HIV 1 Reverse Transcriptase Assay Kit

Catalog Number: RT-1000

For Research Use Only. Not Used for Diagnostic Testing

www.xpressbio.com

INTRODUCTION

Human Immunodeficiency 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 isolated from 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 quantitative, 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 evaluate 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
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 reaction buffer 1, reaction buffer 2, 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
- 4° ultracentrifuge **(optional for viral particle isolation)**
- A 96-well plate reader capable of reading ODs at wavelengths 405 nM

NOTES BEFORE STARTING

General Comments. Carefully review the protocol before beginning as 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.

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 a RT assay, 500 µl of reaction buffer 1 and 500 µl 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 -20°C 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 microwell 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)$_{12\text{-}18}$ 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 over time with an ELISA plate reader set at an Optical Density (OD) of 405 nM. The reaction can also be terminated by adding 25 µl of assay stop solution and reading at an OD of 405 nM. The OD values directly correlate to the level of HIV RT activity in the sample (see Figure 1.)

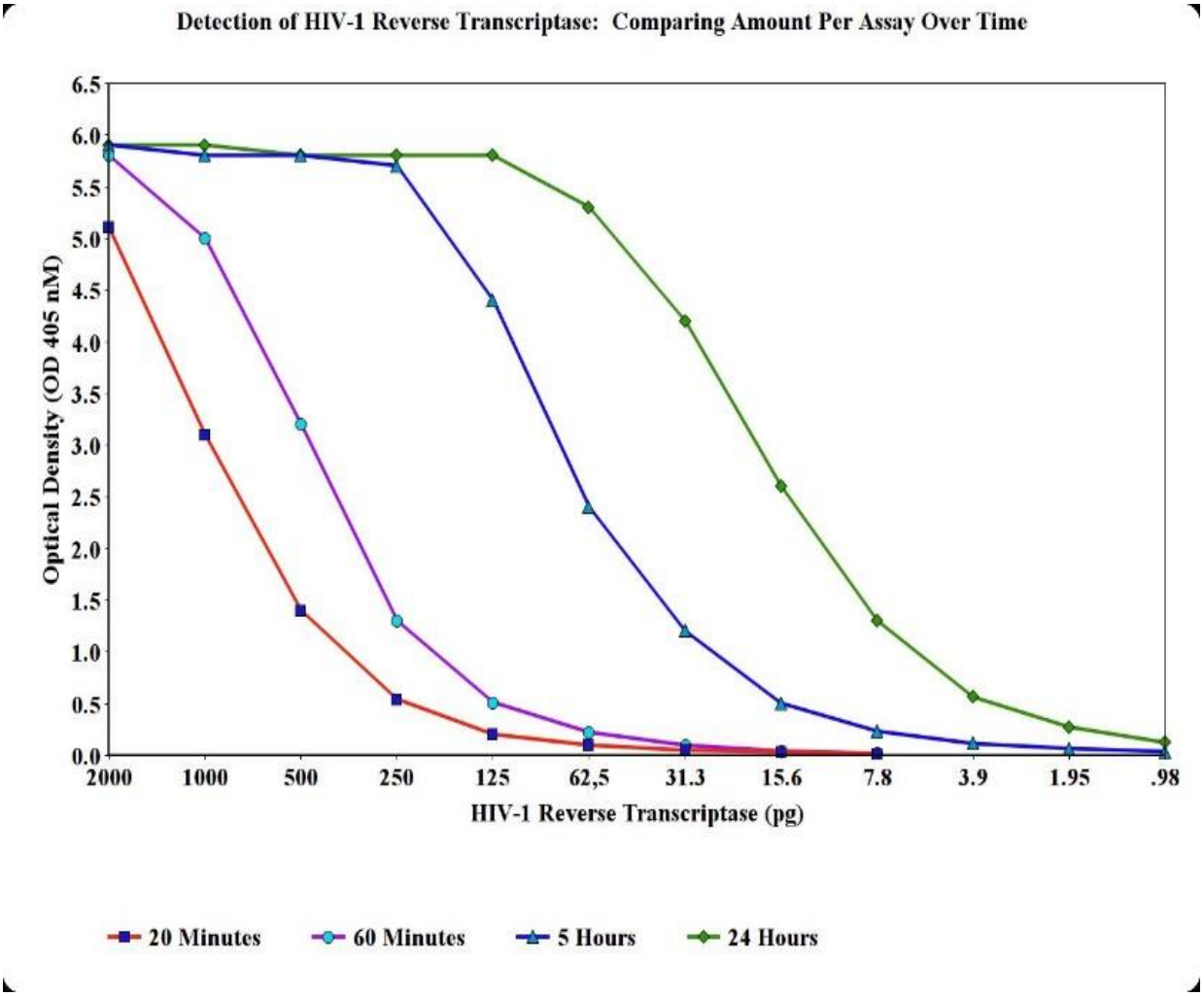

Figure 1. **Time- and Dose-Response Curves for HIV RT**: **The level of detectability of HIV RT activity reaches 1 pg when the incubation time of the assay is 24 hours.**

Test Strip to Familiarize User with Protocol (Optional)

We have included a test strip in which, upon completion, the user will be able to produce a HIV RT standard curve and mimic an inhibitory drug experiment with the addition of azide. Experienced users may skip this section and proceed directly to the experimental protocols located on page 8 for drug compound studies or page 12 for evaluation of RT in cell culture supernatants.

The RT assay requires two sets of tubes which are incubation tubes (1) and dilution tubes (2). Briefly, incubation tubes will receive 40 ul of RB or RB plus azide and dilution tubes will receive 80 ul of HIV RT in lysis buffer. The addition of RB and RB plus 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 2.

1) Prepare the 8 incubation tubes, as shown in Fig. 2. 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. 2, tube A) and RT incubation tubes (Fig. 2, tubes B-F) each receive 40 ul of RB. Fig. 2 also illustrated how to produce the inhibitory 0.21% and 0.5% azide in RB.

