Performance of commercially available serological screening tests for human T-cell lymphotropic virus infection in Brazil

Short title: Performance of screening tests for HTLV infection

Vanessa da Silva Brito,[a] Fred Luciano Neves Santos,[b] Noilson Lazaro Sousa Gonçalves,[a,b] Thessika Hialla Araujo,[a] Davi Silva Vale Nascimento,[a] Felicidade Mota Pereira,[c] Ney Cristian Amaral Boa-Sorte,[a] Maria Fernanda Rios Grassi,[a,b] Adele Caterino-de-Araujo,[d] Bernardo Galvão-Castro[a,b#]

[a]Integrated and Multidisciplinary HTLV Center, Bahiana School of Medicine and Public Health (EBMSP), Salvador, Bahia, Brazil

[b]Advanced Laboratory of Public Health, Gonçalo Moniz Institute (IGM), FIOCRUZ-BA, Salvador, Bahia, Brazil

[c]Gonçalo Moniz Public Health Central Laboratory (LACEN-BA), Bahia State Department of Health, Bahia, Brazil

[d]Adolfo Lutz Institute, São Paulo, São Paulo, Brazil

#Address correspondence to Bernardo Galvão-Castro, bgalvao@bahiana.edu.br.

VSB and FLNS contributed equally to this work.

KEYWORDS HTLV; Screening tests; Diagnostic reagent kits

ABSTRACT Serological screening for HTLV-1 is usually performed using enzyme-linked immunosorbent assay, particle agglutination or chemiluminescence assay kits. Due to antigen matrix improvement entailing the use of new HTLV-antigens and changes in the format of HTLV screening tests, as well as newly introduced CLIAs, a systematic evaluation of the accuracy of currently available commercial tests is warranted. We aimed to assess the performance of commercially available screening tests for HTLV diagnosis. A diagnostic accuracy study was conducted on a panel of 397 plasma samples: 200 HTLV-negative, 170 HTLV-positive and 27 indeterminate under Western blotting analysis. WB-indeterminate samples (i.e. those yielding no specific bands for HTLV-1 and/or HTLV-2) were assessed by PCR and results were used to compare agreement among the commercially available ELISA screening tests. For performance analysis, WB-indeterminate samples were excluded, resulting in a final study panel of 370 samples. Three ELISA kits (Murex HTLV-1/2, anti-HTLV-1/2 SYM Solution and Gold ELISA HTLV-1/2) and one CLIA kit (Architect r-HTLV-1/2) were evaluated. All screening tests demonstrated 100% sensitivity. Concerning the HTLV-negative samples, SYM Solution and Gold ELISA kits had specificity values >99.5%, while the Architect r-HTLV-1/2 test presented 98.1% specificity, followed by Murex (92.0%). Regarding the 27 samples with WB-indeterminate results, after PCR confirmation, all ELISA kits showed 100% sensitivity, but low specificity. Accuracy findings were corroborated by Cohen's Kappa, which evidenced slight and fair agreement between PCR analysis and ELISA tests for HTLV diagnosis. Based on the data, we believe that all evaluated tests can be safely used for HTLV-infection screening.

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Human T-cell lymphotropic virus type 1 (HTLV-1) and type 2 (HTLV-2) were identified in 1980 and 1982, respectively (1, 2). Subsequently, HTLV-3 and HTLV-4 were discovered in 2005 (3, 4). It has been estimated that at least 10 million people harbor HTLV-1 worldwide (5). Large foci of this infection exist in Japan, Africa, the Caribbean Islands, Melanesia, Australia, the Mashhad area of northeastern Iran and South America (5–7). HTLV-1 is associated with, or causes, a broad range of inflammatory conditions and a severe proliferative disease (5, 8–14).

HTLV-2 infection is endemic in native Amerindian populations in both North and South America, certain tribes of Pygmies in Africa and in intravenous drug users (IDUs) in urban areas around the world (15, 16). In contrast to HTLV-1, this type rarely is associated with neurological or lymphoproliferative disorders (17). HTLV-3 and HTLV-4 are restricted to Western Africa and have not yet been associated with any diseases (3, 4).

Brazil, a country of 200 million inhabitants, has a population of 800,000 who potentially harbor HTLV-1, representing one of the largest endemic areas for the virus and its associated diseases anywhere in the world (5). The virus is disseminated throughout the country, with higher rates found in the Northeast and Northern regions compared with the South and Southeast (18, 19). HTLV-2 is present mainly in the North, among indigenous populations and in IDUs in urban centers (17).

