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Telomerase Assays

A review and comparison of several telomerase activity assays.

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Abstract

Telomerase is a protein that is required by immortalized cells and cancer cells to continue their unlimited division. In this paper, a number of assays for detecting telomerase activity are reviewed. This is done in order to assess their viability both as a tool for the quick and accurate diagnosis of cancer, which in turn yields better tools for testing existing cancer treatments. The techniques are also assessed for their ability to study telomerase's role in every living cell. We conclude that it is difficult to directly compare all techniques. *Zhao & Qi et al.*'s[35] multiple amplification techinques is concluded as the best technique for cancer diagnosis and using surface plasmon resonance is concluded as the best technique for further study of telomerase.

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1 Summary

Telomerase is a protein that is required by immortalized cells and cancer cells to continue their unlimited division. In this paper, a number of assays for detecting telomerase activity are reviewed. This is done in order to assess their viability both as a cancer biomarker and their ability to analyze telomerase as part of a larger biological system. We conclude that while it is difficult to directly compare all techniques, a few of them stand out for high sensitivity. The technique of using surface plasmon resonance to measure telomerase activity stands out as the most sensitive. *Zhao & Qi et al.*'s[35] method utilizing multiple amplification techinques is the quickest method that is still usefully accurate for the detection of tumerious cells.

2 Introduction

Telomeres are the tips of chromosomes that guard them against damage which would be caused by over-replication. The enzyme telomerase rebuilds these tips. In healthy cells this system prevents damage to the genome of the cell. Telomerase is active in cancer cells and thereby it plays a key role in cancer cell immortality. Therefore telomeres and telomerase are interesting as biomarkers for cancer. But there are many unanswered questions about telomerase, requiring extensive research.

To study an object you must first know how to find it. There are many ways to detect telomerase activity, and each of them has it's own positive attributes, negative attributes and recurring problems. Here we look at a number of techniques for detecting telomerase activity. The techniques have been evaluated on their both cancer detection ability and as a tool to study the activity of the protein itself. This review focuses on methods that are in use or under development during the year 2012 and 2013; techniques that have not been mentioned in any publications during those years or since were probably abandoned due to lack of promise or application.[1]

In the first part the structure of the telomeres and telomerase activity and the importance of telomere and telomerase research are discussed/described. Subsequently there is the section listing the various methods and techniques for telomerase activity detection employed in 2012 and 2013, followed by a discussion on their merits and weaknesses. Finally some concluding remarks based on the findings are made.

3 Telomeres & Telomerase

All hereditary information of a living organism is stored in the form of a double stranded polymer of four specific nucleotides (DNA), Alanine (A), Tyrosine (T), Cytosine (C) and Guanine (G). When these strands are replicated, the strand serving as a template cannot be fully copied because DNA-replicase needs room to bind the strand. This means that every time a strand is replicated, a few nucleotides will not be copied, and the new strand will be shorter than the original. If nothing is done to prevent this shortening from happening, the DNA degradation would start to damage important genes and coding sequences on the chromosome, eventually resulting in cell death. Therefore the tips of a chromosome do not contain any genetic code. It is a repeating sequence of nucleotides: telomeres. In humans the sequence consists of six nucleotides: TTAGGG.[2]

Telomeres themselves are stable molecules, but they are further stabilized by forming complex structures. Telomeres in all species have a repeating sequence rich in guanine.[1] Four guanine bases (not in secuence on the same strand) can combine to form a square planar structure, and two or more of these can stack on top of each other to form a G-quadruplex (Illustration 1). The quadruplex structure is further stabilized by the presence of a cation, which sits in a central channel between each set of four guanines.[3] In vivo, the resulting complex is further stabilized by several ligands. This structure is a telltale marker for the presence of telomeres. It can even be enzymatically active and can metabolize hydrogen-peroxide, due to the ability to bind an ion.

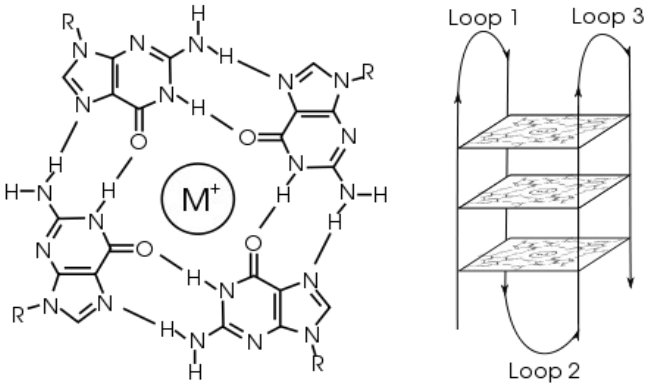

Illustration 1: Guanines in Quadruplex formation. Left: a square planar structure of four guanines. Right: three planes stacked into a single Quadruplex. Image by Julian Huppert*, Nucleic Acids Research. Copy available at wikimedia commens.*

3.1 Telomerase

With each chromosome replication, a part of the telomere is lost. The enzyme telomerase extends from the 3' end of the DNA strand, restoring a segment of the six nucleotides. This as opposed to DNA-replicase which only elongates the strand from the opposite 5' end, and uses the complimentary strand of DNA as a template.

Telomerase is a ribonucleoprotein complex; the active enzyme consists of a protein part and a ribonucleic part. The telomerase reverse transcriptase protein (TeRT) binds to a telomerase RNA component (TeRC) and the resulting enzyme binds to the 3' end of a strand of DNA. Then the RNA template is reverse transcribed for synthesis of one telomeric repetition. After the sequence is fully elongated, the TeRC dissociates from the site, but the DNA strand remains bound to the TeRT to allow for repeated DNA synthesis on the same strand. The TeRC rebinds the strand at the next repetition, which then rejoins the TeRT to add another telomere sequence.[2]

This elongation mechanism for telomeres allows a cell to grow and divide without being stopped by its natural division limit (also known as the Hayflick limit).[2] This is why stem cells can keep dividing to reach the numbers necessary to form a fetus and keep the body supplied with replacement cells to repair damage. In somatic cells (cells that have differentiated into a specific function), telomerase is absent or at least not very active and once these cells reach the Hayflick limit, they will stop dividing, unless something goes wrong. In cancer cells some things go very wrong, allowing them to multiply indefinatly. In approximately 90% of human tumors excessive telomerase activity has been observed[4] [5]. The telomeres in cancer cells remain relatively short compared to those in healthy cells in spite of the

abundance of active telomerase.[5] This makes telomerase a very promising biomarker for the detection of cancer as well as a target for drugs, while the telomeres themselves lack the necessary abundance for detection.

The way the protein and RNA components work is still mostly unknown. There is currently no crystallographic data available for telomerase. The function of telomere length beyond staving off the division limit is also a subject of debate. There is no indication for alternative pathways for telomere extension, although there are immortal cell strains which seem to function without telomerase.[6] This suggests that there is more to the division limit than is currently understood.

