RetroSys™
C-type RT Activity Kit

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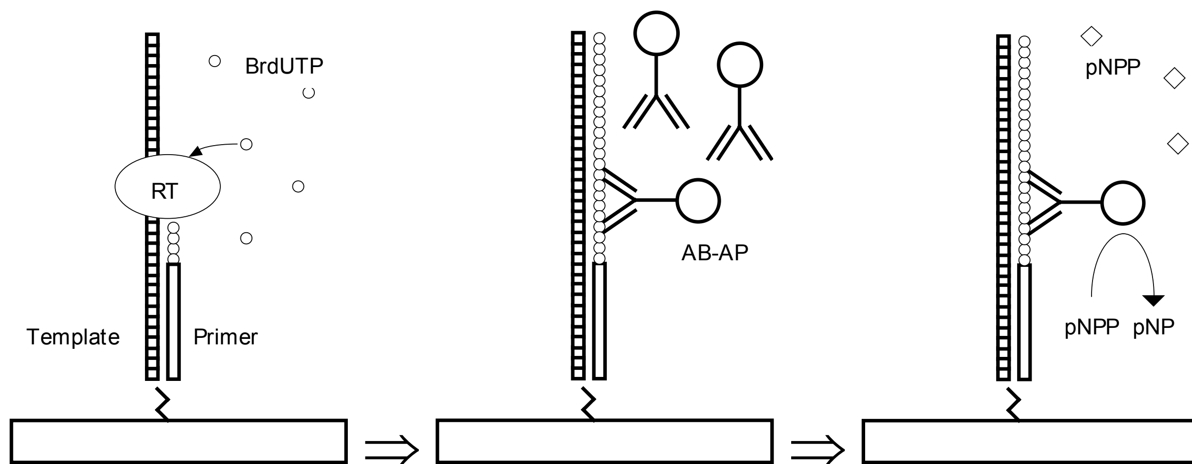
Principles of the RetroSys™ kit.

The *RetroSys*™ 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.



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
- Dilution Buffer, 25 ml in three bottles labelled ……………………… **B**
- C-type RT Reaction Components, lyophilised in two vials labelled **C**
- MuLV RT standard, lyophilised in one vial labelled ……………….. **D**
- Concentrated Washing Buffer, 20 ml in one bottle labelled ……….. **E**
- 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 H_2O of high purity e.g. MilliQ water.
- Incubator cabinet for 33°C.
- Micropipettes, 10-1000 µl.
- 8 and 12 multichannel micropipettes, 10-200 µl.
- Microtitre plate reader.
- Microtitre plate washing system.
- Orbital shaker suitable for microtitre plates.
- 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 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.

Wash protocol

When an automated plate washer is used the following protocol is recomended.

Aspirate 2 sec
Dispense 350 µl
Aspirate 2 sec
Dispense 350 µl
Aspirate 2 sec
Dispense 350 µl
Soak 10 sec
Aspirate 2 sec
Dispense 350 µl
Aspirate 4 sec

Protocol for quantification of RT activity.

20 cell supernatant samples in four dilutions may be analysed on one plate.

1 Prepare the reaction mixture.

- Add 12 ml of Dilution Buffer (B) to one vial containing C-type RT Reaction Components (C). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Add 6 ml of distilled water and mix thoroughly.

2 Prepare the PolyA Plates.

- Take out the PolyA Plate (A) from its foil pouch.
- Add 150 µl of reaction mixture to each well of the plate.
- Seal the plate with adhesive tape.
- Incubate the plate at 33°C for 20-60 minutes on an orbital shaker set at gentle agitation.

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

- Add 4 ml of Dilution Buffer (B) to the vial with MuLV RT standard (D). Make sure that the lyophilised material dissolves properly and mix thoroughly.
- Add 250 µl of Dilution Buffer (B) to seven test tubes.
- Add 250 µl of MuLV RT standard (D) to the first of the seven test tubes.
- Transfer 250 µl from the first test tube to the second.
- Transfer 250 µl from the second test tube to the third and so on until a series of seven dilutions has been obtained.

4 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 mictrotiter 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.

5 Start the RT reaction.

- Take out the PolyA Plate from the incubator.
- Transfer 50 µl of each sample dilution from the sample preparation plate to each corresponding well in columns 1-10 of the PolyA Plate.
- Add 50 µl of Dilution Buffer (B) to wells H11-H12.
- Add 50 µl of the first standard dilution to well A11 and A12.
- Add 50 µl of the second standard dilution to well B11 and B12 and so on until the seventh standard dilution is set in wells G11 and G12.
- Seal the PolyA Plate with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plate at 33°C for 3 hours on an orbital shaker set at gentle agitation.

6 Prepare the wash fluid.

- Add 15 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 2 ml of Concentrated Washing Buffer (E) to a 2 litre container.
- Add the dissolved Triton X-100 to the 2 litre container.
- Adjust the volume to 2 litres with distilled water and mix thoroughly.

7 Prepare the RT Product Tracer.

- Add 12 ml of distilled water to one vial containing RT Product Tracer (O). Make sure that the lyophilised material dissolves properly and mix thoroughly.

8 Stop the RT reaction.
After 3 hours of incubation the RT reaction is stopped by washing.

- Wash the PolyA Plate according to wash protocol.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.

9 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 the PolyA Plate.
- Seal the plate with adhesive tape. Press the tape down firmly over each well to ensure proper sealing.
- Incubate the plate for 90 minutes at 33°C on an orbital shaker set at gentle agitation.

10 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.

11 Remove excess tracer.
After 90 minutes of incubation, excess tracer is removed by washing.

- Wash the PolyA Plates according to wash protocol.
- Remove residual fluid in the wells by tapping the plate upside down on absorbing cloth or paper.

12 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 the PolyA Plate.
- Cover the plate with a plastic lid and incubate at room temperature under dark cover on an orbital shaker set at gentle agitation.

13 Read the plates.
Set the filter of the plate reader to 405 nm.

- Read the absorbance of the plate after 1 hour of incubation.
- Read the absorbance of the plate a second time after 2 hours of incubation.
- If necessary, read the absorbance of the plate after prolonged incubation.

14 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 the mean value of each standard dilution (wells A11-A12 to G11-G12) against its concentration of MuLV RT (see table below). Preferentially use a computer software to calculate the best regression line utilising the mean A_{405} of the background controls (wells H11-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
µU/well	1000	500	250	125	62,5	31,25	15,625
µU/ml	20000	10000	5000	2500	1250	625	312,5

Ordering information

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