Fluorescence Detection of Telomerase in Cancer Cells via a Modified Telomeric Repeat Amplification Protocol Assay

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Abstract

The enzyme telomerase is responsible for repairing the ends of DNA (telomeres) damaged by replication, but is not expressed in cells after a certain stage in development. However, mutations can lead to its expression in cancer cells, immortalizing them. Thus, detection of telomerase could potentially become an important step in diagnosing cancer. In this experiment, the PCR step of a TRAP assay is eliminated via the use of a molecular beacon, and detection limits are improved by coupling telomeric strand primers to gold nanoparticles. Through this method, it is possible to detect the presence of telomerase in samples as small as 10 cells, although a relationship between the number of cells and fluorescence intensity could not be established.

Introduction

Telomerase is a recently discovered ribonucleoprotein that allows cells to become immortalized and continue to divide long past when they normally should stop.[1] Telomerase lengthens telomeres, which protect the ends of DNA during replication to prevent damage. In healthy cells, telomeres are shortened with each successive replication, and eventually become too short to allow the cell to continue dividing, causing many of the effects of old age, including a decreased rate of healing.[1] Cancer cells often express telomerase even though their healthy counterparts should not, which allow them to divide many times more than they normally should.[2] Telomerase is present in approximately 85% of human tumors of any type, but not present in normal human somatic cells, and thus is a widely present marker for tumor detection and could be vital for cancer diagnosis and treatment.[2]

This project involves combining two methods of detecting the presence of telomerase in cancer cells. In the first, a standard method of detecting the presence of telomerase, a Telomeric Repeat Amplification Protocol (TRAP) assay, is modified by attaching thiol-modified telomeric strand (TS) primers to gold nanoparticles (AuNP) to lower detection limits and decrease the amount of interferants present.[3] A TRAP assay involves providing isolated telomerase with telomere strand primers.

Telomerase elongates these products, which are amplified by PCR, and finally polyacrylamide gel electrophoresis (PAGE) is run to quantify results. However, because the procedure still relies on polymerase chain reaction (PCR), PCR artifacts still can cloud results. To correct this, a molecular beacon (MB) is used to detect the presence of telomerase, thus making the amplification step unnecessary.[2, 4]

The MB is a hairpin loop of DNA with a fluorophore (Cy3) at the 5' end, and a quencher (DAB) at the 3' end. Alone, the quencher prevents the fluorescence of the dye. However, when TS primers associate with the loop and are elongated by telomerase, the loop opens, the dye and the quencher separate, and the dye can fluoresce. This fluorescence can be detected by a fluorimeter and used to determine the presence of telomerase in the sample.[2] An additional improvement upon a traditional TRAP assay in this experiment is the use of AuNP. TS primers are conjugated to AuNP, which has been reported to increase the signal by Xiao *et al*.[4]

This technique also as the potential for further improvement, including the study of different methods of synthesizing AuNP, such as citrate-capped AuNP, rather than the protein-conjugated AuNP used in this study.[5] Potential cost-reduction steps are also possible. Zhao *et al* have developed a method of using paper bioassays using AuNP as probes that could potentially eliminate the need for the use of a fluorimeter.[6]

Experimental Methods

Materials

Table 1: DNA Sequences (5' to 3')	
Molecular Beacon	Cy3-TCTTGGACACACTAACCCTAACCCTAACCCTAACTCTGCTCGACGGATTTGTGTCCAAGA-DAB
TS Primer	$HS(CH_2)_6$TTTTTTTTTTTTTTTTTTAATCCGTCGAGCAGAGTTAGGGTTAGGGTTAGGGTTAGTT

MB and TS primer were obtained from Integrated DNA Technologies. Chloroauric acid and tris were obtained from Fisher Scientific. Bovine serum albumin (BSA) was obtained from EMD Chemicals.

Fluorescence spectra were obtained on an LS 55 Fluorescence Spectrometer from PerkinElmer by exciting at 515 nm and scanning from 530-700 nm. Cells used in this experiment were human multiple myeloma cells provided by Dr. DeCicco-Skinner, American University Department of Biology.

Synthesis and Modification of AuNP

AuNP were synthesized by combining chloroauric acid and BSA in a mole ratio of 70 Au/BSA in water. The mixture was then placed in a convection oven at 70°C for 4 hours. The presence of AuNP in solution could be visually determined by a color change from colorless to purple and from the detection of a UV/Vis absorbance peak at 535 nm using a Shimadzu UV 2550 with tungsten and deuterium lamps, scanning from 200-800 nm. Approximate concentration of AuNP was determined using data reported by Bakshi *et al*.[7] TS primers were added at an approximate concentration of 50 TS primers per AuNP.

