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Review

# Assaying and investigating Alternative Lengthening of Telomeres activity in human cells and cancers

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ABSTRACT

**Alternative Lengthening of Telomeres (ALT) activity can be deduced from the presence of telomere length maintenance in the absence of telomerase activity. More convenient assays for ALT utilize phenotypic markers of ALT activity, but only a few of these assays are potentially definitive. Here we assess each of the current ALT assays and their implications for understanding the ALT mechanism. We also review the clinical situations where availability of an ALT activity assay would be advantageous. The prevalence of ALT ranges from 25% to 60% in sarcomas and 5% to 15% in carcinomas. Patients with many of these types of ALT[+] tumors have a poor prognosis.**


## 1. Introduction

All immortalized human cell lines analyzed to date have a telomere length maintenance mechanism (TMM) to compensate for the telomere shortening that accompanies cellular proliferation [1]. In many cell lines, the mechanism involves a ribonucleoprotein enzyme complex, telomerase, that utilizes its integral RNA molecule as a template for reverse transcription of new telomeric DNA [2]. Other cell lines use a non-telomerase mechanism, referred to as Alternative Lengthening of Telomeres (ALT) [3]. Although the definition of ALT encompasses any non-telomerase TMM [4,5], so far there is no clear evidence that there is more than one ALT mechanism in human cells. In contrast to RNA-templated DNA synthesis by telomerase, synthesis of new telomeric DNA by ALT involves the use of a DNA template [6]. As these TMMs are either undetectable or have low levels of activity in normal somatic cells and are relied upon by the vast majority of cancers [7,8], they provide important targets for detection and treatment of cancer cells. ALT is being found in an increasing number of cancer types, including some that have a very poor prognosis with currently available treatments (as discussed below).

Telomerase-independent telomere length maintenance was first described in yeast, where genetic analyses demonstrated its dependence on homologous recombination (HR) [9]. The existence of an ALT mechanism in mammalian cells was deduced from the observation that telomere lengths were maintained for hundreds of population doublings (PD) in telomerase-negative immortalized human cell lines [3,10]. Showing that a cell line can maintain the length of its telomeres in the absence of telomerase for a suitable number of PD (e.g., 20–30) is still the only definitive test for ALT activity, but in a number of regards it is an unsatisfactory assay. First, it is not suitable for detecting ALT activity in human tumors, where it is usually not practicable or ethical to obtain serial samples. Second, the need to demonstrate that the cells or tumors are telomerase-negative means that this assay is not suitable for detecting ALT in situations where both ALT and telomerase may be present. Although there are no known examples of ALT and telomerase being activated spontaneously in the same cells, it has been shown experimentally that both mechanisms can co-exist in cells [11–14]. Third, it takes a long time – 20 PD may take more than a month – and, fourth, this test can only determine whether ALT activity is present and is unable to measure the amount of activity; both of these aspects make it very unsuitable as an assay to screen large numbers of chemical compounds to find ALT inhibitors, and thereby find anticancer treatments that target this mechanism. Finally, the need to demonstrate that telomere length is maintained excludes the possibility of detecting ALT activity levels that are insufficient to completely prevent telomere shortening. The ability to do this will be essential for understanding the role of ALT in normal biology and the potential side-effects of anti-ALT treatments.

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In contrast, there are activity assays for telomerase (the most widely used being the TRAP assay [15]), which was originally detected by its enzymatic activity [16]. This means that it can be detected in tumor samples and cell lines regardless of whether ALT is also present. It can also be detected in some types of normal cells, where the level of telomerase activity is insufficient to completely prevent telomere shortening. Telomerase activity assays are also rapid and can be quantitative. Ideally, assays for ALT activity would be able to provide the same type of information. Telomerase activity assays, however, simply indicate the presence and amount of active enzyme and yield no information about its activity at telomeres [17]. The activity of telomerase in vivo appears to be regulated by the accessibility of telomere termini and by *cis*-acting telomeric factors [18]. In this important regard, analyses of telomerase activity are currently no more advanced than analyses of ALT, because the only way of assessing telomerase activity at human telomeres in vivo is to do medium- to long-term studies of cultured cells to analyze telomere lengths for a suitable number of PD.

Without the availability of an ALT activity assay, the presence of ALT in tumors has been inferred from phenotypic features that are characteristic of ALT[+] cell lines. There is a growing list of these phenotypic characteristics, but in most cases the nature of their relationship to ALT activity is unclear, which means that their use as a surrogate for ALT activity when telomere-related factors are perturbed experimentally is fraught with difficulties of interpretation. However, a few ALT markers may directly assay an aspect of the ALT mechanism or could be based on an intermediary molecule, and hence might be able to be used as a definitive assay for ALT.

