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Introduction

Earlier systems for RT measurement using radioactivity gave low levels of sensitivity. *RetroSys*TM is a non-radioactive method for measuring RT activity with very high sensitivity (5).

The *RetroSys*TM RT activity kit is optimised for the quantification of HIV RT activity in cell culture media. HIV cell cultures are important for routine laboratory diagnosis and therapy resistance analysis, as well as for research. Using cell culture media or pelleted particles, HIV-1, HIV-2 and SIV multiplication can be monitored by measuring the activity of their RT.

When it comes to detection of HIV-1 presence in samples, the *RetroSys*TM RT activity kit is superior to the conventional p24 assays (5). Due to its inherent sensitivity, it is also useful for quantification of RT in plasma during primary viremia in SIV infected monkeys.

The *RetroSys*TM RT activity kit has been proven valuable for determining the level of inhibition of RT activity by various substances and for studies of their mode of action.

The *RetroSys*TM RT activity kit is very useful for determination of HIV-1 RT activity blocking antibody (RTb-ab). Due to its high sensitivity, a small amount of RT can be used and thus the blocking effect of a small amount of RTb-ab present in serum can be measured.

Results obtained earlier, with a less sensitive prototype than the current *RetroSys*TM, showed that sera from HIV-1 infected individuals have high prevalence of HIV-1 RTb-ab. With the prototype test it was found that analysis for RTb-ab was equally sensitive as tests for gp41-antibody (2). Sera from 200 blood donors and 200 patients with various virus infections were all negative. 4/200 sera from patients with malignant disease and 12/200 sera from pregnant women gave borderline values (2,3).

Extended analyses show that the titres of RTb-ab in HIV-1 positive individuals may exceed 1000 000 and remain fairly constant during asymptomatic infection. The titres start to decline at the end of the asymptomatic period and reach low levels at terminal AIDS (3). Ongoing studies with the current *RetroSys*TM kit show, that it has a higher detection sensitivity for HIV-1 RTb-ab in human sera than the prototype. In addition "false positives" are fewer. These are possibly caused by cross-reacting ab induced by other retroviral RT, e.g. HIV-2. The clinical experience of the assay is still too limited to support clinical use.

*RetroSys*TM RT activity kit belongs to a series of new kits for different retroviral purposes. Soon available are the *RetroSys*TM RT antibody kit and the *RetroSys*TM RT inhibitor kit. The former is optimised for screening and quantification of different types of retroviral RTb-ab, while the latter is optimised for screening and quantification of RT inhibitory substances and for studies of their mode of action. The *RetroSys*TM RT inhibitor kit, is also useful for studies on mutated RT. For measurements of other retroviral RT, e.g. Moloney murine leukaemia virus RT, other reaction conditions for optimal quantification are required. Therefore, a separate kit, the *RetroSys*TM RT vector kit, will become available for work with this retrovirus and with vectors.

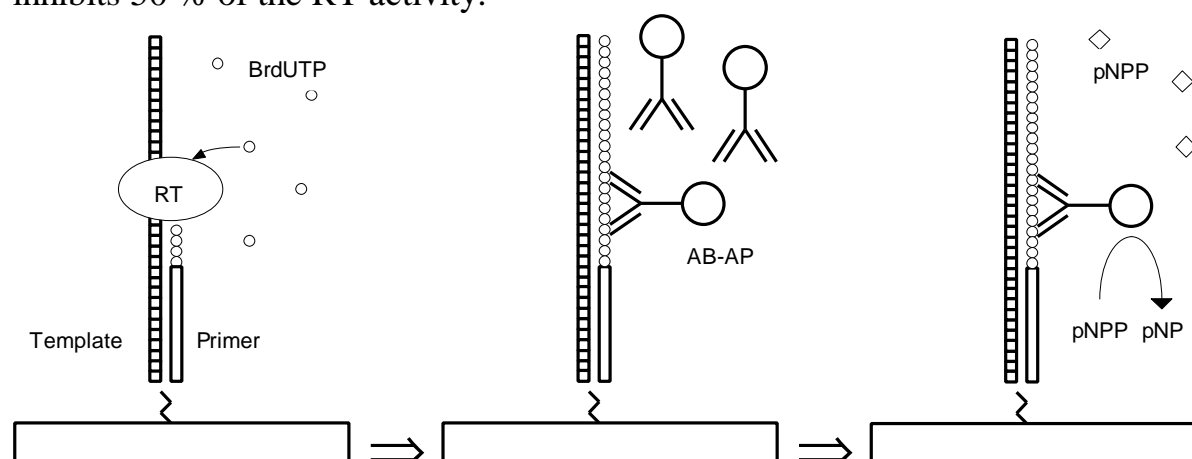
Principles of the *RetroSys*TM kit.

The *RetroSys*TM RT activity assay determines the RT activity in a sample. The procedure consists of two steps, the RT reaction and the product quantification.

The RT in the sample synthesises a DNA strand. Bromo-deoxyuridine triphosphate (BrdUTP) in the reaction mixture is incorporated into the immobilised template/primer construct.

The incorporated BrdUMP is quantified by a RT Product Tracer (BrdU binding antibody conjugated to alkaline phosphatase). The alkaline phosphatase activity of the bound tracer is colourimetrically measured and is proportional to the RT activity in the sample.

When the assay is used for determination of RT inhibitors the compounds being tested should be included in the reaction mixture. When the assay is used to quantify RT activity blocking antibody (RTb-ab) titres, a small amount of RT is first incubated with a serially diluted serum. The residual RT activity is then determined. The titre of RTb-ab is defined as the dilution at which the serum inhibits 50 % of the RT activity.



