

1 **Immunoblots may not be effective in confirming the recency of HIV-1 infection**

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Abstract

Recently, immunoblots (IBs) have tended to substitute Western blots (WBs) for HIV infection diagnosis. Several studies have confirmed IBs' high sensitivity to confirm HIV infection for every stage. Since the nature and pattern of the antigens of IBs are different from those of WB, the abilities of IBs and WBs to distinguish the stages of recent seroconversion and open-ended chronic infection might differ. We aimed to evaluate the performance of two IBs (INNO-LIA™ HIVI/II, Fujirebio, and Geenius™ HIV1/2 Confirmatory assay, Bio-Rad) to define the stage of infection. We studied 53 patients from the French ANRS CO6 PRIMO cohort. IBs have higher positive rates than WB. However, Geenius was less sensitive than WB and INNO-LIA to detect antibodies to p31 (0% vs 22.6% and 15.1%, respectively), so it could wrongly label late Fiebig stage and open-ended chronic infections as recent infections (n=5/53). For the first time, we provide evidence that centralized WBs associated with an enzyme immunoassay for the identification of recent HIV-1 infection support the establishment of a more accurate diagnosis of primary HIV infection to improve the accuracy of enrollments in cohorts of recent HIV infections useful for epidemiological studies, pathogenesis studies or therapeutic trials.

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42 **Keywords:**

43 HIV primary infection

44 Immunoblot

45 Western blot

46 HIV diagnosis

47 Geenius

48 INNO-LIA.

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50 **Highlights :**

51 • Evaluation of Immunoblots to precise the recency of HIV infection.

52 • 53 patients from the ANRS PRIMO cohort.

53 • Geenius is not efficient at detecting anti-p31 antibodies in Fiebig VI.

54 • Immunoblots could wrongly label late Fiebig stages as more recent infections.

55 • Centralized WBs improve the accuracy of enrollments in primary infections cohorts.

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59 **Introduction:**

60 Diagnosis of human immunodeficiency virus (HIV) infection as soon as the primary HIV
61 infection (PHI) occurs is essential for early initiation of combined antiretroviral therapy
62 (cART) to preserve immune function, limit the viral reservoir, and prevent transmission. The
63 biological characterization of PHI is also useful for epidemiological studies as well as
64 enrollment in cohorts dedicated to pathogenesis studies or therapeutic trials (Colby et al.,
65 2018; Dong et al., 2018).

66 For the laboratory diagnosis of HIV infection, reactive serological testing with a 4th
67 generation enzyme immunoassay (EIA) must be confirmed. The confirmatory assays can be
68 Western blots (WBs) using viral antigens, which are considered the gold standard to confirm
69 HIV infection or immunoblots (IBs) using recombinant or synthetic antigens. In case of
70 discrepancy between EIA and confirmatory assays, HIV-RNA plasma detection is
71 recommended.

72 PHI infection usually refers to acute HIV infection and the early stage of HIV infection up to
73 about six months but definitions and biological markers may differ across study. French
74 guidelines defined PHI as detectable HIV-RNA associated with a negative 4th generation EIA
75 or a positive EIA with ≤ 5 HIV-specific antibodies on WB (Morlat, 2019). WB results are
76 then useful to precisely determine the stage of PHI based on Fiebig's classification (Fiebig et
77 al., 2003). Six stages are defined according to the dynamics of HIV-RNA, p24 antigen and
78 antibody seroconversions observed from WB results. This broadly used classification
79 distinguishes acute infection (stages I–III), recent seroconversion (stages IV–V), and open-
80 ended chronic infection (stage VI). Stages I–III are defined by a negative WB. Stage IV is
81 defined as the presence of HIV-1-specific bands that fail to meet criteria for a reactive WB,
82 identified by the US Food and Drug Administration as reactivity to 2 of the following

83 antigens: p24, gp41, and gp120/160. Fiebig stage V is defined as a reactive pattern (presence
84 of at least 2 antigens) but lacking p31 reactivity. Stage VI is defined as full reactivity,
85 including a p31 band.

