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Not for use in diagnostic procedures.

Reverse Transcriptase Assay, colorimetric

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Colorimetric enzyme immunoassay for the quantitative determination of retroviral reverse transcriptase activity by incorporation of digoxigenin- and biotin-labeled dUTP into DNA

Cat. No. 11 468 120 910 1 kit
200 tests

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	violet	Reverse Transcriptase Assay, colorimetric, HIV-1 reverse transcriptase	▪ Recombinant HIV-1-RT ▪ Lyophilized from potassium phosphate buffer, pH 7.4, containing 0.2% bovine serum albumin (BSA, special quality for molecular biology). ▪ Specific activity of HIV-1-RT is >5 U/µg (one unit is the amount of enzyme required for the incorporation of 1 nmol of labeled dNTP in 10 minutes at +37°C using poly (A) × $(dT)_{15}$ as template/primer hybrid).	1 bottle, 500 ng
2	blue	Reverse Transcriptase Assay, colorimetric, Incubation buffer	▪ Ready-to-use solution. ▪ 50 mM Tris buffer, containing 319 mM potassium chloride, 33 mM magnesium chloride, 11 mM DTT; pH 7.8.	1 bottle, 5 ml
3	blue	Reverse Transcriptase Assay, colorimetric, Nucleotide mixture	50 mM Tris-HCl, pH 7.8 with DIG-dUTP, biotin-dUTP, and dTTP.	4 bottles, 100 µl each
4	colorless	Reverse Transcriptase Assay, colorimetric, Template [poly (A) × $(dT)_{15}$]	▪ Template/primer hybrid poly (A) × $(dT)_{15}$ (9 $A260_{nm}$/ml). ▪ Lyophilized	1 bottle, 3.8 $A_{260\ nm}$
5	colorless	Reverse Transcriptase Assay, colorimetric, Lysis buffer	▪ Ready-to-use solution. ▪ Contains Tris buffer: 50 mM Tris, 80 mM potassium chloride, 2.5 mM DTT, 0.75 mM EDTA, and 0.5% Triton X-100; pH 7.8.	1 bottle, 25 ml
6	red	Reverse Transcriptase Assay, colorimetric, Anti-DIG-POD	▪ Anti-DIG-POD polyclonal antibody from sheep. ▪ Lyophilized	1 bottle, 10 U
7	colorless	Reverse Transcriptase Assay, colorimetric, Washing buffer, 10x conc.	For preparation of washing solution.	3 bottles, 25 ml each
8	red	Reverse Transcriptase Assay, colorimetric, Conjugate dilution buffer	▪ Ready-to-use solution. ▪ Sodium phosphate buffer, pH 7.4, containing a blocking reagent.	1 bottle, 75 ml
9	green	Reverse Transcriptase Assay, colorimetric, Substrate buffer	ABTS substrate buffer, containing sodium perborate and citric acid/phosphate buffer.	1 bottle, 125 ml
10	green	Reverse Transcriptase Assay, colorimetric, ABTS substrate	Each tablet sufficient for 5 ml Substrate solution.	1 bottle, 20 tablets
11	white	Reverse Transcriptase Assay, colorimetric, Substrate enhancer	Powder *Use the substrate enhancer only if the reverse transcriptase activity is low.*	1 bottle, 150 mg
12	foil bag	Reverse Transcriptase Assay, colorimetric, Microplate modules	▪ Precoated with streptavidin and postcoated with blocking reagent. ▪ Shrink-wrapped with a desiccant capsule (4 × 8 wells, 224 wells per kit).	7 bags, 4 modules of 8 wells each

13	–	Reverse Transcriptase Assay, colorimetric, Strip frame	▪ Holds a maximum of 12 Microplate modules (total of 96 wells). ▪ Ensures the correct fitting and a tight support of the Microplate modules.	1 frame
14	–	Reverse Transcriptase Assay, colorimetric, Self-adhesive Plate Cover Foil	Prevents evaporation. ⚠ ***Cover the Microplate modules with the Cover Foils during each incubation step.***	10 foils

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1	violet	HIV-1 reverse transcriptase	Store at −15 to −25°C.
2	blue	Incubation buffer	Store at +2 to +8°C.
3	blue	Nucleotide mixture	Store at −15 to −25°C.
4	colorless	Template [poly (A) × $(dT)_{15}$]	
5	colorless	Lysis buffer	Store at +2 to +8°C.
6	red	Anti-DIG-POD	Store at −15 to −25°C.
7	colorless	Washing buffer, 10x conc.	
8	red	Conjugate dilution buffer	
9	green	Substrate buffer	
10	green	ABTS substrate	Store at −15 to −25°C. ⚠ ***Keep protected from light.***
11	white	Substrate enhancer	Store at −15 to −25°C.
12	foil bag	Microplate	Store at +2 to +8°C.
13	–	Strip frame	
14	–	Self-adhesive Plate Cover Foil	

