Research Article
Volume 9:6, 2023

Immunochemistry & Immunopathology

ISSN: 2469-9756 **Open Access**

Performance Characteristics of a Novel, Fully Automated Planar Microarray Immunoassay for the Detection of Antibodies against Centromere Protein B

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Abstract

Introduction: Autoantibody testing, including against Centromere Proteins (CENP), is important in the identification of autoimmune diseases. Test methods may be manual, slow, labor-intensive and/or fragmented. Development of highly automated tools is needed. We aimed to evaluate the performance of MosaiQ® CENP-B (CENP-B-MA), a planar microarray immunoassay designed for use with the fully automated, continuous random access, high-throughput MosaiQ System, for the qualitative serological detection of anti-CENP-B autoantibodies (ACA-B) as an aid in the diagnosis of systemic sclerosis.

Methods: A comparator study was performed at Hôpital Pitié-Salpêtrière, Paris, France using anonymized serum samples, characterized as ACA-B non-reactive or reactive with CE-marked devices. Reproducibility and repeatability evaluations were also performed.

Results: After resolver testing, CENP-B-MA identified 96/99 samples characterized as reactive and all 199 non-reactive samples: positive percent agreement: 97% (95% CI, 91.4%, 99.4%), negative percent agreement: 100% (95% CI, 98.2%, 100%), overall percent agreement: 99% (95% CI, 97.1%, 99.8%). Reproducibility and repeatability evaluation showed, respectively, overall agreement of 99.7% (95% CI, 99.4%, 99.9%) and 100%.

Conclusion: Performance of CENP-B-MA shows high concordance with other CE-marked assays for detecting ACA-B, with a high degree of reproducibility and repeatability.

Keywords: Anti-centromere auto-antibodies • Systemic sclerosis • Immunoassay • Microarray

Introduction

Centromere Proteins (CENP) play an important role in cell division [1-3]. Autoantibodies targeting these proteins are associated with Autoimmune Disease (AID) [4]. At least six CENP, designated CENP-A through CENP-F, have been described in association with an AID such as Systemic Sclerosis (SSc) [4], with CENP-B considered as primary target of the B cell anti-CENP response [3,5-7]. Anti-CENP Autoantibodies (ACA) are found with low prevalence in different autoimmune Connective Tissue Diseases (CTD) (1-10%) and also in healthy individuals (<3%) [8]; however, they occur in 20–40% patients with SSc [9], its main clinical association, a systemic AID characterized by progressive fibrosis of the skin and internal organs, small vessel vasculopathy and antibody production [10-13]. Presence of ACA is included in the joint SSc classification criteria of the American College of Rheumatology (ACR) and the European Alliance of Associations for Rheumatology (EULAR) [14]. ACA are associated with the subset Limited cutaneous SSc (lcSSc), risk for Pulmonary Hypertension (PAH) [5,15] and with a better prognosis and survival than other SSc antibodies, like anti-Scl-70 [4,6,15], particularly in those without PAH [6].

Autoantibody testing is a valuable tool supporting both the diagnosis and prognosis assessment in SSc and other CTD. Indirect Immunofluorescence Assay (IFA), using HEp-2 cells as a substrate [16,17], is commonly used as the initial screening of Antinuclear Antibodies (ANA). Other methods include Enzyme-Linked Immunosorbent Assay (ELISA), immunodiffusion and immunoblotting [5], each having advantages and limitations [8,16,18,19]. Different current test methods differ in performance, are slow, manual, fragmented, labor-intensive, have a low throughput, require special resources, or do not facilitate the simultaneous detection of several autoantibodies that could reduce time to provide comprehensive results to clinicians. Although there are currently available automated or semi-automated platforms using various technologies, the development of highly automated testing tools is still needed.

We report the performance characteristics of MosaiQ® CENP-B Microarray (CENP-B-MA) (AliveDx, Eysins, Switzerland), a new solid phase immunoassay microarray designed for use with the fully automated, continuous random access, high throughput MosaiQ System (AliveDx, Eysins, Switzerland), for qualitative serological detection of autoantibodies directed against CENP-B; compared with selected CE-marked devices.

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Received: 24 November, 2023, Manuscript No. icoa-23-121048; **Editor assigned:** 27 November, 2023, Pre QC No. P-121048; **Reviewed:** 11 December, 2023, QC No. Q-121048; **Revised:** 16 December, 2023, Manuscript No. R-121048; **Published:** 23 December, 2023, DOI: 10.37421/2469-9756.2023.9.207

Materials and Methods

Ethical considerations

All samples used in this study were de-identified in accordance with local institutional requirements. Neither personal health information nor linkage to sample identification was provided to the Study Sponsor. The study was conducted under Good Clinical Practices and in compliance with the Declaration of Helsinki.

