

DIAGNOSTICA E RICERCA DI LABORATORIO

DIAGNOSTIC AND LABORATORY RESEARCH

AP 1800_{HCD}: towards automation of the CLA assay

AP 1800_{HCD}: l'automazione del metodo CLA

O. RUZZENENTE, G. LIPPI, F. DIMA, A. BERTASINI, G.C. GUIDI

Istituto di Chimica e Microscopia Clinica, Dipartimento di Scienze Morfologico-Biomediche, Università di Verona

Key words

Allergology • Automation • Chemiluminescent assay • Recombinant allergens • Panel • Cross-reactivity

Parole chiave

Diagnostica allergologica • Chemiluminescenza • Allergeni ricombinanti • Pannello di allergeni • Cross-reattività

Summary

Laboratory automation is always a key step towards innovation in diagnostic, especially when the final objective is the improvement of methods already suited for routine laboratory testing. The laboratory approach to the diagnosis of IgE-dependent allergy relies on the direct demonstration of specific IgE by several immunoenzymatic methods. The AP 1800, a novel automated allergy diagnostic system, allows the rapid determination of a unique allergy panel format with strong correlation with established *in vitro* assays. Aim of the present investigation is the validation of the analytical performances of the AP 1800, according to validated protocols. Results of the evaluation, compared to those of the Immulite 2000 as the reference system, allowed the formulation of novel considerations on allergy testing, in the perspective of emerging scenarios disclosed by advances in molecular biology.

Riassunto

L'automazione è sempre un evento importante nella diagnostica di laboratorio, soprattutto se si propone come perfezionamento ad un metodo già presente. L'approccio laboratoristico alla diagnostica allergologica si basa sulla dimostrazione diretta della presenza di anticorpi specifici di classe IgE mediante tecniche immunoenzimatiche. AP 1800, un innovativo sistema per la diagnostica allergologica *in vitro*, consente la determinazione rapida di un pannello esclusivo di allergeni, i cui risultati mostrano una ottima correlazione con metodi diagnostici *in vitro* preesistenti. Lo scopo di questo lavoro è la valutazione delle performance analitiche dell'AP 1800, seguendo protocolli validati. I risultati della valutazione, confrontati con quelli dell'Immuline 2000 come riferimento, hanno consentito di formulare nuove considerazioni sulla diagnostica allergologica *in vitro*, anche in prospettiva degli scenari emergenti a seguito dei continui progressi della biologia molecolare.