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- Autoclaved or heat-sterilized labware (reaction vials, pipettes, and pipette tips)
- Multichannel pipettes
- Water bath (+37°C)
- Microplates (96 well)
- Microplate shaker (optional)
- Microplate ELISA reader
- Gloves

Preparation of kit working solutions

- Sterile, autoclaved and/or DEPC- or Velcorin-treated double-distilled water

For preparation of reaction mixture using template of choice (optional)

See section, **Working solution***, solution 3b.*

- dATP (2′-deoxy-adenosine-5′-triphosphate*), dCTP (2′-deoxy-cytidine-5′-triphosphate*), dGTP (2′-deoxy-guanosine-5′-triphosphate*)

For virus isolation by PEG-precipitation (optional)

- Polyethylene glycol (M_r 6,000 to 8,000)

For quantification of the inhibitory effect of RT inhibitors (optional)

- Reverse Transcriptase, HIV-1, recombinant and Reverse Transcriptase inhibitors

For determination of Mn2+-dependent reverse transcriptase (e.g., M-MuLV), the Incubation buffer (solution 2) must be prepared by the customer. For additional information and support, contact the Technical Support staff.

The kit contains all the reagents needed to perform the Reverse transcriptase reaction and ELISA assay.

1.4. Application

The Reverse Transcriptase Assay, colorimetric is designed for use in research studies in the following applications:

- Quantitative determination of reverse transcriptase (RT) activity in cell cultures and other biological samples.
- Determination of RT activity derived from a variety of retroviruses, including HIV-1, HIV-2, SIV-1, AMV, and M-MuLV.
- Determine the propagation of retroviruses in retrovirus-infected mammalian cell cultures.
- Research tool for *in vitro* screening for RT inhibitors.

1.5. Preparation Time

Assay Time

- Approximately 4 hours for an enzyme reaction of 1 hour.
- Approximately 18 hours for an enzyme reaction of 15 hours.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

The Reverse Transcriptase Assay can be used with cell culture supernatants and other biological samples, and RT inhibitors, together with retroviral reverse transcriptase.

Safety Information

For customers in the European Economic Area

Contains SVHC: octyl/nonylphenol ethoxylates. For use in research and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation.

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche.

Working Solution

Preparation of working solutions

⚠ ***Label each solution with the appropriate solution number (solutions 1 to 8). Keep all solutions on ice while performing the Reverse Transcriptase Assay, colorimetric.***

Solution	Content	Reconstitution/Preparation of Working Solution	Storage and Stability
1	HIV-1 reverse transcriptase (Bottle 1)	▪ Reconstitute the lyophilizate in 250 µl autoclaved double-distilled water (final concentration 2 ng/µl, corresponding to 10 mU/µl). ▪ One calibration curve in duplicate can be established with 10 µl of HIV-1-RT, see section **Preparation of HIV-1 reverse transcriptase working dilutions**.	Store stock solution for 12 months, or until the kit expiration date stored in aliquots at −70°C. ⚠ ***Avoid repeated freezing and thawing.***
2	Incubation buffer (Bottle 2)	Ready-to-use solution.	Store at +2 to +8°C until the kit expiration date.

