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Validation of Bet v 1 ELISA 2.0 EP and Comparability to the Method Published in the European Pharmacopoeia

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Project Overview

InBio's ELISA 2.0 platform utilizes 96-well microtiter plates coated with capture antibody followed by treatment with stabilizing and blocking agent. The pre-coated plates are preserved in sealable pouches with desiccant and have an 18-month shelf-life. Assay calibration standards are formulated from InBio's purified natural or recombinant allergens. A mixture of detection antibody and peroxidase-conjugate are combined with TMB substrate to generate a measurable signal for allergen quantification. The assay can be completed in approximately two hours.

An EDQM version of InBio's Bet v 1 ELISA 2.0 has been developed (Bet v 1 ELISA 2.0 EP). The assay uses monoclonal antibodies 5B4 and 6H4 developed by Stallergenes. These antibodies were tested as part of the BSP090 Biological Standardization Program[1,2], which InBio has been involved with since 2009. New batches of the antibodies have since been produced and are used in the Bet v 1 ELISA 2.0 EP. InBio's Bet v 1 calibration standard is formulated from recombinant Bet v 1 and standardized against the EDQM's recombinant Bet v 1 Chemical Reference Substance (CRS Y0001565)[3,4], however, for this study the EDQM Bet v 1 CRS was used as the standard for both ELISA 2.0 and the Stallergenes assay.

The method for the Stallergenes Bet v 1 ELISA was adopted into the European Pharmacopoeia as monograph 2.7.36 in January 2023.[5] Prior to this, InBio conducted a study to demonstrate comparability of the Bet v 1 ELISA 2.0 EP to the Stallergenes method for the quantitative measure of Bet v 1 in birch pollen extracts. The Stallergenes ELISA was performed exactly as described in the protocol provided for the international ring trial run under the auspices of the EDQM in 2011.

Validation of InBio's Bet v 1 ELISA 2.0 EP assay was based on the following parameters:

- pre-coated plate batch consistency
- comparability to the Stallergenes Bet v 1 assay
- plate and reagent stability during shipping
- matrix spike recovery data
- curve parallelism for the EDQM Bet v 1 CRS and birch pollen extract
- assay limit of detection (LOD) and lower limit of quantification (LLOQ)

Note: this study was completed prior to the publication of general chapter 5.27, *Comparability of Alternative Analytical Procedures*, in Supplement 11.5 of the European Pharmacopoeia.[6]

Evaluation of Birch Pollen Extracts

Six commercial birch pollen extracts acquired from four different manufacturers were tested using ELISA 2.0 and the Stallergenes assay (Table 1). Extracts 1, 2, and 6 were provided as lyophilized powder or raw material and reconstituted in sterile water; extracts 3a, 4, and 5 were provided as glycerol preparations. All extracts were diluted in assay buffer (PBS-Tween 20, 0.05%/BSA, 1%), ranging from 1:500 to 1:320,000 to achieve concentrations in the linear part of the calibration curve.

Table 1. Birch pollen extracts

Extract	Dilution Range
#1	10,000-320,000
#2	500-16,000
#3a	2,000-64,000
#4	2,000-64,000
#5	1,000-32,000
#6	5,000-160,000

Table 2 shows Bet v 1 levels measured by the Stallergenes assay and ELISA 2.0. The Stallergenes assay was performed in the US by two different operators. Runs 2 and 3 were requested by the Paul-Ehrlich-Institute (PEI) after the initial evaluation (Run 1) and required new preparations of Bet v 1 CRS and sample extracts. The ELISA 2.0 assay was performed by three different operators: two in the US (runs 1-4) and one in the UK (runs 5-7).

Variability (coefficient of variation, %CV) ranged from 2-30% for the Stallergenes assay and 7-23% for ELISA 2.0.

Table 2. Bet v 1 levels in birch pollen extracts

	Result (ug/mL)													
	Stallergenes					**ELISA 2.0**								
Extract	Run 1	Run 2	Run 3	Avg	**%CV**	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Avg	**%CV**
#1	183.0	120.0	117.0	140.0	**27**	194.5	180.2	167.2	181.8	190.8	158.9	175.6	178.4	**7**
#2	4.9	5.7	4.9	5.2	**8**	4.9	4.8	4.7	4.8	3.3	3.0	3.1	4.1	**23**
#3a	94.6	133.9	106.5	111.7	**18**	96.7	84.4	86.6	76.5	90.6	70.6	75.7	83.0	**11**
#4	145.9	199.2	171.1	172.1	**16**	128.7	118.7	127.2	121.0	118.5	103.7	107.8	118.0	**8**
#5	18.3	17.7	17.9	18.0	**2**	18.1	17.3	18.2	18.9	14.0	12.4	13.6	16.1	**16**
#6	160.9	93.5	103.8	119.4	**30**	177.6	174.5	182.8	198.4	157.1	128.5	133.6	164.6	**16**