The MosaiQ system

The MosaiQ system (Figure 1) consists of the MosaiQ 125 instrument, CENP-B Magazine, containing 250 CENP-B-MA and associated reagents (sample diluent, wash buffers, detection reagent and enhancement reagents; featuring Radio Frequency Identification [RFID]) needed to run the assay. Instrument set-up is approximately 35 min and includes loading of up to four magazines (i.e., 1,000 individual tests), buffers, reagents, independent internal quality controls and primary patient serum sample tubes. Fully automated tests steps include mixing of serum with sample diluent, addition to microarray, incubation for 11 min, series of wash steps, addition of detection reagent, incubation for 16 min, wash (x^2), addition of enhancement reagent 1, then enhancement reagent 2 (to nucleate silver around gold nanoparticles of the secondary antibody), incubation for 3.5 min and final wash. The reaction generates immunoassay spot signals that are interpreted by the instrument using a proprietary image analysis algorithm (Figure 2).

Each CENP-B-MA (Figure 2) consists of two sides containing coated-glass chips. CENP-B antigen is printed on one side of the glass with appropriate test controls, leaving the other side without printed probes and available for future addition of antigens. Assay's analytical characteristics were established in accordance with the European Union *in vitro* diagnostics regulations [Regulation (EU) 2017/746 (EU IVDR)].

Internal quality control

The CENP-B-MA contains eight internal reactive and two non-reactive control probes: Four reactive control spots printed with BSA-gold, located in the corner positions of the microarray panel are used to define the location of the printed antigen probes within the microarray to enable processing of the image. Two reactive control spots printed with BSA-gold are present to demonstrate the addition of enhancement reagents. Two immunoglobulin reactive control spots are used as a process control for addition of the detection reagent. Finally, two non-reactive control spots composed of the same print buffers used for formulation of target-specific antigen spots are used to demonstrate that no non-specific results occur due to print matrix. All internal controls must give the appropriate rection for a valid CENP-B-MA result to be generated.

Figure 1. A) MosaiQ CENP-B microarray, **B)** MosaiQ CENP-B magazine and **C)** MosaiQ 125 instrument.

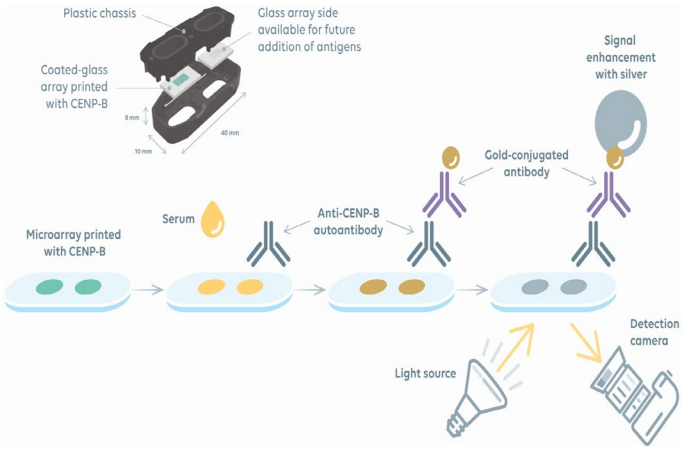

Figure 2. MosaiQ CENP-B microarray and illustration of the assay's reactions.

Independent internal quality controls

CENP-B-MA independent internal Quality Controls (QC) consist of one vial made of pooled human sera reactive for ACA-B titrated to an appropriate level and one pooled serum non-reactive for ACA-B (Theradiag, Croissy Beaubourg, France). Quality control was performed daily and reactions validated prior to sample testing.

Performance evaluation- comparator study

Following positive preliminary results with the CENP-B-MA prototype [20], a performance evaluation in a clinical laboratory testing environment was performed.

Samples: Previously characterized ACA-B reactive or ACA-B non-reactive serum samples, collected and tested for routine diagnostic purposes, were provided by and tested with CENP-B-MA at the Hôpital Pitié-Salpêtrière. A total of 103 samples ACA-B reactive by routine testing was randomly selected from a sample bank stored at ≤ -20 °C (frozen) until CENP-B-MA testing. Fresh samples (n=200) characterized as non-reactive for ACA-B by routine testing were stored at 2-8 °C for up to 5 days until tested with the CENP-B-MA. Acceptable specimens were limited to serum, with adequate volume to conduct study testing with CENP-B-MA, comparator devices and additional discordant investigations using different testing methods (if required). Frozen samples had no more than three freeze-thaw cycles. Samples were excluded in case of inadequate volume, had signs of contamination or were inadequately stored. Samples were retained at 2-8 °C until all discordant test results were investigated and resolved. Specimens were anonymized according to institutional policies and no additional samples were collected.