3	Reaction mixture (Bottles 2, 3, and 4)	The template/primer hybrid and the nucleotides required for the reaction mixture are supplied as separate components. This allows a flexible choice of using either the template/primer hybrid poly (A) × $(dT)_{15}$ supplied with the kit, or a template/primer hybrid of individual choice (e.g., of retroviral origin) for the Reverse Trancriptase Assay, colorimetric. If required, the kit concept also allows the use of a modified reaction buffer of individual choice.	–
3a	Reaction mixture when using poly (A) × $(dT)_{15}$	▪ Reconstitute the template (Bottle 4) in 430 µl of autoclaved double-distilled water. ▪ Add 1 ml Incubation buffer (solution 2) per bottle of nucleotides (Bottle 3). ▪ Add 100 µl of the reconstituted template to each bottle containing the diluted nucleotide solution to obtain a reaction mixture that is ready to use. Final concentration: 46 mM Tris-HCl, 266 mM potassium chloride, 27.5 mM magnesium chloride, 9.2 mM DTT, 10 µM dUTP/dTTP, template/primer hybrid, 750 $mA_{260\ nm}$/ml.	Store reaction mixture for 12 months, or until the kit expiration date stored in aliquots at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.***
3b	Reaction mixture when using a template/ primer hybrid of individual choice	▪ To obtain a template/nucleotide mixture, adjust the template/primer hybrid of choice to a concentration of approximately 9 $A_{260\ nm}$/ml in autoclaved double-distilled water containing dATP, dGTP, and dCTP, 30 µM, each. ▪ Add 1 ml Incubation buffer (solution 2) per bottle of nucleotides (Bottle 3). ▪ Add 100 µl of the chosen template/primer/ nucleotide mixture to each bottle containing the diluted nucleotides to obtain a reaction mixture of individual choice that is ready to use. One calibration curve in duplicate can be established with 280 µl of the reaction mixture. For every additional sample, 20 µl per well is required.	Store reaction mixture for 12 months, or until the kit expiration date stored in aliquots at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.***
4	Lysis buffer (Bottle 5)	Ready-to-use solution.	Store at +2 to +8°C until the kit expiration date.
5	Anti-DIG-POD (Bottle 6)	Reconstitute the lyophilizate in 0.5 ml autoclaved double-distilled water. ⚠ ***Do not add sodium azide.***	Store the undiluted solution for 6 months, or until the kit expiration date at +2 to +8°C. ⚠ ***Do not freeze.***
5a	Anti-DIG-POD, working dilution	Dilute the reconstituted Anti-DIG-POD (solution 5) with Conjugate dilution buffer (solution 8) to a final concentration of 200 mU/ml (e.g., 50 µl antibody solution and 4.95 ml of conjugate dilution buffer).	Unstable, prepare immediately before use.
6	Washing buffer, 1x conc. (Bottle 7)	To prepare 1x Washing buffer, add 225 ml of autoclaved double-distilled water per bottle of Washing buffer, 10x conc. (Bottle 7); mix thoroughly.	Store at +2 to +8°C until the kit expiration date.

7	ABTS substrate solution (Bottles 9 and 10)	▪ Dissolve one ABTS tablet (Bottle 10) in 5 ml Substrate buffer (Bottle 9) and mix by stirring. ▪ Equilibrate to +15 to +25°C before use. ▪ ⚠ ***Keep protected from light.*** ▪ The content of Bottle 10 (20 tablets) is sufficient for 100 ml working solution. The solution is ready to use.	Store 2 months, or until the kit expiration date at +2 to +8°C. ⚠ ***Keep protected from light.***
7a	ABTS substrate solution containing substrate enhancer (Bottle 11)	▪ If a low signal is expected, take an appropriate aliquot of ABTS substrate solution (solution 7) and add 1 mg of substrate enhancer (Bottle 11) per ml of ABTS substrate solution. ▪ Mix by stirring for 15 minutes at +15 to +25°C.	The solution is only stable for 4 hours; prepare immediately before use.
8	Conjugate dilution buffer (Bottle 8)	▪ Ready-to-use solution. ▪ Mix thoroughly before use. ⚠ ***Do not add sodium azide.***	Store 2 months at +2 to +8°C, or for long-term storage, aliquot and store at −15 to −25°C.
–	Microplate modules	Use only the Microplate modules required for the particular experiment. Close the foil bag containing the remaining modules and the desiccant capsule tightly with adhesive tape. ⓘ *The Microplate modules are ready-to-use and do not need to be rehydrated before use.*	Once the foil bag is opened, store Microplate modules for approximately 12 months, or store desiccated at +2 to +8°C until the kit expiration date.
–	Polyethylene glycol (PEG)	▪ For the **Isolation of virus particles by PEG precipitation**. ▪ Prepare 30% polyethylene glycol (M_r 6,000 to 8,000) (w/v), 1.2 M sodium chloride, sterile filtered.	–

2.2. Protocols

Quantitative determination of retrovirus progeny in cell culture supernatant

Perform the Reverse Transcriptase Assay, colorimetric in accordance with commonly used good laboratory practices, especially if used for the determination of reverse transcriptase activity of potentially pathogenic viruses. For HIV-1, incubation of the virus in a lysis buffer containing 0.5% Triton X-100 for 30 minutes has been used for inactivation of the virus.

⚠ ***Any solution or equipment that has been in contact with the sample containing the particular retrovirus can be potentially pathogenic and should be treated accordingly.***

Virus isolation

For quantification of retroviruses in cell culture supernatants, an enrichment of the virus particles prior to performing the Reverse Transcriptase Assay, colorimetric is required. This is particularly true for serum containing cell culture supernatants, as serum constituents may interfere with the assay. Depending on the equipment available, use one of two protocols: **Isolation of virus particles by ultracentrifugation** or **Isolation of virus particles by PEG precipitation**.

