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Protocol

Evaluation of different RT enzyme standards for quantitation of retroviruses using the single-tube fluorescent product-enhanced reverse transcriptase assay☆

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PCR-based reverse transcriptase (RT) assays are highly sensitive for broad detection of retroviruses. These assays are currently used for demonstrating the absence of retroviral contamination in vaccines and can also be applied to clinical and laboratory research to investigate low-virus replication. A single-tube fluorescent product-enhanced reverse transcriptase assay (STF-PERT) has been published that was highly sensitive for retrovirus detection (<10 virions), with enhanced reproducibility and increased efficiency [Sears, J.F., Khan, A.S., 2003. Single-tube fluorescent product-enhanced reverse transcriptase assay with AmpliWax (STF-PERT) for retrovirus quantitation. J. Virol. Meth. 108, 139–142]. In this report, the step-by-step setup and performance of the STF-PERT assay is described and sensitivity, reproducibility and specificity of the assay reported using three different RTs as standards: avian myeloblastosis virus (AMV) RT, murine leukemia virus (MMLV) RT, and human immunodeficiency virus type 1 (HIV-1) RT. Evaluation of virus stocks showed about 1–2 logs difference in RT detection and retrovirus quantitation with the different RT enzyme standards; in general, virus determination using HIV-1 RT was comparable to using the relevant virus RT.

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1. Type of research

All retroviruses contain RNA-dependent DNA polymerase termed reverse transcriptase (RT), which is essential in the retrovirus life cycle; therefore, assays for RT activity can broadly detect different types of retroviruses from various species. In contrast to the conventional RT assays, which detect 10^4–10^6 particles with a short linear range (Sears et al., 1999), PCR-based or PCR-enhanced RT assays (designated as PBRT or PERT assays) can detect 1–10 virions in a broad linear range (6–8 logs) (Heneine et al., 1995; Pyra et al., 1994; Silver et al., 1993). The modification of the PCR-based assays to real-time, fluorogenic PERT assays (Arnold et al., 1998; Lovatt et al., 1999; Maudru and Peden, 1998; Sears and Khan, 2003) has provided an important tool for evaluating the safety of biological products and enhancing virus detection in clinical samples and laboratory research. A single-tube, fluorescent PERT assay (STF-PERT) was described for quantitation of retrovirus particles using avian myeloblastosis virus (AMV) RT (Sears and Khan, 2003). In this paper, the assay is evaluated with other RT enzymes [murine leukemia virus (MMLV); and human immunodeficiency virus type 1 (HIV-1)] for accurate quantitation of different retrovirus types. Additionally, a detailed protocol is presented to facilitate easy establishment of the assay for broad use.

2. Time required

(i) Preparation of buffers: 4–5 h.
- NZ buffer; autoclave (20 min at 121 °C), cool to room temperature and store at −20 °C;
- 10× RT buffer and 10× PCR buffer; aliquot each buffer and store at −20 °C;
- can be stored up to 6 months.

(ii) Preparation of enzyme dilutions for standards: 45 min.
- Make RT dilutions in NZ/DTT buffer;
- if storing diluted enzyme at −20 °C, use within 7 days; if aliquoted and stored at −80 °C, use within 1 month.

(iii) Assay setup: 3 h.
- Prepare working stock reagents, PCR cocktail and RT reaction cocktail;

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- add PCR cocktail, then wax to each well, melt and cool wax;
- add RT reaction cocktail, diluted RT enzyme or sample on top of wax in each well.

(iv) Assay run: 3 h 24 min.
(v) Data analysis: up to 4 h.

3. Materials

3.1. RT enzyme

- AMV (10 U/μl; Catalogue No. N2111: specific activity 73,260 U/mg, Tables 3 and 4, Fig. 1; Catalogue No. M5108: specific activity 40,715, Fig. 1; Catalogue No. M5108: specific activity 36,500 U/mg, Tables 5 and 6, Fig. 2 and specific activity 80,300 U/mg, for SIVmac239 in Table 6; Promega Corp, Madison, WI 53711).
- MMLV (10 U/μl; Catalogue No. M6125H: specific activity 47,619 U/mg, Tables 3 and 4, Fig. 1; specific activity 85,000 U/mg, Tables 5 and 6, Fig. 2; specific activity 33,000 U/mg, for SIVmac239 in Table 6; Epicentre Biotechnologies, Madison, WI 53713).
- HIV-1 (27 U/μl; Catalogue No. RTHIV; Specific activity 21,600 U/mg; Worthington Biochemical Corp, Lakewood, NJ 08701).

