HIV 1 Reverse Transcriptase Assay Kit

Catalog Number RT-1000

INTRODUCTION

Human Immunodeficient Virus 1 (HIV 1) is a retrovirus that contains, as part of its genome, a reverse transcriptase (RT) gene, that when translated into protein, reverse transcribes HIVs RNA genome into a transducing DNA copy (cDNA). This HIV RT activity is critical during early viral infection. HIV 1 infection results in host cell domination, viral replication, and host cell death. HIV RT is an optimistic target for blocking the virus life cycle and the spread of the virus to neighboring cells. In addition, HIV RT in viral conditioned medium directly correlates with virus propagation in cell-mediated *in vitro* experimental model systems, therefore HIV RT activity can be used as a marker for HIV 1 growth.

XpressBio has developed a 2-hour HIV RT assay for drug screening and a 24-hour assay to detect as little as 1 pg of HIV RT in viral conditioned media. An assay incubation time of 5-6 hours allows the experiment to be completed in one day and will detect 2 pg of HIV RT. The RT assay is non-radioactive, colorimetric, and can be used to determine if test articles, compounds, or peptides can inhibit HIV RT activity. The assay can also be used to quantitate HIV RT activity in experimental samples.

The HIV RT Assay Kit can be used to detect other retroviruses including HIV-2, M-MuLV, AMV, and SIV-1.

KIT CONTENTS

Product	Catalogue	Per Kit
Reaction Buffer 1	RT1001	2x 1.3 mL
Reaction Buffer 2	RT1002	2x 1.3 mL
HIV-1 Reverse Transcriptase	RT1003	25 µL
Lysis Buffer	RT1004	12 mL
HRP Anti-Digoxigenin Conjugate	RT1005	12 mL
Dilution Plate	RT1006	1 plate
Sodium Azide (20%)	RT1007	1 mL
Streptavidin-coated 96-well Plate	RT1008	1 plate
Wash Buffer Concentrate 20X	82710	60 mL
ABTS Peroxidase Substrate	515-419	12 mL
Stop Solution	515-417	10 mL
Instruction Manual	RT1000	1

TECHNICAL ASSISTANCE

Please refer any technical questions to XpressBio at info@xpressbio.com.

SAFETY INFORMATION

Sodium azide may react with lead and copper plumbing to form explosive azide compounds. When disposing of reagents, flush with copious quantities of water. The MSDS for this kit is available online at www.xpressbio.com.

STORAGE CONDITIONS

The streptavidin-coated plate, lysis buffer, 20% sodium azide, HRP anti-digoxigenin conjugate, ABTS, 20x wash buffer, and stop solution are stored at 4-8ºC. Reaction buffer 1, reaction buffer 2, and the HIV 1 RT enzyme are stored at -20ºC. The kit is stable for at least one year under these conditions. Microwell strips of the streptavidin-coated plate that are not used after opening the foil pouch should be returned to the pouch along with the sachet of desiccant, close the zip lock flap on the pouch and store at 4-8ºC. The majority of the kit components are shipped at room temperature, except for the DS oligo, TS oligo, and the HIV-1 RT enzyme that are shipped on dry ice. All components should be stored under the conditions specified above.

REAGENTS AND EQUIPMENT SUPPLIED BY THE USER

- Pipettors, Multi-channel and sterile tips
- Disposable gloves
- 1.2 ml dilution tubes
- Paper towels
- Sterile distilled (deionized) water
- A 37ºC incubator
- A 96-well plate reader capable of reading ODs at wavelengths 405 nm and 450 nM

NOTES BEFORE STARTING

General Comments. Carefully review the protocol before beginning, small deviations may lead to discrepancies in the results. The HIV RT Kit provides the user with an extra 8-well strip and reagents to run an initial test, helping to familiarize the user with the protocol. The RT assay requires three sets of tubes for RT dilution, test compound dilution, and RT incubation. Every incubation tube will receive 40 ul of RB (RB + test compound) and 80 ul of lysis buffer (lysis buffer + RT). The addition of RB and RB + test compound to the incubation tubes should be completed before diluting the RT enzyme. A generalized procedure summary for an 8-well strip is outlined below and in Figure 1.