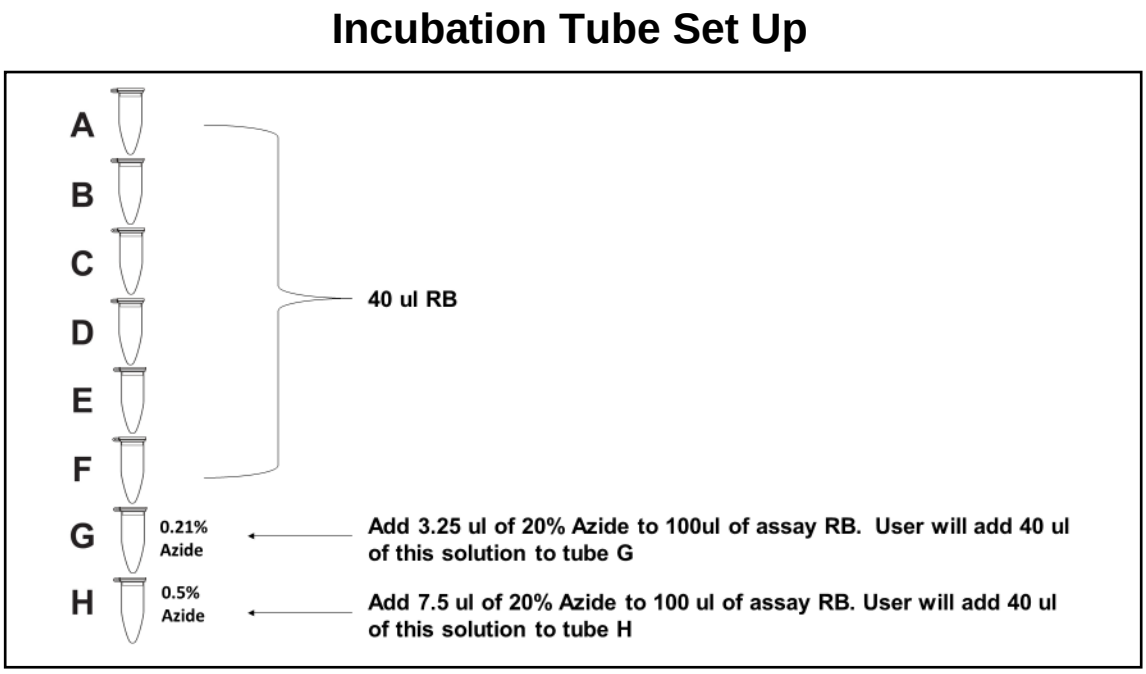

Figure 2. Set up for Incubation Tubes for Standard Curve and Azide Experiment

2) Figure 3 highlights the generation of the 8 dilution tubes. As depicted, Fig 3, tube A is the lysis blank, tube B-F is the HIV standard curve, and tubes G and H will contain 1 ng of HIV RT for use in the inhibitory azide experiment.

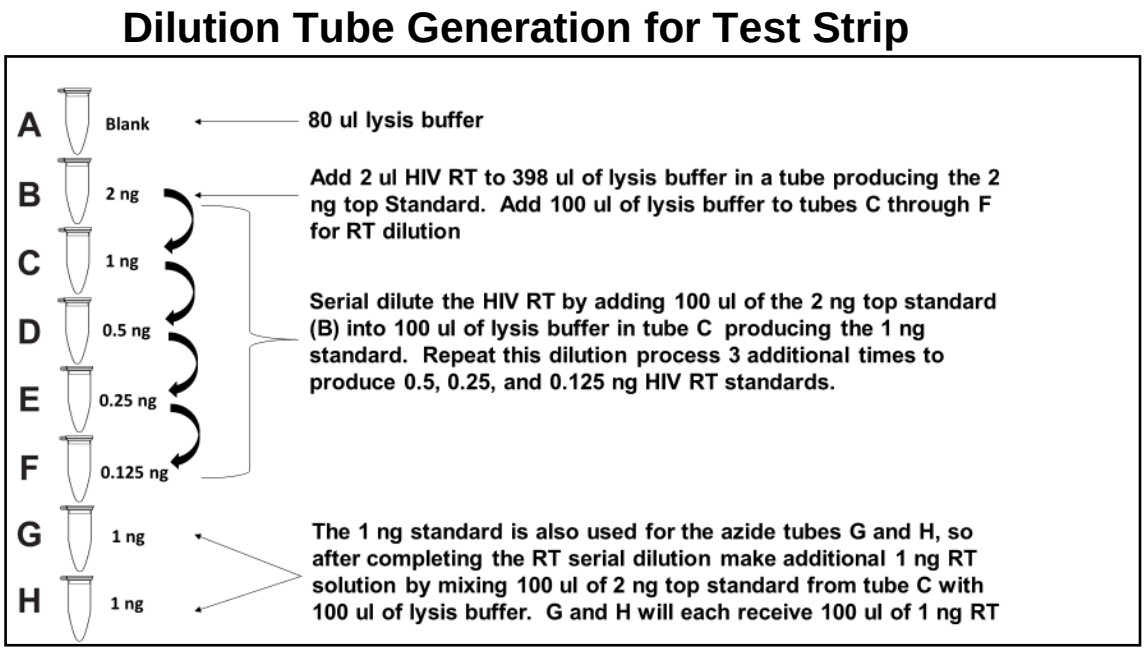

Figure 3. Set up for Dilution Tubes for Standard Curve and Azide Experiment

3) Once incubation and dilution tubes are completed, transfer 80 µl of solution from the dilution tubes into their respective incubation tubes for a total of 120 µl. All incubation tubes are placed at 37ºC for 20 minutes.

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

5) 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.

6) 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.

7) Use an ELISA plate reader to directly measure the optical density (OD) set at 405 nM. If necessary, the reaction can be halted by adding 25 µl of stop solution per well and read the OD set at 405 nM.

