Nanofluidic point-of-care IgE test for subtropical grass pollen for rapid diagnosis of allergic rhinitis

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Introduction

Chronic allergic diseases affect as many of 500 million people across the globe and grass pollens (GP) are a major outdoor allergen trigger. Whilst subtropical zones are widening[1], and climate change may be increasing pollen allergenicity[2] and GP exposure[3], most of the allergen components, or allergen extracts, available to support clinical diagnosis of allergic respiratory diseases, including allergic rhinoconjunctivitis (AR) and asthma, are sourced from temperate regions. Allergen components offer more precise diagnosis and personalized management of allergic conditions[4], but a bias towards temperate allergen sources may affect accurate allergy diagnosis in subtropical regions closer to the equator.

The composition of allergens of subtropical GP differ from those of temperate GP[5-7], and species-specific immune recognition of subtropical GP allergens may depend on biogeographical region of origin[8, 9]. Subtropical Panicoideae Bahia (*Paspalum notatum*) GP were the most frequently recognized allergen in Texas[10], and lacked cross-reactivity with the temperate Pooideae Timothy (*Phleum pratense*) GP in nasal challenges studies with AR patients in Florida[11]. Moreover, only one of three immunodominant T cell epitopes of the major subtropical GP allergens; Pas n 1 of Bahia GP[12], and Cyn d 1[13] of the Chloridoideae Bermuda (*Cynodon dactylon*) GP, is shared with major temperate GP allergens; Lol p 1 from Ryegrass (*Lolium perenne*) pollen[14] and Phl p 1 from Timothy GP[15]. Regional variation in specific IgE profiles with allergen components[8, 16, 17] indicates a need for targeted allergen component resolved diagnostics.

In a high throughput assay using streptavidin ImmunoCAP, biotinylated natural (n) Pas n 1 showed high sensitivity and specificity in Australian patients with AR patients sensitized to Bahia GP[18]. However, 8% of control patients with allergies other than GP also showed IgE reactivity with natural Pas n 1, suggesting that cross-reactivity with cross reactive

carbohydrate determinants (CCD) contributed to false positive reactions[18]. Allergen extracts and natural allergen components both present issues with CCD, batch to batch consistency, and unspecified non-allergenic material[19, 20]. In unselected allergy patients from the Philippines, without clinically confirmed history of GP allergy, serum IgE reactivity with nCyn d 1 could be inhibited competitively by nPhl p 4, and was abolished by deglycosylation of the allergen, indicating CCD contributed to IgE binding with natural Cyn d 1[21].

Despite the increasing prevalence of allergic diseases in our society[22, 23] and the global burden on our healthcare systems[24], there remains suboptimal access to allergy diagnosis. This is particularly problematic for the management of AR patients with a risk of suboptimal self-medication, leading to poor disease control and worsening of comorbidities including asthma[25]. Decentralized testing may expand the reach of allergy diagnosis, particularly in rural and remote areas with limited access to specialist medical care[26].

Targeted point-of-care (POC) allergen arrays offer more accessible diagnostics options including clinical decision support[27, 28]. Semi-quantitative lateral flow technologies have been trialled for allergy diagnostics, none are routinely used clinically[29, 30]. Recent advancements in nanotechnologies underpinned development of immunoglobulin E (IgE) serologic assays[31, 32], allowing for high specificity for the test allergen using small quantities of allergens, blood and reagents[32]. POC nanofluidic IgE immunoassay for a small array of aeroallergens including Timothy allergen Phl p 5 showed good analytical agreement with a high-throughput pathology laboratory assay (PLA)[33].

The goal of this research was to evaluate the analytical and diagnostic performances of recombinant (r) Pas n 1 and Cyn d 1 as novel target allergen components for testing of serum specific (sp) IgE with a medium-scale cohort of well-characterized participants previously recruited for a clinical GP allergy survey using this POC benchtop nanofluidic device[33].

Methods

Grass Pollen Allergy Survey cohort

Participants recruited for the multicenter Grass Pollen Allergy Survey (GPAS)[8] with informed consent (Metro South Health HREC/2009/QPAH/296). Sera were available from non-atopic healthy control donors (n=23), other allergy control participants (n=49) with asthma or allergic sensitization other than GP (frequently house dust mite (HDM); 63%, or cat dander; 37%), and patients with a confirmed history of AR and sensitization to GP based on skin prick testing (SPT) (n=139). Participant clinical history of AR and asthma and location of origin were collected by the treating clinical immunologist. Sensitization to four GP extracts of interest; *P. notatum* (g2), *C. dactylon* (g10), *Sorghum halepense* (g17) and *L. perenne* (g5) pollen extracts as well as HDM, cat dander, *Alternaria* and *Aspergillus* mold spores; Hollister Steir, Spokane, WA, United States of America) were assessed according to national guidelines[34]. Serum concentrations of tIgE and spIgE to these same GP (g2, g10, g17 and g5) were measured by routine PLA (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden) by Sullivan Nicolaides Pathology, Queensland, Australia. Serum spIgE concentrations with biotinylated natural purified allergen components including nPas n 1[18] and nCyn d 1 (unpublished), were measured with streptavidin ImmunoCAPs on a ImmunoCAP 100 (Sullivan Nicolaides Pathology, Queensland, Australia).

Subtropical GP allergens rPas n 1 and rCyn d 1 were produced and purified as described[35]. In brief, sequences of Pas n 1.0101 and Cyn d 1.0203 were constructed in pET28a(+) vector to enhance solubility and yield of protein expressed with an amino (N)-terminal hexahistidine tag in BL21-DE3(RIPL) *E. coli*[36, 37]. The use of these modified allergens for immunodiagnosis was filed in a provisional patent application lodged by the Australian Patent Office (2022903711). Recombinant (r) proteins extracted in urea from inclusion

bodies were purified by nickel affinity chromatography under denaturing conditions and refolded with reducing-oxidizing conditions to maximize recovery in phosphate buffered saline (PBS)[38, 39]. After size exclusion chromatography[40], mass spectrometry and circular dichroism (CD) spectroscopy were performed to analyse observed protein secondary structure similarity between recombinant and natural allergens (Jasco J-1500 spectropolarimeter, Jasco, Hachioji, Tokyo, Japan). Protein secondary structure content of recombinant allergens was predicted from observed CD spectra and compared with the primary sequence, and knowledge of the structure of orthologous maize allergen Zea m 1 using SOMSpec and SOM-SSNN with concentration optimisation[41, 42]. Immunoreactivity of rPas n1 and rCyn d 1 was assessed with custom allergen-specific monoclonal (Cyn d 1; 6C6[6], and Pas n 1; mAb-RB2), and serum IgE of 12 non-atopic and 28 GP-allergic patients, were compared with natural purified allergen coated at 1 µg/ml by ELISA[35].

