

# HS-Mn RT Activity Kit

## Instructions for Use

For Research Purposes Only



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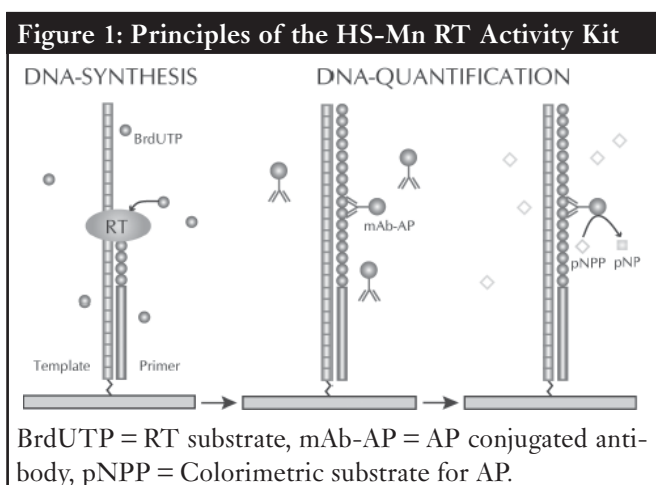
### Intended Use

The HS-Mn RT Activity kit is intended for detection or quantification of the activity of Mn<sup>2+</sup>-dependent Reverse Transcriptase (RT) enzyme from retroviruses like Murine leukemia virus (MuLV), Porcine endogenous retrovirus (PERV), Feline leukemia virus (FeLV) and Spumavirus.

### Principles of the HS-Mn RT Activity Kit

The HS-Mn RT Activity kit determines the RT activity in a sample. The procedure consists of two steps, the DNA synthesis and the DNA quantification. The principle is illustrated in figure 1. The figure shows a well in the 96-well RT Reaction Plate with the poly(A) RNA template bound to the bottom.

A reaction mixture, containing primer and a nucleotide (BrdUTP), is added to the plate as well as the sample to be analysed. The RT in the sample will synthesise a DNA-strand. An alkaline phosphatase (AP) conjugated  $\alpha$ -BrdU antibody binds to the double stranded DNA/RNA molecule. The product is quantified by addition of a colorimetric AP substrate. The AP activity is proportional to the RT activity in the sample.



## Applications

The HS-Mn RT Activity kit instructions for use describes two kit applications, quantification and screening for RT activity. The measuring range of the assay is 4 to 4500  $\mu\text{U RT/ml}^*$ .

### Protocol A: Quantification of RT activity

MAXIMUM NUMBER OF SAMPLES ANALYSED IN ONE KIT: 20 (40\*\*)

TYPE OF SAMPLE: Cell culture supernatant                      SAMPLE VOLUME: 35  $\mu\text{l}$

When analysing crude samples, RT inhibitory factors may be present. To ensure a quantitative determination 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 level of RT activity in the sample is calculated from the MMuLV rRT Standard set on each plate.

The measuring range corresponds to 0.2 to 225  $\mu\text{U}$  of recombinant MMuLV RT per well. The lower detection limit corresponds to the presence of 20  $\mu\text{U/ml}$  of active RT in undiluted cell supernatant, if diluted 1:5 as described in the protocol. Other recombinant  $\text{Mn}^{2+}$ -dependent RT may also be used instead of the included MMuLV rRT.

### Protocol B: Screening for RT activity

MAXIMUM NUMBER OF SAMPLES ANALYSED IN ONE KIT: 80 (160\*\*)

TYPE OF SAMPLE: Cell culture supernatant                      SAMPLE VOLUME: 10  $\mu\text{l}$  + 10  $\mu\text{l}$

When processing a 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 assay. An example is when analysing cell supernatants from virus isolation cell cultures for presence of MMuLV.

Protocol B differs from Protocol A in that a small amount of crude undiluted sample is used. The small sample volume is necessary to avoid disturbances.

\*The measuring range is based on the calculations described in the Data processing section on page 9, using the included MMuLV rRT Standard as reference enzyme. It can, however, be improved further by using a reference wavelength of 630 nm or using kinetic readings.

