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Validation of ELISA methods for quantification of the major birch allergen Bet v 1 (BSP090)

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ABSTRACT

To date, the potency of allergen products in Europe is expressed in manufacturer-specific units relative to a product-specific in-house reference. Consequently, cross-product comparability of allergen products from different manufacturers with respect to strength and efficacy is impossible. The Biological Standardisation Programme (BSP) project BSP090 addresses this issue via the establishment of reference standards in conjunction with ELISA methods for the quantification of major allergens in allergen products. Since the initiation of BSP090, the recombinant major allergen Bet v 1 has been adopted by the European Pharmacopoeia Commission as a Chemical Reference Substance (CRS). In parallel, two sandwich ELISA systems for quantification of Bet v 1 were found suitable in preliminary phases of BSP090 to

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be validated in a large collaborative study. In this study, the candidate ELISA systems were compared with respect to accuracy, precision and variability. Thirteen participating laboratories tested model samples containing the CRS as well as spiked and unspiked birch pollen extracts. Both in pre-testing and in the collaborative study, the 2 candidate ELISA systems confirmed their suitability to quantify recombinant and native Bet v 1. As no clear-cut decision for one of the ELISA systems could be made based on the results of the collaborative study, a post-study testing was performed. Bet v 1 content of 30 birch pollen allergen products was determined in parallel in both ELISA systems. Consequently, 1 candidate ELISA system was selected to be proposed as the future European Pharmacopoeia standard method for Bet v 1 quantification.

KEYWORDS

Major allergen, Bet v 1, biological standardisation, collaborative study, Chemical Reference Substance, ELISA.

1. INTRODUCTION

In accordance with the European Pharmacopoeia (Ph. Eur.), allergen products have to be characterised with respect to their protein and allergen profile as well as total protein content and potency [1]. Potency may be determined as total allergenic activity, expressing the potential to bind allergen-specific immunoglobulin (Ig) E antibodies in a patient serum pool. Alternatively, potency can be expressed based on the content of relevant single allergens. Despite the implementation of these standardised requirements and the resulting adaptations in manufacturing of allergen products [2], the use of company-specific assays and potency units in combination with product-specific in-house reference preparations prevents cross-product comparability in Europe [3–5]. Hence, a clinician cannot decide for one or the other allergen product for a patient by comparing the content of the active ingredient. To explore this problem, the project ‘Development of Certified Reference Materials for Allergen Products and Validation of Methods for their Quantification’ (CREATE) had been initiated in 2001. CREATE was a European Union-funded multidisciplinary project, the aim of which was to establish the quantification of major allergens in allergen standardisation, together with appropriate reference materials and methods [6–8]. When CREATE ended in 2005, the Allergen Standardization Subcommittee of the International Union of Immunological Societies (IUIS) and the World Health Organization (WHO) decided to continue the work in a new project as part of the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines & HealthCare (EDQM). The aims of the BSP090 project are the establishment of reference standards for 2 major allergens, namely Bet v 1 from birch pollen and Phl p 5a from Timothy grass pollen [9]. In conjunction, reference ELISA methods for quantification of these allergens in natural allergen extracts and recombinant allergen products should be identified and, if possible, validated. The project has passed through 3 phases, including an initial feasibility evaluation (Phase I) as well as an extended feasibility study (Phase II) followed by an international collaborative study (Phase III). During Phases I and II, the recombinant (r) candidate reference standards have been thoroughly characterised using physico-chemical and biological tests [9–11]. Hereupon, the 2 Chemical Reference Substances (CRSs) rBet v 1 and rPhl p 5a were adopted by the Ph. Eur. Commission in 2012. Regarding the selection of the corresponding reference methods, 2 candidate Bet v 1 ELISA systems provided by ALK-Abelló A/S (Hørsholm, Denmark) and Stallergenes Greer (Antony, France) were found suitable in Phases I and II to be further examined with the aim of selecting the most appropriate system [9]. The validation of the 2 candidate ELISA systems for Bet v 1 quantification was performed between December 2010 and March 2011, including a pre-testing of samples and materials in the project leader’s laboratory (S. Vieths, Paul-Ehrlich-Institut (PEI), Langen, Germany). Activities directed towards the validation of a Phl p 5-specific ELISA system are still ongoing and will be reported separately in due course.

After completion of the subsequent collaborative study, a post-study testing was initiated to simulate the future use of the ELISA methods as Ph. Eur. standard methods. This parallel

assessment of Bet v 1 concentration in 30 birch pollen allergen products was likewise performed at the PEI. Here we present the results of the pre-testing, the collaborative study and the post-study testing. Note that the dataset presented in this publication is identical to the dataset underlying the publication by Kaul *et al.* (2016) [12]. The combination of all these results provided the basis for selection of a Bet v 1-specific ELISA system to be proposed as Ph. Eur. standard method.

2. PARTICIPANTS

Fourteen laboratories enrolled in the collaborative study, of which 13 submitted results (Table 1). Throughout this publication, laboratories are referred to by code numbers (1-13) allocated at random. Parallel testing of birch pollen allergen products was subsequently performed at the PEI.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Allergens and allergen products

Test samples and controls were prepared for the collaborative study (Table 2), pretested and provided to the participants as lyophilisates [12]. Unused test material was to be discarded by the participants after each assay. The controls as well as the reference standards were based on the rBet v 1 recombinant major allergen CRS (Y0001565) [9, 11]. For the controls, the re-suspended CRS was diluted to the target concentrations with formulation buffer and frozen at -20°C . Test samples were prepared by reconstitution of birch pollen extract (Allergopharma, Reinbek, Germany) and spiking with a known concentration of the corresponding rBet v 1 CRS. Samples were then frozen at -20°C . Test samples and controls were then lyophilised by Virion-Serion GmbH (Würzburg, Germany). All lyophilised samples, including the CRS for preparation of the standard curve, were to be re-suspended just before use by participants in ultra-pure, pyrogen-free water, while further dilutions were to be performed with the corresponding ELISA buffer.

For the post-study testing, 30 birch pollen allergen products including prick test solutions (PTS, $n=10$), products for sublingual immunotherapy (SLIT, $n=11$) and products for subcutaneous immunotherapy (SCIT, $n=9$) from 6 manufacturers were selected (ALK-Abelló Arzneimittel GmbH, Hamburg, Germany; Allergopharma, Reinbek, Germany; Bencard Allergie GmbH, München, Germany; HAL Allergie GmbH, Düsseldorf, Germany; Leti Pharma GmbH, Witten, Germany; Stallergenes Greer, Kamp-Lintfort, Germany) [12]. For chemically modified or adsorbed SCIT products, a native intermediate was assessed. For each product, total allergenic activity was determined as described elsewhere [13]. Each product was diluted to fit the measurement range of the respective system (ELISA-A: 0.1-6.6 ng/mL Bet v 1; ELISA-B: 0.2-10.0 ng/mL Bet v 1).

