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Kerstin Malm, Torbjörn Hans Kjerstadius, Sören Andersson. Evaluation of a new screening assay for HTLV-1 and -2 antibodies for large-scale use.. Journal of Medical Virology, 2010, 82 (9), pp.1606. 10.1002/jmv.21867 . hal-00556039

HAL Id: hal-00556039

<https://hal.science/hal-00556039>

Submitted on 15 Jan 2011

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**Evaluation of a new screening assay for HTLV-1 and -2
antibodies for large-scale use.**

Journal:	<i>Journal of Medical Virology</i>
Manuscript ID:	JMV-09-1694.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	06-May-2010
Complete List of Authors:	Malm, Kerstin; Örebro University Hospital, Dept of Laboratory Medicine Kjerstadius, Torbjörn; Karlstad Central Hospital, Dept of Clinical Microbiology Andersson, Sören; Swedish Institute for Infectious Disease Control, Dept of Virology
Keywords:	HTLV-1/2, Assay evaluation, Sensitivity, Specificity

Table 1. Dilution series of two HTLV-positive specimens

Dilution	RPR207-09 HTLV-1 Signal/cut-off ratio	RPR207-08 HTLV-2 Signal/cut-off ratio
1/1	149.60	113.53
1/10	115.26	83.09
1/100	74.82	32.47
1/1000	34.06	5.88
1/10,000	7.74	1.40
1/100,000	2.65	0.63 ^a
1/1,000,000	1.03	0.67 ^a

a) Samples with s/co < 1.0 are rated as non-reactive

Table 2. Sensitivity and specificity for the Abbott ARCHITECT *r*HTLV assay*a) Sensitivity*

Sample population	n	Number reactive	Sensitivity (95% CI)
HTLV-1 positive	74	74	100% (94.95–100)
HTLV-2 positive	28	28	100% (86.83–100)
Quality control samples, HTLV positive	26 (18 HTLV-1, 7 HTLV-2, 1 HTLV-1/2)	26	100% (85.81–100)
Commercial panel PRP207	14 (7 HTLV-1, 7 HTLV-2)	14	100% (73.65–100)
Overall sensitivity	142	142	100% (97.38–100)

b) Specificity

			Specificity (95% CI)
Blood donors, HTLV- negative	504	0	100% (99.27–100)
Serum samples with rheumatoid factor	36	0	100% (89.75–100)
Serum samples with autoimmune antibodies	37	0	100% (90.03–100)
Serum samples with antibodies to viral infections ^a	30	0	100% (87.70–100)
Previously “false- reactive” samples ^b	16	4	75% (53.80–96.20)
Overall specificity	623	4	99.36% (98.35–99.83)
Samples from routine diagnostics analysed on ARCHITECT only ^c	1412	0	100% (99.74–100)
Overall specificity including samples from routine diagnostics	2035	4	99.80% (99.50–99.95)

a) HBV n=10, HCV n=10, CMV n=5, EBV n=5; **b)** Reactive by the Murex HTLV I/II assay, 12 negative and 4 indeterminate by confirmatory testing; **c)** Samples analysed between 8 Dec. 2008 and 1 Oct. 2009 at USÖ (analysed fresh).

Table 3. Previously false-reactive samples, results when tested with the *r*HTLV I/II assay.

Nr	Sample origin	<i>r</i> HTLV s/co ratio	Murex s/co ratio	Confirmatory result
1	New blood donor	0.21	2.08	Negative
2	New blood donor	0.25	1.03	Negative
3	New blood donor	0.57	1.9	Negative
4	Regular blood donor	0.25	1.1	Negative
5	New blood donor	0.19	1.48	Negative
6	New blood donor	8.75	3.95	Indeterminate
7	New blood donor	44.51	11.47	Indeterminate
8	Unknown	0.82	1.63	Negative
9	New blood donor	1.47	4.10	Negative
10	New blood donor	0.26	1.32	Negative
11	Breast milk donor	0.23	2.48	Negative
12	New blood donor	0.25	1.39	Indeterminate ^a
13	New blood donor	0.23	10.43	Negative
14	New blood donor	0.37	1.05	Negative
15	New blood donor	1.17	1.28	Indeterminate
16	New blood donor	0.67	2.45	Negative

Bold letters indicate reactive result.

^a) This sample had weak reactivities for p24 and gp21 in western blot. A follow up sample one year later had only a weak p24 band. The samples were considered non-infected, but with indeterminate reactivities

Figure 1. Distribution of signal/cut-off values for 504 negative samples from blood donors

Samples with s/co < 1.0 are rated as negative.

