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Standrd operating procedure for HIV-1 drug-resistance

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We use this protocol and it's working

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Abstract

This operating procedure aims to provide standardized guidance for HIV-1 drug resistance testing within the HIV Drug Resistance Laboratory of Chongqing Public Health Medical Center. It is intended for use by laboratories equipped with appropriate experimental conditions. Users should possess relevant professional knowledge and skills and strictly adhere to laboratory safety regulations. The authors and issuing organization shall not be held liable for any direct or indirect damages resulting from the use of this procedure.

Guidelines

- Amplified products were verified by 1% agarose gel electrophoresis (with GoodView nucleic acid dye) using a 100 bp DNA Marker.
- Target bands (~1500 bp for protease + reverse transcriptase region, ~1200 bp for integrase region) were observed.
- Positive controls should show clear bands, while negative controls showed no bands (otherwise, the experiment was repeated).
- Qualified products were sent to Chongqing Qingke Biotechnology Co., Ltd. for Sanger double-ended sequencing.

2. Identification of HIV-1 Genotypes

- The obtained sequences in ABI format were quality-assessed, trimmed, and assembled using ChromasPro software to remove low-quality terminal sequences, followed by sequence editing and alignment with BioEdit software.
- HIV-1 genotyping was performed using the Viral Genotyping Tool from the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>) with the HXB2 strain (GenBank accession number: K03455) as the reference sequence, and supplemented by verification with the REGA HIV-1 Subtype Analysis Tool 3.0.
- Sequence alignment was conducted using the CLUSTAL W tool, and homology analysis was performed with subtype reference sequences from the Los Alamos National Laboratory HIV Sequence Database.
- To further confirm genotypes and exclude contamination, a phylogenetic tree was constructed using MEGA 6.0 software with the Neighbor-Joining method, 1000 Bootstrap replicates, and the Kimura two-parameter model, with a genetic distance scale of 2%.

3. Drug Resistance Analysis

- The edited and assembled HIV-1 pol gene sequences were submitted to the Stanford University HIV Drug Resistance Database (<https://hivdb.stanford.edu/>) for identification of drug resistance mutations (DRMs) and analysis of drug resistance levels.
- Drug resistance was classified into five grades according to the database criteria: susceptible (S, score 0-9), potential low-level resistance (P, score 10-14), low-level resistance (L, score 15-29), intermediate resistance (I, score 30-59), and high-level resistance (H, score ≥ 60).
- Low-level resistance and above (L, I, H) were defined as drug-resistant.



Materials

- Viral nucleic acid extraction kit (Jiangsu Shuoshi Biotechnology Co., Ltd.)
- HiScript® II One Step RT-PCR Kit (Nanjing Vazyme Biotech Co., Ltd.)
- Ace Taq Kit (Nanjing Vazyme Biotech Co., Ltd.)
- 10×buffer
- MgCl₂
- dNTP
- Upstream primers (2029F, 4007F, 2249F, 4063F)
- Downstream primers (3529R, 5219R, 3521R)
- Reverse transcriptase
- Taq enzyme
- RNase inhibitor
- RNase-free water
- RNA template

Safety warnings

- ! - Store extracted RNA at -80°C with no more than 3 freeze-thaw cycles to avoid RNA degradation.
Ethical Compliance Notice: The collection of human plasma samples for the purposes of HIV-1 drug resistance testing, as described in this protocol, must obtain prior approval from the user's Institutional Review Board (IRB), Independent Ethics Committee (IEC), or equivalent ethics review body. Users are solely responsible for ensuring that their sample collection activities comply with all applicable ethical guidelines and regulations in their respective institutions and countries/region.