3.1.2. ELISA materials

The necessary assay reagents, including antibodies and pre-coated microtiter plates, were provided by two co-operation partners (ALK-Abelló A/S, Hørsholm, Denmark, ELISA-A; Stallergenes Greer, Antony, France, ELISA-B) to the EDQM for further distribution to the participants (Table 3). Streptavidin-peroxidase (POD) and enzyme substrates were provided by the EDQM. Like samples and controls, all specific reagents were pre-tested at the PEI. Participants of the collaborative study were provided with assay reagents, Streptavidin-POD and enzyme substrates sufficient for 5 plates per ELISA system. Commercially available reagents for preparation of buffers and working solutions were purchased by the participants (Table 4). The instruments used by the laboratories participating in the collaborative study were appropriately qualified or calibrated in advance.

3.2. Methods

3.2.1. ELISA systems

Similar to previous phases of BSP090, 2 Bet v 1 specific monoclonal antibody-based sandwich ELISAs (ALK-Abelló A/S, Hørsholm, Denmark, ELISA-A; Stallergenes Greer, Antony Cedex, France, ELISA-B) were included in the collaborative study [9, 12]. Each ELISA was performed according to the respective co-operation partners' protocol. The 2 ELISA systems remained unchanged compared to Phases I and II [9]. The 96-well microtiter plates were either provided pre-coated with allergen-specific antibodies (ELISA-A) or were freshly coated by the participants (ELISA-B). After blocking, serial dilutions of references, test samples and run controls were incubated in duplicates. Following detection and quantification of the colour reaction, the Bet v 1 concentration was determined by interpolation with the reference curve. General validity of the independent assays was assessed according to the ELISA-specific criteria defined based on pretesting results as well as according to generally accepted validity parameters such as a maximum coefficient of variation (CV) between duplicates of 20 % (Table 5).

3.2.2. Study design

During pretesting, the complete sample set (Table 2) was tested in 5 independent assays on different days by 2 operators at the PEI. In the collaborative study, the sample set was measured by 1 operator in the 2 ELISA systems at least 3 times independently on 3 different days. Participants were requested to comply with the plate layout provided with the study protocol. Samples and controls were tested in duplicates. During the post-study testing of birch pollen allergen products at the PEI, 2 operators performed at least 3 independent experiments on different days. Raw data were submitted to the EDQM for statistical analysis.

3.2.3. Statistical evaluation

Statistical analysis was performed as previously described [12]. In accordance with the general method described in Ph. Eur. general chapter 5.3 [14], raw data, i.e. optical density (OD) values, of reference curves, blanks, controls and test samples were submitted to 4-parameter logistic curve analysis using the CombiStats software [15]. When fitting the logistic curves through the respective data points, curves were only shifted horizontally to each other while slope and asymptotes of all preparations had to be identical. The respective equation used is:

$$y = d + (a - d)f(b(x - c_i)) + \varepsilon$$

where y = observed ODs; d = common lower asymptote; a = common upper asymptote; b = common slope factor; $x = \ln(\text{dose})$; c_i = points of inflexion of preparations; ε = error term with constant variance and expectation 0; f = logistic function defined by $f(x) = (1 + e^{-x})^{-1}$.

In the case of Laboratory 2, the applied model had to be reduced to 3 parameters because the number of data points submitted for ELISA-B were not sufficient to estimate the upper asymptote:

$$y = d + f(b(x - c_i)) + \varepsilon$$

where f = exponential function defined by $f(x) = e^x$; other parameters as described above.

The calculation of the relative allergen content in a sample was based on:

$$\exp(c_i - c_s)$$

where c_s = point of inflexion of the standard; c_i = point of inflexion of the respective test sample.

All results were calculated irrespective of the information provided in the ELISA-specific protocols [9, 12], but for sake of comparison, ELISA-specific calculations were performed using the SAS System [16].

The geometric mean (GM) of the assays per laboratory as well as the geometric coefficient of variation (gcv) was determined for each sample. The gcv was calculated based on:

$$gcv = \sqrt{(\exp(v) - 1)} \times 100\%$$

where v = sample variance of the log-transformed activities.

In addition, for the collaborative study, the overall GM and gcv were also determined and accuracy was calculated as previously described [9, 12]:

$$Recovery = \left[\frac{S - U}{T} \right] \times 100$$

where S = concentration of spiked sample; U = concentration of unspiked sample; T = theoretical spike concentration.

To allow an estimation of accuracy, empirical acceptance limits of 70 and 130 % around the theoretical spike concentration were defined. The respective calculations were not only performed for individual estimates but also for the overall GM values in order to estimate overall spike recovery. In addition, 2 precision levels were predefined, corresponding to 20 % and 30 % around the median of all estimates from all laboratories for a given sample. Based on the approximate standard deviation (SD) of the estimated value, intra-assay variability was defined as the median width of the confidence intervals expressed as percentage of the estimate divided by 4. The median gcv of the estimates within single laboratories was used to calculate inter-assay variability, while inter-laboratory variability was defined as the gcv of the GM within laboratories. The Student's t-test was used to assess statistical significance of differences between the two ELISA methods with regard to precision as well as estimates. Pearson's correlation coefficient (r) was determined using the software GraphPad Prism (La Jolla, USA; version 6.07).

4. RESULTS AND DISCUSSION

4.1. Pretesting of samples and run controls

Pre-tests were performed at the PEI to verify sample suitability and establish acceptance ranges for all controls to be included in the collaborative study. Both ELISA systems showed high consistency of standard curves in 5 independent tests (Figure 1 a/b) as well as satisfactory determination of the theoretical Bet v 1 content in all samples and controls (Figure 1 c/d). Only the concentration of Sample 1, containing 6 ng/mL Bet v 1 in birch pollen extract according to previous measurements, was underestimated by both systems. In contrast, 6 ng/mL rBet v 1 in Control 2 were accurately determined by both ELISA systems. Regarding the recovery of rBet v 1 in the spiked samples, ELISA-A and ELISA-B exhibited persistently good results close to 100 % recovery (Figure 1 e/f). Inter-assay variability was found to be adequately low in both systems with the exception of Sample 1 in ELISA-B, for which the predefined gcv limit of 15 % was surpassed slightly (Table 6). Overall, a good consistency of concentration estimates and spike recovery was observed throughout pretesting, confirming the positive results of the previous phases of BSP090. On this basis, acceptance criteria for the controls were defined for each ELISA system and included in the validity criteria for the assays (Table 5).

