# Multicenter prospective study on multivariant diagnostics of autoimmune bullous dermatoses using the BIOCHIP technology

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***Background:*** The current standard in the serologic diagnosis of autoimmune bullous diseases (AIBD) is a multistep procedure sequentially applying different assays. In contrast, the BIOCHIP Mosaic technology combines multiple substrates for parallel analysis by indirect immunofluorescence.

***Methods:*** Sera from 749 consecutive, prospectively recruited patients with direct immunofluorescence–positive AIBD from 13 international study centers were analyzed independently and blinded by using (1) a BIOCHIP Mosaic including primate esophagus, salt-split skin, rat bladder, monkey liver, monkey liver with serosa, recombinant BP180 NC16A, and gliadin GAF3X, as well as HEK293 cells expressing recombinant desmoglein 1, desmoglein 3, type VII collagen, and BP230 C-terminus and (2) the conventional multistep approach of the Department of Dermatology, University of Lübeck.

***Results:*** In 731 of 749 sera (97.6%), specific autoantibodies could be detected with the BIOCHIP Mosaic, similar to the conventional procedure (725 cases, 96.8%). The Cohen $\kappa$ for both serologic approaches

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ranged from 0.84 to 1.00. In 6.5% of sera, differences between the 2 approaches occurred and were mainly attributed to autoantigen fragments not present on the BIOCHIP Mosaic.

***Limitations:*** Laminin 332 and laminin γ1 are not represented on the BIOCHIP Mosaic.

***Conclusions:*** The BIOCHIP Mosaic is a standardized time- and serum-saving approach that further facilitates the serologic diagnosis of AIBD. (J Am Acad Dermatol 2020;83:1315-22.)

***Key words:*** autoimmune bullous diseases; biochip; immunofluorescence; pemphigoid; pemphigus.

**CAPSULE SUMMARY**

- Parallel multivariant analysis of serum autoantibodies for the diagnosis of autoimmune bullous diseases (AIBD) is not routinely used. In this large, prospective multicenter study, an extended multivariant BIOCHIP Mosaic was compared with results by direct immunofluorescence and the conventional multistep procedure.
- The extended BIOCHIP Mosaic is a time- and serum-saving approach that allows the serologic diagnosis of AIBD in nearly 98% of patients and has a high agreement with the conventional multistep approach.

Autoantibodies (Aab) against structural proteins of the epidermis or the dermoepidermal junction of skin and mucous membranes define the heterogeneous group of about a dozen autoimmune bullous disorders (AIBDs).[1,2] Pemphigoid diseases are characterized by subepidermal split formation and, clinically, by tense blisters and/or erosions. The target antigens and distribution of lesions can be used to distinguish bullous pemphigoid (BP), mucous membrane pemphigoid, anti-p200/laminin γ1 pemphigoid, pemphigoid gestationis, linear IgA disease, and epidermolysis bullosa acquisita (EBA).[1] In pemphigus, Aab are directed against desmoglein (Dsg) 3 (pemphigus vulgaris, mucosal type), Dsg3 and Dsg1 (pemphigus vulgaris, mucocutaneous type), or, in pemphigus foliaceus, against Dsg1 alone.[2] In dermatitis herpetiformis, epidermal and/or tissue transglutaminase are targeted.[1]

Diagnosis of AIBD requires knowledge of the clinical picture and detection of tissue-bound and circulating Aab. Although the visualization of Aab in the skin/mucous membranes by direct immunofluorescence (IF) is still regarded as the diagnostic criterion standard in AIBD, recent advances in the serologic analyses allow the identification of the distinct target antigens and, therefore, accurate classification of the disease entity.[3-6] Prognoses of the various AIBD greatly differ and, because the therapeutic armamentarium and our knowledge of how to apply it have increased, exact diagnosis of AIBD is becoming more relevant in clinical practice. Examples include paraneoplastic pemphigus and anti-laminin 332 mucous membrane pemphigoid, which are associated with malignancies in 100% and 25% of patients, respectively, and require tumor searches.[7-9] On the other hand, linear IgA disease and anti-p200/laminin γ1 pemphigoid often have a benign course and can be treated with relatively little immunosuppression.[1,10]

At present, an increasing number of sophisticated, sensitive, and specific tools for the serologic diagnosis of AIBD are available, such as indirect IF-based assays on different substrates and commercial enzyme-linked immunosorbent assay (ELISA) systems. In addition, Western blotting and immunoprecipitation based on cellular or cell-derived target antigens are performed in specialized laboratories. These test systems are usually applied in a multistep process.[5,11,12] Recently, multivariant assays based on indirect IF and ELISA techniques have become widely available and have allowed parallel testing of several Aab specificities.[13-15]

In the present study, an indirect IF test based on the BIOCHIP Mosaic technology was compared in parallel with the conventional multistep approach.

