

# Validation of a Serotonin ELISA Kit with Blood Samples from Three Domestic Animal Species

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# SEROTONIN ELISA KIT (ADI-900-175)

### HIGHLIGHTS

- · Enzo's Serotonin ELISA Kit was tested with dog serum, horse plasma, and pig serum
- · Precision, accuracy, parallelism, and linearity under dilution were evaluated
- Precision and accuracy of Enzo's Serotonin ELISA Kit were validated for the analysis of serotonin in these three sample types
- · Dilutional linearity was achieved with all three sample types
- Enzo's Serotonin ELISA Kit is able to recognize and bind in a specific manner the serotonin present in these three sample types similarly to the serotonin from the standard

### **INTRODUCTION**

The Phérosynthèse research and development laboratory was created in 1995, specializing in chemical communication within the living world. In 2010, Phérosynthèse became the Research Institute in Semiochemistry and Applied Ethology (IRSEA). By identifying chemical signals that play a role in the life of animals, the development of new therapeutic and zootechnical tools that respect man, animals, and the environment are possible. Dr. Cécile Bienboire-Frosini is the head of the Department of Physiological and Behavioral Mechanisms of Adaptation and as such, is in charge of several transversal projects on chemical communication and the mechanisms of action of semiochemicals. One of her projects is to adapt and validate commercial immunoassays for the study of their animals of interest. For example, a method combining a pre-extraction step and Enzo's Oxytocin ELISA Kit was recently developed and validated for the determination of oxytocin in plasma samples from seven domestic animal species. The results of this study, published in Frontiers in Neuroscience (Bienboire-Frosini *et al.*, 2017), could prove critical for the study of oxytocin and its involvement in behaviors and emotions.

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine biochemically-derived from tryptophan. It is found in the central nervous system, gastrointestinal tract, and blood. It has broad physiological functions as a neurotransmitter, in gastric motility, hemostasis, and cardiovascular integrity (Pineyro *et al.*, 1999). Effects are mediated through the activation of 5-HT receptors. Serotonergic neurons have axons, which project towards many different parts of the brain. A portion of the serotonin that has been produced and released by serotonergic neurons triggers a neuronal impulse capable of affecting many different behaviors. The rest is captured by the presynaptic serotonergic neurons and used in a negative feedback loop. In humans, a decrease in serotonin signaling has been linked with neurodegenerative disorders such as Parkinson's disease (Fox, *et al.*, 2009), but also complex behavioral disorders such as aggression (Young and Moskowitz, 2005) and depression (Murphy *et al.*, 2008). Low serotonin levels are believed to be the cause of many cases of mild-to-severe depression, which can lead to symptoms of anxiety, apathy, fear, insomnia, and fatigue.



The knowledge gained in humans can be directly applied to animals. Indeed, the study of serotonin levels in animals is clinically-relevant and can be indicative of welfare. Diet, environment, exercise, housing, animal-human interactions, and weaning were shown to influence serotonin concentration in dog serum (Alberghina *et al.*, 2014; Park *et al.*, 2014; Alberghina *et al.*, 2017), horse (Bruschetta *et al.*, 2014; Alberghina *et al.*, 2013). Low levels of serotonin were found to be associated with distress and behavioral disorders such as impulsiveness and aggression in some dogs (Leon *et al.*, 2012; Amat *et al.*, 2013), cribbing in horses (Lebelt *et al.*, 1998), and tail biting in pigs (Ursinus *et al.*, 2014). As a precursor of serotonin, tryptophan can be envisaged as a supplement to diet to attenuate these aggressive behaviors, control stress, and improve wellbeing (DeNapoli *et al.*, 2000; Shen *et al.*, 2012). The data obtained in dog, horse, and pig suggest that serotonin could be a key biomarker for the monitoring of their overall welfare and/or for helping the clinical assessment of some behavioral disorders and therapies. Using a similar approach to the one used for the Oxytocin ELISA Kit, the researchers at IRSEA evaluated Enzo's Serotonin ELISA Kit for the measurement of serotonin in these three species.