4.2. Phase III: Collaborative study

Overall, both ELISAs confirmed the positive results from preceding phases of BSP090 as summarised in the publication by Kaul *et al.* [12]. The collaborative study included 14 laboratories in Europe and the USA, involving Official Medicines Controls Laboratories (OMCLs), manufacturers and academic laboratories alike. All participants were provided with identical sample sets (Table 2). The results from 13 participating laboratories were evaluated, of which one (Laboratory 12) had to be regarded as a systematic outlier. For ELISA-B, all OD values in Laboratory 12 were at baseline and had thus to be excluded entirely from the evaluation. In comparison, assays performed with ELISA-A in Laboratory 12 could be evaluated, but results were excluded from the general evaluation due to the unusually high variability (Table 7). When Laboratory 12 was excluded, none of the assays performed in the course of the collaborative study with ELISA-A surpassed the limit for the correlation coefficient

or exceeded the predefined range of 80-125 % for the 95 % confidence interval (CI) of the estimated concentrations (Table 7). In comparison, 6 assays performed with ELISA-B did not comply with the predefined 95 % CI range, although this was limited to Control 1 in all cases (Table 8). Furthermore, 1 assay performed with ELISA-B in Laboratory 8 did not comply with the general validity criterion for the correlation coefficient of ≥ 0.995 .

4.2.1. Assay precision

To estimate assay precision, the median concentrations were calculated for samples and controls for each performed assay (Table 7 and 8). Subsequently the deviations from the median were checked for compliance with 2 precision levels of $< 20\%$ and $< 30\%$ deviation from median. For ELISA-A, 98.6 % of sample and control estimates were within 30 % around median and 92.7 % within 20 % (Table 7). For ELISA-B, precision was lower: 87.4 % of all estimates were within 30 % around median and 68.9 % within 20 % around median (Table 8). Thus, ELISA-A was found to be more precise in total Bet v 1 determination than ELISA-B.

Interestingly, comparison of single estimates as well as GMs determined by the 2 ELISA systems showed that values from ELISA-B tended to be higher than values from ELISA-A (Table 7, Table 8). Although especially the estimates of Sample 1 and 4 were markedly lower in ELISA-A, the trend could be observed for all samples and controls throughout the collaborative study.

4.2.2. Assay accuracy

Assay accuracy was assessed on the basis of samples containing rBet v 1 in defined concentrations. This included the 2 controls with a theoretical concentration of 2 and 6 ng/ml rBet v 1 on the one hand and the spiked samples containing 60, 20 and 5 ng/ml rBet v 1 on the other hand. In all cases, a range of $\pm 30\%$ around the theoretical rBet v 1 concentration was the predefined accuracy limit. For ELISA-A, good compliance with this range of maximum deviation could be observed (Figure 2a). Taking together control estimates and spike recovery results, only 6 single estimates did not meet this criterion. However, when comparing the overall GMs determined with ELISA-A with the theoretical rBet v 1 concentration in controls and spikes, it became apparent that the content was systematically underestimated. The situation was found to be reversed for ELISA-B (Figure 2b). Here the overall GMs and the theoretical rBet v 1 content were in very good agreement. In return, 32 single estimates exceeded the range of $\pm 30\%$ around the theoretical rBet v 1 content. It can therefore be concluded that ELISA-A was more precise in determination of rBet v 1 in controls and spiked samples, while ELISA-B gave more accurate results.

4.2.3. Assay variability

For each control and sample, the parameters intra-assay, inter-assay and inter-laboratory variability were assessed to estimate repeatability, intermediate precision and reproducibility (Table 9). The two ELISA systems showed comparably low intra-assay and inter-assay variability. All results were clearly below the limit of 15 % gcv. Inter-laboratory variability was likewise low for ELISA-A, while this parameter was found to be increased for ELISA-B for all controls and samples.

4.2.4. Comparison of calculation methods

Analysis of the data also encompassed the comparison of different calculation schemes [17]. The general method described in general chapter 5.3 of the Ph. Eur. was used as reference and results were calculated using CombiStats [15]. Especially in case of ELISA-B, this analysis was regarded as important, because the assay had been originally designed to be evaluated after logarithmic transformation. However, since the ultimate goal is to incorporate the method in a monograph, it is not desirable that method-dependent calculations need to be described. In order to demonstrate that the general model yields equally reliable outcomes for all methods, the concentrations were also calculated on the basis of the descriptions using the SAS System for each method according to protocols given by the manufacturers of the respective ELISA systems [16]. Importantly, concordance plots showed a very good agreement between the

general method and the collaboration partners' calculation methods for both ELISA systems (Figure 3).

4.3. Post-study testing of birch pollen allergen products

In the collaborative study, both ELISA systems proved their suitability to quantify Bet v 1 in a standardised set of samples. To gain more insight into performance of the assays under conditions mimicking the future use as Ph. Eur. standard method, a post-study testing was initiated. This encompassed the parallel measurement of 30 birch pollen allergen products from 6 different manufacturers in both ELISA systems. Products were selected to cover 3 allergen product types: PTS, SLIT products and SCIT products.

In accordance with the observations of the collaborative study, intra-assay variability of both ELISA systems was low (Table 10). However, inter-assay variability was higher for ELISA-A, with a maximum gcv of 39.6 % and gcv values > 20 % for several products from all assessed product groups. In comparison, the maximum gcv value determined for ELISA-B was 19.3 %. These results imply a superior intermediate precision of ELISA-B in a setting resembling the future use as Ph. Eur. standard method.

The trend of continuously higher Bet v 1 estimates of ELISA-B compared to ELISA-A persisted in the post-study testing (Figure 4). Only for 1 out of 30 tested products, the mean Bet v 1 content determined by ELISA-A was higher than the value determined by ELISA-B. Nearly half of the mean Bet v 1 estimates deviated significantly between the 2 ELISA systems, especially in products with high Bet v 1 concentration. Using antibody-based techniques it is not possible to quantify allergens in extracts in an absolute manner relative to a gold standard. Therefore, it cannot be determined which ELISA method is more accurate with respect to the total Bet v 1 content of a birch pollen allergen product. In search of a potential surrogate parameter, correlation of total allergenic activity of the tested products and Bet v 1 content determined by ELISA-A and ELISA-B was investigated (Figure 5). As expected, correlation between major allergen content and IgE binding capacity was strong, but no significant difference could be observed between the two ELISA methods. Eventually, analysis of the co-operation partner providing ELISA-A revealed that the monoclonal antibody used in this system has a lower dissociation constant for rBet v 1 in comparison to natural Bet v 1 (determined using Biolayer Interferometry, Octet Platform, Pall ForteBio Europe, Portsmouth, UK; unpublished). It is therefore likely that the Bet v 1 content of birch pollen extracts is underestimated in relation to a standard curve consisting of rBet v 1 in ELISA-A. As already outlined though, an absolute quantification of allergens is not the goal when using monoclonal antibody-based ELISA systems. Hence, underestimation *per se* is not to be regarded as an exclusion factor for a candidate Ph. Eur. standard method for allergen quantification.

As a consequence, further criteria had to be evaluated when deciding for 1 of the ELISA methods to be proposed as Ph. Eur. standard method. Understandably, long-term availability of all assay components is one of the main prerequisites for a future standard method. ELISA-B is based on 2 monoclonal antibodies, which can be produced in comparable quality from hybridoma cell lines. In contrast, ELISA-A utilises the combination of a monoclonal antibody and a polyclonal rabbit serum. Hence, long-term availability requires a new immunisation of rabbits and constant quality of the antiserum cannot be assured. This is not only a source of potential fluctuation, but also conflicts with the principles of the 3Rs (replacement, reduction and refinement of animal experimentation [18]). Thus, the design of ELISA-B was regarded to better ensure long-term availability of assay reagents while being also line with the requirements of Directive 2010/63/EC regarding the use of animals for scientific purposes.

