Nonradioactive direct telomerase activity detection using biotin-labeled primers

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Abstract
Background: Telomerase is a ribonucleoprotein enzyme responsible for maintenance of telomere length which expressed in more than 85% of cancer cells but undetectable in most normal tissue cells. Therefore, telomerase serves as a diagnostic marker of cancers. Two commonly used telomerase activity detection methods, the telomerase repeated amplification protocol (TRAP) and the direct telomerase assay (DTA), have disadvantages that mainly arise from reliance on PCR amplification or the use of an isotope. A safe, low-cost and reliable telomerase activity detection method is still lacking.

Method: We modified DTA method using biotin-labeled primers (Biotin-DTA) and optimized the method by adjusting cell culture temperature and KCl concentration. The sensitivity of the method was confirmed to detect endogenous telomerase activity. The reliability was verified by detection of telomerase activity of published telomerase regulators. The stability was confirmed by comparing the method with TRAP method.

Results: Cells cultured in 32°C and KCl concentration at 200 mM or 250 mM resulted in robust Biotin-DTA signal. Endogenous telomerase activity can be detected, which suggested an similar sensitivity as DTA using radioactive isotope markers. Knockdown of telomerase assembly regulator PES1 and DKC1 efficiently reduced telomerase activity. Compared with TRAP method, Biotin-DTA assay offers greater signal stability over a range of analyte protein amounts.

Conclusion: Biotin-labeled, PCR-free, and nonradioactive direct telomerase assay is a promising new method for the easy, low-cost, and quantitative detection of telomerase activity.

Keywords
biotin-labeled primers, direct telomerase activity, nonradioactive
1 INTRODUCTION

Telomerase is a reverse transcriptase that consists of the protein subunit, TERT, and the RNA subunit, TERC. The main function of telomerase is to use its TERC as a template to add a repeated sequence (TTAGGG) to the end of the telomeres. This prevents the telomeres from shortening during cell division and thereby maintains telomere stability. Telomerase is undetectable in the majority of normal somatic cells, while it can be activated in more than 85% of tumor cells. Therefore, telomerase activity can be used as a marker to assist in the early diagnosis of tumors, to determine the prognosis of patients, and to detect recurrence. Thus, detection of telomerase activity has attracted much attention and a sensitive and reliable method is important for the clinical diagnosis and treatment evaluation of tumors.

The polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP) is currently the most commonly used method for telomerase detection because TRAP has extremely high sensitivity as a result of PCR amplification of the signal. However, TRAP faces several drawbacks. For example, cellular biomolecules such as bile salts, hemoglobin, heparin, and lactoferrin can inhibit Taq polymerase in the PCR reaction, thereby causing false-negative results. Additionally, PCR carries the risk of amplification errors, it is a time-consuming process, and while differences between samples are obvious at low levels of telomerase activity, this is not the case at high levels. Thus, TRAP cannot accurately reflect the continuous binding ability of telomerase. Therefore, many research teams continue to explore alternative methods for detecting telomerase, most of which are based on nanoparticle materials. These include colorimetry based on gold nanoparticles, exponential isothermal amplification of telomere repeat (EXPIATR), electrochemistry, fluorescence, surface-enhanced Raman scattering (SERS), surface plasmon resonance (SPR), and the intracellular telomerase assay. Although these methods do not incorporate PCR, and are sensitive, they still have their shortcomings including complicated manipulation, time-consuming procedures, or the need for elaborate instruments and expensive fluorescent labels. With the development of nanotechnology, a variety of biosensors based on nanomaterials are widely used in biomedical field. For the detection of telomerase activity, those methods have special sensitivity. However, there are many reasons to limit the application of nanomaterials in normal biochemistry laboratories. Firstly, nanomaterials need careful design. Secondly, the state of nanoparticles might be affected by proteins in cell lysate and resulted nonspecific interference. Finally, the detection of signals needs special instruments. The PCR-free direct telomerase assay (DTA) has attracted increasing attention in recent years. This method can accurately quantify telomerase activity and offers several advantages for telomerase research in the fields of cell biology and in vitro biochemistry. However, the DTA method uses radioactive isotope markers and is, therefore, subject to the risk of researcher exposure and environmental contamination. Besides, each experiment should use from 100–200µCi α-32P, and the associated costs are relatively large.