Comparator devices: Samples were previously characterized as ACA-B reactive or ACA-B non-reactive with one or more of the following CE-marked Comparator devices: ANA-Ro IgG FLUORESCENT HEp-2000® (ANA-IFA) (Immuno Concepts, Hannover, Germany), ANAscreen (ANA-ELISA) (ORGENTEC Diagnostika GmbH, Mainz, Germany) and FIDIS™ Connective Profile (FIDIS, a semi-quantitative fluorescent-based microparticles immunoassay using flow cytometry readings; Theradiag, Croissy Beaubourg, France), according to local diagnostic testing algorithms. Resolver testing with Immunoblot (EUROLINE Systemic sclerosis, Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany) was used when required. All samples were initially screened by ANA-IFA. Reactive samples by ANA-IFA were tested using FIDIS and Immunoblot (when indicated). Samples ANA-IFA non-reactive was tested with ANA-ELISA. If both ANA-IFA and ELISA were non-reactive, no further comparator testing was performed.

Disposition of samples for CENP-B-MA in the comparator study: Samples (n=103) were characterized as ACA-B reactive using a Composite Comparator Result (CCR), as follows: ANA-IFA reactive/FIDIS ACA-B reactive; ANA-IFA reactive/FIDIS ACA-B non-reactive/ANA pattern consistent with ACA-B and/or Immunoblot reactive for ACA-B. Samples (n=200) were characterized by CCR as ACA-B non-reactive as follows: ANA-IFA non-reactive and ANA-ELISA non-reactive.

CENP-B-MA testing protocol: Samples were processed in accordance with the CENP-B-MA Instructions For Use (IFU). CENP-B-MA results were reported as: Reactive (R): Testing completed, no further testing required; Non-Reactive (NR) no further testing required; Indeterminate (IND): No result determined; Data Reduction Error (DRE); failure to detection localization controls; Processing Errors (PE): Related to the sample (e.g., presence of bubbles or fibrin) rendering no result. Samples reported as IND, DRE or PE

was retested once, within 24 hours of initial testing. Samples yielding a double IND, PE or DRE were excluded from the data analysis.

Definition of CENP-B-MA samples: Samples both CENP-B-MA and CCR ACA-B non-reactive were considered CENP-B-MA true non-reactive. Samples both CENP-B-MA and CCR ACA-B reactive were considered CENP-B-MA true reactive. Samples CENP-B-MA ACA-B non-reactive and CCR ACA-B reactive were considered CENP-B-MA false non-reactive. Samples CENP-B-MA ACA-B reactive and CCR ACA-B non-reactive were considered CENP-B-MA false reactive. For frozen samples, due to a potential for sample degradation during storage, CENP-B-MA results discordant with FIDIS were retested on this comparator. If the FIDIS retest was invalid the sample was excluded from the analysis. If a discordant CENP-B-MA result was found to be concordant upon FIDIS retest, the sample would be included in the analysis with the final concordant result. If a discordant CENP-B-MA result was confirmed discordant with FIDIS retest, when indicated, resolver testing using immunoblotting techniques was performed and research team adjudicated the results by examining the immunofluorescence pattern on HEp-2 cells. For fresh ACA-B non-reactive samples: If a discordant CENP-B-MA result was determined *vs.* FIDIS, resolver testing using immunoblotting techniques were performed when indicated and the research team adjudicated the results by examining the immunofluorescence pattern on HEp-2 cells.

Reproducibility and repeatability studies

Designed based on the recommendations of guideline EP05-A3 by the Clinical and Laboratory Standards Institute (CLSI) [21], a reproducibility evaluation was conducted to assess lot-to-lot, day-to-day, operator-to-operator, intra run-to-run, inter run-to-run and instrument-to-instrument variability, whereas repeatability evaluation assessed variability among replicate measurements of a sample under experimental conditions held as constant as possible. A reproducibility panel was composed of three samples: One ACA-B non-reactive sample, one ACA-B low reactive sample and one ACA-B high reactive sample, made from pooled human sera non-reactive for ACA-B or containing ACA-B spiked at two different levels into pooled human sera. CENP-B-MA testing was performed at AliveDx (Eysins, Switzerland) over five days using three different MosaiQ 125 instruments, three different operators and three different CENP-B-MA Magazine lots. Each lot was tested twice per day on all three instruments. Ten replicates of each panel member were tested in each run. On each day of testing, 180 tests were performed, 900 tests in total performed on each instrument, for a final total of 2,700 tests performed. The repeatability study considered the data generated on one instrument and one lot, based on the premises of no variability expected between instruments and lots. A total of 300 tests were performed.

Statistical analysis

Concordant, discordant and total results of CENP-B-MA *vs.* CCR were evaluated. Target positive (reactive) Percent Agreement (PPA), Negative (non-reactive) Percent Agreement (NPA) and Overall Percent Agreement (OPA) with CCR was set ≥ 90% with a double-sided 95% Confidence Interval (CI) calculated using Clopper-Pearson Exact Method. Reproducibility and repeatability study results with 95% CI were calculated using Clopper-Pearson Exact Method.

Results

Performance evaluation- comparator study

A total of 103 sera ACA-B reactive and 200 sera ACA-B non-reactive as per CCR were enrolled in the study. Four samples were initially excluded due to double DREs (three ACA-B reactive and one ACA-B non-reactive by CCR) for a final total of 299 comparable results (199 ACA-B non-reactive and 100 ACA-B reactive).