Matrix and Spike Recovery

Extract #5 was selected as a matrix sample to be spiked with three different concentrations of the Bet v 1 CRS. A single stock preparation of the diluted extract and each of the spike samples was prepared in assay buffer. Diluted stocks were stored at 4°C and used over the course of four weeks (separate samples were prepared in the UK). Samples were applied to the assay plate per the ring trial format provided by the PEI (Figure 1).

Materials

- Birch pollen extract #5 ≈ 18µg/mL (determined by ELISA 2.0) diluted 1:1,000 in PBS-Tween 20, 0.05%/BSA, 1% to achieve ≈ 18ng/mL
- Bet v 1 CRS reconstituted in sterile water at 10µg/mL
- 96-well microtiter plates pre-coated with mAb 5B4 at 1µg/mL (lots 39304 and 37357)
- Biotin-conjugated 6H4 detection antibody

Calibration Standard

Reconstituted Bet v 1 CRS diluted in PBS-Tween 20, 0.05%/BSA, 1% (conc. range: 100ng/mL to 0.19ng/mL)

Samples

Matrix = diluted birch pollen extract (≈18ng/mL)
Spike 1 = Bet v 1 CRS (25ng/mL)
Matrix + Spike 1 = 50% extract + 50% spike 1
Spike 2 = Bet v 1 CRS (10ng/mL)
Matrix + Spike 2 = 50% extract + 50% spike 2
Spike 3 = Bet v 1 CRS (5ng/mL)
Matrix + Spike 3 = 50% extract + 50% spike 3

Table 3 shows results from ten assays. Robustness was evaluated through spike recovery tests performed by three different operators (two in the US, one in the UK) and by using two different batches of pre-coated plates. Recovery ranged from 80 to 127% for each matrix and spike sample, with an average recovery range of 97 to 109%. Calculations were based independently on the measured Bet v 1 content in the matrix sample from each respective run.

Figure 1. Plate layout for matrix and spike tests

	1	2	3	4	5	6	7	8	9	10	11	12
A	CRS										blank	blank
	Stock	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7	Dilution 8	Dilution 9		
B	CRS										blank	blank
	Stock	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7	Dilution 8	Dilution 9		
C	Sample 1: Matrix						Sample 2: Matrix + Spike 1					
	undiluted	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	undiluted	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
D	Sample 1: Matrix						Sample 2: Matrix + Spike 1					
	undiluted	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	undiluted	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
E	Sample 3: Matrix + Spike 2						Sample 4: Matrix + Spike 3					
	undiluted	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	undiluted	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
F	Sample 3: Matrix + Spike 2						Sample 4: Matrix + Spike 3					
	undiluted	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	undiluted	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
G	Control 1: Spike 1				Control 2: Spike 2				Control 3: Spike 3			
	undiluted	Dilution 1	Dilution 2	Dilution 3	undiluted	Dilution 1	Dilution 2	Dilution 3	undiluted	Dilution 1	Dilution 2	Dilution 3

Table 3. Birch pollen extract matrix spike recovery

	Plate Lot										
	39304	39357	39304*	39304	39357	39304	39304*	39304	39304	39304**	
Sample	**%Recovery**										**Average**
Matrix + Spike 1	113	97	103	94	103	93	101	92	89	80	**97**
Matrix + Spike 2	99	108	111	102	110	105	98	108	100	98	**104**
Matrix + Spike 3	107	98	107	93	100	96	98	95	92	88	**97**
Spike 1	99	117	118	118	117	115	94	104	94	100	**108**
Spike 2	97	116	119	110	127	113	111	106	96	96	**109**
Spike 3	100	120	120	114	118	115	115	108	83	97	**109**

*Operator 2

**Operator 3 (UK)

Calibration Curves

Figures 2 and 3 show representative calibration curves for the Stallergenes and ELISA 2.0 assays using EDQM Bet v 1 CRS as standard. Parallelism in ELISA 2.0 was demonstrated by running quadruplicate curves of the Bet v 1 CRS and extract #5 on the same assay plate. Figure 4 shows the average of each set of four curves (replicate variability is represented by error bars).

