Incorporation of Biotinylated Nucleotides for the Quantification of PCR-Amplified HIV-1 DNA by Chemiluminescence

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Summary: Chemiluminescent detection of polymerase chain reaction (PCR)-amplified DNA was used as a quantitative method for detecting HIV-1. For this purpose, biotinylated dUMP was directly incorporated into the amplified DNA during the PCR reaction. Biotinylation was visualized in an enzymatic reaction using avidine-conjugated alkaline phosphatase and its chemiluminescent 1,2-dioxetane substrate AMPPD, which decomposes upon dephosphorylation and emits light. Light emission was either detected with X-ray films or quantified with a single-photon counting camera connected to a computer imaging system. The specificity of the method was shown by hybridization with a biotinylated or radiolabelled HIV-1-specific oligonucleotide probe. Besides being quantitative, this method represented a non-hazardous, rapid and sensitive technique for the detection of HIV-1 DNA.

Introduction
The polymerase chain reaction (PCR) has become a widespread technique for amplifying HIV-1-specific segments of DNA in vitro (1). DNA is amplified by the thermostable Taq DNA polymerase, using specific oligonucleotide primers that hybridize to the opposite strands and flank the region of DNA to be amplified. Repetitive cycles, involving template denaturation, primer annealing and extension of the annealed primers by the DNA polymerase results in exponential accumulation of amplified DNA segments. The amplified DNA is usually detected and analysed by gel electrophoresis and subsequent Southern blot hybridization with a radiolabelled sequence-specific oligonucleotide probe (2).

Alternatively, DNA probes can be labelled with biotin and visualized by chemiluminescence via an avidine-conjugated alkaline phosphatase and its substrate AMPPD (3- [2'-spiroadamanantane-4-methoxy-4'-(3'-phosphoryloxy)phenyl] -1,2-dioxetane) (3–5). AMPPD has been developed by the company Tropix Inc., (Bedford, MA) (3). It is a stable 1,2 dioxetane, which upon dephosphorylation becomes an unstable phenolate anion (AMPD), which decomposes with light emission at 477 nm.

As a rapid, sensitive, quantitative and non hazardous HIV-1 detection method we modified the PCR amplification method by directly incorporating biotinylated nucleotides during the PCR amplification procedure. The resulting amplified DNA can then be visualized directly without requiring subsequent hybridization with a specific oligonucleotide probe.

Material and Methods
HIV-1-PCR amplification
The HIV-1 plasmid (pBT-1) was used (6). The HIV-1 gag region was amplified in the presence of 20 pmol of the primer pair SK38/SK39 (1) in 50 mmol/l Tris-HCl pH 8.3, 2 mmol/l MgCl₂, 40 mmol/l KCl, 1 mmol/l dithioerythritol and 170 μg/ml bovine serum albumin, using 2 U Taq polymerase (Boehringer Mannheim, Germany) and 200 μmol/l of each the nucleotides dATP, dCTP, dGTP and dTTP. PCR conditions were 25 cycles of 1 min denaturation at 92 °C, 1 min annealing at 54 °C and 2 min polymerization at 72 °C. The DNA Thermal Cycler of Perkin Elmer (Cetus Corporation, Emerville CA) was used.

To directly label amplified DNA with biotin, the PCR reaction was performed as described above, but 150 μmol/l dTTP plus 50 μmol/l Bio-16-UTP (Boehringer) were used instead of 200 μmol/l dTTP.

Amplified DNA was analysed by electrophoresis on a 2% agarose gel in buffer (89 mmol/l Tris-borate, 2.5 mmol/l EDTA, pH 8.0) and transferred to a Biodyne (Pall) nylon membrane by the method of Southern (7). DNA was fixed to the membrane by UV irradiation (8).

Detection by chemiluminescence
The membranes were developed according to the procedure recommended by the company Tropix (Bedford, MA) (9). Briefly, the membranes were blocked with "I-Light Blocking" buffer of Tropix, incubated with 1:30 000 dilution of the avidine-conjugated alkaline phosphatase (Tropix) and washed in

1) Enzyme: Alkaline phosphatase (EC 3.1.3.1)
a buffer containing 2.7 mmol/l KCl, 137 mmol/l NaCl, 1.5 mmol/l KH₂PO₄, 7.75 mmol/l Na₂HPO₄, pH 7.4, 3 ml/l Tween 20. The alkaline phosphatase substrate AMPPD (Tropix) was added at a concentration of 0.25 mmol/l in 50 mmol/l sodium carbonate-bicarbonate buffer pH 9.5. Ten minutes after addition of AMPPD and up to 24 hours later, chemiluminescence of the membranes can be detected, either by exposing the membranes for 10 minutes on X-ray films or by quantifying the emitted light with a photon counting camera connected to a computer image acquisition system (Hamamatsu C1966, Herrsching, Germany) for 30 s to 60 s (10, 11).

Hybridization

The oligonucleotide probe SK19 (1) was either labelled with radioactive α³²P-dATP using T4 polynucleotide kinase (Biofinex, Fribourg, Switzerland) or labelled with biotinylated dUTP (Bio-16-UTP, Boehringer) using the terminal transferase (12).

One hour prehybridization and overnight hybridization with 40 pmol/l radioactive probe were performed in 1 mol/l NaCl, 250 ml/l formamide, 100 g/l dextran sulphate, 10 g/l SDS, 200 mg/l salmon sperm DNA at 54 °C. Membranes were washed 2 times for 30 min at 54 °C in a solution containing 0.3 mol/l NaCl, 30 mmol/l sodium citrate, 10 g/l SDS and once at room temperature in a solution containing 0.015 mol/l NaCl, 1.5 mmol/l sodium citrate.

Results and Discussion

We adapted the method described by Y.-M. Lo et al. (13) for biotinylating probes by adding biotinylated dUTP into the PCR reaction buffer. The resulting biotinylated dUMP's were directly incorporated into the amplified DNA during the PCR reaction and could be visualized by chemiluminescence after incubation with avidine-conjugated phosphatase and its light emitting substrate AMPPD. This methodology allowed us to quantify the amplification by measuring the emitted light as photon counts.
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Fig. 3. Comparison of PCR’s performed in the presence or the absence of biotinylated UTP

20 ng of HIV-1 plasmid gag region were amplified with the primer pair SK38/39 in presence (upper panel) or absence (lower panel) of 50 μmol/l biotinylated dUTP (Bio-16-UTP). After amplification 2.5, 1.25, 0.62, 0.31, and 0.15% of the PCR reaction volume (total volume = 50 μl) was analysed by gel electrophoresis and transferred to nylon membranes by the method of Southern. The upper Southern blot was directly developed according to the chemiluminescence protocol, while the lower Southern blot was first hybridized with the HIV-1 gag-specific biotinylated SK19 oligonucleotide probe. X-ray films exposed for 4 minutes are shown for both blots.

Panel 1); if amplified in the presence of biotinylated dUTP’s, amplified DNA was first hybridized with a biotinylated oligonucleotide probe, which was then visualized by chemiluminescence (fig. 3, lower panel). Identical detection sensitivities were obtained with both amplification techniques.

Thus, direct incorporation of biotinylated nucleotides during PCR represented a sensitive and rapid method, which allowed direct quantification of the amplified product. Additionally, it not only avoids the handling of hazardous radioactivity, but also circumvents hybridization procedures.

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