A. Quantification of RT activity.

When using crude samples RT activity inhibitory factors may be present. In order to ensure a quantitative determination of enzyme activity in such samples, it is important to establish that the enzyme reaction is linear with time. It is also important to verify that the measured RT activity is proportional to the concentration of the sample in the assay. Thus samples to be quantified should be analysed at several dilutions as well as at different RT reaction times. The measurement should be carried out on serially diluted samples set on two plates. For one of the plates the RT reaction is stopped by washing after three hours of incubation, while the other is incubated overnight.

On day two the plates are incubated with the RT Product Tracer. After removing excess tracer by washing the plates, the amount of bound tracer is determined by an alkaline phosphatase / pNPP colour reaction measured at different times. The level of RT activity in the sample is calculated from the HIV-1 rRT standard dilutions set on each plate.

The measuring range of the assay is the activity of 0.01-50 pg recombinant HIV-1 RT per well. The lower detection limit corresponds to the presence of 1 pg/ml of active RT in undiluted cell supernatant, if the analysis is performed with the cell supernatant diluted 1/5, which is the highest concentration that can be used without major disturbances (Ekstrand et al 1996).

For comparison the activity of 1 pg of the supplied HIV-1 rRT-standard is equal to the activity of 4 μ U of the rRT from Boehringer Mannheim.

B. Screening for RT activity in cell supernatants.

When processing large series of samples, of which only a few are expected to be RT positive, it may be advantageous to perform a semi quantitative screening. For instance when analysing cell supernatants from virus isolation cell cultures for presence of HIV.

Screening differs from quantification in that a small amount of crude undiluted sample is used. The small sample amount is necessary in order to avoid disturbances. As a result a larger number of samples can be analysed per kit. In addition sample handling becomes more convenient. However, it also means that the level of accuracy in measuring RT activity is reduced.

In all other relevant aspects this application follows the same procedure as for quantification of RT activity. The assay is performed by using two incubation times in the RT reaction step, three hours and overnight.

C. Determination of RT activity blocking antibody.

The sensitivity of an assay for RTb-ab depends on the amount of RT used. Since the *RetroSys*TM assay uses a small amount of RT and overnight polymerisation reaction, it gives a very high sensitivity for RTb-ab detection and measures a broad range of titres. When monitoring titre changes it is advised to include the previous sample from the individual together with the new one that is to be analysed. This is to authenticate the significance of any changes in titre.

Each serum is analysed using seven tenfold serial dilutions. A standardised small amount of recombinant HIV-1 RT is mixed with each serum dilution. RTb-ab negative reference and background controls are included. The samples are incubated for 90 minutes in order to let the antibodies bind the enzyme. After the incubation the remaining RT activity in each sample is determined by starting the RT reaction. For both plates the RT reaction is stopped by washing after overnight incubation.

Only the samples containing unblocked RT have now synthesised a DNA strand containing BrdUMP. The product is quantified by the addition of the RT Product Tracer to all wells. After 90 minutes of incubation, excess tracer is removed and the amount of bound tracer is determined by an alkaline phosphatase / pNPP colour reaction measured at different times. The measured residual RT activity for each serum dilution is corrected for background signal and then calculated as a percentage of the RT activity in the RTb-ab negative reference. The titre of RTb-ab in the serum is expressed as the dilution at which the serum inhibits 50% of the RT activity.

The kit can be used to determine RTb-ab in 23 sera and one sample with known titre. Titres in the range of 1:1 to 1:1 000 000 or greater, are detected using the protocol given. The lowest amount of RTb-ab detected, i.e. the titre 1:1, corresponds to the blocking of 50% of the activity of 2.4 pg of HIV-1 rRT by incubation with 8 µl of undiluted serum.

The kit can also be used to screen sera for HIV-1 RTb-ab presence. In this case 84 sera can be analysed in duplicate using an amount corresponding to 8 µl of undiluted serum.

D. Determination of IC₅₀ value of RT activity inhibiting substances.

The *RetroSys*TM RT activity kit can be used to study RT inhibiting substances. In order to obtain optimal sensitivity the analysis should be done in a system where the concentrations of substrate and primer are close to the K_m value.

When determining IC₅₀ values the substances that are to be analysed are serially diluted. The diluted substances are then added to a plate with reaction mixture. After 30 minutes of preincubation at 33°C, the reaction is started by the addition of a standardised amount of RT. The RT will now incorporate BrdUMP depending on the level of inhibition.

The reaction is stopped by washing the plate. The product is quantified by the addition of the RT Product Tracer which binds to the incorporated BrdUMP. After removing excess tracer the amount of bound tracer is determined by an alkaline phosphatase / pNPP colour reaction.

After correction for background signal, the measured residual RT activity for each substance dilution is calculated as a percentage of the measured RT activity in absence of inhibiting substances. The inhibitory effect of each substance is expressed as an IC₅₀ value i.e. the concentration at which 50 % of the RT activity is inhibited.

The kit can also be used for screening a larger number of substances for inhibitory effects. In this case the substance is not serially diluted but used at one concentration. A protocol for this is not provided.

It should be noted that this test is not sensitive to all kinds of inhibitors. For instance, inhibition by substances competing with dNTPs other than dTTP are not detected with this assay as it is based on the use of prA/odT as template/primer.

Components of the kit

Each kit contains two 96-well PolyA Plates microtitre RT assay plates and all reagents necessary to support any of the applications described in this booklet. *The kit does not contain any material of human origin.*

Materials provided

- PolyA Plate, two pieces in foil pouches labelled **A**
- Adhesive tape, six pieces
- Plastic lid for microtitre plates, two pieces
- Sample Dilution Buffer, 30 ml in bottle labelled **B**
- RT Reaction Components, lyophilised in two vials labelled **C1**
- Reconstitution Buffer, 25 ml in one bottle labelled **C2**
- HIV-1 rRT-standard, lyophilised in one vial labelled **D**
- Concentrated Washing Buffer, 20 ml in one bottle labelled **E**
- Incubation Buffer, 50 ml in one bottle labelled **F**
- RTb-ab Negative Reference, 0.6 ml in one tube labelled **G**
- RT Product Tracer, lyophilised in two vials labelled **O**
- AP Substrate Tablets, two tablets in one tube labelled **P1**
- AP Substrate Buffer, 60 ml in one bottle labelled **P2**

Materials required but not provided

- Distilled H₂O of high purity e.g. MilliQ water.
- Glass beakers 50 ml and 100 ml.
- Incubator cabinet for 33°C.
- Micropipettes, 10-1000 µl.
- 8 and 12 multichannel micropipettes, 10-200 µl.
- Microtitre plate reader.
- Microtitre plate washing system.
- 2 orbital shakers suitable for microtitre plates.
- 2 standard 96-well microtitre plates for titrations.
- Reservoirs for multichannel pipetting.
- Test tubes.
- Triton X-100.

