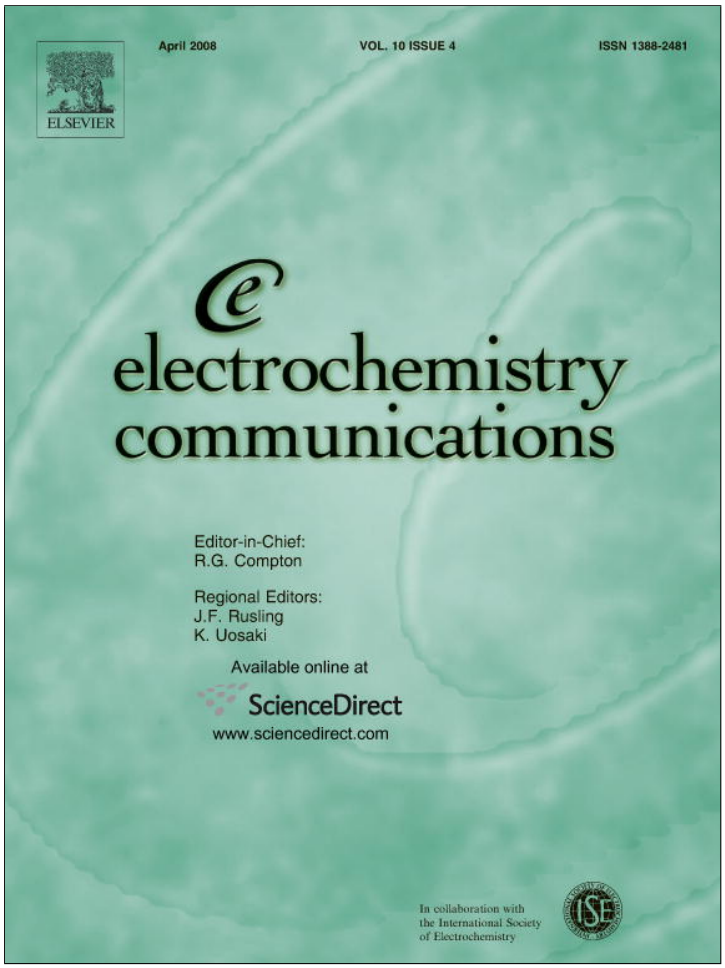

Available online at www.sciencedirect.com
ScienceDirect
Electrochemistry Communications 10 (2008) 564–567
electrochemistry communications
www.elsevier.com/locate/elecom

Magnetic beads-based electrochemiluminescence assay for rapid and sensitive detection of telomerase activity

Xiaoming Zhou, Da Xing *, Debin Zhu, Li Jia

MOE Key Laboratory of Laser Life Science and Institute of Laser Life Science, South China Normal University, Guangzhou 510631, China

Received 23 December 2007; received in revised form 29 January 2008; accepted 30 January 2008
Available online 8 February 2008

Abstract

Telomerase is a potential cancer marker. We developed a new and robust telomerase activity assay which combines the modified telomere repeat amplification protocol (TRAP) with magnetic beads-based electrochemiluminescence (ECL) detection. The high performance of this assay is related to the determination of telomerase activity from single cell levels, and ECL intensity is linear over the range of 1–1000 HeLa cell equivalents. The proposed telomerase assay offers a highly cost- and time-effective alternative to presently available telomerase assays, which are limited by tedious and complicated post-PCR detection.

Keywords: Telomerase activity; TRAP; Electrochemiluminescence; Magnetic beads

1. Introduction

Human telomerase is a ribonucleoprotein complex which is inactivated in most somatic cells and reactivated in most cancer cells [1]. The strong association of telomerase activity with tumors establishing it is valuable for cancer diagnosis and prognosis [2]. TRAP is a standard assay for telomerase activity analysis [3]. This assay and its modified versions [4–6] use slab-gel electrophoresis to size and semiquantify the PCR products, which is time-consuming and adds variables during analysis of the PCR products, make the accurate measurement of telomerase activity difficult. Some alternatives for post amplification detection have been reported with the TRAP assay as a foundation [7–11]. However, methods mentioned above either need elaborate instruments or required use of harmful radioactive and expensive florescent substances. Thus, a highly sensitive, yet simple, safe, and quantitative approach for telomerase activity detection is expected.