In view of the importance of telomerase activity detection, we intended to establish a simple and reliable method. Here, we explored a nonradioactive direct telomerase activity detection method, which we termed “Biotin-DTA.” This method does not pose any risks of radioactive contamination, and it is a promising method for the easy, low-cost, and reliable quantitative detection of telomerase activity.

2 MATERIALS AND METHODS

2.1 Plasmids, siRNAs, and reagents

PcDNA3.0-Flag-hTERT, pcDNA3.0-hTR, siPES1, siDKC1, and siNC were described previously. Lentiviral vectors for Flag-hTERT overexpression were obtained by inserting PCR-amplified gene fragments into pCDH-EF1-MCS-T2A-Puro (System Biosciences). Anti-Flag M2 agarose (A2220) was obtained from Sigma-Aldrich. The biotinylated telomeric DNA substrate Bio-L18GGG was synthesized and purified by Biomed Biotech with the sequences [5′-biotin-CTAGACCTGTGATCA(TTAGGG)3′-3′]. dNTPs, gel electrophoresis loading buffer, and ladder DNA were purchased from Sangon Biotech. D-biotin (#B4639) was purchased from Sigma.

2.2 Cell culture, transfection, and RNA interference

Two hundred and ninety-three T cells (human embryonic kidney cells) and liver cancer cell lines HepG2 were purchased from the American Type Culture Collection and have been previously tested for mycoplasma contamination. Cells were routinely cultured in Dulbecco’s Modified Eagle Medium (Macgene) with 10% fetal bovine serum (HyClone). PEI (Polysciences) was used for 293 T cell transient transfection. After transfection, cells were cultured in 37°C, 5% CO2 incubator for 6 h and then transferred to 32°C, 5% CO2 incubator for 48–72 h as indicated. RNAiMax (Invitrogen) was used for RNA interference according to the instructions.

2.3 Biotin-DTA

Cells were lysed with CHAPS lysis buffer (10 mM Tris–HCl at pH 7.5, 1 mM MgCl2, 1 mM EGTA, 0.5% 3-[(3-cholamidopropyl) dimethylammonio] propane sulfonate (CHAPS), 10% glycerol, 1 mM phenyl methanesulfonyl fluoride (PMSF), 1 mM DL-Dithiothreitol (DTT)]. Briefly, 1.0×10^6 cells were suspended in 1 ml of CHAPS, and then 5 µl RNase inhibitor (TAKARA) was added and rotated in a 4°C refrigerator for 1 h. Then, 150 µl of 2 M KCl was added into the cell lysates. Subsequently, the cell lysates were centrifuged for 30 min at 4°C (12,470 g). The supernatant was transferred into a new tube, quickly frozen in liquid nitrogen, and stored at −80°C for further use. Besides, 20 µl of supernatant could be aliquoted as “input” for
detecting efficiency of immunoprecipitation by western blot. For exogenous telomerase, the cell lysates were mixed with 60 µl of anti-Flag M2 agarose for 3 h. For endogenous telomerase, cell lysate was mixed with 20 µg of anti-hTERT antibody (abx120550, Abbexa) and rotated in a 4°C refrigerator for 2 h. Twenty microliters of Protein G agarose (Santa Cruz Biotechnology) was added and rotated for 1 h. After washing of the immunoprecipitates with 500 µl of CHAPS lysis buffer containing 300 mM KCl, telomerase was eluted with 3 µl of Flag peptide (5 mM solution in DEPC-treated H2O) or telomerase peptide antigen (N-ARPAAEATLSLEGALSGTRH-C). Then, immunoprecipitation eluate (IP eluate) containing purified telomerase was obtained. A telomerase substrate, 5′end labeled with biotin, was used for extension reaction at the presence of telomerase and dNTP. Each telomerase reaction solution contained 2–50 µl of IP eluate, 5 µl of 10×Telomerase reaction buffer (200 mM Tris–HCl pH 8.3, 15 mM MgCl2, 630 mM KCl, 0.5% Tween 20, 10 mM EDTA), 123–350 mM KCl, 0.5 µM Bio-L-18GGG, 1 mM dNTPs, 10 mM DTT, 10% glycerol and moderate amount of DEPC-treated H2O. Each mixture was incubated at 37°C overnight. For control experiments, CHAPS lysis buffer was used as negative control. After telomerase reaction was stopped by 25 µl of Stop Mix (20 mM EDTA), 20 µl of Dynabeads M-280 Streptavidin solution (Invitrogen) was added and rotated for 1 h. At the same time, 1×TBE (90 mM Tris base, 90 mM boric acid, and 1 mM EDTA) was used as electrode buffer for pre-electrophoresis, and 8 M urea and 10% polyacrylamide gels were run at 400 V for 1–1.5 h to make the electrophoresis temperature rise to more than 50°C, and could remove some impurities in the gel. Magnetic beads were collected by magnetic rack to remove supernatant, washed with 0.5 ml of Bind/Wash buffer (10 mM Tris–HCl at pH 7.5, 1 mM EDTA, and 2 M NaCl) three times and 0.5 ml of TE buffer (10 mM Tris–HCl at pH 7.5, 1 mM EDTA) one time, then mixed with 10 µl of Elution mix buffer (90% v/v formamide, 90 mM Tris base, 90 mM boric acid, 10 mM EDTA, 0.01% w/v xylene cyanol and 0.01% w/v bromophenol blue), 10% v/v D-Biotin solution (5 mM in Buffer TE)]. After treatment at 90°C for 10 min, centrifuged for 15 s at 2000 rpm, then placed on the magnetic rack for electrophoresis. After pre-electrophoresis, extension products were loaded onto the gel in 1×TBE buffer and the gel was run at 200 V constant voltage for about 38 min. Then the extension products were transferred to the nylon membrane from the gel by vacuum at the pressure of 0.8 bar for 50 min. The film crosslinked by UV at 150 mJ. Signal was detected according to the instructions of Chemiluminescent Nucleic Acid Detection Module Kit (89880, Thermo Fisher Scientific). The 6-bp telomerase ladder products were clearly observed on the membrane.