Storage

The kit should be stored at +4 - +8°C, and will be stable until the expiry date.

The RT Product tracer can be kept in refrigerator for up to a week after it has been reconstituted. After reconstitution it should not be frozen.

Precautions

- The kit is intended for *in vitro* use only.
- The kit is intended for research purposes only.
- The components are not to be used after their expiry date.
- Components from kits with different lot numbers should not be interchanged.
- Some components contain hazardous and toxic substances.
- The components contain sodium azide used as a preservative and are hazardous to ingest. Contact with skin and mucosa should be avoided.
- Do not pipette by mouth.
- Do not eat, drink or smoke in areas in which specimens or kit reagents are handled.
- The kit itself contains no material of human origin. However, when using it with possibly infectious samples, especially those of human origin, handling must be in accordance with safety regulations of the laboratory.

Sample collection and preparation

Cell supernatants

Cell supernatants for RT analysis should be sampled carefully to avoid transferring of cells. For repeated analysis or later typing experiments, duplicate samples may be extracted, aliquoted and frozen at -20 °C. This facilitates the later experiments as the RT activity of the frozen aliquots will be known. It is recommended that the frozen samples are used within two weeks. For longer storage, keep the RT samples at -70°C.

Serum samples

Serum samples for RTb-ab analysis should be prediluted by using 5 % v/v Triton X-100 in distilled water, and adding a volume corresponding to 25 % of the serum volume. Thereafter a heat inactivation of the diluted serum is performed at 56 °C for 30 minutes.

The diluted serum may be used directly in the assay or it can be frozen at -20°C for later use.

A. Protocol for quantification of RT activity.

20 cell supernatant samples in four dilutions may be analysed with one kit.

1 Collect kit components.

- Take out the following components from the refrigerator:

- 2 PolyA Plates
- 1 Sample Dilution Buffer (bottle B)
- 2 RT Reaction Components (vial C1)
- 1 Reconstitution Buffer (bottle C2)
- 1 HIV-1 rRT-standard (vial D)
- 1 Concentrated Washing Buffer (bottle E)
- 4 pieces of adhesive tape

2 Prepare the reaction mixture.

- Add 12 ml of Reconstitution Buffer (C2) to each of the two vials containing RT Reaction Components (C1). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Transfer the contents of both C1 vials to a 50 ml glass beaker.
- Add 12 ml of distilled water and mix thoroughly.

3 Prepare the PolyA Plates.

- Take out both PolyA Plates from their foil pouches. Save one foil pouch for later use.
- Add 150 μ l of reaction mixture (beaker) to each well of both plates.
- Seal the plates with adhesive tape.
- Incubate the plates at 33°C for 20-60 minutes.

4 Dilute the HIV-1 rRT standard.

When preparing the standard dilutions, mix thoroughly and change pipette tips between each transfer.

- Add 6 ml of Sample Dilution Buffer (B) to the vial with HIV-1 rRT-standard (D). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Add 250 μ l of Sample Dilution Buffer (B) to twelve test tubes.
- Add 100 μ l of HIV-1 rRT-standard (D) to the first of the twelve test tubes.

- Transfer 200 µl from the first test tube to the second.
- Transfer 200 µl from the second test tube to the third and so on until a series of twelve dilutions has been obtained.

5 Dilute the samples.

Avoid aspirating cells when sampling cell culture samples.

- Add 160 µl of Sample Dilution Buffer (B) to all wells in columns 1-10 of a sample preparation plate (96-well microtiter plate, not supplied).
- Add 40 µl of the first sample to be analysed to well A1.
- Add 40 µl of the second sample to be analysed to well A2 and so on until all 20 samples have been added to wells A1-10 and E1-10.

Dilution of samples in well A1-10.

Mix thoroughly and change pipette tips between each transfer.

- Transfer 40 µl from wells A1-10 down to their corresponding B-wells.
- Transfer 40 µl from wells B1-10 down to their corresponding C-wells.
- Transfer 40 µl from wells C1-10 down to their corresponding D-wells.

Dilution of samples in well E1-10.

Mix thoroughly and change pipette tips between each transfer.

- Transfer 40 µl from wells E1-10 down to their corresponding F-wells.
- Transfer 40 µl from wells F1-10 down to their corresponding G-wells.
- Transfer 40 µl from wells G1-10 down to their corresponding H-wells.

6 Start the RT reaction.

- Take out the PolyA Plates from the incubator.
- Transfer 50 µl of each sample dilution from the sample preparation plate to each corresponding well in columns 1-10 of both PolyA Plates.
- Add 50 µl of Sample Dilution Buffer (B) to wells E12-H12 of both plates.
- Add 50 µl of the first standard dilution to well A11 of both plates.
- Add 50 µl of the second standard dilution to well B11 and so on until all twelve standard dilutions are set on both plates.
- Seal the PolyA Plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.

- Incubate the plates at 33°C on an orbital shaker set at gentle agitation.
The first plate is incubated for 3 hours and the second overnight.