## MATERIALS AND METHODS

### Sera

Sera from 749 consecutive patients with AIBD with positive direct IF were prospectively collected after informed consent before systemic treatment was initiated. Thirteen study centers from 12 countries participated in this study from June 2012 to December 2015. By direct IF, pemphigus was diagnosed in 333 (44.5%) patients, pemphigoid disease in 413 (55.1%), dermatitis herpetiformis in 2 (0.3%), and bullous systemic lupus erythematodes in 1. Ages of patients with AIBD ranged from 7 to

*Abbreviations used:*

Aab: autoantibodies
AIBD: autoimmune bullous disorder
BP: bullous pemphigoid
Dsg: desmoglein
EBA: epidermolysis bullosa acquisita
IF: immunofluorescence

98 years (mean ± standard deviation, 63.7 ± 18.3 years); 57% were female, and 43% were male (Table I).

All serum samples were shipped on dry ice and stored at -80°C (-112°F) until used. Sera from 50 blood donors (mean age, 29.4 ± 9.3 years; 36% females, 64% males) were used as additional controls. The study was performed according to the Declaration of Helsinki and was approved by the ethics committee of the University of Lübeck (11-078) and the local ethic committees of all study centers.

### Indirect IF–based BIOCHIP assay

The BIOCHIP Mosaic assembled 12 miniature biochips (primate esophagus; salt-split primate skin; recombinant BP180 NC16A; human HEK293 cells expressing recombinant Dsg1, Dsg3, type VII collagen, and the BP230 C-terminus[15,16]; recombinant gliadin analogue fusion peptide [GAF3X]; rat bladder; monkey liver; monkey liver with serosa and HEK293 cells transfected with a negative control vector) in 1 incubation field of 9 × 7 mm on a standard laboratory slide (EUROIMMUN, Lübeck, Germany) (Fig 1). Sera were diluted 1:10 and 1:100 in phosphate-buffered saline–Tween and analyzed as described previously,[15] with monoclonal mouse anti-IgG, anti-IgA, and anti-IgG supplemented with anti-IgG4 as secondary antibody used separately (EUROIMMUN).

Diagnoses were based on at least 2 compatible results, such as intercellular epithelial staining on monkey esophagus and rat bladder urothelial fluorescence for paraneoplastic pemphigus, or blister floor staining on salt split skin and reactivity with type VII collagen. In the case of strong IgG reactivity (at a dilution of 1:100) with the blister roof on salt-split skin, the diagnosis of BP (IgG) was made. Sera with exclusive IgA reactivity on monkey esophagus and/or salt-split skin were diagnosed a linear IgA dermatosis.

### Conventional multistep approach

The multistep approach was performed according to the algorithm of the routine laboratory of the Department of Dermatology, University of Lübeck, as described previously.[15,17]

The indirect IF-based BIOCHIP assay and the conventional multistep approach were performed independently and blinded including repeated testing of sera with discrepant results.

### Statistical analysis

Statistical analysis was performed using IBM (Armonk, NY) SPSS Statistics software, version 23. A Cohen kappa ($\kappa$) value above 0.81 was classified as an almost perfect congruence of results.[18]

**Table I.** Age and sex distribution of patients

| Diseases | n | Age, y | | Sex, male/female ratio |
|---|---|---|---|---|
| | | Mean ± SD | Range | |
| Based on direct IF | | | | |
| Pemphigus | 333 | 52.6 ± 16.1 | 15-90 | 0.91 |
| Pemphigoid | 413 | 72.6 ± 14.9 | 7-98 | 0.67 |
| Based on the BIOCHIP Mosaic | | | | |
| Pemphigus vulgaris | 257 | 51.4 ± 15.4 | 16-88 | 0.80 |
| Pemphigus foliaceus | 68 | 57.0 ± 17.8 | 15-90 | 1.27 |
| Paraneoplastic pemphigus | 4 | 52.5 ± 15.3 | 32-68 | 3.00 |
| Bullous pemphigoid | 371 | 73.1 ± 14.9 | 7-98 | 0.64 |
| Anti-p200* pemphigoid | 17 | 74.9 ± 8.6 | 60-94 | 3.25 |
| Anti-laminin 332 pemphigoid* | 4 | 69.3 ± 16.6 | 54-86 | 3.00 |
| Linear IgA diseases | 6 | 66.7 ± 11.2 | 48-79 | 0.20 |
| Epidermolysis bullosa acquisita | 4 | 52.0 ± 23.1 | 25-77 | All women |
| Dermatitis herpetiformis | 1 | 73 | — | 1.00 |