2.4 Western blot analysis

Western blot analysis was performed according to the standard procedure. Anti-hTERT (ab32020) and anti-DKC1 were purchased from Abcam. Anti-Flag (A8592) was obtained from Sigma-Aldrich; anti-PES1 (sc-166300) and β-actin (sc-47778HRP) were purchased from Santa Cruz Biotechnology; anti-hTERT (abx120550) was purchased from Abbexa. Samples were separated on SDS-polyacrylamide gels and transferred to NC membranes. The membranes were incubated with corresponding antibodies. Immunoreactive proteins were visualized using the ChemiDoc™ Imaging System (Bio-Rad) with a Super Signal™ West Pico PLUS Chemiluminescent Substrate Kit (Thermo). Gray value was analyzed using Scion Image software, and the effect was judged by comparing the ratio of two groups.

2.5 TRAP assay

The TRAP assay was performed according to protocol (S7700, Millipore). Briefly, cultured cell pellets were lysed with 1 × CHAPS lysis buffer for 30 min on ice. After centrifugation at 10,625 g for 20 min, the total protein concentration was determined by the Bio-Rad Protein Assay Kit. Indicated amounts of samples were mixed with 2 µl TRAP buffer, 0.4 µl TS primer, 0.4 µl primer mix, 0.4 µl dNTPs, 0.2 µl RNase inhibitor, 0.2 µl Taq DNA polymerase, 15.4 µl DEPC-treated H2O, with a total volume of 20 µl. The solution was incubated at 30°C for 30 min, then at 94°C for 30 s to terminate the reaction, 25–30 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min; 72°C for 5 min. PCR products were loaded onto a 10% polyacrylamide gel (29:1 acryl/bisacryl) in 0.5 × Tris–borate–EDTA (TBE). Gels were run at room temperature for 50 min at 200 V. The gel was photographed by Gel Image System (Tanon).

2.6 Quantification of signals and statistical analyses

The intensity of telomerase activity signal was analyzed using Image J software. The calculation of relevant telomerase activity was as follows: detect the average gray value of repeated bands (biotin-TS band without extension was not included) and then subtract the background gray value of the area with the same size. The relative telomerase activity of each lane was determined relative to the control lane or the first lane. Statistical analysis was performed using SPSS 22.0 (IBM). Continuous parameters, which were expressed as mean ± standard deviation, were compared by using the t test. p < 0.05 was considered statistically significant.