7 Prepare the wash fluid.

- Add 75 ml of Triton X-100 to 1 litre of distilled water and mix thoroughly with a magnetic spin bar.
Make sure that the Triton X-100 dissolves completely which takes approximately 10 minutes.
- Add 10 ml of Concentrated Washing Buffer (E) to a 10 litre container.
- Add the dissolved Triton X-100 to the 10 litre container.
- Adjust the volume to 10 litres with distilled water and mix thoroughly.
The residual Concentrated Washing Buffer (E) is used on day two and should be stored in the refrigerator until then.

8 Stop the RT reaction of the first plate.

After 3 hours of incubation the RT reaction of the first plate is stopped by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash the PolyA Plate twice. Turn the plate 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.
- Return the plate to its foil pouch and store it at -20°C.

Day two

9 Collect kit components.

- Take out the kit box from the refrigerator and take out the following components:
 - 1 Concentrated Washing Buffer (bottle E)
 - 2 RT Product Tracer (vials O)
 - 1 AP Substrate Tablets (tube P1)
 - 1 AP Substrate Buffer (bottle P2)
 - 2 pieces of adhesive tape
 - 2 plastic lids

10 Prepare the wash fluid.

Follow the procedure for this as described in step 7.

11 Prepare the RT Product Tracer.

- Add 12 ml of distilled water to each of the two vials containing RT Product Tracer (O). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Transfer the contents of both O vials to a 50 ml glass beaker.

12 Stop the RT reaction of the second plate.

After overnight incubation the RT reaction of the second plate is stopped by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash the PolyA Plate twice. Turn the plate 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.
- Collect the first PolyA Plate from the freezer.

13 Start the binding of RT Product Tracer.

When dispensing the tracer, pipette tips should not touch the bottom of the wells.

- Add 100 µl to each well of both PolyA Plates.
- Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plates for 90 minutes at 33°C on an orbital shaker set at gentle agitation.

14 Prepare the AP substrate solution.

- Add the AP Substrate Tablets (P1) to the AP Substrate Buffer (P2).
- Shake the bottle occasionally and the tablets will dissolve completely in approximately twenty minutes.

15 Remove excess tracer.

After 90 minutes of incubation, excess tracer is removed by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash both PolyA Plates four times. Turn the plates 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.

16 Start alkaline phosphatase reaction.

The AP substrate solution must be mixed thoroughly and equilibrated to room temperature before it is added to the PolyA Plates. When dispensing the substrate solution, pipette tips should not touch the bottom of the wells.

- Add 200 µl of AP substrate (P2) solution to each well of both PolyA Plates.
- Cover the plates with plastic lids and incubate them at room temperature under dark cover on an orbital shaker set at gentle agitation.

17 Read the plates.

Set the filter of the plate reader to 405 nm.

- Read the absorbance of both plates after 30 minutes of incubation.
- Read the absorbance of both plates a second time after 2 hours of incubation.
- Read the absorbance of both plates a last time after overnight incubation.

18 Process the data.

The RetroSys™ RT assay gives a linear relationship between A_{405} and RT activity if the readings are within the linear measuring range of the plate reader used

When calculating the results the data from each reading time should be processed separately, i.e. values for standard curve and samples should be from the same reading time.

- Plot the A_{405} of each standard dilution (wells A11-D12) against its concentration of HIV-1 rRT (see table below). Preferentially use a computer program to calculate the best regression line utilising the mean A_{405} of the background controls (wells E12-H12) as y-axis intercept. Only use readings within the linear measuring range of the microtiter plate reader.
- Determine the RT activity of each well with the aid of the regression line. Only determine RT activity for wells giving an A_{405} within the linear measuring range of the reader and greater than two times the mean of the background controls.
- Calculate the RT activity for undiluted sample by compensating for the dilution used in the assay.
- Calculate the mean RT activity of the sample. Should significantly lower values be obtained for higher sample concentrations, this is due to disturbing factors in the sample. Values obtained from these dilutions should be excluded from the calculation.

Standard dilution	1	2	3	4	5	6	7	8	9	10	11	12
pg/well	61.9	27.5	12.2	5.34	2.42	1.07	0.477	0.212	0.094	0.042	0.019	0.008
pg/ml	1240	550	245	109	48.3	21.5	9.54	4.24	1.88	0.838	0.372	0.165

B. Protocol for screening for RT activity in cell supernatants.

80 samples set at two assay durations may be analysed with one kit.

1 Collect kit components.

- Take out the following components from the refrigerator:

- 2 PolyA Plates
- 1 Sample Dilution Buffer (bottle B)
- 2 RT Reaction Components (vial C1)
- 1 Reconstitution Buffer (bottle C2)
- 1 HIV-1 rRT-standard (vial D)
- 1 Concentrated Washing Buffer (bottle E)
- 4 pieces of adhesive tape

2 Prepare the reaction mixture.

- Add 12 ml of Reconstitution Buffer (C2) to each of the two vials containing RT Reaction Components (C1). Make sure that the lyophilised material dissolves properly.
- Transfer the contents of both C1 vials to a 50 ml glass beaker.
- Add 12 ml of distilled water.
- Add 12 ml of Sample Dilution Buffer and mix thoroughly.

3 Prepare the PolyA Plates.

- Take out both PolyA Plates from their foil pouches. Save one foil pouch for later use.
- Add 200 µl of reaction mixture (beaker) to each well of the plates.
- Seal the plates with adhesive tape.
- Incubate the plates at 33°C for 20-60 minutes.

4 Dilute the HIV-1 rRT-standard.

When preparing the standard dilutions, mix thoroughly and change pipette tips between each transfer.

- Add 1.2 ml of Sample Dilution Buffer (B) to the vial containing lyophilised HIV-1 rRT-standard (D). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Add 250 µl of Sample Dilution Buffer (B) to twelve test tubes.
- Add 100 µl of HIV-1 rRT standard (D) to the first of the twelve test tubes.
- Transfer 200 µl from the first test tube to the second.
- Transfer 200 µl from the second test tube to the third and so on until a series of twelve dilutions has been obtained.