5. CONCLUSION

Both Bet v 1-specific ELISAs included in Phase III of BSP090 could confirm the positive results from earlier phases of the project. Based on the results from pretesting and from the collaborative study, both systems could have been recommended to become the Ph. Eur. standard method. While ELISA-A was superior with respect to precision and reproducibility, ELISA-B exhibited superior accuracy. Post-study testing mimicking the future use as Ph. Eur.

standard method again confirmed the suitability of both methods, but also indicated superiority of ELISA-B with respect to intermediate precision in this setup. Further criteria were taken into account to select one of the methods, such as the long-term availability of assay material, which was regarded to be more critical for ELISA-A due to the use of a rabbit serum. All in all, the analysis of all available data from the different phases of BSP090 [9, 12], in combination with the considerations regarding long-term availability of ELISA components and 3R considerations, led to the selection of the ELISA-B to be proposed as the Ph. Eur. standard method. The process of incorporating the method as appropriate in the Ph. Eur. texts has been initiated.

6. ACKNOWLEDGEMENTS

The BSP090 project was organised in the framework of the Biological Standardisation Programme, a joint programme of the Council of Europe and the EU Commission. We thank all participating laboratories for their contribution and the technical staff for timely and reliable work at the bench. Grateful acknowledgements go to the companies ALK-Abelló A/S, Allergopharma and Stallergenes Greer for providing materials, SOPs and technical support throughout the project. Permission of the publisher of [12] was granted for reproduction of tables and figures.

7. PARTICIPANTS

For a list of participants in alphabetical order by country, see Table 1.

8. ABBREVIATIONS

BSP: Biological Standardisation Programme; Bet v: *Betula verrucosa* (silver birch); CREATE: Development of Certified Reference Materials for Allergen Products and Validation of Methods for their Quantification; CRS: Chemical Reference Substance; EDQM: European Directorate for the Quality of Medicines & HealthCare; gcv: geometric coefficient of variation; GM: geometric mean; IUIS: International Union of Immunological Societies; LCL: lower confidence limit; MAb: monoclonal antibody; Ph. Eur.: European Pharmacopoeia; Phl p: *Phleum pratense* (Timothy grass); OD: optical density; OD_{max}: maximum OD value; OPD: o-Phenylenediamine dihydrochloride; POD: Peroxidase; PTS: prick test solution; r: correlation coefficient; R²: coefficient of determination; rBet v 1: recombinant major allergen of *Betula verrucosa* pollen; rPhl p 5a: recombinant group 5 allergen of *Phleum pratense* pollen; SD: standard deviation; SCIT: subcutaneous immunotherapy; SLIT: sublingual immunotherapy; UCL: upper confidence limit; WHO: World Health Organization.

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ANNEX: TABLES AND FIGURES

Table 1 – *Participating laboratories in collaborative study (adapted from [12])*

Collaborator	Institute/company/agency	Country
E. Sandberg, L. Husager	Danish Medicines Agency	Denmark
J. Dayan-Kenigsberg, A. Bertocchi, G. Chaudemanche	Agence nationale de sécurité du médicament et des produits de santé (ANSM)	France
P. Moingeon, E. Nony, M. Hrabina	Stallergenes Greer	France
H. Fiebig, G. Reese, B. Weber	Allergopharma	Germany
M. Raulf, I. Sander	Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA)	Germany
T. Holzhauser	Paul-Ehrlich-Institut (PEI)	Germany
C. Pini, P. Iacovacci	Istituto Superiore di Sanità, (ISS CNCF)	Italy
J.A. Asturias, M.C. Arilla	Roxall Medicina España (formerly BIAL Industrial Farmacéutica)	Spain
D. Barber, A. Ledesma, M. Lombardero	ALK-Abelló S.A.	Spain
J.A. Carnés, M.T. Gallego	Laboratorios Leti S.L.	Spain

Collaborator	Institute/company/agency	Country
R. Van Ree, J. Akkerdaas	Academic Medical Centre	The Netherlands
M. Chapman, B. Smith	INDOOR Biotechnologies Inc.	United States of America
C. Valerio	Food and Drug Administration (FDA)	United States of America

Table 2 – Sample composition in collaborative study (reprint from [12])

Sample	rBet v 1 [ng/mL] ^a	Extract [ng/mL] ^b	Theoretical concentration [ng/mL]
Control 1	2	–	2
Control 2	6	–	6
Sample 1	–	6	6
Sample 2	60	6	66
Sample 3	20	6	26
Sample 4	5	6	11
rBet v 1 CRS	n/a	n/a	n/a

^a Concentration determined prior to lyophilisation based on amino acid analysis.

^b Concentration determined prior to lyophilisation as the mean of several measurements in both candidate ELISAs. CRS: Chemical Reference Substance.

Table 3 – ELISA-specific reagents used in the collaborative study

ELISA-A

Product	Manufacturer batch number	Concentration/content	Storage
Anti-Bet v 1 MAb pre-coated plates	3/10	–	+ 5 °C
Anti-Bet v 1 polyclonal rabbit serum	1/08	80 µL/vial	– 20 °C
Goat anti-rabbit IgG-POD	D00084897	80 µL/vial	+ 5 °C
OPD tablets (Dako)	00051439	20 tablets/vial	+ 5 °C

ELISA-B

Product	Manufacturer batch number	Concentration/content	Storage
Anti-Bet v 1 capture MAb 5B4	E7053	2.946 mg/mL	– 20 °C
Anti-Bet v 1-biotin detection MAb 6H4	E2010/066	0.472 mg/mL	+ 5 °C
Streptavidin-POD	126K6084	250 µg/mL	+ 5 °C
SigmaFast OPD tablets	019K8214	5 or 1 OPD/pack	+ 5 °C

Table 4 – Further reagents used in the collaborative study

Product	Supplier	Catalogue no.	Purity
Bovine serum albumin (BSA)	Sigma	A7030/A7888	analysis
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma	D5652	analysis
Dipotassium hydrogen phosphate	VWR	26931.263	analysis

Suppliers were recommended.

Reagents from alternative suppliers could be used provided the required grade of purity was ensured.

Product	Supplier	Catalogue no.	Purity
Disodium hydrogen phosphate dihydrate	Merck	106580	analysis
Ethylenediaminetetraacetic acid (EDTA)	VWR	20302.18	analysis
Hydrochloric acid 37 %	VWR	20252.29	analysis
Hydrogen peroxide	Merck	108597	analysis
Phosphate Buffered Saline (PBS)	Sigma	P4417	analysis
Sodium azide	Merck	106688	analysis
Sodium chloride	VWR	27810.295	analysis
Sodium dihydrogen phosphate monohydrate	Merck	106346	analysis
di-Sodium hydrogen phosphate dodecahydrate	Merck	106579	analysis
Sulfuric acid	Sigma	339741	–
Thiomersal	Sigma	T5125	–
Tween 20	VWR	28829.296	analysis
Ultra pure water	–	–	pyrogen-free
pH universal indicator pH 0-14	Merck	109535	–

Suppliers were recommended.
Reagents from alternative suppliers could be used provided the required grade of purity was ensured.