Preparation of nanofluidic IgE assay and measurement procedure

Other proteins including rPhl p 1, rPhl p 5 and the capture antibody for the tIgE assay were purchased from commercial sources). The total protein concentration was standardized by bicinchroninic acid assay (Thermo Fisher Scientific). Native proteins were stored below –15 °C with monitoring of freeze-and-thaw cycles. rCyn d 1 and rPas n 1 were compared with their natural purified allergen form[18] for spIgE reactivity using sera or plasma from GP sensitized (n = 10) and non-allergic (n = 8, per allergen) patients by enzyme-linked immunosorbent assay (ELISA) in 96 well microtiter plates. The spIgE concentrations were extrapolated from a standard curve made with preparations of total (t) IgE.

Capture molecules labelled with biotin were then coated onto the sensing surface present in the biofunctionalized nanosensors (biosensors) for the specific binding of IgE[32]. The biofunctionalization process of the sensor was optimized for each allergen and antibody

component (data not shown). Two lots of rPas n 1 and rCyn d 1 were prepared and dispatched from the academic laboratory to the industry laboratory and performance of labelled allergen batches were evaluated.

Dose-response calibration curves

Each lot of tests was calibrated using calibrators directly traceable to the 3rd International Reference Preparation (11/234) of Human Serum Immunoglobulin E from the World Health Organisation[43] using a weighted 4-parameter logistic curve.

Testing procedure

Sera were incubated for five minutes with a detection reagent containing a fluorescently labeled anti-human IgE antibody in a Tris-buffered saline solution at pH 7.4 supplemented with Tween-20% at 1% (v/v) and ProClin300 at 0.04% (v/v). The sample reaction mixture was loaded onto the capsule of the kit and drawn through the biosensor by capillary action. The serum spIgE-detector antibody complex bound the allergen components coated on the biosensor's read-out surface area.

The capsule contained biosensors individually prepared with rCyn, d 1, rPas n 1, rPhl p 1 and rPhl p5 in triplicate, as well as two biosensors with an anti-IgE antibody (tIgE assay) for measurement of the tIgE.

The device automatically processed the measured relative fluorescence in kU/L using information embedded within the capsule chip [32]. A region of interest (ROI) along the nanochannel was tailored for each test and consisted of the capture surface zone where the signal changes were the highest according to the nanofluidic principles.

Analytical performances characterization

Test performances and specificity of biosensors coated with rCyn d 1 and rPas n 1 were verified by a competitive inhibition study using two pools of human plasma or sera with known sensitization to Bermuda GP (nCyn d 1; g216) or Bahia GP (g7). Each sample pool was in the class 4 range[44] measured by ImmunoCAP. Each sample pool was incubated for 25°C for 4h with increasing molar excesses up to 128× of capture reagents (rCyn d 1 or rPas n 1) over the sIgE analytes for each test (n = 12, per condition). The sample was mixed in a 1:1 volumetric ratio with a detection reagent and analyzed by fluorescence microscopy. The signal was calculated using the mean signal per biosensor in relative fluorescence unit (RFU) of five ROI. The signal-to-baseline ratio was verified using an IgE-low plasma (n=5).

Assay linearity

The assay linear ranges of the rCyn d 1 and rPas n 1 tests were assessed on the prototype device through a dilution-recovery study using a pool of human plasma or sera of patients sensitized to Bermuda (nCyn d 1; g216) or Bahia GPs (g7).

Sample pools were serially diluted with allergen-spIgE negative plasma (< 0.1 kU_A/L, as per ImmunoCAP). Individual samples spanned the higher analytical range of interest for the tests. The mean intensity in RFU of the ROI using at least 3 replicates per dose. The average of triplicate measurements of each tested concentration was compared to the concentration expected by the serial dilution.

Repeatability

Panels of sera pools; non-atopic and non-GP allergic; and a high, moderate, and low GP allergic sera, were repeatedly tested in the device weekly. The inter-assay coefficient of variation (CoV; standard deviation/mean) for five repeat tests was determined.

Stability upon transportation

A subset of 22 GPAS sera sent to the industry laboratory in Switzerland were tested in the POC device with capsules loaded with rPas n 1 and rPhl p 1 biosensors, and with the t IgE test. The same set of GPAS sera was tested with another of the POC device in the academic research laboratory in Australia with the same capsule batch.

Inter-method agreement

The agreement of test results between the nanofluidic POC prototype with the reference PLA was evaluated in duplicates using 10 negative samples and 15 or 11 positive samples for the rCyn d 1 and rPas n 1 test, respectively. Each replicate was considered as a test result. The inter-method agreement was evaluated using the positive (PPA), negative (NPA) and overall percentage agreement (OPA).

Statistics and data analysis

The signal processing algorithm of the POC device [31] included control checks that invalidate results when a technical issue occurred. Data verification were performed in post-processing and the number of invalidated test results was recorded. If the CoV between triplicates was over 29%, then the outlier value was excluded from analysis of the remaining two replicates.

The threshold for positivity on the nanofluidic POC device was set at 0.7 kU_A/L[33]. As the cohort data distributions were not normal (Kolmogorov Smirnoff test), correlations were analysed using Spearman's ranked sum. The 95% confidence intervals for simpler linear regressions were modelled. The Kruskal-Wallis assay with a Dunn's correction for multiple comparisons was used to analyse differences between multiple groups. The PPA and NPA of classes between the device and spIgE to Bahia GP (g17) and Bermuda GP (g2) extract, and custom spIgE to biotinylated purified natural allergens; nPas n 1 and nCyn d 1 measured by streptavidin-ImmunoCAP, and SPT results for pollen extracts. Cohan's Kappa nominal values were used to evaluate overall agreements[45].

Results

Newly generated and purified rCyn d 1 and rPas n 1 displayed similar CD spectra to the corresponding purified natural allergens by ELISA (eFigure 1A), and the predicted secondary structure (eFigure 1B). Polyclonal rabbit anti-nPas n 1 serum showed reactivity to both recombinant allergens, as well as their corresponding natural allergen. The mAb 6/C6 generated against Bermuda GP reacted with rCyn d 1 and nCyn d 1 of Bermuda GP, while mAb RB2 specific to natural Pas n 1, showed reactivity to rPas n 1 and nPas n 1 of Bahia GP (eFigure 1C). In the academic laboratory, serum specific IgE reactivity with rCyn d 1 and rPas n 1 were highly correlated for a panel of AR and healthy donor sera with natural purified allergens with IgE to nCyn d 1($r = 0.972$) and nPas n 1 ($r = 0.986$), respectively (eFigure 1D). By mass spectrometry, peptides encompassing coverage of 86% and 88% respectively of the sequence of rCyn d 1 and rPas n 1 were detected, verifying composition of the recombinant allergens (eFigure 2).

Assay development and verification

The nanofluidic POC assay included subtropical and temperate GP allergens coated onto biosensors in the test capsule (Table 1). The recombinant allergens also showed good correlation by the industry laboratory for spIgE reactivity with nPas n 1 and nCyn d 1 by ELISA (eFigure 3). The coated biosensors showed a dose-dependent inhibition of the spIgE signal with increasing molar excess of capture reagents reaching a signal inhibition of 81% and 76% for the highest molar excess tested for rCyn d 1 and rPas n 1, respectively, confirming the binding to rCyn d 1 and rPas n 1-specific IgE (data not shown). Two batches of the recombinant rCyn d 1 and rPas n 1 showed comparable performances in biosensors following labelling with biotin (eFigure 4).

