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1 Performance assessment of a *Trypanosoma cruzi* chimeric antigen in multiplex liquid
2 microarray assays
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16
17 Short title: LMA-based CD detection using chimeric antigens

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18 **Abstract**

19 Diagnosing chronic Chagas disease (CD) requires antibody–antigen detection methods,
20 traditionally based on enzymatic assay techniques whose performance depend on the
21 type and quality of antigen used. Previously, 4 recombinant chimeric proteins from
22 Instituto de Biologia Molecular do Paraná (IBMP-8.1 to -8.4) comprising immuno-
23 dominant regions of diverse *Trypanosoma cruzi* antigens showed excellent diagnostic
24 performance in enzyme-linked immunosorbent assays. Considering that next-generation
25 platforms offer improved CD diagnostic accuracy with different *T. cruzi*-specific
26 recombinant antigens, we assessed the performance of these chimeras in liquid
27 microarrays (LMAs). The chimeric proteins were expressed in *Escherichia coli* and
28 purified by chromatography. Sera from 653 chagasic and 680 healthy individuals were
29 used to assess the performance of these chimeras in detecting specific anti-*T. cruzi*
30 antibodies. Accuracies ranged from 98.1–99.3%, and diagnostic odds ratio values were
31 3,548 for IBMP-8.3, 4,826 for IBMP-8.1, 7,882 for IBMP-8.2, and 25,000 for IBMP-
32 8.4. A separate sera bank (851 samples) was employed to assess cross-reactivity with
33 other tropical diseases. *Leishmania* spp., a pathogen with high genome sequence similar
34 to *T. cruzi,* showed cross-reactivity rates ranging from 0–2.17%. Inconclusive results
35 were negligible (0–0.71%). Bland–Altman and Deming regression analysis based on
36 200 randomly selected CD-positive and -negative samples demonstrated
37 interchangeability with respect to CD diagnostic performance in both singleplex and
38 multiplex assays. Our results suggested that these chimeras can potentially replace
39 antigens currently used in commercially available assay kits. Moreover, the use of a
40 multiplex platform, like LMA assays employing 2 or more IBMP antigens, would
41 abrogate the need for 2 different testing techniques when diagnosing CD.

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43 **Keywords**: Human Chagas disease; *Trypanosoma cruzi*; Chimeric antigens; Liquid

44 microarray; Singleplex and Multiplex assays

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45 Chagas disease (CD) is a life-threatening neglected tropical condition affecting
46 approximately 5.7 million people in 21 Latin America countries, of which Brazil,
47 Mexico, and Argentina are home to >60% of the estimated total number of infected
48 individuals (1). Human migration has contributed to the worldwide distribution of
49 infection, transforming this disease into a global health problem (2, 3). The vector-borne
50 protozoan parasite *Trypanosoma cruzi* is the causative agent of CD, whose transmission
51 occurs mainly when contaminated urine/feces of hematophagous insects of the
52 Triatominae family enters a bite site wound or mucosal membrane, blood transfusions,
53 and the consumption of contaminated beverages or food (4).

54 Two distinct stages occur during the natural course of CD progression. Initially,
55 an acute phase presents as a non-specific oligosymptomatic febrile illness, lasting for
56 approximately 2–3 months with abundant parasitemia. A small number of cases are
57 accompanied by myocarditis and other lethal complications. This parasite can only be
58 observed by staining thick and thin blood smears during the initial phase. During the
59 lifelong chronic stage, parasites remain hidden in target tissues, notably in the digestive
60 system and cardiac muscles. This phase is initially characterized by an asymptomatic
61 clinical course lasting 2–3 decades, after which approximately 10% and 20% of infected
62 individuals develop digestive and heart complications, respectively (5). Due to low
63 parasitemia and high levels of specific anti-*T. cruzi* antibodies, diagnosis in the chronic
64 phase is traditionally performed by serological methods, including enzyme-linked
65 immunosorbent assays (ELISAs), indirect immunofluorescence assays, and indirect
66 hemagglutination inhibition assays (6). Because no standardized reference test is
67 commercially available, the World Health Organization advises the use of two distinct
68 techniques for CD diagnosis (7), and the Brazilian Health Ministry recommends 2
69 serological methods involving distinct antigen preparations, both of which must be

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70 performed concomitantly (6). Next-generation diagnostic platforms have improved the
71 accuracy of CD diagnosis by using different *T. cruzi*-specific recombinant proteins in a
72 variety of detection systems, such as chemiluminescence (8), surface plasmon resonance
73 (9, 10), and bead-based technologies, including cytometry bead arrays (11) and liquid
74 microarrays (LMAs) (12).