5 Prepare the samples.

Avoid aspirating cells when sampling cell culture supernatants.

- Collect the samples to be analysed. 2 x 10 µl of each sample is required.

6 Start the RT reaction.

When adding samples to the plates, change pipette tips between each sample.

- Take out the PolyA Plates from the incubator.
- Add 10 µl of the first sample to well A1 of both PolyA Plates.
- Add 10 µl of the second sample to well B1 and so on until all 80 samples are set on both plates.
- Add 10 µl of Sample Dilution Buffer (B) to wells E12-H12 of both plates.
- Add 10 µl of the first standard dilution to well A11 of both plates.
- Add 10 µl of the second standard dilution to well B11 and so on until all twelve standard dilutions are set on both plates.
- Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plates at 33°C on an orbital shaker set at gentle agitation.
The first plate is incubated for 3 hours and the second overnight.

7 Prepare the wash fluid.

- Add 75 ml of Triton X-100 to 1 litre of distilled water and mix thoroughly with a magnetic spin bar.
Make sure that the Triton X-100 dissolves completely which takes approximately 10 minutes.
- Add 10 ml of Concentrated Washing Buffer (E) to a 10 litre container.
- Add the dissolved Triton X-100 to the 10 litre container.
- Adjust the volume to 10 litres with distilled water and mix thoroughly.
The residual Concentrated Washing Buffer (E) is used on day two and should be stored in the refrigerator until then.

8 Stop the RT reaction of the first plate.

After 3 hours of incubation the RT reaction of the first plate is stopped by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash the PolyA Plate twice. Turn the plate between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.
- Return the plate to its foil pouch and store it at -20°C.

Day two

9 Collect kit components.

- Take out the kit box from the refrigerator and take out the following components:
 - 1 Concentrated Washing Buffer (bottle E)
 - 2 RT Product Tracer (vials O)
 - 1 AP Substrate Tablets (tube P1)
 - 1 AP Substrate Buffer (bottle P2)
 - 2 pieces of adhesive tape
 - 2 pieces of plastic lids

10 Prepare the wash fluid.

Follow the procedure for this as described in step 7.

11 Prepare the RT Product Tracer.

- Add 12 ml of distilled water to each of the two vials containing RT Product Tracer (O). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Transfer the contents of both O vials to a 50 ml glass beaker.

12 Stop the RT reaction of the second plate.

After overnight incubation the RT reaction of the second plate is stopped by washing. Use approximately 6 ml per well and wash.

- Wash the PolyA Plate twice. Turn the plate 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.
- Collect the first PolyA Plate from the freezer.

13 Start the binding of RT Product Tracer.

When dispensing the tracer, pipette tips should not touch the bottom of the wells.

- Add 100 µl to each well of both PolyA Plates.
- Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plates for 90 minutes at 33°C on an orbital shaker set at gentle agitation.

14 Prepare the AP substrate solution.

- Add the AP Substrate Tablets (P1) to the AP Substrate Buffer (P2).
- Shake the bottle occasionally and the tablets will dissolve completely in approximately twenty minutes.

15 Remove excess tracer.

After 90 minutes of incubation, excess tracer is removed by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash both PolyA Plates four times. Turn the plates 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.

16 Start the alkaline phosphatase reaction.

The AP substrate solution must be mixed thoroughly and equilibrated to room temperature before it is added to the PolyA Plates. When dispensing the substrate solution, pipette tips should not touch the bottom of the wells.

- Add 200 µl of AP substrate solution to each well of both PolyA Plates.
- Cover the plates with plastic lids and incubate them at room temperature under dark cover on an orbital shaker set at gentle agitation.

17 Read the plates.

Set the filter of the plate reader to 405 nm.

- Read the absorbance of both plates after 30 minutes of incubation.
- Read the absorbance of both plates after 2 hours of incubation.
- Read the absorbance of both plates after overnight incubation.

18 Process the data.

The RetroSys™ RT assay gives a linear relationship between A_{405} and RT activity if the readings are within the linear measuring range of the plate reader used

When calculating the results the data from each reading time should be processed separately, i.e. values for standard curve and samples should be from the same reading time.

- Plot the A_{405} of each standard dilution (wells A11-D12) against its concentration of HIV-1 rRT (see table below). Preferentially use a computer program to calculate the best regression line utilising the mean A_{405} of the background controls (wells E12-H12) as y-axis intercept. Only use readings within the linear measuring range of the microtiter plate reader.

- Determine the RT activity of each well with the aid of the regression line. Only determine RT activity for wells giving an A_{405} within the linear measuring range of the reader and greater than two times the mean of the background controls.
- Compare RT activity values for long and short RT assay incubations for each sample.
 - a) If the RT values are similar then calculate the mean value for the sample.
 - b) If a higher value is obtained for the short RT reaction time then this is the value of the sample. The lower value obtained with the long RT reaction time is normally due to RT product degradation.
 - c) If a significant value only is obtained for the long RT reaction time then this is the value of the sample.

For samples where the first colour reading gives an A_{405} above the linear measuring range of the plate reader, the RT activity is higher than the highest HIV-1 rRT activity included in the standard curve. If an accurate value for such a sample is desired, the sample should be re-analysed using several dilutions of the sample (see Protocol for quantification of RT activity).

Standard dilution	1	2	3	4	5	6	7	8	9	10	11	12
pg/well	61.9	27.5	12.2	5.34	2.42	1.07	0.477	0.212	0.094	0.042	0.019	0.008
pg/ml	6190	2750	1220	534	242	107	47.7	21.2	9.42	4.19	1.86	0.83

C₁. Protocol for determination of RT activity blocking antibody titres.

23 serum samples and one sample with known titre may be analysed with one kit.

