Published in final edited form as:

Methods Mol Biol. 2016 ; 1354: 61–70. doi:10.1007/978-1-4939-3046-3_5.

HIV-1 Reverse Transcriptase based assay to determine cellular dNTP concentrations

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Summary

Deoxynucleoside triphosphates (dNTPs) are the building blocks of DNA and their biosynthesis are tightly regulated in the cell. HPLC-MS and enzyme-based methods are currently employed to determine dNTP concentrations from cellular extracts. Here, we describe a highly efficient, HIV-1 reverse transcriptase (RT)-based assay to quantitate dNTP concentrations. The assay is based on the ability of HIV-1 RT to function at very low dNTP concentrations, thus providing for the high sensitivity of detection.

Keywords

dNTP concentration; HIV-1; reverse transcriptase; deoxynucleoside triphosphates

1. Introduction

DNA polymerases use deoxynucleoside triphosphates (dNTPs) as substrates during DNA replication. dNTPs are generated by either the *de novo* pathway using ribonucleotide reductase, or by the deoxynucleoside salvage pathway. The functional activities of DNA polymerases are dependent on cellular dNTP concentrations, meaning that enzymes with high steady state K_m and presteady state K_d values (low dNTP binding affinity) require high dNTPs concentrations in order to function efficiently. In normal replicating cells, chromosomal DNA synthesis by DNA polymerase occurs during the S phase of cell division, when dNTP biosynthesis is most active and cellular dNTP concentrations are highest. For cancer cells and transformed cell lines, cellular dNTP concentrations are increased due to their uncontrolled cell division. In primary terminally differentiated, non-dividing cells, such as macrophages or neurons, have very low dNTP concentrations due to their lack of robust dNTP biosynthesis. Measuring the cellular dNTP concentrations in these cell types requires a highly sensitive and reliable assay to accurately detect the small quantities of dNTPs present. Indeed, high performance liquid chromatograph-mass spectrometry (HPLC-MS) and polymerase-based dNTP assay have been developed to

determine cellular dNTP concentrations, which will be described in this section. For HPLC-MS, a standard curve for each dNTP needs to be routinely generated to validate the assay and then be used to quantitate dNTP concentrations for samples. Although HPLC-MS is very accurate and quantitative, major drawbacks of the method are: 1) the requirement of enough biomass to detect dNTPs over background noise, 2) the time required for sample collection on the machine 3) matrix effect (contaminants may change the profile) and 4) time required for data analysis. Several polymerase-based dNTP assays have been developed using DNA polymerase I (Klenow fragment) (1), *Taq* DNA polymerase (2) or human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) (3). The ability to detect very low concentrations of dNTPs will depend upon the K_d for the particular enzyme used in a given assay. Klenow has a K_d of 18 μ M (4), whereas the K_d of HIV-1 RT ranges between 0.3 and 3.9 μ M (5), allowing it to function under low substrate conditions.

2. Materials

2.1. Cell Lysis

1. Prepare 65% v/v methanol and store at -20°C before use.
2. PBS without magnesium chloride or calcium chloride.

2.2 Primer and Template Labeling

1. DNA primer sequence is 5'-GTCCCTCTTCGGGCGCCA-3'
2. DNA template sequences are: 5'-ATGGCGCCCGAACAGGGAC-3', 5'-TTGGCGCCCGAACAGGGAC-3', 5'-GTGGCGCCCGAACAGGGAC-3', and 5'-CTGGCGCCCGAACAGGGAC-3'.
3. T4 Polynucleotide kinase (PNK) enzyme (10,000 units/ml)
4. 10 $\times$ PNK buffer: (700 mM Tris-HCl, 100 mM MgCl_2 , and 50 mM dithiothreitol. pH at 25°C : 7.6).
5. Gamma-[^{32}P] ATP (see Note 1).
6. Sodium chloride-Tris-EDTA (STE) buffer (10 $\times$): 5 M NaCl, 1 M Tris-HCl (pH 7.5), and 0.5 M EDTA.
7. Geiger counter.
8. Pipettes (P20 and P1000) and tips.

2.3 Reverse Transcription

1. Reconstitute the 18-mer oligo dT at 200 μ M in buffer: 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.
2. RT reaction buffer (4 $\times$): 100 mM Tris-HCl (pH 8.0), 400 mM KCl, 8 mM dithiothreitol, 20 mM MgCl_2 , and 0.4 mg/ml bovine serum albumin.

¹Institutional radiation training using radioactive isotopes should be completed before starting any experiments. Radiation safety procedures should be followed at all times. Proper personally protective equipment to limit exposure to radioactive materials should be worn at all times.