Evaluation of a new screening assay for HTLV-1 and -2 antibodies for large-scale use

Kerstin Malm¹, Torbjörn Kjerstadius², Sören Andersson³

¹Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro, Sweden;

²Department of Clinical Microbiology, Karlstad Central Hospital, Karlstad, Sweden;

³Departement of Virology, Swedish Institute for Infectious Disease Control, Solna Sweden

Running title: **HTLV-1/2 antibody assay evaluation**

Key words: HTLV-1/2, assay evaluation, sensitivity, specificity

Correspondence to: Kerstin Malm, Department of Laboratory Medicine, Microbiology, Örebro University Hospital, 701 85 Örebro, Sweden

E-mail: Kerstin.malm@orebroll.se

Telephone: +46 19 602 11 34

Fax: +46 19 12 74 16

Abstract

Laboratory testing for Human T-lymphotropic Virus type 1 and 2 (HTLV-1 and-2) infections has become routine in blood transfusion, tissue transplantation and clinical diagnoses in many countries worldwide. Screening is usually based on the detection of antibodies to HTLV-1 and/or -2. The number of commercially available assays is limited, and among them, ELISA tests based on microtiter format are most commonly used. Recently, the new *r*HTLV-I/II assay (Abbott Laboratories, Abbott Park, IL, USA) was released; this assay was developed for an automatic large-scale screening platform. This assay was evaluated using pre-characterised serum panels and routine samples from the clinical laboratory. The sensitivity was 100% for HTLV-1 and -2 (99/99 and 42/42, respectively, including one sample that was dually reactive, HTLV-1+2). To test assay specificity, panels of blood donor sera, specimens from patients with autoimmune diseases and some viral infections were used. False-reactive samples from previous HTLV diagnoses were also included. With these panels, the specificity was 99.4% (619/623). However, the four false-reactive samples all belonged to the group of samples that were previously considered as false-reactive for HTLV-antibodies. All other samples were negative by the *r*HTLV-I/II assay, and thus 100% specificity was obtained. The 1 412 samples tested in the clinic by this assay in routine use were all negative (100% specificity). Taken together, the overall specificity was 99.8%. The assay was sensitive, specific and appropriate for the large-scale screening of samples for HTLV-1/2 antibodies.

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Introduction

The human T-lymphotropic viruses type 1 and 2 (HTLV-1 and -2) are effectively transmitted via blood and blood products [Hall et al., 1996, Verdonck et al., 2007]. Therefore, many countries have introduced mandatory screening for HTLV infection of blood and plasma donors. The most cost-efficient mode of screening is by the detection of HTLV-1 or -2 antibodies. Enzyme immuno assays (EIAs) have been used for this screening, and the majority of these EIA screening assays have been based on a microtiter plate format [Andersson et al., 1999; Berini et al., 2008]. Confirmation of screening-reactive specimens has usually been accomplished using immunoblot assays (western blot or line immunoassays) [Thorstensson et al., 2002]. The number of commercially available antibody-screening assays is limited, and the development and release of new assays have been rather few over a period of several years.

Instruments have become increasingly common for use in large-scale screening of blood samples for blood-borne infectious diseases, and assays have been developed for the most prevalent microorganisms and adapted for these instruments [Malm et al., 2009; Kim et al., 2008; Marangoni et al., 2009]. Until recently, no assay for HTLV-1 or -2 has been available on such instruments. With the release of the *r*HTLV-I/II assay (Abbott Laboratories, Abbott Park, IL, USA) it may be possible to perform a package of screening assays for blood-borne infections that, in many countries, would be specific for microorganisms of regional priority. This will allow an important rationalisation of testing procedures in laboratories that have suitable equipment. It is also the first new commercially available screening assay for HTLV-1 and -2 in many years. This new automated assay for detection of antibodies to HTLV-1 and HTLV-2, *r*HTLV-I/II, is an immunoassay using a chemiluminescent microparticle technique [Qiu et al., 2008].