1 Collect kit components.

- Take out the following components from the refrigerator:
 - 1 HIV-1 rRT-standard (vial D)
 - 1 Incubation Buffer (bottle F)
 - 2 pieces of plastic lids

2 Dilute the serum samples.

*The serum samples should be pre-treated according to **Sample collection and preparation**, page 8. When diluting the serum samples, change pipette tips and mix thoroughly between transfers.*

- Add 89 µl of Incubation Buffer (F) to wells A1-12 of two sample preparation plates (96-well microtiter plates, not supplied).
- Add 90 µl of Incubation Buffer (F) to each of the remaining wells of both plates.
- Add 11 µl of the first serum sample to be analysed to well A1.
- Add 11 µl of the second serum sample to be analysed to well A2 and so on until the serum samples have been added to wells A1-12 of both plates.
- Transfer 10 µl from wells A1-12 down to their corresponding B-wells.
- Transfer 10 µl from wells B1-12 down to their corresponding C-wells and so on until the G-rows of both plates are reached.
- Discard 10 µl from each well in the G-rows of both plates.
The H-rows are used for controls.

3 Dilute the HIV-1 rRT-standard.

- Add 7.5 ml of Incubation Buffer (F) to the vial containing lyophilised HIV-1 rRT-standard (D). Make sure that the lyophilised material dissolves properly.
- Add 20 ml of Incubation Buffer (F) to a 50 ml glass beaker.
- Transfer 200 µl of HIV-1 rRT-standard (D) to the beaker and mix thoroughly.

4 Start the sample incubation.

- Transfer 90 µl of standard enzyme (beaker) to all wells except H8-12 of both sample preparation plates.
Remove the beaker from the working area. This beaker should not be used any further in this assay.
- Add 90 µl of Incubation Buffer (F) to wells H8-12 of both plates.
- Cover the plates with plastic lids and incubate them at 33°C for 90 minutes on an orbital shaker set at gentle agitation.

5 Collect kit components.

- Take out the following components from the refrigerator:
 - 2 PolyA Plates
 - 2 RT Reaction Components (vial C1)
 - 1 Reconstitution Buffer (bottle C2)
 - 2 pieces of adhesive tape

6 Prepare the reaction mixture.

- Add 12 ml of Reconstitution Buffer (C2) to each of the two vials containing RT Reaction Components (C1). Make sure that the lyophilised material dissolves properly.
- Transfer the contents of both C1 vials to a 50 ml glass beaker.

7 Prepare the PolyA Plates.

- Take out both PolyA Plates from their foil pouches.
- Add 100 µl of reaction mixture (beaker) to each well of both plates.
- Seal the plates with adhesive tape.
- Incubate the plates at 33°C for 20-60 minutes.

8 Start the RT reaction.

When transferring start with the H-row and work upwards through the plate as this prevents the need to change pipette tips. The pipette tips should however be changed between plates.

- Take out the PolyA Plates from the incubator.
- Transfer 100 µl from each well of the first sample preparation plate to its corresponding well of the first PolyA Plate.
- Transfer 100 µl from each well of the second sample preparation plate to its corresponding well of the second PolyA Plate.
- Seal the PolyA plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plates overnight at 33°C on an orbital shaker set at gentle agitation.

Day two

9 Collect kit components.

- Take out the kit box from the refrigerator and take out the following components:
 - 1 Concentrated Washing Buffer (bottle E)
 - 1 RT Product Tracer (vials O)
 - 1 AP Substrate Tablets (tube P1)
 - 1 AP Substrate Buffer (bottle P2)
 - 2 pieces of adhesive tape

10 Prepare the wash fluid.

- Add 150 ml of Triton X-100 to 1 litre of distilled water and mix thoroughly with a magnetic spin bar.
Make sure that the Triton X-100 dissolves completely which takes approximately 10 minutes.
- Add 20 ml of Concentrated Washing Buffer (E) to a 20 litre container.
- Add the dissolved Triton X-100 to the 20 litre container.
- Adjust the volume to 20 litres with distilled water and mix thoroughly.

11 Prepare the RT Product Tracer.

- Add 12 ml of distilled water to each of the two vials containing RT Product Tracer (O). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Transfer the contents of both O vials to a 50 ml glass beaker.

12 Stop the RT reaction of the plates.

After overnight incubation the RT reaction of both plates is stopped by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash both PolyA Plates twice. Turn the plates 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.

13 Start the binding of RT Product Tracer.

When dispensing the tracer, pipette tips should not touch the bottom of the wells.

- Add 100 µl to each well of both PolyA Plates.
- Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plates for 90 minutes at 33°C on an orbital shaker set at gentle agitation.

14 Prepare the AP substrate solution.

- Add the AP Substrate Tablets (P1) to the AP Substrate Buffer (P2).
- Shake the bottle occasionally and the tablets will dissolve completely in approximately twenty minutes.

15 Remove excess tracer.

After 90 minutes of incubation, excess tracer is removed by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash both PolyA Plates four times. Turn the plates 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.

16 Start the alkaline phosphatase reaction.

The AP substrate solution must be mixed thoroughly and equilibrated to room temperature before it is added to the PolyA Plates. When dispensing the substrate solution, pipette tips should not touch the bottom of the wells.

- Add 200 µl of AP substrate solution to each well of both PolyA Plates.
- Cover the plates with plastic lids and incubate them at room temperature under dark cover on an orbital shaker set at gentle agitation.

17 Read the plates.

Set the filter of the plate reader to 405 nm.

- Read the absorbance of both plates after 30 minutes of incubation.
- Repeat the reading of the plates at intervals until wells H1-7 give values of approximately 75% of the maximum value within the linear reading range of the plate reader.

18 Process the data.

Use an alkaline phosphatase reading time at which wells H1-7 give approximately 75% of the maximum value within the linear measuring range of the plate reader. Follow the instructions for each plate separately.