3 RESULTS

3.1 Detection of telomerase activity by Biotin-DTA

The principle of the Biotin-DTA method is illustrated in Figure 1A. Generally, the assay includes the following steps: (i) Telomerase is purified by immunoprecipitation to remove the cellular factors that inhibit telomerase activity; (ii) A biotinylated substrate (Bio-L-18GGG)
(A) Immunoprecipitation, telomerase reaction, captured by magnetic beads, elution, incubated with an HRP-conjugated avidin antibody and detected by luminol chemiluminescence, electrophoresis and transfer to membrane.

(B) IB: Flag, IB: Actin

(C) TRAP, Biotin-DTA

(D) IP

(E) IB: TERT (Abcam)
is elongated by telomerase in the presence of dNTP; (iii) The biotin-labeled extension products are immobilized on streptavidin-coated magnetic beads via the interaction between biotin and streptavidin; (iv) The extension products are eluted from the magnetic beads, separated by polyacrylamide gel electrophoresis (PAGE), transferred to a nylon membrane, and fixed on the membrane by UV cross-linking; (v) The extension products on the membrane are incubated with an HRP-conjugated avidin antibody. Finally, the signal is amplified and detected by luminol chemiluminescence. Using this method, the 6-bp repeat telomerase ladder products were clearly observed on the membrane. Telomerase activity was determined according to the number and signal strength of the bands in the product ladders.

We first applied the Biotin-DTA method to the detection of exogenous telomerase activity. The expression and subcellular localization of Flag-hTERT were detected by western blot and immunofluorescence in HepG2 cells (Figure 1B). Telomerase activity was higher in the HepG2-Flag-TERT (HepG2-FT) cells than the bulk HepG2 cells as determined with the TRAP assay. Importantly, Biotin-DTA detected six bands of decreasing molecular weight and increasing signal intensity in the Flag-hTERT group (Figure 1C), although the signals were weak. There were no signals in the control groups (Flag only) or buffer-only group (without the IP product). These results suggest that Biotin-DTA can detect telomerase activity.

To detect endogenous telomerase activity, HepG2 cells were divided into two groups: one group used an anti-hTERT antibody for immunoprecipitation, while the second group used IgG. Because the amount of endogenous telomerase is very small, 33 80 µl IP eluate was used in the telomerase extension reaction. The other experimental steps were the same as those used for the detection of exogenous telomerase activity. The results showed that the IP eluate of the anti-hTERT group yielded a weak signal, while the control group exhibited no signal (Figure 1D). The expression of hTERT in the IP eluate was also detected by western blot (Figure 1E). Therefore, endogenous telomerase can be detected with the Biotin-DTA method, which indicates that this new method has a similar sensitivity as DTA using radioactive isotope markers.13

![FIGURE 1 Establishment of the Biotin-DTA assay. (A) Illustration of the biotin-labeled direct telomerase assay (Biotin-DTA). (B) HepG2 cells were used to stably express Flag-hTERT. The expression (left panel) and localization (right panel) of Flag-hTERT were detected by western blot and immunofluorescence, respectively. (C) Telomerase activity in the HepG2-Flag-hTERT cell line was detected using TRAP (left panel) and Biotin-DTA (right panel). Cells with (Flag-hTERT) or without the TERT construct (Flag) were immunoprecipitated with anti-Flag, followed by telomerase activity detection. Only cells expressing Flag-hTERT exhibited telomerase activity (Chaps and buffer both indicate the CHAPS lysis buffer). (D) The endogenous telomerase activity of the HepG2 cells was detected with the Biotin-DTA method. (E) The expression of hTERT in the IP eluate was detected by western blot.](image)

3.2 | Optimization of Biotin-DTA

To improve the signal intensity, we next explored optimization of the experimental conditions. According to previous studies,35 culture incubation at 32°C increases telomerase activity. HepG2-FT cells were, therefore, divided into two groups. One group was incubated at 37°C for 3 days, while the other group was incubated at 32°C for 3 days. The telomerase activity of the two groups was detected with the Biotin-DTA method (Figure 2A). The 32°C group showed higher activity than that of the 37°C group. The expression of Flag-hTERT and the efficiency of immunoprecipitation were confirmed by western blot (Figure 2B). These findings indicate that cells can be cultured in a 32°C incubator for 2–3 days to obtain higher yields of telomerase activity.