Table 5 – *Validity criteria for ELISA assays in collaborative study (adapted from [12])***General**

Parameter	Specification
Geometric coefficient of variation (gcv) between duplicates	< 20 %
Correlation coefficient (r)	≥ 0.995

ELISA-A

Parameter	Specification
Control 1 (Bet v 1 concentration)	1.3-2.4 ng/mL
Control 2 (Bet v 1 concentration)	3.9-7.3 ng/mL

ELISA-B

Parameter	Specification
Control 1 (Bet v 1 concentration)	1.4-2.6 ng/mL
Control 2 (Bet v 1 concentration)	4.5-8.3 ng/mL

Table 6 – *Inter-assay variability during pretesting*

Geometric coefficient of variation [%]	Control 1	Control 2	Sample 1	Sample 2	Sample 3	Sample 4
ELISA-A	7.0	8.7	4.1	10.2	4.6	7.2
ELISA-B	4.7	4.5	16.6	10.3	7.3	8.4

Table 7 – Estimated Bet v 1 concentrations for ELISA-A (adapted from [12])

Lab	Plate	Corr. r	Sample 1			Sample 2			Sample 3			Sample 4			Sample 5			Sample 6		
			Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL
1	1	0.998	4.3	93-107	66.2	94-106	28.0	94-106	9.9	94-107	2.1	91-110	5.8	93-108						
	2	0.999	3.6	90-111	59.2	93-108	23.1	92-108	8.5	92-109	1.9	86-116	5.5	90-111						
	3	0.999	4.0	97-103	64.7	97-103	24.2	97-103	9.1	97-103	1.9	96-104	5.5	96-104						
2	1	0.998	3.2	88-113	58.2	91-110	19.8	90-111	8.4	90-111	1.3	83-120	4.7	87-115						
	2	0.999	2.9	96-104	48.9	97-103	20.0	97-103	6.9	97-103	1.6	94-106	4.5	96-104						
	3	1.000	3.5	96-104	53.5	97-103	20.6	97-103	7.2	96-104	2.0	94-106	4.5	95-105						
3	1	0.998	3.7	96-104	59.8	97-104	21.7	97-104	8.5	96-104	1.7	96-104	6.3	94-106						
	2	0.999	3.6	96-105	59.6	96-105	21.1	96-105	8.7	96-105	1.8	95-106	4.7	94-106						
	3	0.998	3.1	95-105	59.8	95-105	20.5	95-105	8.3	95-105	1.7	94-106	5.0	93-108						
4	1	0.998	3.1	91-110	51.2	91-109	23.9	91-109	8.1	91-109	1.7	90-111	5.4	86-117						
	2	0.998	4.1	91-110	64.3	91-110	25.4	91-110	8.6	91-110	1.7	89-112	5.0	86-117						
	3	0.998	3.5	91-110	63.8	91-110	22.7	91-110	8.2	91-110	1.7	90-111	6.3	81-124						
5	1	0.997	2.3	88-113	52.0	90-111	22.1	91-110	6.7	91-110	1.6	85-117	3.8	88-113						
	2	1.000	3.7	96-105	55.7	96-104	21.4	96-104	7.5	96-105	2.0	94-106	5.8	95-106						
	3	1.000	3.7	96-105	55.7	96-104	21.4	96-104	7.5	96-105	2.0	94-106	5.8	95-106						
6	1	1.000	3.0	97-103	48.7	97-103	17.7	97-103	6.8	97-103	1.5	96-104	4.8	96-104						
	2	0.999	3.2	96-104	54.9	97-104	20.2	97-104	7.1	96-104	1.7	95-105	5.3	95-105						
	3	1.000	3.5	98-102	58.9	98-102	22.0	98-102	8.5	98-102	1.8	98-102	5.3	98-102						
7	1	0.998	3.7	95-105	55.4	95-105	24.4	95-105	8.2	93-107	1.8	94-107	6.1	92-109						
	2	0.998	4.0	95-105	55.8	95-105	23.1	95-105	8.9	95-105	1.7	94-106	6.4	91-110						
	3	0.998	3.8	95-106	56.0	95-106	23.1	95-106	8.7	95-106	1.7	93-107	6.3	88-113						
8	1	0.999	3.5	95-105	63.6	96-104	21.4	96-104	8.9	96-104	1.9	95-106	5.2	95-106						
	2	0.999	3.1	93-107	63.2	95-105	21.4	95-105	8.5	95-105	1.8	94-107	5.4	94-107						
	3	0.999	3.5	96-104	52.0	97-103	19.8	97-103	7.9	97-103	1.8	96-104	5.4	96-104						

Corr. = Correlation coefficient |r|, Est. = Estimated concentration, 95 % CL = 95 % Confidence Limit; expressed as percentage of the estimate, GM = geometric mean.

Correlation coefficients < 0.995 are printed in bold.

Cells coloured mid-blue indicate confidence intervals wider than 80 % to 125 % of the estimate.

Dark-blue cells and light-blue cells represent concentrations deviating > 30 % and > 20 % from the median respectively.

Lab	Plate	Corr. r	Sample 1			Sample 2			Sample 3			Sample 4			Sample 5			Sample 6		
			Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL
9	1	0.999	3.4	95-105	48.0	96-104	21.8	96-104	7.0	96-104	7.0	96-104	1.6	94-107	4.9	95-105				
	2	0.999	3.6	97-103	49.8	98-102	20.8	98-102	7.0	98-102	7.4	98-102	1.8	96-104	4.9	97-103				
	3	0.999	3.5	97-103	55.2	98-102	22.3	98-102	7.4	98-102	7.6	98-102	1.9	96-104	5.0	97-103				
10	1	0.999	3.4	96-104	55.8	97-103	21.5	97-103	7.6	97-103	7.6	97-103	1.8	94-106	5.1	96-104				
	2	0.999	3.7	96-104	61.7	97-103	22.4	97-103	8.1	97-103	8.1	97-103	1.8	95-106	4.9	96-104				
	3	0.998	3.1	93-107	56.3	94-106	19.6	94-106	7.3	94-106	7.3	94-106	1.7	91-110	4.9	93-107				
11	1	0.999	4.0	96-104	58.6	96-104	24.1	96-104	8.4	96-104	8.4	96-104	1.9	95-105	5.7	94-106				
	2	0.999	3.9	97-103	50.0	97-103	23.1	97-103	7.3	97-103	7.3	97-103	1.8	97-104	7.0	94-106				
	3	1.000	3.5	97-103	56.0	97-103	22.7	97-103	7.9	97-103	7.9	97-103	1.9	97-103	5.8	96-104				
12	1	0.984	1.5	77-129	15.9	77-129	8.0	78-128	2.9	78-128	2.9	78-128	0.5	65-150	0.4	58-167				
	2	0.997	2.3	88-113	42.0	90-111	14.2	90-111	5.5	90-111	5.5	90-111	1.2	85-117	3.6	88-114				
	3	0.998	2.5	97-103	41.6	97-103	15.1	97-103	5.2	97-103	5.2	97-103	1.1	96-104	3.2	97-103				
13	1	0.998	4.1	95-105	—	—	25.5	95-105	—	—	—	—	1.6	94-106	5.4	92-109				
	2	0.998	3.5	96-104	59.4	96-104	21.8	96-104	7.5	96-104	7.5	96-104	4.8	92-109	6.6	89-113				
	3	0.998	2.5	97-103	50.5	97-103	20.6	97-103	6.1	97-103	6.1	97-103	1.2	97-103	5.2	93-108				
4	0.998	3.2	97-103	54.9	97-103	21.9	97-103	7.5	97-103	7.5	97-103	1.3	96-104	5.2	92-108					
	GM [ng/mL]		3.5		56.4		22.0		7.9		1.8									