⚠ ***Avoid filtration of the cell culture supernatants containing the retrovirus particles.***

Isolation of virus particles by ultracentrifugation

1. Centrifuge suspension cells at 250 × *g* for 10 minutes in a refrigerated centrifuge at +4°C.
   – Transfer supernatant into a fresh centrifugation tube.
   The initial centrifugation step at 250 × g is particularly important when only reverse transcriptase activity that is released into the culture supernatant is to be measured. For adherent cells, the initial centrifugation step may be omitted.
   – If required, resuspend the cells in culture medium according to the particular cell type's media needs for further cultivation.

2. Centrifuge supernatant at 2,000 × *g* for 30 minutes or 8,000 × *g* for 10 minutes in a refrigerated centrifuge at +4°C to remove cellular debris.

3. Transfer the supernatant containing retroviral particles into an ultracentrifugation tube and centrifuge in a swinging-bucket rotor at 100,000 × *g* for 10 minutes.
   – Alternatively, perform centrifugation at 40,000 × *g* for 1 hour, or 22,000 × *g* for 2 hours at +4°C.
   – After this centrifugation step, no clearly visible pellet will have formed since virus particles will be present in only low concentration. Therefore, always label the centrifugation tubes with a pen and orient the tubes so that the approximate position of the pellet containing the viral particles is known.

4. Discard the supernatant and proceed as described in the section, **Reverse transcriptase assay**.
   – Alternatively, store the pellets at −60°C or below.

Isolation of virus particles by PEG precipitation

1. Centrifuge suspension cells at 250 × *g* for 10 minutes in a refrigerated centrifuge at +4°C.
   – Transfer supernatant into a fresh centrifugation tube.
   The initial centrifugation step at 250 × g is particularly important when only reverse transcriptase activity that is released into the culture supernatant is to be measured. For adherent cells, the initial centrifugation step may be omitted.
   – If required, resuspend the cells in culture medium according to the particular cell type's media needs for further cultivation.

2. Centrifuge supernatant at 2,000 × *g* for 30 minutes or 8,000 × *g* for 10 minutes in a refrigerated centrifuge at +4°C.

3. Transfer supernatant into a fresh centrifugation tube.
   – Add 1 ml PEG solution per 2 ml supernatant.
   – Mix the resulting mixture thoroughly and incubate overnight on ice.

4. Centrifuge at 800 × *g* for 45 minutes or 8,000 × *g* for 10 minutes in a refrigerated centrifuge at +4°C.

5. Discard supernatant; carefully remove the last drops of resting PEG solution and proceed as described in the section, **Reverse transcriptase assay**.
   – Alternatively, store the pellets at −60°C or below.

Total removal of the PEG solution is essential. Residual PEG solution may inhibit RT activity, interfering with the reverse transcription.

For reasons that are not clearly understood, some batches of PEG are not suitable for virus isolation as they produce a pellet that is difficult to resuspend. Therefore, always test the particular batch prior to use.

Preparation of HIV-1 reverse transcriptase working dilutions

To produce a calibration curve for reverse transcriptase, use duplicates of the seven concentrations listed below. The appropriate amount of HIV-1 reverse transcriptase working dilution, prepared as described, is diluted in Lysis buffer and used in the Reverse Transcriptase Assay, see section **Reverse transcriptase assay**.

1. Prepare the HIV-1-RT working dilutions in sterile reaction tubes in 1:2 dilution steps as shown in the table.
   – To avoid carryover of the higher concentrated solution to the lower concentrated samples, use a fresh pipette tip for each dilution step.
   Prepare HIV-1-RT dilutions in duplicates to ensure accurate results.

Step	HIV-1-RT [µl]	Lysis Buffer (solution 4) [µl]	HIV-1-RT Concentration [ng/well]
0	0	150	0
1	10 (solution 1)	390	2.0
2	150 of step 1	150	1.0
3	150 of step 2	150	0.5
4	150 of step 3	150	0.25
5	150 of step 4	150	0.125
6	150 of step 5	150	0.0625

2. Transfer 40 µl of each working dilution into a reaction tube.
   – The sample containing no HIV-1-RT (Step 0) serves as a negative control.

Reverse transcriptase assay

Lysing the retroviruses

1. Resuspend the pellet in 40 µl Lysis buffer (solution 4) and transfer the suspension to a fresh reaction tube.

2. Incubate at +15 to +25°C for 30 minutes to completely solubilize viral particles.
   For cell cultures producing high levels of virus progeny, dilute lysate with lysis buffer immediately before use in the Reverse transcriptase assay. Perform the Reverse transcriptase assay with 40 µl of the diluted solution.

3. Store solubilized pellets at +2 to +8°C for approximately 1 week or at −60°C or below for approximately 1 month.

4. If a large number of samples is to be processed, transfer the pellets into a sterile 96-well microplate to perform the lysis of virus particles and the RT reaction.
   – This allows for easy transfer of the samples into the Microplate modules using a multichannel pipette.