As stated, the enzyme telomerase is activated in 80-90% of all human malignancies and immortal cell lines.[4 5] This indicates that the enzyme contributes to making cancer-cells immortal, which makes telomeres and telomerase interesting as biomarkers for cancer. At the same time, telomere degradation is an important component of cellular aging[7]. Proper understanding of telomerase not only improves our understanding of both, it will also define the healthy balance between them.

As such a lot of research into telomeres and telomerase is needed, and for that, good methods and assays to detect telomeres and telomerase activity are needed. This paper will focus on important telomerase activity analysis methods used in 2012 and 2013. There are many more techniques to study telomerase, but due to practical constraints not all of them can be listed.

4 Telomerase Detection Methods

It is important to separate the stages of an assay into a few steps. If a sample is prepared from living tissue, it it is pertinent to purify it to expose the actual telomerase activity from the rest of the cell. But isolating a protein does not mean that it is active. Telomerase will only function if the TeRT and TeRC parts are both present and undamaged. To make sure the right protein is isolated, it is necessary to check for telomere extension capacity.

The detection of the elongation of a few strands in a solution can not be seen directly. So this process will need to be visualized or detected electronically. If the signal is weak, it can be amplified with additional reactions.

4.1.1 Telomerase purification

In a regular healthy cell there is only a very small amount of any specific protein present. Telomerase is no exception; the concentration in somatic cells is almost nonexistent. In stem- and tumor cells more telomerase is produced, but it is still a very small amount compared to the total content of a cell. Ergo, it pays off to concentrate telomerase activity in the sample used for detection.

On the cellular level, there are ways to concentrate the telomerase from a tissue sample by separating healthy cells from cancerous ones using flow cytometry. Flow cytometry separation is based on cancer stereotypes, such as: larger size, deformability, antibodies for cancer antigens, affinity of various other

materials etc.[8] Since cancer cells will have higher telomerase activity than normal cells, this is an easy way to increase the concentration of telomerase in a sample and to reduce the amount of throughput needed to analyze it.

On the protein level *Cohen & Reddel et al.*[9] developed a two-step purification technique for telomerase based on simple antibody affinity. The telomerase antibodies are bound to Protein-G agarose beads. The proteins are released by washing the beads with an antigen similar to telomerase. In the next step the telomerase is allowed to bind to immobilized DNA strands (as per natural affinity). Before the second washing step, dTTP and dATP are added, which will allow the enzyme to elongate the strands. This will reduce its binding affinity resulting in reducing the half-life of their bond from roughly 10 hours down to 5 minutes (as part of the cycle of elongation) releasing the telomerase. This method allows the sample to be concentrated up to 10^8 times of the original sample.

4.2 Direct Measurement Methods for Telomerase Activity

There are many ways to isolate a set of molecules from a cell, but that does not necessarily mean one has certainty which ones have been isolated. The only thing we know for certain is that telomerase extends strands of DNA by adding a specific sequence. Most enzymes and structure stabilizers will remain active even if the cell around them is destroyed. This means that if telomerase is active in the cell, it will also be active in the cell's lysate. So instead of measuring the presence of telomerase directly, many techniques use the process of telomere elongation on a primer strand to detect its presence. This has the advantage that there can be no question or doubt about what is detected: molecules with telomerase activity. Below are the steps and techniques for directly assessing the presence of (active) telomerase in a sample. These techniques explicitly do not rely on other reactions to subsequently amplify the signal; those techniques are discussed in the next section.

4.2.1 Optical detection

Direct optical detection of telomerase activity is difficult, so some sort of beacon or chemiluminescence has to be used to facilitate measurements.

In the set-up developed by *Kha & Zhou et al.*[10] , the telomerase-extended biotinylated primer was hybridized to a digoxigenin-labeled telomere anti-sense DNA probe. This was followed by an enzyme-linked immunosorbent assay (ELISA), in which the biotinylated hybrid is captured on streptavidin-coated microtiter plates. The immobilized hybrid was probed with alkaline phosphatase–antidigoxigenin and detected through chemiluminescent readout. The assay could detect approximately 37 500 telomerase-positive cells under sub-optimal conditions.[7]

Similar results have been achieved with assorted “molecular beacons”, molecules which have been designed to remain inactive until they bind to a specific substrate and become fluorescent. Most of the telomerase beacons do not actually bind the enzyme itself, but are activated by telomeres created by telomerase.[30]
The molecular beacon for a telomere consists of three parts. First a target sequence of nucleotides

complementary to the DNA/RNA has to be researched, in this case the nucleotide sequence CCCTAA. This strand has a nucleotide sequence on one side and its complementary sequence on the other side as the second part. These two sections will bind together locking the strand into a hairpin-structure, forcing the ends together. Finally, the third part, on the ends of the chain, there are a fluophore and a matching quencher. With the hairpin intact, the quencher is close enough to the fluophore to quench its signal. But if the target sequence binds to the DNA of interest, it will make the hairpin straight and inflexible, forcing it to unfold from its closed structure. In its open configuration the Fluophore is distanced from the quencher, allowing the signal to be observed.

4.2.2 Array and biosensor chip

The most standard assay is a simple primer elongation experiment. *Cristofari & Reichenbach, et al.*[11] have developed a microarray for this purpose, named Telospot. After the elongation, the telomerase elongation products were transferred to a nylon membrane with a spot array and hybridized with radiolabelled telomeric probes. Then the array was examined using a PhosphorImager. The level of detected radioactivity was proportional to the telomerase activity.
The assay can be done by hand or by machines, for low-throughput and high-throughput formats respectively. The assay relies on radioactive probes from visualization, rather than on radioactive nucleotides, which ensures a high fidelity to natural levels of processivity, in other words, the average amount of telomeric repetitions added by a single telomerase protein during one cycle of binding, extending and unbinding. However, the assay was developed to screen for telomerase inhibitors, not to quantify telomerase. As such there are no exact figures to absolutely quantify telomerase activity.

A different approach is the use of surface plasmon resonance (SPR), a light absorption based measurement technique which utilizes the phenomenon of collective oscillation of electrons, known as a plasmon wave, in a substance stimulated by incident light (see Illustration 2 for more details). *Sharon & Freeman, et al.*[12], have tested the technique for measuring telomerase activity. They allowed a small amount of telomerase primers to adhere to a gold surface, which was then analyzed with SPR. If the primers are elongated by the enzyme, the dielectric properties of the surface change, which is reflected in the properties of the plasmon wave. The method was able to detect telomerase activity from eighteen 293T cells per microliter (transformed human embryonic kidney cells).

