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Kerstin Malm, Torbjörn Hans Kjerstadius, Sören Andersson

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

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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).

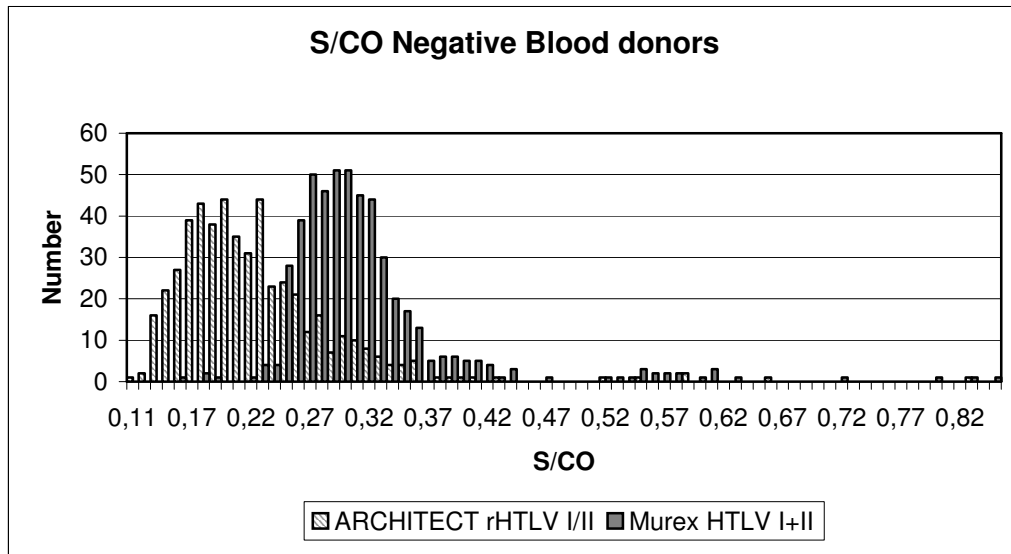
Table 3. Previously false-reactive samples, results when tested with the *r*HTLV I/II assay.

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

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3 **Figure 1. Distribution of signal/cut-off values for 504 negative samples from blood**
4 **donors**
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26 Samples with s/co < 1.0 are rated as negative.
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3 1 **Evaluation of a new screening assay for HTLV-1 and -2 antibodies for large-scale use**
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8 3 Kerstin Malm¹, Torbjörn Kjerstadius², Sören Andersson³
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11
12 5 ¹Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro,
13
14
15 6 Sweden;

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17 7 ²Department of Clinical Microbiology, Karlstad Central Hospital, Karlstad, Sweden;

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19 8 ³Departement of Virology, Swedish Institute for Infectious Disease Control, Solna Sweden
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24 10 Running title: **HTLV-1/2 antibody assay evaluation**
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33 14 Key words: HTLV-1/2, assay evaluation, sensitivity, specificity
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48 20 Correspondence to: Kerstin Malm, Department of Laboratory Medicine, Microbiology,
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51 21 Örebro University Hospital, 701 85 Örebro, Sweden
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53 22 E-mail: Kerstin.malm@orebroll.se
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55 23 Telephone: +46 19 602 11 34
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1
2
3 26 **Abstract**
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5 27 Laboratory testing for Human T-lymphotropic Virus type 1 and 2 (HTLV-1 and-2) infections
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7 28 has become routine in blood transfusion, tissue transplantation and clinical diagnoses in many
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10 29 countries worldwide. Screening is usually based on the detection of antibodies to HTLV-1
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12 30 and/or -2. The number of commercially available assays is limited, and among them, ELISA
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14 31 tests based on microtiter format are most commonly used. Recently, the new *r*HTLV-I/II
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16 32 assay (Abbott Laboratories, Abbott Park, IL, USA) was released; this assay was developed for
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18 33 an automatic large-scale screening platform. This assay was evaluated using pre-characterised
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20 34 serum panels and routine samples from the clinical laboratory. The sensitivity was 100% for
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22 35 HTLV-1 and -2 (99/99 and 42/42, respectively, including one sample that was dually reactive,
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24 36 HTLV-1+2). To test assay specificity, panels of blood donor sera, specimens from patients
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26 37 with autoimmune diseases and some viral infections were used. False-reactive samples from
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28 38 previous HTLV diagnoses were also included. With these panels, the specificity was 99.4%
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30 39 (619/623). However, the four false-reactive samples all belonged to the group of samples that
31
32 40 were previously considered as false-reactive for HTLV-antibodies. All other samples were
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34 41 negative by the *r*HTLV-I/II assay, and thus 100% specificity was obtained. The 1 412
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36 42 samples tested in the clinic by this assay in routine use were all negative (100% specificity).
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38 43 Taken together, the overall specificity was 99.8%. The assay was sensitive, specific and
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40 44 appropriate for the large-scale screening of samples for HTLV-1/2 antibodies.
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50 **Introduction**