- Calculate the mean A_{405} of the background controls (wells H8-12).
- Subtract the mean A_{405} of the background controls from the obtained A_{405} of the other wells.
- Calculate the mean A_{405} of the HIV-1 rRT standard (wells H1-7).
- Express the RT activity for wells containing serum dilutions as a percentage of the mean A_{405} HIV-1 rRT standard.
- Plot the percentage of residual RT activity against the serum concentrations for each sample.

The titre of the sample is the serum concentration giving 50% inhibition of the RT activity and is determined with the aid of the obtained graph.

19 Interpret the data.

Sera taken from HIV infected individuals three months after primary infection normally give titres of 1:1000 - 1:1 000 000. Lower titres, i.e. 1:10-1:1000, are related to sampling shortly after primary infection or possibly due to cross reacting RTb-ab induced by other retroviruses, e.g. HIV-2. Such low titres can also be found in sera taken from individuals in the late stages of AIDS.

C₂. Protocol for screening for RT activity blocking antibody.

84 serum samples set in duplicate may be analysed with one kit.

1 Collect kit components.

- Take out the following components from the refrigerator:
 - 1 HIV-1 rRT-standard (vial D)
 - 1 Incubation Buffer (bottle F)
 - 1 RTb-ab Negative Reference (tube G)
 - 2 pieces of adhesive tape
 - 2 pieces of plastic lids

2 Dilute the samples.

*The samples should be pre-treated according to **Sample collection and preparation**, page 8.*

- Add 10 µl of the first serum sample to be analysed to well A1 of two sample preparation plates (96-well microtiter plate, not supplied).
- Add 10 µl of the second serum sample to be analysed to well A2 and so on until the serum samples have been added to wells A1-G12 of both plates.
- Add 10 µl of RTb-ab Negative Reference (G) to wells H1-12 of both plates.

3 Dilute the HIV-1 rRT-standard.

- Add 7.5 ml of Incubation Buffer (F) to the vial containing lyophilised HIV-1 rRT- standard (D). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Add 38 ml of Incubation Buffer (F) to a glass beaker.
- Transfer 200 µl of HIV-1 rRT-standard (D) to the beaker and mix thoroughly.

4 Start the sample incubation.

- Add 170 µl of standard enzyme (beaker) to all wells except H8-12 of both sample preparation plates.
Remove the beaker from the working area. This beaker should not be used any further in this assay.
- Add 170 µl of Incubation Buffer (F) to wells H8-12 of both plates.
- Cover the plates with plastic lids and incubate them at 33°C for 90 minutes on an orbital shaker set at gentle agitation.

5 Collect kit components.

- Take out the following components from the refrigerator:
 - PolyA Plates
 - RT Reaction Components (vial C1)
 - Reconstitution Buffer (bottle C2)

6 Prepare the reaction mixture.

- Add 12 ml of Reconstitution Buffer (C2) to each of the two vials containing RT Reaction Components (C1). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Transfer the contents of both C1 vials to the beaker.

7 Prepare the PolyA Plates.

- Take out both PolyA Plates from their foil pouches.
- Add 100 µl of reaction mixture (beaker) to each well of the plates.
- Seal the plates with adhesive tape.
- Incubate the plates at 33°C for 20-60 minutes.

8 Start the RT reaction.

When transferring samples, change pipette tips between each transfer.

- Take out the PolyA Plates from the incubator.
- Transfer 100 μ l from each well of the first sample preparation plate to its corresponding well in the first PolyA Plate.
- Transfer 100 μ l from each well of the second sample preparation plate to its corresponding well in the second PolyA Plate.
- Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plates overnight at 33°C on an orbital shaker set at gentle agitation.

Day two

Follow the procedures as described in **C₁. Protocol for determination of RT activity blocking antibody titres**, steps 9-17, pages 23 through 25.

18. Process the data.

Use an alkaline phosphatase reading time at which wells H1-7 give approximately 75% of the maximum value within the linear measuring range of the plate reader. Follow the instructions for each plate separately.

- Calculate the mean A_{405} of the background controls (wells H8-12).
- Subtract the mean A_{405} of the background controls from the obtained A_{405} of the other wells.
- Calculate the mean A_{405} of the HIV-1 rRT standard incubated with the RTb-ab negative reference (wells H1-7).
- Express the RT activity for wells containing serum samples as a percentage of the mean A_{405} HIV-1 rRT standard incubated with the RTb-ab negative reference.
- Compare the percentage of residual RT activity obtained from the two plates for each serum sample. If they are similar then calculate a mean value. If they are significantly different then re-analyse the serum sample.

19. Interpret the data.

Strong RTb-ab positive serum samples leave less than 25% residual RT activity compared to the negative controls. Serum samples that leave 50% residual RT activity or less are considered RT-blocking. However, as for all HIV-1 ab assays, sera from persons not known to be HIV-1 infected, should be verified by an alternative HIV-1 assay. In addition it is recommended for serum samples leaving 30-50% residual RT activity to perform an analysis for HIV-2, as weak cross-reactivity between HIV-1 and HIV-2 occurs.

D. Protocol for determination of IC₅₀ value of RT activity inhibiting substances.

23 substances and one reference sample may be analysed with one kit.

1 Collect kit components.

- Take out the following components from the refrigerator:
 - 2 PolyA Plates
 - 1 Sample Dilution Buffer (bottle B)
 - 1 RT Reaction Components (vial C1)
 - 1 Reconstitution Buffer (bottle C2)
 - 1 HIV-1 rRT-standard (vial D)
 - 1 Concentrated Washing Buffer (bottle E)
 - 4 pieces of adhesive tape

2 Prepare the diluted sample dilution buffer.

- Label a 100 ml glass beaker "1".
- Add 30 ml of Sample Dilution Buffer (B) to beaker 1.
- Add 30 ml of distilled water to beaker 1.

3 Dilute the substances.

When diluting the substances, mix thoroughly and change pipette tips between each transfer.