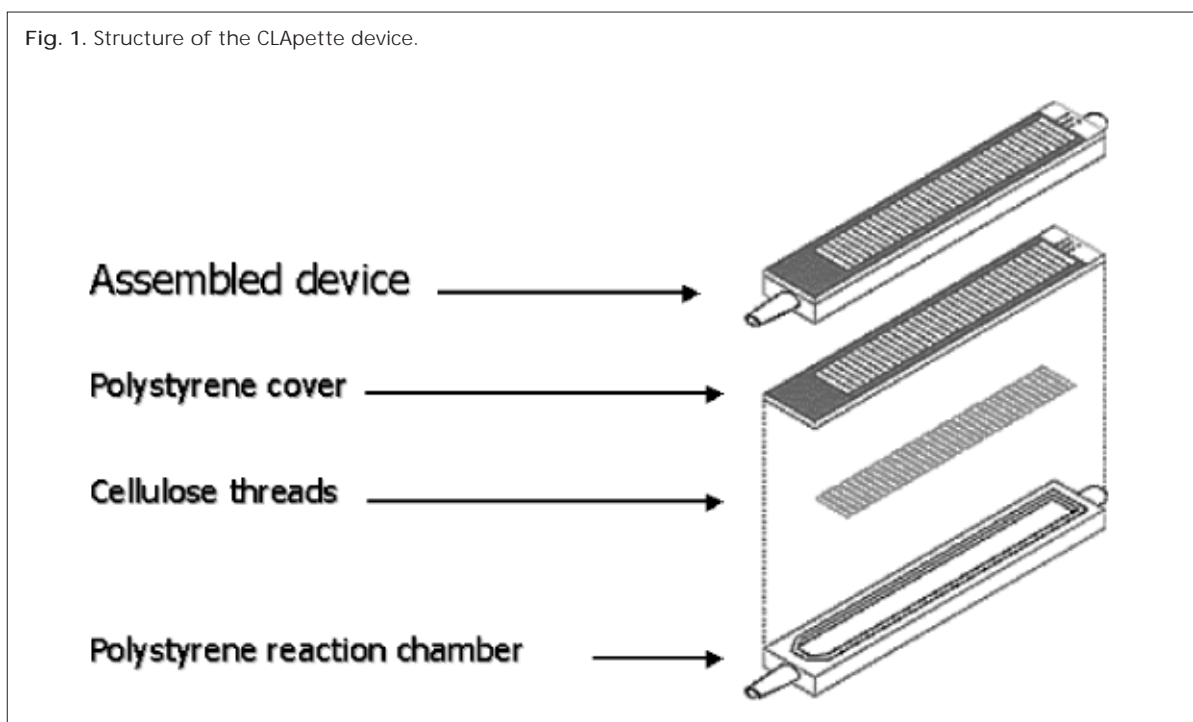
Introduction

The delivery of dedicated automation always represents a pivotal step, not only for commercial purposes, but also and especially for diagnostics. The design of an automated immunoenzymatic system joins several aspects, including design, diagnostic technology, development of reagents, detection and elaboration of the signal developed during the immunoreaction. Ideally, each of these aspects should be harmonized within a unique diagnostic system, maintaining elevated analytical performances, joined to easy utilization¹. Such a project usually requires total revision of the original methods and techniques, introducing novel electronic technology that should ideally maintain or enhance the analytical performances. The analytical performances following automation of the immunoreaction are influenced by factors that characterize both the homogeneous and heterogeneous phases (reactant metering, timing, temperature,

reagents concentration), but also by the separative and detection techniques of the device.

In the United States of America, Europe and Japan, over 20% of the population suffer from some form of allergy. Of those, nearly 50% tolerate symptoms or self-treat with over-the-counter medications, 40% are treated symptomatically, without establishing the primary cause of the allergy, and the remaining 10% are investigated by skin testing or *in vitro* or blood testing. Although both testing methods tend to give comparable results, the blood test has significant advantages, as it is cheaper, less time-consuming, it is barely influenced by drugs, does not require suspension of therapies with anti-histamines and has no risk for the patient health. Finally, results of *in vitro* testing are not dependent on the technique used to perform the skin test and might be more comparable among laboratories employing different assay techniques. The most common methodologies for *in vitro* testing are the radioimmunoassay (RIA), frequently known

Fig. 1. Structure of the CLApette device.



as radioallergosorbent test (RAST), and the enzyme immunoassay (EIA), which consists basically on the adaptation of a multiple allergosorbent test system (MAST) to a chemiluminescent assay (CLA)^{2,3}. The automation of the CLA technique, through analytical systems principally developed for the simultaneous semiquantitative determination of many allergens, represents a rational and suitable approach to the growing demand of allergy *in vitro* testing, allowing a faster and more efficient production of results⁴. The introduction of a diagnostic approach mainly based on panels of allergens allowed to recognize a frequency of polysensitization much higher than that earlier observed (45%) in studies on patients suffering from atopic dermatitis, highlighting the problem of the contextual cross-reactivity⁵. However, such an innovation in allergy testing should not fall within “diagnostic abuses or misuses” of diagnostic resources; in fact, laboratory automation should ease but not revolutionize the correct diagnostic approach to allergy, which must never disregard an accurate anamnesis.

The CLA system allows the simultaneous determination of specific IgE by an immunoenzymatic technique based on chemiluminescent detection. Core of the system is the “CLApette” device, a little, rigid and flat rectangular plastic column (Fig. 1). The CLApette includes a reaction chamber, containing segments of cellulose thread as solid phase, each with an allergen chemically bonded to it. Each

CLApette contains three control cellulose threads: the first, uncoated, is the negative control and allows detection of the aspecific binding of the serum; the second is coated with human IgE and represents the positive control to verify proper function of reagents and system; the third thread, allocated to the upper extremity of the reaction chamber, is conjugated with Protein G and testifies for device filling, following adequate serum aspiration. The variable association of allergens in the CLApette device generates three major profiles or panels: inhalants, foods and combined. The final choice of the panel usually depends on the geographic area. In brief, the patient’s serum is incubated with the solid phase. After first washing, peroxidase-labeled anti-IgE antibodies and photoreagents are added. The developing chemical reaction between the antibody-antigen complex and the photoreagents produces a light signal measured by a luminometer, which intensity is directly proportional to the amount of bound IgEs and is finally expressed in Luminometer Units (LU)⁶⁻⁸.