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

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

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3 74 The rHTLV-I/II assay has been evaluated using well-characterised panels of sera from
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5 75 HTLV-1 or HTLV-2 infected individuals and various HTLV-negative specimens. The assay
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8 76 showed high sensitivity and specificity, and it is appropriate for use in a large-scale screening
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10 77 package.
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78 **Material and Methods**

79 *Screening immunoassays*

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

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

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3 103 gp 46 peptides. Sample and reagent addition steps, as well as washing steps, were performed
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5 104 manually with the use of a washing instrument (Anthos Fluido, Anthos Nordstrasse 4,
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8 105 Eugendorf, Austria); samples were read manually using a spectrophotometer (Multiskan
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10 106 Ascent, Thermo Labsystems, P.O. Box 208, Helsinki, Finland). The conjugate in this assay
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12 107 was horseradish peroxidase-labelled HTLV-1 p21 recombinant antigen, HTLV-1 and -2 gp
13
14 108 46 peptides. The resulting absorbance values were divided by the cut-off value, like the
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16 109 ARCHITECT results, to give an s/co ratio. The cut-off value is determined by adding 0.200 to
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18 110 the mean value of the negative control. All experimental protocols and analyses were
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20 111 performed according to the manufacturers' directions.
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24 112 *Serum panels*

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27 113 The following samples were used for the evaluation of assay sensitivity:
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- 29 114 a) Serum panels kept at the Swedish institute for Infectious Disease Control, Stockholm,
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31 115 Sweden (SMI) including HTLV-1 positive samples from Guinea-Bissau (n=63 and
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33 116 Sweden (n=11) and HTLV-2 positive samples from Sweden (n=28). The HTLV-1
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35 117 positive samples from Guinea-Bissau had been collected in previous field studies
36
37 118 described elsewhere [Andersson et al 1999, Norrgren et al., 1995, Andersson et al.,
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39 119 1997]. They included samples from occupational cohorts, pregnant women,
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41 120 outpatients clinics and hospitals. The HTLV-2 positive specimens were obtained from
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43 121 intravenous drug users in Stockholm, Sweden, also described previously [Andersson
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45 122 et al., 1995];
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50 123 b) Confirmed positive quality-control samples, (26 total; 18 HTLV-1, 7 HTLV-2, and 1
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52 124 HTLV-1/2). These samples were sent to clinical laboratories for quality evaluation of
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54 125 the laboratory methods during the years 2001–2008. The samples were prepared by
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56 126 the External Quality Assurance in Laboratory Medicine in Sweden (Equalis AB, Box
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3 127 977, Uppsala, Sweden). Twenty-three of these samples were undiluted, while one
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5 128 sample was diluted 1/50, one 1/100 and one 1/1000;
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8 129 c) A commercial panel consisting of 14 reactive (7 HTLV-1, 7 HTLV-2) and one non-
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10 130 reactive sample (PRP207, BBI diagnostics, West Bridgewater, Ma, USA). Two of
11
12 131 these panel members (one HTLV-1, PRP207-09 and one HTLV-2, PRP207-08) were
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14 132 also serially diluted 10-fold into six dilutions (1/10 to 1/10⁶).

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17 133 The following samples were tested to evaluate the specificity of the assay:

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20 134 d) 504 samples from non-reactive blood donors that had tested HTLV-negative using the
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22 135 Murex assay in the past;
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24 136 e) 36 samples from patients with positive rheumatoid factor;
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27 137 f) 37 samples from patients with antibodies associated with autoimmune disease (anti-
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29 138 nuclear antibodies, SS-A antibodies and/or native DNA-antibodies);
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31 139 g) 30 samples with antibodies/antigens to some viral infections, namely, Hepatitis B
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33 (HBV, n=10), Hepatitis C (HCV, n=10), Cytomegalovirus (CMV, n=5) and Epstein-
34 140 Barr virus (EBV, n=5);
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38 142 h) 16 samples (14 clinical samples and 2 quality-control samples) that were repeatedly
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40 143 reactive with the Murex assay but regarded as false-reactive, as they could not be
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42 144 confirmed positive with confirmatory assays (12 negative and 4 indeterminate);
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45 145 i) 1 412 routine samples that were analysed at the Department of Laboratory Medicine,
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47 146 Microbiology, Örebro University Hospital, Örebro, Sweden (USÖ), from December
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49 147 2008 to October 2009; these samples were analysed only with the ARCHITECT
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51 148 *r*HTLV I/II assay.
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55 149 The samples in groups c and h were analysed at the Department of Laboratory Medicine,
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57 150 Karlstad Hospital, Karlstad, Sweden (KH), as were 10 samples from group e and 10 samples
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59 151 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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8 154 ARCHITECT instrument.