All fresh samples 199 of 199 (100%) pre-defined as ACA-B non-reactive by CCR resulted non-reactive with CENP-B-MA. Of the 100 frozen samples pre-defined as ACA-B reactive by CCR, 96 resulted ACA-B reactive and four resulted ACA-B non-reactive with CENP-B-MA, demonstrating an initial PPA of 96.0% (95% CI, 90.1%, 98.9%), NPA of 100% (95% CI, 98.2%, 100%) and OPA of 98.7% (95% CI, 96.6%, 99.6%) (Table 1).

As per protocol, the four frozen samples with discordant ACA-B false non-reactive results with CENP-B-MA were retested on FIDIS, to determine if frozen storage may have impacted the stability of the sample leading to a false non-reactive CENP-B-MA result. One discordant sample gave an invalid repeat FIDIS result and was, therefore, excluded from the analysis, leaving 99 pre-characterized ACA-B reactive samples for evaluation. The remaining three discordant samples were found to be FIDIS repeat ACA-B reactive and were considered as CENP-B-MA false non-reactive (Table 2). The three remaining discordant CENP-B-MA false non-reactive samples were repeated on CENP-B-MA and were found to be reactive. The repeat CENP-B-MA data was not included in the analysis and samples remained classified as CENP-B-MA false non-reactive.

The three CENP-B-MA false non-reactive (FIDIS reactive) samples were further examined to verify coherence between the results obtained with FIDIS based upon ANA-IFA pattern on HEp-2 cells and resolver testing with immunoblot. Both ANA-IFA and resolver testing confirmed the CENP-B-MA results were false non-reactive. Without and with resolver testing, the final results were the same as shown in Table 2. CENP-B-MA PPA was 97% (95% CI, 91.4%, 99.4%), NPA was 100% (95% CI, 98.2%, 100%) and OPA was 99% (95% CI, 97.1%, 99.8%).

FIDIS comparison to composite comparator results

FIDIS results were independently compared to CCR. Overall, FIDIS was false non-reactive for four samples (Table 3) which was confirmed by ANA-IFA. Additionally, the four FIDIS false non-reactive samples were reported as CENP-B-MA reactive. PPA and OPA for FIDIS were 96% (95% CI, 90.0%, 98.9%) and 98.7% (95% CI, 96.6%, 99.6%), respectively. FIDIS demonstrated a 100% NPA (95% CI, 98.2%, 100%).

Correlations with clinical diagnosis and ANA-IFA patterns

The demographic characteristics of the 99 ACA-B reactive samples included in the analysis were as follows, median age 57 years (standard deviation 15.25 years, range 8 to 88 years), 94 (94.95%) females. Clinical diagnosis information was available for all 99 (100%) reactive samples (Table 4). Of those, 37 (37.37%, 35 female) were directly related to SSc (recorded as CREST: 27, SSc: 9, Reynolds syndrome: 1); 5 (5.05%, all female) were recorded as Raynaud's phenomenon or disease. Sjögren's syndrome was the diagnosis in 11 (11.11%, 10 female) samples; 7 (7.07%, all female) had mixed

Table 1. Initial testing: MosaiQ CENP-B-MA *vs.* composite comparator results[a].

	MosaiQ CENP-B-MA			
Composite Comparator Results	**R**	**NR**	**IND[b]**	**Total**
R	96	4	0	100
NR	0	199	0	199
Total	96	203	0	299

MosaiQ CENP-B-MA *vs.* Composite Comparator Results	**Percent Agreement (%)**	**95% CI[c]**	-	-
Positive	96.0	(90.1, 98.9)	-	-
Negative	100	(98.2, 100)	-	-
Overall	98.7	(96.6, 99.6)	-	-

Abbreviations: R: Reactive; NR: Non-Reactive; IND: Indeterminate; CI: Confidence Interval

a. Composite comparator results included: ANA-IFA, ANA-ELISA, FIDIS and immunoblot (if indicated).
b. Counts of Double Indeterminate (IND) and data reduction error results not included in % agreement calculations.
c. Two-sided 95% cI using Clopper-Pearson exact method.

Table 2. MosaiQ CENP-B-MA *vs.* composite comparator results[a] after repeating testing of MosaiQ CENP-B-MA discordant samples with FIDIS™ Connective Profile.

		MosaiQ CENP-B-MA		
Composite Comparator Results	**R**	**NR**	**IND[b]**	**Total**
R	96	3[c]	0	99
NR	0	199	0	199
Total	96	202	0	298

MosaiQ CENP-B-MA *vs.* Composite Comparator	**Percent Agreement (%)**	**95% CI**	-	-
Positive	97.0	(91.4, 99.4)	-	-
Negative	100	(98.2, 100)	-	-
Overall	99.0	(97.1, 99.8)	-	-

Abbreviations: R: Reactive; NR: Non-Reactive; IND: Indeterminate; CI: Confidence Interval

a. Composite comparator results included: ANA-IFA, ANA-ELISA, FIDIS and immunoblot (if indicated).
b. Counts of Double Indeterminate (IND) and data reduction error results not included in % agreement calculations.
c. One sample was excluded from analysis as per protocol due to invalid repeat on FIDIS.
d. Two-sided 95% cI using Clopper-Pearson exact method.