Here we review these phenotypic characteristics of ALT-positive cell lines and tumors, the extent to which they can be relied on for detecting the presence of ALT activity, and progress towards an ALT activity assay. We also briefly reflect upon the implications these ALT characteristics have for understanding the mechanism of ALT, models of which have been presented in a recent review [19]. Finally, we summarize the increasing number of cancer types in which ALT activity has been detected, and clinical situations where availability of an ALT activity assay would be advantageous.

## 2. ALT assays

### 2.1. The "gold standard": maintenance of telomeres in the absence of telomerase activity

The only test for ALT that can be regarded as definitive at present is to determine whether telomere length is maintained in the absence of telomerase activity [3], although as a practical assay this has the drawbacks listed above. It can only be used for cell lines (or animal model systems) where telomerase is absent, and it is a medium- to long-term assay that is essentially non-quantitative. Even in clonally derived cell lines, there can be minor fluctuations in telomere length from passage to passage, so in practice it is necessary to culture cells for at least 20–30 PD in order to be confident that telomere length is being maintained, and ideally the absence of telomerase activity should be demonstrated at multiple PD levels. Because maintenance of telomere length in the absence of telomerase has never been demonstrated in non-immortalized human cells, a useful confirmatory test is to demonstrate that the cells being studied are in fact immortalized. This is usually done by determining whether the cells are able to grow in culture for >100 PD.

Limited quantitative information can be extracted from this assay in a carefully controlled context. In an ALT[+] cell line, inhibition of ALT can be detected by demonstrating that telomeres shorten steadily with increasing PD [20–22]. For example, in an ALT[+] fibroblast cell line in which the MRE11/RAD50/NBS1 (MRN) complex was sequestered following expression of high levels of SP100 protein, telomere length declined linearly (over several time-points) at a rate of approximately 120 base pairs (bp)/PD. This is within the range of telomere attrition rates in normal human fibroblasts that do not have any detectable telomere maintenance mechanism, and is consistent with ALT having been almost completely inhibited in these cells [20]. The rate of telomere attrition following treatment with a candidate ALT inhibitor can yield quantitative information about the extent of inhibition. However, the length of time required for this assay results in the possibility of mutations or other adaptations occurring in the cultured cells that confound the results. Moreover, it may not be possible to use this assay to demonstrate that a candidate has ALT inhibitor activity if it also has effects that result in cell death or senescence within that time period, which may explain the difficulty demonstrating telomere shortening in some circumstances [23–25]. A more practical assay is clearly required.

### 2.2. Telomere length heterogeneity

One of the first hallmarks found in immortalized human cell lines that use the ALT TMM was a characteristic pattern of telomere lengths (as analyzed by terminal restriction fragment [TRF] Southern blots), ranging from very short to extremely long, and with a modal length approximately twice that in comparable telomerase[+] or normal cells [3,26]. The telomere length heterogeneity can also be visualized in metaphase spreads by telomere fluorescence in situ hybridization (FISH) [14]. Telomere lengths in ALT cells are not normally distributed, in contrast to telomerase[+] cells, most of which regulate their telomere lengths about a mean of 5–10 kb [27,28]. Analyses of single telomeres in ALT[+] cells revealed sporadic, unsynchronized increases and decreases in telomere lengths of a variable amount (that are sometimes very large – up to ~20 kb) on a background of gradual erosion (~50 bp/PD) [29], which appear to be responsible for the broad distribution of ALT telomere lengths [28]. This pattern is established within one PD (equivalent to approximately 17 cell generations, which are required for one clone to overgrow a culture that is in crisis) of ALT being activated during immortalization in vitro [30].

The long heterogeneous telomere length pattern remains the best-established marker for ALT in human cells, including tumors archived under conditions where it is possible to extract high-quality genomic DNA [5,31]. In tumor specimens the distinction between ALT[+] and ALT[−] is sometimes less obvious due to tumor heterogeneity and non-tumor cells in the specimen [31]. Tumor length heterogeneity needs to be interpreted cautiously, especially under experimentally perturbed conditions, as this characteristic may not be completely ALT-specific. Long and/or heterogeneous telomeres are seen in telomerase[+] cell lines when expression of hTR or hTERT at supra-physiological levels challenges the telomere length regulatory mechanism with excessive telomere lengthening [32,33]. Conversely, it may not be necessary for cells using the ALT mechanism to have long telomeres [34].

### 2.3. Telomere length fluctuation

Because rapid, unsynchronized changes in telomere length cause telomere length heterogeneity [29], detection of these length changes may be regarded as closely related to detection of length heterogeneity. However, detection of ongoing fluctuations in individual telomere lengths may be useful in the situation where length heterogeneity has already been established by ALT activity, and it is necessary to determine whether an experimental manipulation has subsequently repressed ALT [14]. This could potentially be done by subcloning followed by Southern blot analysis of individual telomere lengths using a probe for a DNA marker inserted in