⚠ ***Do not use the streptavidin-coated Microplate supplied with the kit for the reverse transcriptase reaction.***

Reverse transcriptase reaction

⚠ ***Perform the reaction under nuclease-free conditions.***

1. Add 20 µl reaction mixture (solutions 3a or 3b) to each reaction tube containing virus lysates or HIV-1-RT standards, prepared as described in sections **Preparation of HIV-1 reverse transcriptase working dilutions** and **Lysing the retroviruses**.

2. Incubate for 1 to 15 hours at +37°C.

ELISA assay

⚠ ***Perform the assay under nuclease-free conditions.***

1. Open enough foil bags for the number of Microplate modules to be used.
   – Place them into the frame in the correct orientation.
   The correct fitting ensures a tight support of the Microplate modules.
   – The Microplate modules are ready to use and do not need to be rehydrated prior to addition of the samples.

2. After finishing the reverse transcriptase reaction, transfer the samples (60 µl) and the HIV-1-RT working dilutions for establishing the calibration curve (60 µl) into the wells of the Microplate modules according to the pipetting scheme below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	Bl	P1	P1	–	–	–	–	–	–	–	–
B	S0	S0	–	–	–	–	–	–	–	–	–	–
C	S1	S1	–	–	–	–	–	–	–	–	–	–
D	S2	S2	–	–	–	–	–	–	–	–	–	–
E	S3	S3	–	–	–	–	–	–	–	–	–	–
F	S4	S4	–	–	–	–	–	–	–	–	–	–
G	S5	S5	–	–	–	–	–	–	–	–	–	–
H	S6	S6	–	–	–	–	–	–	–	–	P40	P40

Legend:
Bl = blank (only ABTS substrate)
S0 to S6 = HIV-1-RT working dilutions
P1 to P32 = samples 1 to 32

– Since the ABTS substrate (solution 7) is slightly green in color, leave one well free in order to determine the blank (baseline) value. Add ABTS substrate into this well for use as a reference when measuring the Microplate modules in the ELISA reader. Most readers can be programmed to automatically substract the blank value from the other samples.

3. Cover the Microplate modules with a Self-adhesive Cover Foil and incubate for 1 hour at +37°C.

4. Remove the solution completely.

5. Rinse 5 times with 250 µl of Washing buffer per well (solution 6) for 30 seconds each.
   – Carefully remove Washing buffer.

6. Add 200 µl of Anti-DIG-POD working dilution (200 mU/ml, solution 5a) per well.

7. Cover the Microplate modules with a Self-adhesive Cover Foil and incubate for 1 hour at +37°C.

8. Remove the solution completely.

9. Rinse 5 times with 250 µl of Washing buffer per well (solution 6) for 30 seconds each.
   – Carefully remove Washing buffer.

10. Add 200 µl of ABTS substrate solution (solution 7) or ABTS substrate solution with substrate enhancer (solution 7a) per well.

11 Incubate at +15 to +25°C until color development (green) is sufficient for photometric detection, approximately 10 to 30 minutes.

12 Using a microplate (ELISA) reader, measure the absorbance of the samples at 405 nm (reference wavelength: approximately 490 nm).

Quantification of the inhibitory effect of reverse transcriptase inhibitors

For the quantification of the inhibitory effect of reverse transcriptase inhibitors, a reverse transcriptase (e.g., the reverse transcriptase, HIV-1 included in the kit or any other reverse transcriptase) can be used in conjunction with the Reverse Transcriptase Assay, colorimetric. All steps of the Reverse Transcriptase Assay, including the RT reaction can be performed directly in the Microplate modules supplied with the kit. Inhibitory activity of reverse transcriptase inhibitors is usually calculated as percent inhibition as compared to a sample that does not contain an inhibitor. Therefore, an HIV-1-RT calibration curve is not required.

ELISA assay

1 Add 4 to 6 ng recombinant HIV-1-RT, diluted in Lysis buffer (20 µl/well) in a separate reaction tube.
– Use Lysis buffer with no HIV-1-RT added as a negative control.
– Add 20 µl of RT inhibitors diluted in Lysis buffer and 20 µl reaction mixture (solutions 3a or 3b) per reaction tube and incubate for 1 hour at +37°C.

2 Open enough foil bags for the number of Microplate modules to be used.
– Place them into the frame in the correct orientation.
i The correct fitting ensures a tight support of the Microplate modules.
– Microplate modules are ready to use and do not need to be rehydrated prior to addition of the samples.

3 Transfer the samples (60 µl) into the wells of the Microplate modules.

4 Cover the Microplate modules with a Self-adhesive Cover Foil and incubate for 1 hour at +37°C.

5 Remove the solution completely.

6 Rinse 5 times with 250 µl of Washing buffer per well (solution 6) for 30 seconds each.
– Carefully remove washing buffer.