75 In endemic countries, the screening of blood donors for *T. cruzi* is mandatory to
76 prevent CD transmission by blood transfusions. Accordingly, numerous tests must be
77 performed on a daily basis in these areas. LMA is considered appropriate for detecting
78 and quantifying multiple analytes in multiplex assays, using relatively small sample
79 volumes with high-throughput potential. Using this technique, it is possible to
80 incorporate up to 500 color-coded fluorescent magnetic bead sets, each with 2 spectrally
81 different fluorophore ratios, making each bead set distinguishable by its fluorescence
82 emission when excited by a laser (13, 14). Because LMA technology permits the
83 detection of many analytes simultaneously in each test sample, this method could
84 potentially be singularly employed for CD diagnosis, as a substitute for ELISAs and
85 other traditional serological methods. These serological assays employ either
86 fractionated lysates of *T. cruzi* at the epimastigote stage or recombinant proteins, which
87 can produce inconclusive results or cross-reactivity with related diseases. Therefore,
88 chimeric proteins have been proposed to improve the assay's accuracy to diagnose
89 chagasic. Recently, a phase I study was performed with 4 chimeric proteins from
90 Instituto de Biologia Molecular do Paraná (IBMP-8.1, -8.2, -8.3, and -8.4) to detect
91 specific anti-*T. cruzi* antibodies using both ELISA and LMA (15), demonstrating that
92 each antigen accurately discriminated CD-positive from CD-negative samples. In
93 addition, no significant differences were observed with respect to the diagnostic
94 performances of the ELISA and LMA test methods. Data from a subsequent phase II

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95 study confirmed the high performance of these proteins in ELISAs (16). In the present
96 study, we aimed to assess the diagnostic performance of the IBMP chimeras to diagnose
97 CD in singleplex and multiplex formats, using LMA.
98

99 **RESULTS**

100 **LMA performance.** Using 1,333 sera from Ch and NCh individuals, the LMA
101 performance and RI distributions of all IBMP chimeras were assessed, as shown in Fig.
102 1. AUC values were >99%, revealing excellent overall diagnostic accuracy. IgG levels
103 in Ch samples were variable, ranging from 4.52 for IBMP-8.3 and 4.98 for IBMP-8.4 to
104 5.19 for IBMP-8.2 and 5.55 for IBMP-8.1. Out of 653 Ch samples, IBMP-8.4-LMA
105 showed 99.1% sensitivity with only 6 cases classified as false negatives; with these
106 samples 4 were also classified as false negatives for all other antigens. Higher numbers
107 of false negatives were observed for IBMP-8.1 (15 cases), IBMP-8.2 (11 cases), and
108 IBMP-8.3 (20 cases), with corresponding sensitivity values of 97.7%, 98.3%, and
109 96.9%, respectively. Nevertheless, no statistically significant differences were detected
110 with respect to IBMP protein sensitivity. Regarding the NCh samples, the IBMP
111 chimeras showed specificity values >99.0%, and RI values ≤0.13 for all chimeras, with
112 statistical differences observed only in relation to IBMP-8.2.

113 Relatively few Ch and NCh samples were considered inconclusive: 3 (0.23%) in
114 the IBMP-8.1 assay, 5 (0.38%) in the IBMP-8.2 assay, 12 (0.90%) in the IBMP-8.3
115 assay, and 3 (0.23%) in the IBMP-8.4 assay. IBMP-8.4 was found to most accurately
116 diagnose CD (99.3%), followed by IBMP-8.2 (99.0%), IBMP-8.1 (98.4%), and IBMP-
117 8.3 (98.1%). The Youden index was the highest for IBMP-8.4 (98.6%), followed by the
118 IBMP-8.2 (97.6%), IBMP-8.1 (96.8%), and IBMP-8.3 (96.1%) proteins. The test

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119 performance was summarized by the DOR value, which reached 25,000 for IBMP-8.4,
120 7,882 for IBMP-8.2, 4,826 for IBMP-8.1, and 3,548 for IBMP-8.3.

121 Potential cross-reactivity (RI ≥ 1.0) of the IBMP chimeras was assessed using
122 serum samples from 851 individuals with unrelated diseases. As shown in Fig. 2, the
123 incidence of cross-reactivity was negligible: 0.12% (1/851) for IBMP-8.1 and IBMP-
124 8.4, 0.24% (2/851) for IBMP-8.2, and 0.59% (5/581) for IBMP-8.3. Moreover, a very
125 low frequency of inconclusive results was observed: 0.12% (1/851) for IBMP-8.1,
126 0.71% (6/851) for IBMP-8.2, and 0.47% (4/851) for IBMP-8.3 (Fig. 2). Notably, we
127 found no inconclusive results in relation to the IBMP-8.4 protein. Regarding the
128 *Leishmania* spp. samples, none exhibited any cross-reactivity with the 4 IBMP
129 chimeras, and only 1 showed an inconclusive result with respect to IBMP-8.3.