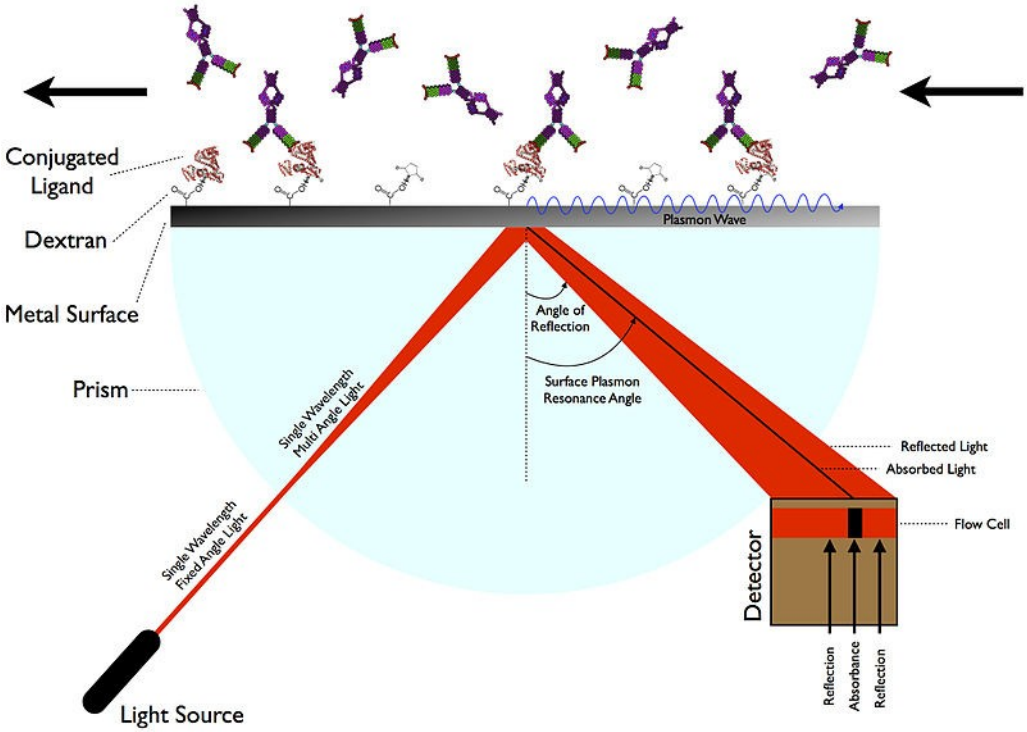

*Illustration 2: A simple picture displaying the most important components of a surface plasmon resonance set-up for measuring Dextran Ligand Antibodies. The detector measures at which angle the laser light is absorbed, and derives data from this measurement.*Sharon & Freeman, et al.[12]

4.2.3 Direct electrochemical strategies

An effective label-free electrochemical method for telomerase activity was developed by *Sato & Takenaka*.[13] With chrono-coulometry (coulometry is the technique of measuring the amount of energy used or released) they measured the amount of hexaammineruthenium(III) chloride (RuHex) that is bound to the phosphate backbone of a DNA strand. They used their technique for quick screening of telomerase inhibiting drugs and where able to achieve results with as few as 5 to 1000 HeLa cells. The method still has the obvious drawback that it is completely nonspecific for telomeres, and instead measures the total amount of DNA in the sample after the telomerase reaction.
Electrochemical methods (EC) use the change of conductivity of a substrate to detect molecules. While methods based on EC require specialized equipment, they can usually be produced inexpensively, while retaining high sensitivity.

4.2.4 Nano-particle assistance

When an object has a size definable on a nano-scale, the material it is made of, can have very different

properties than an object of the same material on a macroscopic scale. Many of these properties neatly lend themselves to highly sensitive detection techniques.

The technique developed by *Zheng & Daniel, et al*, relies on four different types of nano-particles to purify telomeres and analyze telomerase.[14] First primers attached to gold nano-particles are elongated, and then picked up by anti-sense strands attached to 'magnetic micro-particles'. The products are isolated with magnetics, attached to a gold surface and then stained with silver nano-particles. The final result is measured based on the array's light scattering. The group was able to detect the activity in as few as 10 HeLa cells. However the sophisticated tools required to perform this assay impede its practical use as a routine method of measurement of a biomarker.

The group of *X. M. Zhou, D. Xing, et al*.[15] has simplified this assay by employing Electrochemiluminescence (ECL). ECL is a broad term for techniques where a reaction or mechanism produces light at the surface of an electrode or via an enzymatic reaction. In this assay, ECL nano-probes were synthesized by making gold nano-particles with both a telomere capture probe and an ECL signal probe. The primers for telomerase were immobilized on to streptavidin coated magnetic beads. The ECL allows non-linear sensing of the telomerase products. This allows activity detection from 500 HeLa cells, without the use of complicated equipment (Illustration 3).

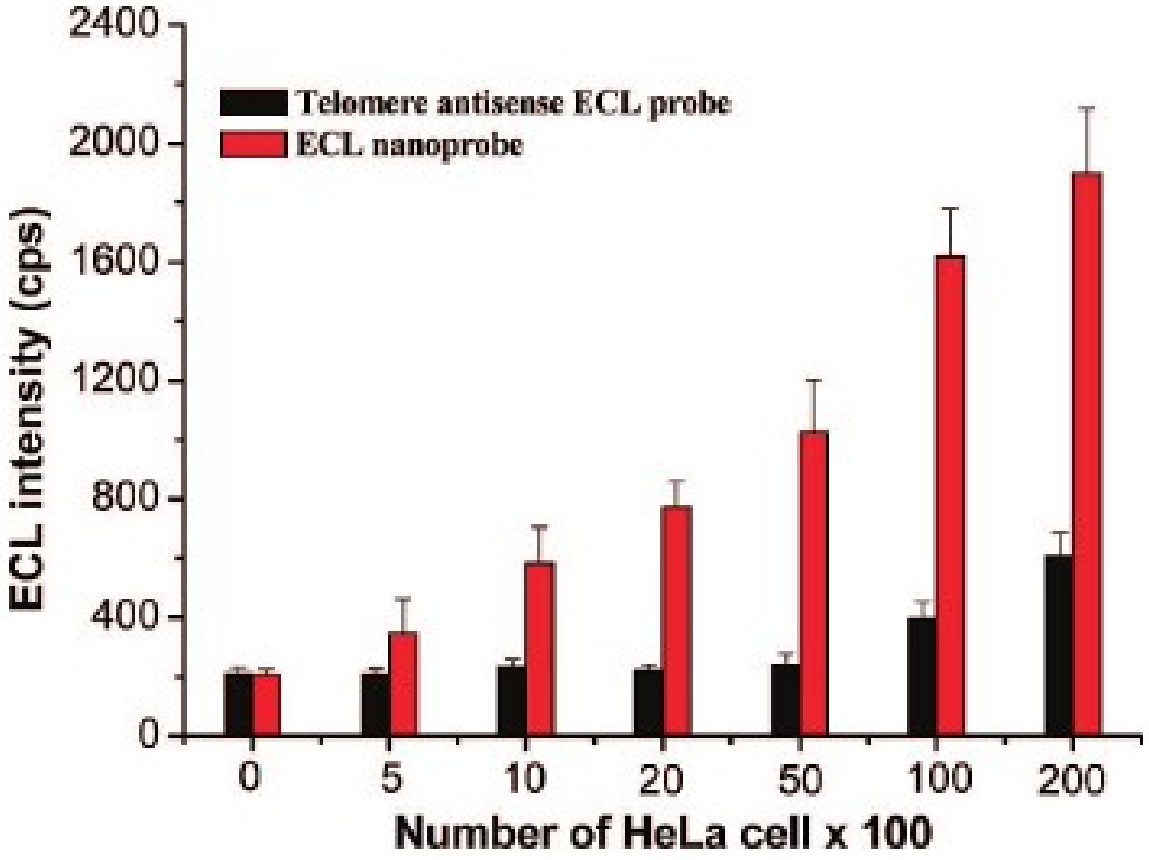

Illustration 3: Sensitivity of Zhou & Zing*'s telomerase assay.*[15] *ECL nanoprobe was used for the analysis of different numbers of HeLa cell extracts and a cellfree extract control (0), respectively. Telomere antisense ECL probes used as a negative control. At 500 HeLa cells there is a noticeable difference in ECL intensity between the sample tested with the actual nanoprobe, and the negative control. Although the intensity of the negative controls increases with the sample size, the intensity of the ECL nanoprobed samples is much greater in all samples, save the zero control.*

