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Published in:
Journal of Clinical Microbiology

Citation for published version (APA):

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Detection of Human Immunodeficiency Virus Type 1 Nucleocapsid Protein p7 In Vitro and In Vivo

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Received 4 June 1998/Returned for modification 19 August 1998/Accepted 25 September 1998

We developed and evaluated an immunoassay for the detection and quantification of human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein p7 using electrochemiluminescence technology. The assay had a dynamic range of 50 to 20,000 pg/ml and a lower detection limit equivalent to approximately \(10^{6.5}\) HIV-1 RNA copies/ml in culture supernatant. In vitro kinetic replication studies showed that the amount of p7 correlated strongly with the amount of p24 \((R^2 = 0.869; P < 0.0001)\) and viral RNA \((R^2 = 0.858; P = 0.0009)\). On the basis of the p7 and RNA concentrations, we calculated the median p7:RNA ratio to be approximately 1,400 p7 molecules per RNA molecule. HIV-1 p7 could be detected and quantified in culture supernatants of both group M subtype A to E viruses and group O viruses. The presence of p7 in vivo was evaluated in 81 serum samples collected from 62 HIV-1-infected individuals. Five samples were p7 positive, whereas 45 samples were HIV-1 p24 positive. Four of the five p7-positive samples were p24 positive as well. p7 could be detected only when serum HIV-1 RNA levels were greater than \(10^{5}\) copies/ml. Anti-p7 antibodies were found in six samples, and all six were p7 negative. In contrast to the in vitro results, it appeared that HIV-1 p7 could not be used as a marker for viral quantification in vivo, since more than 90% of the serum samples were p7 negative. In combination with the low prevalence of anti-p7 antibodies, this may, in turn, be advantageous: the p7 assay may be a good alternative to the p24 assay as the readout system for determination of neutralizing activity against HIV-1 in serum or other fluids containing anti-p24 antibodies.

Materials and Methods

Antisera and antigens. The characteristics of the antisera used in our experiments are summarized in Table 1. Total immunoglobulin G (IgG) was purified from all sera and fluids [ImmunoPure (G) IgG Purification Kit; Pierce, Rockford, Ill.] prior to labelling. Part of the purified IgGs were labelled with biotin and part with ruthenium (Ru)-Tris \([1,1\text{-bispyrimidyl according to the instructions of the label manufacturer (IGEN International Inc., Gaithersburg, Md.)}].

Samples. Infectious subtype A to E virus stocks were collected by the UN-AIDS Network for HIV Isolation and Characterization (20). Expanded stocks of virus were produced by a previously described protocol (30) by inoculation of \(4.0 \times 10^3\) phylomegaglutinin-simulated donor peripheral blood mononuclear cells with supernatant from cultures of the primary isolate. After incubation and washing, the cells were resuspended in culture medium and were incubated at 37°C for 3 days. The cultures were then split into two. After continued incubation, cell-free supernatant was harvested at day 6 to 7 from one culture and at day 10 to 11 from the other culture to determine which would yield the highest p24 values.

The same protocol was performed with a biological clone (clone BC617), the culture supernatant of which was used for determination of the reproducibility of the HIV-1 p7 ECL immunoassay. As a reference, in the same experiments a virus stock (HXB3) was used (24). To test the reproducibility of the p7 assay, the ratios of the ECL signal between that for the reference strain and those for the three other subtype B viruses were independently determined five times.

Serum samples collected from 62 HIV-1-positive patients in the Amsterdam, The Netherlands, cohort studies on HIV infection and AIDS (3) were used as well.