Recently the use of surface-functionalized magnetic beads in bioaffinity assays has increased in popularity due to its ability to selectively bind low-abundance target analytes (DNA, bacteria, protein) and pre-concentrate them and to discard the sample matrix prior to the detection step. Magnetic beads have proven valuable in immune and nucleic acid assays with improved sensitivity and selectivity [12–14]. ECL is a general term used to describe a reaction or mechanism, which produces light at the surface of an electrode. The most common ECL luminophore is *tris*(2,2-bipyridine)ruthenium(II)($Ru(bpy)3^{2+}$), and tripropylamine (TPA) is the most efficient known coreactant and showed that femtomolar concentrations of the ruthenium chelate could be detected under conditions that were compatible with antibody and polynucleotide binding reactions [15–17]. Here we present a new and robust method for telomerase activity analysis. This strategy combines the modified TRAP with magnetic beads-based ECL detection that allows rapid, sensitive and accurate determination of telomerase activity from cancer cells. Fig. 1 shows the basic principle of the novel TRAP–ECL method. Biotinylated TS primer (B-TS) is interacted with cancer cell extract in the presence of the nucleotide mixture dNTPs,

* Corresponding author. Tel.: +86 20 85210089; fax: +86 20 85216052.
E-mail address: xingda@scnu.edu.cn (D. Xing).

1388-2481/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.elecom.2008.01.039

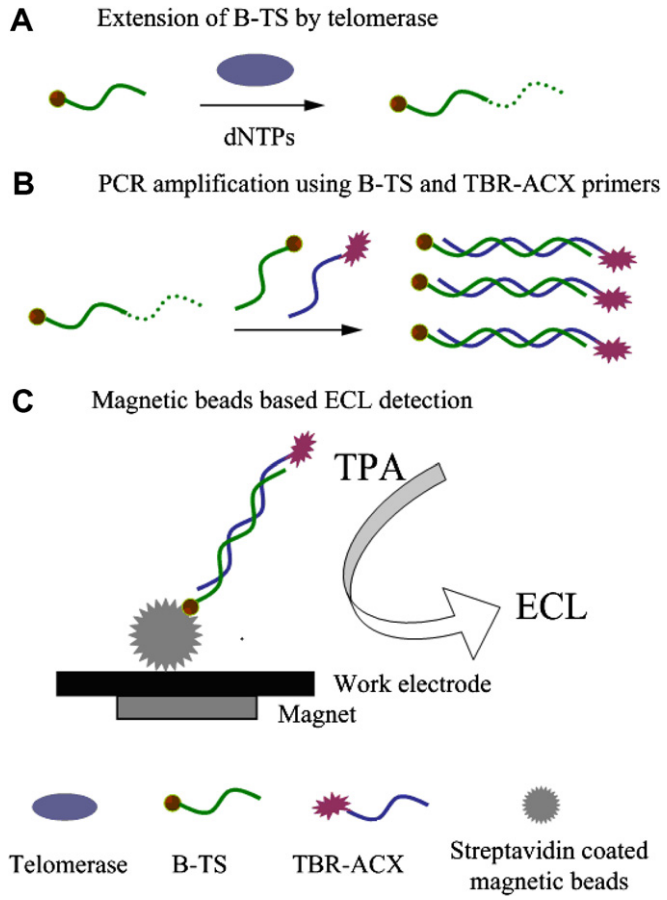

Fig. 1. The basic principle of the magnetic beads based TRAP–ECL method for detection of telomerase activity.

and telomerase bind to the B-TS primer and synthesize telomere TTAGGG repeats sequence (A). The extension products serve as the templates for PCR amplification which uses B-TS primer as forward primer and TBR-ACX primer as reversed primer (B). Amplified products are linked on to the surface of streptavidin-coated magnetic beads through biotin–streptavidin linkage. The amount of PCR products are determined by measuring the ECL signal generated from the electrochemical reaction of TBR and TPA in the reaction cell (C).