There is another type of nanomaterial worth mentioning here, superparamagnetic nanoparticles. In nano-scale ferro magnetic particles, magnetization can randomly flip direction under the influence of temperature, complete with an average amount of time to flip, Néel relaxation time.[16] Usually their magnetization will average to zero, but in the presence of a magnetic field, they are paramagnetic. *Grimm & Perez, et al*. attached anti-sense telomere strands to these nanoparticles, allowing their use for telomerase activity detection.[17] The particles would adhere to any telomerase products, causing them to cluster together, which influences their Néel relaxation time. This effect can be measured through the changes in magnetization. Grimm's group used telomerase from an isolated protein stock, rather than cell extracts and was able to detect about 10 attomoles of isolated telomeres (thirty base pairs) on well plates. Tests done on various types of immortalized cells (cultures and tissue samples etc.) showed a high level of correlation with the tests done with the photometric-based ELISA method (r2=0.94), but the assay's ability to detect cancer cells depends on the concentration of telomerase in the cell line.

4.3 Indirect Measurement Methods

4.3.1 Enzymatic G-quadruplex method

As mentioned in the introduction, long telomeres will form G-quadruplex structures in the presence of cations. These structures can be used as a method for indirect telomerase detection. The cation at the center of a G-quadruplex can metabolize hydrogen-peroxide (H_2O_2) by oxidizing organic molecules. The probe used by *Freeman & Sharon, et al*. in their telomerase detection strategy is one such molecule.[18] In their assay telomerase first extends a set of TS-primers with the telomere sequence. Then the sample's conditions are changed to allow the resulting telomere products to assemble into a quadruplex structure, enabling the catalytic function that metabolizes hydrogen-peroxide. Finally the peroxide and the probe are added. If there had been telomerase present in the original sample, the probe is oxidized by the quadruplex. This results in a change in the absorbence spectrum of the probe.
With this method it was possible to detect telomerase activity from 100 HeLa cells.[19]

4.3.2 Indirect electrochemical methods

In environments relatively caustic to natural biological conditions, alkaline phosphatase is a hydrolase enzyme which removes phosphate groups from many types of molecules. *Pavlov & Willner, et al* have used antisense telomeric probes with attached phosphatase.[20] When the probe hybridizes with telomerase products, the phosphatase can react with 5-bromo-4-chloro-3-indolyl phosphate, resulting in an insoluble precipitation, which can be detected by electrodes. This method allows the detection of telomerase activity from 1000 HeLa cancer cells.
Note the contrast between using alkaline phosphatase to produce visual effects, used by *Kha & Zhou et al*.[10] (described on page 7-8) and this method, which uses alkaline phosphatase to create a different substance that can be detected electrically.

4.4 Amplification & Amplification-based Measurement Methods

As mentioned before, studying a specific protein is difficult, especially when working with cell lysates. There is only a very small amount of telomerase present in healthy somatic cells. Even in tumor cells, which usually have much more telomerase, it is only a small part of the whole cell. Telomerase detection through its activity has the advantage that even a small amount of the enzyme can be detected by allowing the reaction to run for a longer time and create more signal strength. However doing so not only takes up time and resources, it also amplifies any false positives and measurement artifacts, resulting in high levels of background noise. This is where signal amplification comes in. Some of the amplification techniques even produce quantifiable results, that can be traced back to the concentration of the telomerase in the sample. Unless specifically mentioned otherwise, all of these techniques are applied to cell lysates, which makes them suitable for patient diagnostics.

4.4.1 Telomere Repeat Amplification Protocol (TRAP)

In 1994 a crude TRAP assay was developed that could detect telomerase activity from a few cancer-cells present in tissue biopsies.[21]

TRAP starts with adding a set of DNA 'forward' primers (or TS primers) to a prepared sample. The primers will be elongated by any telomerase present. This is followed by a standard Polymerase Chain Reaction (PCR), in which a set of complementary reverse primers binds to the resulting telomeres. This double strand will be extended and then copied by TAQ polymerase. Finally the results are analyzed with gel electrophoresis.

This method has a substantial number of problems. The PCR step takes a long time to complete and the results are very inaccurate due to PCR-related errors. Most notable is the problem that the repeat sequence of telomere base-pairs allows a reverse primer to bind at a random point on the elongated telomere, which means the extended strands will not have a consistent length, which is vital for gel analysis. Furthermore the sample contains all the other proteins that were present and active, not just in the nucleus, but in the entire cell. Many of these proteins interfere with DNA extension, and others inhibit or even destroy telomerase and polymerase. Various solutions are reported to reduce these problems such as: redesigning the primers, adding internal controls and adopting end-point detection modes[22] [23] [24]. But they do not fully remove or account for them. Many refinements and variations of the assay have been developed (described below), which address some of the problems at each step.

4.4.2 Improved primers for TRAP

The first problem encountered during the TRAP assay is that telomeres are extended from another telomere with the same sequence on the primer. This sequence repeats itself every six nucleotides. This means that during PCR, a poorly designed reverse primer can bind halfway down the telomere, rather than at the tips. Worse yet a reverse primer can bind to the unextended tip of a forward primer just as easily as an extended one. So on top of the unknown processivity of telomerase, there will also be an inconsistent shortening caused by the reverse primers which bind at a wrong location.
To solve this issue *Szatmari & Aradi* have developed a modified TRAP assay.[25]
They used two different reverse primers, RPC3g and RP. The 5' tip of the strand has a 20 nucleotide tag sequence. This means that TAQ polymerase will extend the telomere with a sequence of 20 nucleotides which can only be annealed by the second reverse primer.
RPC3g's 3' tip consists of two GG dinucleotides, rather than 2 trinucleotides, which means that even while it is bound to the telomere, it can not be extended.
During the first and second annealing steps of the PCR, a temperature lower than the standard PCR temperature ensures that only if RPC3g binds to the tip of the telomere, it will be extended.

4.4.3 G-Quadruplex inhibition of telomerase and polymerase

Another problem of TRAP in its basic form is that it does not unfold the G-Quadruplex, preventing

telomerase from binding the telomere. On top of that cell lysates will contain proteins and other ligands which further stabilize G-Quadruplexes. To make matters worse, these complexes also inhibit polymerization during PCR and without some system to distinguish one from the other, reliable telomere elongation and thus telomerase activity measurements are impossible.
By adding an extra step to the PCR, which employs a commercially available 'QIA quick nucleotide purification kit' *Reed & Gunaratnam, et al.*[26] were able to remove the Quadruplex stabilizing ligands, leaving the Quadruplex sufficiently weakened for telomerase to unfold and elongate them. This made the modified assay sufficiently reliable for measurements of telomerase activity.