Achieving an accurate diagnosis of HTLV infection is a complex task. Serological screening for HTLV-1 is usually performed using enzyme-linked immunosorbent assay (ELISA), particle agglutination testing or chemiluminescence assay (CLIA) kits. The Brazilian Ministry of Health recommends the use of ELISA or particle agglutination tests as a screening protocol. Western blotting (WB) or immunoblot is used for confirmation, and polymerase chain reaction (PCR) is employed in the case of inconclusive confirmatory test

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results (20). Among the screening options, ELISA is used most extensively due to an elevated level of automation, simplicity and low cost. ELISA performance depends on antigen composition and assay format (21–24). Tests providing low accuracy present a public health problem, as false-positive results can have a negative impact, not only economically due to the need for confirmation by WB, but also on individuals' quality of life.

In light of this scenario, we endeavored to conduct a systematic evaluation of the commercial screening test kits for HTLV diagnosis. Statistical tools were used to obtain a robust assessment of the performance of each molecule by determining the following diagnostic test parameters: sensitivity (probability of test being positive in the presence of infection) and specificity (probability of test being negative in the absence of infection).

MATERIAL AND METHODS

Ethical considerations. The present research protocol was approved by the Institutional Research Board (IRB) of the Bahiana School of Medicine and Public Health (EBMSP) in Salvador (protocol no. 464.286). All procedures were performed in accordance with the principles established in the Declaration of Helsinki and its subsequent revisions.

Sample selection. The present diagnostic accuracy study was carried out between February 2015 and December 2017 using anonymous plasma samples obtained from the biorepository of the Integrated and Multidisciplinary HTLV Center (CHTLV) at EBMSP. CHTLV is an outpatient clinic, open to the public, that provides inter-disciplinary care and services, including general medical treatment, laboratory diagnosis, psychological counseling and physical therapy. All included plasma samples had been previously screened for antibodies against HTLV-1/2 using an enzyme-linked immunosorbent assay (Ortho® HTLV-1/HTLV-2 Ab-Capture ELISA Test Systems, Ortho-Clinical Diagnostic, Raritan,

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95 USA), and reactive samples were retested by Western Blot (HTLV Blot 2.4, Genelabs
96 Diagnostics®, Singapore). Test results were interpreted according to the stringent criteria
97 indicated by the manufacturer and in accordance with the guidelines established by the
98 Brazilian Ministry of Health (20).

99 The panel consisted of 397 samples: 200 HTLV-negative, 170 HTLV-positive (122
100 HTLV-1, 31 HTLV-2, 5 HTLV-1+HTLV-2, and 12 HTLV), and 27 WB-indeterminate.
101 Briefly, HTLV-negative samples were defined as those lacking reactivity to HTLV-specific
102 proteins; HTLV-1-positive samples were defined as reactive to GAG (p19 with or without
103 p24) and two ENV (GD21 and rgp46-I); HTLV-2-positive samples were reactive to GAG
104 (p24 with or without p19) and two ENV (GD21 and rgp46-II); HTLV seropositive samples
105 were reactive to GAG (p19 and p24) and ENV (GD21); samples were considered
106 indeterminate when no HTLV specific bands were detected, i.e. the criteria for HTLV-I,
107 HTLV-II or HTLV were not satisfied. Indeterminate samples were assessed by PCR analysis
108 and the obtained results were used to compare agreement with ELISA screening test results.
109 For performance analysis, the WB-indeterminate samples were excluded, forming a final
110 study panel of 370 samples (Fig. 1).

111 Alternatively, 217 plasma samples (112 positive, 105 negative) were also assessed by
112 chemiluminescence assay - CLIA (Architect rHTLV-1/2, Abbott Diagnostics Division,
113 Wiesbaden, Germany).