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5 75 HTLV-1 or HTLV-2 infected individuals and various HTLV-negative specimens. The assay
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Material and Methods

Screening immunoassays

This study evaluated an automated, chemiluminescent immunoassay assay for the detection of antibodies to HTLV-1 and HTLV-2 (*r*HTLV-I/II). All assay steps were performed by the automated ARCHITECT *i* system immunoanalyser (Abbott Laboratories). The solid phase of the assay consisted of paramagnetic microparticles coated with three different antigens; HTLV-1 gp 46 syntetic peptide, HTLV-2 gp 46 syntetic peptide and HTLV-2 gp 21 recombinant antigen. The samples were each mixed with assay diluent, and an aliquot of the mix was combined with the microparticles. After incubation and washing, an acridinium-labelled conjugate with HTLV-1 gp 21 recombinant antigen, HTLV-1 gp 46 and HTLV-2 gp 46 syntetic peptides were added. After another incubation and wash cycle, pre-trigger (H₂O₂) and trigger (NaOH) were added. This resulted in a chemiluminescent reaction, which was measured by the instrument's optical system as relative light units (RLU). The presence or absence of anti-HTLV antibodies was analysed by comparing the chemiluminescent signal to the cut-off signal determined by a calibration step. The ARCHITECT *i* System calculates the cutoff (CO) by using the mean chemiluminescent signal (RLU) from three replicates of the Calibrator 1 multiplied by 0.25. The result is shown as a signal-to-cut-off ratio (s/co). Samples with s/co ratios ≥ 1.0 are considered reactive.

The immunoanalyser used, ARCHITECT i2000 (Abbott Laboratories), is a fully automated instrument that performs all the assay steps, from sample collecting to reporting of the result [Hendriks et al., 2000]. The samples that were analysed in this evaluation were also analysed with the Murex HTLV I/II assay (Murex Biotech Ltd, Dartford, United Kingdom), which was the routine assay used by a large proportion of laboratories in Sweden prior to the introduction of the *r*HTLV-I/II assay. Murex HTLV I/II is a microtiter plate EIA assay . The microtiter-plate wells are coated with HTLV-2 p21 recombinant antigen and HTLV-1 and -2

gp 46 peptides. Sample and reagent addition steps, as well as washing steps, were performed manually with the use of a washing instrument (Anthos Fluido, Anthos Nordstrasse 4, Eugendorf, Austria); samples were read manually using a spectrophotometer (Multiskan Ascent, Thermo Labsystems, P.O. Box 208, Helsinki, Finland). The conjugate in this assay was horseradish peroxidase-labelled HTLV-1 p21 recombinant antigen, HTLV-1 and -2 gp 46 peptides. The resulting absorbance values were divided by the cut-off value, like the ARCHITECT results, to give an s/co ratio. The cut-off value is determined by adding 0.200 to the mean value of the negative control. All experimental protocols and analyses were performed according to the manufacturers' directions.

Serum panels

The following samples were used for the evaluation of assay sensitivity:

- a) Serum panels kept at the Swedish institute for Infectious Disease Control, Stockholm, Sweden (SMI) including HTLV-1 positive samples from Guinea-Bissau (n=63 and Sweden (n=11) and HTLV-2 positive samples from Sweden (n=28). The HTLV-1 positive samples from Guinea-Bissau had been collected in previous field studies described elsewhere [Andersson et al 1999, Norrgren et al., 1995, Andersson et al., 1997]. They included samples from occupational cohorts, pregnant women, outpatients clinics and hospitals. The HTLV-2 positive specimens were obtained from intravenous drug users in Stockholm, Sweden, also described previously [Andersson et al., 1995];
- b) Confirmed positive quality-control samples, (26 total; 18 HTLV-1, 7 HTLV-2, and 1 HTLV-1/2). These samples were sent to clinical laboratories for quality evaluation of the laboratory methods during the years 2001–2008. The samples were prepared by the External Quality Assurance in Laboratory Medicine in Sweden (Equalis AB, Box

977, Uppsala, Sweden). Twenty-three of these samples were undiluted, while one sample was diluted 1/50, one 1/100 and one 1/1000;

c) A commercial panel consisting of 14 reactive (7 HTLV-1, 7 HTLV-2) and one non-reactive sample (PRP207, BBI diagnostics, West Bridgewater, Ma, USA). Two of these panel members (one HTLV-1, PRP207-09 and one HTLV-2, PRP207-08) were also serially diluted 10-fold into six dilutions (1/10 to 1/10⁶).

The following samples were tested to evaluate the specificity of the assay:

d) 504 samples from non-reactive blood donors that had tested HTLV-negative using the Murex assay in the past;

e) 36 samples from patients with positive rheumatoid factor;

f) 37 samples from patients with antibodies associated with autoimmune disease (anti-nuclear antibodies, SS-A antibodies and/or native DNA-antibodies);

g) 30 samples with antibodies/antigens to some viral infections, namely, Hepatitis B (HBV, n=10), Hepatitis C (HCV, n=10), Cytomegalovirus (CMV, n=5) and Epstein-Barr virus (EBV, n=5);

h) 16 samples (14 clinical samples and 2 quality-control samples) that were repeatedly reactive with the Murex assay but regarded as false-reactive, as they could not be confirmed positive with confirmatory assays (12 negative and 4 indeterminate);

i) 1 412 routine samples that were analysed at the Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro, Sweden (USÖ), from December 2008 to October 2009; these samples were analysed only with the ARCHITECT rHTLV I/II assay.