3.2. Special equipment and computer software

(i) ABI PRISM® 7700 or 7900HT Sequence Detection System and Sequence Detection Software (SDS1.9.l for PRISM 7700 and SDS2.2.2 or higher, for 7900HT (Applied Biosystems, Foster City, CA 94404).
(ii) PCR workstation (Model#: P-036, C.B.S. Scientific Co. Del Mar, CA 92014).
(iii) Biosafety cabinet (class II Type A/B3, Model No. NU-407-400; NUAIRE, Plymouth, MN 55447).
(iv) Designated "closed" areas for preparation of solutions/buffers; RT standards; test samples; and assay run.

3.3. Chemicals, reagents and materials

- 1 M Tris–HCl, pH 7.5 (DNase, RNase and Protease-free; Catalogue No. 351-006-131; Quality Biological Inc., Gaithersburg, MD 20879).
- 2 M $MgCl_2$ (DNase, RNase and Protease-free; Catalogue No. 340-034-721; Quality Biological Inc., Gaithersburg, MD 20879).
- 0.5 M EDTA, pH 8.0 (DNase, RNase and Protease-free; Catalogue No. 351-027-101; Quality Biological Inc., Gaithersburg, MD 20879).
- IGEPAL CA-630 (nonionic detergent; Catalogue No. I-3021; Sigma-Aldrich, St. Louis, MO 63178).
- Triton X-100 (nonionic surfactant; Catalogue No. TRX10001; MP Biomedicals, Irvine, CA 92618).
- UltraPure™ Glycerol (Catalogue No. 15514-011; Invitrogen, Carlsbad, CA 92008).
- UltraPure™ Dithiothreitol (DTT) (0.1 M; Catalogue No. 15508-013; Invitrogen, Carlsbad, CA 92008).
- UltraPure™ DEPC-treated water (Pyrogen and DNA/RNAase Free; Catalogue No. 750024; Invitrogen, Carlsbad, CA 92008).

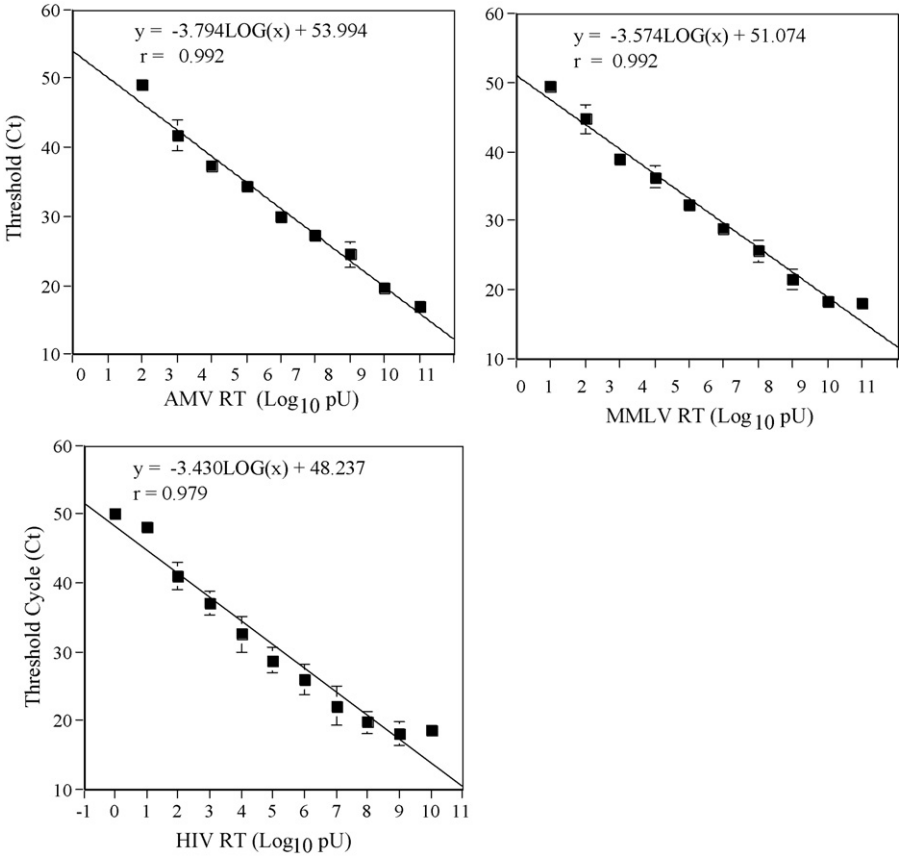

Fig. 1. Sensitivity and reproducibility of RT detection by STF-PERT assay. Ten-fold serial dilutions of AMV RT, MMLV RT and HIV-1 RT (in range of 10^0–10^{10} pU) were assayed. The results were analyzed using the same threshold for each enzyme. Linear standard curves are shown. The threshold cycle (Ct) of each dot is plotted against the RT concentration ($\log_{10}$ pU). Each dot represents mean ± S.D. of three assays with triplicate samples in each for HIV-1 RT and five assays with triplicate samples in each for AMV RT and MMLV RT (in case of MMLV RT, the Ct for the highest concentration is based upon two assays).

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