114 **Immunoassays.** Three HTLV1/2-specific enzyme immunoassay kits, all
115 commercially available in Brazil, were employed in this study: Murex HTLV-1/2 (DiaSorin
116 S.p.A., Dartford, UK), anti-HTLV-1/2 Sym Solution (Symbiosis Diagnostica LTDA, Leme,
117 Brazil) and Gold ELISA HTLV-1/2 (Rem Indústria e Comércio LTDA, São Paulo, Brazil).
118 Cut-off values, as well as gray zones, were calculated for each test as follows: by adding 0.2

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to the mean of the negative control replicates for Murex HTLV-1/2; adding 0.18 to the mean of the negative control replicates for Anti-HTLV-1/2 Sym Solution; by adding 0.25 to the mean of the negative control replicate for Gold ELISA HTLV-1/2. For data normalization, all results were expressed by plotting values in an indexed format, calculated as the ratio between a given sample's optical density (OD) and the cut-off OD values respective to each assay. Under this index, referred to as a reactivity index (RI), all results <1.00 were considered negative. When a sample's RI value was 1.0 ± 10%, the result was considered as indeterminate (i.e. in the grey zone), and these samples were deemed inconclusive.

HTLV-1/2 molecular detection. Peripheral blood mononuclear cells (PBMC) from 27 patients with WB-indeterminate results were obtained from EDTA blood samples under density gradient centrifugation; DNA was extracted using a spin column kit (Qiagen, Hilden, Germany). DNA samples were submitted to nested-PCR using the HTLV-1 long terminal repeat (LTR) 5' region primers as described previously (25), outer primers BSQF6/BSDR3 and inner primers BSQF2/BSDR4, to amplify a 672-bp fragment in the HTLV-2 LTR region (26). All amplified products were submitted to electrophoresis on a 1% agarose gel with Syber Safe DNA (Invitrogen).

Statistical analysis. Data were encoded and analyzed using scatterplot computer graphic software (Prism version 7; GraphPad, San Diego, CA). Descriptive statistics are presented as geometric means ± standard deviation. To test data normality, the Shapiro-Wilk test, followed by Student's t-test, were used. When assumed homogeneity was not confirmed, Wilcoxon's signed rank test was used. All analyses were two-tailed, and p-values under 5% were considered significant ($p < 0.05$). Enzyme immunoassay test performance was computed using a dichotomous approach and compared in terms of sensitivity, specificity, accuracy, likelihood ratio (LR) and diagnostic odds ratio (DOR). Additionally, receiver

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operating characteristic (ROC) curves were constructed and the areas under these curves were used as a global measure of test performance. Confidence intervals (CI) were employed at a confidence level of 95%. The strength of agreement between screening commercial tests and PCR results was assessed by Cohen's Kappa coefficient (κ) (27), which accounts for agreement occurring only by chance beyond simple percentage agreement calculations. κ values are interpreted as poor ($\kappa \leq 0$), slight ($0 < \kappa \leq 0.20$), fair ($0.21 < \kappa \leq 0.40$), moderate ($0.41 < \kappa \leq 0.60$), substantial ($0.61 < \kappa \leq 0.80$) and almost perfect agreement ($0.81 < \kappa \leq 1.0$). A flowchart (Fig. 1) have been provided the Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines (28).

RESULTS

Assay performance. Using plasma from 170 HTLV-positive individuals, ELISA and CLIA performance were assessed, as shown in Fig. 2. The area under the curve (AUC) values were >99%, demonstrating excellent overall diagnostic accuracy for all kits tested. RI values for HTLV-1/2-positive samples were variable, ranging from 14.2 for SYM Solution, 14.5 for Gold ELISA to 16.8 for Murex. In addition, Architect r-HTLV-1/2 yielded the highest RI value (>90).

As all kits test demonstrated 100% sensitivity, no statistically significant differences were detected. Regarding the HTLV-1/2-negative samples, SYM Solution and Gold ELISA presented specificity values >99%. Architect r-HTLV-1/2 showed a specificity of 98.1%, followed by Murex at 92.0%. Differences in specificity and RI were not statistically significant between the SYM Solution and Gold ELISA kits. With respect to HTLV-negative samples, the maximum RI value was obtained using Murex (RI = 0.30) (Fig. 2). Considering

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RI values of 1.0 ± 0.10 as inconclusive, i.e. falling in the gray zone, we verified that truly positive HTLV-1/2 samples were conclusively diagnosed by the Gold ELISA, Murex, SYM Solution and Architect r-HTLV-1/2 tests. As regards the HTLV-negative samples, one fell in the gray zone using the Gold ELISA test. With respect to diagnostic accuracy, Gold ELISA, SYM Solution, and Architect rHTLV-1/2 tests demonstrated the highest accuracy (>99.1%), while Murex presented the lowest result (95.6%). DOR scores, based on likelihood ratios, were 524,000 for Architect rHTLV-1/2, 338,200 for Gold ELISA, 168,254 for SYM Solution and 19,552 for Murex HTLV-1/2. Among the ELISA kits evaluated, Gold ELISA offered the best performance, as evidenced by ROC analysis and, notably, the exceptionally high diagnostic odds ratio produced by this test (Fig. 2). No significant differences in RI signal were observed with regard to the different types of seroreactivity (HTLV-1 vs. HTLV-2 vs. HTLV-1/2 and HTLV).