9
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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14
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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20 159 Diagnostics.

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3 161 **Results**

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5 162 *Sensitivity evaluation*

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8 163 a) The 102 clinical samples that were confirmed HTLV-positive were all reactive with
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10 164 the ARCHITECT *r*HTLV assay. The mean s/co ratio for these samples when analysed
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12 165 with the ARCHITECT *r*HTLV assay was 70.42 (73.96 for HTLV-1, 61.61 for HTLV-
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15 166 2), with s/co ratios ranging from 1.17 to 149.33 (14.88–149.88 for HTLV-1 and 1.17–
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17 167 87.16 for HTLV-2). Except for one sample ratio (1.17), the s/co ratios for the positive
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20 168 samples were at least five times higher than cut-off. The Murex assay's mean s/co
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22 169 ratio for 93 of these (available) samples was 10.56 (12.10 for HTLV-1, 7.08 for
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24 170 HTLV-2) with s/co ratios ranging from 1.12 to 15.82 (1.40-15.82 for HTLV-1 and
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26
27 171 1.12-15.00 for HTLV-2).

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29 172 b) All 26 HTLV-positive quality-control samples analysed were reactive with the new
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31 173 ARCHITECT assay. The mean s/co ratio for these samples when analysed with the
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33 174 ARCHITECT *r*HTLV assay was 97.90 (115.36 for HTLV-1, 51.34 for HTLV-2) with
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36 175 s/co ratios ranging from 35.60 to 151.12 (35.60-151.12 for HTLV-1, n=16 and 48.79
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38 176 to 53.41 for HTLV-2, n=6, undiluted samples only). With the Murex assay the mean
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41 177 s/co ratios for the same samples was 14.39 (15.09 for HTLV-1, 12.55 for HTLV-2)
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43 178 with s/co ratios ranging from 10.16 to 23.43 (10.16-23.43 for HTLV-1 and 10.72-
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46 179 16.76 for HTLV-2).

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48 180 c) All 14 (seven HTLV-1, seven HTLV-2) expected positive members of the commercial
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50 181 panel were reactive when tested with the ARCHITECT *r*HTLV assay. The non-
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52
53 182 reactive panel member was non-reactive with the ARCHITECT *r*HTLV assay. The
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55 183 mean s/co ratio for the panel samples when analysed with ARCHITECT *r*HTLV assay
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57 184 was 83.19 (89.05 for HTLV-1, 77.32 for HTLV-2) with s/co ratios ranging from 47.20
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60 185 to 149.60 (47.20-149.60 for HTLV-1, and 66.26 to 71.36 for HTLV-2, positive

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3 186 samples only). With the Murex assay the mean s/co ratios for the same samples was
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5 187 11.64 (11.68 for HTLV-1, 11.60 for HTLV-2) with s/co ratios ranging from 11.60 to
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8 188 11.80.

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10 189 The two panel members that were serially diluted 10-fold six times showed an end-
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12 190 point dilution for HTLV-1 of $1/10^6$ and for HTLV-2 of $1/10^4$ (final sample still reactive; Table
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15 191 1). In all, 141 (98 HTLV-1, 42 HTLV-2 and one HTLV-1/2) samples that were confirmed
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17 192 positive for HTLV-1 or/and HTLV-2 were also positive with the new *r*HTLV assay. Thus, the
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20 193 sensitivity for the *r*HTLV assay was 100% in this study (Table 2).
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24 195 *Specificity evaluation*

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27 196 d) The 504 previously HTLV-negative blood donors were non-reactive when analysed
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29 197 with the ARCHITECT *r*HTLV-I/II assay. The mean s/co ratio for these samples was
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31 198 0.22, compared to 0.32 when analysed with the Murex assay. In Figure 1, the
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34 199 distributions of s/co ratios for these samples are shown.
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36 200 e) The 36 samples from patients with rheumatoid factor antibodies were all non-reactive
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38 201 with the ARCHITECT *r*HTLV-I/II assay.
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41 202 f) The 37 samples from patients with anti-nuclear antibodies, SS-A antibodies and/or
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43 203 native DNA-antibodies were all non-reactive with the ARCHITECT *r*HTLV assay.
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46 204 g) The 30 samples with antibodies/antigens to viral infections were all non-reactive with
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48 205 the ARCHITECT *r*HTLV-I/II assay.
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51 206 h) Of the 16 samples that were previously regarded as false-reactive, 12 were non-
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53 207 reactive when tested with the ARCHITECT *r*HTLV assay. Among these, 11 were
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55 208 negative with confirmatory assays for HTLV-1/2, while one sample was
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58 209 indeterminate. Four samples were still reactive when tested with the ARCHITECT
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60 210 *r*HTLV assay. Three of these were indeterminate with confirmatory tests, one was