# **MATERIALS AND METHODS**

## **Animal Studies**

Three adult mammal species of both sexes were used in this study. Dogs *(Canis familiaris)*, horses *(Equus caballus)*, and pigs *(Sus scrofa)* were from IRSEA breeding facilities. Housing, husbandry, and use of animals described in this document were carried out following the French and European legislation and in compliance with the principles of replacement, reduction, and refinement. All procedures were performed with approval from the Ethics Committee C2EA125, in concordance with French and European legislation.

# **Blood Sampling**

Blood samples were collected from the jugular vein of animals. Dog blood was transferred into a dry tube containing a gel separator (Vacuette, 8287165). Horse blood was collected in EDTA tubes (Vacuette, 8287248). Pig blood was transferred into a dry tube (Vacuette, 8287142). Blood samples were centrifuged at 4° C at 1800 x g for 12 minutes. Dog and pig serum samples and horse plasma samples were pipetted, aliquoted, and stored at -20° C until further use.

# Serotonin ELISA Kit

Serotonin (5-HT) was measured using Enzo's Serotonin ELISA Kit (ADI-900-175) following the manufacturer's instructions. This kit has been used in previous studies assaying 5-HT in a variety of animal species including cow (Laporta *et al.*, 2013), dog (Park *et al.*, 2014), human (Wannhoff *et al.*, 2016), mouse (Homma *et al.*, 2016), pig (Willemen *et al.*, 2012), rat (Laporta *et al.*, 2013), and sheep (Lacerda *et al.*, 2012). The kit's sensitivity is 0.293 ng/mL with an assay range between 0.49 and 500 ng/mL. It can be defined as two standard deviations from the mean signal at zero, which was determined from seven independent standard curves and 14 zero standard duplicates. Intra-assay precision at low, medium, and high concentrations is 11.0, 5.8, and 4.2%, respectively. It was determined by assaying 20 replicates of three buffer controls containing serotonin in a single assay. Finally, inter-assay precision at low, medium, and high concentrations is 12.7, 18.4, and 16.2%, respectively. It was determined by measuring buffer controls of varying serotonin concentrations in multiple assays over several days.

# **Serotonin Concentration**

In this study, sample dilution in Assay Buffer was required for dog (1 in 20), horse (1 in 10), and pig (1 in 50) to remove matrix interference and obtain concentration values within the kit's standard range. Diluted samples were run in triplicates.

# **Assessment of Validation Criteria**

According to the EMA Guideline on bioanalytical method validation (2011), commercial kits need to be revalidated when the biological matrix is changed to ensure that the sample analysis is performed accurately and precisely. For the validation procedure with dog serum, horse plasma, and pig serum, a partial validation was performed and the following parameters were checked: precision, accuracy, parallelism, and linearity under dilution.

# RESULTS Precision

The precision of an ELISA can be defined as "the closeness of agreement between a series of measurements obtained under the prescribed conditions". Repeated measurements are performed with the same sample under specific and identical conditions. The precision is expressed as the coefficient of variation (%CV). The intra-assay precision of Enzo's Serotonin ELISA Kit was determined by measuring several samples in replicates at three levels of 5-HT concentrations in order to cover the entirety of the standard curve: a concentration representing approximate-ly three times the lower limit of quantification (low concentration), one near the middle of the standard curve (medium concentration), and one close to the upper limit of the standard curve (high concentration).

The lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample, which can be quantified reliably, with an acceptable accuracy and precision. The lowest calibration standard is deemed to be the LLOQ. The LLOQ for Enzo's Serotonin ELISA Kit is 0.49 ng/mL. The LLOQ should not be confused with the limit of detection or sensitivity, which is outside the standard range (i.e. 0.293 ng/mL). Conversely, the highest calibration standard is referred to as the upper limit of quantification (ULOQ). The ULOQ for Enzo's Serotonin ELISA Kit is 500 ng/mL.