Table 3. FIDIS *vs.* composite comparator results[a] after repeating test of MosaiQ CENP-B-MA discordant samples with FIDIS™ Connective Profile.

		FIDIS	
Composite Comparator Results	**R**	**NR**	**Total**
R	95	4	99[b]
NR	0	199	199
Total	95	203	298

FIDIS *vs.* Composite Comparator Results	**Percent Agreement (%)**	**95% CI[c]**	-
Positive	96.0	(90.0, 98.9)	-
Negative	100	(98.2, 100)	-
Overall	98.7	(96.6, 99.6)	-

Abbreviations: R: Reactive; NR: Non-Reactive; IND: Indeterminate; CI: Confidence Interval

a. Composite comparator results included: ANA-IFA, ANA-ELISA, FIDIS and Immunoblot (if indicated).
b. One sample was excluded from analysis as per protocol due to invalid repeat on FIDIS.
c. Two-sided 95% CI using Clopper-Pearson exact method.

Table 4. Clinical diagnosis, gender and age range of ACA-B reactive samples.

Diagnosis	**Cases n (%)**	**Females n (%)**	**Age Range (years)**
Systemic sclerosis	9 (9.09)	8 (88.88)	8 – 86
CREST syndrome[a]	27 (27.27)	26 (96.29)	33 – 78
Reynolds syndrome[b]	1 (1.01)	1 (100)	-
Raynaud's phenomenon/disease	5 (5.05)	5 (100)	43 – 80
Sjögren's syndrome	11 (11.11)	10 (90.91)	51 – 88
Sharp's syndrome[c]	7 (7.07)	7 (100)	40 – 82
Lupus	7 (7.07)	7 (100)	22 – 73
Idiopathic inflammatory myopathies	6 (6.06)	6 (100)	35 – 76
Others	26 (26.26)	24 (92.31)	29 – 76
Total	99 (99.99)[d]	94 (94.95)	8 – 88

Abbreviation: n: number

a. Also referred to as lcSSc.
b. Association of Primary Biliary Cirrhosis (PBC) with lcSSc.
c. Refers to mixed connective tissue disease.
d. Result is 100% when including all decimals.

connective tissue disease (MCTD, recorded as Sharp's syndrome: 7 or Sharp's scleroderma: 1); 7 (7.07%, all female) had lupus; and 6 (6.06%, all female) had Idiopathic Inflammatory Myopathies (IIM). Diagnosis in the remaining 26 (26.26%) samples included multiple different conditions varying from other autoimmune diseases to cancer. In the 99 ACA-B reactive included samples, the centromere ANA-IFA pattern was the most frequent, observed as primary pattern in 86 (86.87%) samples and as secondary pattern in 6 (6.06%) samples and it was the primary pattern in all specimens with diagnosis of SSc, CREST, Reynolds syndrome, Raynaud's disease and Raynaud's phenomenon; except for a sample with diagnosis of SSc in which the primary pattern was speckled and centromere, the secondary pattern.

CENP-B-MA reproducibility study

Overall, from the 2,700 expected results, 2,572 were included in the analysis, with 128 excluded from the analysis due to single IND (n=1) or single DRE (n=127). Agreement with expected results was observed in 2,565 of the 2,572 included tests, for an overall reproducibility agreement with expected results by Instrument, Lot and Operator of 99.7% (2,565/2,572 tests; 95% CI, 99.4%, 99.9%) (Table 5). Low reactive ACA-B sample agreement was 99.2% (846/853 tests; 95% CI, 98.3%, 99.7%), whereas non-reactive ACA-B sample agreement and high reactive ACA-B sample agreement were both 100%, in 867/867 and 852/852 tests, respectively (95% CI, 99.6%, 100%) (Table 5).

CENP-B-MA repeatability study

Repeatability was calculated on the data generated on one Instrument and one CENP-B-MA Magazine lot. After excluding from the analysis 25 results due to single DRE (as per protocol), a total, 275 results were included, out of

Table 5. Reproducibility agreement with expected results by instrument, lot, operator.

