Nucleic Acids Research, 1993, Vol. 21, No. 15 ***3593–3594***

An RT-PCR assay for the enzyme activity of reverse transcriptase capable of detecting single virions

Jonathan Silver, Thomas Maudru, Kazunobu Fujita and Roy Repaske
Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Received May 25, 1993; Accepted June 20, 1993

Abstract. We developed a sensitive assay for the enzyme activity of reverse transcriptase (RT) by setting up an RT-PCR assay containing an ample amount of exogenous RNA template but no added RT except that provided in the sample to be tested. This assay can detect less than 100 molecules of RT, which is approximately 10^6 times more sensitive than a conventional RT assay. The assay detects the RT activity present in single virus particles. Because the assay is general for most, if not all, RTs and is several orders of magnitude more sensitive than ELISA or infectivity assays, it also has promise as a tool for discovery of new retroviruses.

RT-PCR is conventionally used for detection of minute amounts of RNA template. In this format, exogenous RT is provided in excess to convert template RNA into amplifiable DNA. We reasoned that the assay could be 'turned around' to detect minute amounts of RT activity by setting up an RT-PCR reaction with adequate amounts of exogenous RNA template but no RT except that provided in the sample to be tested. The RNA template we used comes from Brome Mosaic Virus (BMV), an RNA virus with no DNA phase in its life cycle; this minimizes the problem of homologous DNA contaminating the template RNA. To make the assay more convenient and reduce the chance of 'carry-over' contamination, we set up the RT and PCR reactions in a single tube but in separate compartments. The reagents for the PCR reaction, minus Mg^{++} and template DNA, are placed in the bottom of a thin walled 'MicroAmp' PCR tube (Perkin Elmer). Above this is a layer of melted, re-congealed wax. Above the wax are the RT reactants including Mg^{++} and sample to be assayed for RT activity. During the 37°C incubation for reverse transcription, the chance for formation of 'primer–dimer' and other amplifiable artifacts is reduced because Taq polymerase is inactive in the absence of Mg^{++}. Following reverse transcription, the heating associated with PCR melts the wax and mixes the two compartments, allowing Taq polymerase to come into contact with Mg^{++} and any template DNA synthesized by RT. The product of the PCR reaction is detected using a radiolabelled internal oligonucleotide which is primer extended on the PCR product in a subsequent reaction and then detected by polyacrylamide gel electrophoresis and autoradiography.

The sensitivity of this assay was investigated using purified RTs and stocks of Human Immunodeficiency Virus (HIV-1) and Friend Murine Leukemia Virus (F-MuLV). The assay detects between 3 and 100 molecules of RT from HIV-1, Moloney MuLV or Avian Myeloblastosis Virus (AMV) (Figure 1, lanes 3, 6 and 9). To determine sensitivity for viral particles, we serially diluted stocks of HIV-1 containing known concentrations of p24 *gag* protein. One virus particle is estimated to contain 0.05 fg of p24 (1). Our assay detects RT activity in samples containing as little as 0.6 to 0.03 fg of p24 (Figure 1, lanes 13 and 15). Similar results to those shown in Figure 1 were obtained with 11 other HIV-1 isolates grown in tissue culture. Standard ELISA assays for p24 are limited in sensitivity to about 5 pg of p24. With HIV-1 or F-MuLV, our assay can detect RT activity in samples containing fewer than 10^{-4} infectious units (Figure 1, lanes 13, 15 and 19); this implies that these virus stocks contain at least 10,000 times more physical particles than infectious particles.

Our assay also detects the RTs of Human T Cell Leukemia Viruses-I and II, Simian Immunodeficiency Virus, Bovine Immunodeficiency Virus, Caprine Arthritis–Encephalitis Virus, Equine Infectious Anemia Virus and Visna Virus (not shown). While many DNA-dependent DNA polymerases, such as those from Taq, T4 and T7, are negative for RT activity when added as 'samples' in this assay, Pol I has a small amount of RT activity. Extracts from some tissue culture cells also have RT activity in this assay, although the levels are orders of magnitude lower than for high titer viral stocks. We have not yet determined whether these cell-associated RT activities reflect the products of endogenous retrovirus-like RT genes or DNA-dependent DNA polymerases.

Because of its tremendous sensitivity and generality, this assay may be very useful as a tool for discovery of new retroviruses.

ACKNOWLEDGEMENTS

We thank Drs N.Salzman, T.Limjoco, M.Gonda, G.Franchini and R.Shibata for virus preparations, Dr A.Buckler-White for oligonucleotides, and Dr M.Martin for helpful discussions.

REFERENCE

1. Layne,S.P., Merges,M.J., Dembo,M., Spouge,J.L., Conley,S.R., Moore,J.P., Raina,J.L., Renz,H., Gelderblom,H.R. and Nara, P.L. (1992) *Virology* **189**, 695–714.