\*\*Once the system is standardised for your application there is no need for analysis with different RT reaction times. The kit can then be used for twice as many samples.

## Components

### Kit components

- 2 RT Reaction Plate (96 wells)
- 1 Base Buffer HS-Mn (58 ml)
- 2 Buffer Components (lyophilised)
- 2 Buffer Components HS-Mn (lyophilised)
- 2 RT Reaction Components HS (lyophilised)
- 1 MMuLV rRT Standard (lyophilised)
- 1 Plate Wash Buffer HS Conc. (50 ml)
- 2 RT Product Tracer (lyophilised)
- 1 Substrate Tablet (15 mg)
- 1 Substrate Buffer (30 ml)
- 4 Pieces of Adhesive Tape
- 2 Plastic Lids for 96-well plate
- 1 MMuLV rRT Standard Sheet for HS-Mn RT Activity Kit

### Equipment required but not provided

- Purified water
- Triton X-100 (laboratory grade or higher)
- Magnetic spinner and spin bars
- Incubator set at 33°C
- ELISA-plate reader with  $A_{405}$  filter
- ELISA-plate washer or 4 wash buckets (approx. 3 litre)
- Orbital shaker suitable for ELISA-plates
- Vortex
- Single-channel pipettes and tips (10-1000  $\mu\text{l}$ )
- Multi-channel pipettes and tips (10-200  $\mu\text{l}$ )
- Reservoirs for multi-channel pipettes
- Measuring cylinders, pipettes etc.
- Bottles for 50 ml and 2 litres
- Container for 10 litres

#### Equipment required for Protocol A only

- 1 Test tube (about 5 ml)
- 1 Standard 96-well microtitre plate with 300- $\mu$ l wells for titrations

#### Equipment required for Protocol B only

- 12 Test tubes (about 1 ml)

#### Description of components

- The *RT Reaction Plate* has pr(A) (polyriboadenosine) strands covalently bound to the wells.
- The *RT Reaction Components HS* contains lyophilised odT (oligodeoxythymidine) primer and BrdUTP (5-bromo-3-deoxyribouridine 5'-triphosphate).
- The *MMuLV rRT Standard* contains lyophilised recombinant MMuLV RT.
- The *RT Product Tracer* contains lyophilised monoclonal  $\alpha$ -BrdUMP (anti bromo-deoxyribouridine monophosphate) antibodies conjugated to alkaline phosphatase.
- The *Substrate Tablet* contains pNPP (paranitrophenylphosphate disodium).

#### Kit Storage and Shelf Life

- Store the kit reagents at between -14 and -25°C until use.
- If delivered unfrozen, the kit should be refrozen and stored between -14 and -25°C or stored at max. 8°C for usage within a week.
- Once reconstituted, lyophilised components should not be refrozen, but can be stored at 4 to 8°C for usage within a week.






#### Sample Collection, Preparation and Storage

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

#### Precautions

- For *in vitro* use only.
- For research purposes only.
- Observe normal precautions required for handling infectious material and laboratory reagents.
- Do not combine components from kits with different LOT numbers.

#### Symbols - used in instructions for use and kit labelling

 Manufacturer	 Keep away from direct light	 Article number
 LOT number	 -25°C / -14°C Temperature limitation	Cat# HS-Mn RT Activity kit article number

## Step-by-Step Instructions

### PROTOCOL A: QUANTIFICATION OF RT ACTIVITY

#### A-1. Collect and thaw components

- Collect the following kit components and thaw the frozen ones at or below 37°C:
  - 2 *RT Reaction Plate*
  - 1 *Base Buffer HS-Mn*
  - 2 *Buffer Components*
  - 2 *Buffer Components HS-Mn*
  - 2 *RT Reaction Components HS*
  - 1 *MMuLV rRT Standard*
  - 1 *Plate Wash Buffer HS Conc.*
  - 2 Pieces of adhesive tape
  - 2 Plastic lids for 96-well plates

#### A-2. Prepare the *Base Buffer HS-Mn*

- a. Add a few millilitres of *Base Buffer HS-Mn* to the two vials of *Buffer Components* and the two vials of *Buffer Components HS-Mn*. Vortex for ten seconds.
- b. Transfer the contents of the four vials back to the *Base Buffer HS-Mn*. Vortex for five seconds.