Calibration curve models demonstrated relative error and within-device imprecision <15% in RFU for all doses within the assay range 0.7 to 61.9 for the rCyn d 1 test and 1.0 to 87.0 except for the calibrator serum pool at 41.3 kU_A/L for the rPas n 1 test (Figure 1). The assay linearity range was 0.5–61.9 and 1.2–83.8 kU_A/l for the rCyn d 1 and for the rPas n 1 spIgE tests on the device, respectively (Figure 1). Linear regression analysis of the relation between expected and measured concentrations of rCyn d 1 and rPas n 1 spIgE showed correlations of R^2=0.96 ($p<0.0001$) and R^2=0.98 ($p<0.0001$), respectively.

Stability and repeatability in academic laboratory

Following assay development, IgE concentrations for rPas n 1 and tIgE from twenty-two sera were measured by the POC device in the industry development laboratory, and then again after transportation on another device at the academic research laboratory. Correlations for rPas n 1 spIgE (r=0.695, P< 0.0005), and tIgE (r=0.945, $p<0.0001$; Figure 2A) were highly significant. However, the slope for rPas n 1 spIgE and tIgE were 2.258 and 1.539, respectively, indicating that IgE reactivity was higher when measured on the device in the industry laboratory. Out of the 44 test results, one was invalidated due to a technical issue.

The CoV for multiple occasions testing over five weeks for specific rPas n 1 IgE showed an average CoV between five samples of 10%, which was similar to the assay for spIgE to rPhl p 1 (11%) (Figure 2B).

Correlations between the point-of-care device with GPAS cohort

For the cohort of 208 participants, correlation between serum rCyn d 1 and rPas n 1 spIgE on the POC with custom biotinylated natural allergen spIgE and whole GP spIgE on the PLA, as well as pollen extract SPT diameters were determined (Figure 3). The correlations between spIgE to rCyn d 1 (r=0.594; CI 0. 0.491 – 0.680) and rPas n 1 (r=0.719; CI 0.639 – 0.783), with biotinylated nCyn d 1 and nPas n 1 spIgE respectively, were highly significant

($p<0.0001$). Similarly, correlations between spIgE to rCyn d 1 (r=0.549; CI 0.441– 0.641) and rPas n 1 (r=0.679; CI 0.594 - 0.749), with Bermuda and Bahia GP spIgE respectively, were highly significant ($p<0.0001$). The correlations between spIgE to rCyn d 1 (r=0.384; CI 0.217 to 0.467) and rPas n 1 (r=0.549; CI 0.441 to 0.641), with Bermuda and Bahia GP SPT diameters were lower but still highly significant. tIgE measured by the nanofluidic device was highly correlated with serum tIgE measured by PLA (r=0.882; CI 0.846 to 0.910; p <0.0001).

There was no difference in tIgE concentrations between the AR patients with other allergies (n=49) compared to GP allergies (n=134), but both of these groups showed high tIgE compared with non-atopic participants (n=23) in the POC device (Figure 4A). There was no difference between the POC tIgE test and the PLA (Figure 4B). Higher rCyn d 1 and rPas n 1 spIgE were detected in the GP allergic patients with clinician diagnosed AR than participants with other or no allergies when analysing RFU values (Figure 4A). When analysed in spIgE units, the median rPas n 1 spIgE in GP allergic AR patients was significantly lower than Bahia GP spIgE and biotinylated nPas n 1 IgE, whilst rPas n 1 spIgE was significantly higher than in participants with no allergy or other allergies (Figure 4B). Similarly, when analysed in spIgE units, the median spIgE in GP allergic AR patients with rCyn d 1 was lower than with nCyn d 1. However, whilst rCyn d 1 spIgE was higher than non-allergic donors, there was no difference detected between GP allergic patients and those with other allergies.

Contingency plots for the spIgE classes for the recombinant allergens in comparison to GP spIgE and biotinylated natural allergen, showed the concordance between the POC and PLA (Figure 5). The PPA and NPA of spIgE classes for rPas n 1 with Bahia GP spIgE was 73% and 82.5%, respectively, and with biotinylated nPas n 1 the PPA and NPA were 71.6% and 81%, respectively (Table 2). For rCyn d 1 spIgE classes the PPA and NPA with Bermuda GP spIgE were 67.8% and 66.3%, and PPA and NPA with biotinylated nCyn d 1 were 66.7% and

67.5%. These agreements between the POC device measurements and PLA were substantial for rCyn d 1 and good for rPas n 1. Receiver operator plots showed that the area under the curves were similar for rCyn 1 and rPas n 1 with 0.777 and 0.772 ($p<0.0001$), respectively (data not shown).

Across 249 capsules including any repeated assays with five tests each, a total of 97 biosensor errors out of 3486 biosensors (2.8%) occurred, which invalidated outcomes of 28 tests out of 1245 results (2.2%). Instances when one triplicate biosensor lay outside of acceptable limits of variation occurred on 7 (2.8%), 15 (6.0%), 35 (14.1%), and 3 (1.2%) of tests for the allergens rCyn d 1, rPas n 1, rPhl p 1, and rPhl p 5, respectively. The average time taken for the device to read a capsule with the 5 tests was 17 minutes.

Discussion

This study evaluated the ability of a nanofluidic rapid POC IgE assay to identify allergic sensitization to major pollen allergens of subtropical grasses. Availability of an accessible POC test designed for diagnosis of AR based on subtropical allergens is likely to become increasingly important as GP allergen exposure in subtropical regions of the world is likely to increase[1, 3]. For instance, in the subtropical region of this study, the magnitude of GP seasonal index and number of high and extreme GP exposure days, have substantially increased in recent environmental monitoring period 2016-2020 compared with 1994–1999[46].

A high correlation between the native and recombinant Pas n 1 and Cyn d 1 allergens was demonstrated by immunoassays in the academic laboratory, which was then confirmed by the industry laboratory, with reproducible results between these settings. Notably, two batches of each allergen produced in the academic laboratory showed remarkably comparable performance, showing reliable delivery of high quality allergen components from the academic laboratory to industry. Whilst biosensors produced in Switzerland with the novel

subtropical GP allergen rPas n 1 and shipped back to Australia for testing in the university laboratory showed lower reactivity, this was also observed for the reference tIgE assay. Inter-laboratory variability, variation between prototype POC devices, and possible loss of function on shipping, may account to these differences. However, there appeared to be good repeatability of testing biosensors in the academic laboratory during the study. Moreover, the new rPas n 1 and rCyn d 1 components performed similarly to previously tested GP allergens (rPhl p 1 and rPhl p 5) in this POC device[33], indicating their technical suitability for further development and translation into practice.

Whilst the correlation values between POC and PLA, and the PPA and NPA for rCyn d 1 were lower than for rPas n1 based on RFU, the POC test for rCyn d 1 could distinguish between patients with AR with clinically confirmed history of GP allergy, and those with other allergies and non-allergic individuals. Such observations indicate a need for further optimization of the calibration strategy to ensure inter-method comparability of Cyn d 1 test results. Secondary analysis of the history of these other allergy participants suggests that the detection of spIgE rCyn d 1 may be a real specific IgE measurement at least in some cases; one participant assigned based on clinical history to the other allergy group showed 6.2 kU_A/L spIgE rCyn d 1 by POC, also had 23.4 kU_A/L BeGP by PLA.