Figure 2. Stallergenes calibration curve; highest concentration 10ng/mL

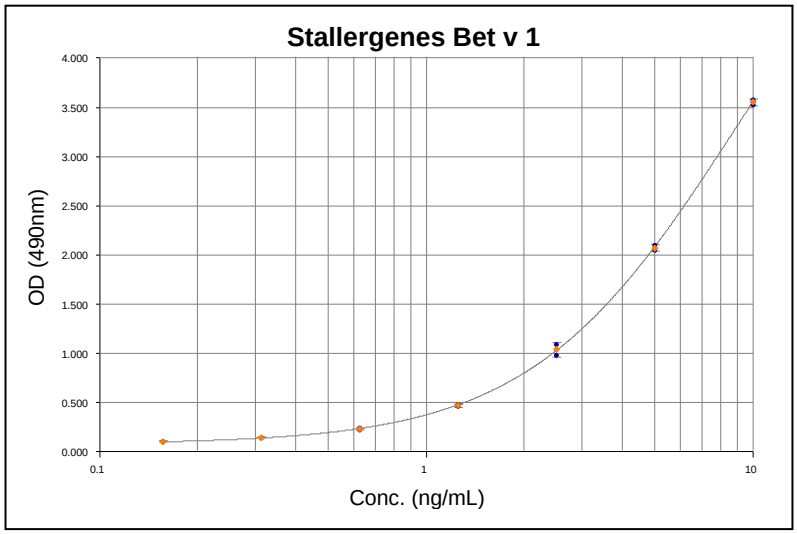


Figure 3. ELISA 2.0 calibration curve; highest concentration 100ng/mL

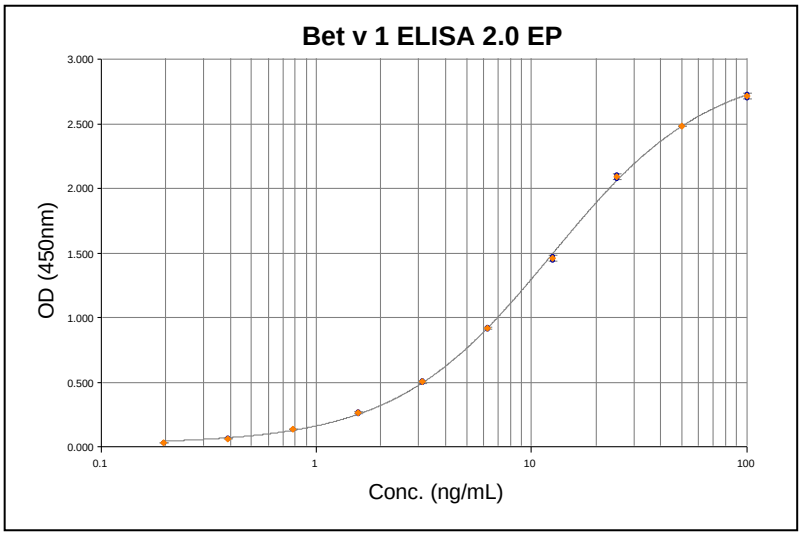
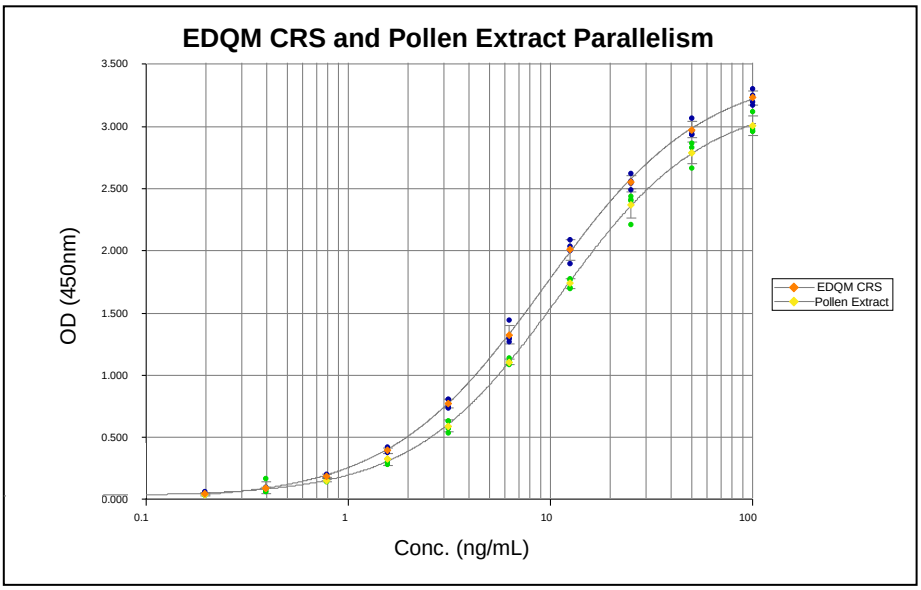