2. Experimental

2.1. Materials and reagents

TPA and the chemicals to synthesize the Ru(bpy)3^{2+} *N*-hydroxysuccinimide ester (TBR-NHS ester) were purchased from Sigma (Louis, MO, USA). RNA Secure was acquired from Ambion. Hot Start Taq DNA polymerase was from TaKaRa Bio. Streptavidin microbeads (2.8 μm diameter) were products of Dynal Biotech (Lake Success, NY, USA). The B-TS primer (5'-biotin-AATCCGTC-GAGCAGAGTT-3') and 5'-amino modified ACX primer (5'-amino-GCGCGGCTTACCCTTACCCTTACCCTAA-CC-3') were synthesized and HPLC purified by SSBE. The 5'-amino modified ACX primer was label with TBR-NHS ester by our lab according to Terpetschnig's paper [18].

2.2. Magnetic beads-based TRAP–ECL assay

Cell was prepared as described in our recent paper [19]. Telomerase extract was executed by the CHAPS lysis buffer method [3]. TRAP assay was modified as follow: TS primer was replaced by its 5'-biotinylated version (B-TS), ACX primer was labeled with TBR, and PCR was executed for 30 cycles. Other components remained unchanged as described previously [20]. Sliver staining experiments was done according to the published protocol [21].

A custom-built ECL detection system was described in detail in our previous research [13]. For the samples analysis, 10 μl PCR products were directly added to 200 μl TE buffer (pH 7.4) containing 10 μl streptavidin coated beads, and incubated this for 20 min at room temperature with gentle shaking. The reaction mixture was separated by using magnetic racks, following washed twice with TE buffer (pH 7.4), resuspended in 100 μl ECL assay buffer containing 0.2 M NaH_2PO_4, 0.04% Tween 20, 0.1 M TPA, pH 8, and was sequentially used for ECL detection. The applied potential of the ECL reaction was fixed at 1.25 V according to the previous optimization [13].

3. Results and discussion

In this modified TRAP, TS primer was replaced by its 5'-biotinylated version (B-TS) which was used for selective immunomagnet capture by streptavidin-coated paramagnetic beads in the detection cell, thus, ensuring the specificity of the assay. ACX primer was labeled with TBR which was used to react with TPA to emit light for ECL detection. The high affinity of the streptavidin/biotin interaction ($K_d = 10^{-15}$) allows for only the DNA samples labeled with both biotin and TBR can be detected in the detection cell.

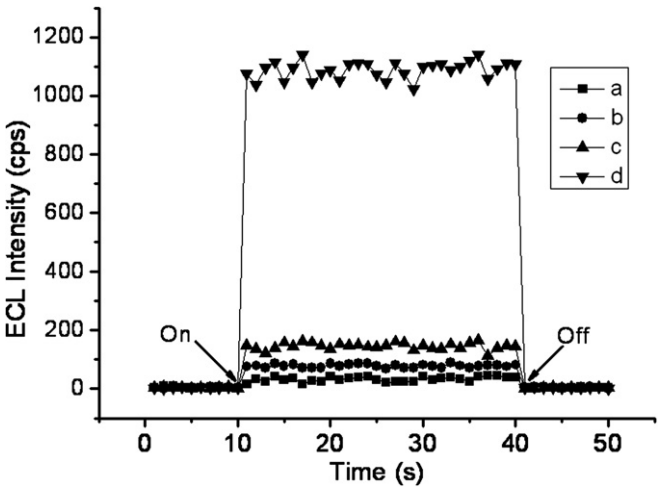

Fig. 2. ECL intensity corresponding to (a) ECL buffer backgroud (TE + TPA), (b) extract free control, (c) RNase inactivated HeLa cell extract (100 cells), and (d) HeLa cell extract (100 cells). On: potentiostat on. Off: potentiostat off.