#### A-3. Prepare the Reaction mixture

- a. Add 12 ml of the *Base Buffer HS-Mn*, prepared in step A-2, to each of the two *RT Reaction Components HS*. Vortex for ten seconds. Save the remains of the *Base Buffer HS-Mn* for later use in step A-5 and A-6.
- b. Transfer the contents of both *RT Reaction Components HS* to a 50-ml bottle, labelled "Reaction Mixture". This makes up the Reaction mixture.

#### A-4. Prepare the *RT Reaction Plates*

- a. Take out both *RT Reaction Plates* from their pouches. Save one pouch for later use in step A-10.
- b. Add 100 µl of the Reaction mixture, prepared in step A-3, to each well of both *RT Reaction Plates*. Put a lid on each *RT Reaction Plate*.
- c. Incubate the plates for 20 to 60 minutes at 33°C. Perform steps A-5 to A-7 during this time.

#### A-5. Prepare the sample preparation plate

- a. Add 140 µl of the *Base Buffer HS-Mn*, prepared in step A-2, to all wells in columns 1 to 10 of a 96-well microtitre plate (not supplied). This is the sample preparation plate.
- b. Add 125 µl of *Base Buffer HS-Mn*, prepared in step A-2, to all wells, EXCEPT A11, in columns 11 to 12 of the sample preparation plate.

#### A-6. Prepare *MMuLV rRT Standard* dilution series

- a. Add 2.1 ml of *Base Buffer HS-Mn*, prepared in step A-2, to the *MMuLV rRT Standard*. Vortex for ten seconds.
- b. Mix 520 µl *Base Buffer HS-Mn* and 20 µl *MMuLV rRT Standard* in a test tube. Transfer 250 µl of the diluted *MMuLV rRT Standard* to well A11 of the sample preparation plate.
- c. Make a dilution series by transferring 100 µl from A11 to B11, change pipette tip

and mix five times. Transfer 100 µl from B11 to C11, change pipette tip and mix five times etc...Transfer 100 µl from H11 to A12 , change pipette tip and mix five times etc. all the way to D12.

#### **A-7. Dilute the samples**

- a. Add 35 µl of the first sample to be analysed to well A1 of the sample preparation plate. Add 35 µl of the second sample to be analysed to well A2 and so on until the first ten samples has been added (wells A1 to A10).
- b. Add 35 µl of the next ten samples to wells E1 to E10, in the same manner as for samples 1 to 10.

##### **Dilution of samples in wells A1 to A10:**

- c. Transfer 35 µl from wells A1 to A10 to their corresponding B wells, change pipette tip and mix five times.
- d. Transfer 35 µl from wells B1 to B10 to their corresponding C wells, change pipette tip and mix five times.
- e. Transfer 35 µl from wells C1 to C10 to their corresponding D wells, change pipette tip and mix five times. Discard the pipette tips.

##### **Dilution of samples in wells E1 to E10:**

- f. Transfer 35 µl from wells E1 to E10 to their corresponding F wells, change pipette tip and mix five times.
- g. Transfer 35 µl from wells F1 to F10 to their corresponding G wells, change pipette tip and mix five times.
- h. Transfer 35 µl from wells G1 to G10 to their corresponding H wells, change pipette tip and mix five times.

#### **A-8. Add samples, buffer blanks and *MMuLV rRT Standard* to *RT Reaction Plates***

See figure 2 on page 10 for an illustration of the set-up of the *RT Reaction Plates*.

- a. Collect the *RT Reaction Plates* from the incubator.
- b. Transfer 50 µl from each well of the sample preparation plate to the corresponding wells in the both *RT Reaction Plates* (A1 to A1, B1 to B1 etc).
- e. Seal both *RT Reaction 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 on gentle agitation. Incubate the first plate for three hours and the second overnight (16 to 24 hours).