Assay agreement. Analysis of the diagnostic accuracy of the three commercial ELISAs with respect to 27 WB-indeterminate samples, considering PCR amplification as a gold standard for HTLV diagnosis, revealed that eight samples were negative (29.6%) and 19 were positive (70.4%) for HTLV-1 (Fig. 3), with all ELISA tests yielding 100% sensitivity. Conversely, all three assays presented specificity inferior to 25%, with Gold ELISA offering just 12.5% specificity. Despite this very low accuracy, both Murex and SYM Solution kits offered higher accuracy than Gold ELISA. Slight and fair agreement (Cohen's Kappa < 0.40) between PCR analysis and the ELISA screening tests was detected with regard to diagnosing HTLV infection. Table 1 details the 27 HTLV-indeterminate profiles that allowed for the identification of distinct patterns. No HGIP (29) or N (30) patterns were observed.

DISCUSSION

The present study found a high diagnostic value for each of the four different evaluated commercially available HTLV screening tests used to detect anti-HTLV antibodies in Brazil. In fact, AUC values greater than 99% demonstrates convincing evidence of the optimal discriminative power of these kits regarding HTLV-positive and HTLV-negative samples. Gold ELISA and Architect rHTLV-1/2 both presented AUC values of 100%. Furthermore, the Murex, SYM Solution and Architect rHTLV-1/2 assays did not show inconclusive results (grey zone) in HTLV antibody screening procedures. Gold ELISA yielded low number of inconclusive results, as only one out of 170 HTLV-positive samples tested using this kit produced an RI value that fell in the grey zone.

All tests displayed 100% sensitivity in diagnosing HTLV-positive samples. RI values were higher than 14 for the ELISA tests and above 90 for the Architect rHTLV-1/2 kit, which corroborates previous reports (31). Regarding the ELISA tests, the highest RI value was achieved by Murex, with statistically significant differences seen compared to Gold ELISA and SYM Solution.

Due to the high number of misdiagnosed samples (4.3%) under the Murex test, its accuracy was significantly lower compared to the other kits. Gold ELISA, SYM Solution and Architect rHTLV-1/2 were all found to be 99% accurate, suggesting that these kits can be safely employed for HTLV infection screening. Although the Murex test is less accurate, it nonetheless returned values above 95%, indicating suitability in the diagnosis of HTLV infection; however, the proportion of samples requiring WB confirmation was greater, which increases the cost of performing diagnosis. In fact, 8% of the HTLV-negative samples assayed with Murex yielded false-positive results, with a specificity of 92%. It is interesting to note that this test's performance has improved over time, as studies performed in 2007 and

2009 described its sensitivity and specificity at 98.2% and 42.6%, respectively (32, 33). Another study conducted in Argentina showed that Murex was 97.2% sensitive and 99.7% specific (34). More recently, other studies have reported high values of specificity, such as those evaluating HIV/HTLV co-infected individuals (99.0%) (31) and blood donors (97.2%) (34). With respect to HTLV-negative samples, the Murex test returned the highest RI value. The observed differences in RI values could arise from variability in antigenic composition. While all tests correctly diagnosed positive samples, it is possible that the antigenic matrix employed in the solid phase of the Murex kit recognized no specific anti-HTLV antibodies, which led to false-positive results or cross-reactions.