130 **Comparison of singleplex vs. multiplex IBMP antigen performance.** No
131 significant differences were observed with respect to LMA performance when assaying
132 100 Ch and 100 NCh samples, in singleplex or multiplex assays (Fig. 3). The AUCs
133 were >99.7%. The level of agreement between the expected results ranged from 95.0%
134 (κ 0.950 [0.907–0.993]) for IBMP-8.1 to 99.0% (κ 0.990 [0.970–1.01]) for IBMP-8.2,
135 while the IBMP-8.3 and-8.4 chimeras showed 100% agreement. Despite the high level
136 of agreement seen and the consistency in performance of the parameters evaluated, NCh
137 samples yielded lower signals when assayed with IBMP-8.1, IBMP-8.3, and IBMP-8.4
138 in the multiplex assay. Regarding the Ch samples, differences in RI values were
139 observed only in the samples assayed by the IBMP-8.1 chimera in multiplex assays. For
140 comparison purposes, ELISA performances are also described in Fig. 3.

141 Fig. 4 graphically illustrates the strength of agreement between the singleplex
142 and multiplex data for each protein assayed by Deming regression fit analysis (left
143 panels) and Bland–Altman plots (right panels). The IBMP-8.1 antigen multiplex aligned

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144 poorly with the singleplex method under Deming regression fit analysis, with an
145 equation of $y = -0.2268 + 2.3293x$, an intercept of -0.2268 (95% CI: -1.0042 to 0.5506),
146 a slope of 2.3293 (95% CI: 1.8478 to 2.8109), and an *R*-squared value of 0.76 (Fig. 4A).
147 The mean bias derived from the Bland–Altman difference plot was -6.6% (95% CI: -
148 19.5 to 5.89%) with the LoA ranging between -183.0% and 169.8%. Although Deming
149 regression fit analysis indicated significant proportional bias, Bland–Altman analysis
150 presented no significant bias with respect to the means, as the line of equality fell within
151 the confidence interval. We observed that all data points fell within the LoAs, which is
152 consistent with the expectation that only 5% would fall outside these limits. The IBMP-
153 8.2 antigen (Fig. 4B) showed good agreement between the singleplex and multiplex
154 assays, with an *R*-squared value of 0.88, an intercept of -0.2306 (95% CI: -0.4291 to -
155 0.0320), and a slope of 1.1441 (95% CI: 1.0589 to 1.2313). The mean bias was 8.01%
156 (95% CI: 2.9 to 13.12%) with LoA values ranging from -64.3% to 80.3%, which
157 indicated statistical significance since the line of equality fell outside the CI. We
158 observed 8 points (4.0%) outside the LoAs, which is consistent with the 5%
159 expectation. Fig. 4C shows a good fit between these 2 methods using IBMP-8.3, with an
160 *R*-squared value of 0.90, an intercept of -0.1099 (95% CI: -0.2751 to 0.0554), and a
161 slope of 0.9814 (95% CI: 0.9081 to 1.0546). The mean bias was -23.36% (95% CI: -
162 28.98 to -17.74%) with LoA values ranging from -102.8% to 56.1%. Despite the
163 absence of significance regarding the slope under Deming regression analysis, the mean
164 bias derived from Bland–Altman analysis indicated that the multiplex results were up to
165 -23.36% less than those obtained with the singleplex method. Nine points (4.5%) fell
166 beyond the LoAs. For IBMP-8.4 (Fig. 4D), the correlation coefficient between the 2
167 singleplex and multiplex tests was 0.77. Deming regression analysis showed a slope of
168 1.5553 (95% CI: 1.3609 to 1.7498) and an intercept of -0.2360 (95% CI: -0.5851 to

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169 0.1131), while the Bland–Altman plot showed a mean bias of -26.42% (95% CI: -36.95
170 to -15.89). The slope value is indicative of significant proportional bias, as evidenced by
171 an up to 20% variation between the results obtained with the singleplex and multiplex
172 techniques. Just 1 point (0.5%) fell outside the LoA.
173
174 **DISCUSSION**
175 The *T. cruzi* IBMP recombinant antigenic proteins employed herein have already been
176 shown to be sensitive and specific for CD diagnosis when assessed by ELISA (16),
177 although their performances using other approaches remains to be elucidated. A phase-I
178 study, previously conducted by our group using ELISAs and LMAs, showed high
179 performance when these antigens were assayed using a small set of samples comprised
180 of only 300 sera from CD-positive and CD-negative individuals (15). Here, we
181 expanded the sample size to 1,333 sera and found AUC values higher than 99% for all 4
182 proteins. These data are in accordance with results from a phase-II study, where these
183 same proteins were tested by ELISA (16), thereby indicating the high discriminative
184 power these antigens potentially possess with respect to other diagnostic platforms.
185 Most importantly, these IBMP chimeric proteins provided much better AUC values than
186 did *T. cruzi* cell lysates, single recombinant proteins, or other recombinant chimeric
187 proteins commonly used in diagnostic kits (17, 18). In addition, differences higher than
188 4.40 were seen between the RI signals from the positive and negative samples for all
189 proteins, providing further evidence of their high discriminatory capability. Moreover,
190 the RI signals obtained from positive samples assayed by LMA were up to 56%
191 stronger than those previously obtained by ELISA (16). Conversely, the average RI
192 signals from negative samples were 32% lower by LMA. Finally, the total number of
193 inconclusive results was very low, ranging from 0.23% to 0.90%, again reinforcing the