Figure 4. EDQM Bet v 1 CRS and pollen extract curve parallelism, Bet v 1 ELISA 2.0 EP



Assay Range and Sensitivity

The assay LOD is determined by the lowest point on the calibration curve that yields an OD value distinguishable from the blank. The LLOQ is defined by the lowest point on the calibration curve that has a recovery between 80 and 120%, and CV less than 10% for duplicates. Table 4 shows statistical data from a typical ELISA 2.0 calibration curve: usable concentration range was 50-0.39ng/mL, LOD is 0.19ng/mL and the LLOQ is 0.39ng/mL.

Table 4. Calibration curve statistics

Standard	Expected (ng/mL)	Blank 450	Measured (ng/mL)	Mean	Std Dev	CV (%)	% Recovery
STD1	100	2.736	>100.000	90.374	?????	?????	NA
	100	2.7	90.374				
STD2	50	2.484	50.039	49.985	0.075	0.151	100
	50	2.483	49.932				
STD3	25	2.11	26.599	26.057	0.766	2.94	104
	25	2.08	25.515				
STD4	12.5	1.449	11.87	12.078	0.294	2.44	97
	12.5	1.479	12.286				
STD5	6.25	0.927	6.297	6.251	0.066	1.05	100
	6.25	0.916	6.205				
STD6	3.125	0.512	3.207	3.158	0.07	2.22	101
	3.125	0.497	3.108				
STD7	1.5625	0.275	1.692	1.649	0.062	3.75	106
	1.5625	0.261	1.605				
STD8	0.78125	0.14	0.842	0.836	0.009	1.09	107
	0.78125	0.138	0.829				
STD9	0.39063	0.069	0.363	0.352	0.015	4.37	90
	0.39063	0.066	0.341				
STD10	0.19531	0.034	<0.195	?????	?????	?????	NA
	0.19531	0.033	<0.195				

Table 5. Bet v 1 ELISA 2.0 EP performance characteristics

Standard range	100-0.19ng/mL
Detection range	50-0.39ng/mL
Sensitivity: LOD **LLOQ**	0.19ng/mL 0.39ng/mL
Recovery: Range **Average**	82-120% 100%
Intra-assay precision (CV): Range **Average**	1-9% 3%
Inter-assay precision (CV): Range **Average**	5-11% 7%

Conclusions

Based on data obtained from validation experiments and assay performance characteristics, InBio's Bet v 1 ELISA 2.0 EP is comparable to the Stallergenes method. Chapter 5.27 of the European Pharmacopoeia (Supplement 11.5), which provides guidelines for making a formal comparison of analytical procedure performance, was published seven years following the completion of this comparability study. However, the study was designed according to parameters outlined by the EDQM and PEI to compare InBio's Bet v 1 ELISA 2.0 EP to Stallergenes' Bet v 1 ELISA (performed exactly as prescribed for the BSP090 ring trial). Both methods use monoclonal antibodies 5B4 and 6H4, providing a consistent, sustained supply of critical reagents (an important factor noted in the BSP090 validation report).

Although an equivalence margin was not established prior to implementing this comparability study, both assays met pre-determined acceptance criteria for reproducibility, which was a CV of less than 30% for replicate measurements of Bet v 1 in commercial birch pollen extracts. Further assessment of InBio's Bet v 1 ELISA 2.0 EP included a measure of accuracy, which was determined from spike recovery tests performed by three operators at two laboratories using two different batches of pre-coated plates. Bet v 1 recovery ranged from 83 to 127%, with an average spike recovery of 109%.

The Stallergenes Bet v 1 assay showed slightly better sensitivity (0.31ng/mL compared to 0.39ng/mL), however, the sigmoidal calibration curve generated by ELISA 2.0 provided a detection range of 50 - 0.39ng/mL compared to 5 - 0.31ng/mL for the Stallergenes assay. In addition to the broader dynamic range, ELISA 2.0 offers several other advantages. ELISA 2.0 requires only water to prepare two buffer concentrates (all other reagents are ready to use), whereas the Stallergenes assay requires preparation of six different buffers and reagents, two of which contain toxic chemicals. Furthermore, the Stallergenes ELISA requires three days to prepare the antibody-coated plate and 4.5 hours to perform the assay. InBio's Bet v 1 ELISA 2.0 EP requires no advanced plate preparation, and the assay can be completed in 2-2.5 hours, providing a convenient method for quantifying Bet v 1 in birch pollen extracts and other allergenic products.

References

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