*IF*, Immunofluorescence; *SD*, standard deviation.
*Based on the conventional multistep procedure.

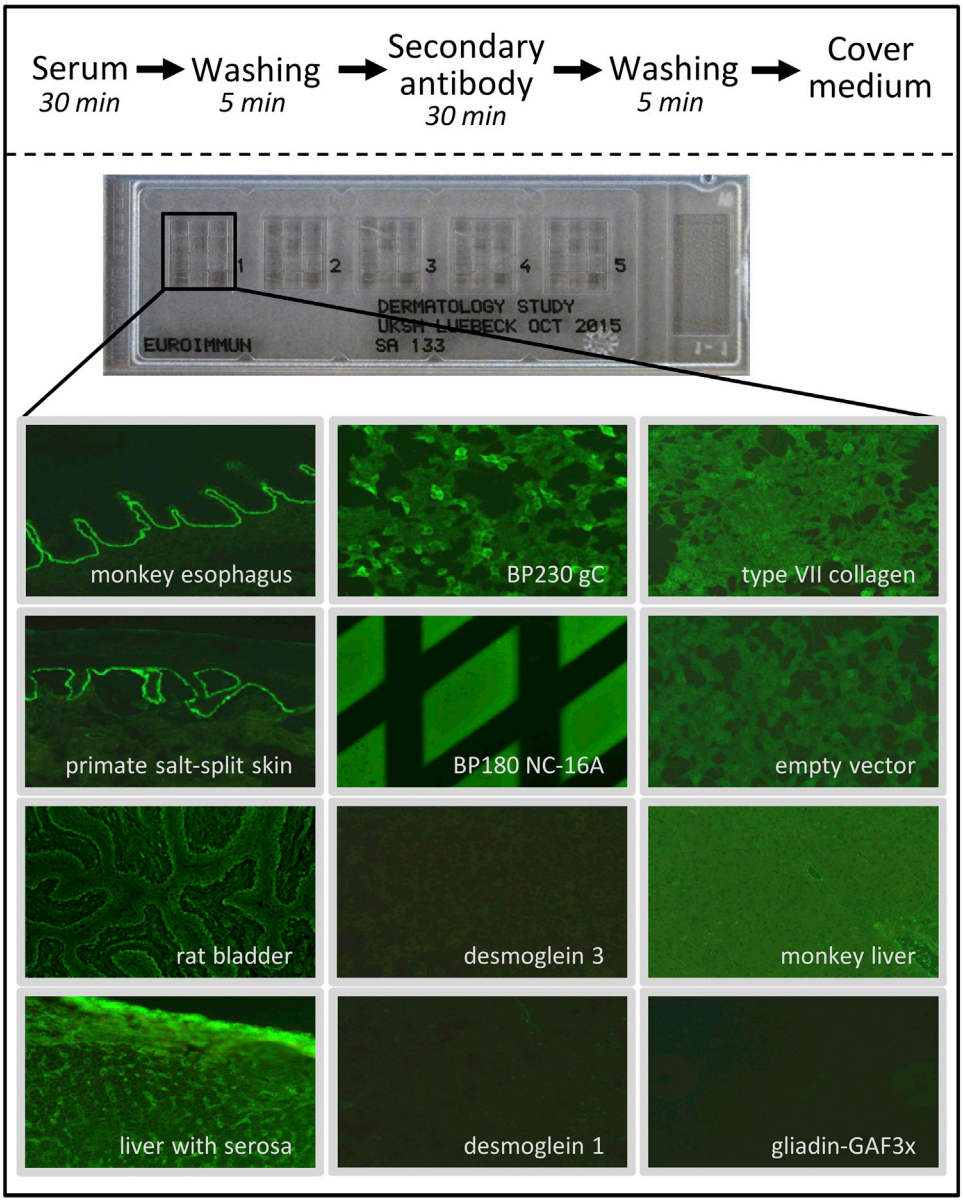


**Fig 1.** The extended BIOCHIP Mosaic for the in-parallel multivariant serologic diagnosis of autoimmune blistering diseases. Representative stainings with linear deposits of IgG and C3 by direct IF and predominant skin lesions was subjected to indirect IF on the BIOCHIP Mosaic containing 12 miniature substrates in 1 incubation field. Reactivity with the basement membrane of monkey esophagus, salt-split skin, and rat bladder, as well as with recombinant BP180 and HEK293 cells expressing BP230, is seen, compatible with the diagnosis of bullous pemphigoid. No reactivity with recombinant gliadin GAF3X, HEK293 cells expressing recombinant desmoglein 1, desmoglein 3, the NC1 domain of type VII collagen, monkey liver, monkey liver with serosa and cells transfected with pTriEx-1 alone (empty vector) is present. Here, 5 incubation fields were placed on a regular-sized laboratory slide. The staining procedure is schematically outlined on top. *IF*, Immunofluorescence.

## RESULTS

### Sensitivities and specificities of the indirect IF BIOCHIP test

Based on the diagnosis by direct IF sensitivities and specificities of the BIOCHIP Mosaic substrates were calculated (Table II).

### The BIOCHIP Mosaic showed a high correlation with direct IF

By the BIOCHIP Mosaic approach, 731 (97.6%) of the 749 patients with AIBD could receive diagnoses in consistency with the direct IF (Fig 2). Of those, BP (Fig 3) was diagnosed in 371 sera (49.5%), of which

**Table II.** Sensitivities and specificities of the indirect IF–based BIOCHIP for IgG secondary autoantibodies compared with IgG/IgG4 in pemphigus (n = 333) and pemphigoid diseases (n = 413)*

| Substrates | IgG | IgG/IgG4 |
|---|---|---|
| Monkey esophagus (intercellular) | | |
| Sensitivity, % | 92.8 | 94.5 |
| Specificity, % | 89.7 | 91.3 |
| Monkey esophagus (basal membrane) | | |