1). Warm reagents and make 1x reaction buffer (RB) by mixing equal volumes of reaction buffer 1 and 2. Incubation tubes require 40 ul of RB, so for the 8-well test strip, make 350 ul of RB, by mixing together 175 ul of reaction buffer 1 and reaction buffer 2. The assay blank (Fig. 1, Tube A) and RT incubation tubes (Fig. 1, Tubes B-F) each receive 40 ul of RB. Figure 1 illustrates the serial dilution of the RT enzyme in lysis buffer and explains how to set-up the inhibitory azide reaction in RB (Fig.1, Tubes G and H).

2). Next perform the HIV RT serial dilution series (Fig. 1) to make extra 1 ng RT solution for drug testing incubation tubes. The user adds 80 µl of HIV RT diluted standards to each incubation tube, except for the assay blank, which receives 80 ul of lysis buffer (Fig. 1, tube A). We recommend adding

the RB and RB + test articles (azide) to each incubation tube first, then use a multichannel pipette to add the diluted HIV RT standards simultaneously to the incubation tubes starting the reaction.

3). All incubation tubes are placed at 37ºC for 20 minutes. Transfer 100 µl of the RT reaction into individual streptavidin-coated microwells (8-well strips) provided and incubate at 37ºC for 20 minutes.

4). Remove the incubation reaction from wells and wash each well 5 times with 1x wash buffer (300 µl per well), then pat dry onto paper towels. Add 100 µl of HRP-digoxigenin conjugate per well and incubate at 37ºC for 45 minutes.

5). Remove the conjugate solution and wash each well 5 times with 1x wash buffer (300 ul per well), then pat dry onto paper towels. Add 100 ul of ABTS substrate solution per well and incubate at room temperature or at 37ºC for 30 minutes.

6). Use an ELISA plate reader to directly measure the optical density (OD) when set at 405 nM or add 100 µl of stop solution per well and read the OD set at 450 nM.

Dilution Tube Generation

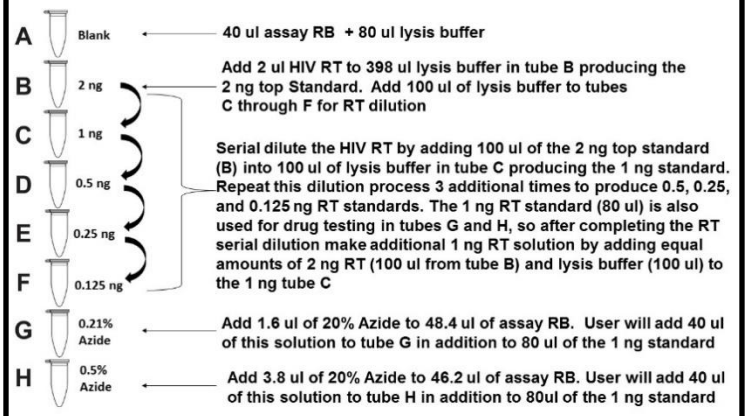

Figure 1. Once dilution tubes are complete (tubes B-F), transfer 80 ul of each solution into their respective incubation tubes, which will contain 40 ul of assay RB. For tubes G and H, the user will transfer 80 ul of the 1 ng HIV RT (Tube C) to incubation tubes G and H in addition to the 40 ul of RB plus azide solution.

Wash Buffer Concentrate. Wash buffer is provided as a 20X concentrate. Before use, mix 50 mL of 20X wash buffer concentrate with 950 mL sterile distilled water to make a 1x wash buffer solution.

Reaction Buffer (RB). Reaction buffer 1 and reaction buffer 2 are mixed together in equal volumes just before use. For example, if 1.0 ml of reaction buffer is required for an RT assay, 500 µl of reaction buffer 1 and 500 ul of reaction buffer 2 are combined to form 1x assay reaction buffer (RB). Each test well requires 40 ul of RB, for 8 wells make 350 µl to ensure coverage of all wells.

HIV 1 Reverse Transcriptase Protein Stability. The HIV RT enzyme should be stored at -20ºC and is stable for at least 6 freeze-thaw cycles. If more cycles are required, aliquot into smaller portions and freeze tubes at -20ºC until needed. Before each use, if the enzyme solution is frozen, it should be thawed in a 37ºC water bath for 10 seconds. After the HIV RT has been diluted for assay, the remaining enzyme solution should be stored at -20ºC.

Plate Washing Steps. Washing of the micro well ELISA plates can be performed manually, with a wash bottle, or with an automatic plate washer. The liquid should be completely removed from the plate wells after each washing step by patting the plate down onto paper towels.