Corr. = Correlation coefficient |r|, Est. = Estimated concentration, 95% CL = 95% Confidence Limit; expressed as percentage of the estimate, GM = geometric mean.

Correlation coefficients < 0.995 are printed in bold.

Cells coloured mid-blue indicate confidence intervals wider than 80% to 125% of the estimate.

Dark-blue cells and light-blue cells represent concentrations deviating > 30% and > 20% from the median respectively.

Table 8 – Estimated Bet v 1 concentrations for ELISA-B (adapted from [12])

Lab	Plate	Corr. r	Sample 1			Sample 2			Sample 3			Sample 4			Sample 5			Sample 6		
			Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL
1	1	0.999	4.8	95-105	67.4	97-104	27.3	97-104	11.0	96-104	2.1	90-111	6.4	96-105						
	2	0.997	4.9	89-112	71.3	92-109	24.9	92-109	10.7	92-109	2.3	79-126	6.8	90-111						
	3	0.998	4.6	95-105	69.9	97-103	24.9	97-103	10.9	97-103	2.3	90-111	6.3	96-104						
2	1	0.998	5.9	96-104	72.5	97-103	28.4	96-104	10.2	98-102	2.8	90-111	5.9	96-104						
	2	0.995	6.5	94-106	51.0	92-108	24.7	92-109	9.9	96-104	1.9	75-131	6.9	94-106						
	3	0.999	5.8	97-103	62.9	97-103	27	97-103	9.9	98-102	1.9	90-111	5.8	97-103						
3	1	0.998	6.8	97-103	59.4	97-103	30.1	97-103	12.5	97-103	2.6	90-111	6.9	97-104						
	2	0.999	6.6	97-103	71.8	98-103	30.5	98-103	13.1	97-103	2.5	90-111	7.5	97-103						
	3	0.998	6.1	97-103	65.1	98-102	27.8	98-102	11.8	98-102	2.6	93-108	7.4	97-103						
4	1	0.998	4.8	95-106	64.9	96-104	24.4	96-104	9.8	96-104	2.0	83-120	4.6	94-106						
	2	0.999	5.7	94-106	66.3	96-105	30.6	96-105	11.5	96-105	2.2	80-125	5.8	94-107						
	3	0.998	4.8	94-106	64.7	96-104	26.7	96-104	9.2	96-104	2.1	84-119	5.9	95-105						
5	1	0.999	4.0	93-107	55.0	96-104	21.4	96-104	8.6	96-104	1.7	84-118	5.2	94-106						
	2	0.998	4.4	97-103	61.7	98-102	24.9	98-102	9.3	98-102	2.2	94-107	5.9	97-103						
	3	0.999	3.5	96-104	41.5	97-103	18.3	97-103	7.7	97-103	2.0	93-108	4.8	96-104						
6	1	0.999	4.8	98-102	64.0	99-101	24.6	99-101	10.3	99-101	2.4	97-103	6.9	98-102						
	2	0.999	5.1	98-102	57.3	99-101	24.1	99-101	10.7	99-101	2.6	97-103	7.4	98-102						
	3	0.999	5.0	99-102	61.5	99-101	24.4	99-101	11.0	99-101	2.7	98-102	7.5	98-102						
7	1	0.999	3.2	97-103	54.6	98-102	20.8	98-102	7.6	98-102	1.6	95-105	5.2	98-103						
	2	0.999	3.9	97-103	51.8	98-102	23.7	98-102	8.4	98-102	1.7	94-107	5.3	97-103						
	3	0.999	2.7	97-104	30.5	97-103	14.5	97-103	5.4	97-103	1.3	92-108	3.1	97-103						
8	1	0.997	6.3	93-107	52.6	94-107	23.7	94-107	10.1	94-107	2.2	77-130	6.3	93-108						
	2	0.998	6.3	95-105	46.5	96-104	23.9	96-104	9.3	96-104	2.1	81-123	5.9	95-105						
	3	0.993	6.1	87-114	22.1	91-110	23.4	91-110	10.4	90-111	1.6	51-195	5.3	85-117						

Corr. = Correlation coefficient |r|, Est. = Estimated concentration, 95 % CL = 95% Confidence Limit; expressed as percentage of the estimate, GM = geometric mean.

Correlation coefficients < 0.995 are printed in bold.

Cells coloured mid-blue indicate confidence intervals wider than 80 % to 125 % of the estimate.

Dark-blue cells and light-blue cells represent concentrations deviating > 30 % and > 20 % from the median respectively.

Lab	Plate	Corr. r	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
			Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL	Est. (ng/mL)	95% CL
9	1	0.999	10.1	94-106	71.2	95-105	36.4	95-105	18.0	95-105	2.3	72-138	7.7	93-108
	2	0.999	10.1	96-105	80.0	96-104	36.2	96-104	17.6	96-104	2.9	83-121	7.8	95-106
	3	0.999	9.2	96-104	73.7	97-104	36.4	97-104	15.7	96-104	3.0	87-115	7.8	95-105
10	1	0.999	3.7	95-105	59.3	95-105	21.6	95-105	8.8	95-105	2.1	93-107	5.7	93-108
	2	0.998	3.8	91-110	58.5	93-108	23.6	93-108	8.8	92-108	2.4	87-115	6.2	91-111
	3	0.995	4.3	87-115	63.1	90-112	26.4	90-112	10.3	90-112	1.8	69-143	4.8	87-115
11	1	0.997	6.0	89-112	71.3	90-112	31.7	90-112	12.3	90-112	2.2	81-122	6.6	88-113
	2	0.999	4.9	96-104	71.5	97-104	32.7	97-104	13.2	97-104	2.0	90-111	5.6	96-104
	3	0.998	4.5	93-107	44.6	94-106	24.7	94-106	6.9	94-106	1.6	86-116	4.7	93-107
13	1	0.999	5.4	96-104	70.4	97-103	28.4	97-103	11.4	97-103	2.6	93-107	7.0	97-104
	2	0.998	5.7	95-105	43.5	97-103	30.1	97-103	13.6	96-104	3.0	92-109	8.4	96-104
	3	0.998	5.5	94-106	65.0	96-104	25	96-104	11.1	96-104	2.8	90-112	7.4	95-105
	4	0.997	5.9	93-107	68.7	95- 105	30.8	95-105	12.4	95-105	2.9	88-113	7.2	94-106
	GM [ng/mL]		5.2		58.6		26.0		10.5		2.2		6.2	

Corr. = Correlation coefficient |r|, Est. = Estimated concentration, 95 % CL = 95 % Confidence Limit; expressed as percentage of the estimate, GM = geometric mean.