| Sensitivity, % | 59.6 | 72.3 |
| Specificity, % | 100 | 100 |
| Salt-split skin (blister roof/blister floor) | | |
| Sensitivity, % | 96.9 | 97.3 |
| Specificity, % | 97.0 | 95.7 |
| Desmoglein 1 and 3 | | |
| Sensitivity, % | 98.2 | 99.7 |
| Specificity, % | 98.3 | 98.3 |
| BP180 and BP230 | | |
| Sensitivity, % | 82.6 | 83.7 |
| Specificity, % | 96.7 | 96.3 |

*Sensitivities and specificities for rat bladder, monkey liver, endomysium, GAF3X, and type VII collagen are not shown because of the low numbers of patients.

344 (92.7% of BP sera) showed reactivity with BP180 NC16A. Pemphigus vulgaris (Fig 3) was diagnosed in 257 sera (34.3%), all of which had Dsg3 reactivity. Paraneoplastic pemphigus was diagnosed in 4 sera by additional reactivity with plakins on rat bladder (Fig 3). EBA was identified in 4 sera (0.5%), linear IgA dermatosis in 6 (0.8%), and dermatitis herpetiformis in 1 (0.1%) (Fig 3). Another 21 sera (2.8%) stained the blister floor of salt-split skin and the basement membrane of monkey esophagus without reactivity against type VII collagen. In 18 sera (from patients with pemphigus [n = 6], pemphigoid [n = 11], and bullous lupus erythematodes [n = 1]), no autoantibodies were detected by the BIOCHIP Mosaic.

### High agreement between the indirect IF-based BIOCHIP Mosaic approach and the conventional multistep procedure

A high diagnostic concordance was seen between the 2 approaches for pemphigus, BP, paraneoplastic pemphigus, epidermolysis bullosa acquisita, and linear IgA dermatosis (Fig 3). In contrast, anti-p200 and anti-laminin 332 pemphigoid could be diagnosed only by the conventional approach (Fig 3), and in 6 pemphigoid sera unreactive in the BIOCHIP Mosaic, immunoblotting showed anti-BP180 reactivity. Twenty-two pemphigus vulgaris sera showed discrete staining in the BIOCHIP Mosaic with Dsg3, while reactivity by Dsg3 ELISA was below the cutoff (Fig 3). Unexplained discrepant results between the 2 approaches are detailed in Table III.