Preparation of Telomerase Extract

Telomerase extract was prepared as recommended by Piatyszek *et al*.[3] A culture of 40 million human multiple myeloma cells were suspended in 200 µL of lysis buffer and incubated on ice for 30 minutes. The cells were then centrifuged at 13200 rpm for 30 minutes using a FormaScientific centrifuge, and the supernatant was collected and frozen at -80°C.

Telomeric Elongation of TS Primers

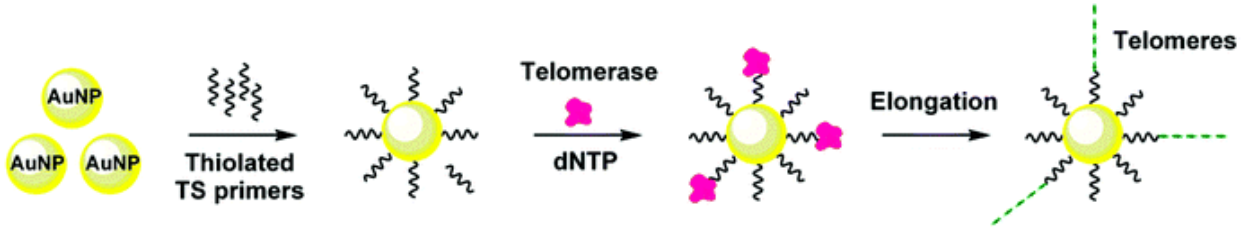

Figure 1: Illustration of AuNP-modified TRAP assay, adapted from Xiao *et al*. Thiol-modified TS primers are attached to AuNP, which serves as a substrate for telomeric elongation.[4]

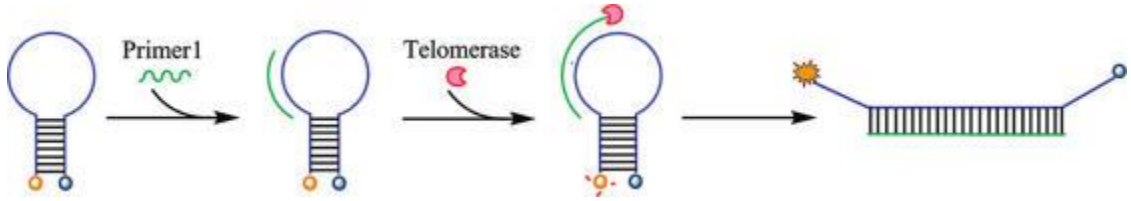

Figure 2: Illustration of MB, adapted from Ding *et al*. TS primers associate with the hairpin loop of the MB, and serve as a substrate for telomeric elongation. As the TS primers are lengthened, the loop splits, and the dye is separated from the quencher, and fluorescence activity can be detected.[2]

3 µL AuNP/TS primer, 0.5 µL 50x dNTP mix, 16 µL sterile water, 2.5 µL 10x PCR buffer, and 2 µL cell extract containing varying concentrations of cells were mixed in a PCR tube and incubated in a heat block at 30°C for 30 minutes, resulting in the elongation of the TS primers on the AuNP by telomerase. Then, MB was added in a ratio 1/1 ratio of MB/TS primer (6.1 µL), and the mixture was incubated in a heat block at 37°C for 60 minutes, causing the opening of the MB, allowing the dye to fluoresce. Fluorescence emission spectra were taken of all samples.