Instrument	Lot	Operator	Agreement[a] Non-reactive (NPA) % (n/N)[b]	Agreement[a] Low reactive (PPA) % (n/N)[b]	Agreement[a] High reactive (PPA) % (n/N)[b]	Agreement[a] Overall (OPA) % (n/N)[b]
SN101	1100049222	1	100 (44/44)	100 (50/50)	100 (43/43)	100 (137/137)
	1100049222	2	100 (48/48)	100 (46/46)	100 (44/44)	100 (138/138)
	1100049232	1	100 (48/48)	100 (48/48)	100 (47/47)	100 (143/143)
	1100049232	2	100 (48/48)	100 (48/48)	100 (47/47)	100 (143/143)
	1100049239	1	100 (49/49)	100 (47/47)	100 (47/47)	100 (143/143)
	1100049239	2	100 (47/47)	100 (47/47)	100 (47/47)	100 (141/141)
SN107	1100049222	1	100 (48/48)	100 (48/48)	100 (50/50)	100 (146/146)
	1100049222	3	100 (49/49)	98.0 (49/50)	100 (46/46)	99.3 (144/145)
	1100049232	1	100 (48/48)	100 (49/49)	100 (48/48)	100 (145/145)
	1100049232	3	100 (47/47)	100 (45/45)	100 (47/47)	100 (139/139)
	1100049239	1	100 (46/46)	91.3 (42/46)	100 (47/47)	97.1 (135/139)
	1100049239	3	100 (49/49)	97.8 (45/46)	100 (50/50)	99.3 (144/145)
SN131	1100049222	2	100 (48/48)	97.9 (47/48)	100 (48/48)	99.3 (143/144)
	1100049222	3	100 (50/50)	100 (46/46)	100 (48/48)	100 (144/144)
	1100049232	2	100 (48/48)	100 (46/46)	100 (47/47)	100 (141/141)
	1100049232	3	100 (50/50)	100 (47/47)	100 (49/49)	100 (146/146)
	1100049239	2	100 (50/50)	100 (47/47)	100 (48/48)	100 (145/145)
	1100049239	3	100 (50/50)	100 (49/49)	100 (49/49)	100 (148/148)
Overall [95% CI[c]]	-	-	100 (867/867) [99.6, 100]	99.2 (846/853) [98.3, 99.7]	100 (852/852) [99.6, 100]	99.7 (2,565/2,572) [99.4%, 99.9%]

Abbreviations: NPA: Negative Percent Agreement; PPA: Positive Percent Agreement; OPA: Overall Percent Agreement; CI: Confidence Interval; SN: Instrument Serial Number

a. Counts of indeterminate and data reduction error results not included in % agreement calculations.
b. % (n/N): Percentage agreement (number correct/number tested).
c. Two-sided 95% CI using Clopper-Pearson exact method.

Table 6. Repeatability agreement with expected results by day (inter-run), run (intra-run) (instrument and magazine lot variability not considered).

Day	Run	Agreement[a] Non-reactive (NPA) % (n/N)[b]	Agreement[a] Low reactive (PPA) % (n/N)[b]	Agreement[a] High reactive (PPA) % (n/N)[b]	Agreement[a] Overall (OPA) % (n/N)[b]
Day 1	Run 1	100 (8/8)	100 (10/10)	100 (8/8)	100 (26/26)
	Run 2	100 (10/10)	100 (9/9)	100 (10/10)	100 (29/29)
Day 2	Run 1	100 (10/10)	100 (10/10)	100 (8/8)	100 (28/28)
	Run 2	100 (10/10)	100 (9/9)	100 (9/9)	100 (28/28)
Day 3	Run 1	100 (10/10)	100 (10/10)	100 (9/9)	100 (29/29)
	Run 2	100 (10/10)	100 (10/10)	100 (9/9)	100 (29/29)
Day 4	Run 1	100 (9/9)	100 (10/10)	100 (9/9)	100 (28/28)
	Run 2	100 (10/10)	100 (10/10)	100 (8/8)	100 (28/28)
Day 5	Run 1	100 (7/7)	100 (10/10)	100 (9/9)	100 (26/26)
	Run 2	100 (8/8)	100 (8/8)	100 (8/8)	100 (24/24)
Overall Day 1 [95% CI[c]]	-	100 (18/18) [81.5, 100]	100 (19/19) [82.4, 100]	100 (18/18) [81.5, 100]	100 (55/55) [93.5, 100]
Overall Day 2 [95% CI]	-	100 (20/20) [83.2, 100]	100 (19/19) [82.4, 100]	100 (17/17) [80.5, 100]	100 (56/56) [93.6, 100]
Overall Day 3 [95% CI]	-	100 (20/20) [83.2, 100]	100 (20/20) [83.2, 100]	100 (18/18) [81.5, 100]	100 (58/58) [93.8, 100]
Overall Day 4 [95% CI]	-	100 (19/19) [82.4, 100]	100 (20/20) [83.2, 100]	100 (17/17) [80.5, 100]	100 (56/56) [93.6, 100]

Overall Day 5 [95% CI]	-	100 (15/15) [78.2, 100]	100 (18/18) [81.5, 100]	100 (17/17) [80.5, 100]	100 (50/50) [92.9, 100]
Overall Run 1 [95% CI]	-	100 (44/44) [92.0, 100]	100 (50/50) [92.9, 100]	100 (43/43) [91.8, 100]	100 (137/137) [97.3, 100]
Overall Run 2 [95% CI]	-	100 (48/48) [92.6, 100]	100 (46/46) [92.3, 100]	100 (44/44) [92.0, 100]	100 (138/138) [97.4, 100]

Abbreviations: NPA: Negative Percent Agreement; PPA: Positive Percent Agreement; OPA: Overall Percent Agreement; CI: Confidence Interval

a. Counts of indeterminate and data reduction error results not included in % agreement calculations.

b. % (n/N): Percentage agreement (number correct/number tested).

c. Two-sided 95% CI using Clopper-Pearson exact method.

the 300 tests performed on Table 6. Expected results for each panel member and overall agreement were tabulated by day (inter-run) and by run (intra-run) for the relevant instrument and magazine lot. The observed repeatability performance across all factors and samples (day and run) was 100% (275 of 275 tests).