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194 optimal discriminatory power of these IBMP proteins combined with next-generation
195 diagnostic platforms.

196 Performance assessments were carried out with the LMA assays to determine the
197 diagnostic sensitivity, specificity, and accuracy for CD. Despite the fact that no
198 differences were observed in sensitivity and specificity, the IBMP-8.4 protein produced
199 more accurate results than IBMP-8.2. Nonetheless, this difference was almost negligible
200 considering that the 95% CI values practically overlapped. LMA assay performance was
201 comparable to previously published data with ELISAs (16). With the exception of the
202 IBMP-8.2 antigen, both testing methods offered similar performance. When evaluated
203 by LMA, the IBMP-8.2 protein showed 99.0% accuracy, while it showed 96.6%
204 accuracy by ELISA. According to a previous study, the lower value obtained by ELISA
205 was probably due to the amino acid sequence of this protein, which impaired its
206 recognition by specific anti-*T. cruzi* antibodies from CD-positive samples collected in
207 distinct geographical regions (16). However, this discrepancy in accuracy may also be
208 the result of characteristics inherent to each diagnostic platform used. Indeed, the MFI
209 of the detection antibody corresponds to an average of 100 bead readings, i.e., a single
210 serum sample is analyzed 100 times per antigen versus just once in an ELISA reaction.
211 This level of precision improves the limit of detection by LMA assays (13, 19). These
212 performance results were corroborated by the J index and DOR. In addition to accuracy,
213 the J index measures the effectiveness of a diagnostic marker by considering the
214 sensitivity and specificity together as a single parameter, and we found that the J index
215 value was >0.96 for all chimeras. The DOR is a global performance parameter that
216 summarizes the diagnostic accuracy of a given testing method (20). It can vary from 0
217 to infinity, with higher values indicating improved discriminatory diagnostic testing.
218 The DOR for IBMP-8.4 (25,000) was greater than that obtained for IBMP-8.1 (4,826),

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219 IBMP-8.2 (7,882), and IBMP-8.3 (3,548). These data agree with data from a previous
220 study using ELISA that highlighted the IBMP-8.4 protein as the best antigen for
221 diagnosing CD (16).

222 Considering the large number of sera from patients with unrelated diseases used
223 to assess cross-reactivity, the small number of samples that cross-reacted was irrelevant.
224 This was expected due to the low similarity between the IBMP sequences and those
225 deposited in the NCBI database for other pathogens, including *Leishmania* spp.
226 Furthermore, cross-reacting samples also presented a weak RI signal. Similarly,
227 inconclusive results using this same panel were statistically irrelevant, particularly with
228 respect to IBMP-8.1 and -8.4. These findings are consistent with previous results
229 obtained when assessing cross-reactivity in ELISAs (16). As such, the authors are
230 confident that all of these chimeric proteins can be safely employed in diagnostic
231 platforms in areas endemic for CD, as well as other infectious diseases.

232 We also comparatively assayed 100 CD-positive and 100 CD-negative samples
233 by the singleplex and multiplex LMA approaches. Both methods were highly efficient
234 in distinguishing CD-positive and CD-negative samples. Regarding the CD-positive
235 samples, a significant difference was seen in the RI signal intensity only with respect to
236 the IBMP-8.1 protein, whereas, in the CD-negative samples, lower RI signals for
237 IBMP-8.1, -8.3, and -8.4 proteins were observed in multiplex assays compared to
238 singleplex assays. Despite these discrepancies, the performance parameters were
239 identical for both methods. Deming regression analysis showed a substantial
240 proportional bias for the IBMP-8.1, -8.2, and -8.4 proteins, suggesting that these
241 methods are not in complete agreement throughout the measurement range involving
242 CD-positive and CD-negative samples, as evidenced by the Bland–Altman plots,
243 especially regarding IBMP-8.1 and IBMP-8.4. This finding indicates a highly linear