Fig. 2 shows the ECL signal obtained from ECL assay buffer backgroud (a), extract free control (b), RNase treated telomerase positive HeLa cells extract control (c), and 100 telomerase positive HeLa cells (d). It shows the light emitted from the system upon analysis of an extract from 100 cells is significantly higher than the ECL signals obtained from extract free control. To determine whether the signals of ECL are dependent on telomerase activity, HeLa cells extract was treated by RNase which destroyed the RNA component of telomerase thus prevented the telomerization reaction. An obvious decrease in the ECL signals was observed. This clearly shows the ECL signals were dependent on telomerase activity. Importantly, the ECL signal for the extract free control was only as small as the ECL assay buffer background, indicated the excellent specificity of this modified TRAP system.

To define if a sample is telomerase-positive, a cut-off value was calculated based on the average ($V_{control}$) and standard deviation ($V_{stdev(con)}$) of the ECL reading from the extract free control samples, shown as formula (1),

$$V_{cutoff} = V_{control} + 3V_{stdev(con)} \quad (1)$$

According to this formula, the cut-off level for telomerase-positive samples was set at 142 cps. ECL signal less than 142 cps should not be indicated as telomerase-positive under our conditions. Under the prerequisite, the sensitivity of the current TRAP–ECL method was evaluated and compared with conventional TRAP sliver staining assay using serial dilutions of extracts of telomerase-positive HeLa cells (1000, 100, 10, and 1 cells). For conventional TRAP-sliver staining assay, a 6 bp DNA ladder bands indicative of telomerase activity were observed in extracts of 100 HeLa cells but not in extracts from 10 and 1 cells (Fig. 3A). In the case of TRAP–ECL, mean ECL signals calculated from three parallel experiments with single-cell samples were higher than the cut-off value (Fig. 3B). It is indicated that the sensitivity of current TRAP–ECL is about 100 fold higher than that of conventional TRAP sliver staining assay.

In current TRAP–ECL method, each molecular of TRAP products incorported a TBR-labeled primer, which would be essential for quantification of telomerase activity. To confirm if this TRAP–ECL method is a cell numbers dependent manner, serial dilutions of extracts from these cells (1000, 500, 100, 50, 10, 1 cells) were evaluated. As shown in Fig. 3C, the TRAP–ECL method showed a tight linear correlation between ECL intensity and the number of analyzed cells, with R values of 0.985. When 5000 or