Indeterminate, data reduction errors and processing error rates

Rates of unreportable results (IND, DRE, PE) were evaluated to assess instrument and assay robustness. In the comparator study, the initial DRE rate was 1.7% (5 of 303 tests) and upon retest one sample resolved for a final DRE rate (no result reported) of 1.3% (4/303). The IND rate upon first test was 0.33% (1/299) that resolved on retest for a final IND rate of 0%. No PE were reported and quality control was consistent and never resulted in failure. In the reproducibility and repeatability study, samples generating a DRE or IND result were not repeated. There was a total of 127 DREs out of 2,700 tests, for a first test DRE rate of (4.7%). There was one indeterminate result (0.04%) recorded in the study.

Safety

No device adverse events were reported.

Discussion

This study describes the performance of a novel immunoassay microarray-based testing system for the qualitative detection of ACA-B in human serum samples and the assay's reproducibility and repeatability.

In the comparator study, after analysis of four CENP-B-MA false non-reactive discordant results, CENP-B-MA correctly identified 96 of the 99 samples characterized as ACA-B reactive by comparators and all 199 non-reactive samples (PPA: 97%, NPA: 100%, OPA: 99%), showing high correlation with composite comparator results. In the reproducibility study, overall agreement with expected results (by instrument, lot and operator) was 99.7%, whereas repeatability evaluation showed 100% agreement, exhibiting the precision of the device.

There is limited data on the performance of stand-alone test devices for detection of autoantibodies against CENP. EliA™ CENP (Phadia GmbH, Freiburg, Germany), is a CE-marked, FDA-cleared fluoroenzyme immunoassay designed for the *in vitro* semi-quantitative measurement of IgG antibodies directed to CENP in human serum and plasma using Phadia™ series instruments. The instructions for use of this device include data on a comparison against a predicate, using 150 samples, showing, respectively, PPA, NPA and OPA in the agreement calculations, with equivocal results scored as negative, of 98.1% (95% CI, 90.1, 100), 100% (95% CI, 96.2, 100) and 99.3% (95% CI, 96.3, 100); and of 71.6% (95% CI, 59.9, 81.5), 100% (95% CI, 95.3, 100) and 86.0% (95% CI, 79.4, 91.1), for the agreement calculations with equivocal results scored as positive [22]. More data available on ACA-B tests come from multiplexed devices, in general, with favorable results for this analyte. In a prospective, multicentric study evaluating ANA by a multiplex immunoassay using fluorescent microspheres (beads) with flow cytometry readings, compared with enzyme immunoassay for the detection of 13 autoantibodies including ACA-B, the authors reported a PPA 97%, NPA 99% and OPA 99% (95% CI, 98, 100) for this analyte [23].

Other studies evaluating multiplex bead-based assays for the quantitative detection of different autoantibodies, including ACA-B, found at least similar performance compared with ANA-IFA [24] or ELISA [25], but reducing time from sample receipt to reporting of results, in one study, from 150 minutes to 90 minutes [24]. In another evaluation comparing a multiplex line immunoassay and a fluorescence enzyme immunoassay, high agreement in the results for ACA-B was reported [26]. Although, apart from the present study, the MosaiQ CENP-B-MA has not been directly compared to other similar available devices, the results of this research suggest that the performance of the MosaiQ CENP-B-MA is comparable to that of other marketed devices. Further studies are required to confirm these observations.

The 4 discordant results in the comparator evaluation, all potential false non-reactive on CENP-B-MA, were retested on FIDIS and one was excluded from analysis after an invalid result upon retest with this comparator. The remaining three discordant samples results were considered as CENP-B-MA false non-reactive. Further examination of the CENP-B-MA images revealed spot reactivity for all three samples despite the ACA-B non-reactive result, suggesting that image analysis could be further improved, an ongoing endeavor at the time of writing this report. Upon CENP-B-MA retest, all three samples were found to be ACA-B reactive (data not included in the final analysis).

The rate of unreportable results (IND, DRE, PE) observed under clinical laboratory testing conditions was low, with no processing errors related to the instrument. The initial DRE rate was 1.7% and was further reduced to 1.3% (4/303) upon retest. 1 sample returned an indeterminate result after the initial test that was resolved upon retest, for a final IND rate of 0%. These results show the robustness of the instrument and the assay and may positively impact laboratory performance, given the low rate of unreportable results and the subsequent need for repeated testing, contributing to reduce costs relating to repeat testing and delays in reporting results to clinicians.