7 Add 200 µl of Anti-DIG-POD working dilution (200 mU/ml, solution 5a) per well.

8 Cover the MicroplIte modules with a Self-adhesive Cover Foil and incubate for 1 hour at +37°C.

9 Remove the solution completely.

10 Rinse 5 times with 250 µl of Washing buffer per well (solution 6) for 30 seconds each.
– Carefully remove washing buffer.

11 Add 200 µl of ABTS substrate solution (solution 7) per well and incubate at +15 to +25°C until color development (green) is sufficient for photometric detection, approximately 10 to 30 minutes.

12 Using a microplate (ELISA) reader, measure the absorbance of the samples at 405 nm (reference wavelength: approximately 490 nm).

i Shaking of microplates at 250 rpm during incubation with substrate solution can be employed to shorten the incubation period, but is not essential. If shaking is not carried out, gently tap on the side of the microplate before measuring absorbance to ensure a homogeneous distribution of the colored reaction product.

2.3. Parameters

Sensitivity

The Reverse Transcriptase Assay detects 20 pg HIV-1 reverse transcriptase in a 1 hour reaction. 1 pg can be detected in an overnight reaction, see Figure 1, section **Results, Interpretation**.

Specificity

The assay specifically detects the activity of natural or recombinant retroviral reverse transcriptase. Anti-DIG-POD is specific for digoxigenin (DIG).

3. Results

Reverse transcriptase assay

Interpretation

The resulting signal intensity is directly proportional to the actual RT activity. Plotting of the averages of the absorbance readings on the Y-axis versus the concentration of the HIV-1-RT working dilutions on the X-axis results in a linear calibration curve, such as in Figure 1. The reverse transcriptase concentration of unknown samples can then be determined by plotting the observed absorbance values on the Y-axis, extrapolating to meet the calibration curve, and reading the RT concentration from the X-axis. To obtain reliable results, the absorbance values of the unknown sample should lie within the linear portion of the calibration curve. Establish a new calibration curve each time the Reverse Transcriptase Assay, colorimetric is performed.

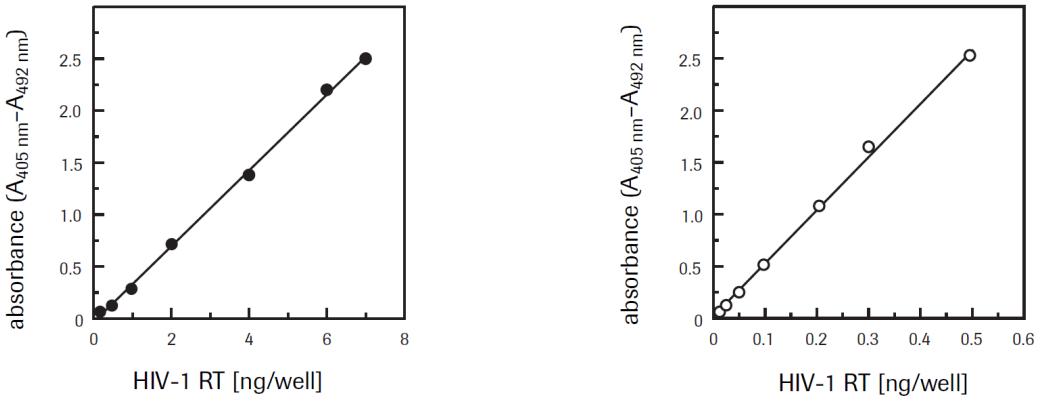

Fig. 1: Typical calibration curve using ABTS substrate without substrate enhancer. After an enzyme reaction of 1 hour (●) or 15 hours (○), respectively, absorbance was determined at 405 nm with a reference wavelength at 492 nm.For a qualitative determination of virus progeny, compare cell cultures that are infected with virus with cell cultures that do not contain virus particles. After 4 to 10 days, infection of HIV-1-permissive cell cultures with HIV-1 leads in most cases to detectable amounts of RT activity in the cell culture supernatant, depending on the cell culture conditions.