The use of an anti-IgG4 combined with an anti-IgG secondary antibody (IgG/IgG4) further enhanced the sensitivity of the BIOCHIP Mosaic compared with anti-IgG alone in particular in tissue substrates and transfected cells (data not shown). Of note, 6 (0.8%) of the 749 sera could be diagnosed using only the IgG/IgG4 conjugate (3 pemphigus vulgaris, 2 pemphigus foliaceus, 1 BP).

## DISCUSSION

Recent advances in the detection of autoantibodies in AIBD, that is, the molecular identification of target antigens and the increasing availability of tissue substrates and ELISA systems, have greatly improved the serologic diagnosis of these diseases.[5,11,12] A caveat of these assays is their application as part of a multistep diagnostic approach that may last several days and requires at least 10 times more serum when including immunoblotting analyses compared with the single-assay approach. Multivariant detection systems have been introduced for the parallel diagnosis of several AIBDs based on the BIOCHIP technology and, more recently, as ELISA systems.[15,19] The initially described BIOCHIP Mosaic containing 6 substrates (salt-split primate skin; monkey esophagus; recombinant BP180 NC16A; and HEK cells expressing the BP230 C-terminus, Dsg1, and Dsg3) as well as mosaics compiled of some of these substrates subsequently, showed good and very good diagnostic accuracies when applied by other investigators in other patient cohorts.[16,20,21] A recent review has shown a comparable diagnostic accuracy of the aforementioned mosaic compared with the existing multistep procedure using indirect IF and ELISA methods.[22] Meanwhile, further miniature substrates have been developed for this technology, including the NC1 domain of type VII collagen for the diagnosis of EBA[14,23]; desmocollins 1, 2, and 3 for the diagnosis of rare pemphigus variants[24]; and, most recently, laminin 332 for mucous membrane pemphigoid.[9]

The present study considerably extended the previous studies: (1) additional substrates were included, such as HEK cells expressing the NC1 domain of type VII collagen (for the diagnosis of EBA), monkey liver, monkey liver with serosa, rat bladder (for paraneoplastic pemphigus), and recombinant gliadin-GAF3X for the diagnosis of dermatitis herpetiformis; (2) all sera were tested for IgA autoantibodies; (3) the use of an anti-IgG spiked with an anti-IgG4 detection antibody was shown to be more sensitive than with anti-IgG alone, and

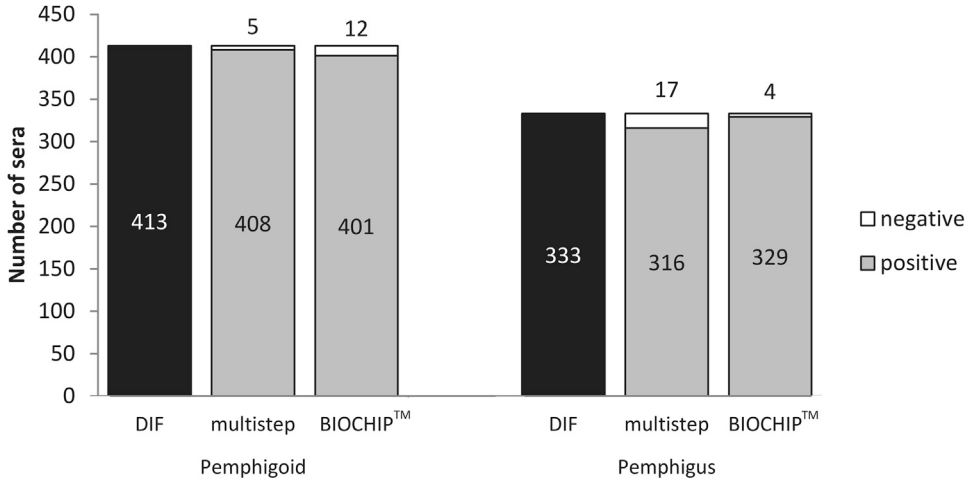


**Fig 2.** High diagnostic accuracy for pemphigus and pemphigoid diseases for both the BIOCHIP Mosaic and the conventional multistep approach compared with results by DIF. Both the extended BIOCHIP Mosaic and the conventional multistep procedure showed a high correlation with the results by DIF (black bars) in patients with both pemphigus (right) and pemphigoid (left). Two patients with dermatitis herpetiformis and 1 with bullous systemic lupus erythematodes are not shown for reasons of simplicity. The number of reactive sera is shown in the corresponding bars (gray columns, positive reactivity; white columns, negative reactivity). *DIF*, Direct immunofluorescence.