#### **A-9. Prepare Plate wash buffer**

- a. Slowly pour 75 ml of Triton X-100 into 1 litre of purified water containing a magnetic spinbar on continuous rotation. Make sure that the Triton X-100 dissolves completely, which takes approximately ten minutes.
- b. Mix 25 ml of *Plate Wash Buffer HS Conc.* and the dissolved Triton X-100 in a 10-litre container. Adjust the volume to 10 litres with purified water and mix thoroughly.
- c. Store the *Plate Wash Buffer HS Conc.* bottle at between 4 to 8°C for later use in step Q-4.

#### **A-10. Stop the RT reaction on the first *RT Reaction Plate* by washing**

If you are uncertain of the performance of your plate washer, try the manual washing procedure described on page 11.

- a. Collect the first *RT Reaction Plate* from the incubator after the three-hour incubation.

- Adjust the plate washer to pass 3 ml of wash fluid per well for each cycle.
- Wash the plate two cycles. 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. Leave the plate to dry upside-down for five to ten minutes.
  - Put the *RT Reaction Plate* back in its pouch and store it between -14 to -25°C.
  - Continue the assay the following day using the Product Quantification Protocol on page 8.

## PROTOCOL B: SCREENING FOR RT ACTIVITY

### B-1. Collect and thaw components

- Collect the following kit components and thaw the frozen ones at or below 37°C:
  - 2 *RT Reaction Plate*
  - 1 *Base Buffer HS-Mn*
  - 2 *Buffer Components*
  - 2 *Buffer Components HS-Mn*
  - 2 *RT Reaction Components HS*
  - 1 *MMuLV rRT Standard*
  - 1 *Plate Wash Buffer HS Conc.*
  - 2 Pieces of adhesive tape
  - 2 Plastic lids for 96-well plates

### B-2. Prepare the *Base Buffer HS-Mn*

- Add a few millilitres of *Base Buffer HS-Mn* to the two vials of *Buffer Components* and the two vials of *Buffer Components HS-Mn*. Vortex for ten seconds.
- Transfer the contents of the four vials back to the *Base Buffer HS-Mn*. Vortex for five seconds.

### B-3. Prepare the Reaction mixture

- Add 17 ml of *Base Buffer HS-Mn*, prepared in step B-2, to each of the two *RT Reaction Components HS*. Vortex for ten seconds. Save the remains of the *Base Buffer HS-Mn* for later use in step B-5 and B-7.
- Transfer the contents of both *RT Reaction Components HS* to a 50-ml bottle, labelled "Reaction Mixture". This makes up the Reaction mixture.

### B-4. Prepare the *RT Reaction Plates*

- Take out both *RT Reaction Plates* from their pouches. Save one pouch for later use in step B-9.
- Add 140 µl of the Reaction mixture, prepared in step B-3, to each well of both *RT Reaction Plates*. Put a lid on each *RT Reaction Plate*.
- Incubate the plates for 20 to 60 minutes at 33°C. Perform steps B-5 and B-6 during this time.

### B-5. Prepare *MMuLV rRT Standard* dilution series

- Add 2.1 ml of *Base Buffer HS-Mn*, prepared in step B-2, to the *MMuLV rRT Standard*.

Vortex for ten seconds.

- b. Take out twelve test tubes. Add 220 µl of *Base Buffer HS-Mn* to the first test tube. Add 250 µl of *Base Buffer HS-Mn* to the following eleven test tubes.
- c. Add 50 µl of *MMuLV rRT Standard* to the first test tube. Change pipette tip and mix five times.
- d. Make a dilution series by transferring 200 µl from the first test tube to the second, change pipette tip and mix five times. Transfer 200 µl from the second test tube to the third, change pipette tip and mix five times etc, all the way to the 12<sup>th</sup> test tube.

#### **B-6. Prepare the samples**

- Collect the samples to be analysed (two 10-µl portions of each sample is required).

#### **B-7. Add samples, buffer blanks and *MMuLV rRT Standard* to *RT Reaction Plates***

See figure 3 on page 10 for an illustration of the set-up of the *RT Reaction Plates*.