Results and Discussion

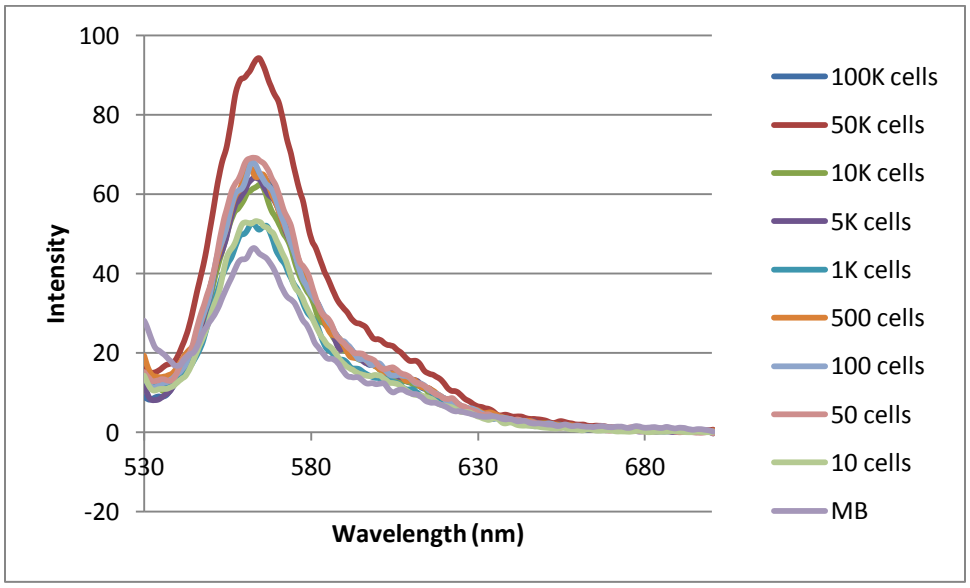

Figure 3: Graph of fluorescence intensity versus wavelength for each cell dilution and the MB alone. All dilutions of cells show at least some fluorescence activity above the MB.

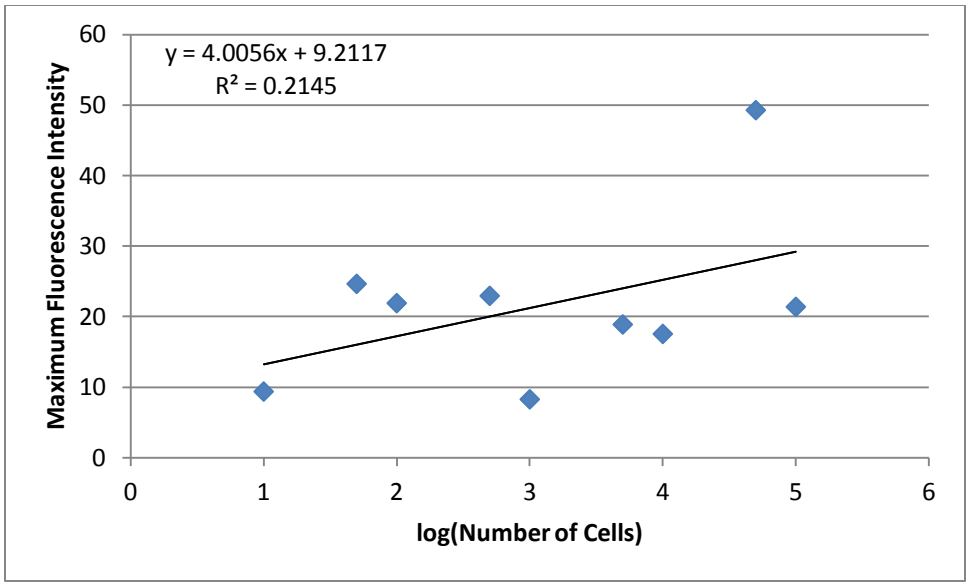

Figure 4: Graph of peak fluorescence intensity (minus the fluorescence due to the MB alone) versus the log base 10 of the number of cells in the sample (for purposes of visualization). Although there is a general trend of increasing peak fluorescence intensity with increasing number of cells, the correlation is very low.

Fluorescence spectra of samples containing 10 to 100,000 cells indicate the presence of telomerase. All samples show the presence of telomerase. A relationship between the number of cells in the sample and the fluorescence intensity was observed in Figure 4. However, this relationship is very weak and is unlikely to be significant. This is contrary to the results reported by Ding *et al* and Xiao *et al*, who report a positive correlation between fluorescence intensity and the number of cells in the sample, although a similarly low detection limit and nonlinear response was observed by each.[2, 4] It is possible that 10 cells contain enough telomerase to extend all of the exposed TS primers, explaining the lack of a relationship between fluorescence and the number of cells in the sample. This could possibly be due to the BSA bioconjugated AuNP used in this experiment. If little of the surface area of the AuNP was exposed to the TS primers, few TS primers would be able to bind to the AuNP, resulting in similar levels of fluorescence regardless of the number of cells in the sample.

This method represents a potential improvement upon the methods created by both Ding *et al* and Xiao *et al*. The use of the MB eliminates the PCR artifacts and the entire elongation step reported by Xiao *et al* and that are necessary in a traditional TRAP assay. In addition, the use of AuNP as a substrate

onto which the TS primers are bound groups the TS primers together so that each enzyme has access to more TS primers, and thus more TS primers are elongated and can be associated with the MB, increasing sensitivity. Further experiments should explore the use of other types of AuNP, such as citrate-capped AuNP, to increase the available surface area for TS primer binding.

Conclusion

This method represents a new way to detect telomerase activity in as few as 10 cells, without the interference issues caused by PCR. The association of the TS primers with the AuNP serves to lower detection limits, while the MB completely eliminates the need for any kind of amplification step that would result in increased background noise or interferences. This technique is relatively quick and simple, and could easily be applied to detect cancer in human tissue.

References

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