86 In recent years, several new IBs, which are faster to use than WBs, have tended to substitute
87 WBs as confirmation assays. IBs assess fewer antibodies than WBs, but their high sensitivity
88 to confirm HIV infection has been reported in several studies (Kondo et al., 2018; Lindman et
89 al., 2019; Serhir et al., 2019; Tinguely et al., 2014). However, their ability to differentiate PHI
90 from later stages needs to be largely evaluated.

91 We aimed to evaluate two IBs frequently used at preinclusion in the French ANRS (Agence
92 Nationale de Recherches sur le SIDA et les Hépatites Virales) CO6 PRIMO cohort, to
93 determine if they are performant to consider a patient as recently HIV infected.

94

95 **Materials and methods**

96 Fifty-three patients from the ANRS PRIMO cohort between November 2018 and May 2019
97 who had frozen blood plasma at inclusion in the cohort participated in this study. The cohort
98 was approved by the Ile-de-France-3 Ethics Committee, and patients gave their informed
99 consent to participate. Participants presenting with acute or recent HIV-1 infection,
100 symptomatic or not, were included if they presented one of the following criteria: 1)
101 detectable p24 antigenemia or plasma viral load, associated with an incomplete confirmatory
102 assay (i.e., indeterminate or positive confirmatory assay with absence of antibodies to p31
103 and/or p68) during the six weeks before inclusion, 2) detectable p24 antigenemia or plasma
104 viral load, with either a negative or weakly reactive EIA or a negative WB or IB during the
105 six weeks before inclusion, or 3) an interval of <3 months between a negative and a positive
106 EIA (Goujard et al., 2006). Preinclusion was based on clinical investigation and routine

107 laboratory assays, which could vary depending on the laboratory. Subsequently, centralized
108 Vidas® HIV-DUO Ultra and WBs were systematically performed on inclusion samples
109 (APHP, Hôpital Necker-Enfants Malades, Paris, France). Vidas® HIV-DUO Ultra, which is
110 an Enzyme Linked Fluorescent Assay (ELIFA), was applied to determine if antibodies against
111 HIV-1 or HIV-2 or p24 antigen were detectable.

112 Bio-Rad HIV-1 WB uses the inactivated HIV-1 native viral antigens Env (gp160, gp120 and
113 gp41), Pol (p68/65, p51 and p31) and Gag (p55, p40/39, p24, and p18/17). The assay was
114 performed according to the manufacturer's recommendations using a visual reading. We
115 interpreted the WBs according to the WHO's recommendations (WHO, 1991): they were
116 considered positive if at least two antibodies against the envelope antigens were detected. The
117 WBs were indeterminate if antibodies were detected without the above criteria of positivity.
118 As a result, we were able to precisely determine the pattern of HIV-specific antibodies on WB
119 to classify the stage of infection with centralized tools.

120 The two IB assays INNO-LIA™ HIVI/II (Fujirebio) and Geenius™ HIV1/2 Confirmatory
121 assay (Bio-Rad) were performed by the same operator on the same inclusion samples and
122 interpreted according to manufacturer recommendations. The INNO-LIA assay is a single-use
123 line immunoassay (LIA) which can detect antibodies against five HIV-1 proteins (gp120,
124 gp41, p31, p24, and p17) and two against HIV-2 envelope proteins (gp105 and gp36). Patients
125 samples were incubated all night with the test strips. Each line's intensity was read visually
126 and compared to control lines. INNO-LIA was interpreted as HIV-1 positive when there were
127 at least two positive anti-HIV-1 antibodies, including one directed against the HIV-1 envelope
128 antigens. The Geenius assay is a single-use immunochromatographic test. It is composed of
129 four HIV-1 antigens (Env gp120 and gp41, Pol p31 and Gag p24 and p17) and two HIV-2
130 antigens (gp36 and gp140). The duration of the assay is less than one hour. In our study,

131 bands were observed visually. Geenius was interpreted HIV-1 positive if the antibody to at
132 least one envelope protein and antibody to another HIV antigen were positive.