Additional recommendations

- Shaking microplates at 250 rpm during incubation with substrate solution can be employed to shorten the incubation period, but is not essential. If shaking is not carried out, gently tap on the side of the microplate before measuring absorbance to ensure a homogeneous distribution of the colored reaction product.
- After 1 hour incubation time with the reaction mixture, reverse transcriptase activity can be detected down to a level of 20 pg per well. For such a standard application (1 hour at +37°C) using HIV-1-RT at a concentration of 2 ng per well, an absorbance of approximately 2.3 absorption units ($A_{405\ nm}$ to $A_{490\ nm}$) is observed after 10 to 20 minutes substrate reaction when using ABTS substrate without substrate enhancer (solution 7).
- Incubation of samples for 2 hours at +37°C results in an increase in sensitivity by a factor of 1.5 to 2. To achieve maximal sensitivity, the incubation of the samples with RT can be prolonged.
- After a 15 hour incubation, 1 to 5 pg RT per well is detectable using ABTS substrate without substrate enhancer (solution 7).
- The use of ABTS substrate containing substrate enhancer (solution 7a) approximately doubles the sensitivity of the assay. If poor results were obtained when using the POD substrate without substrate enhancer (solution 7), remove solution 7 and replace with solution 7a. To do this, wash each well twice with Washing buffer (solution 6) and incubate with ABTS substrate solution with substrate enhancer (solution 7a) as described above to increase sensitivity. Using the same procedure, an incubation with any of the substrate solutions may be followed by incubation with another substrate solution to adapt sensitivity. An additional substrate reaction can only be performed when the preceding incubation step has not been stopped (e.g., using sulfuric acid).

Quantification of the inhibitory effect of reverse transcriptase inhibitors

Interpretation

The resulting signal intensity is directly proportional to the actual RT activity. To compare the inhibitory activity of RT inhibitors, calculate percent inhibition as compared to a sample that does not contain an inhibitor.

4. Additional Information on this Product

4.1. Test Principle

Retroviruses are widespread in nature, and the many hundreds of isolates can be classified into groups according to biological properties, morphology, and genome structure. The entire family is characterized by the presence of the enzyme reverse transcriptase in the virions. Reverse transcriptase is required for the unique retroviral type of multiplication. After entering the cell, the uncoated viral RNA is transcribed into a double-stranded DNA copy by reverse transcription. This copy is then integrated into the cellular DNA as a provirus. The provirus is transcribed by the cellular DNA-dependent polymerase II into the viral messenger RNA.
Assaying for RT activity has been broadly applied in testing retroviral propagation *in vitro*. RT is required for early proviral DNA synthesis and is therefore a prime target for anti-retroviral therapy. Reverse transcriptases generally use RNA that is complexed with various primers as a template for DNA synthesis. Classically, for detection or quantification of RT activity, the amount of incorporated radioactively labeled nucleotides is measured. For the detection and quantification of retrovirus progeny in cell cultures and the screening of large numbers of antiviral compounds or RT inhibitors, a non-isotopic RT assay was developed.

Principle

The Reverse Transcriptase Assay, colorimetric takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly (A) × $(dT)_{15}$. It avoids the use of [^{3}H]- or [^{32}P]-labeled nucleotides employed for the setup of the classical RT assay. In place of radiolabeled nucleotides, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule, which is freshly synthesized by the RT. Alternatively, the flexibility of the assay allows for the use of a template/primer hybrid of individual choice, such as a viral template in place of the template/primer hybrid supplied with the kit.
See Figures 2 and 3 for comparisons of the Reverse Transcriptase Assay, colorimetric versus using radioactively labeled nucleotides.

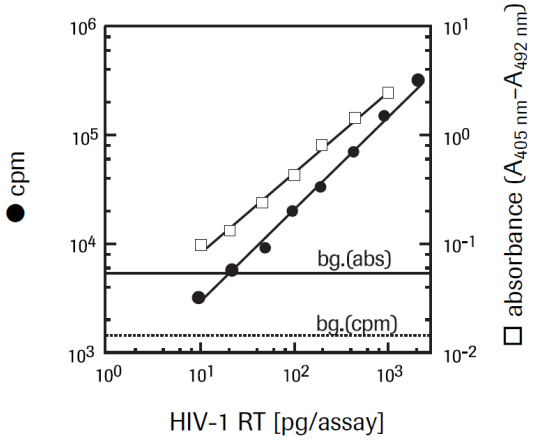

Fig. 2: Comparison of the Reverse Transcriptase Assay, colorimetric with the traditional radioisotopic assay. Various concentrations of recombinant HIV-1 reverse transcriptase were assayed with the Reverse Transcriptase Assay, colorimetric without substrate enhancer, using the protocol in the Instructions for Use (□), or with a standard RT assay, measuring the incorporation of the radioisotope [^{3}H]-dTTP (●) into the template/primer hybrid poly (A) x oligo $(dT)_{15}$ supplied with the kit. Both assays were performed following an enzyme reaction of 1 hour and allowed for the specific detection of approximately 10 pg HIV-1 reverse transcriptase per sample. The nonspecific background activity determined in samples without reverse transcriptase was at an absorbance of 0.054 for the Reverse Transcriptase Assay, colorimetric [bg (abs)] and at 1266 cpm for the isotopic RT assay [bg (cpm), dashed line]. The detection limit was defined to be a signal level of twice the background.