3. Recombinant HIV-1 Reverse Transcriptase (RT) (see Note 2).
4. Dialysis buffer (5×): 1 M Tris-HCl (pH 7.5), 0.5 M EDTA, 5 M NaCl, 50% glycerol.
5. 50 µM dNTPs (positive control) – dilute the 100 mM stocks from commercial supplier in water.
6. Stop dye: 99% formamide, 40 mM EDTA, 0.003 g/ml bromophenol blue and 0.003 g/ml xylene cyanol.

2.4 Urea Polyacrylamide Gel

1. Part A reagent: 20% acrylamide/bis solution (19:1), 8 M urea, 0.1 M Tris, 0.08 M borate, 1 mM EDTA and 0.075% TEMED.
2. Part B diluent: 8 M urea, 0.1 M Tris, 0.08 M borate, 1 mM EDTA and 0.075% TEMED.
3. Ammonium persulfate - 10% solution in water.
4. 10× Tris-Borate-EDTA (TBE) buffer (890 mM Tris, 890 mM boric acid, 20 mM EDTA. pH at 25 °C: 8.0).
5. Whatman filter paper (No 1) (46 × 57 cm sheets).
6. Plastic wrap (18 inches wide).
7. Gel dryer.
8. Radioactive waste containers – liquid and dry.
9. Protective beta radiation shielding.
10. Beta radiation microcentrifuge tube rack.

2.5 Data Capture and Analysis

1. Phosphorimager screen.
2. Phosphorimager instrument.
3. Data analysis software such as QuantityOne from BioRad Imagine.

3. Methods

3.1 Processing cells for dNTPs

3.1.1 A) Working with non-adherent cells

1. Determine the number of cells/ml and resuspend cells at a final of 2×10^6 cells/ml (see Note 3).

²Recombinant HIV-1 reverse transcriptase can be obtained by commercial sources such as Chimex and EMD Millipore. We overexpress the His-tagged p66 subunit from HIV-1 strain HXB2 in *E. coli* and purify it using a nickel column chromatography.

³If using cancer cell lines, 600,000 cells can be used since the dNTP concentrations are very high. Importantly, empirical analysis may be required to determine the minimum number of cells needed for a given cell type. For example, primary human monocytes have very

2. Transfer 2×10^6 cells to a 1.5 ml eppendorf tube and close the top.
3. Microcentrifuge the eppendorf tube at 2000 *g* for 15 seconds.
4. Remove supernatant and wash cell pellet with 1 ml of PBS.
5. Pellet cells by centrifugation the tube at 2000 *g* for 15 seconds.
6. Carefully remove PBS and do not disrupt the cell pellet.
7. The cells are lysed by quickly adding 200 μ l of ice-cold 65% methanol to the tube.
8. Vigorously vortex sample for 2 minutes.
9. Completely lyse the cells by incubating the tube at 95 °C for 3 minutes. Make sure an eppendorf tube lid lock is securely in place to prevent the lid from opening during the 95 °C incubation. Do not use Parafilm M to seal the tube since it may melt at 95 °C.
10. Chill the tube on ice for 1 minute to prevent burning your hands and loss of material due to lid opening.
11. The tube is centrifuged for 3 minutes at 18000 *g*. Next, the 65% methanol solution is transferred to a new labeled tube. Discard the tube with the cell pellet in proper waste receptacle.
12. Speed vacuum the tube until the liquid is completely evaporated. This processes usually takes 1 to 2 hours at 55 °C.
13. Store the tube at –80 °C until you are ready to perform the HIV-1 RT-based dNTP assay (see Note 4).

3.1.2 B) Working with adherent cells

1. Wash cell monolayer twice with PBS (see Note 5).
2. Lyse the cells by quickly adding 200 μ l of ice cold 65% methanol for a 6-well plate. Larger volumes of methanol can be used for petri dishes, however try and keep the volumes under 1.5 ml to fit into an eppendorf tube.
3. Use a cell scraper to remove cells from plate. Wash the 6-well by adding another 200 μ l of ice cold 65% methanol in order to recover all the biomaterial. Place all the material into one tube. (Place the tubes on ice if processing multiple samples.)
4. Follow steps 8–13 as indicated for the non-adherent cells protocol above.

3.2 Primer labeling

To determine the concentration of each dNTP, four separate primer-template combinations are needed. The template provides specificity by having one additional nucleotide (Section

low dNTPs and even 5 million cells are just at the level of detection for this assay. Therefore, we recommend 2×10^6 cells/ml to provide enough biomaterial for dNTP analysis for most cell types.

⁴Dried sample pellets can be stored indefinitely at –80 °C.