133 Fiebig's classification (Fiebig et al., 2003) was used to differentiate acute and early infection
134 according to Vidas® HIV-DUO Ultra, WB and IB assays.

135 Finally, a centralized EIA was also performed for the identification of recent HIV-1 infection
136 (EIA-RI) to clarify doubtful cases. It was based on the quantification of antibodies binding to
137 synthetic antigens of the immunodominant epitope of gp41 and the V3 region of gp120 (Barin
138 et al., 2005). A score <0.5 was in favor of infection of fewer than 180 days, as previously
139 described (Barin et al., 2005).

140 Fisher and χ^2 tests were used to compare WB and IBs and to evaluate the relationship
141 between WB or IB results and Fiebig stages (Prism version 8.0, GraphPad).

142

143 **Results**

144 Fifty-three patients were tested with WB and two IBs. The positive rate of WB (13.2%) was
145 lower than that of INNO-LIA (66.0%, $p<0.0001$) and Geenius (62.3%, $p<0.0001$). INNO-LIA
146 and Geenius showed positive results for most of the indeterminate WB samples, 87.1%
147 ($n=27/31$) and 77.4% ($n=24/31$), respectively (Table 1). Seven samples showed concordant
148 positive results with the three assays ($n=7/53$, 13.2%).

149 Antibody patterns help to distinguish acute, recent, and established infections. Antibodies to
150 p24 and envelope glycoproteins are the earliest to develop during seroconversion. WB and
151 INNO-LIA better detected antibodies to p24 than Geenius (66.0% vs 9.4%, $p<0.0001$ and
152 62.3% vs 9.4%, $p<0.0001$, respectively). Detection of antibodies to Env antigens was higher
153 with Geenius than with WB (gp120/160: 62.3% vs 30.2%, $p=0.0017$; gp41: 84.9% vs 13.2%,
154 $p<0.001$). Antibodies to gp120/160 were more often detected with Geenius than with INNO-
155 LIA (62.3% vs 32.1%, $p=0.0033$). Antibodies to p31 appear later during the course of

156 infection and are used to define Fiebig stage VI. WB detected antibodies to p31 in twelve
157 samples (n=12/53, 22.6%), including eight samples that were also p31 positive with INNO-
158 LIA (n=8/53, 15.1%). Both WB and INNO-LIA better detected antibodies to p31 than
159 Geenius, which did not detect any antibodies to p31 in any sample (WB vs. Geenius: p =
160 0.0002; INNO-LIA vs Geenius: p=0.0059) (Table 1).

161 Fiebig classification applied to Vidas® HIV-DUO Ultra combined with either WB, INNO-
162 LIA, or Geenius showed different distributions of Fiebig stages (p<0.0001) (Table 1).
163 Differences were particularly observed for the latest Fiebig stages IV to VI. Fiebig
164 classification according to WB ranked more samples in Fiebig stage IV (n=31/53, 58.5%)
165 than both INNO-LIA (n=9/53, 17.0%) and Geenius (n=12/53, 22.6). Conversely, the IBs
166 ranked more samples in Fiebig stage V than WB (INNO-LIA n=27/53, 50.9%, Geenius
167 n=33/53, 62.3% vs WB n= 1/53, 1.9%). Due to the difference in detecting antibodies to p31,
168 WB and INNO-LIA classified some samples in Fiebig stage VI (n=6/53, 11.3%, and n=8/53,
169 15.1%, respectively), whereas Geenius did not.