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244 nature of multiplex assays, compared with the singleplex approach. Regression analysis
245 also showed a systematic negative bias only with respect to the IBMP-8.2 protein,
246 indicating that results obtained using this antigen under multiplex assays were slightly
247 higher, by a constant amount, than those produced by the singleplex method. This was
248 probably due to inadequate blanking, a mis-set 0 calibration point, or some other type of
249 interference in the assay (21). Although this bias seems to indicate a substantial
250 difference between the singleplex and multiplex LMA techniques, it does not affect
251 diagnostic accuracy, i.e., both methods are sufficiently interchangeable for CD
252 diagnosis. As multiplex assays inherently involve similar analysis time and serum
253 volumes when compared to singleplex methods, multiplex methods are crucial for
254 assessing outbreaks involving the screening of large populations, as well as for routine
255 testing at blood donation centers. Furthermore, multiplex approaches can be used to
256 effectively screen for several diseases concomitantly, differently from traditional
257 serological testing, in which only 1 condition is evaluated. Thus, multiplexing not only
258 reduces costs, analysis time, and the serum volume required, but it also enables the
259 incorporation of multiple markers for infectious diseases (14, 22, 23), cancer, and other
260 conditions (24-26). Although LMA-based technology offers several advantages, it
261 nonetheless requires a significant laboratory infrastructure, a well-trained workforce,
262 and substantial financial investment.

263 In conclusion, the results described herein indicate that these 4 *T. cruzi* IBMP
264 recombinant antigenic proteins can be safely used for CD diagnosis in both LMA
265 platforms evaluated, as well as in ELISA-based assays (16). Moreover, the accuracy of
266 LMA was shown not to vary among these IBMP antigens, regardless of using
267 singleplex or multiplex techniques, suggesting that these chimeras can potentially
268 replace those currently used in commercially available assay kits. Accordingly, a

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269 multiplex LMA assay employing 2 or more IBMP antigens would abrogate the need for
270 using 2 different tests when diagnosing CD.
271

272 **MATERIALS AND METHODS**

273 **Ethical considerations.** The Institutional Review Board (IRB) for Human Research at
274 the Aggeu Magalhães Institute of the Oswaldo Cruz Foundation (Recife, Pernambuco-
275 Brazil) provided ethical approval to conduct this study (CAEE: 15812213.8.0000.5190).
276 To protect patients' privacy, the IRB required that samples be coded to mask patient
277 identification, thus eliminating the need for verbal or written consent.

278 **Subjects and sample collection.** Human sera, previously collected, were
279 provided by the biorepositories of the Hemope Foundation (Recife, Pernambuco), the
280 Central Laboratory for Public Health-LACEN (Recife, Pernambuco), the Reference
281 Laboratory for Chagas Disease (Fiocruz-Recife, Pernambuco), the Molecular Biology
282 Institute of Paraná (IBMP-Paraná), and the Laboratory for Research on Chagas Disease
283 (Federal University of Goiás-Goiás). Samples from 653 chagasic (Ch) and 680 non-
284 chagasic (NCh) individuals were utilized to assess the performance of *T. cruzi* IBMP
285 chimeras in diagnosing CD by LMA. This panel was composed of samples from
286 endemic and non-endemic Brazilian states (Bahia-BA, Minas Gerais-MG, Goiás-GO,
287 Pernambuco-PE, and Paraná-PR), as well as from Brazilian and international
288 commercial suppliers (National Panel for Blood Screening Quality Control, Fiocruz, RJ,
289 Brazil; Boston Biomedical Inc., Norwood, MA, USA; SeraCare Life Sciences Inc.,
290 Milford, MA, USA). Samples from individuals with dengue virus (n = 50), hepatitis B
291 virus (n = 160), hepatitis C virus (n = 98), human immunodeficiency virus (n = 144),
292 human T-cell lymphotropic virus (n = 109), leishmaniasis (n = 18), leptospirosis (n =
293 92), rubella virus (n = 15), measles (n = 21), and syphilis (n = 144) were used to assess

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294 cross-reactivity between the IBMP chimeras and proteins associated with unrelated
295 diseases. Before LMA analysis, all sera were re-evaluated using 2 commercial ELISA
296 tests, namely the Imuno-ELISA Chagas test (Wama Diagnostica, São Paulo, Brazil;
297 batch 14D061) and the ELISA Chagas III test (BIOSChile, Ingeniaría Genética S.A.,
298 Santiago, Chile; batch 1F130525) (27). Each sample was assigned a numeric code in the
299 laboratory to ensure a blinded analysis.

300 **Acquisition of recombinant chimeric proteins.** Immuno-dominant sequence
301 selection, synthetic gene construction, and recombinant chimeric protein expression
302 were performed, as previously described (15). Briefly, *T. cruzi* synthetic gene constructs
303 were obtained from a commercial supplier (GenScript, Piscataway, NJ, USA) and
304 subcloned into the pET28a expression vector (Novagen, Madison, WI, USA). Chimeric
305 antigens were expressed as soluble proteins in *Escherichia coli* BL21-Star (DE3) cells
306 grown in LB medium supplemented with 0.5 M isopropyl-β-D-1-thiogalactopyranoside
307 (IPTG). Recombinant expression of the chimeras was checked by sodium dodecyl
308 sulfate-polyacrylamide gel electrophoresis (28). Chimeras were purified by both ion-
309 exchange and liquid affinity chromatography. Concentrations were determined by
310 performing a fluorimetric assay (Qubit 2.0, Invitrogen Technologies, Carlsbad, CA,
311 USA).

