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The C-Circle Assay for alternative-lengthening-of-telomeres activity

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ABSTRACT

The C-Circle Assay has satisfied the need for a rapid, robust and quantitative ALT assay that responds quickly to changes in ALT activity. The C-Circle Assay involves (i) extraction or simple preparation (Quick C-Circle Preparation) of the cell's DNA, which includes C-Circles (ii) amplification of the self-primed C-Circles with a rolling circle amplification reaction and (iii) sequence specific detection of the amplification products by native telomeric DNA dot blot or telomeric qPCR. Here we detail the protocols and considerations required to perform the C-Circle Assay and its controls, which include exonuclease removal of linear telomeric DNA, production of the synthetic C-Circle C96 and modulation of ALT activity by γ-irradiation.

1. Introduction

The C-Circle Assay (CCA) [1] has become the front-line measure for the alternative-lengthening-of-telomeres (ALT) mechanism [2,3]. It has satisfied the need for a rapid, robust and quantitative ALT assay that responds quickly to changes in ALT activity. ALT is relied on as the telomere maintenance mechanism for 10% of cancers (on average) [4] and there is increasing interest in understanding the ALT mechanism and developing ALT-targeted therapeutics. The CCA has a role to play in facilitating this, and here we provide a detailed protocol for the appropriate implementation of this assay and its controls.

The CCA is based on detection of the C-Circle biomarker [1], which is the only known ALT specific molecule. We have defined C-Circles as self-primed templates for rolling circle amplification that have a telomeric C-strand template sequence [3] (Fig. 1). The fact that C-Circles are self-primed is a key factor in the success of the CCA because it means that no primer hybridization is required. This allows the CCA to avoid detection of double-stranded telomeres and other non-ALT-specific telomeric DNA [5]. The CCA's use of rolling circle amplification provides a sensitive, linear and isothermal amplification of the biomarker. The linear amplification allows the CCA to be robustly quantitative, which combined with the rapid response of C-Circles to changes in ALT activity (within 24 h [1]), means that the CCA is well suited to screen for ALT inhibitors. The CCA can detect as few as 100 ALT+ cells [1] and therefore can be performed with a limited amount of tumor sample. The isothermal amplification reaction allows the CCA to be performed in a water bath or heating block, which can be useful if thermocyclers are not available or being used for other purposes.

An overview of the CCA is presented in Fig. 1. Performing the CCA consists of three main steps: (i) extracting the DNA (including C-Circles) from the specimen (Section 3), followed by quantitation of the DNA (Section 4), (ii) amplifying the C-Circle sequence by rolling circle amplification (Section 5) and (iii) detecting the CCA (rolling circle amplification) products (Section 6). The CCA signal