Despite studies showing AR patients in subtropical regions have higher levels of allergic sensitization to subtropical GPs including Bahia and Bermuda grasses[8, 9, 11, 47, 48], there is currently no allergen component test available for clinical diagnostic use for any Panicoideae allergen. Although Panicoideae and Chloridoideae grasses are both subtropical, and are often considered similar, the primary sequence of Pas n 1 and Cyn d 1 differ by as much as over 30%[35].

The concordance between allergen spIgE classes for rCyn d 1 and rPas n 1 indicated a considerable number of participants showed class 3 or more on either the POC or PLA. Whilst it is tempting to speculate that some discrepancies between results may be due to CCD cross-reactivity in natural allergens[49], this is unlikely to explain all differences. Theoretically, the function of the nanofluidic POC device with low allergen concentration and low contact time should favour binding of high affinity spIgE[32], whereas the reference PLA assay has high antigen density of cellulose discs favouring binding of all spIgE[28]. These differences in assay features mean that analytical outcomes could not be expected to be directly comparable, nor interchangable.

The strengths of this research are that this study included twice as many participants as previous studies of this POC device[33] and this was the first trial of this nanofluidic device outside the country of development, and in an academic setting. It is relevant that many of this well-characterized cohort came from a subtropical climate zone where the subtropical grass are the predominant grass families to which the population is sensitized and exposed [7, 8]. Among the study limitations we note there was no assessment for any interference of serum factors with allergen spIgE measurement, which can occur in some cases[28]. Notably, in the POC device there are no wash steps to remove any factors in sera that could interfere with measurement of allergen spIgE, whereas the high throughput PLA has stringent washing. Whilst this study utilized stored sera, the POC is designed for use with whole blood from a finger prick. The performance of the device, and these new subtropical allergen component tests, should be evaluated for potential real-world clinical utility with whole blood.

A benchtop nanofluidic POC device could allow for rapid serological testing in primary care or specialist clinics, particularly if skin prick testing is not indicated or inaccessible, enabling immediate decision-making on referrals or treatment options. Moreover, with telehealth services[50], access to a rapid POC device could enable allergy testing in regional and rural

312 general practice clinics. A rapid POC with rPas n 1 and rCyn d 1 tests could improve access
313 to clinical diagnosis for allergy patients in subtropical regions.

314

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457 **Table 1.** Allergens coated onto biosensors within the test capsule

Tests	**Interpretation**
rCyn d 1	Major allergen of Bermuda grass *(Cynodon dactylon)* pollen
rPas n 1	Major allergen of Bahia grass *(Paspalum notatum)* pollen
rPhl p 1	Major allergen of Timothy grass (*Phleum pratense*) pollen
rPhl p 5	Major allergen of Timothy grass (*Phleum pratense*) pollen
Anti-IgE	Total IgE level

458 Abbreviations: r; recombinant, IgE; immunoglobulin of type E

Table 2. Analysis of positive and negative percentage agreements between point-of-care and pathology laboratory assays

Test	Comparator	n	P	N	NPA	PPA	OPA	Kappa
rCyn d 1	BeGP	208	110	98	66.3%	67.8%	67%	substantial
	nCyn d 1	208	110	98	67.5%	66.7%	67.1%	substantial
rPas n 1	BaGP	207	107	100	82.5%	73.0%	75.3%	good
	nPas n 1	207	107	100	81.0%	71.6%	76.7%	good

Natural allergens were biotinylated and tested in a custom pathology laboratory assay. Cohen's Kappa values for overall agreement are noted.

Abbreviations: NPA; negative percentage agreement, PPA; positive percentage agreement, OPA, overall percentage agreement, n; natural; P; positive, N; negative

Figure Legends

Figure 1. Linearity of spIgE with rCyn d 1 and rPas n 1 on the point-of-care (POC) device. Example calibration curves for rCyn d 1 (A) and rPas n 1 (C) relative to pathology laboratory assay (PLA) values. Linear regression with 95% confidence intervals (C and D). Linear ranges delimited in grey.

Figure 2. Stability of biosensor capsules. A. Allergen and tIgE tested in industry and academic laboratories (Spearman's r, best fit with 95% confidence intervals). B. Repeated measurements of individual and pooled sera (Relative Fluorescence Units; RFU, standard error of three biosensors; rPas n 1 and rPhl p 1, two biosensors; tIgE).

Figure 3. Correlation between serum rCyn d 1 and rPas n 1 spIgE, and total IgE, measured in nanofluidic point-of-care device (POC) with serological pathology laboratory assays (PLA), and with SPT (Spearman's correlation coefficient and p value).

Figure 4. Detection of allergic rhinitis patients with GP allergy using the point-of-care (POC) device compared with pathology laboratory assays (PLA). Serum tIgE, rCyn d 1 and rPas n 1 spIgE data shown as RFU (A) and kU/l (B). NA; non-atopic (blue), OA; other allergy (orange), GP; GP allergic (green).

Figure 5. Agreement between GPAS cohort by point-of-care (POC) device for recombinant allergens compared with pathology laboratory assays (PLA) with natural allergens and GP extract. Missing; missing data. Data shown in spIgE Classes, 0; < 0.35, 1; 0.35-0.7, 2; 0.7-3.5, 3; 3.5-17.5, 4; 17.5-52.5, 5; 52.5-100, 6; >100.