KIT PROTOCOL – EVALUATING REVERSE TRANSCRIPTASE INHIBITOR COMPOUNDS

General Comments. 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).

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. Generation of 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. As stated above, the user will mix their test compound in the RB up to 40 μl total for the each experimental well. 40 ul of RB or RB plus test compound will be added to their respective incubation tubes.

3. Generation of the HIV RT Standard Curve

After RB or RB plus test compound has been added to the incubation tubes for the experiment, label separate RT dilution tubes 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 HIV RT tube 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 using a multi-channel pipettor.

4. Generation of 1 ng HIV RT Stock for Inhibitor Testing

Enough HIV RT is provided to produce 100 wells worth of 1 ng standard for the testing of HIV RT inhibitory compounds. The quantity of 1 ng HIV RT to make per experiment will depend on how many samples the user has. The kit is designed to use 1 µl of stock HIV RT for every 399 µl of lysis buffer to produce a 1 ng concentration. As previously stated, each test well will require 80 µl of 1 ng HIV RT. Therefore, if the user wants to test 10 different concentrations of a compound, it will require, 800 µl worth of 1 ng HIV RT, which equates to 2 µl of HIV RT stock and 796 µl of lysis buffer.

5. Addition of HIV RT and RB

Once the HIV RT (standard and/or 1 ng HIV RT stock) has been produced, add 80 µl of this solution into their respective RB incubation tubes which should contain 40 µl of RB or RB plus test article. Incubate this mixture for 20 minutes at 37 °C.

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

1. After the HIV RT reaction has incubated at 37º C for 20 minutes, remove 100 µl from each reaction incubation tube and add it to a streptavidin-coated micro well (RT1008).

2. 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.

3. 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.

4. Add 100 µl of ABTS substrate solution (515-419) and incubate at room temperature for 30 minutes. The plate can also be incubated at 37º C, which will greatly speed up the reaction. The development of color can by monitored over time with an ELISA plate reader set to an OD of 405 nM or the RT reaction can be terminated by adding 25 µl of Stop Solution (515-417) and reading the OD at 405 nM.

5. 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.

USAGE OF TEST ARTICLES: EFAVIRENZ INHIBITS 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. Therefore, 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 4. 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 4).