For the precision to be accepted, %CV must not exceed 20% except for LLOQ where it must not exceed 25%. As the concentrations of 5-HT in samples were unknown, samples were randomly chosen in the hope of covering the entire assay range. A precision profile representing %CV against 5-HT concentration was established and only three samples (2 from horses and 1 from pig) were found to have %CV above the acceptance criteria (**Fig. 1**). Precision evaluation for dog, horse, and pig were summarized in tables 1, 2, and 3, respectively. Based on these results, the precision of Enzo's Sero-



Figure 1: Precision profile of Enzo's Serotonin ELISA Kit with dog and pig serum as well as horse plasma.

tonin ELISA Kit was validated for the determination of 5-HT concentration in dog, horse, and pig.



		Precision				Aco	curacy					
	Mean Concentration (Pg/mL) (n=3)	Intra-batch Precision (%CV)	Precision Acceptability Criteria	Supple- mentation level	Expected Concentration (pg/mL)	Measured Concentration (pg/mL) (n=3)	Intra-batch Precision (%CV)	Precision Acceptability Criteria	Recovery (%)	± Bias (%)	Total error (%)	Acceptability Criteria
		1	\u000	30	162.4	166.5	-	< 20%	103	3	4	< 30%
Sample I	04.2		% <b>77</b> %	1.5	67.4	62.4	3.3	< 20%	93	7	10	< 30%
Samula 2	30.4		%0UC /	30	130.4	118.1	7.2	< 20%	91	6	16	< 30%
	t.00	F	0/07 /	1.5	35.4	36.4	7.1	< 20%	103	3	10	< 30%
Sample 3	31.5	18.8	< 20%	9	51.5	55.8	2.8	< 20%	108	8	11	< 30%
Sample 4	35.9	3	< 20%	9	55.9	53.8	4	< 20%	96	4	8	< 30%
Sample 5	17.5	15.9	< 20%	9	37.4	40.9	8	< 20%	109	6	17	< 30%
Sample 6	15.4	7.3	< 20%	9	35.4	36.8	10	< 20%	104	4	14	< 30%
Sample 7	40.2	1.4	< 20%	9	60.2	69.4	3.6	< 20%	115	15	19	< 30%
Sample 8	25.8	24.7	> 20%	9	45.8	46.9	19	< 20%	102	2	21	< 30%
Sample 9	22.7	18.2	< 20%	9	42.6	42.4	2.1	< 20%	66		3	< 30%
Sample 10	23.6	1.2	< 20%	9	43.5	47.6	2.6	< 20%	109	6	12	< 30%
Sample 11	48.9	1.1	< 20%	9	68.9	61.7	5.1	< 20%	06	10	15	< 30%
Sample 12	75.2	8.3	< 20%	9	95.2	95.6	2.8	< 20%	100	0	3	< 30%

Table 1: Validation criteria of precision and accuracy for Enzo's Serotonin ELISA Kt in dogs.

		Precision				Aco	suracy					
	Mean Concentration (Pg/mL) (n=3)	Intra-batch Precision (%CV)	Precision Acceptability Criteria	Supple- mentation level	Expected Concentration (pg/mL)	Measured Concentration (pg/mL) (n=3)	Intra-batch Precision (%CV)	Precision Acceptability Criteria	Recovery (%)	± Bias (%)	Total error (%)	Acceptability Criteria
				5	206.6	189.66	7.06	< 20%	92	8	15	< 30%
Sample 1	203.72	11.32	< 20%	20	215.6	211.66	8.72	< 20%	98	2	11	< 30%
			<u> </u>	100	263	259.97	8.67	< 20%	66	-	10	< 30%
				5	145.8	143.86	9.43	< 20%	99	1	10	< 30%
Sample 2	142.22	16.01	< 20%	20	156.6	127.13	5.39	< 20%	81	19	24	< 30%
				100	213.8	150.74	8.18	< 20%	71	29	37	> 30%
				5	99.2	103.81	20	< 20%	105	5	25	< 30%
Sample 3	95.1	10.03	< 20%	20	111.2	133.29	5.5	< 20%	120	20	26	< 30%
				100	176.06	168.74	20.21	> 20%	96	4	24	< 30%
				5	90.2	80.84	16.84	< 20%	90	10	27	< 30%
Sample 4	85.96	5.22	< 20%	20	102.52	130.76	2.1	< 20%	128	28	30	< 30%
				100	168.8	151.63	7.6	< 20%	90	10	18	< 30%
				5	26.2	18.13	13.76	< 20%	69	31	45	> 30%
Sample 5	21.51	35.16	> 20%	20	40.6	48.06	6.82	< 20%	118	18	25	< 30%
				100	117.2	66.28	5.21	< 20%	57	43	48	> 30%
				5	19.4	5.608	ND	ND	29	71	DN	> 30%
Sample 6	14.55	31.7	> 20%	20	34	43.86	30.42	> 20%	129	29	59	> 30%
				100	111.6	86.23	9.05	< 20%	77	23	32	> 30%