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Figure

Figure 1

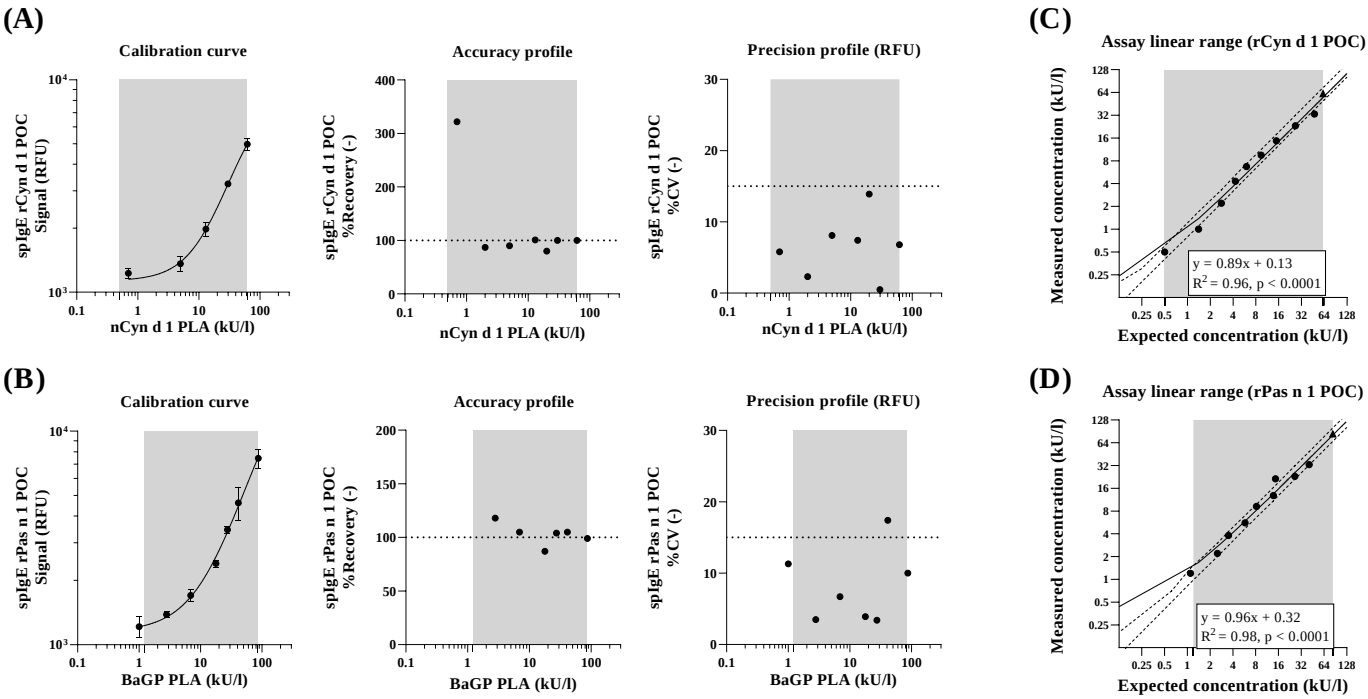
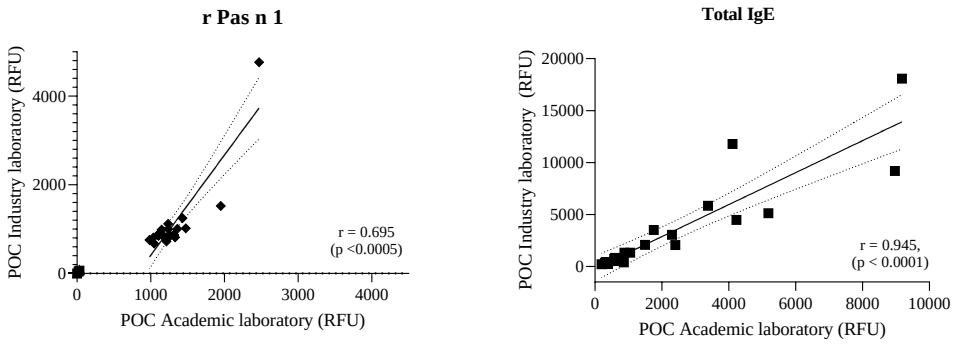


Figure 2

(A)



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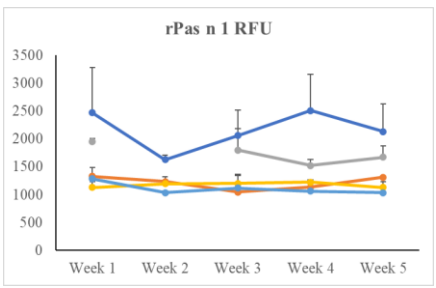
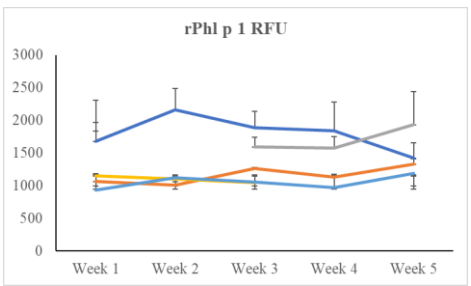
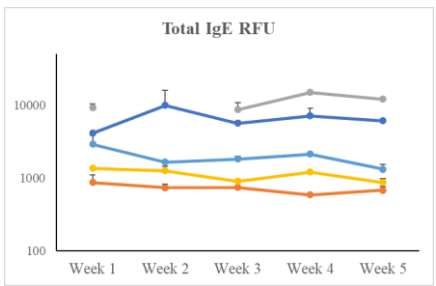






Sample	Mean	SD	Number	CoV
405046	2160	357	5	17%
405047	1214	121	5	10%
407025	1737	183	4	11%
NA Pool	1176	43	5	4%
NGP Pool	1109	105	5	9%
Average				10%

Sample	Mean	SD	Number	CoV
405046	1798	273	5	15%
405047	1162	133	5	11%
407025	1736	180	4	10%
NA Pool	1068	77	4	7%
NGP Pool	1054	107	5	10%
Average				11%

Sample	Mean	SD	Number	CoV
405046	6586	2160	5	33%
405047	723	101	5	14%
407025	11227	2906	4	26%
NA Pool	1121	222	5	20%
NGP Pool	1968	597	5	30%
Average				25%