312 **IBMP antigen coupling to microsphere beads and in-house LMA**
313 **procedures.** The IBMP antigen-coupling protocol employed herein was performed as
314 previously described (15). Briefly, 2×10^6 microsphere beads were washed with
315 activation buffer (100 mM sodium phosphate, pH 6.3) and chemically activated using 1-
316 ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-
317 hydroxysulfosuccinimide (Sigma, St. Louis, MO, USA), both diluted to 50 mg/mL of
318 ultrapure water (18.2 MΩ). Activated beads were subsequently incubated with 200 μL

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319 of antigen diluted in coupling buffer at previously determined concentrations (15).
320 These suspensions were incubated at 250 rpm under horizontal agitation for 2 h at 37°C.
321 Next, the beads were washed 3 times with wash buffer (PBS, containing 1% BSA,
322 0.05% Tween 20). The final bead suspensions were adjusted to a concentration of 50 ×
323 10^3 microspheres/mL in wash buffer and stored overnight at 2–8°C in low-binding
324 tubes. For LMA analysis, a previously prepared *E. coli* lysate (diluted to 2%) (29) was
325 mixed with 50 µL of serum sample (diluted 1:200) and 50 µL of bead suspension,
326 placed in a 96-well plate, and incubated under agitation for 15 min at 37°C. The beads
327 were then washed twice. Phycoerythrin-conjugated, goat anti-human IgG (Moss
328 Substrates, Pasadena, MA, USA), diluted 1:1,000, was added and the plates were
329 incubated under agitation for 15 min at 37°C. The beads were then washed with sheath
330 fluid and resuspended in 200 µL of the same solution. For the multiplex LMA assay,
331 2,500 beads of each set were mixed together in a final volume of 50 µL/well, following
332 the assay protocol described above. The results were interpreted using a Luminex 200
333 BioAnalyzer (Luminex Corp. Austin, TX, USA) with xPONENT software (version
334 3.1.871.0). For bead identification, a minimum of 100 beads bearing a unique
335 fluorescent signature was detected per region, measured in terms of the median
336 fluorescence intensity (MFI) per sample in accordance with the manufacturer
337 instructions.

338 **Singleplex vs. multiplex LMA.** A total of 100 Ch and 100 NCh samples were
339 randomly selected to compare the performance and concordance among the IBMP
340 chimeric antigens, either singleplexed (assayed individually using a single bead type) or
341 multiplexed (each antigen assayed together with different bead types).

342 **Data analysis.** Data were encoded and analyzed using Prism graphing software,
343 version 6 (GraphPad, San Diego, CA, USA). Descriptive statistics are presented as

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344 geometric means ± standard deviation (SD). The Shapiro–Wilk test was used to test data
345 normality, and homogeneity of variance was verified using Levene's test. When these 2
346 assumptions were confirmed, Student's t test was used for sample comparisons;
347 otherwise, the Wilcoxon signed-rank test was employed. All analyses were 2-tailed, and
348 a $p < 0.05$ was considered significant. Cut-off point analysis was used to establish a
349 maximum MFI to distinguish positive and negative samples. The threshold was set by
350 determining the greatest area under the receiver operating characteristic (ROC) curve.
351 Data are displayed via scatter plot and are presented in terms of the reactivity index (RI,
352 i.e., ratio of the sample MFI to the cut-off MFI), with results ≥ 1.00 considered positive.
353 RI values within 1.0 ± 10% were considered indeterminate and deemed as inconclusive
354 (shown as a gray zone). LMA performance was evaluated using a dichotomous
355 approach with respect to sensitivity, specificity, accuracy, Youden index (J), the
356 likelihood ratio, and the diagnosis odds ratio (DOR) (30). Confidence intervals (CI)
357 were calculated to assess the precision of these parameters, with a confidence level of
358 95%. Singleplex vs. multiplex LMA results were compared using Cohen's kappa
359 coefficient (κ), the Bland–Altman plot, and Deming regression analysis. The strength of
360 agreement was interpreted as nearly perfect ($0.81 < \kappa \leq 1.0$), substantial
361 ($0.61 < \kappa \leq 0.80$), moderate ($0.41 < \kappa \leq 0.60$), fair ($0.21 < \kappa \leq 0.40$), slight ($0 < \kappa \leq 0.20$),
362 or poor ($\kappa \leq 0$) agreement (31). Bland–Altman plots with limits of agreement (LoAs)
363 were generated to assess the variability and magnitude between the singleplex and
364 multiplex assays (32). Deming regression was used to mathematically determine the
365 agreement between the singleplex and multiplex techniques, as well as proportional bias
366 (slope, 95% CI) and systematic bias (intercept, 95% CI). Deming regression analysis
367 revealed a null hypothesis when the intercept and slope were 0 and 1, respectively. A