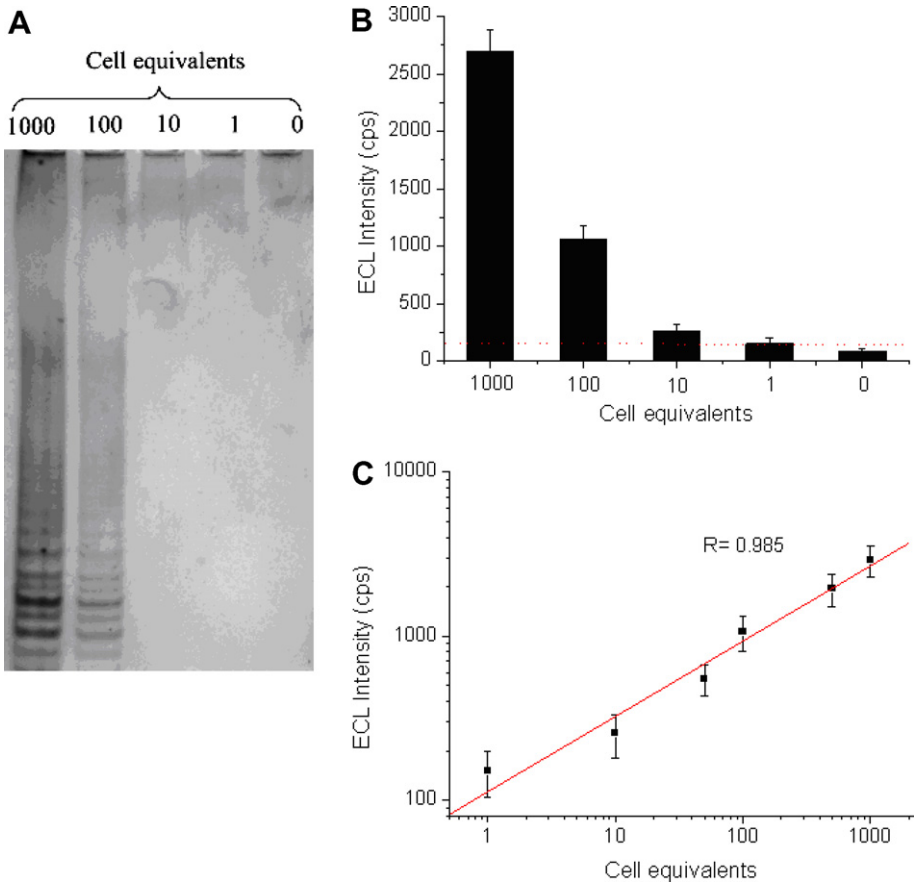

Fig. 3. Sensitivity and linearity of TRAP–ECL method. Protein extracts equivalent to the indicated numbers (1000, 100, 10, 1) of HeLa cells were used for conventional TRAP with sliver staining (A) and TRAP–ECL (B). Linearity of ECL intensities was observed in the equivalent of 1000, 500, 100, 50, 10, 1 cells with TRAP–ECL (C). Each data point represents an average response from three parallel assays. The red dash line represents the cut-off value for telomerase positive samples.

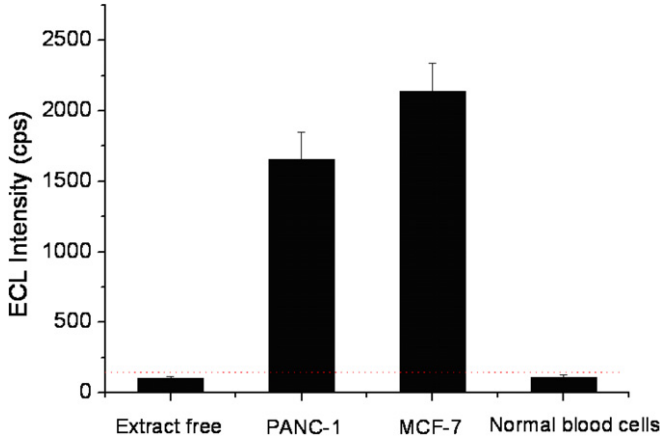

Fig. 4. ECL intensities obtained by analyzing extracts from extract free control, PANC-1 cancer cells (1000 cells), 1000 MCF-7 cancer cells (1000 cells), and normal blood cells (1000 cells). The red dash dot lines represents the cut-off value which was calculated based on formula (1).

more cells were analyzed, an inhibition was observed (data not shown), which may be accounted for by an increased concentration of PCR inhibitors present in tumor cells.

To investigate accuracy of the proposed assay, coefficient of variation (CV) was determined by measuring ECL signal with six replicates for 100 and 500 HeLa cells equivalent, CV values were 12.6% and 9.7%, respectively. We noted that in conventional TRAP telomerase assay the CV values are usually varied from 30% to 40% [22]. The high variability of the conventional TRAP is partly resulted from the samples handling, and partly resulted from multi-stage post-PCR detection. In this study, the improvement in reproducibility should be entirely derived from the use of magnetic beads based ECL technology.

The proposed method was also implemented in the analysis of MCF-7 human breast adenocarcinoma cell lines, PANC-1 human pancreatic cancer cell lines and normal blood cells. As shown in Fig. 4. The ECL signals obtained from both MCF-7 and PANC-1 carcinoma cells were significantly higher than the cut-off value which was calculated based on formula (1), whereas no activity could be detected in normal blood cell extracts from a healthy volunteer used as control. The results indicated that the proposed method can be applied to telomerase activity analysis from other cancers.