Figure 3

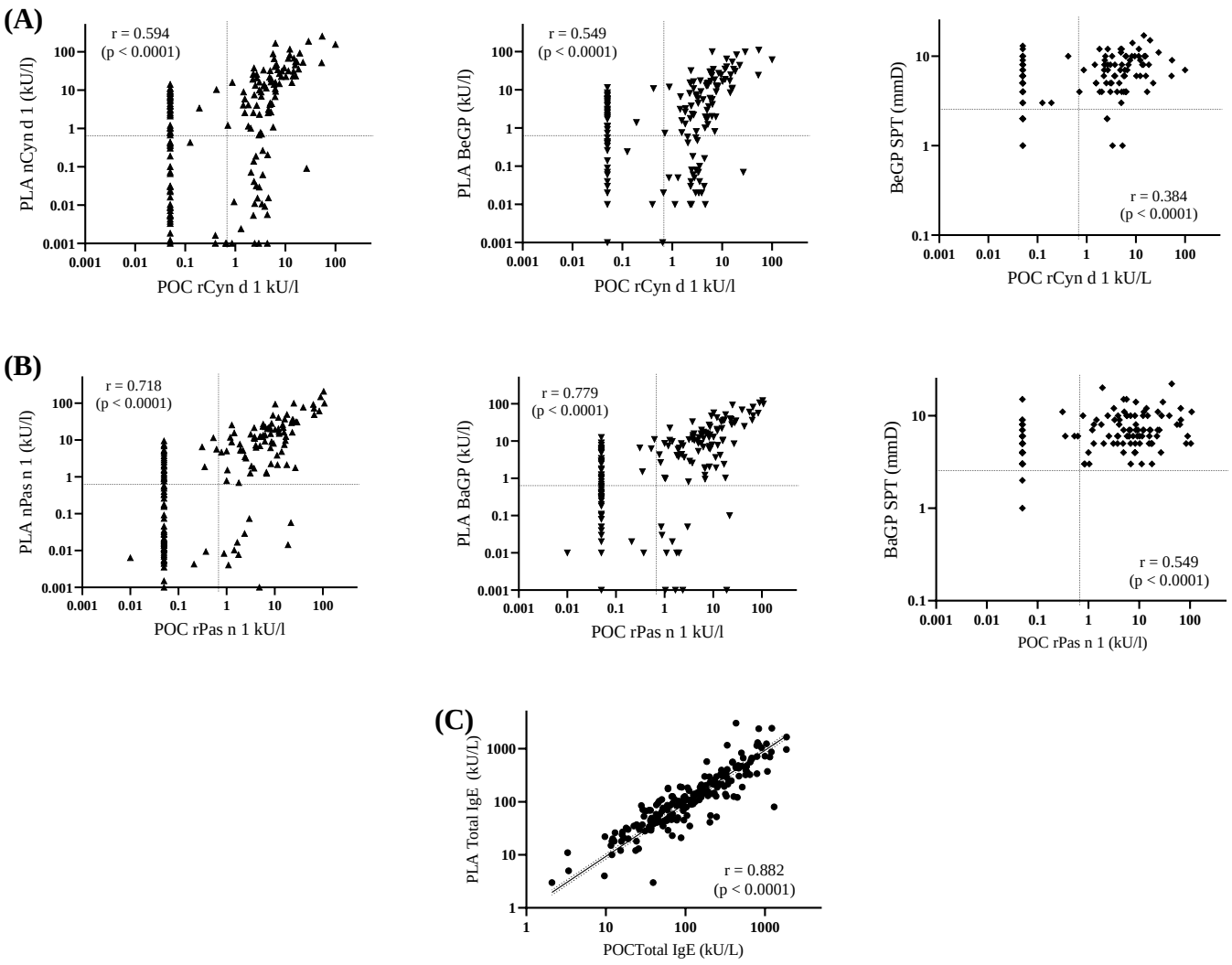

(A)
r = 0.594
(p < 0.0001)
PLA nCyn d 1 (kU/l)
POC rCyn d 1 kU/l
r = 0.549
(p < 0.0001)
PLA BeGP (kU/l)
POC rCyn d 1 kU/l
r = 0.384
(p < 0.0001)
BeGP SPT (mmD)
POC rCyn d 1 kU/L
(B)
r = 0.718
(p < 0.0001)
PLA nPas n 1 (kU/l)
POC rPas n 1 kU/l
r = 0.779
(p < 0.0001)
PLA BaGP (kU/l)
POC rPas n 1 kU/l
r = 0.549
(p < 0.0001)
BaGP SPT (mmD)
POC rPas n 1 (kU/l)
(C)
r = 0.882
(p < 0.0001)
PLA Total IgE (kU/L)
POCTotal IgE (kU/L)

Figure 4

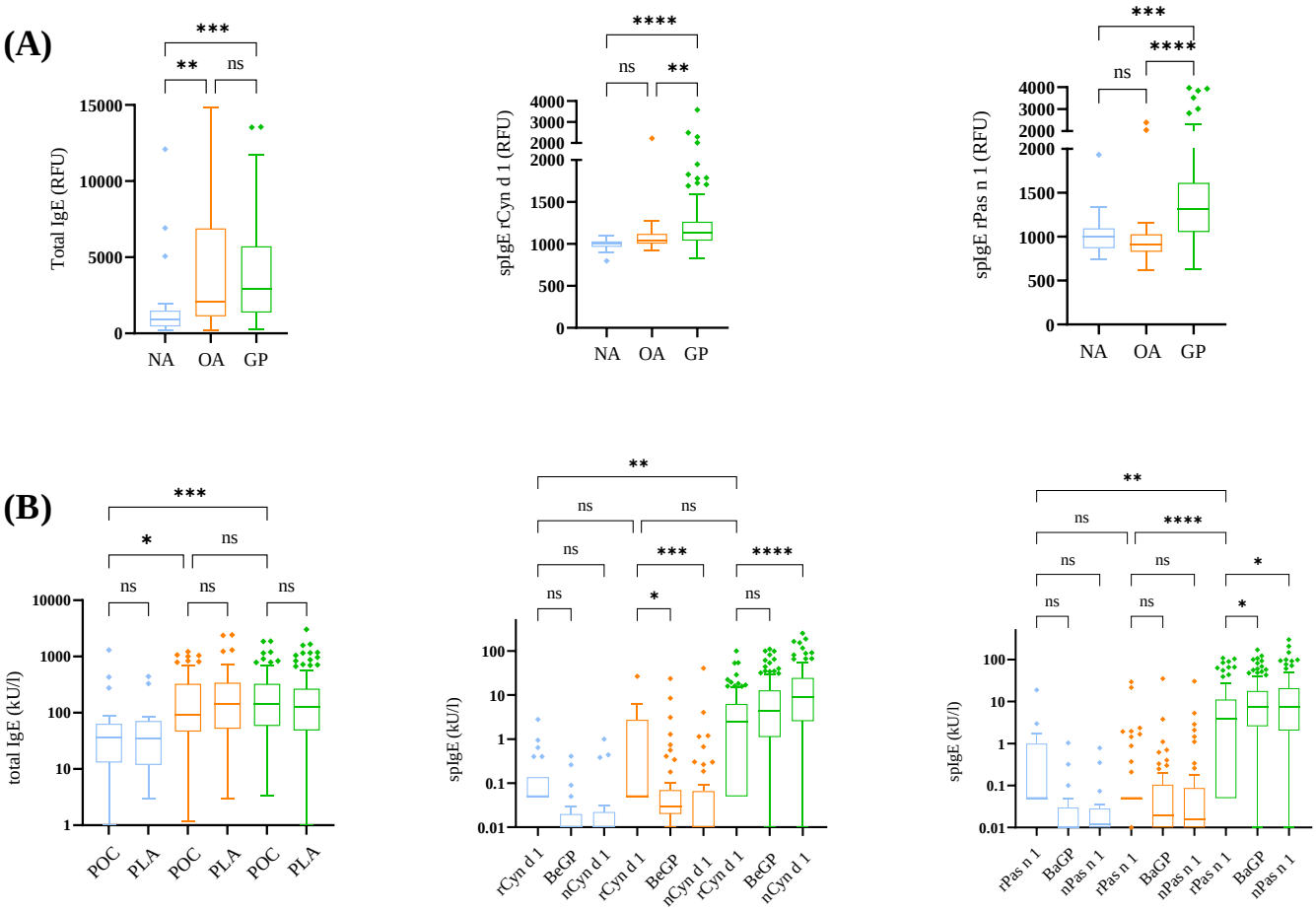

(A)
Total IgE (RFU)
15000
10000
5000
0
NA
OA
GP
***
**
ns
spIgE rCyn d 1 (RFU)
4000
3000
2000
2000
1500
1000
500
0
****
ns
**
spIgE rPas n 1 (RFU)
4000
3000
2000
2000
1500
1000
500
0
***
****
ns
(B)
total IgE (kU/l)
10000
1000
100
10
1
POC
PLA
***
*
ns
spIgE (kU/l)
100
10
1
0.1
0.01
rCyn d 1
BeGP
nCyn d 1
**
ns
*
***
****
spIgE (kU/l)
rPas n 1
BaGP
nPas n 1
**
****
*

Figure 5

(A)