The analytical performances of the manual method, originally introduced in 1984, have been fully evaluated and compared with those of alternative immunoenzymatic techniques and with results of skin prick testing⁸⁻¹⁰. Aim of the present investigation is the evaluation of the analytical performances of the CLA method, adapted to the novel AP 1800 automated allergy diagnostic system (Hitachi Chemical Diagnostic, HCD).

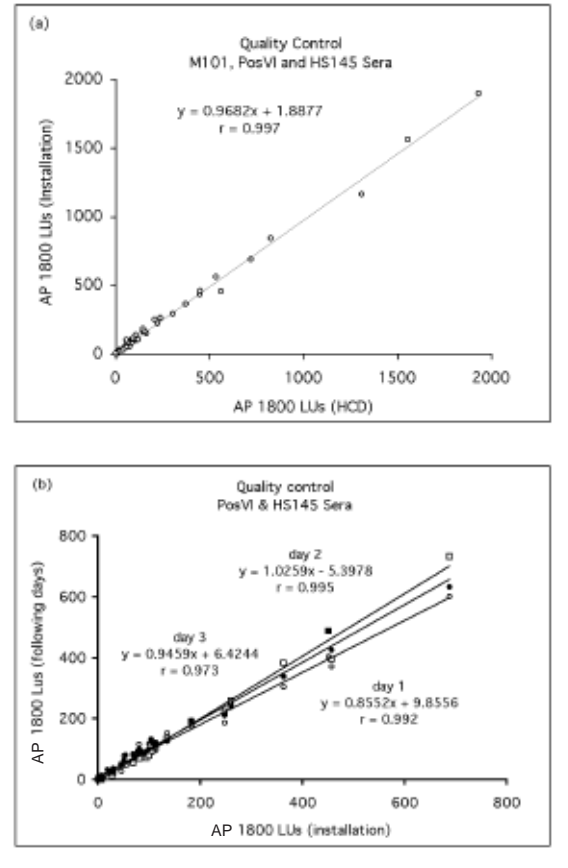
Materials and Methods

The purpose of a procedural control is to confirm that the various steps in the analytical procedure have occurred correctly. Therefore, the study protocol was carried out with the primary aim to verify proper installation and functioning of the instrument. Samples directly produced by HCD and earlier tested in our laboratory on an identical instrument were re-tested on daily basis for three consecutive days: M101, POSVI and HS145 sera were used. Each of these samples exhibits different degrees of immunoreactivity towards common allergens included in the panel of the specific CLApette. The evaluation of the analytical performance of the AP 1800 system comprised analysis of imprecision, accuracy, linearity and detection of the lower detection limit. Dedicated CLApettes were assembled, containing five filaments for each D1 (*d. pteronyssinus*), G6 (timothy), E1 (cat) allergens together with standard internal control filaments. Testing sera were obtained from samples earlier testes and stored in our laboratory, which were classified within three classes according to the degree of immunoreactivity for a certain allergen: Low (classes 0-1), Medium (class 2) and High (classes 3-4). Accuracy testing for each specific allergen was performed by serial dilution of pools of high-reactive sera (class 4) with an IgE negative serum (HCD). The accuracy testing for the E1 allergen was performed on a single high-reactive serum, as the relative pool immunoreactivity was unsatisfactory. Results of the novel AP 1800 were further compared to those obtained on the Immulite 2000 (DPC), assaying 235 consecutive serum samples, earlier classified according to their mono or polyspecific immunoreactivity. The comparison evaluation consisted on 10 allergens: M3 (*aspergillus*), D1 (*d. pteronyssinus*), D2 (*d. farinae*), E1 (cat), G6 (timothy), T3 (birch), F1 (egg white), F2 (milk), F4 (wheat) and F13 (peanut). The statistical evaluation to establish sensitivity, accuracy and diagnostic efficiency for either pooled data or single allergens was performed by the use of ROC curves according to Galen and Gambino^{3,11} and class agreement between methods was further analyzed by chi-square test.

Results and Discussion

Results of imprecision and accuracy testing carried on in the first phase of the evaluation by evaluation of intra and intrerassay variability are presented in Figure 2 and Table I. Linear regression analysis and correlation coefficient calculation were excellent, testifying for proper installation and good quality of the system. Accordingly, the calculation of the relative coefficients of variation was rather satisfactory.

Fig. 2. Evaluation of the proper function of the AP 1800 instrument and reagents by repeated measurement of quality controls. Results of measurement are compared with either values obtained by HD (Fig. 2a) or values obtained on the day of the AP 1800 system installation (Fig. 2b).