170 We further performed EIA-RI to assess discrepancies. Out of 53 samples, 48 were classified
171 as recent infection shorter than 180 days (median score: 0.01, range [0.01-0.25]), one
172 equivocal and four long-term (>180 days). These five cases are detailed on Table 2. The only
173 sample presenting Fiebig stage V characteristics on WB showed an index of EIA-RI at 0.99 in
174 favor of an infection longer than 180 days. The IBs also classified this sample in Fiebig stage
175 V. INNO-LIA had been used to confirm the diagnosis at preinclusion for this participant and
176 presented negative for antibodies to p31 and positive for antibodies to p24. There was no
177 previous negative HIV serology and no p24 detection, and the CD4/CD8 T cell ratio was 0.2,
178 with 4.7 log copies HIV-RNA/mL at inclusion. Immunosuppression in late chronic infection
179 leading to loss of antibodies to Pol and Gag might have distorted the confirmatory assay and
180 mislead the Fiebig classification.

181 Three patients out of six classified in Fiebig stage VI with WB had an elevated index of EIA-
182 RI >0.8 in favor of infection longer than 180 days. They all had been diagnosed on the basis
183 of the results of a Geenius assay at preinclusion (no antibodies to p24 or p31), and two of
184 them were positive for p24 antigen at preinclusion. Curiously, one patient had very low HIV-
185 RNA and HIV-DNA and a CD4/CD8 T cell ratio conserved >1 at inclusion fourteen days
186 later. This patient could be a natural HIV controller. The fifth patient presented a doubtful
187 EIA-RI index (0.44) and was positive for all three assays but with antibodies to p31 detected
188 only with WB. The patient had no past serology and no p24 antigen detected, and WB was
189 complete except for gp110.

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191 **Discussion**

192 Different manufacturers have approved several IB assays as confirmation assays, without any
193 doubt regarding their performance (Kondo et al., 2018; Serhir et al., 2019; Tinguely et al.,
194 2014), but few studies concern their ability to distinguish infection stages. Therefore, our aim
195 was to focus on acute and recent stages.

196 The present study on a large number of samples clearly shows that the ability to detect each
197 antibody differed between the IBs and WB. The higher ability of the IBs to detect anti-Env
198 antibodies led to a higher positive rate of these assays than that of WB, which is advantageous
199 to confirm HIV diagnosis as soon as the earliest Fiebig stages of seroconversion. WBs were
200 then more often indeterminate, with a profile suggesting recent infection. Moreover, Gag and
201 Pol antibodies are essential to distinguish acute from recent and chronic infections,
202 particularly anti-p31 antibodies, which characterize Fiebig stage VI. Our study showed that
203 Geenius is not efficient at detecting antibodies to p31 in Fiebig stages VI, so Geenius could
204 wrongly label late Fiebig stage and chronic infections as more recent infections. These data

205 are in agreement with the lower capacity of Geenius to detect anti-p24 and anti-p31 antibodies
206 observed during chronic HIV infection (Tuaille et al., 2017). An alternative method based on
207 the quantitative measurements of antibody band intensities determined by the automated
208 Geenius optical reader may have an interest to distinguish between recent and long-standing
209 HIV infection, as previously suggested (Keating et al., 2016).

210 Overall, we provide evidence that Geenius and INNO-LIA may not be assays of choice to
211 stage infections following Fiebig classification. As the latest stages or some rare cases, such
212 natural HIV controllers, can be confusing for both WB and the IBs, the EIA-RI result,
213 interpreted in association with other assays, can be an additional argument to confirm or reject
214 recent infection.

215 In conclusion, our study highlights the difficulties of providing consistent results for
216 determining the stage of HIV infection when antibodies are already detectable. Clinical
217 laboratories must keep in mind that Geenius might mistakenly refer to recent infection, so if a
218 recent infection is suspected, additional investigations are needed. In our study, we showed
219 that a centralized WB confirmation with a complementary assay of recent infection combined
220 with assays realized at preinclusion are beneficial to confirm the recency of HIV infection and
221 reassure enrollment criteria in PHI cohorts.

222

223 **Funding sources**

224 The ANRS PRIMO cohort is sponsored by the French National Agency for Research on
225 AIDS and Viral Hepatitis (ANRS). This work was funded by the ANRS. The funders of the
226 study had no role in study design, data collection, data analysis, data interpretation, report
227 writing, or decision to submit for publication.