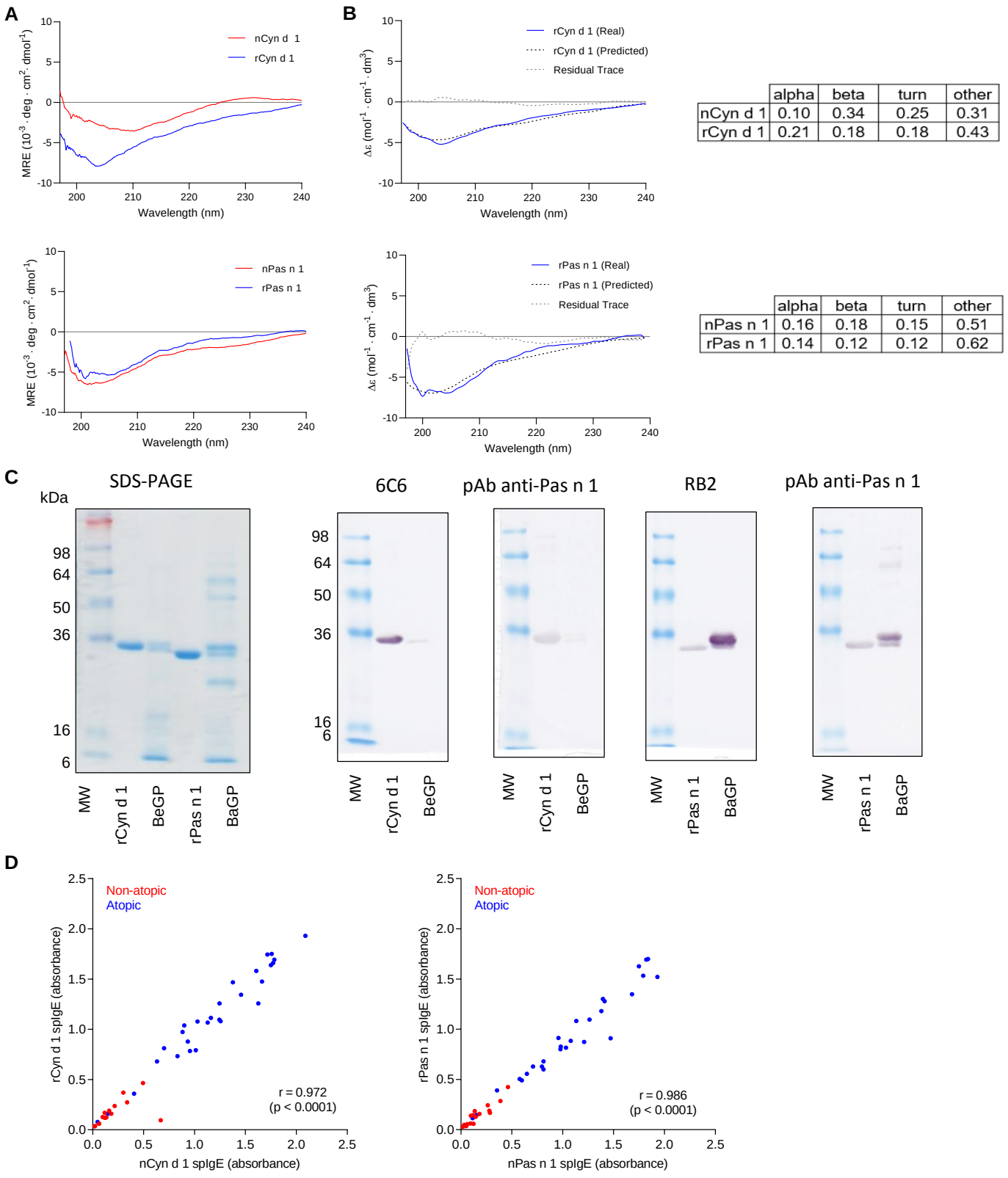
rCyn d 1 class	nCyn d 1 class								
	0	1	2	3	4	5	6	Missing	Total
0	46	6	15	23				3	93
1	4			1					5
2	18	1	8	12	6			1	46
3	7		4	14	18	7	2	4	56
4	1				1	2	1		5
5					1		1		2
6							1		1
Missing									
Total	76	7	27	50	26	9	5	8	208

rCyn d 1 class	BeGP class								
	0	1	2	3	4	5	6	Missing	Total
0	51	6	20	16					93
1	4			1					5
2	18	4	11	12	1				46
3	8		9	22	13	2	1	1	56
4	1				2	2			5
5					1		1		2
6						1			1
Missing									
Total	82	10	40	51	17	5	2	1	208

(B)

rCyn d 1 class	nPas n 1 class								
	0	1	2	3	4	5	6	Missing	Total
0	58	5	18	13				3	97
1	1			2					3
2	9	1	6	10	2			3	31
3	2		5	24	15	1		5	52
4	2		2	1	7	2		1	15
5	1				1	3	1		6
6						1	2		3
Missing									
Total	73	6	31	50	25	7	3	12	**207**

rPas n 1 class	BaGP class								
	0	1	2	3	4	5	6	Missing	Total
0	58	7	21	11					97
1	1			2					3
2	10		5	14	2				31
3	1		8	28	13	1		1	52
4	3		1	1	8	2			15
5						3	1		6
6							3		3
Missing									
Total	73	7	35	56	25	6	4	1	**207**



	alpha	beta	turn	other
nCyn d 1	0.10	0.34	0.25	0.31
rCyn d 1	0.21	0.18	0.18	0.43

	alpha	beta	turn	other
nPas n 1	0.16	0.18	0.15	0.51
rPas n 1	0.14	0.12	0.12	0.62

eFigure 1. CD-spectra of newly generated recombinant allergens rPas n 1 and rCynd 1 compared to purified natural allergen (A) and compared to the predicted spectra (B). Tables show the percentage of secondary structures ; MRE, mean residual ellipticity. Coomassie stained SDS-PAGE of recombinant allergens and pollen extracts, and immunoblotting with monoclonal (6C6 and RB2) and polyclonal (pAb) anti-Pas n 1 antibodies (C). Testing in academic laboratory of human sera IgE immunoreactivity with natural and recombinant allergen (D); 12 non-atopic (red) and 28 grass pollen allergic (blue) sera were tested against nCyn d 1 and rCyn d 1, and nPas n 1 and rPas n 1. Spearman ranked sum correlation coefficients are also shown.