Replicates. We recommend that all samples and controls are tested using at least duplicate wells for each experimental data point. The use of additional replicates may reduce variability in the assay, especially when the assay is used as a research tool for *in vitro* screening of HIV 1 RT inhibitors.

Assay Summary. The assay components are taken from the refrigerator and warmed to room temperature. Reaction buffer 1 and reaction buffer 2 are removed from the -20ºC freezer and thawed. The experiment is designed, and the appropriate amount of assay RB is mixed. The HIV RT assay measures the synthesis of cDNA starting from an RNA template primed with Oligo(dT)$_{12-18}$ in a buffered solution of digoxigenin-labeled and biotin-labeled nucleotides. Quantification of the biotin-tagged cDNA synthesized by the HIV RT is achieved using an ELISA protocol. The newly synthesized biotin-tagged cDNA is bound by streptavidin that has been coated onto plastic microwell strips. A detector antibody specific for digoxigenin and conjugated with peroxidase, binds to the digoxigenin-labeled cDNA. The peroxidase substrate, ABTS, is added to initiate the develop of a colorimetric reaction product that can be monitored with an ELISA plate reader set at an Optical Density (OD) of 405 nM. The reaction can be terminated by adding 100 µl of assay stop solution (515-417) and reading at an OD of 450 nM. The OD values directly correlate to the level of HIV RT activity in the sample (see Figure 2.)

KIT PROTOCOL
HIV 1 Reverse Transcriptase Assay

RT Assay Set-up, Binding to Streptavidin-Plate, Antibody-specific Detection and Colorimetric Development.

1. Thaw and Prewarm Reagents

Place reaction buffer 1 (RT1001) and reaction buffer 2 (RT1002) in a 37ºC water bath for 5 minutes to thaw. Prewarm the other kit components, except for the HIV 1 RT enzyme (RT1003), by placing them at room temperature. The HIV RT should be stored at -20ºC except during dilution into lysis buffer.

2. Experimental Design

The quantitative portion of the RT assay occurs in micro-well strips containing 8 wells, so experiments can be designed in multiples of 8. An experiment to generate a standard curve for the HIV RT requires single or duplicate wells of zero HIV RT (assay blank) and HIV RT at 2 ng, 1 ng, 0.5 ng, and 0.25 ng per well. Each reaction well requires 40 µl of RB, 8 wells x 40 µl = 320 µl, however mix 350 µl to ensure enough RB for each incubation tube. An incubation tube is required for each test well and additional dilution tubes are needed for the titration of the HIV RT and for test compound dilutions.

When performing screening of test articles in the assay, design the experiments so that the highest concentration of test article requires a volume addition of no more than 20 µl into 20 µl of RB (do not dilute reaction buffer by more than 50% with test articles, 20-40% dilutions are best). The highest concentration for each test article may be different and require some experimentation to determine. The assay incubation tubes contain 40 µl of test article in RB, 80 µl of HIV RT in lysis buffer is added to start the RT reaction, so the test article concentration is diluted to 1/3 of the concentration prepared in RB. For example, Efavirenz was made-up to 50 µM, a serial dilution performed, and the assay run before the 1/3 adjustment of the concentration was made. The highest concentration dilution tube of 50 µM was really an effective concentration in the assay of 16.7 µM (Figure 3).

3. Reaction Buffer (RB)

Reaction buffer (RB) addition to assay incubation tubes illustrates the general layout for the experiment design. Label tubes for the incubation of RB with lysis buffer (assay negative control) or lysis buffer with a serial dilution of the HIV RT (see Figure 1 and section 4). RB is also used to perform the final dilution of test articles being evaluated in the assay for RT inhibition potential. The concentration of the test article should be high enough so that only 1-20 µl of test article is added to 39-20 µl of RB. Make extra test article solutions to ensure a complete fill to all incubation tubes. If the assay reactions are performed in duplicate, make 90-100 µl of each test article concentration.

4. Generation of the HIV RT Standard Curve

The HIV RT is diluted into lysis buffer, add 4 µl of RT (RT1003) solution to 796 µl of lysis buffer (RT1004). This dilution generates an RT solution that when 80 µl is added to 40 µl of RB, the amount of HIV RT in 100 µl of this solution is approximately 2 ng. Serial dilution of the 2 ng RT solution results in a standard curve of HIV RT activity per ng of RT added. For example, if the standard curve is generated in duplicate wells, 160 µl of each HIV RT concentration would be needed for the assay. Make extra volume to ensure a complete 80 µl fill into the incubation tubes, so prepare 180 µl of solution for each HIV RT concentration.