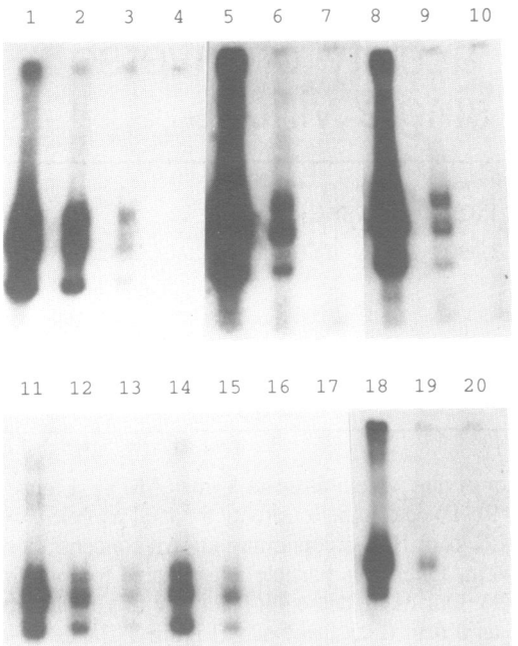

Figure 1. RT-PCR reactions were set up by adding one 'Ampliwax' pellet (Perkin Elmer) to 25 μl of PCR mix containing 2.5 U Taq polymerase in 10 mM Tris−HCl pH 8.3, 50 mM KCl, 1 μM each PCR primer 5′-CGTGGTTGACACGCAG-ACCTCTTAC-3′ and 5′-TCAACACTGTACGGCACCCGCATTC-3′, 0.2 mM each dATP, dCTP, dGTP and dUTP. After brief heating and cooling to melt and resolidify the wax above the aqueous phase containing the PCR reactants, we added 25 μl containing 10 μl of sample to be assayed for RT activity plus 15 μl of RT reaction mix. The RT reaction mix contained 21 ng BMV RNA (Promega) and 8 U RNasin RNAse inhibitor (Promega) in 16.7 mM Tris−HCl pH 8.3, 83.3 mM KCl, 8.3 mM $MgCl_2$, 0.167 mM each dATP, dCTP, dGTP and dUTP, 3.3 mM DTT, 0.17% NP-40, and 18 nM RT primer 5′-GGTCT-CTTTTAGAGATTTACAGTG 3′. The mixture was incubated at 37°C for 1 h, then 94°C×1′, then 40 cycles of 94°C×15', 56°C×15', 72°C×15', then 72°C×5′. To detect the PCR product, 8 μl of reaction mixture was combined with 2 μl of 10 mM Tris−HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each dATP, dCTP, dGTP, dUTP, 0.5 U Taq polymerase and about 20 fmol (50,000 to 100,000 cpm) of 5′-^{32}P-labelled probe oligonucleotide 5′-GCCTTTGAGAGTTACTCTTTG-3′, and incubated at 94°C×1′, then 4 cycles of 94°C×30', 56°C×30', 72°C×30', then 72°C×5′. The probe-sample mix was electrophoresed on 8% polyacrylamide gels in 0.5×TBE buffer and autoradiographed. Lanes 1−10: dilutions of HIV-1 RT (American Bio-Technologies, Inc), Moloney MuLV RT and AMV RT (Life Technologies) in 10 mM Tris−HCI pH 8.3. Lanes 1−3: 10^{-10}, 10^{-11} and 10^{-12} dilutions of HIV-1 RT, containing 10^4 to 10^2 molecules, respectively; lanes 5−6: 10^{-10} and 10^{-12} dilutions of Mo-MuLV RT, containing 4000 and 40 molecules; lanes 8 and 9: 10^{-10} and 10^{-12} dilutions of AMV RT, containing 300 and 3 molecules; lanes 4, 7 and 10: Tris diluent alone. Lanes 11−20: serial 10-fold dilutions of HIV-1 and F-MuLV virus stocks. The HIV stocks were grown in peripheral blood mononuclear cells (PBMC) and the F-MuLV was a 10% (wt/vol) spleen extract from a mouse infected with Friend virus complex. Lanes 11−13 and 14−16: 10^{-6} to 10^{-8} dilutions of two different HIV-1 isolates, the undiluted stocks of which contained $3-6\times10^5$ pg/ml of p24 and 10^2 to 10^3 $TCID_{50}$/ml of infectious virus (assayed on PBMC); lanes 18−19: 10^{-7} and 10^{-8} dilutions of a stock of F-MuLV which contained 10^6 XC infectious units/ml; lanes 17 and 20: Tris diluent alone.