Of note, the sensitivity, specificity, and accuracy values associated with diagnostic tests are unsatisfactory in terms of influencing clinical decisions (35). A diagnostic test can only be considered valid if the results produced modify the probability of disease occurrence. Likelihood Ratio (LR) measurements can be helpful in describing a test's discriminatory power and determining the possibility of a particular result occurring among infected individuals, as opposed to the probability of the same result being obtained among healthy individuals (36). In our study, Gold ELISA had a positive LR of 201, indicating that an HTLV-infected person is approximately 201 times more likely to be diagnosed with this infection if evaluated with this kit. The lowest positive LR value was observed with the Murex test (12.6), indicating a low probability for an HTLV-infected person to be accurately diagnosed. Conversely, a study performed in 2008 found a positive LR of 326.5 for Murex (34). HTLV-negative samples returned LR values lower than 0.001 under all of the evaluated tests. There is a consensus that positive LRs above 10 and negative LRs below 0.1 contribute substantially to diagnosis (36). DOR, calculated as the ratio between positive and negative LR values, is considered a global performance parameter that summarizes the diagnostic test

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accuracy. DOR values describe the probability of receiving a positive result for a person with infection, as opposed to someone who is uninfected (35). The DOR for Architect rHTLV-1/2 (524,000) was the highest among the screening tests evaluated, followed by Gold ELISA (338,200), SYM Solution (168,254) and Murex (19,552). These findings suggest that Architect rHTLV-1/2 and Gold ELISA offer superior performance to SYM Solution and Murex. LR and DOR determination are relevant and stable tools, since these parameters remain independent of the prevalence of disease (37). The HTLV-1 and HTLV-2-seroindeterminate WB patterns observed herein were similar to those reported by other studies. However, no HGIP or N patterns were identified.

It is important to note that, concerning the Architect rHTLV-1/2 test, our findings are in agreement with those reported by other studies. In fact, identical values of sensitivity (100%) and specificity (>99%) have been described in both samples from blood donors and hospitalized patients (38). Similar results were demonstrated by Malm et al. (39) (Sen 100%; Spe 99.8%), as well as by Qiu et al. (40) (Sen 100%; Spe 99.98%) in general populations of the USA, Japan and Nicaragua. Although the present study was unable to assess other screening tests, the literature indicates the high performance of both the Elecsys HTLV-I/II and Abbott Prism HTLV-I/HTLV-II kits (Sen 100%; Spe > 99%) in samples from both blood donors and other obtained from a routine diagnostic service (41). The DiaSorin LIAISON® XL recHTLV-I/II kit was also evaluated elsewhere, with high sensitivity and specificity values reported, similarly to the Architect rHTLV-1/2 test (42–44).

The results presented herein indicate that all evaluated kits can safely be used for HTLV-infection screening. However, it is important to note that the high sensitivity offered by these kits may lead to false-positive results, which could increase cost as a result of WB confirmation requirements. From the perspective of large diagnostic centers and blood banks,

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proper screening method selection can substantially reduce costs associated with confirmatory testing. In an effort to reduce costs and assure correct diagnosis, a new diagnostic protocol for HTLV-infection diagnosis was proposed by Costa et al. (45), who suggested the use of two ELISA tests for screening purposes, followed by real-time PCR. In this case, WB confirmation would only be indicated in cases of negative PCR results. Herein, when the 27 WB-indeterminate samples were analyzed by PCR, all HTLV-1 positive samples demonstrated agreement with results from each of the three ELISA tests evaluated. On the other hand, overall agreement was slight or fair due to the high number of false-positive results obtained using ELISA. Moreover, it has been demonstrated that the INNO-LIA HTLV I/II Ab serological confirmatory assay for HTLV yielded results for most of the samples considered indeterminate or positive, but untypeable, in WB assays (31, 46). These data suggest the costs associated with HTLV-infection diagnosis could be lowered by using molecular biology-based methodologies, or INNO-LIA HTLV, as a confirmatory assay in place of WB. In the context of low-income countries, such as those in Africa and Latin America, we suggest that CLIA represents a suitable screening strategy for blood banks due to the high DOR values found herein. However, in countries lacking the necessary infrastructure, the use of an ELISA offering a high DOR value, e.g. Gold Elisa, seems to be a satisfactory alternative.

Despite the scarcity of studies evaluating the diagnostic performance of screening tests in diagnosing HTLV-infection by employing LR, DOR and AUC as performance parameters, we evaluated three ELISA tests and one CLIA used for HTLV-infection screening. Based on the present findings, we conclude that all of the 3rd generation commercially available kits employed herein presented high sensitivity and specificity values compared to previous

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studies. Among the ELISA tests evaluated, the Gold HTLV-1/2 kit offered the best performance parameters, while the ARCHITECT rHTLV-1/2 demonstrated the highest performance of all the assays considered. High sensitivity values produced by screening tests could lead to high proportions of false-positive results. Thus, we reinforce our previous suggestion and urge the consideration of a new protocol employing molecular biology or line immune assay (INNO-LIA HTLV) techniques as a first choice for confirmatory testing in place of WB.

