 1 [Homo sapiens]	4.26E-01	0.12	10.09	95007026	No match except m/z 1016.6, 777.4, 690.5, 660.5 and 460.3	NLFYPNPTALL KLLKYIEVISNK
		2	dipeptidylpeptidase IV [Homo sapiens]	7.31E-01	0.00	8.06	18765694	No match except m/z 1180.6, 1115.6, 1015.9, 690.5, 660.5 and 460.4	KYPLLLDV YAGPCSQK
		3	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	9.02E-01	0.09	8.08	86788132	No match	EHIVDLEMLTVS SIGFTRTSSVLR
		4	PREDICTED: hypothetical protein [Homo sapiens]	1.00E+00	0.00	10.06	88959018	No match	SEREGKVY GQAEAAIGK
	Equine	1	CAL1_HORSE Calcitonin gene-related peptide I precursor (CGRP-I) (Alpha-type CGRP)	9.98E-01	0.24	10.05	27734224	No match except m/z 965.7, 559.4, 540.1	FSPFLPLSILVLY QVGIIQAAPFR
		2	IATR_HORSE Inter-alpha-trypsin inhibitor (ITI) (HI-14) (Inhibitory fragment of ITI)	9.98E-01	0.01	8.02	124017	No match except m/z 1180.6, 1106.6	AFIRLWAFDAAQ GKCVLFTYGGCR
		3	seahorse [Danio rerio]	9.98E-01	0.00	8.02	50539688	No match	TRVLQQE TKYLEER
		4	PERC_ARMRU Peroxidase C1C precursor	9.99E-01	0.00	10.02	129816	No match	DAFGNANSA RGFPVVDR
T9 of DPO	Human	1	leucine zipper domain protein [Homo sapiens]	4.15E-02	0.03	10.08	13375779	No match except m/z 1020.2, 857.3, 800.0	WLLHDEVHA VLKQLQDILK
		2	bicaudal D homolog 1	7.04E-01	0.04	8.08	51093830	No match except	AVVTNVQAEN

			isoform 2 [Homo sapiens]	-02				m/z 1056.5, 1020.2, 963.3, 857.3	ERLTAVVQDLK
		3	PREDICTED: similar to G protein-coupled receptor 158 isoform a [Homo sapiens]	3.01E-01	0.01	10.05	113427390	No match	IRDKSEAG DSVEAR
		4	alpha-kinase 3 [Homo sapiens]	4.02E-01	0.01	8.05	115511036	No match	GRAARGPG SSGTDSTR
Equine		1	TRFE_HORSE Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal binding globulin)	9.39E-01	0.00	10.03	136190	No match except m/z 907.5	KSVTEFK SCYLAR
		2	CYTB_10543 cytochrome b [Equus caballus]	1.00E+00	0.01	10.04	5835120	No match except m/z 1020.2	YLHANGASM FFICLFHVGRR
		3	putative equine mast cell protease-1 [Equus caballus]	1.00E+00	0.01	8.03	43823414	No match	TQVRPGEVCSV AGWGQLAPKGR
		4	HPL1_HORSE Hyaluronan and proteoglycan link protein 1 precursor (Proteoglycan link protein) (Cartilage link protein) (LP)	1.00E+00	0.00	8.02	2499842	No match	FLRDPTA FGSGTHK
T6	Human	1	erythropoietin precursor [Homo sapiens]	3.01E-03	0.84	10.12	62240997	Match to m/z 828.4, 714.4, 567.4, 404.3, 361.2, 333.3, 214.1	VNFIYAWK
		2	15 kDa selenoprotein isoform 1 precursor [Homo sapiens]	7.76E-03	0.00	10.06	42741648	No match except m/z 828.4, 715.4, 567.2, 479.2	LFRGLQ IKYVR
		3	PREDICTED: hypothetical protein [Homo sapiens]	8.89E-02	0.00	8.05	113418319	No match	NFRFEA PQLSGK
		4	hypothetical protein LOC79919 [Homo sapiens]	2.58E-01	0.13	8.08	13376290	No match	VNHESWR

	Equine	1	MM13_HORSE Collagenase 3 precursor (Matrix metalloproteinase-13) (MMP-13)	9.97E -01	0.02	10.02	5921828	No match except m/z 361.2	DRFFWR
		2	TGF1_HORSE Transforming growth factor beta 1 precursor (TGF-beta 1)	1.00E +00	0.00	8.02	3024717	No match except m/z 764.3, 464.4, and 333.3	YSNNSWR
		3	A36034 fibrinogen alpha chain - horse (fragment)	1.00E +00	0.01	10.03	108195	No match	KEYHTG KLVTSK
		4	MT3_HORSE Metallothionein-III (MT-III) (Growth inhibitory factor) (GIF)	1.00E +00	0.00	8.02	585521	No match	KSCCSCC PAECEK
T17	Human	1	erythropoietin precursor [Homo sapiens]	2.58E -02	0.11	8.07	62240997	Matches to m/z 799.5, 636.4, 549.4, 288.3, 263.1, 235.1	VYSNFLR
		2	LPS-responsive vesicle trafficking, beach and anchor containing [Homo sapiens]	3.62E -01	0.20	10.08	16904381	No match except m/z 799.5, 549.4, 391.4,235.1	VYSTDTGR
		3	G protein-regulated inducer of neurite outgrowth 1 [Homo sapiens]	9.19E -01	0.01	8.05	112821681	No match	YSGKEH PVSSEK
		4	PREDICTED: similar to Zinc finger CCHC domain-containing protein 4 isoform 5 [Homo sapiens]	9.99E -01	0.02	10.05	88979556	No match	RFYACS ACRDR
	Equine	1	TPM4_HORSE Tropomyosin alpha 4 chain (Tropomyosin 4) (Platelet beta tropomyosin)	8.77E -01	0.01	10.02	20178270	No match except m/z 637.4, 391.3, 262.6	YSEKEDK
		2	S25828 serpin II - horse (fragment)	9.32E -01	0.00	8.01	108207	No match except m/z 391.9, 262.9, 236.3	KAPNEAIR

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3	HS9A_HORSE Heat shock protein HSP 90-alpha (HSP 86)	1.00E +00	0.01	10.02	17865490	No match	HFSVEG QLEFR
4	HS9B_HORSE Heat shock protein HSP 90-beta (HSP 84)	1.00E +00	0.01	10.02	20177936	No match	TLTLVDT GIGMTK

^a SEQUEST search parameters used were: 2.0 a.m.u. for precursor peptide mass tolerance, and 1.0 a.m.u. for fragment ion tolerance. Protein databases searched were: human reference protein FASTA database (March 2007) included in Bioworks software (v. 3.3.1), and an equine protein FASTA database (April 2004) included in Bioworks software (v. 3.3.1).