⁵Cell count is required in order to calculate the concentration of dNTPs per cell. Always have an extra experimental well for adherent cells to do cell counts for each experimental group.

2.2). Therefore, we ^{32}P -radiolabel the primer in four separate tubes and then add one of the four templates to the tube.

1. In four 1.5 ml eppendorf tubes, combine 4 μl of 20 μM primer, 23 μl of water, 4 μl of 10 $\times$ PNK buffer. This can be done at the bench. If only one of the four dNTPs will be evaluated, then only one tube with reaction components is needed.
2. Next, move the tubes behind a beta shield. While wearing proper protective equipment open the radiation container and add 5 μl of gamma- ^{32}P ATP to the first tube. Discard pipet tip in the solid radioactive waste. Move to the next tube and repeat until all the tubes have radiation added.
3. Add 2 μl of PNK enzyme to each tube. Change the pipet tip between tubes.
4. Incubate the tubes in a heating block at 37 $^{\circ}\text{C}$ for 30 minutes.
5. Add an addition 2 μl of PNK enzyme to each tube. Remember to change the pipet tip between tubes and discarding the tips in the solid radioactive waste container. Incubate the tubes for another 30 minutes at 37 $^{\circ}\text{C}$.
6. Stop the PNK enzyme reaction by placing the tubes in a heating block set at 95 $^{\circ}\text{C}$ for 10 minutes.
7. Removed tubes from the 95 $^{\circ}\text{C}$ heating block. Allow the tubes to cool to room temperature behind beta radiation shielding. Cooling usually takes about 5 minutes.
8. Add 12 μl of 20 μM of template to the tube. Each tube with ^{32}P -labeled primer will have a different template added to it. Make sure to identify the template used and the date of ^{32}P -labeling on the tubes.
9. Add 10 μl of 10 $\times$ STE, 38 μl of water and 700 μl of 1 $\times$ STE.
10. To anneal the different primer/templates, place a lid lock on the tubes and then incubate tubes in a heating block set at 95 $^{\circ}\text{C}$ for 10 minutes.
11. Remove the block from the heating apparatus. Allow the block with the tubes in it to cool to room temperature. This usually takes about 1 hour to cool down to room temperature.
12. The different primer/templates are now ready to be used in the HIV-1 RT-based dNTP assay (see Note 6). Place the tubes containing the ^{32}P -labeled primer/templates in a beta radiation microcentrifuge tube rack. Store the radioisotope as per your institute radiation safety guidelines.
13. Clear up your area using the Geiger counter to detect any radioactive contamination.

⁶One can store the primer/template at room temperature at room temperature for several weeks. If one freezes the ^{32}P -labeled primer/template, reannealing it is required. This is accomplished by heating the tube, with a lid lock on, in a heating block to 95 $^{\circ}\text{C}$ for 5 minutes. Next, remove the block from the apparatus and allow it to slowly cool to room temperature, which usually takes about one hour.

3.3 HIV-1 RT-based dNTP assay

1. If the sample tubes were stored at -80°C (Section 3.1.), allow the tubes to equilibrate to room temperature for 5 minutes before adding 20 μl of RNase/DNase free water to each tube. Vortex the tube for 30 seconds to suspend the pellet. Next, microcentrifuge the tube for 1 minute at 18000 g to pellet cellular debris, dNTPs will be in solution. Small batches of tubes (1–20 tubes) can be kept at room temperature on the bench if the reactions are done that day, otherwise store them at -80°C (see Note 4).
2. Mark reaction tubes for samples, and include both positive and negative control tubes for each ^{32}P -labeled primer/template.
3. Prepare reaction master mix. Calculate the appropriate volumes for each of the components below while have enough for two additional reactions. The reaction master mix for one sample volume is 5 μl of 4 $\times$ RT reaction buffer, 1 μl of oligonucleotide dT, 6 μl of water and 2 μl of ^{32}P -radiolabeled primer/template. Remember to work behind protective shielding and be wearing proper protective equipment when handling the ^{32}P -radiolabeled primer/templates
4. Aliquot 14 μl of the reaction master mix into each of the marked tubes. Next, add 2 μl of the sample to the proper tube. The negative control tube has 2 μl of water added, while the positive control has 2 μl of 50 nM dNTPs added to it. The next set is to prepare for the enzymatic reaction. Set a timer for 5 minutes. To the first tube add 4 μl of HIV-1 RT enzyme (see Note 2) and place the tube at 37°C . Discard the pipet tip in the solid radiation waste and then start the timer. Move to the second tube and allow a 5 second interval before adding the enzyme. Place the tube in the heating block. Repeat the process until all the sample reactions have enzyme added.
5. At the end of five minutes, stop the reactions by adding 10 μl of stop buffer and then placing the tube in a heating block set at 95°C . Stop the reaction in the order in which they were started. After 5 minutes, remove the tubes from the heating block and allow them to cool to room temperature behind proper shielding. Samples are now ready to be resolved on a 14% urea-PAGE. Alternatively, reactions can be stored at 4°C for several weeks in a beta radiation microcentrifuge tube rack, but remember the decay of the radioisotope is occurring and this will lead to longer imaging screen exposure times.