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Fig 1 Flowchart depicting study design in accordance with the Standards for Reporting of Diagnostic Accuracy studies (STARD) guidelines.

Fig 2 Reactivity index of screening assays obtained in positive (red dots) and negative (blue dots) plasma samples under HTLV-1/2 WB analysis. The cut-off value was IR = 1.0

and the area delimited by lines represents the indeterminate zone (RI ± 10%). Numbers shown for each group are representative of geometric means (± 95% CI); AUC (Area Under Curve); Sen (Sensitivity); Spe (Specificity); Acc (Accuracy); LR (Likelihood Ratio); DOR (Diagnostic Odds Ratio).

Fig 3 Analysis of WB-indeterminate samples using PCR as a gold standard. Acc (accuracy); CI (confidence interval); κ (Cohen's Kappa coefficient); PCR (polymerase chain reaction); Sen (sensitivity); Spe (specificity).

517 TABLE 1. Indeterminate HTLV patterns in samples from Brazil

WB Pattern	***n (%)***	**Gold[b]**	**Murex[c]**	**SYM[d]**
gd21 alone	7 (25.9)	7/7	6/7	6/7
gd21+p19	7 (25.9)	7/7	7/7	7/7
gd21+synthetic peptides (46I or 46II)	5 (18.5)	4/5	4/5	4/5
Others[a]	8 (29.7)	8/8	8/8	8/8
HGIP (29)	0	-	-	-
N (30)	0	-	-	-
Total	27 (100)	26/27	25/27	25/27

518 [a]One band each for gd21 plus p19 plus p28, gd21 plus p19 plus p26 plus p28 plus p32, gd21
519 plus p19 plus p28 plus p36, gd21 plus p19 plus p26 plus p28 plus p36, plus gd21 plus p19
520 plus p26 plus p28 plus p32 plus p36, p19 plus p21 plus p26 plus p28 plus p32 plus p36 plus
521 MTA-1 plus pr53, p19 plus p26 plus p28, synthetic peptide 46II alone

522 [b]Gold ELISA HTLV-1/2 (Rem Indústria e Comércio LTDA, São Paulo, Brazil);

523 [c]Murex HTLV-1/2 (DiaSorin S.p.A., Dartford, UK);

524 [d]Anti-HTLV-1/2 Sym Solution (Symbiosis Diagnostica LTDA, Leme, Brazil)