- a. Collect the *RT Reaction Plates* from the incubator.
- b. Add 10 µl of sample 1 to well A1 of each *RT Reaction Plate*. Change pipette tip and add 10 µl of sample 2 to well B1 and so on until all 80 samples have been added to each plate. DO NOT use any wells in columns 11 or 12.
- c. Add 10 µl of *Base Buffer HS-Mn* to wells E12 to H12 of both *RT Reaction Plates*.
- d. Transfer 10 µl of the diluted *MMuLV rRT Standard* in the first test tube to well A11 of both *RT Reaction Plates*. Change pipette tip and transfer 10 µl of each dilution to the corresponding well in both plates (test tube 2 to well B11, test tube 3 to C11...test tube 8 to H11, test tube 9 to A12...test tube 12 to D12).
- e. Seal both *RT Reaction 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 on gentle agitation. Incubate the first plate for three hours and the second overnight (16 to 24 hours).

#### **B-8. Prepare Plate wash buffer**

- a. Slowly pour 75 ml of Triton X-100 into 1 litre of purified water containing a magnetic spinbar on continuous rotation. Make sure that the Triton X-100 dissolves completely, which takes approximately ten minutes.
- b. Mix 25 ml of *Plate Wash Buffer HS Conc.* and the dissolved Triton X-100 in a 10-litre container. Adjust the volume to 10 litres with purified water and mix thoroughly.
- c. Store the *Plate Wash Buffer HS Conc.* bottle at between 4 to 8°C for later use in step Q-4.

#### **B-9. Stop the RT reaction on the first *RT Reaction Plate* by washing**

If you are uncertain of the performance of your plate washer, try the manual washing procedure described on page 11.

- a. Collect the first *RT Reaction Plate* from the incubator after the three-hour incubation. Adjust the plate washer to pass 3 ml of wash fluid per well for each cycle.
- b. Wash the plate two cycles. 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. Leave the plate to dry upside-down for five to ten minutes.
- c. Put the *RT Reaction Plate* back in its pouch and store it between -14 to -25°C.
- d. Continue the assay the following day using the Product Quantification Protocol on page 8.

## PRODUCT QUANTIFICATION PROTOCOL FOR PROTOCOLS A AND B

### Q-1. Collect and thaw components

- Collect the following kit components and thaw the frozen ones at or below 37°C:
  - 1 *Plate Wash Buffer HS Conc.*
  - 2 *RT Product Tracer*
  - 1 *Substrate Tablet*
  - 1 *Substrate Buffer*
  - 2 Pieces of adhesive tape
  - 2 Plastic lids for 96-well plates

### Q-2. Prepare Tracer

- a. Prepare about 30 ml of purified water containing 1% Triton X-100.
- b. Add 12 ml of the 1% Triton X-100 solution to each *RT Product Tracer* and vortex for ten seconds. Pool contents of both *RT Product Tracer* into a 50-ml bottle, labelled "Tracer".

### Q-3. Prepare *Substrate Buffer*

- Add the *Substrate Tablet* to the thawed *Substrate Buffer*. Store it in the **dark** until use.

### Q-4. Prepare Plate wash buffer

- a. Slowly pour 75 ml of Triton X-100 into 1 litre of purified water containing a magnetic spinbar on continuous rotation. Make sure that the Triton X-100 dissolves completely, which takes approximately ten minutes.
- b. Mix 25 ml of *Plate Wash Buffer HS Conc.* and the dissolved Triton X-100 in a 10-litre bucket. Adjust the volume to 10 litres with purified water and mix thoroughly.

### Q-5. Collect *RT Reaction Plate* from freezer

- Collect the first *RT Reaction Plate* from the freezer. Please note that this plate should not be washed in step Q-6.

### Q-6. Stop the RT reaction by washing

If you are uncertain of the performance of your plate washer try, the manual washing procedure described on page 11.

- a. Collect the second *RT Reaction Plate* from the incubator. Adjust the plate washer to pass 3 ml of wash fluid per well for each cycle.
- b. Wash the plate two cycles. 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. Leave the plate to dry upside-down for five to ten minutes.