RNA Extraction and Nested PCR

- 1 After plasma separation by centrifugation at 3000×g for 15 minutes, HIV-1 total RNA (including integrase gene transcripts) was extracted using a viral nucleic acid extraction kit (Jiangsu Shuoshi Biotechnology Co., Ltd.) following the manufacturer's instructions. During extraction, positive controls (known HIV-1-positive plasma) and negative controls (HIV-1-negative plasma) were set up simultaneously to exclude contamination or RNA degradation. The extracted RNA was immediately stored at -80°C with no more than 3 freeze-thaw cycles to avoid RNA degradation.
- 2 Nested PCR was performed to simultaneously amplify three key functional regions of the HIV-1 pol gene: protease (4-99 aa), reverse transcriptase (38-320 aa), and integrase (1-288 aa). The total length of the amplified fragment covered ~2300 bp, with the integrase region fragment being 1157-1213 bp (referring to HXB2 strain, GenBank accession no. K03455, nucleotide positions 4063-5219).
- 3 First-round RT-PCR: The HiScript® II One Step RT-PCR Kit (Nanjing Vazyme Biotech Co., Ltd.) was used, with separate reaction systems (25 µL total volume per system) for the "protease + reverse transcriptase region" and "integrase region":
 - 3.1 For protease + reverse transcriptase region: 2.5 µL of 10×buffer, 5.0 µL of MgCl₂, 2.5 µL of dNTP, 0.5 µL of upstream primer (2029F: 5'-TGGAAGTGGTGRAAGGAAGGAC-3'), 0.5 µL of downstream primer (3529R: 5'-GCTAyyAAGTCTTTTGATGGGTCAT-3'), 0.5 µL of reverse transcriptase, 0.5 µL of Taq enzyme, 0.5 µL of RNase inhibitor, 7.5 µL of RNase-free water, and 5.0 µL of RNA template;
 - 3.2 For integrase region: 2.5 µL of 10×buffer, 5.0 µL of MgCl₂, 2.5 µL of dNTP, 0.5 µL of upstream primer (4007F: 5'-GCAGGATTCRGGATYAGAAGTAAA-3'), 0.5 µL of downstream primer (5219R: 5'-CCTAGTGGGATGTGTACTTCTGAAC-3'), 0.5 µL of reverse transcriptase, 0.5 µL of Taq enzyme, 0.5 µL of RNase inhibitor, 7.5 µL of RNase-free water, and 5.0 µL of RNA template[3].
- 4 Both systems shared the same program: 50°C for reverse transcription (30 min), 95°C for pre-denaturation (5 min), followed by 30 cycles of 94°C (30 s), 55°C (30 s), and 72°C (2.5 min), with a final extension at 72°C (10 min) and holding at 4°C.
- 5 Second-round nested PCR: The Ace Taq Kit (Nanjing Vazyme Biotech Co., Ltd.) was used, with 50 µL reaction systems for the two target regions:
 - 5.1 For protease + reverse transcriptase region: 25 µL of amplification mixture, 1.0 µL of upstream primer (2249F: 5'-CTTCCCTTCARATCACTCT-3'), 1.0 µL of downstream primer (3521R: 5'-GTCTTTTGTATGGGTCATA-3'), 18 µL of enzyme-free water, and 5.0 µL of first-round RT-PCR product;

- 5.2 For integrase region: 25 µL of amplification mixture, 1.0 µL of upstream primer (4063F: 5'-TCATTCA RGCACAACCAG-3'), 1.0 µL of downstream primer (5219R: same as first round), 18 µL of enzyme-free water, and 5.0 µL of first-round RT-PCR product.
- 6 The reaction program was: 94°C for pre-denaturation (5 min), followed by 30 cycles of 94°C (30 s), 63°C (30 s), and 72°C (2.5 min), with a final extension at 72°C (10 min) and holding at 4°C.
- 7 Amplified products were verified by 1% agarose gel electrophoresis (with GoodView nucleic acid dye) using a 100 bp DNA Marker. Target bands (~1500 bp for protease + reverse transcriptase region, ~1200 bp for integrase region) were observed; positive controls should show clear bands, while negative controls showed no bands (otherwise, the experiment was repeated). Qualified products were sent to Chongqing Qingke Biotechnology Co., Ltd. for Sanger double-ended sequencing.

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