- Add 135 µl of the diluted sample dilution buffer (beaker 1) to each well of two sample preparation plates (96-well microtiter plate, not supplied).
- Add 15 µl of the first substance to be analysed to well A1.
- Add 15 µl of the second substance to be analysed to well A2 and so on until the substances have been added to wells A1-12 of both plates.
- Transfer 15 µl from wells A1-12 down to their corresponding B-wells.
- Transfer 15 µl from wells B1-12 down to their corresponding C-wells of both plates and so on until the G-rows of both plates are reached.
The H-row is used for references and background controls.

4 Prepare the reaction mixture.

- Add 2 ml of Reconstitution Buffer (C2) to the vial containing RT Reaction Components (C1). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Transfer 200 µl of the dissolved material back to the Reconstitution Buffer (C2) and mix thoroughly.

5 Prepare the PolyA Plates with samples.

When transferring samples start with the H-row and work upwards through the plate as this prevents the need to change pipette tips. The pipette tips should however be changed between plates.

- Take out both PolyA Plates from their foil pouches.
- Add 100 µl of reaction mixture (C2) to each well of both plates.
- Transfer 50 µl from each well of the first sample preparation plate to its corresponding well of the first PolyA Plate.
- Transfer 50 µl from each well of the second sample preparation plate to its corresponding well of the second PolyA Plate.
- Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the PolyA plates for 30 minutes at 33°C.

6 Dilute the HIV-1 rRT-standard.

- Add 5 ml of diluted sample dilution buffer (beaker 1) to the vial containing lyophilised HIV-1 rRT-standard (D). Mix thoroughly and make sure that the lyophilised material dissolves properly
- Label a 50 ml glass beaker "2".
- Add 12 ml of diluted sample dilution buffer (beaker 1) to beaker 2.
- Add 1 ml of HIV-1 rRT-standard (D) to beaker 2 and mix thoroughly.

7 Start the RT reaction.

- Take out the PolyA Plates from the incubator.
- Add 50 µl of standard enzyme (beaker 2) to all wells except H8-12 of both PolyA plates.
- Add 50 µl of diluted sample dilution buffer (beaker 1) to wells H8-12 of both plates.
- Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plates for three hours at 33°C on an orbital shaker set at gentle agitation.

8 Collect kit components.

- Take out the kit box from the refrigerator and take out the following components:
 - 1 Concentrated Washing Buffer (bottle E)
 - 2 RT Product Tracer (vials O)
 - 1 AP Substrate Tablets (tube P1)
 - 1 AP Substrate Buffer (bottle P2)
 - 2 pieces of adhesive tape
 - 2 pieces of plastic lids

The remaining components are not used in this application and may be discarded after performing the assay.

9 Prepare the wash fluid.

- Add 150 ml of Triton X-100 to 1 litre of distilled water and mix thoroughly with a magnetic spin bar.
Make sure that the Triton X-100 dissolves completely which takes approximately 10 minutes.
- Add 20 ml of Concentrated Washing Buffer (E) to a 20 litre container.
- Add the dissolved Triton X-100 to the 20 litre container.
- Adjust the volume to 20 litres with distilled water and mix thoroughly.

10 Prepare the RT Product Tracer.

- Add 12 ml of distilled water to each of the two vials containing RT Product Tracer (O). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Transfer the contents of both O vials to a 50 ml glass beaker.

11 Stop the RT reaction of the plates.

After three hours of incubation the RT reaction of the plates is stopped by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash both PolyA Plates twice. Turn the plates 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.

12 Start the binding of RT Product Tracer.

When dispensing the tracer, pipette tips should not touch the bottom of the wells.

- Add 100 µl to each well of both PolyA Plates.
- Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plates for 90 minutes at 33°C on an orbital shaker set at gentle agitation.

13 Prepare the AP substrate solution.

- Add the AP Substrate Tablets (P1) to the AP Substrate Buffer (P2).
- Shake the bottle occasionally and the tablets will dissolve completely in approximately twenty minutes.

14 Remove excess tracer.

After 90 minutes of incubation, excess tracer is removed by washing. Use approximately 6 ml of wash fluid per well and wash.

- Wash both PolyA Plates four times. Turn the plates 180° between washes if possible.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.

15 Start the alkaline phosphatase reaction.

The AP substrate solution must be mixed thoroughly and equilibrated to room temperature before it is added to the PolyA Plates. When dispensing the substrate solution, pipette tips should not touch the bottom of the wells.

- Add 200 µl of AP substrate solution to each well of both PolyA Plates.
- Cover the plates with plastic lids and incubate them at room temperature under dark cover on an orbital shaker set at gentle agitation.

16 Read the plates.

Set the filter of the plate reader to 405 nm.

- Read the absorbance of both plates after 30 minutes of incubation.
- Repeat the reading of the plates at intervals until wells H1-7 give values of approximately 75% of the maximum value within the linear reading range of the plate reader.

17 Process the data.

Use an alkaline phosphatase reading time at which wells H1-7 give approximately 75% of the maximum value within the linear measuring range of the plate reader. Follow the instructions for each plate separately.

- Calculate the mean A_{405} of the background controls (wells H8-12).
- Subtract the mean A_{405} of the background controls from the obtained A_{405} of the other wells.
- Calculate the mean A_{405} of the HIV-1 rRT standard (wells H1-7).
- Express the RT activity for wells containing substance dilutions as a percentage of the mean A_{405} HIV-1 rRT standard.
- Plot the percentage of residual RT activity against the concentrations of the substance dilutions for each of the tested substances.

The IC_{50} value is the substance concentration giving a 50% inhibition of the RT activity and is determined with the aid of the obtained graph.

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Ordering information

General inquiries and orders for the *RetroSys*[™] RT activity kit may be put to Innovagen. Users of this kit are advised to turn to this instance as well for technical support.

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