Quantitative ECL immunoassay for HIV-1 p7. After testing of the various antisera and reaction conditions (see Results section), the p7 ECL immunoassay was evaluated on culture supernatants and sera and consisted of the following format: 50 µl of binding buffer (phosphate-buffered saline [PBS], 2% normal...
goat serum [NGS], 2% Tween 20, 100 mM NaCl containing 4 μg of bio- 
tin-labelled g#1098 antibody per ml, and 5 μg of Ru-labelled g#1113 antibody per 
ml was added to 25 μl of the sample. The mixture was diluted 10 times in 
PBS-2% NGS-0.5% percent Nonidet P-40 (NP-40) and heat inactivated for 30 
min at 56°C to inactivate infectious HIV particles. After 30 min of incubation 
at room temperature, 25 μl of a 10-μg/25-μl suspension of streptavidin-coated 
M-280 Dynabeads (Dynal, Oslo, Norway) in bead diluent (IGEN International 
Inc., Glastonbury, Md.) was added, and the mixture was incubated for possible, 
15 min at room temperature with gentle shaking. After the addition of 200 μl of 
asay buffer (IGEN International Inc.), the samples were analyzed in the ORI-
GEN analyzer (IGEN International Inc.).

Immunos assay for anti-HIV-1 p7. Each well of microtiter plates was coated 
with 100 μl of 100 ng of recombinant p7 in PBS (Gibco BRL) per ml. After 
overnight coating, the wells were blocked for 1 h at 37°C with 150 μl of PBS–3%
bovine serum albumin BSA (Boehringer Mannheim, Mannheim, Germany)-
0.1% Tween 20 (Merck, Darmstadt, Germany). A total of 100 μl of serum 
samples diluted 1:100 in blocking buffer was added to the wells, and the plates 
were incubated for 2 h at 37°C. After extensive washing three times with PBS–
0.1% Tween 20, each well was incubated for 1 h at 37°C with 100 μl of horse-
radish peroxidase-conjugated goat anti-human IgG (Gibco BRL) diluted 1:5,000 
in blocking buffer. A total of 100 μl of substrate (o-phenylenediamine; Abbott 
Laboratories) was added after five extensive washings, and the reaction was 
stopped after 10 min of incubation at room temperature with 50 μl of 0.5 M 
H2SO4. The optical density was read at 450 nm. Samples which had an optical 
density of the mean for 21 blank samples plus 2 times the standard deviation 
were considered positive.

Quantitative assays for HIV-1 p24. The HIV-1 p24 antigen concentration was 
measured in cell culture supernatants and human sera by a commercially avail-
able immunoassay (Abbott Laboratories, Abbott Park, Ill.) and an in-house 
assay. The in-house assay, described by Moore et al. (27), was optimized for 
HIV-1 subtype B in order to achieve results comparable to those achieved by 
the commercial assay.

Quantitative assay for anti-HIV-1 p24. Anti-p24 responses were measured by a 
commercially available assay (Wellcozyme HIV-1 anti-p24; Murex Diagnostics 
Ltd., Dartford, Great Britain).

Quantitative assay for HIV-1 RNA. For determination of the HIV-1 RNA 
concentration in cell culture supernatant and sera, we used the commercially 
available HIV-1 NucleSens assay (Organon Teknika, Biotest, The Netherlands) 
as instructed by the manufacturer.

Statistical analysis. Correlation analyses were performed with SigmaStat for 
Windows, version 1.0 (Jandel Corporation, San Rafael, Calif.).

RESULTS

Development of ECL immunoassay for HIV-1 p7. The IgG anti-p7 fractions labelled with biotin and the Ru tag were tested in all combinations at a starting concentration of 2 μg/ml each (25 μl) to detect p7 as sensitively as possible. Combinations were further tested with respect to concentra-
tions, incubation time and temperature, the antiserum dilution buffer, and the sample dilution buffer. PBS and citrate buffers containing different concentrations of NGS, detergents (Twee 20, NP-40, Triton X-100, Brij 58, Ipegal CA-630, so-
dium dodecyl sulfate), and NaCl were tested to achieve optimal conditions. The preferred antiserum diluent turned out to be PBS, 2% NGS, 2% Tween 20, and 100 mM NaCl in combi-
nation with sample diluent containing PBS, 2% NGS, and 0.5% NP-40. The antiserum diluent was optimal for the best antiserum combination, which was biotinylated g#1098 and 
Ru tag-labelled g#1113. The culture supernatant samples had to be diluted at least 10-fold, most likely due to the biotin in the cell culture medium, since biotin can react with streptavidin and disturb the later steps of the assay. Infectious HIV 
particles in the diluted samples were heat inactivated for 30 
min at 56°C, after which 50 μl of 4 μg of g#1098 per ml and 5 μg of g#1113 per ml dissolved in antiserum diluent were added. After 30 min of incubation at room temperature, streptavidin-coated magnetic beads were added and the mix-
ture was incubated before the samples were analyzed. The dynamic range of the assay with regard to these antiseras was 
between 50 and 20,000 pg of p7 per ml when recombinant p7 
was used in 90% sample diluent and 10% cell culture medium.