Previous studies have shown that the concentration of KCl influences the activity of telomerase during the telomerase extension reaction.35 We, therefore, explored the effect of different concentrations of KCl on the activity of telomerase. Telomerase reactions were performed using different concentrations of KCl with the same volume of IP eluate (10 µl), and the telomerase extension products were detected (Figure 2C). The number of bands resulting from the Biotin-DTA method decreased as the KCl concentration increased. Further, for increasing concentrations of KCl from 123 to 250 mM, the band intensities for the short repeats increased gradually; however, when the KCl concentration exceeded 250 mM, the band intensities decreased. Telomerase activity at the different KCl concentrations suggested that inhibition may occur at high concentrations of KCl of >250 mM (Figure 2C). Thus, the concentration of KCl is key for optimizing telomerase activity, and a concentration of 200–250 mM is optimal for the telomerase extension reaction to yield a strong signal. The concentration of 200 mM KCl was used for all subsequent experiments.

According to the previous results, the primer signal at the front of each lane was relatively strong, indicating that most of the primers could not participate in telomerase extension reaction. Therefore, we designed telomerase extension reaction with different amount of primers to evaluate telomerase activity. The result shows that higher signal intensity was observed in group with 0.25 µl primers (Figure 2D). The time of telomerase extension reaction may affect the amount of products and ultimately affect the signal changes. Therefore, we designed telomerase reaction experiments with different incubation time (Figure 2E). The result shows that the signal gradually increased with the extension of reaction time. Then, we set the telomerase extension reaction time at 14 h, that is, overnight, to achieve the best signal display effect. To test the repeatability of the methods, a series of Biotin-DTA assays with the same amounts of samples were used and displayed a similar signal intensity (Figure 2F).

3.3 | Effect of analyte protein amount and PES1 knockdown on Biotin-DTA

To evaluate whether the Biotin-DTA method could reliably quantify telomerase activity over a broad analyte range, we detected the telomerase activity of a dilution series of IP eluate. We found that with
increasing amount of the IP eluate, the signals from the telomerase activity increased gradually (Figure 3A).

Next, we detected the effect of the knockdown of PES1, a telomerase assembly factor, on telomerase activity as determined with Biotin-DTA. HepG2-FT cells were divided into two groups: (1) transfected with siNC (negative control siRNA), and (2) transfected with siPES1 to silence the expression of PES1. The cells were collected to detect the telomerase activity with Biotin-DTA (Figure 3B) and the effect of PES1 siRNA on PES1 expression was determined by western blot (Figure 3C). The results showed that the telomerase activity of the siPES1 group was significantly lower than that of the control group.

### 3.4 Stability of Biotin-DTA

TRAP is currently the most widely used method for the detection of telomerase activity. To illustrate the advantages of the Biotin-DTA method, we compared it with TRAP. Two sets of cultures of 293 T cells were used for this purpose. The first group was transfected with siNC and the second group was transfected with siDKC1, a siRNA targeted toward the DKC1 telomerase assembly factor. The 293 T cells were then transfected with Flag-hTERT and hTR after siRNA transfection and the cells were cultured at 32°C for 2 days. After the cells were lysed, the total protein concentrations were determined, and a series of protein amount were subjected to TRAP (Figure 4A) or Biotin-DTA (Figure 4B). As expected, the telomerase activity levels were much higher for the negative control compared with the siDKC1 group for both the TRAP and Biotin-DTA methods. However, with the TRAP method, the differences in signal between the siDKC1 and siNC samples varies at different protein amounts. For example, when the protein amount was 5 ng, the signal difference was 82%, while the difference was 57% for 10 ng and 50% for 15 ng. In contrast, with the Biotin-DTA method, these signal differences were relatively stable: 69% for 10 µl, 62% for 20 µl, and 66% for 30 µl. Therefore, the Biotin-DTA method
Endogenous human telomerase is scarce because the number of TERT-hTR complexes per cell is estimated at only 250 in even the most highly telomerase-positive tumor cell lines. To amplify telomerase activity signals, here we established a direct telomerase activity assay using biotin-labeled primers. The sensitivity of Biotin-DTA is based on high efficiency of product collection (due to strong interaction between streptavidin and biotin) and high sensitivity in detection of biotin-labeled nucleic acids. The streptavidin–biotin system is widely used in chemistry, biology, biotechnology, and medicine. For example, it has been used in live cell imaging, the identification of proteins that undergo posttranslational modification (PTM), the identification of RNA-binding proteins, the detection of in vivo protein–DNA interactions, and drug delivery for cancer and gene therapy, and the development of novel sensors. Here we show that Biotin-DTA is sensitive enough to detect endogenous telomerase activity in tumor cells.