After RB or RB + test article has been added to the incubation tubes for the experiment, label 4 RT dilution tubes

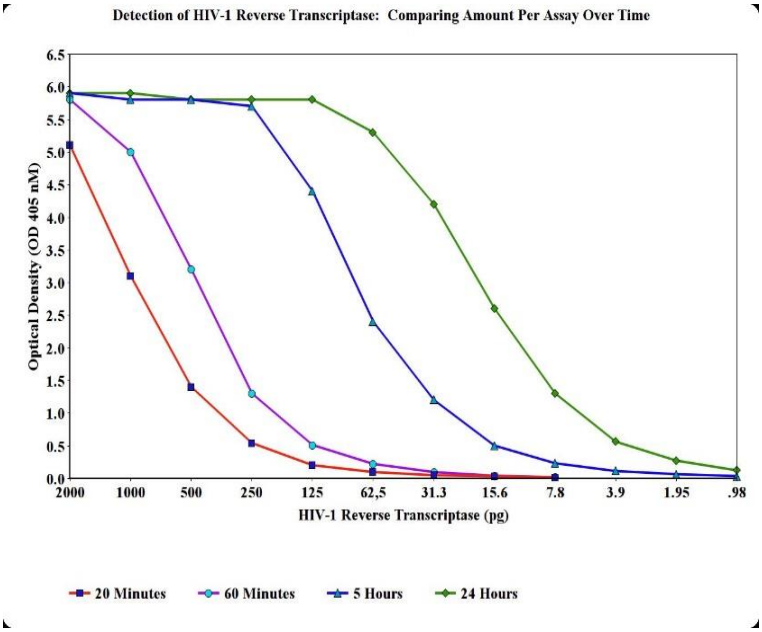

Figure 2. **Time- and Dose-Response Curves for HIV RT**: **The level of detectability of HIV RT activity reaches 1 pg when the incubation time of the assay is 24 hours.**

2 ng, 1 ng, 0.5 ng, and 0.25 ng (0.125 ng and so on) and add 796 µl of lysis buffer (RT1004) to the 2-ng tube and 180 µl of lysis buffer to the 1 ng, 0.5 ng, and 0.25 ng tubes. Remove the HIV RT enzyme solution (RT1003) from the -20ºC freezer. If the solution is frozen place it in a 37ºC water bath for 10 seconds, mix and centrifuge for 3 seconds. Add 4 µl of HIV RT to the 2-ng tube, mix by vortex (setting 5-6) for 5-10 seconds, remove 180 µl and add that to the 1 ng tube, vortex, and remove 180 µl and add it to the 0.5 ng tube and so on. For smaller experiments where less volume of enzyme is needed, for the 2-ng tube, add 2 µl of HIV RT to 398 µl of lysis buffer. For larger experiments 6 µl of HIV RT can be added to 1194 µl of lysis buffer.

The assay negative control incubation tube containing 40 µl of RB and 80 µl lysis buffer can be set up before the addition of stock HIV RT into the 2-ng dilution tube. Add 80 µl of each HIV RT serial dilution to the corresponding incubation tube, one incubation tube per Streptavidin-coated well (see below) using a multi-channel pipettor.

5. Test Articles: Efavirenz and Azide Inhibit the HIV RT Reaction

The HIV RT assay can be used to determine the potential of test articles, compounds, or peptides to block RT activity. Test articles diluted with DMSO to 10-30 mM should be diluted further with a low ionic strength buffer (0.5x PBS) to a concentration of approximately 100-300 µM. Do not use the RB supplied with the kit for these large volume dilutions. A dose-response curve for any inhibitor can be generated by diluting the compound at various concentrations into RB and running the HIV RT assay. For example, our dose curve for Efavirenz started at a concentration of 16.7 µM so to make 100 µl (enough for duplicate wells) at this concentration 16.7 µl of

stock (100 μM) Efavirenz was added to 83.3 μl of RB. The other Efavirenz concentrations tested in the HIV RT assay are shown in Figure 3. Efavirenz was shown, using the RT assay, to have an inhibitory concentration of 50% (IC50) in the HIV RT assay of approximately 1.4 μM while the IC90 was found to be approximately 11.7 μM (Figure 3).