Reproducibility of the ECL immunoassay for HIV-1 p7 in 
viral cultures. The absolute ECL counts for HXB3 ranged 
between 42,203 and 56,158. The ratios between the counts for 
the reference strain and those for the other viruses were calculated. If the assay was reproducible, the ratios had to be 
constant. As shown in Table 2, the reproducibility of the p7 
assay was high, with standard deviations of between 0.05 and 0.12.

HIV-1 p7 versus p24 and RNA in subtype B viral culture. 
The amounts of HIV-1 p7, HIV-1 p24, and HIV-1 RNA were 
quantified in two parallel cultures of HIV-1 subtype B viruses 
(7). For comparison of the p7 and p24 concentrations, we took 
samples at eight time points after infection, and the results 
(Fig. 1A and B) showed that the amounts of p7 and p24 
paralleled each other closely in time.

The p7 and p24 levels in all the samples together showed a 
correlation coefficient of 0.869 (P < 0.0001). Theoretically, the 
mass ratio between p24 and p7 is approximately 3.5:1, since the 
molar ratio is 1:1 and since both proteins are formed out of one 
precursor molecule, Pr55pol. The mass ratios found at most 
sampling points approached this ratio of 3.5:1.

<table>
<thead>
<tr>
<th>Virus stock</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
<th>Exp 4</th>
<th>Exp 5</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HXB3</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>BC617</td>
<td>0.82</td>
<td>0.84</td>
<td>0.79</td>
<td>0.73</td>
<td>0.78</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td>BR21</td>
<td>0.90</td>
<td>0.77</td>
<td>0.90</td>
<td>1.04</td>
<td>0.90</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>TH14</td>
<td>1.05</td>
<td>0.97</td>
<td>1.00</td>
<td>1.13</td>
<td>1.26</td>
<td>1.08 ± 0.12</td>
</tr>
</tbody>
</table>

TABLE 2. Ratios of ECL counts for HXB3 compared with those 
for other viral strains

Antiserum (reference)*
<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Antigen used (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g#900, g#923, g#969, g#980, g#1098, g#1113</td>
<td>Goat</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>R-D-20750</td>
<td>Rabbit</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>R-D-25974</td>
<td>Rabbit</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>R-DJ-31677</td>
<td>Rabbit</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>R-DJ-37545</td>
<td>Rabbit</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>R195</td>
<td>Rabbit</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>HH3 (29)</td>
<td>Mouse</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>58/TE3 and 4D1</td>
<td>Mouse</td>
<td>Monoclonal</td>
</tr>
</tbody>
</table>

* All antiseras except R195, R196, and HH3 were kindly provided by L. O. Arthur (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, Md.). R195, R196, and HH3 were gifts from J.-L. Darlix (Laboratoire de Virologie Humaine, Ecole Normale Superieure de Lyon, Lyon, France).
If most of the p7 and p24 measured is bound to the viral particle, then the p7 and p24 levels would also correlate with the viral RNA level in the culture supernatant. The viral RNA levels were measured in five of the eight samples, and the log10 p7 concentration (in picograms per milliliter) was plotted against the log10 viral RNA level (in numbers of copies per milliliter) (Fig. 2A and B). Taking all samples together, we found a correlation coefficient between the RNA level and the p7 level of 0.858 ($P = 0.0009$). To test the sensitivity of the p7 ECL assay and compare it to the viral RNA level, a sample was drawn from the culture just before passaging. From a 10-fold serial dilution series of this sample, it appeared that p7 could be detected when RNA levels were $10^{6.5}$ copies/ml.