4.4.4 Gold nanoparticle assistance for PCR in TRAP

In standard TRAP, the initial forward primers can dimerize with the reverse primers, even before any elongation occurs. These dimers do not contribute to the PCR reaction and interfere with the observation of the shortest PCR-amplified telomerase products on agarose gels.
The sensitivity of a PCR reaction can be greatly improved by the addition of Gold nanoparticles (AuNP). *Xiao & Dane, et al.*[27] It is not clear how this works, but by allowing the forward primers to bind to the AuNP, they will not dimerize (Illustration 4).

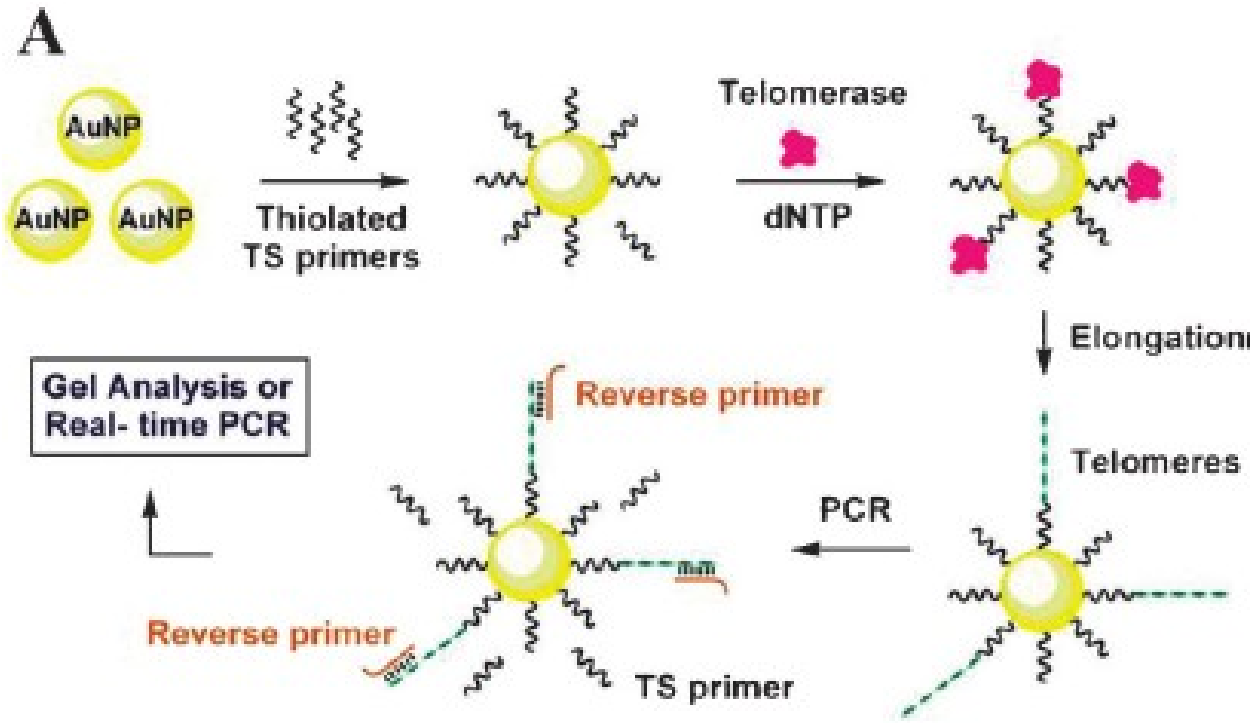

Illustration 4: A schematic depicting the amplification cycle of AuNP-modified primer-based TRAP. The TS-primers attach themselves to the nanoparticles (top left). In the presence of assorted nucleotides, telomerase can extend the primers (right). The extended primers can now bind reverse primers, and serve as a template for PCR amplification (bottom). Xiao & Dane, et al.[27]

In classic TRAP the TS primers float freely in the sample.[14] The addition of gold nanoparticles prevents the primers from dimerizing.

4.4.5 Capillary electrophoresis for TRAP

Traditional gel electrophoresis relies on the friction of the molecules on the gel substrate (which can vary based on size, affinity to the substrate, charge, etc.) versus its charge to separate the components of a sample. In a standard TRAP the results are visualized by staining the DNA strains with for example SYBR Green I. The results are analyzed by comparing the distance from the start to the end point of the components of the sample moved over time with a set of standard calibration molecules, placed in a separate lane on the flat gel.

Capillary electrophoresis works in a physically similar manner, but instead of a slab of agarose gel, a single capillary tube is used to separate the molecules. The components are distinguished by the amount of time spent moving through the capillary until they reach the detection module. Detection is done by measuring light absorbency (of ultraviolet or visible light) in a specific section of the tube.

Because the analysis is done by a detection mechanism, rather than by visual cues, capillary electrophoresis is not only more sensitive than gel electrophoresis (and is capable of detecting telomerase in as few as 5 to 15 cells), it is also more reproducible and since the whole process can be automated, a lot less laborious to use. Many researchers have used the technique to successfully detect active telomerase.[28] [29] [30] They were able to complete the whole assay in times varying from 2 hours for a laboratory study to 2 minutes for a modified TRAP assay chip.

4.4.6 Real time quantitative TRAP (RTQ-TRAP)

In parallel with the development of normal TRAP, the quantitative measurement of telomerase through this assay has been studied. However the ability of TRAP to measure telomerase quantitatively in real time is still limited. Real Time Quantitative-TRAP (RTQ-TRAP) combines the advances made quantitative PCR with telomerase detection. The first assay was a variation of real-time PCR, in which 'SYBR Green I' would bind to double-stranded DNA chains and then become fluorescent. Since SYBR Green I does not bind to a specific DNA sequence, the assay suffers from non-specific amplification and strong background signals.[31]
A better assay has been developed by *Fajkus & Elmore et al*.[32] by utilizing two spectrally distinct fluorophores to label hairpin primers, allowing simultaneous detection of the telomerase products and the internal control. However, in this assay fluorescent signals strictly depend on the primer specificity.[32]
The results of RTQ-TRAP in general are also limited due to the inability to handle tissue samples or blood samples which may contain only a few cancer cells. There are techniques to get around these problems, such as concentrating the cancer cells in the sample as mentioned, but these can be used with any assay, many of which have more to offer than RTQ-TRAP.

4.4.7 Amplification assays utilizing molecular beacons

As stated, the molecular beacon is especially useful for telomerase studies. Since the enzyme continually tacs on telomerase onto the primer, any lack of sensitivity can be overcome by increasing the incubation time. But as the signal is amplified, so are various effects which will only contribute noise to the final analysis. It is therefore imperative to speed up the assay by employing additional amplification reactions. These amplifications greatly increase the amount of end products, which can be detected quickly and easily.