(4) the by far largest prospectively collected multicenter cohort of more than 700 patients with AIBD with positive direct IF from 13 centers in Europe, Asia, and Australia was used. Of note, sera were taken before systemic therapy was initiated. The aim of the study was to determine the diagnostic accuracy of the extended BIOCHIP Mosaic, the sensitivity and specificity of the individual substrates, and the value of testing for IgA and IgG/IgG4 autoantibodies.

By this extended BIOCHIP Mosaic, diagnoses in 98.8% if pemphigus and 97.3% of pemphigoid sera were agreement with the direct IF results. This finding was rather surprising and challenges the current guidelines that recommend direct IF as diagnostic criterion standard for AIBD.[3-6] The present data show that in cases where biopsy may be inappropriate for medical or practical reasons, analysis of serum autoantibodies by the BIOCHIP Mosaic or the multistep procedure is nearly equivalent to direct IF microscopy. Of note, only 4 (1.2%) of the 333 pemphigus sera were unreactive with both the Dsg1 and Dsg3 biochips. In a previous study, 2 of these 4 sera were shown to react with recombinant desmocollin 1, 2, or 3 expressed on HEK293 cells also applying the BIOCHIP technology.[24]

The second part of the study analyzed the concordance between results by the multivariant BIOCHIP Mosaic and those obtained by the conventional multistep approach. Here, a very high agreement between the 2 approaches was observed in patients with pemphigus vulgaris, pemphigus foliaceus, paraneoplastic pemphigus, BP, EBA, linear IgA disease, and dermatitis herpetiformis. The majority of discrepancies were due to the lack of laminin 332 and p200/laminin γ1 in the BIOCHIP Mosaic. Very recently, a widely available BIOCHIP Mosaic with the recombinant laminin 332 trimer showed a sensitivity of 84% and a specificity of 100% in a multicenter study.[9] Development of a biochip with recombinant laminin γ1 for the serologic diagnosis of anti-p200/laminin γ1 pemphigoid is in progress. The discrepancies in 22 pemphigus sera that showed weak labeling of Dsg3-expressing cells in the BIOCHIP Mosaic but reactivity below the cutoff by Dsg3 ELISA can be explained by the slightly higher sensitivity of the Dsg3 biochip (6 of the 413 pemphigoid sera reacted in the Dsg3 biochip, data not shown). In only 5 (0.7%) of the 749 sera, discrepant results between the single-step and multistep approaches could not be explained (Table III).

In summary, the extended and modified AIBD-specific BIOCHIP Mosaic detected serum Aab in nearly 98% of AIBD sera of a large multicenter cohort and showed a very high diagnostic agreement with the conventional multistep approach. The current biochip-based assay is a time- and serum-saving, standardized, widely available, and easy-to-use tool for the serologic diagnosis of AIBD. Further efforts aim at including additional target antigens, such a laminin γ1 and the BP180 ectodomain, and providing an automated readout.