The ratio of p7 molecules per RNA molecule was calculated from the concentration of p7 and the RNA levels. For all seven samples in which both p7 and viral RNA could be detected, the median was approximately 1,400 molecules of p7 per RNA molecule, with a range of approximately 1,000 to 5,250 molecules of p7 per RNA molecule or 12 to 60 pg/10^6 molecules of RNA (the molecular mass of p7 is approximately 6,850 g/mol).

**HIV-1 p7 in cultures of different viral subtypes.** Finally, a collection of 21 viral isolates comprising HIV-1 subtypes A to E of the M group (20) and 6 viral isolates of the HIV-1 O group (4) was analyzed, and p7 could be detected in all culture supernatants.

**ECL immunoassay for HIV-1 p7 with clinical serum samples.** Eighty-one serum samples taken from 62 HIV-1-infected and nontreated individuals were analyzed for p24, anti-p24, p7, and anti-p7 responses, as well as RNA levels. Of these samples, 45 (56%) were positive for p24 and 46 (57%) reacted positively in the anti-p24 assay. Of all samples, six reacted positively in the enzyme-linked immunosorbent assay for anti-HIV-1 p7, suggesting that in approximately 7% of the samples a possible interference of anti-p7 antibodies in a p7 detection assay is to be expected. In five other samples (6%), p7 could be detected (Table 3). All five samples were anti-p7 negative. Four of the

<table>
<thead>
<tr>
<th>No. of serum samples with the following Ab or Ag status:</th>
<th>+ (45)</th>
<th>- (36)</th>
<th>+ (5)</th>
<th>- (76)</th>
<th>+ (6)</th>
<th>- (75)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-p24 Ab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ (46)</td>
<td>11</td>
<td>35</td>
<td>1</td>
<td>45</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>- (35)</td>
<td>34</td>
<td>1</td>
<td>4</td>
<td>31</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>Anti-p7 Ab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ (6)</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td></td>
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<tr>
<td>- (75)</td>
<td>43</td>
<td>32</td>
<td>5</td>
<td>70</td>
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<tr>
<td>p7 Ag</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ (5)</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- (76)</td>
<td>41</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses are numbers of serum samples in that group. Ab, antibody; Ag, antigen.
five p7-positive samples were positive for p24 and negative for anti-p24. The remaining p7-positive sample was positive for anti-p24 and negative for p24 (Table 4), as were five of the six anti-p7-positive serum samples. The HIV-1 RNA levels in the five p7-positive serum samples were high, ranging from $10^{6.041}$ to $10^{8.991}$ copies/ml (Table 4), corresponding to the lower detection limit of the assay for p7 in viral cultures.

**DISCUSSION**

An immunoassay based on ECL technology was developed to detect the HIV-1 nucleocapsid Gag protein p7. This assay for p7 was a capture assay with two antisera directed against p7, one labelled with biotin and the other labelled with Ruthenium[1,1]bispyrimidyl. The antibody-complexed antigen was detected by using streptavidin beads in an ECL system (18).

Evaluation of the p7 ECL assay showed that it could detect and quantify p7 in cultures of various virus subtypes and also, but less so, in HIV-1-positive sera. The amounts of p7 measured in two parallel viral cultures correlated well with the amounts of p24, as could be expected, since both proteins are cleaved from the same p55 Gag precursor (11, 12). Furthermore, viral RNA production correlated well with p7 production, suggesting that in these cultures most of the p7 (and p24) was in viral particles. Theoretically, since p7 has a binding region of seven nucleotides of RNA (31), approximately 1,300 molecules of p7 per RNA molecule are present if the whole genome is covered with p7 (22, 31). We calculated a ratio of approximately 1,400 molecules of p7 per RNA molecule, which corresponds well with the theoretical figure. The correlation between p7, p24, and RNA indicated that the p7 assay is well able to monitor viral infections in culture.