### Q-7. Add Tracer to *RT Reaction Plate*

- a. Vortex the Tracer bottle for five seconds. Transfer 100 µl from the Tracer bottle to each well of both *RT Reaction Plates*, without touching the bottom of the wells.
- b. Seal the plates with adhesive tape. Press the tape down firmly over each well to ensure proper sealing. Incubate for 90 minutes at 33°C, on an orbital shaker set on gentle agitation.

### Q-8. Remove excess Tracer by washing

If you are uncertain of the performance of your plate washer, try the manual washing procedure described on page 11.

- a. Collect the *RT Reaction Plates* from the incubator after the 90 minute-incubation.

Adjust the plate washer to pass 3 ml of wash fluid per well for each cycle.

- b. Wash the plates eight cycles. 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. Leave the plate to dry upside-down for five to ten minutes.

#### Q-9. Add *Substrate Buffer*

- Vortex the *Substrate Buffer*, prepared in step Q-3, for five seconds and add 100 µl to each well of both *RT Reaction Plates*, without touching the bottom of the wells. Remove bubbles, if any, in the wells with a clean pipette tip for each well. Cover the plates with plastic lids and incubate in the **dark** at room temperature.

#### Q-10. Read the *RT Reaction Plates*

- Read the plates at  $A_{405}$  30 minutes after addition of the substrate. Repeat the reading at appropriate times, depending on the development of the colour signal. Readings after two hours and the following day is recommended. Incubate the plates in the **dark** at room temperature between the readings.

## DATA PROCESSING

The manual calculations described below can be programmed for automatic execution in e.g. an Microsoft Excel® spreadsheet.

### Protocol A: Quantification of RT activity

Please note that the HS-Mn RT Activity kit only gives a linear relationship between  $A_{405}$  and RT activity if the readings are within the linear range of the plate reader used.

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

- a. Plot the  $A_{405}$  of each MMuLV rRT Standard dilution (wells A11 to D12) against the concentration of MMuLV rRT present. The MMuLV rRT values are found on the Standard sheet included in the kit. Calculate the best regression line utilising the mean  $A_{405}$  of the buffer blanks (wells E12 to H12) as y-axis intercept.
- b. 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 range of the reader and having signals greater than two times the mean value of the buffer blanks.
- c. Compare the RT activity values for long and short RT assay incubation for each sample using the information below:
  - If the RT values are similar - calculate the mean value for the sample.
  - If a higher value is obtained for the short RT reaction time - use this value. The lower value obtained with the long RT reaction time is normally due to RT product degradation.
  - If a significant value is obtained for the long RT reaction time only - use this value.

- d. Calculate the RT activity for undiluted samples by compensating for the dilution used in the assay.
- e. Calculate the mean RT activity of the sample. Should significantly lower values be obtained for higher sample concentrations, this could be due to disturbing factors in the sample. Values obtained from these dilutions should be excluded from the calculation.

**Figure 2: Set-up of the RT Reaction Plates for Protocol A**

		1	2	3	4	5	6	7	8	9	10	11	12
Conc. 1	A	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	Ⓢ1	Ⓢ9
Conc. 2	B	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	Ⓢ2	Ⓢ10
Conc. 3	C	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	Ⓢ3	Ⓢ11
Conc. 4	D	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	Ⓢ4	Ⓢ12
Conc. 1	E	⑪	⑫	⑬	⑭	⑮	⑯	⑰	⑱	⑲	⑳	Ⓢ5	Ⓢb
Conc. 2	F	⑪	⑫	⑬	⑭	⑮	⑯	⑰	⑱	⑲	⑳	Ⓢ6	Ⓢb
Conc. 3	G	⑪	⑫	⑬	⑭	⑮	⑯	⑰	⑱	⑲	⑳	Ⓢ7	Ⓢb
Conc. 4	H	⑪	⑫	⑬	⑭	⑮	⑯	⑰	⑱	⑲	⑳	Ⓢ8	Ⓢb

① = Sample number      Ⓢb = Buffer blank  
 Ⓢ1 = MMuLV rRT Standard

### Protocol B: Screening for RT activity

Please note that the HS-Mn RT Activity kit only gives a linear relationship between  $A_{405}$  and RT activity if the readings are within the linear range of the plate reader used.