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368 checklist and flowchart (Fig 5) are provided according to the Standards for the
369 Reporting of Diagnostic accuracy studies (STARD) guidelines (33).
370

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510 **Fig 1 Singleplex IBMP chimeric antigen assay of serum samples from chagasic (Ch)**
511 **and non-chagasic (NCh) individuals.** The cut-off value was established as reactivity
512 index = 1.0, and the shadowed area represents the gray zone (RI = 1.0 ± 0.10). Geometric
513 means (±95% CI) are represented by horizontal lines with corresponding results for each
514 group. Acc, accuracy; AUC, area under the curve; DOR, diagnostic odds ratio; J index,
515 Youden index; LR, likelihood ratio; Sen, sensitivity; Spe, specificity.
516
517 **Fig 2 Analysis of IBMP chimera cross-reactivity with sera from individuals with**
518 **unrelated diseases.** The cut-off value was established as reactivity index = 1.0, and the
519 shadowed area represents the gray zone (RI = 1.0 ± 0.10). Geometric means (±95% CI) are
520 represented by horizontal lines, with the corresponding results shown for each group. CR,
521 cross-reaction); DENG, Dengue; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV,
522 human immunodeficiency virus; HTLV, human T-cell lymphotropic virus; IR, inconclusive
523 results; LEIS, leishmaniasis; LEPT, leptospirosis; MEAS, measles; RI, reactivity index;
524 RUBE, rubella virus; SYPHI, syphilis.
525
526 **Fig 3 Singleplex and multiplex IBMP chimeric antigen assays of serum samples from**
527 **chagasic (Ch) and non-chagasic (NCh) individuals.** The cut-off value was established as
528 reactivity index = 1.0, and the shadowed area represents the gray zone (RI = 1.0 ± 0.10).
529 Geometric means (±95% CI) are represented by horizontal lines, with the corresponding
530 results shown for each group. Acc, accuracy; AUC, area under the curve; EIA, ELISA;
531 LMA-M, multiplex liquid microarray; LMA-S, singleplex liquid microarray; RI, reactivity
532 index; Sen, sensitivity; Spe, specificity.
533

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534 **Fig 4 Deming regression fit (left) and Bland–Altman plots (right) comparing single-**
535 **and multiplex methods of detecting anti-*T. cruzi* IgG, using the IBMP-8.1 (A), IBMP-**
536 **8.2 (B), IBMP-8.3 (C), and IBMP-8.4 (D) chimeras.**
537
538 **Fig 5 STARD flowchart.** Standards for the Reporting of Diagnostic Accuracy Studies
539 (STARD) description of the study design.

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IBMP-8.1

Reactivity index

	RI (95%CI)	CR (%)	IR (%)
DENG	0.10 ± 0.02	0	0
HBV	0.08 ± 0.01	0	0
HCV	0.07 ± 0.01	0	0
HIV	0.07 ± 0.01	1 (0.69%)	1 (0.69%)
HTLV	0.06 ± 0.01	0	0
LEIS	0.06 ± 0.03	0	0
LEPT	0.05 ± 0.01	0	0
RUBE	0.04 ± 0.02	0	0
MEAS	0.08 ± 0.02	0	0
SYPHI	0.08 ± 0.02	0	0
TOTAL	0.07 (±0.01)	1 (0.12%)	1 (0.12%)

IBMP-8.2

Reactivity index

	RI (95%CI)	CR (%)	IR (%)
DENG	0.08 ± 0.02	0	0
HBV	0.12 ± 0.02	0	1 (0.63%)
HCV	0.09 ± 0.02	0	0
HIV	0.14 ± 0.02	0	2 (1.39%)
HTLV	0.15 ± 0.03	0	1 (0.92%)
LEIS	0.06 ± 0.04	0	0
LEPT	0.13 ± 0.02	0	0
RUBE	0.04 ± 0.02	0	0
MEAS	0.07 ± 0.03	0	0
SYPHI	0.19 ± 0.03	2 (1.39%)	2 (1.39%)
TOTAL	0.12 ± 0.01	2 (0.24%)	6 (0.71%)