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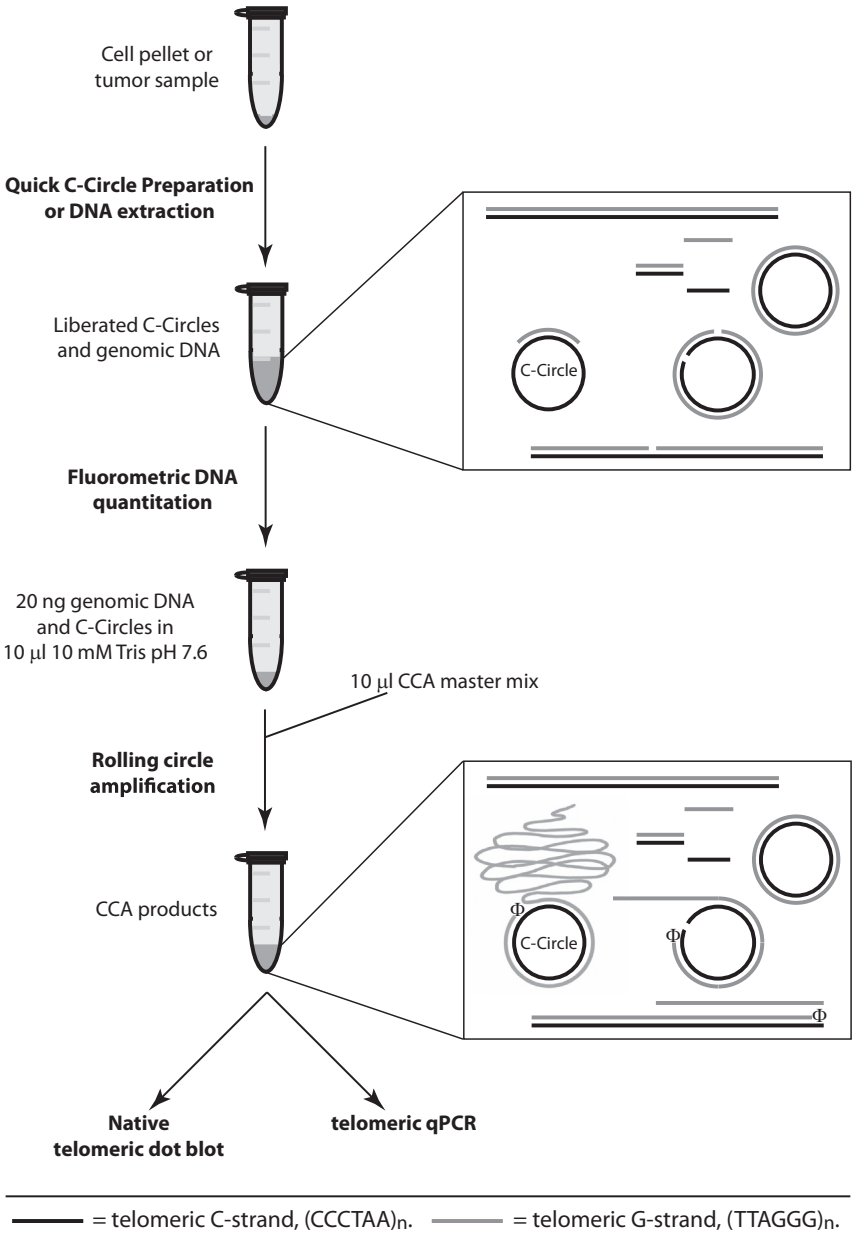

Fig. 1. Overview of the CCA. The first step of the CCA is to perform a "Quick C-Circle Preparation or DNA extraction" of the DNA from the sample. This DNA includes a diverse range of extra-chromosomal telomeric DNA molecules, including circular and linear species. C-Circles are telomeric DNAs that have an uninterrupted circular telomeric C-strand (template strand for the CCA), with at least one telomeric G-strand 3′ end available for self-priming a rolling circle reaction. Aliquots of the samples, each with the same amount of genomic DNA (in the order of 20 ng), are loaded into the CCA's "rolling circle amplification" reaction that only amplifies self-primed templates (marked with a "Φ" in the "CCA products" diagram). Native telomeric dot blot detects single-stranded telomeric G-strand products of the CCA. Low levels of CCA signal need appropriate controls (Section 7); because low signal could arise from non-C-Circle sources, such as pre-existing single-stranded telomeric G-strand, linear telomeric double-stranded DNA that have nicks or gaps in their G-strand and telomeric circles that are nicked/gapped in both strands (topologically linear). Telomeric qPCR can also be used to detect CCA products by determining the change in total telomeric signal, which usually increases five-fold after the CCA (for an average ALT+ cell line).

is usually reported relative to the corresponding amount of genomic DNA. We also present the preliminary considerations (Section 2), controls (Section 7) and post-CCA analysis (Section 6) necessary for appropriate use of the CCA.

2. Preliminary considerations

One specific consideration is that the CCA requires the circular (telomeric C-strand) template to be complete and factors that may cause DNA nicks should be avoided for all stages prior to the CCA's rolling circle amplification reaction (Section 4). This includes avoiding nuclease activity, freeze-thaw cycles, acid hydrolysis and shearing.

2.1. Temperature and storage

All samples prior to amplification should be stored in a freezer without a defrost cycle. To minimize nuclease activity, processed samples such as extracted DNA should not be kept unfrozen for longer than necessary. This includes keeping samples on ice or refrigerated, which should be kept to less than one day. Although room temperature should be avoided where possible, we commonly find mixing of viscous samples and centrifugation of ethanol precipitated DNA works best at room temperature and we have not noticed nuclease activity if time at room temperature is kept to a minimum. DNA should not be heated over 85 °C or background signal will be significantly increased.

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