We could detect p7 in culture supernatants of both the M and the O groups of HIV-1 isolates, which is in accordance with the conserved nature of the protein (19). The in-house p24 assay (27) did not detect p24 in group-O isolates, whereas the commercially available assay did. This confirms that p7 is highly conserved among the HIV-1 subtypes.

We were not able to show the clinical utility of p7 as a possible marker for viral replication in vivo, since p7 could be measured in only a few serum samples. In culture supernatants, p7 was detected only when the HIV-1 RNA level was greater than $10^{8.5}$ copies/ml. The RNA levels in the p7-positive sera were greater than $10^{6}$ copies/ml. Since mean viral RNA levels in sera are approximately $10^{3}$ to $10^{5}$ copies per ml (9, 13–15, 25, 26), the current format of the assay has limited clinical utility. As was shown in this study, antibody reactivity to p7 was limited. Antibodies to p7 could be detected in only 7% of the samples, in accordance with previous studies (2a). Nevertheless, p7 appeared to be immunogenic, because we have observed that mice, rabbits, and goats develop an immune response to p7 after immunization (although it is in the presence of an adjuvant). Therefore, the limited immune response in vivo may be the result of the strong particle-associated nature of p7 (11, 12), which keeps it shielded from the immune system.

In summary, we developed an ECL-based p7 immunoassay with a dynamic range of 50 to 20,000 pg of p7 per ml. It was well suited to compete with p24 assays to monitor standard viral infections, independent of which HIV-1 subtype is cultured. Compared to standard enzyme-linked immunosorbent assay-based assays for the detection of p24 antigen, the p7 assay was faster. Its lower detection limit is higher than that in a standard p24 immunoassay, but this was a negligible disadvantage when assaying the production of viral antigens in tissue cultures. An assay for p7 may be of use in neutralization studies, in which antisera are tested for their ability to prevent HIV-1 from infecting cells. Currently, these studies are performed by adding serum to cell cultures that are exposed to HIV-1. Viral production is then measured by determining p24 production, and cultures must be washed extensively after incubation with virus and serum to remove antibodies against p24, which interfere with the assay. Since little or no p7 or antibody response to p7 is detectable in serum, the p7 assay would eliminate the washing step and the p7 produced by the cells in culture could be measured directly in the culture supernatant. The omission of the washing step would probably improve the reproducibilities of the neutralization assays.

Therefore, in addition to the monitoring of HIV-1 replication in viral cultures, the p7 assay described here may be a good alternative to the p24 assay as the readout system of neutralizing activity of serum antibodies.

**ACKNOWLEDGMENTS**

We thank L. O. Arthur for providing recombinant p7, goat-anti-p7 antisera, and monoclonal antibodies. We thank J.-L. Darlix and V. Tanchou from the Laboratoire de Virologie Humaine, Ecole Normale Superieure de Lyon, Lyon, France, for providing some of their rabbit-anti-p7 antisera and monoclonal antibodies. We thank M. Bakker, M. Van Putten, E. Van Egmond, and E. Hogevorst for excellent technical assistance; Paul Converse and M. Kibbey of IGEN International Inc. for useful input; and Lucy Phillips for editorial review.

This work was financially supported by IGEN International Inc. and by a grant (V/E) from the Dutch Ministry of Public Health.

**REFERENCES**


**TABLE 4.** Anti-p24, p24, and anti-p7 status of and RNA levels in p7-positive sera

<table>
<thead>
<tr>
<th>Serum sample no.</th>
<th>Status for the following:</th>
<th>RNA level (log_{10} copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R9600215</td>
<td>+</td>
<td>8.991</td>
</tr>
<tr>
<td>R960383</td>
<td>+</td>
<td>8.000</td>
</tr>
<tr>
<td>R9609726</td>
<td>+</td>
<td>6.084</td>
</tr>
<tr>
<td>R9708740</td>
<td>+</td>
<td>6.041</td>
</tr>
<tr>
<td>R9710811</td>
<td>+</td>
<td>7.114</td>
</tr>
</tbody>
</table>

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