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229 **Declarations of interest:** none
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1 Table 1. Comparison of positive antibodies and stages of Fiebig's classification between Western blot (WB), Geenius, and INNO-LIA

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N (%)	Assay results			Positive antibodies				Fiebig stages					
	Positive	Indeterminate	Negative	GP160/120	GP41	P24	P31	I	II	III	IV	V	VI
Western blot, Bio-Rad	7 (13.2)	31 (58.5)	15 (28.3)	16 (30.2)	7 (13.2)	35 (66.3)	12 (22.6)	0 (0.0)	10 (18.9)	5 (9.4)	31 (58.5)	1 (1.9)	6 (11.3)
Immunoblot, INNO-LIA HIV 1/2	35 (66.0)	9 (17.0)	9 (17.0)	17 (32.1)	44 (83.0)	33 (62.3)	8 (15.1)	0 (0.0)	7 (13.2)	2 (3.8)	9 (17.0)	27 (50.9)	8 (15.1)
Immunoblot, GEENIUS HIV 1/2	33 (62.3)	12 (22.6)	8 (15.1)	33 (62.3)	45 (84.9)	5 (9.4)	0 (0.0)	0 (0.0)	6 (11.3)	2 (3.8)	12 (22.6)	33 (62.3)	0 (0.0)
EIA-RI index for Fiebig's stages defined with WB: Median (Ranges)									0.01 (0.01-0.04)	0.01 (0.01-0.01)	0.02 (0.01-0.19)	0.99	0.63 (0.16-0.99)

EIA-RI: Enzyme immunoassay for identification of recent HIV-1 infections, threshold to discriminate between < or > 180 days: 0.50.

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Fiebig's Stages defined according to each confirmation assay	Preinclusion		Inclusion										
	p24 antigen	Confirmation test	Delay from preinclusion	CD4 T cell count (/mm ³)	CD4/CD8 ratio	HIV-RNA (log copies/mL)	HIV-DNA (log copies/10 ⁶ PBMCs)	Antibodies, DUO ULTRA VIDAS	p24 antigen, DUO ULTRA VIDAS	WB, VIH1	INNO-LIA	Geenius	EIA-RI index
WB: V INNO-LIA: V Geenius: V	nr	INNO-LIA: gp120, gp41, P24	22	247	0.2	4.7	3.57	21.08 Positive	Negative	Positive gp160, gp110, p68, p55, gp41, p24+, p18±	Positive gp120, gp41, p24	Positive gp160, p24±, gp41	0.99
WB: VI INNO-LIA: VI Geenius: V	Positive	Geenius: gp160, gp41	14	602	1.5	1.53	1.0	13.71 Positive	Negative	Positive complete	Positive gp120, gp41, p31, p24, p17	Positive gp160, p24, gp41	0.83
WB: VI INNO-LIA: VI Geenius: V	Positive	Geenius: gp160, gp41	13	nr	nr	4.85	3.09	10.03 Positive	Negative	Positive gp160, p68, p55, gp41, p31, p24,	Positive gp120, gp41, p31, p24±	Positive gp160, gp41	0.99
WB: VI INNO-LIA: VI Geenius: V	nr	Geenius: gp160, gp41	13	307	0.6	6.09	3.41	13.92 Positive	Negative	Positive gp160, gp110, p68, p55±, p31, p24±, p18	Positive gp120, gp41, p31, p24±	Positive gp160, gp41	0.99
WB: VI INNO-LIA: V Geenius: V	nr	Geenius: gp160, gp41, p24	15	451	0.3	5.57	3.21	8.36 Positive	Negative	Positive gp160, p68, p55, gp41, p40, p31, p24, p18	Positive gp120, gp41, p24, p17	Positive gp160, p24, gp41	0.44

nr: not realized.
WB: Western blot.
Antibodies, DUO ULTRA VIDAS: relative fluorescence value (RVF) of antibodies ≥ 0.25: positive.
EIA-RI: Enzyme immunoassay for identification of recent HIV-1 infections, threshold to discriminate between < or > 180 days: 0.50.