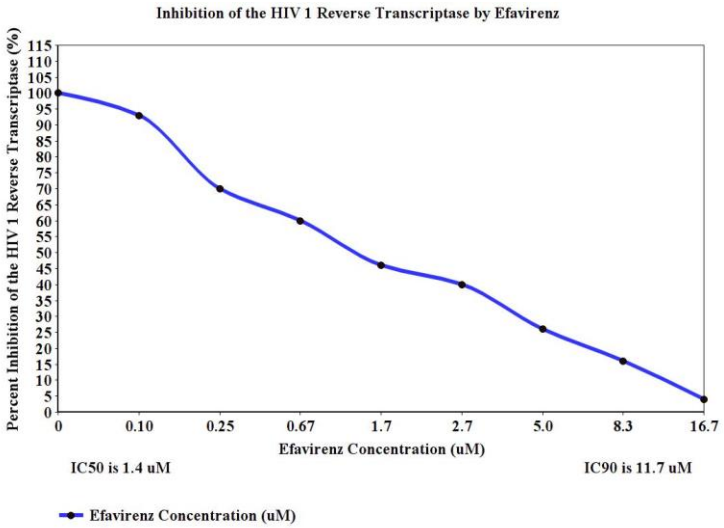

Figure 3. Dose-response Curve for the Inhibition of the HIV RT Activity by Efavirenz.

After all incubation tubes have 40 μl of RB or RB + test article, remove the HIV RT from the -20ºC freezer and check to see if the solution is frozen, if frozen place in a 37ºC water bath for 10 seconds. Add 4 μl of the HIV RT enzyme to 796 μl of lysis buffer, mix well and perform a serial dilution series of the enzyme as described in section 4 and then add 80 μl of enzyme to the appropriate labeled incubation tubes. Incubate the covered reaction tubes for 20 minutes to 24 hours depending upon the purpose of the assay.

We have determined that azide acts to inhibit the HIV RT enzyme in a dose-dependent manor, like Efavirenz. The effects of azide on the HIV RT reaction is shown in Table 1.

Table 1. Azide an Effective Inhibitor of HIV RT Activity

% Azide	20% Az (μl)	RB (μl)	Mean OD	% Inhibition
0.000	0.00	100	2.18	0
0.083	1.25	98.75	1.66	24
0.217	3.25	96.75	1.02	53
0.317	4.75	95.25	0.59	73
0.500	7.5	92.5	0.19	92

The assay was run in duplicate with HIV RT at 1 ng per reaction.

The 20% solution of sodium azide (RT1007) was used to make the 0.25%, 0.65%, 0.95%, and 1.5% dilutions (Table 1). After 40 μl of RB plus azide was added to incubation tubes, 80 μl of HIV RT in lysis buffer was added, so the concentration of azide was diluted to 1/3 of the concentration in RB or to 0.083%, 0.217%, 0.317%, and 0.50% respectively. Azide is a convenient inhibitor (most likely working by binding to heavy metal ions needed for RT catalysis) of the HIV RT reaction and can be used to rapidly test the assay system for RT inhibition.

6. The Quantitation of the HIV RT Assay Using a Standard ELISA Platform

After the HIV RT reaction has incubated at 37º C for the appropriate amount of time (20 minutes to 24 hours), remove 100 μl from each reaction incubation tube and add it to a streptavidin-coated micro well (RT1008). Incubate the ELISA plate at 37º C for 20 minutes. Remove the reaction mixture from the wells and wash each well 5 times with 1x wash buffer (see plate washing section). Add 100 μl of HRP Anti-Digoxigenin Conjugate (RT1005) and incubate at 37º C for 45 minutes. Remove the conjugate from the wells and wash each well 5 times with 1x wash buffer. Add 100 μl of ABTS substrate solution (515-419) and incubate at room temperature or at 37º C for 30 minutes. The development of color can by monitored with an ELISA plate reader set to an OD of 405 nM or the RT reaction can be terminated by adding 100 μl of Stop Solution (515-417) and reading the OD at 450 nM.

The average assay negative control OD value should be subtracted from the OD values of the HIV RT and HIV RT plus test article wells, before calculating RT activity and percent inhibition of RT activity by a test compound.

CONTRACT RESEARCH

Need a hand with your research? Would you like an independent confirmation of your results? Why not let us perform the HIV 1 reverse transcriptase assay for you? Contact us for more information.

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