The analytical performances, further evaluated according to the standardized protocols of imprecision testing followed during the evaluation are excellent, as the observed coefficients of variation (CVs) are always lower than 20%, the maximum allowed limit suggested by the National Committee for Clinical Laboratory Standards (NCCLS) (Fig. 3). Higher degrees of variability were observed for a minority of data, however, those samples were characterized by a very low immunoreactivity, thus producing feeble luminometric signals, which commonly fell at the lower linearity limits of the assay. The ROC curve analysis displayed in Figure 4, shows the overall sensitivity, accuracy and diagnostic efficiency for measurement of specific IgEs on the 10 allergens tested (pooled data), using the Immulite 2000 as reference system. After ranking results of 235 consecutive serum samples among three major classes of im-

Tab. I. Evaluation of the proper function of the AP 1800 instrument and reagents by repeated measurement of quality controls for three standard allergens: G6 (timothy), D1 (*d. pteronyssinus*) and E1 (cat). Results are expressed in terms of mean \pm standard deviation; the coefficient of variation is reported in brackets.

	Intra-assay		
	Low	Medium	High
G6	539 \pm 36 (6.8%)	1088 \pm 72 (6.6%)	1465 \pm 58 (4.0%)
D1	701 \pm 50 (7.2%)	1066 \pm 28 (2.6%)	1832 \pm 29 (1.6%)
E1	140 \pm 18 (4.0%)	182 \pm 27 (14.8%)	482 \pm 70 (14.6%)
	Inter-assay		
	Low	Medium	High
G6	542 \pm 35 (6.4%)	1061 \pm 86 (8.1%)	1529 \pm 119 (7.8%)
D1	715 \pm 61 (8.6%)	1154 \pm 102 (8.8%)	1831 \pm 73 (4.0%)
E1	160 \pm 28 (17.6%)	191 \pm 28 (14.8%)	509 \pm 94 (18.5%)

munoreactivity for both systems (low, medium and high), the overall concordance was excellent, with a final agreement of 91% (Tab. II). Eight samples (3%) classified in the lower immunoreactivity class by the Immulite 2000 were classified in the medium class by the AP 1800, whereas thirteen (6%) samples classified in the higher immunoreactivity class by the Immulite 2000 were classified in the medium class of immunoreactivity by the AP 1800. Such a good agreement was further confirmed by result of the chi square test analysis ($p = 0.168$). Sensitivity, accuracy, positive and negative predictive values, overall diagnostic efficiency and results of ROC curve analysis for the measurement of specific IgEs for each single allergen are shown in Table III. In synthesis, the overall diagnostic efficiency, as compared to the Immulite 2000 as reference system, was substantially acceptable, ranging from 65% for E1 (cat) to 92% for G6 (timothy); the relative areas under the curve were also satisfactory, comprised between 0.718 and 0.967.

The modest results achieved in the samples' comparison for the E1 allergen were further confirmed by the accuracy testing on pooled sera. In fact, results were excellent for D1 and G6, but not for E1 (Fig. 5); the accuracy test for E1, reproduced on the same an-

Fig. 3. Precision profile obtained by the intra and inter-assay measurements, as elaborated by the NCCLS EPS5-A protocol, which allows a maximum total imprecision expressed in term of coefficient of variation (CV) lower than 20%.

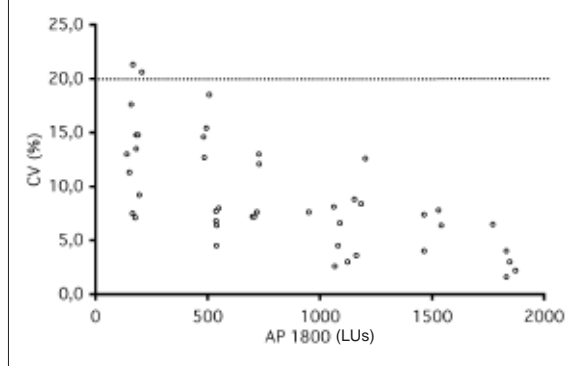
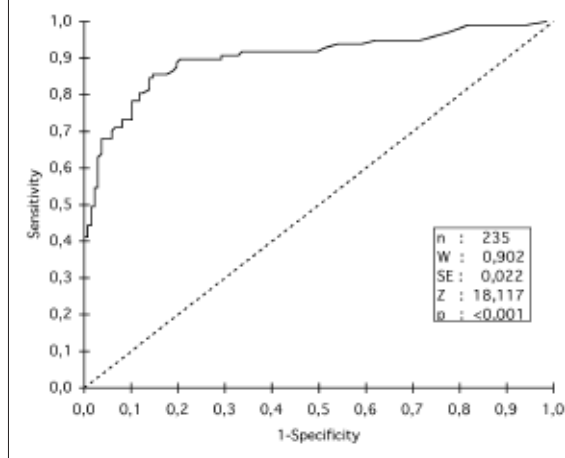