Correlation coefficients < 0.995 are printed in bold.

Cells coloured mid-blue indicate confidence intervals wider than 80 % to 125 % of the estimate.

Dark-blue cells and light-blue cells represent concentrations deviating > 30 % and > 20 % from the median respectively.

Table 9 – Variability parameters during collaborative study (adapted from [12])

	gcv [%]	Sample 1	Sample 2	Sample 3	Sample 4	Control 1	Control 2
ELISA-A	intra-assay	2.3	2.0	2.1	2.0	2.9	3.0
	inter-assay	8.6	7.7	4.0	6.4	5.1	4.6
	inter-laboratory	7.8	6.1	7.2	8.6	5.7	9.1
ELISA-B	intra-assay	2.3	1.8	1.8	1.8	5.4	2.3
	inter-assay	6.2	13.6	8.3	8.2	12.9	8.9
	inter-laboratory	29.3	19.4	16.7	21.9	18.0	17.4

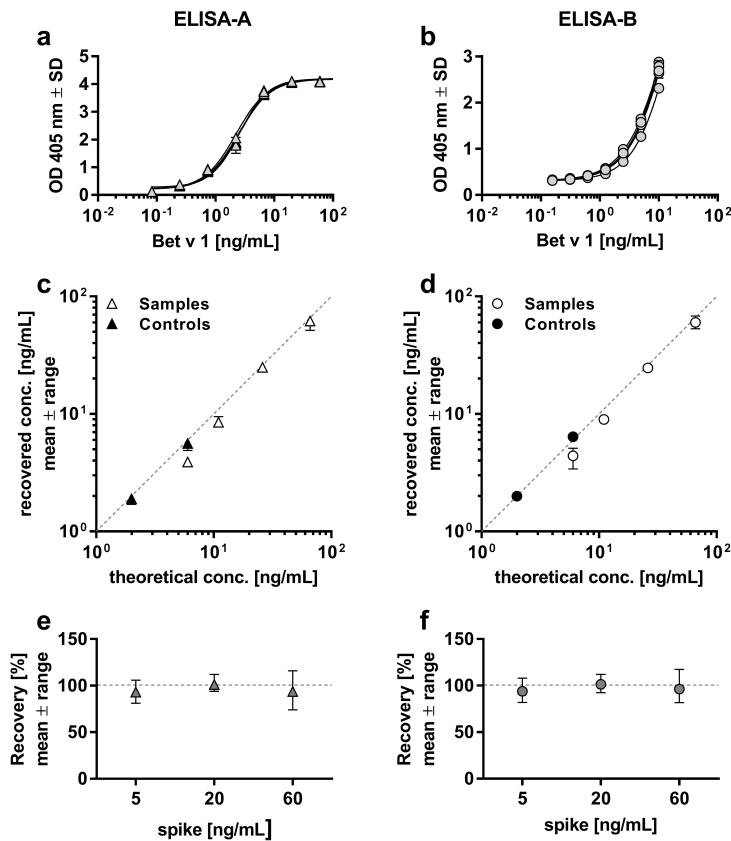
gcv = geometric coefficient of variation

Table 10 – Variability parameters during post-study testing (adapted from [12])

gcv [%] (min – max)	intra-assay	inter-assay
ELISA-A	2.2	12.0
	(1.43 – 5.00)	(3.81 – 39.64)
ELISA-B	1.7	8.2
	(0.92 – 7.10)	(2.39 – 19.29)

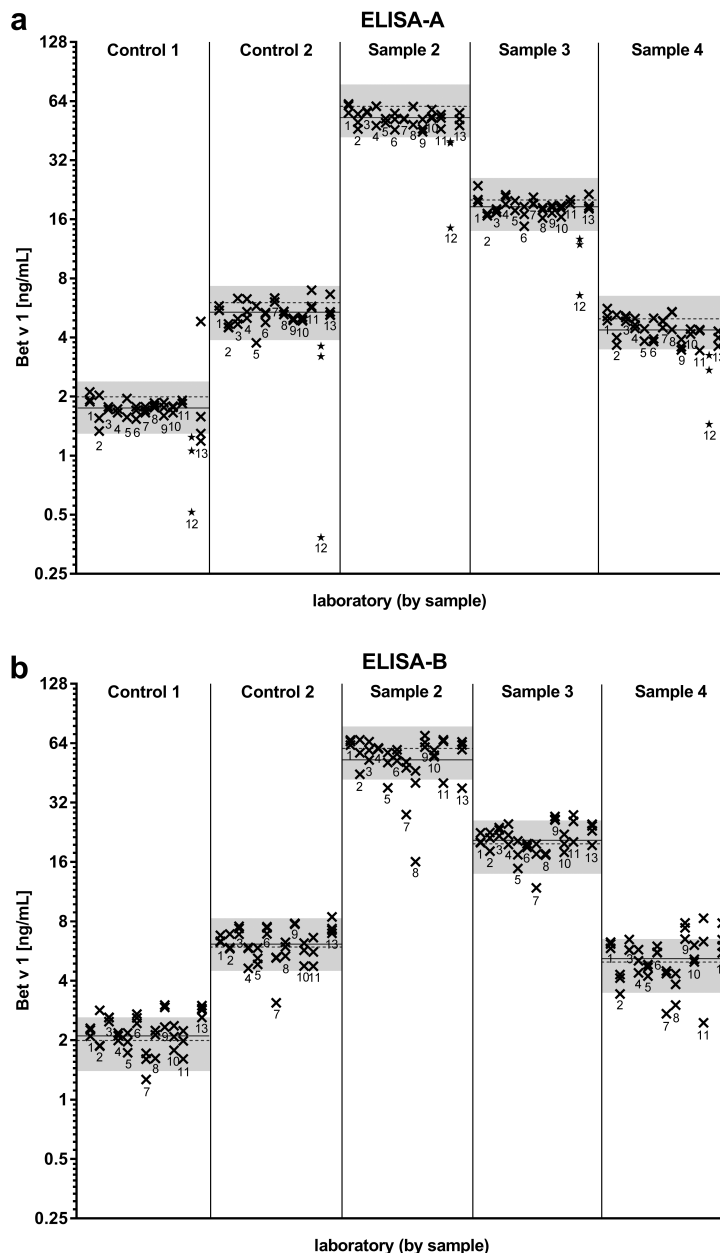
gcv = geometric coefficient of variation