The samples in groups c and h were analysed at the Department of Laboratory Medicine, Karlstad Hospital, Karlstad, Sweden (KH), as were 10 samples from group e and 10 samples from group f. The other sample groups were tested at USÖ. Sample groups e and f were

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3 152 analysed with the Murex assay during this study, while the other samples were tested
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5 153 previously with the Murex assay. All samples were stored at -20°C prior to analysis on the
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10 155 The confirmatory assays used in this study were a line immunoblot assay; INNO-LIA
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12 156 HTLV I/II Score (Innogenetics NV, Technologiepark 6, Gent, Belgium) and a western blot
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15 157 assay; HTLV Blot 2.3 (Genelabs Diagnostics, Singapore). The confirmatory assays were
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17 158 performed at SMI, except for the commercial panel, where the assays were performed at BBI
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Results

Sensitivity evaluation

- a) The 102 clinical samples that were confirmed HTLV-positive were all reactive with the ARCHITECT *r*HTLV assay. The mean s/co ratio for these samples when analysed with the ARCHITECT *r*HTLV assay was 70.42 (73.96 for HTLV-1, 61.61 for HTLV-2), with s/co ratios ranging from 1.17 to 149.33 (14.88–149.88 for HTLV-1 and 1.17–87.16 for HTLV-2). Except for one sample ratio (1.17), the s/co ratios for the positive samples were at least five times higher than cut-off. The Murex assay's mean s/co ratio for 93 of these (available) samples was 10.56 (12.10 for HTLV-1, 7.08 for HTLV-2) with s/co ratios ranging from 1.12 to 15.82 (1.40-15.82 for HTLV-1 and 1.12-15.00 for HTLV-2).
- b) All 26 HTLV-positive quality-control samples analysed were reactive with the new ARCHITECT assay. The mean s/co ratio for these samples when analysed with the ARCHITECT *r*HTLV assay was 97.90 (115.36 for HTLV-1, 51.34 for HTLV-2) with s/co ratios ranging from 35.60 to 151.12 (35.60-151.12 for HTLV-1, n=16 and 48.79 to 53.41 for HTLV-2, n=6, undiluted samples only). With the Murex assay the mean s/co ratios for the same samples was 14.39 (15.09 for HTLV-1, 12.55 for HTLV-2) with s/co ratios ranging from 10.16 to 23.43 (10.16-23.43 for HTLV-1 and 10.72-16.76 for HTLV-2).
- c) All 14 (seven HTLV-1, seven HTLV-2) expected positive members of the commercial panel were reactive when tested with the ARCHITECT *r*HTLV assay. The non-reactive panel member was non-reactive with the ARCHITECT *r*HTLV assay. The mean s/co ratio for the panel samples when analysed with ARCHITECT *r*HTLV assay was 83.19 (89.05 for HTLV-1, 77.32 for HTLV-2) with s/co ratios ranging from 47.20 to 149.60 (47.20-149.60 for HTLV-1, and 66.26 to 71.36 for HTLV-2, positive

samples only). With the Murex assay the mean s/co ratios for the same samples was 11.64 (11.68 for HTLV-1, 11.60 for HTLV-2) with s/co ratios ranging from 11.60 to 11.80.

The two panel members that were serially diluted 10-fold six times showed an end-point dilution for HTLV-1 of $1/10^6$ and for HTLV-2 of $1/10^4$ (final sample still reactive; Table 1). In all, 141 (98 HTLV-1, 42 HTLV-2 and one HTLV-1/2) samples that were confirmed positive for HTLV-1 or/and HTLV-2 were also positive with the new *r*HTLV assay. Thus, the sensitivity for the *r*HTLV assay was 100% in this study (Table 2).