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3 211 negative. If these four samples were considered false-reactive, the specificity for the
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5 212 ARCHITECT *r*HTLV-I/II assay in this evaluation is 99.36% (Table 2). More detailed
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8 213 data on the 16 previously false-reactive samples (with the Murex assay) are shown in
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10 214 Table 3.
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12 215 i) When the samples from the routine screening at USÖ (n=1 412) were included, the
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15 216 overall specificity was 99.80% (100% if the preselected Murex false-reactive samples
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17 217 were excluded) (Table 2).
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3 218 **Discussion**
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5 219 The new ARCHITECT *r*HTLV-I/II fully automated assay for large scale screening of
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8 220 antibodies to HTLV-1 or -2 has been evaluated. The sensitivity of this assay was high for both
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10 221 HTLV-1– and HTLV-2–positive specimens. The specificity of this assay was high for HTLV-
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12 222 negative blood donor specimens as well as for samples collected from individuals with
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14
15 223 various autoimmune disorders or infections known to influence the results of other screening
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17 224 assays, such as EBV infections.
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22 226 The number of commercially available assays for HTLV-1 and HTLV-2 antibody screening is
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24 227 limited. This new assay will be a useful alternative to the available assays. It is performed on
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26 228 the ARCHITECT *i* system immunoanalyser, which is designed for large-scale screening of
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28 229 blood samples. As this instrument also has assays for other markers for blood-borne infectious
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30 230 diseases, this assay will facilitate the screening process of blood donations and clinical
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32 231 samples, especially in laboratories with large volumes of samples. With just one sample tube
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34 232 it is possible to analyse all the markers needed.
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41 234 It has been shown previously that for several ELISAs used to detect HTLV-1 and -2
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43 235 antibodies it is possible to dilute or pool serum samples up to at least 1/10 and maintain a
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45 236 positive signal [Andersson et al., 2001; Chang et al., 2002]. This may be useful, for example,
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47 237 in studies of the seroepidemiology of large number of samples. Some countries are also using
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49 238 pools of samples established for nucleic acid testing (NAT) screening of blood donors (HIV,
50
51 239 hepatitis B and C) for serological HTLV screening. The limited dilution experiment in this
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53 240 study indicates that a pooling strategy, combining 5–10 samples, can be used with the *r*HTLV
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55 241 I/II assay. However, it is important to realise that this strategy should not be used for clinical
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3 242 diagnoses. An extended dilution experiment is also needed in order to conclude the utility of
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5 243 the Architect *r*HTLV-I/II assay for analyses of pooled samples.
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10 245 When the screening of large numbers of negative samples is one of the main objectives in the
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12 246 assay, it is important to have a clear separation between the negative values and the cut-off
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15 247 value. This will minimise the risk of scoring samples as reactive when they are not actually
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17 248 positive. As shown in Figure 1, for the negative blood donor samples analysed in this study,
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19 249 the sample to cut-off (s/co) ratios for the *r*HTLV I/II assay are lower than for the Murex
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21 250 assay. Moreover, the mean s/co values for the positive samples are at least 10 times higher
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23 251 than the cut-off level for both HTLV-1 and HTLV-2. Thus, there is a wide separation of s/co
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25 252 ratios between positive and negative samples, which is in accordance with the high specificity
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27 253 observed in this evaluation.
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33 255 The only samples that were designated false-reactive in this evaluation came from a group of
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35 256 samples that were reactive in prior tests using the Murex HTLV-I/II assay. It cannot be
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37 257 excluded that some of these samples were actually positive for HTLV-1 or -2 as follow-up
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39 258 samples were not available for all of them. For instance, it is known that HTLV-2 may be
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41 259 associated with low viral load and low antibody reactivity, which at times may give
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43 260 indeterminate results upon testing [Thorstensson et al., 2002]. Recent reports have also
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45 261 suggested the existence of HTLV-3 and possibly HTLV-4, which may also give indeterminate
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47 262 reactions in common assays for HTLV-1 and -2 [Calattini et al ,2005, Switzer et al, 2006,
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49 263 Wolfe et al 2005]. Of the 16 samples that were previously designated as indeterminate, 12
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51 264 were negative by the *r*HTLV-I/II assay. Although this may be interpreted as a result of higher
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53 265 specificity for the *r*HTLV-I/II assay, it is important to remember that other samples with false
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55 266 reactivities may be selected with the *r*HTLV-I/II assay.
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6 268 In conclusion, the *r*HTLV-I/II assay performed well on the serum panels used in this
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8 269 evaluation, and with its design for large-scale screening, it may be a novel tool of high value
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10 270 in the diagnostic arsenal for blood-borne infections.
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