Figure 1 – Pretesting of samples and run controls for collaborative study



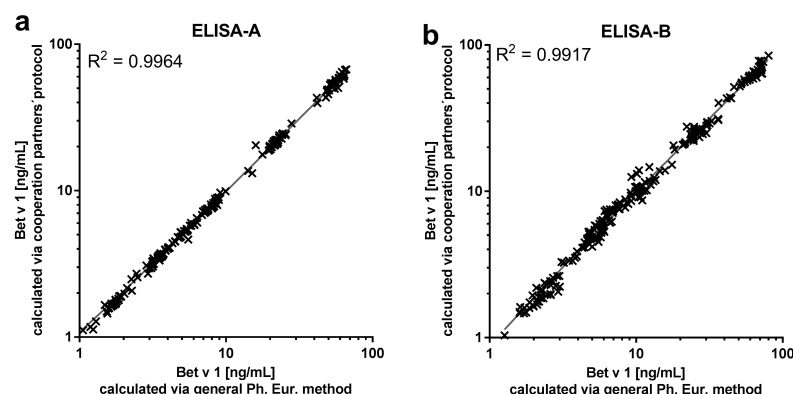
Four samples and 2 controls with predefined allergen concentrations were measured in 5 independent tests in the 2 ELISA systems (a/c/e ELISA-A b/d/f ELISA-B). a/b) For each ELISA, the standard curves of all assays performed during pretesting were superimposed. c/d) For all samples and controls, the mean detected allergen concentrations were compared to the theoretical allergen concentration in a two-way plot. e/f) In addition, the mean recovery of the recombinant spikes was calculated for the 3 spiked samples for each ELISA.

Figure 2 – Accuracy of Bet v 1-specific ELISAs in collaborative study (adapted from [12])



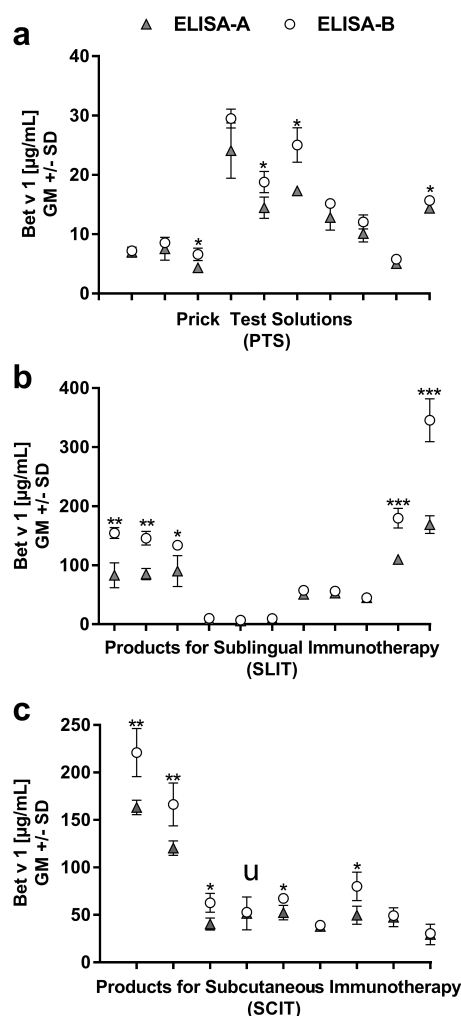
The distribution of Bet v 1 estimates is presented for **a)** ELISA-A and **b)** ELISA-B, grouped by laboratories, numbered 1-13. All Bet v 1 estimates of the 2 controls and the 3 spike estimates are depicted as crosses, only results obtained with ELISA-A in laboratory 12 are shown as stars. Solid lines represent the respective geometric mean, while dashed lines mark the theoretical rBet v 1 concentration in the controls and spikes respectively. The grey boxes indicate the pre-defined range of 70-130 % variation around the respective theoretical content.

Figure 3 – *Comparison of calculation methods for Bet v 1 determination (adapted from [17])*



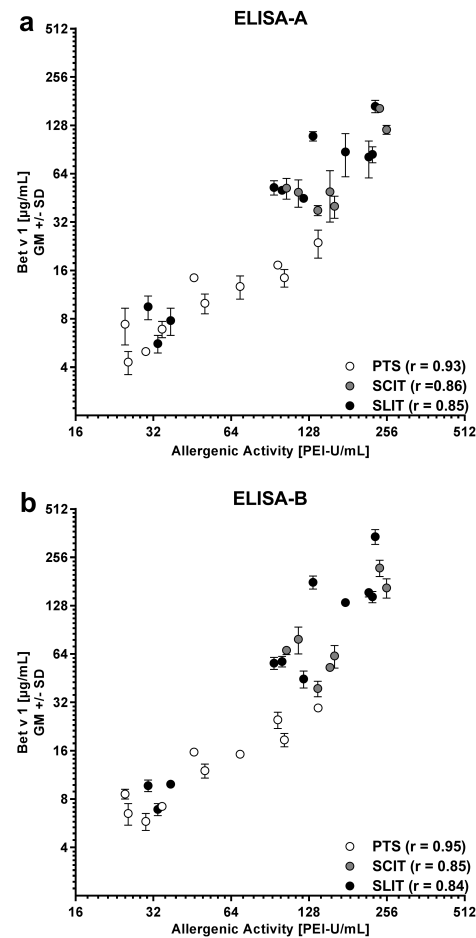
The complete set of raw data obtained during the collaborative study was analysed either via the general method laid down in the Ph. Eur. or by the ELISA-specific protocols of the collaboration partners providing **a)** ELISA-A and **b)** ELISA-B. For the resulting linear regressions, the coefficient of determination (R^2) is given.

Figure 4 – *Parallel assessment of Bet v 1 content in birch pollen allergen products in candidate ELISA systems (adapted from [12])*



In a post-study testing, Bet v 1 content was determined in **a)** 10 prick test solutions (PTS), **b)** 11 products for sublingual immunotherapy (SLIT) and **c)** 9 products for subcutaneous immunotherapy (SCIT). The geometric mean (GM) is depicted with the standard deviation (SD) of at least 3 independent experiments. Significant differences between the estimates of ELISA-A and ELISA-B are marked as * $p \geq 0.05$, ** $p \geq 0.01$, *** $p \geq 0.001$ based on Student's T-Test.

Figure 5 – Correlation of Bet v 1 content and total allergenic activity of birch pollen allergen products (reprint from [12])



The total allergenic activity (determined in official batch release) was plotted against the Bet v 1 content of each birch pollen allergen product as determined in **a)** ELISA-A and **b)** ELISA-B respectively. Results for prick test solutions (PTS, n = 10) are presented in white, products for subcutaneous immunotherapy (SCIT, n = 9) in grey and products for sublingual immunotherapy (SLIT, n = 11) in black. The correlation coefficient (Pearson's r) was calculated for each product group.