Specificity evaluation

- d) The 504 previously HTLV-negative blood donors were non-reactive when analysed with the ARCHITECT *r*HTLV-I/II assay. The mean s/co ratio for these samples was 0.22, compared to 0.32 when analysed with the Murex assay. In Figure 1, the distributions of s/co ratios for these samples are shown.
- e) The 36 samples from patients with rheumatoid factor antibodies were all non-reactive with the ARCHITECT *r*HTLV-I/II assay.
- f) The 37 samples from patients with anti-nuclear antibodies, SS-A antibodies and/or native DNA-antibodies were all non-reactive with the ARCHITECT *r*HTLV assay.
- g) The 30 samples with antibodies/antigens to viral infections were all non-reactive with the ARCHITECT *r*HTLV-I/II assay.
- h) Of the 16 samples that were previously regarded as false-reactive, 12 were non-reactive when tested with the ARCHITECT *r*HTLV assay. Among these, 11 were negative with confirmatory assays for HTLV-1/2, while one sample was indeterminate. Four samples were still reactive when tested with the ARCHITECT *r*HTLV assay. Three of these were indeterminate with confirmatory tests, one was

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211 negative. If these four samples were considered false-reactive, the specificity for the
212 ARCHITECT *r*HTLV-I/II assay in this evaluation is 99.36% (Table 2). More detailed
213 data on the 16 previously false-reactive samples (with the Murex assay) are shown in
214 Table 3.
215 i) When the samples from the routine screening at USÖ (n=1 412) were included, the
216 overall specificity was 99.80% (100% if the preselected Murex false-reactive samples
217 were excluded) (Table 2).

Discussion

The new ARCHITECT *r*HTLV-I/II fully automated assay for large scale screening of antibodies to HTLV-1 or -2 has been evaluated. The sensitivity of this assay was high for both HTLV-1– and HTLV-2–positive specimens. The specificity of this assay was high for HTLV-negative blood donor specimens as well as for samples collected from individuals with various autoimmune disorders or infections known to influence the results of other screening assays, such as EBV infections.

The number of commercially available assays for HTLV-1 and HTLV-2 antibody screening is limited. This new assay will be a useful alternative to the available assays. It is performed on the ARCHITECT *i* system immunoanalyser, which is designed for large-scale screening of blood samples. As this instrument also has assays for other markers for blood-borne infectious diseases, this assay will facilitate the screening process of blood donations and clinical samples, especially in laboratories with large volumes of samples. With just one sample tube it is possible to analyse all the markers needed.

It has been shown previously that for several ELISAs used to detect HTLV-1 and -2 antibodies it is possible to dilute or pool serum samples up to at least 1/10 and maintain a positive signal [Andersson et al., 2001; Chang et al., 2002]. This may be useful, for example, in studies of the seroepidemiology of large number of samples. Some countries are also using pools of samples established for nucleic acid testing (NAT) screening of blood donors (HIV, hepatitis B and C) for serological HTLV screening. The limited dilution experiment in this study indicates that a pooling strategy, combining 5–10 samples, can be used with the *r*HTLV I/II assay. However, it is important to realise that this strategy should not be used for clinical

diagnoses. An extended dilution experiment is also needed in order to conclude the utility of the Architect *r*HTLV-I/II assay for analyses of pooled samples.

When the screening of large numbers of negative samples is one of the main objectives in the assay, it is important to have a clear separation between the negative values and the cut-off value. This will minimise the risk of scoring samples as reactive when they are not actually positive. As shown in Figure 1, for the negative blood donor samples analysed in this study, the sample to cut-off (s/co) ratios for the *r*HTLV I/II assay are lower than for the Murex assay. Moreover, the mean s/co values for the positive samples are at least 10 times higher than the cut-off level for both HTLV-1 and HTLV-2. Thus, there is a wide separation of s/co ratios between positive and negative samples, which is in accordance with the high specificity observed in this evaluation.

The only samples that were designated false-reactive in this evaluation came from a group of samples that were reactive in prior tests using the Murex HTLV-I/II assay. It cannot be excluded that some of these samples were actually positive for HTLV-1 or -2 as follow-up samples were not available for all of them. For instance, it is known that HTLV-2 may be associated with low viral load and low antibody reactivity, which at times may give indeterminate results upon testing [Thorstensson et al., 2002]. Recent reports have also suggested the existence of HTLV-3 and possibly HTLV-4, which may also give indeterminate reactions in common assays for HTLV-1 and -2 [Calattini et al, 2005, Switzer et al, 2006, Wolfe et al 2005]. Of the 16 samples that were previously designated as indeterminate, 12 were negative by the *r*HTLV-I/II assay. Although this may be interpreted as a result of higher specificity for the *r*HTLV-I/II assay, it is important to remember that other samples with false reactivities may be selected with the *r*HTLV-I/II assay.

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In conclusion, the *r*HTLV-I/II assay performed well on the serum panels used in this evaluation, and with its design for large-scale screening, it may be a novel tool of high value in the diagnostic arsenal for blood-borne infections.

For Peer Review

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