# Table 2: Validation criteria of precision and accuracy for Enzo's Serotonin ELISA Kit in horses.

# APPLICATION NOTE



		Precision				Acc	suracy					
	Mean Concentration (Pg/mL) (n=3)	Intra-batch Precision (%CV)	Precision Acceptability Criteria	Supple- mentation level	Expected Concentration (pg/mL)	Measured Concentration (pg/mL) (n=3)	Intra-batch Precision (%CV)	Precision Acceptability Criteria	Recovery (%)	± Bias (%)	Total error (%)	Acceptability Criteria
	C C L	0		10	72.8	83.8	0.7	< 20%	115	15	16	< 30%
sample 1	97.7 <u>c</u>	21	< 20%	2.5	57.8	59.8	17.1	< 20%	103	3	20	< 30%
C of second		T C T	,0000 ·	10	39.9	31.2	2.5	< 20%	78	22	25	< 30%
Sample 2	19.9	13.1	~ ZU%	2.5	24.9	17.5	16.6	< 20%	70	30	37	> 30%
		1	)0 <b>00</b>	10	42.6	32.1	5.1	< 20%	75	25	30	< 30%
	0.77	,	0/NZ >	2.5	27.6	26.4	65.1	> 20%	95	5	70	> 30%
Commo 1	0 10	T U	\0 <b>00</b>	10	41.8	38.4	11	< 20%	91	6	20	< 30%
odilipie 4	0.12	0.4	0∕. <b>∩</b> 2 >	2.5	26.8	26.5	12	< 20%	66	-	13	< 30%

Table 3: Validation criteria of precision and accuracy for Enzo's Serotonin ELISA Kit in pigs.

# Accuracy

The accuracy of an ELISA can be defined as "the closeness of the determined value to the value which is accepted either as a conventional true value or an accepted true value". Repeated measurements are performed with samples spiked with known amounts of the analyte of interest. The accuracy is determined using a minimum of three measurements per concentration and a minimum of four different concentrations within the assay range is recommended. For the accuracy to be accepted, %CV must not exceed 20%. An accuracy profile representing %CV against 5-HT concentration was established and only three samples were found to have %CV above the acceptance criteria (**Fig. 2**).

The total error expresses the combination of random error and systematic error. It is the sum of the %CV (random error) from the precision measurement and the bias (systematic error) from the accuracy estimation. It must not exceed 30% of the true value, even at LLOQ. Accuracy evaluation for dog, horse, and pig were summarized in tables 1, 2, and 3, respectively. Despite six samples of horse plasma and a few samples of pig serum with a total error greater than 30%, all the remaining samples tested in this study gave acceptable values. Since at least 67% of the samples in each species gave total error within the limits of acceptance, the accuracy of Enzo's Serotonin ELISA Kit was validated for the determination of 5-HT concentration in dog, horse, and pig.