3.4 Urea-PAGE

1. Clean both the large and the small glass plates with ethanol for the apparatus. Treat the small plate with a thin layer of silicone-based rain repellent and assemble PAGE apparatus.
2. Cast a 14% urea-PAGE. Volumes of Part A reagent and Part B diluent will depend on apparatus size. Refer to manufactures recommendations for total volume required. Wait 30 minutes for gel polymerization to occur.
3. Remove the comb and place the glass plates into the gel apparatus. Add 1 $\times$ TBE running buffer to the top and bottom reservoirs. Flush the wells out with running

buffer using a 20 ml syringe with an 18-gauge needle. Then pre-warm the gel (100 watts) for 10 minutes. Next, flush the wells again with running buffer. Count the number of wells needed and position your samples in the middle of the gel. The first and last wells should contain 4 μ l of stop reaction buffer, with 4 μ l of the samples loaded in between. Run the urea-PAGE until the bromophenol blue dye runs into the lower reservoir (15 inches from the bottom of the comb well), to provide enough separation between the primer and the primer +1 product (extended product).

4. Carefully disassemble the apparatus to remove the glass plates. Use a plastic wedge to separate the two plates, being careful to keep the gel intact on one plate (usually the large, non-silicone-based treated plate). The plate should be on the counter top with the gel on top. To remove the gel from the glass plate, cover the gel with seal wrap and fold over the edges back onto the seal wrap. Turn the plate over and using the plastic wedge carefully, remove the gel from the plate, which should be sticking to the plastic wrap.
5. Detect where the radiation is using a hand-held Geiger counter. Cut a piece of Whatman paper length-wise so it is longer than the first and last stop dyes and about 3 inches wide. One can use the xylene cyanol dye as a reference point in order help position the Whatman paper. Press the Whatman paper on the gel. Use scissors to cut the edge of the Whatman paper, removing the remaining non-radioactive gel to be discarded in the dry radioactive waste container. Dry the gel using a vacuum gel dryer for at least 45 minutes. A very large gel can take up to 3 hours to dry.
6. Clean up area by discarding the remaining gel in the solid radioactive waste and the running buffer in the liquid radioactive waste. Clean the PAGE apparatus with water and allow it to air-dry.

3.5 Phosphorimaging and Data Analysis

1. After the gel has dried onto the Whatman paper, expose a phosphorimaging screen. The duration of time for exposure will be dependent on the amount of radioactivity. When using freshly ^{32}P -labeled primer/template, screen exposure time will be for 1–2 hours to prevent oversaturation. Capture the data using phosphorimager instrument. We use the program software to ensure that the screen is not oversaturated; otherwise a shorter exposure is required. Figure 1 shows a typical gel.
2. Data analysis for dNTPs is done using the vendor's software. As depicted in Figure 1, a rectangle is set and then applied to the negative control Primer +1. This accounts for the amount of background noise. Apply this same rectangle to Primer and Primer +1 bands for the samples. The densities allow for determining the amount of extension over the total amount of primer in that lane. Table 1 shows how the dNTP concentrations are calculated. As shown in Table 1, a box is set in the negative control Primer +1 region, which is used for an internal blank to be subtracted from all the samples (Table 1, Column D). The amount of extension is

calculated by Primer +1/ (Primer +1 and Primer) (Column E). This is converted to a percentage (Column F). Values below 2% are below the linear range of the assay and need to be discarded. Extensions over 36% require additional dilution of that sample (3) (see Note 7). Column G is the amount of volume the sample was suspended in, which is typically 20 μL . Column H is the determined cell number at the time of dNTP harvesting. The fmol/reaction is calculated using the following formula: $((200 \text{ fmol of Primer} \times \% \text{ Extension} \times \text{Volume Suspended cell pellet} \times \text{Dilution})/(\text{Volume added to the reaction}))$ and displayed in Column K. The fmol/Cell Number is then calculated by dividing the fmol/Rxn by the cell count (Column L). If the cell volume is known then the molar concentration can be determined as depicted in Column M. The cell volumes for macrophage, resting CD4⁺ T cells and activated CD4⁺ T cells are 2660, 186, and 320 μm^3 , respectively (3). For dendritic cells (6) and HeLa cells (7) the cell volumes are 1000 and 2600 μm^3 , respectively (see Note 8).