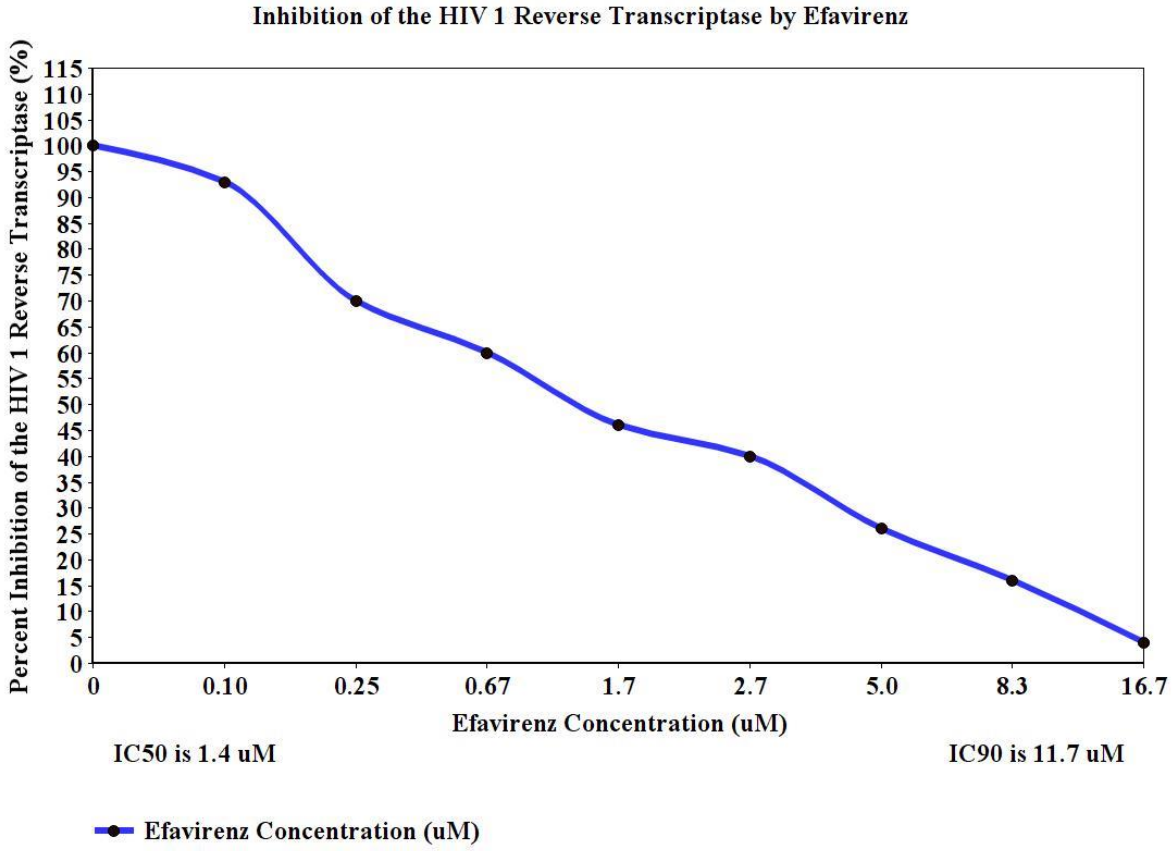

Figure 4. Dose-response Curve for the Inhibition of the HIV RT Activity by Efavirenz.

USAGE OF TEST ARTICLES: AZIDE INHIBITS THE HIV RT REACTION

Similar to Efavirenz, we have determined that azide acts to inhibit the HIV RT enzyme in a dose-dependent manor. 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.

KIT PROTOCOL – EVALUATING REVERSE TRANSCRIPTASE IN CELL CULTURE SUPERNATANT

General Comments. A qualitative analysis of HIV RT can be performed using the HIV 1 RT assay kit. However, it is necessary to isolate HIV viral particles from cell culture supernatant in order to produce a reliable signal. This is particularly true for cell cultures containing serum as it interferes with this assay.

1. Ultracentrifugation of Viral Particles from Cell Culture Supernatant

1. Centrifuge cell culture supernatant at 300 x g for 5 minutes in a 4°C centrifuge to pellet cells
2. Transfer supernatant into a fresh centrifuge tube
3. Centrifuge supernatant at 2500 x g in a 4°C centrifuge. This will eliminate cellular debris
4. Transfer supernatant into an ultracentrifuge tube and centrifuge at 4°C in a swing-out rotor at either:
    a. 100,000 x g for 10 minutes
    b. 25,000 x g for 2 hours
5. The viral pellet will likely not be visible to the eye, so mark the ultracentrifuge tube where the pellet should form
6. Decant the supernatant away from the pellet marking
7. Resuspend the pellet in 80 µl of lysis buffer

2. Generation of 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.

3. Addition of Viral Particles and RB

Once the viral particles have been isolated and resuspended in lysis buffer, add 80 µl of each solution into their respective RB incubation tubes which will also contain 40 µl of RB. Incubate this solution for 20 minutes to 24 hours at 37 °C. Exact incubation time will depend on the amount of viral particles in the sample and needs to be determined by the end user.

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

1. After the HIV RT reaction has incubated at 37º C from 20 minutes to 24 hours, remove 100 µl from each reaction incubation tube and add it to the streptavidin-coated micro well (RT1008).

2. 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.

3. 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.

4. Add 100 µl of ABTS substrate solution (515-419) and incubate at room temperature for 30 minutes. The plate can also be incubated at 37º C, which will greatly speed up the reaction. The development of color can by monitored over time with an ELISA plate reader set to an OD of 405 nM or the RT reaction can be terminated by adding 25 µl of Stop Solution (515-417) and reading the OD at 405 nM.

5. 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.

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