To achieve this *Ding & Li, et al*.[33] have added a second primer and polymerase to the assay mixture, which will bind to an unfolded beacon strand and then replicate it. This will displace the telomere strand, while creating a new stronger beacon-binding strand. The telomere will be free to unfold other beacons (Illustration 5).[33] This system still has some drawbacks, most importantly the limited sensitivity as the lower limit of telomerase activity detection requires 40 to 1000 detectable cancer cells.

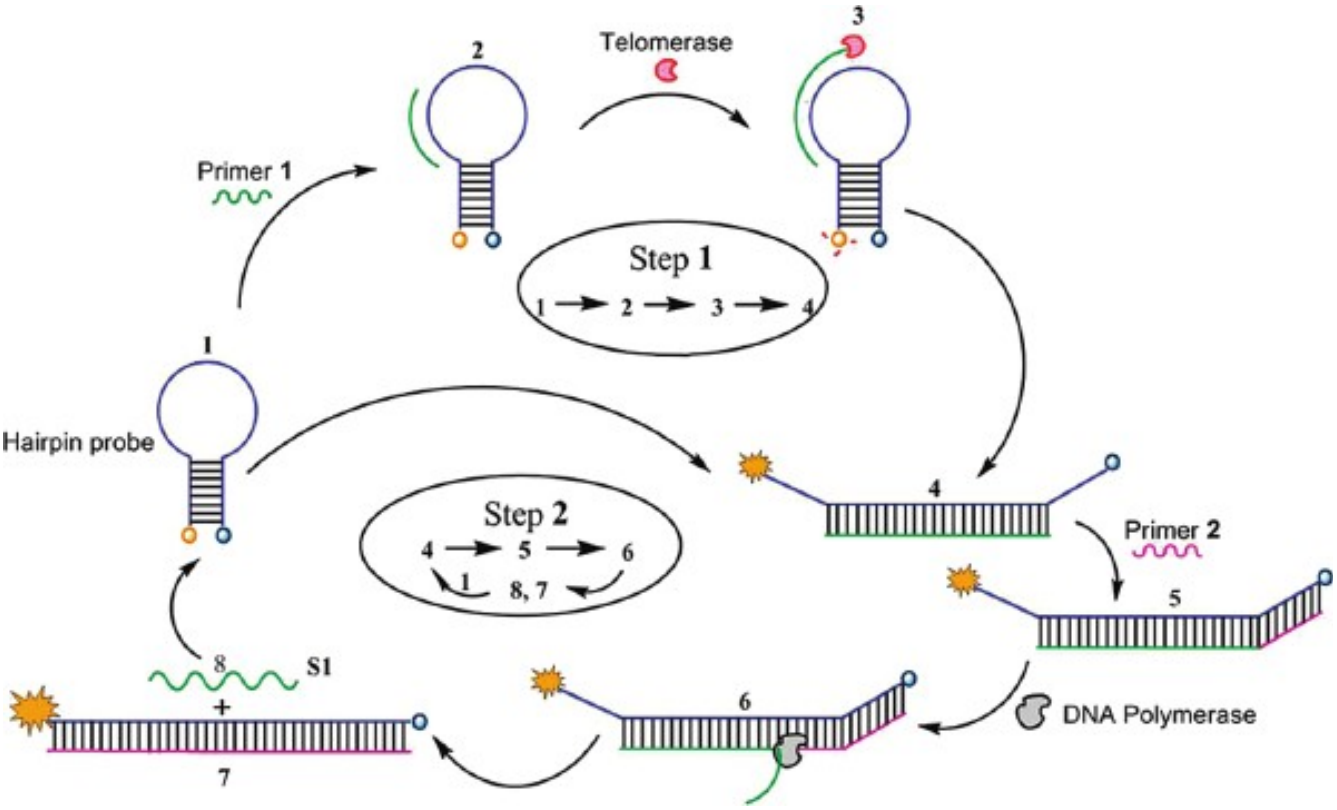

Illustration 5: A schematic illustration of the mechanism of the molecular beacon (Step 1) and the way Ding & Li, et al.*'s[33] second primer and polymerase enhance the signal (Step 2). (**1**) The molecular beacon in its hairpin formation. Note that the fluophore (yellow dot) is in close proximity to the quencher (blue dot). If an unextended TS-primer binds the probe, the hairpin will not unfold (**2**). If the TS-primer is extended by telomerase (**3**), it will force the beacon into it's unfolded state (**4**). At this point, the second primer can bind the beacon (**5**), which can then be extended by DNA Polymerase (**6**). This results in the beacon being permanently activated (**7**), while extended TS-primer will be able to unfold another molecular beacon (**8** & **4**).*

4.4.8 DNAzyme

Telomeres are made of DNA and as such they can be manipulated using assorted DNA targeting enzymes and bind complimentary DNA strands. *Wang & Donovan et al.*[34], have developed a telomerase assay based on the specific cleavage activity of PbII-dependent 8–17 DNAzyme combined with another complimentary DNA strand molecular beacon for detection. They tested it on prepared samples with known telomerase concentrations.

In the assay the telomerase from the sample will again elongate a primer with the telomere sequence. However, this primer has been linked to a PbII-dependent 8–17 DNAzyme section. If a lead-ion is bound to the DNAzyme domain, the DNAzyme will cut off the elongated sequence, without any additional enzymes. This freed strand can now bind to its complementary sequence on the molecular

beacon (Illustration 6).

This assay has been tested on cancer-cell lysates, but it has not yet been tested on mixed tissue samples. It was able to quantifiably detect 0.1–1 mg of telomerase, and the reports indicate it could detect it if one cell out of 200 is cancerous.[34]