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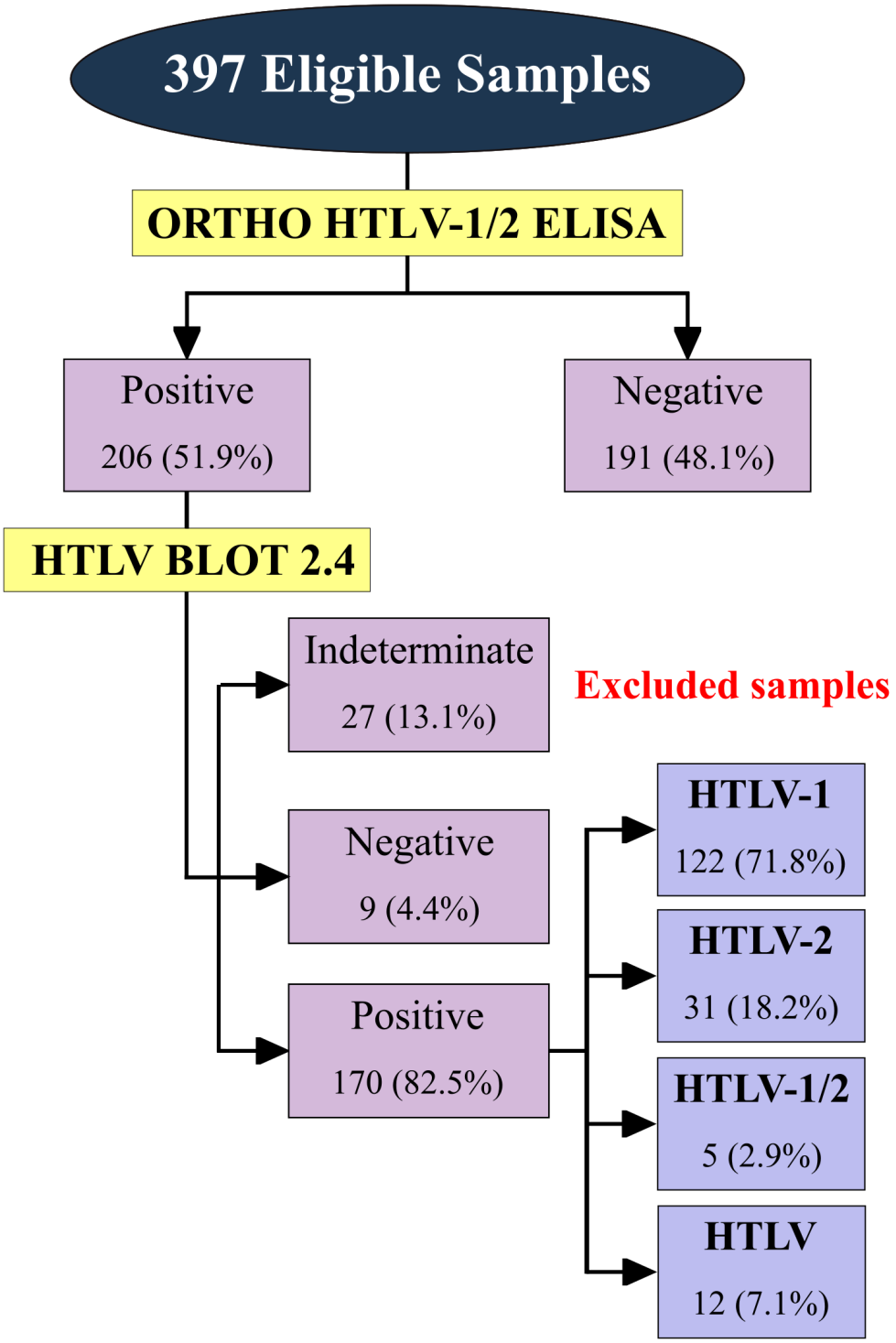
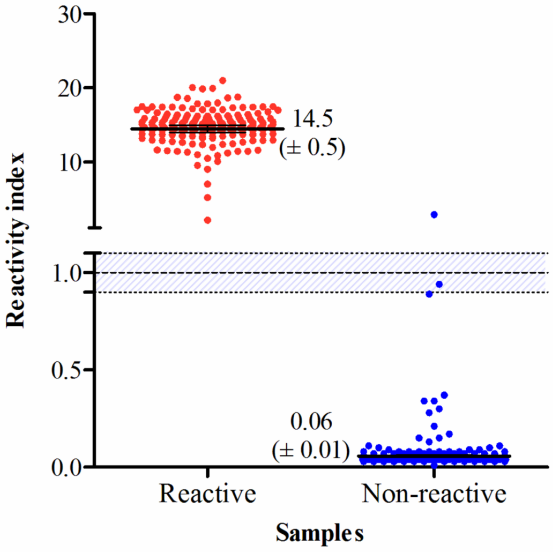
Study design to evaluate the performance of commercial HTLV screening tests



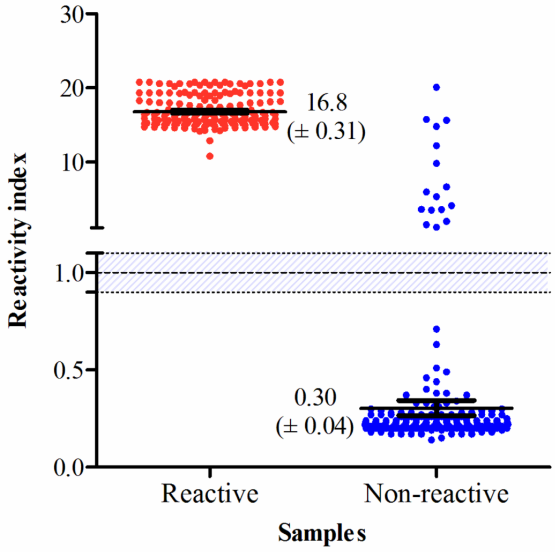
GOLD ELISA HTLV-1/2



AUC: 1.0000
Sen (%): 100 (97.8-100)
Spe (%): 99.5 (97.2-99.9)
Acc (%): 99.7 (98.5-100)