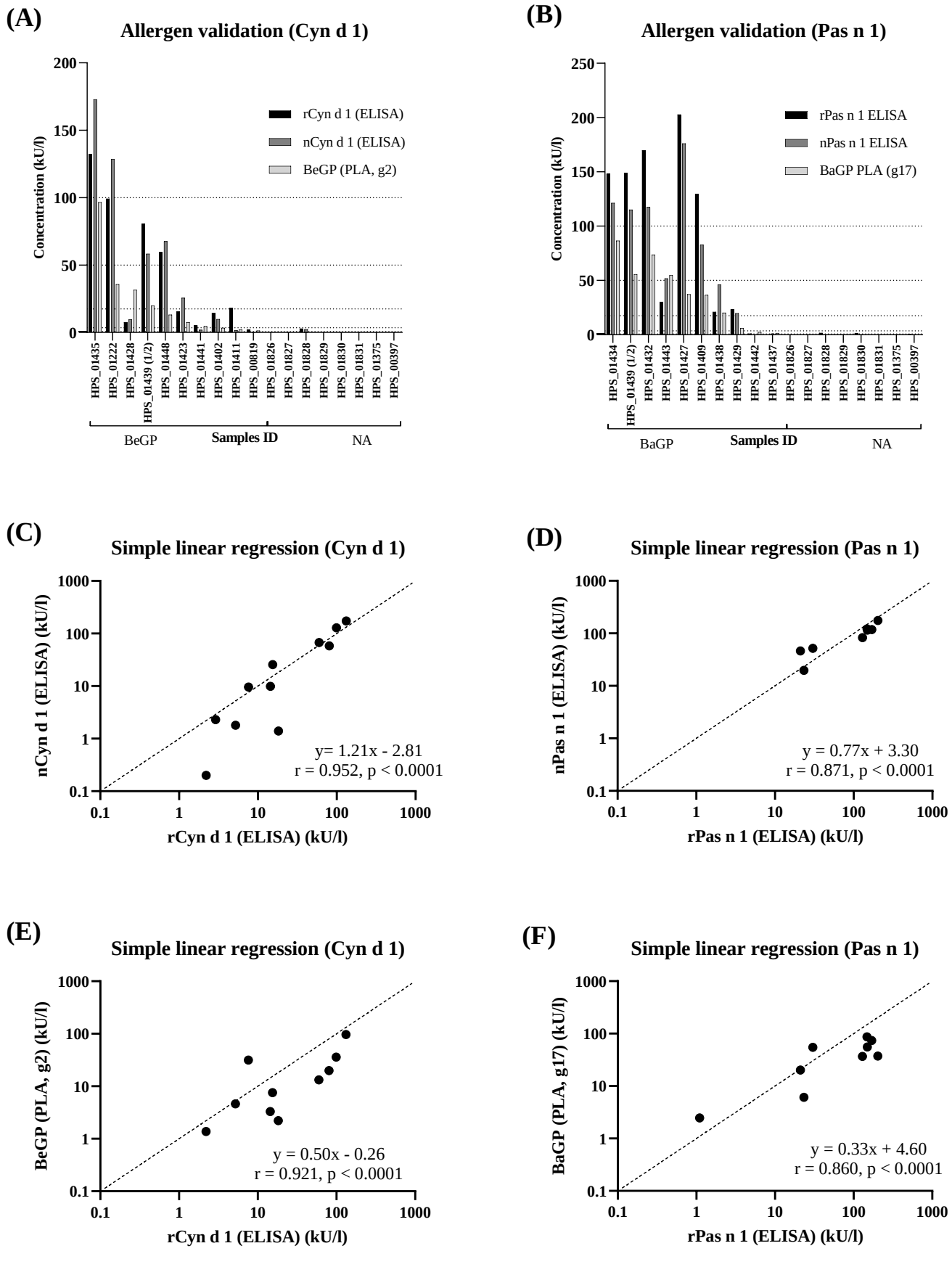
A

MGSSHHHHHHSSGLVPRGSHMAMGDK**S**
GPNITATYGDKWLDAKATFYGSDPRGAA
PDDHGGACGYKDVDKAPFDSMTGSGNEP
IFKDGLGR**GSCYEIKCKEPAECSGEPVLIKI**
TDKNYEHIAAYHFDLSGKAFGAMAKKGEE
DKLR**KAGELMLQFR**R**VKCEYPSDTKIAFH**
VEKGSNPNYLALLVKYAAGDGNIVSVDIKS
KGSDEFLPMKSSWGAIWRIDPPKPLKGPF
TIRLTSESGGHVEQEDVIPEDWKPDTVYKS
KIQF*

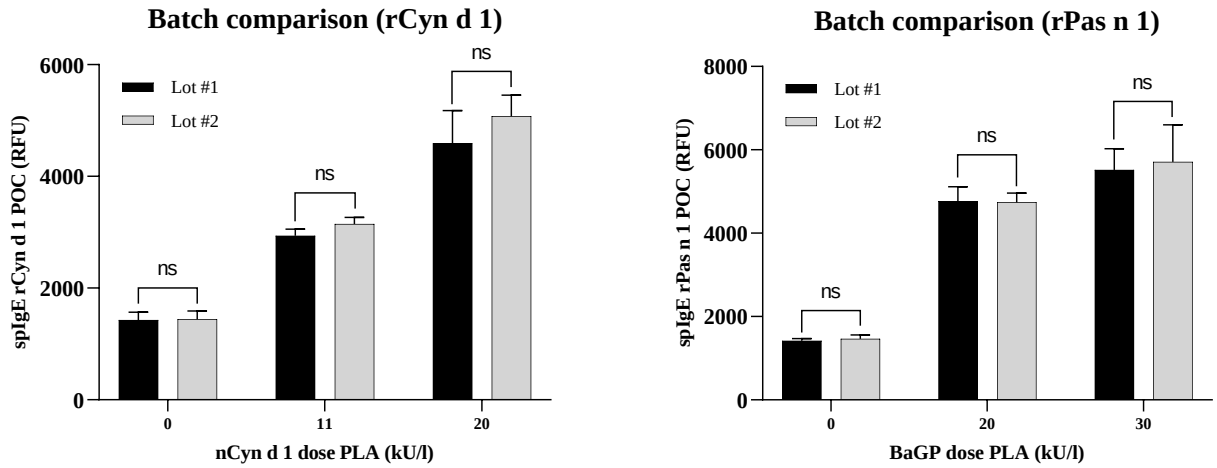
B

M**GSSHHHHHHSSGLVPRGSHMGPSKV**
PSGPNITTNYNGKWLPAKATWYGQPNG
AGPDDNGGACGIKNVNLPPYNGFTASG
NPPIFKDGKGCGSCYEIRCNKPECSGQP
VTVFITDMNYEPIAPYHFDLSGKAFGAM
AKPGLNDKLRHYGIFDLEFRRVRCKYQ
GGQK**IVFHVEKGSNPNYLAMLVKFVAD**
DGDIVLMELKEKSSDWKPMK**LSWGAIW**
RMDTPK**ALVPPFSIR**LTSESGK**KVIAQDV**
IPVNWKPDTVYNSNVQF*

eFigure 2 Validation of recombinant protein sequence by mass spectrometry. Amino acid sequence of rCyn d 1 (A) and rPas n 1 (B) showing amino acids detected in tryptic peptide fragments resolved by 1D-nano LC-MS/MS analysis. Coverage of amino acids that matched the peptide sequence are shown in bold.



e**Figure 3.** Allergen validation by ELISA; Evaluation by industry laboratory of recombinant (r) and natural (n) Cyn d 1 and Pas n 1 for spIgE reactivity to Bermuda (BeGP) or Bahia grass pollen (BaGP) allergic and non-allergic (NA) subjects in comparison with whole GP extract in pathology laboratory assay (PLA).



eFigure 4. Comparability of performance between lots of recombinant (r) allergens; two lots of rCyn d 1 and rPas n 1 generated by the academic laboratory compared by the industry laboratory upon labelling using the point-of-care (POC) device. Non-significant (ns) difference in relative fluorescent unit (RFU) for each dose according to t-tests, displaying mean and standard-deviation, $\alpha = 0.05$).