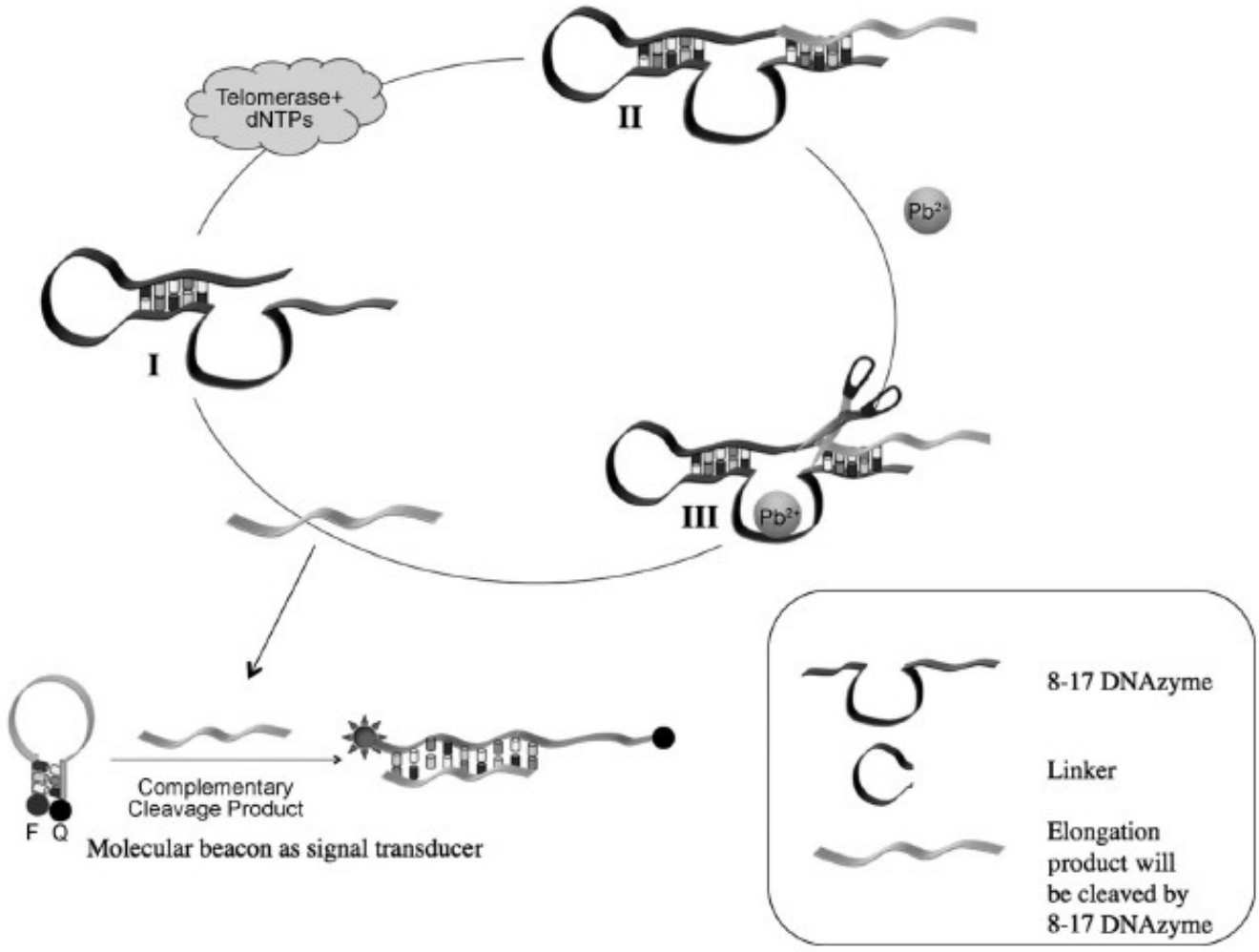

Illustration 6: DNAzyme in action. Telomerase elongates the strand (step II), which is then cut off by the DNA-enzyme (step III). The elongation product can then bind the molecular beacon to provide signal transduction. Wang & Donovan et al.[34]

Zhao & Qi, et al.[35] have developed a similar assay utilizing an isothermal signal amplification cascade (Illustration 7). As usual telomerase extends the TS-primer (7A). But rather than try to measure the telomeres directly, in the next step the telomerase products are combined with 3WJ-primers and -templates which can bind a telomerase elongated TS primer to form a stable complex. An enzym, named a Klenow Fragment, will replicate the 3WJ-template and the extended strand can be cut by a

DNAzyme (Nt.BbvCI) generating lots of short oligonucleotides. The oligonucleotides stabilize a second set of primers by means of a phenomenon known as based stacking hybridization. These primers will then be copied, with one product further stabilizing the hybrid, further amplifying the process and the other attaching to the actual molecular beacon (Illustration 7C).[35]

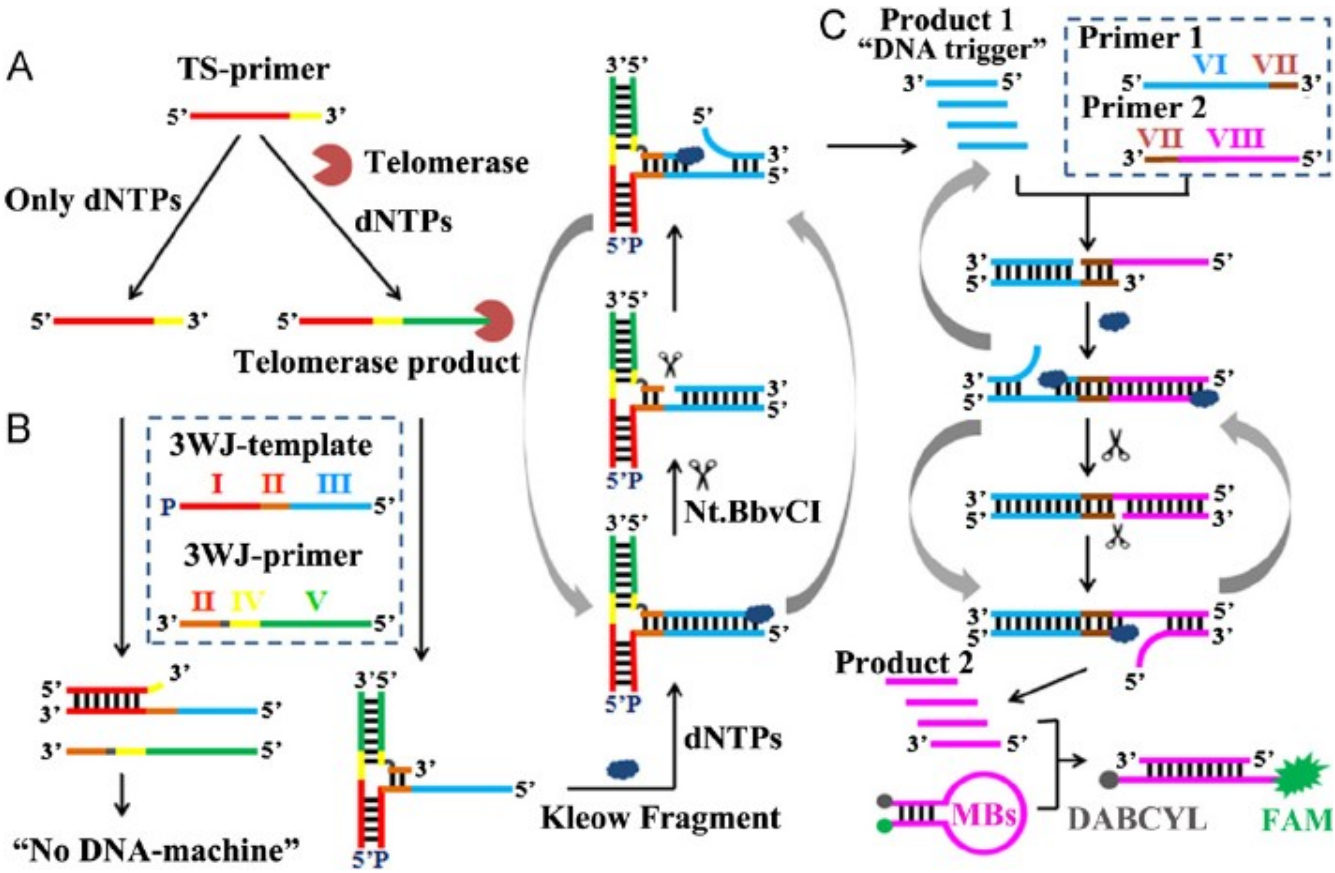

Illustration 7: The three steps of the isothermal signal amplification cascade. First the TS-primer is extended by telomerase (or it is not) (A). After this the 3WJ-primers and -templates are added (B). In samples without telomerase products, nothing happens (bottom left), but in samples where the TS-primer was extended, it will now bind the two 3WJ-stands to form a stable complex, which serves as a template for the "DNA-trigger" (middle column). The DNA-trigger now starts a third reaction sequence (right column) consisting of several more PCR reactions and finally activation of the ~~M~~ molecular beacon (bottom right). Zhao & Qi, et al.[35]

The assay was completed in 3 hours and is sufficiently sensitive to detect telomerase activity in ranges cell numbers down to 3 cells for HeLa, and achieving similar sensitivity with other cell lines. However *Zhao & Qi, et al.*[35] do not mention any opportunities for quantitative measurement of activity. While it is not impossible to calculate the level the artificial amplification caused by this method, and derive the amount of telomerase present, it would require extensive research to make it possible.