IBMP-8.3

Reactivity index

	RI (95%CI)	CR (%)	IR (%)
DENG	0.13 ± 0.04	0	0
HBV	0.14 ± 0.02	2 (1.25%)	0
HCV	0.09 ± 0.02	0	1 (1.02%)
HIV	0.07 ± 0.01	1 (0.69%)	0
HTLV	0.09 ± 0.02	0	2 (1.83%)
LEIS	0.07 ± 0.05	0	1 (5.56%)
LEPT	0.14 ± 0.03	2 (2.17%)	0
RUBE	0.08 ± 0.02	0	0
MEAS	0.15 ± 0.06	0	0
SYPHI	0.15 ± 0.02	0	0
TOTAL	0.11 ± 0.01	5 (0.59%)	4 (0.47%)

IBMP-8.4

Reactivity index

	RI (95%CI)	CR (%)	IR (%)
DENG	0.02 ± 0.01	0	0
HBV	0.06 ± 0.01	0	0
HCV	0.05 ± 0.01	0	0
HIV	0.05 ± 0.01	0	0
HTLV	0.07 ± 0.01	0	0
LEIS	0.07 ± 0.03	0	0
LEPT	0.05 ± 0.01	0	0
RUBE	0.03 ± 0.01	0	0
MEAS	0.05 ± 0.03	0	0
SYPHI	0.10 ± 0.02	1 (0.69%)	0
TOTAL	0.06 ± 0.01	1 (0.12%)	0

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I B M P - 8 . 1

	Singleplex	Multiplex	ELISA
RI (95%CI) - NCh	0.15 ± 0.02	0.05 ± 0.01	0.27 ± 0.02
RI (95%CI) - Ch	3.75 ± 0.50	8.29 ± 1.28	3.03 ± 0.26
AUC % (95%CI)	99.8 (99.6-100)	100 (99.9-100)	100 (99.9-100)
Sen % (95%CI)	96.0 (90.2-98.4)	100 (96.3-100)	100 (96.3-100)
Spe % (95%CI)	99.0 (94.6-99.8)	100 (96.3-100)	100 (96.3-100)
Acc % (95%CI)	97.5 (94.3-98.9)	100 (98.1-100)	100 (98.1-100)

I B M P - 8 . 2

	Singleplex	Multiplex	ELISA
RI (95%CI) - NCh	0.08 ± 0.02	0.09 ± 0.02	0.36 ± 0.02
RI (95%CI) - Ch	7.51 ± 0.98	7.83 ± 1.15	2.19 ± 0.20
AUC % (95%CI)	100 (99.9-100)	100 (99.9-100)	100 (99.9-100)
Sen % (95%CI)	99.0 (94.6-99.8)	98.0 (93.0-99.4)	95.0 (88.8-97.8)
Spe % (95%CI)	100 (96.3-100)	100 (96.3-100)	100 (96.3-100)
Acc % (95%CI)	99.5 (97.2-99.9)	99.0 (96.4-99.7)	97.5 (94.3-98.8)

I B M P - 8 . 3

	Singleplex	Multiplex	ELISA
RI (95%CI) - NCh	0.05 ± 0.01	0.04 ± 0.01	0.28 ± 0.02
RI (95%CI) - Ch	6.83 ± 0.81	6.47 ± 0.79	2.51 ± 0.17
AUC % (95%CI)	100 (99.9-100)	100 (99.9-100)	100 (99.9-100)
Sen % (95%CI)	98.0 (93.0-99.4)	98.0 (93.0-99.4)	99.0 (94.6-99.8)
Spe % (95%CI)	100 (96.3-100)	100 (96.3-100)	100 (96.3-100)
Acc % (95%CI)	99.0 (96.4-99.7)	99.0 (96.4-99.7)	99.5 (97.2-99.9)

I B M P - 8 . 4

	Singleplex	Multiplex	ELISA
RI (95%CI) - NCh	0.13 ± 0.02	0.05 ± 0.01	0.13 ± 0.01
RI (95%CI) - Ch	5.76 ± 0.62	8.60 ± 1.00	2.36 ± 0.13
AUC % (95%CI)	100 (99.9-100)	100 (99.9-100)	100 (99.9-100)
Sen % (95%CI)	100 (96.3-100)	100 (96.3-100)	100 (96.3-100)
Spe % (95%CI)	100 (96.3-100)	100 (96.3-100)	100 (96.3-100)
Acc % (95%CI)	100 (98.1-100)	100 (98.1-100)	100 (98.1-100)

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Study Design for Testing IBMP *T. cruzi* Chimeras by LMA

	Singleplex	Multiplex
IBMP-8.1	97 Ch + 103 NCh	100 Ch + 100 NCh
IBMP-8.2	101 Ch + 99 NCh	98 Ch + 102 NCh
IBMP-8.3	98 Ch + 102 NCh	98 Ch + 102 NCh
IBMP-8.4	100 Ch + 100 NCh	100 Ch + 100 NCh

*Chagasic individuals
**Non-Chagasic individuals

1

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