Compared with cells cultured at 37°C, those cultured at 32°C are reported to produce fivefold higher levels of the active telomerase complex. When cultured at 32°C, cells can sustain the expression of proteins during the G1 stage and produce a large number of Cajal bodies, which support maturation of telomerase complex. Hence, during our experiments to detect exogenous telomerase activity.

4 | DISCUSSION

Endogenous human telomerase is scarce because the number of TERT-hTR complexes per cell is estimated at only 250 in even the most highly telomerase-positive tumor cell lines. To amplify telomerase activity signals, here we established a direct telomerase activity assay using biotin-labeled primers. The sensitivity of Biotin-DTA is based on high efficiency of product collection (due to strong interaction between streptavidin and biotin) and high sensitivity in detection of biotin-labeled nucleic acids. The streptavidin–biotin system offers greater signal stability over a range of analyte protein amount compared with traditional TRAP method.
activity, after the transfection of the plasmids, the cells were cultured at 32°C for 2–3 days to increase the yield of telomerase. The western blot results showed that after the cells were cultured at 32°C, the expression of Flag-hTERT was significantly higher than that obtained with conventional cultures at 37°C. Whether culture temperature also affects endogenous telomerase expression still needs further exploration.

Using our method in the presence of low concentrations of KCl (123–200 mM), the extension products showed a continuous increase in the number of repeat sequences, indicating improved continuous telomerase binding ability. At higher concentrations of KCl (250–350 mM), the continuous binding ability of telomerase was decreased, no further bands were observed for the 6-bp repeats, and similar results were detected by α-32P-mediated DTA assays.³⁵ The presence of high concentrations of KCl not only reduces the repeat addition processivity (RAP) of telomerase but also causes the participation of more substrates in the extension reaction. Studies have shown that after the primer is prolonged by telomerase, a single-stranded DNA molecule is first formed.⁴⁷ In the presence of KCl, the telomere 6-bp repeat unit can form the structure of a G-quadruplex.¹² Whether the formation of too many of these G-quadruplex structures affects the continued extension of primers requires further study.

TRAP has been widely used for telomerase activity detection for its high sensitivity. However, the accurate differences between two samples may be ambiguous because of inhibitory molecules which existed in cell lysates and saturation effect of PCR. As shown in (Figure 4A), the signal intensities could not elevate collaboratively with increasing of cell lysates, especially siNC group, which possesses a higher activity. However, Biotin-DTA detected a relative stable difference before and after knockdown of DKC1 (Figure 4B).

Recently, different methods and strategies of detection of telomerase activity have been reported.⁴⁸,⁴⁹ Compared with the traditional methods, the advantages of biotin-labeled primer-based nonradioactive detection method are simple operation, reliable results, accurate quantification, and easy promotion in ordinary laboratories. However, it is time-consuming and not high-throughput.

In summary, we established a biotin-labeled, PCR-free, nonradioactive direct telomerase biopsy assay that is practical and stable. It can accurately respond to changes in telomerase activity in cells after the intervention, and it can be applied to various avenues of research involving the study of telomerase. One limitation of our method is that telomerase activity can only be detected in cell lysates at present, and telomerase activity in tissues has not been
tested. We are currently working to further optimize the experimental conditions and to explore the application of this method to the assay of telomerase activity in clinical tissues.

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CONFLICT OF INTEREST
The authors have declared that no conflict of interest exists.

DATA AVAILABILITY STATEMENT
All data needed to evaluate the conclusions in the paper are present in the paper. Additional data related to this paper may be requested from the authors.

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