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

- a. Plot the  $A_{405}$  of each standard dilution (wells A11 to D12) against the concentration of MMuLV rRT present. The MMuLV rRT values are found on the Standard sheet included in the kit. Calculate the best regression line utilising the mean  $A_{405}$  of the buffer blanks (wells E12 to H12) as y-axis intercept.
- b. 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 range of the reader and having signals greater than two times the mean value of the buffer blanks.
- c. Compare the RT activity values for long and short RT assay incubation for each sample using the information below:
  - If the RT values are similar - calculate the mean value for the sample.
  - If a higher value is obtained for the short RT reaction time - use this value. The lower value obtained with the long RT reaction time is normally due to RT product degradation.
  - If a significant value is obtained for the long RT reaction time only use this value.
- d. For samples where the first reading gives an  $A_{405}$  above the linear measuring range of the plate reader, the RT activity is above the highest MMuLV rRT activity in the standard curve. If an accurate value for such a sample is desired, the sample should be reanalysed using several dilutions (see Protocol A).

**Figure 3: Set-up of the RT Reaction Plates for Protocol B**

		1	2	3	4	5	6	7	8	9	10	11	12
A	①	⑨	⑰	⑲	⑳	㉑	㉒	㉓	㉔	㉕	㉖	Ⓢ1	Ⓢ9
B	②	⑩	⑱	㉒	㉓	㉔	㉕	㉖	㉗	㉘	㉙	Ⓢ2	Ⓢ10
C	③	⑪	⑲	㉒	㉓	㉔	㉕	㉖	㉗	㉘	㉙	Ⓢ3	Ⓢ11
D	④	⑫	⑲	㉒	㉓	㉔	㉕	㉖	㉗	㉘	㉙	Ⓢ4	Ⓢ12
E	⑤	⑬	⑳	㉒	㉓	㉔	㉕	㉖	㉗	㉘	㉙	Ⓢ5	Ⓢb
F	⑥	⑭	㉑	㉒	㉓	㉔	㉕	㉖	㉗	㉘	㉙	Ⓢ6	Ⓢb
G	⑦	⑮	㉑	㉒	㉓	㉔	㉕	㉖	㉗	㉘	㉙	Ⓢ7	Ⓢb
H	⑧	⑯	㉑	㉒	㉓	㉔	㉕	㉖	㉗	㉘	㉙	Ⓢ8	Ⓢb

① = Sample number      Ⓢb = Buffer blank  
 Ⓢ1 = MMuLV rRT Standard

## PROTOCOL FOR MANUAL WASHING OF PLATES

Automatic plate washers can sometimes give too high  $A_{405}$  values in the buffer blanks and/or negative samples due to inadequate washing. If you are uncertain of the performance of your plate washer, try the following manual washing procedure. It can be used as a standard for calibration and control of automatic washing procedures.

- a. Use four buckets of approximately 3 litres for the washing procedure. Prepare the Plate wash buffer as described in previous protocols. Pour 1 litre of the Plate wash buffer into each bucket (up to two plates can be washed simultaneously with this amount of buffer).
- b. Collect the *RT Reaction Plate/RT Reaction Plates* from the incubator. Remove the tape carefully, pulling it diagonally from one corner to the other. Secure the strips with a rubber band. Empty the plate into the waste sink.
- c. Put the plate in the first bucket with the wells facing up. Pick up the plate and empty it over the bucket. Repeat this action 15 times before moving the plate to the second bucket. Repeat the procedure in the second bucket and move the plate to the third bucket and so on.
- d. After washing in four buckets, tap the plate upside-down on dry absorbing paper. Leave to dry upside-down on the paper for five to ten minutes. Tap the plate again to make all bubbles in the wells disappear. Do not forget to take off the rubber band before proceeding to the next step.
- e. Throw away the used Plate wash buffer and rinse out the buckets, first with tap water and then with purified water. DO NOT use the same buffer for the two different wash steps (after RT reaction incubation and after RT Product Tracer incubation, respectively).

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