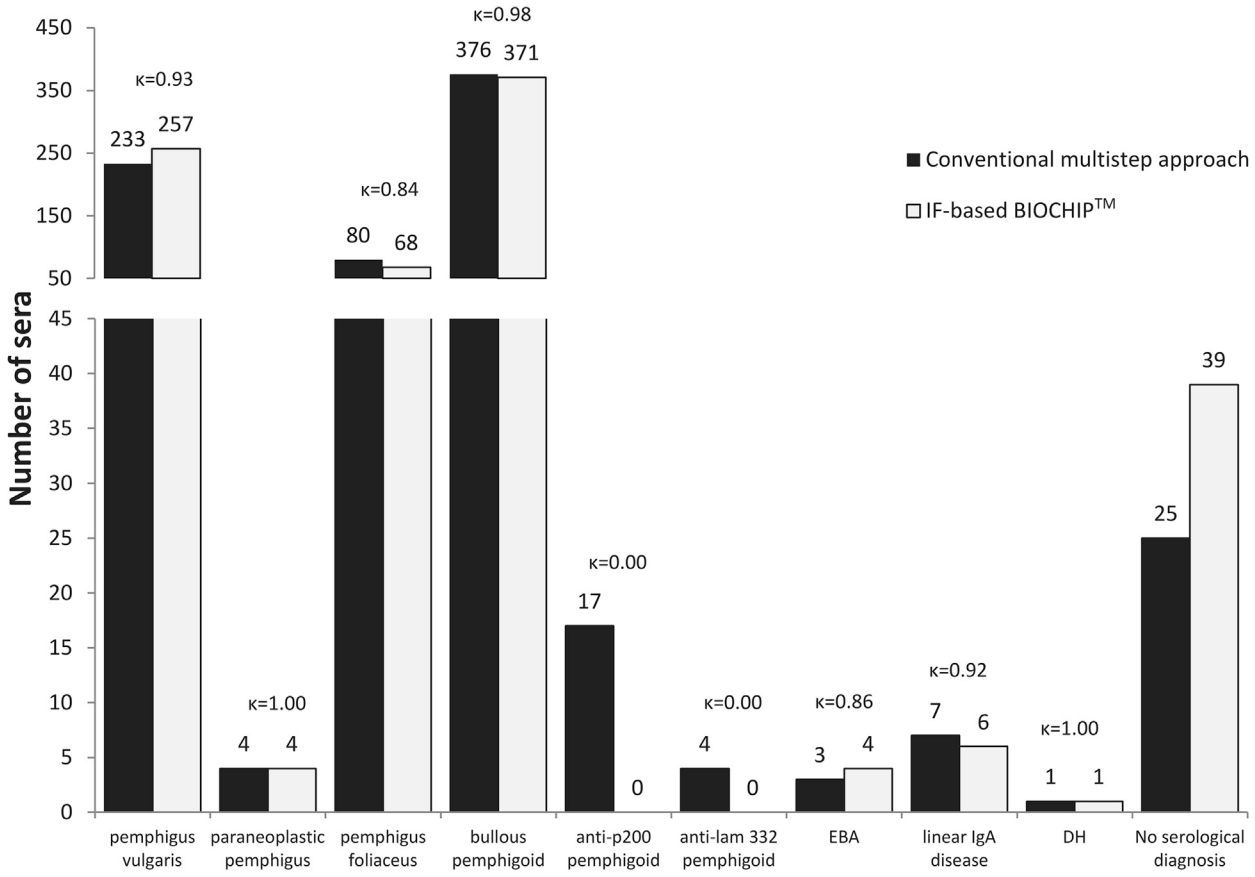


**Fig 3.** High diagnostic agreement between the extended BIOCHIP Mosaic and the conventional multistep procedure. A high agreement between the indirect IF–based BIOCHIP (white bars) and the conventional multistep procedure (black bars) was observed when applying sera from 749 consecutive patients with AIBD with positive direct IF. The degree of agreement is indicated by Cohen $\kappa$ values. A Cohen $\kappa$ value of 0.41-0.60 is regarded as moderate concordance, $\kappa$ values of 0.61-0.80 as substantial, and $\kappa$ values of 0.81 or greater as almost perfect concordance.[18] One serum with features of BP and PV is shown in both groups, resulting in a total of 750 diagnoses for 749 sera. *BP*, Bullous pemphigoid; *DH*, dermatitis herpetiformis; *EBA*, epidermolysis bullosa acquisita; *IF*, immunofluorescence; *LAD*, linear IgA disease; *lam*, laminin; *PF*, pemphigus foliaceus; *PNP*, paraneoplastic pemphigus; *PV*, pemphigus vulgaris.

**Table III.** Unexplained discrepant results between the BIOCHIP Mosaic and the conventional multistep approach

| Disease based on direct IF | Number of patients | BIOCHIP Mosaic | Multistep diagnostics |
|---|---|---|---|
| Pemphigoid | 2 | BP180$^{+}$ | BP180$^{-}$ |
| | 1 | IgA col VII$^{+}$ | IgA NC16A$^{+}$ |
| Pemphigus | 2 | Dsg 3$^{+}$ | Dsg 3$^{-}$ |

*BP180,* Recombinant BP180 NC16A; *Dsg 3*, desmoglein 3; *IgA col VII*, IgA reactivity against type VII collagen; *IgA NC16A*, IgA reactivity in immunoblotting with BP180 NC16A.

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