4. Conclusions

Telomerase activity detection with conventional TRAP assay has not been introduced widely into large analysis or the clinical setting partly because of its complex, time consuming, and labor-intensive nature. It is proved that the developed method has more advantageous than the conventional TRAP assay in terms of the clinical use because this assay provides sensitive results without the need for radioactive materials. In addition, compared to traditional TRAP assay, the assay required only 30 min to complete ECL detection and generates more accurate and thus more reliable telomerase activity data. Importantly, with the use of magnetic beads, the method should can be easily extended to high-throughput and automatic screening format. These characteristics demonstrated that the proposed TRAP–ECL method could potentially serve as a first line assay for the clinical diagnosis and prognosis of cancer.

Acknowledgments

This research is supported by the National Natural Science Foundation of China (30600128; 30470494; 30700155) and the Natural Science Foundation of Guangdong Province (7005825; 7117864).

References

[1] J. Lingner, J.P. Cooper, T.R. Cech, Science 269 (1995) 1533.
[2] N.W. Kim, Eur. J. Cancer 33 (1997) 781.
[3] N.W. Kim, M.A. Piatyszck, K.R. Prowse, C.B. Harley, M.D. West, P.L.C. Ho, G.M. Coviello, W.E. Wright, S.L. Weinrich, J.W. Shay, Science 266 (1994) 2011.
[4] W.E. Wright, J.W. Shay, M.A. Piatyszek, Nucleic Acid Res. 23 (1995) 3794.
[5] G. Krupp, K. Kuhne, S. Tamm, W. Klapper, K. Heidron, A. Rott, R. Parwaresch, Nucleic Acids Res. 25 (1997) 919.
[6] N.W. Kim, F. Wu, Nucleic Acid Res. 25 (1997) 2595.
[7] E. Savoysky, K. Akamatsu, M. Tsuchiya, T. Yamazaki, Nucleic Acids Res. 24 (1996) 1175.
[8] A.J. Cheng, R.P. Tang, J.Y. Wang, J.T. Chang, T.C.V. Wang, Jpn. J. Cancer Res. 90 (1999) 280.
[9] M. Hirose, J. Abei-Hashimoto, K. Ogura, H. Tahara, T. Ide, T. Yoshimura, J. Cancer Res. Clin. Oncol. 123 (1997) 337.
[10] S. Xu, M. He, H. Yu, X. Cai, X. Tan, B. Lu, B. Shu, Anal. Biochem. 299 (2001) 188.
[11] S. Gelmini, A. Caldini, L. Becherini, S. Capaccioli, M. Pazzagli, C. Orlando, Clin. Chem. 44 (1998) 2133.
[12] G.H. Yan, D. Xing, S.C. Tan, Q. Chen, J. Immunol. Meth. 288 (2004) 47.
[13] D.B. Zhu, D. Xing, X.Y. Shen, J.F. Liu, Biochem. Biophys. Res. Commun. 324 (2004) 964.
[14] Y.Y. Lin, G.D. Liu, C.M. Wai, Y.H. Lin, Electrochem. Commun. 9 (2007) 1547.
[15] J.K. Leland, M.J. Powell, J. Electrochem. Soc. (137) (1990) 3127.
[16] G.F. Blackburn, H.P. Shah, J.H. Kenten, J. Leland, R.A. Kamin, J. Link, J. Peterman, M.J. Powell, A. Shah, D.B. TaHey, S.K. Tyagi, E. Wilkins, T.G. Wu, R.J. Massey, Clin. Chem. 37 (1991) 1534.
[17] W.J. Miao, A.J. Bard, Anal. Chem. 76 (2004) 5379.
[18] E. Terpetschnig, H. Szmacinski, H. Malak, J.R. Lakowicz, Biophys. J. 68 (1995) 342.
[19] Y.H. Pei, D. Xing, X.J. Gao, L. Liu, T.S. Chen, Apoptosis 12 (2007) 1681.
[20] J.P. Jakupciak, P.E. Barker, W. Wang, S. Srivastava, D.H. Atha, Clin. Chem. 51 (2005) 1443.
[21] B.J. Bassam, P.M. Gresshoff, Nat. Protoc. 2 (2007) 2649.
[22] Y.B. Gan, J. Lu, A. Johnson, M.G. Wientjes, D.E. Schuller, J. L.-S. Au, Pharm. Res. 4 (2001) 488.