4. Additional Information on this Product

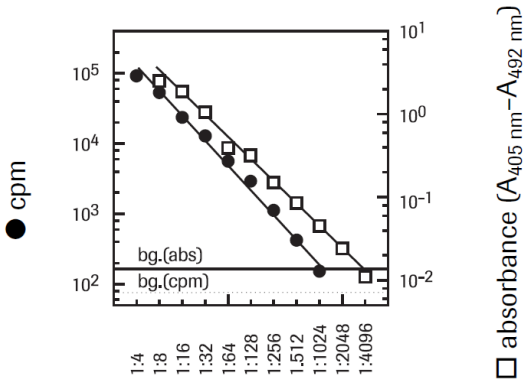

Fig. 3: Comparison of reverse transcriptase levels in various dilutions of HIV-1 enriched by PEG precipitation from 1 ml culture supernatant of HIV-1-infected HUT 78 cells. RT activity was determined with the Reverse Transcriptase Assay, colorimetric without substrate enhancer, using the protocol in the Instructions for Use (□), or with a standard RT assay measuring the incorporation of the radioisotope [^{3}H]-dTTP (●) into the template/primer hybrid poly (A) × oligo $(dT)_{15}$ supplied with the kit. The background activity with analogous preparations of the culture supernatant of non-HIV-1-infected HUT 78 cells (diluted 1:8) after subtraction of the nonspecific background activity was at an absorbance of 0.01 for the Reverse Transcriptase Assay, colorimetric [bg (abs.)] and at 153 cpm for the isotopic RT assay [bg (cpm), dashed line]. With the detection limit defined to be a signal level of twice the background, RT activity was detected with both methods in culture supernatant at a dilution of 1:1024.
The detection and quantification of synthesized DNA as a parameter for RT activity is based on the a sandwich ELISA protocol (Fig. 4).

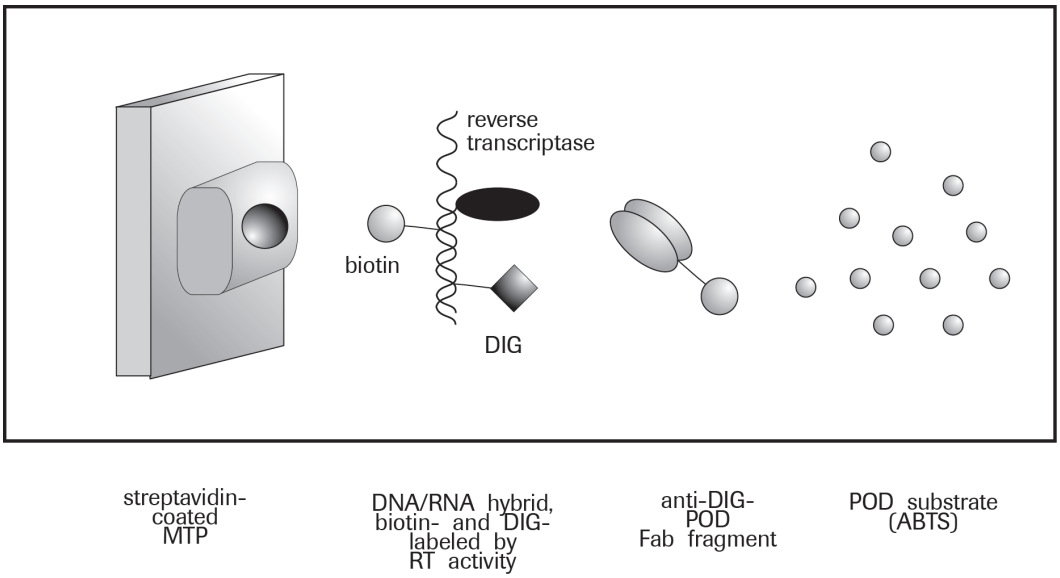

Fig. 4: Test principle

1. Biotin-labeled DNA binds to the surface of the Microplate modules that have been precoated with streptavidin.

2. An antibody to digoxigenin conjugated to peroxidase (Anti-DIG-POD) binds to the digoxigenin-labeled DNA.

3. The peroxidase substrate ABTS is added.
   – The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product.

4. The absorbance of the samples can be determined using a microplate (ELISA) reader and is directly correlated to the level of RT activity in the sample, see Figure 1, section **Results, Interpretation**.

5. Supplementary Information

5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

5.2. Changes to previous version

Update to include new safety Information to ensure handling according controlled conditions.

5.3. Trademarks

ABTS is a trademark of Roche.
All other product names and trademarks are the property of their respective owners.

5.4. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

5.5. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

5.6. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

5.7. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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