Fig. 4. Evaluation of the overall concordance by the ROC curve analysis performed according to Galen and Gambino^{3,11} of the specific IgE measurement of 235 consecutive serum samples on the AP 1800 and the Immulite 2000 systems.



alytical conditions, confirmed the poor trend (Fig. 5c). However, data obtained replacing the pooled sera with a single donor high-reactive serum (earlier labeled as serum 14558) were more satisfactory and substantially acceptable (Fig. 5d). It is conceivable that, in some circumstances, the use of pooled sera for the accuracy testing might be misleading. In fact, the whole immunoreactivity of the pool depends on the combination of the affinities of each single serum for the antibodies used, which might be finally influenced by dilution. Crystallographic studies on Fel d1,

Tab. II. Evaluation of the overall concordance of specific IgE measurement on 235 consecutive serum samples determined by the AP 1800 and the Immulite 2000 allergy diagnostic systems.

		157	29	49	235
AP 1800	High	0	0	36	36
	Medium	8	29	13	50
	Low	149	0	0	149
		Low	Medium	High	
		Immulinite 2000			

- b) conformational epitopes play a more determinant role than the sequential ones in the binding with specific IgEs; the tertiary and quaternary structures of the molecule are responsible for both biochemical and immunochemical characteristics;
- c) natural allergen extracts not always have an adequate and steady content in allergenic molecules;
- d) the remarkable discrepancy between immunoassays and bioassays emphasizes the problematic standardization of the natural allergen extracts.

Taken together, results of the comparison between the AP 1800 and the Immulite 2000 systems are substantially acceptable; diagnostic sensitivity, sensibility and efficiency are almost equivalent for the ma-

Tab. III. Evaluation of the concordance for specific allergens measurement on 235 consecutive serum samples determined by the AP 1800 and the Immulite 2000 allergy diagnostic systems.

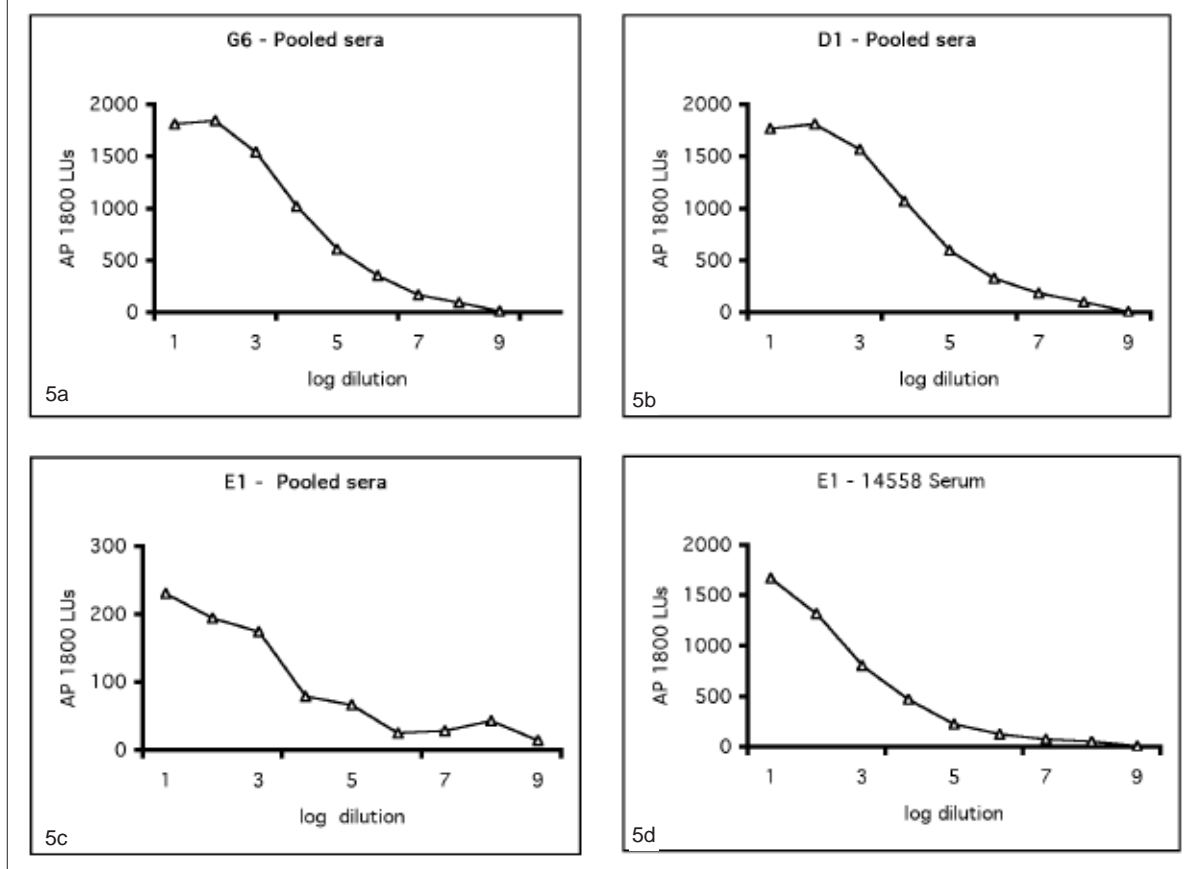
Allergen	Area under the curve	z	LUs cut off	Diagnostic sensitivity	Diagnostic specificity	Positive predictive value	Negative predictive value	Diagnostic efficiency
M3	0.778	2.405	48	83%	61%	42%	92%	67%
D1-D2	0.956	16.628	46	93%	75%	84%	88%	85%
G6	0.967	13.089	14	93%	89%	93%	89%	92%
E1	0.718	1.844	65	56%	71%	56%	71%	65%
T3	0.950	8.891	37	92%	80%	86%	89%	87%
F1	0.856	3.209	15	80%	87%	67%	93%	86%
F2	0.747	1.430	29	80%	84%	57%	94%	83%
F4	0.918	5.732	29	87%	81%	70%	93%	83%
F13	0.938	8.407	19	87%	87%	78%	93%	87%