Acknowledgments

This work was supported by National Institutes of Health grants AI049781 and GM104198 (B.K.).

References

1. Ferraro P, Franzolin E, Pontarin G, Reichard P, Bianchi V. Quantitation of cellular deoxynucleoside triphosphates. *Nuc Acids Res.* 2010; 38:e85.
2. Wilson PM, Labonte MJ, Russell J, Louie S, Ghobrial AA, Ladner RD. A novel fluorescence-based assay for the rapid detection and quantification of cellular deoxyribonucleoside triphosphates. *Nuc Acids Res.* 2011; 39:e112.
3. Diamond TL, Roshal M, Jamburuthugoda VK, Reynolds HM, Merriam AR, Lee KY, Balakrishnan M, Bambara RA, Planelles V, Dewhurst S, Kim B. Macrophage tropism of HIV-1 depends on efficient cellular dNTP utilization by reverse transcriptase. *J Biol Chem.* 2004; 279:51545–51553. [PubMed: 15452123]
4. Reineks EZ, Berdis AJ. Evaluating the contribution of base stacking during translesion DNA replication. *Biochem.* 2004; 43:393–404. [PubMed: 14717593]
5. Santos-Velazquez J, Kim B. Deoxynucleoside triphosphate incorporation mechanism of foamy virus (FV) reverse transcriptase: implications for cell tropism of FV. *J Virol.* 2008; 82:8235–8238. [PubMed: 18508890]
6. de Baey A, Lanzavecchia A. The role of aquaporins in dendritic cell macropinocytosis. *J Exp Med.* 2000; 191:743–748. [PubMed: 10684866]
7. Zhao L, Kroenke CD, Song J, Piwnicka-Worms D, Ackerman JJ, Neil JJ. Intracellular water-specific MR of microbead-adherent cells: the HeLa cell intracellular water exchange lifetime. *NMR Biomed.* 2008; 21:159–164. [PubMed: 17461436]

⁷Samples with over 36% extension require sample diluted and retested in order to bring them within the linear range of the assay.

⁸Conversion of literature reported cell volumes (μm^3). $1.0 \mu\text{m}^3 = 1 \times 10^{-15} \text{ L}$

Figure 1. HIV RT-based dNTP urea-PAGE analysis

Sample reactions were resolved on a 14% urea-PAGE. Imaging Screen K (BioRad) was exposed to the dried gel. The image data were captured using PharosFX Plus Molecular Imager (BioRad). Data analysis was accomplished by using QuantityOne software (BioRad). The unextended primer (Primer) and the extended primer (Primer +1) are shown. Lanes 1 and 2 are the negative and positive controls, respectively. Lane 3 was left empty. Lanes 4–8 were the samples to be quantitated. A quantity box was set to capture the raw data. U1, negative control (Primer +1 area) was used to subtract background. Boxes U2–U6 were for the unextended Primer values, whereas boxes U7–U11 captured data for extended Primer +1 values.

Table 1

Calculating dNTP concentrations

A	B	C	D	E	F	G	H	I	J	K	L	M
		CNT*mm2	(-) Blank	Extended	Percent Extended	Volume Resuspended (ul)	Cell Number	Vol/Rxn	Dilution	fmol/Rxn	fmol/cell number	nM
	Neg. Control	17681										
Sample 1	Primer	1138268	1120587	0.01	1.4	20	2000000	2	1	28.7	1.4E-05	5.5
Sample 2	Primer	1015468	997787	0.11	11.2	20	2000000	2	1	223.3	1.1E-04	42.9
Sample 3	Primer	97371	79690	0.93	92.9	20	2000000	2	1	1858.0	9.3E-04	357.3
Sample 4	Primer	981895	964214	0.13	13.0	20	2000000	2	1	260.8	1.3E-04	50.2
Sample 5	Primer	1033627	1015946	0.15	14.6	20	2000000	2	1	292.3	1.5E-04	56.2
Sample 1	Primer +1	34020	16339									
Sample 2	Primer +1	143072	125391									
Sample 3	Primer +1	1060461	1042780									
Sample 4	Primer +1	162278	144597									
Sample 5	Primer +1	191546	173865									

Extended: (Primer +1/(Primer + Primer +1/))
 fmole/rxn: ((200 × Extended × Volume Resuspended × Dilution)/(Volume/Rxn))
 fmol/cell number: ((fmol/Rxn)/Cell Number)
 nM: (fmol/cell number)/Cell Volume