5 Discussion

For the comparison of the described methods we are not only interested in the capability of detecting telomerase activity, but also in being able to do it quantitatively. Clearly there is also great interest in detecting telomerase accurately. Moreover, ideally it must be possible to do the assay quickly enough to diagnose a patient with cancer in a reasonable amount of time. There are a few problems that obstruct a direct application of any one of the mentioned methods for clinical diagnosis of cancer.

5.1.1 Telomerase detection standards

It is clear from the erratic listing of results that there is no consensus on what standards to use for defining the sensitivity of a telomerase assay. Each research group has devised their own standard. Most assays, like *Zhao & Qi et al.*[35] and *Wang & Donovan, et al.*[34], list the number of HeLa cells that need to be lysed in order to amass a sufficient amount of telomerase to detect its activity. These numbers are effectively meaningless if there is no estimate of the amount of telomerase present in a HeLa cell in comparison to normal healthy cells. There have been studies into this subject[36] [37], which show that tumorous tissue has roughly 3 to 5 times higher telomerase activity then healthy tissue. As such, the assays can be used to diagnose cancer. But the amount of activity can vary greatly between different immortalized cell lines. So the techniques that are defined by their cell number requirements cannot be used to quantify the actual amount of telomerase, especially when cells do not match a cell line previously researched. Nor can they be used to establish the amount of cancerous cells in a sample containing both healthy and tumorous tissue. They can still be used to qualitatively determine the presence of (active) telomerase, and establish a telomerase activity ratio between different cell lines, for distinguishing cancerous or immortalized cells from somatic or growth limited cells.

Other techniques, like *Grimm & Perez, et al.*[17], list the concentration of telomerase or the amount in micrograms in test samples, prepared from a purified protein stock. However presence of telomerase components does not necessarily equate to high levels of activity.[38] While telomerase activity is confirmed within the confines of each assay, these systems of measurement do not carry over to cell lysates for the purpose of cancer detection, due to risk of denaturization and the large variation of telomerase activity in strains of tumorous cells. In order to make this possible, the techniques would have to researched and gauged to various cell lines.

5.1.2 Which assay should one use for what research?

So there are many ways to detect telomerase activity in tissue samples and cell lysates, but which assay is the best depends more on what is being studied and which resources are available, rather than actual detection sensitivity. When researching telomerase as a component of a biological pathway, an assay needs to accurately gauge telomerase activity. Assays that can only detect a unquantified amount 'relative to another cell line' would quickly become cumbersome to execute and verify.
This means that all amplification techniques are out of the question, unless highly accurate values for the processivity of each step in the amplification process is established.
Of the remaining direct techniques, Surface Plasmon Resonance is shown to be the most sensitive, but requires specialized equipment to be performed.

The assay developed by *Sato & Takenaka*[13] based on chrono-coulometry is less demanding, but also less sensitive. Ergo, coulometry would be the most suitable assay if high sensitivity is required, but not to the extent that it could overwhelm the budget of a study.

For the purpose of cancer detection it is more important that the assay reliably notes elevated telomerase activity compared to healthy cells of similar tissue and that this can be done quickly. The ability to quantify telomerase is not relevant, as the telomerase in cells will vary based on the type of cancer.
For this purpose an assay based on gold nano particles, an amplification method and molecular beacons would be most suitable. The amplification and beacon components are biomolecules which can be synthesized by expression in bacteria. This allows any laboratory equipped for biochemistry to synthesize the required molecules. It guaranties that the assays can be done at low costs, either by in-house synthesis, or outsourced to a business.
Zhao & Qi et al.'s[35] technique with multiple amplification mechanisms seems to be the most promising, as the technique is the most sensitive. Although the technique has been tested with various cell lines, they do not make any claims about samples from tissue biopties or other patient extracts. The development of this assay for diagnosis would be an interesting subject for future studies.

5.1.3 Does telomerase activity remain the same in cell lysates?

The methods discussed are clearly able to determine telomerase activity in cell lysates. However it is unclear to what extent the determined telomerase activity in cell lysates reflects the activity in living cells. The assays mentioned specifically look for the activity of telomerase in cell lysates, but say nothing about the presence of telomerase in states that are inactive, deactivated by natural ligands, deactivated by ligands which do not naturally encounter telomerase, denatured telomerase components, etc. It can be argued that this telomerase is irrelevant for studies into telomerase activity. If you look for its activity, in cancer cells compared to healthy cells or in telomerase suppressed cells compared to normal cells, the amount of properly deactivated telomerase is not relevant. But the issue becomes relevant when one is trying to quantitatively establish the total amount of telomerase in respect to its other biochemical functions, while its telomere extending activity is irrelevant. Although no direct conclusions can be drawn on the amount of telomerase based soly on these assays, and there are assays which do accurately asses this, the detected levels of activity are still indicative of the amount of telomerase present in the samples.

6 Concluding Remarks

There is still a lot of work to be done to get the most out of each assay. Almost every single technique mentioned is being applied for the purpose for which it was developed or is being researched for improved potential. Some methods clearly do not measure up to other mentioned methods. TRAP in particular, has been outclassed in all respects, except technique familiarity.

However the lack of uniform criteria of detection makes it impossible to select one assay as superior to all others. In order to determine which technique is the best, a system for comparison will need to be established, based perhaps on a number of frequently used cell lines, such as HeLa cells. Subsequently each technique will need to be rated according to this system.

For the purpose of cancer detection, there are many viable telomerase assays and techniques that meet the criteria of being able to distinguish cancer-cells from negative controls, and most of them have been tested against somatic or growth limited cells as well. At the time of writing this review, telomerase has been accepted as a biomarker for bladder cancer[39] [40], but there is no consensus as to what assay to use to test for telomerase activity.
With improved cancer detection also come more opportunities to test for cancer, and better abilities to see if a specific therapy works. Telomerase is also being researched as a drug target, but this line of research is still in its infancy.[41] This research will also benefit from improved telomerase detection assays, not only to verify that the treatment is working as intended, but also by providing better tools for understanding what is going on in the telomere restoration process.

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