the major E1 epitope responsible for 60-90% of the overall individual immunoreactivity to cat epithelia, demonstrated that the recombinant protein is characterized by three separate epitopes for the IgE binding on its surface, one of which is recognized by about 46% of the antibodies of the samples tested¹². Recombinant proteins have several advantages over the traditional extracts in allergology, both for diagnostic and therapeutic purposes. The almost absolute purity of recombinants allows the performance of accurate crystallographic studies, providing valuable information on structure, folding and antigen expression of the protein¹³. These aspects are all essential in diagnostic allergology, especially to reach standardization and harmonization of results between different analytical and commercial methods. Such an observation deserves further regards, that should be taken in consideration while evaluating and comparing allergy diagnostic systems.

- a) the individual immunoreactivity towards different epitopes on the same allergen molecule is rather heterogeneous;

majority of the allergens tested. Eventual discrepancies, observed only for some allergens, basically E1, might be due to the heterogeneity allergen extracts rather than to analytical disagreement between the methods. In conclusion, based on proven CLA reagent technology, the novel AP 1800 allergy diagnostic system provides a high correlation with established *in vitro* methods, over an extended reporting range. The AP 1800 combines several practical and analytical advantages of existing manual and automated techniques in diagnostic allergology. First, the CLApette device has built-in positive and negative controls, which are useful both for comparison purposes and to confirm that the device has worked correctly. Then, the volume requirement of the system is rather limited (up to 1.5 ml of serum), which may be of relevance to pediatric patients. Other practical advantages are the comprehensive panels, which include inhalants and foods, the simplification of the test request, due to the availability of pre-selected panels, and the cost effectiveness. The simple access to each part of the system, including the easy loading

Fig. 5. Accuracy testing by measurement of serial dilutions of pooled sera for the G6 (Fig. 5a), D1 (Fig. 5b) and E1 (Fig. 5c) allergens and serial dilutions of serum labeled 14558 for E1 allergen (Fig. 5d).



of samples and reagents, the presence of a clear, comprehensible and intelligible software further con-

firm the suitability of the novel AP 1800 allergy diagnostic system for routine allergy laboratory testing.

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■ Corrispondenza: dott. Giuseppe Lippi, Istituto di Chimica e Microscopia Clinica, Dipartimento di Scienze Morfologico-Biomediche, Università di Verona, Ospedale Policlinico "G.B. Rossi", piazzale Scuro 10, 37134 Verona - Tel. +039 045 8074516 - Fax +039 045 8201889 - E-mail: ulippi@tin.it