In total, 94 of 99 (94.95%) reactive samples included in the analysis belonged to females. Without additional details on severity or organ involvement or criteria used, diagnosis information was available for all 99 ACA-B reactive samples, of which at least 73 (73.73%) corresponded to CTD, with 37 (37.37%) diagnoses directly related to SSc (all displaying centromere ANA-IFA pattern), ACA's main clinical association (particularly lcSSc). These patients have better prognosis, but also a higher risk of pulmonary hypertension. In five (5.05%) samples, the diagnosis was Raynaud's phenomenon/disease, which in presence of ACA-B is recognized as prognostic for onset of lcSSc [27] and together would account for 6 points in the ACR/EULAR SSc classification criteria, in which individuals with a score ≥ 9 would be classified as having definite SSc, provided that inclusion/exclusion criteria are met [14]. In at least 31% of samples, the diagnosis was a CTD different from SSc. ACA are present in Sjögren's syndrome (5-10%), systemic lupus erythematosus (2-5%), MCTD (2-5%) and IIM (1-3%) [8]. The diagnosis in 11 (11.11%) ACA-B reactive samples in this study was Sjögren's syndrome, a condition in which ACA positivity is associated with a more severe presentation [3,27,28]. Evidence from an international registry of Sjögren's syndrome showed that presence of ACA is independently associated with more severe exocrine glandular dysfunction [28], which further highlights the importance of autoantibody testing in CTD. Notably, centromere ANA-IFA pattern was not observed in 7 (7.07%) ACA-B reactive samples included in the analysis, suggesting that ACA-B can be found in the absence of its characteristic ANA-IFA pattern, results that are in agreement with previous findings using ELISA [29].

The MosaiQ System has several characteristics that could have a positive impact for laboratories, physicians and patients. Up to four magazines can be loaded on the instrument, each containing up to 250 microarrays, for a total of up to 1,000 individual tests. The system features fully automated, continuous random access, with high throughput. Every CENP-B microarray is printed with reactive and non-reactive control probes, primary patient serum tubes can be used. The system includes security features such as RFID and when magazines and reagents are loaded on the instrument, key information (i.e., lot number, expiry date and volume) is transmitted to it to determine whether all resources loaded are adequate to perform the test order selected. Redundant safety codes (numeric, color and geometrical shapes) are in place to prevent errors (e.g., the system will not allow to insert a reagent in an incorrect port of the instrument). The instrument will monitor reagent volumes and alert users when bottles need to be replaced and discarded to waste. An intuitive user-friendly interphase allows easy use.

Different autoantibodies are associated with specific CTD and its detection aid in the identification of these conditions. Testing of combinations of autoantibodies is usually performed in a tiered fashion (reflexing to a subsequent tier of autoantibodies depending on the results of the previous one) and frequently using individual assays per analytes, which can be time/resource consuming, though some multiplex devices are already available. A limitation of the present CENP-B-MA is that does not include detection of other commonly ordered autoantibodies for CTD; however, the CENP-B assay is part of a multiplexed panel under development for the simultaneous detection of different specific autoantibodies associated with CTD, allowing for the testing of several tiers of analytes at the same time, with the possibility of reflex reporting, meaning that results can be made available only for the requested autoantibodies, enabling the report of timely and comprehensive, yet tailored, results to clinicians. Additional studies are required to demonstrate the MosaiQ potential to multiplex and improve laboratory workflow.

Conclusion

MosaiQ CENP-B microarray shows high concordance with other CE-marked assays for detecting autoantibodies against CENP-B, with demonstrated reproducibility and repeatability. MosaiQ System has the potential to improve laboratory efficiency and productivity, with the ability to multiplex the detection of various autoantibodies on a single microarray and a capacity to automatically process samples, delivering a large number of results per day. This may contribute to the prompt and comprehensive provision of results to clinicians and could accelerate time to diagnosis and treatment. Additional studies are required to demonstrate the potential of the MosaiQ system to improve laboratory workflow in autoimmune disease testing.

Acknowledgment

This research was funded by AliveDx Suisse as part of the requirements for CE mark under IVDR.

Conflict of Interest

Rocio Pasion-Galvan, Gerber Gomez, Daphne Bijlsma, Ewa Lukasik, Valeria Botti, Emmanuel Moreau, Christine C. Ginocchio and Michael Hausmann are employees of AliveDx or were so at the time the study was conducted. Makoto Miyara has participated as external advisor in Scientific Advisory Board meetings for AliveDx.

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How to cite this article: Ghillani-Dalbin, Pascale, Christel Daubrosse, Valérie Mercier and Rocio Pasion-Galvan, et al. "Performance Characteristics of a Novel, Fully Automated Planar Microarray Immunoassay for the Detection of Antibodies against Centromere Protein B." *Immunochem Immunopathol* 9 (2023): 207.