LR+: 201
LR-: 0.001
DOR: 338,200

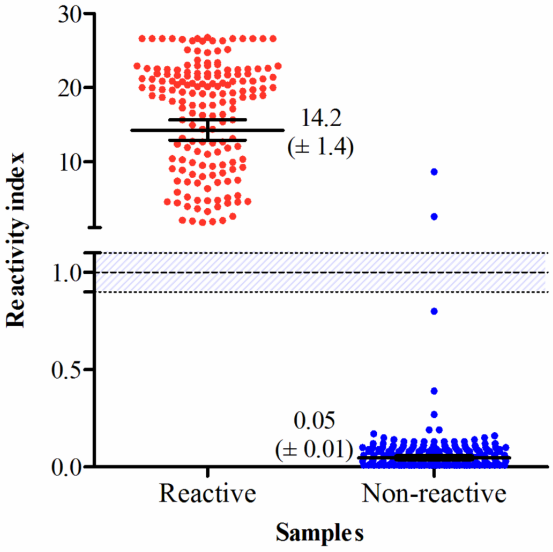
MUREX HTLV-1/2



AUC: 0.9915 (± 0.0098)
Sen (%): 100 (97.8-100)
Spe (%): 92.0 (87.5-95.0)
Acc (%): 95.7 (93.1-97.3)

LR+: 12.6
LR-: 0.001
DOR: 19,552

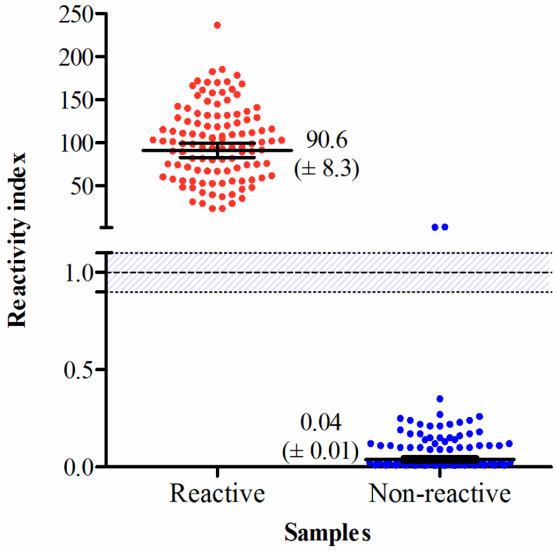
ANTI-HTLV-1/2 SYM SOLUTION



AUC: 0.9989 (± 0.0021)
Sen (%): 100 (97.8-100)
Spe (%): 99.0 (96.4-99.7)
Acc (%): 99.5 (98.1-99.9)

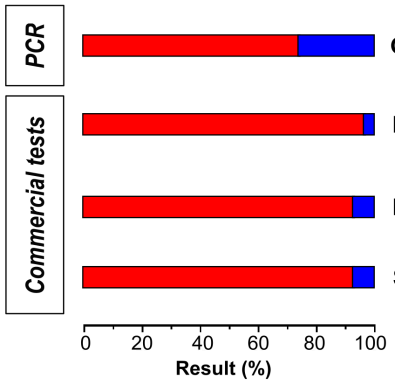
LR+: 100.4
LR-: 0.001
DOR: 168,254

ARCHITECT rHTLV-1/2



AUC: 1.0000
Sen (%): 100 (96.7-100)
Spe (%): 98.1 (93.3-99.5)
Acc (%): 99.1 (96.7-99.7)

LR+: 52.4
LR-: 0.0001
DOR: 524,000



	κ (95%CI)	Sen (95%CI)	Spe (95%CI)	Acc (95%CI)
Gold Standard →	1.00	100	100	100
ELISA GOLD →	0.17 (-0.36-0.70)	100 (83.2-100)	12.5 (2.2-47.1)	74.1 (55.3-86.8)
Murex →	0.32 (-0.16-0.80)	100 (83.2-100)	25.0 (7.1-59.1)	77.8 (59.2-89.4)
SYM →	0.32 (-0.16-0.80)	100 (83.2-100)	25.0 (7.1-59.1)	77.8 (59.2-89.4)

LEGEND Positive Negative