### **Linearity and Parallelism**

The selectivity of an ELISA is its ability to measure and differentiate unequivocally the analyte of interest in the presence of other components habitually present in the biological matrix such as degraded proteins, impurities, metabolites, and other constituents of the matrix. Selectivity issues may arise from matrix interferences unrelated to



Figure 2: Accuracy profile of Enzo's Serotonin ELISA Kit with dog and pig serum as well as horse plasma.

the analyte of interest and to a lesser extent, substances that are physicochemically-similar to the analyte (e.g. cross-reactants). Matrix interference can affect an ELISA assay either positively (i.e. components from the matrix causing background or binding to the antibody in an unspecific manner) or negatively (i.e. components from the matrix preventing binding of the analyte).

The study of linearity is meant to assess the selectivity of the assay by investigating the relationship between expected theoretical values and observed measured values. The biological matrix is spiked at a concentration falling close to the top of the curve (i.e. greater than ULOQ) to ensure that even the highest dilution will not fall below the lower end of the curve. Serial dilutions of the analyte are then prepared in Assay Buffer.



Samples previously used in the study of repeatability were serially diluted using two-fold dilutions (e.g. 1:2.5, 1:5, 1:10, 1:20, 1:40, and 1:80). Dilutional linearity was demonstrated in all three species by representing measured values against expected theoretical values. Regression lines show acceptable linearity under dilution with a slope of 1. Correlation coefficients (i.e. R<sup>2</sup> values) are also significant with a value close to 1 indicating the absence of any scattering around the regression line for dog (**Fig. 3A**), horse (**Fig. 3B**), and pig (**Fig. 3C**).



The calculation of recovery percentages between expected values and measured values demonstrates the correlation of the results even when the dilution factor is important. Furthermore, the recovery percentage at working dilution (i.e. 1:20 for dog serum; 1:10 for horse plasma; and 1:50 for pig serum) is correct and within the accepted range (i.e. between 80 and 120%) **(Table 4)**.

Der	Dilution	5	10	20	40	80
Dog	Recovery (%)	97.8	87.8	97.1	115.1	110.6
Here a	Dilution	5	10	20	40	80
Horse	Recovery (%)	ND	115.5	104.1	118.6	115.5
D:	Dilution	12.5	25	50	100	200
Pig	Recovery (%)	ND	81.1	88.3	74.1	59.8

Table 4: Percentage of recovery when using Enzo's Serotonin ELISA Kit with diluted dog serum, horse plasma, and pig serum.

Selectivity of the assay can be further looked at by conducting a study of parallelism and comparing serially diluted samples with the standard curve. Of note, conversely to the dilutional linearity study, samples used for the parallelism study are not spiked. The different dilution factors chosen for the study were shown as a function of 5-HT concentration for dog (Fig. 4A), horse (Fig. 4B), and pig (Fig. 4C). Regardless of the sample origin, the dilution curves obtained with the samples are parallel to the dilution curve obtained with the standard indicating the ability of the antibody used in this kit to recognize and bind in a specific manner the serotonin present in the samples in a similar way as the serotonin from the standard.



# CONCLUSION

Enzo's Serotonin ELISA Kit has been validated by the manufacturer for the measurement of serotonin in a variety of biological fluids including human plasma, serum, and urine. For use with dog serum, horse plasma, and pig serum, a partial validation had to be undertaken and a number of performance criteria had to be checked including precision, accuracy, linearity, and parallelism. The last two parameters, in particular, allow the end user to assess both the selectivity of the assay and the matrix effect. All these criteria were validated as evidenced by the results highlighted in this application note. This kit was chosen to assay serotonin in dog serum with a 1 in 20 dilution, horse plasma with a 1 in 10 dilution, and pig serum with a 1 in 50 dilution. Enzo's Serotonin ELISA Kit was deemed to be adapted to the analysis of serotonin in different clinical trials or research projects in veterinary science (Marcet Rius *et al.*, 2018).



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Visit <u>www.enzolifesciences.com</u> for more information about Enzo's Serotonin ELISA Kit:

- References
- Cited Samples
- Other Application Notes





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