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A novel method for the sensitive detection of mutant proteins using a covalent-bonding tube-based proximity ligation assay

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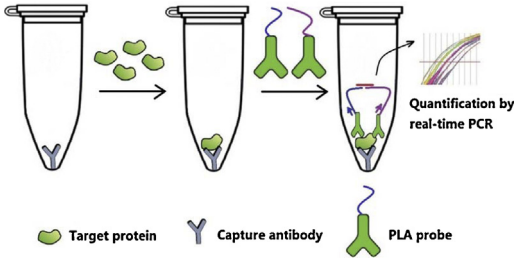
HIGHLIGHTS

- We developed a covalent-bonding tube-based proximity ligation assay (TB-PLA).
- TB-PLA was a specific, highly sensitive and inexpensive protein-detection method.
- We measured p53 mutants in serum using sensitive TB-PLA for the first time.
- TB-PLA can detect a 500-fold lower concentration of mutant p53 than ELISA.
- TB-PLA was suitable for the early clinical diagnosis and prognosis of cancer.

GRAPHICAL ABSTRACT



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ABSTRACT

Tumorigenesis is the cumulative result of multiple gene mutations. The mutant proteins that are expressed by mutant genes in cancer cells are secreted into the blood and are useful biomarkers for the early diagnosis of cancer. However, some difficulties exist; for example, the same gene will express different protein mutants in different patients, and early tumors secrete only small amounts of mutant protein. Thus, the presence of mutant proteins in plasma has not previously been exploited for the early diagnosis of cancer. Proximity ligation assay is a protein-detection method that has been developed in recent years and has been widely used because of its high sensitivity. However, this approach still suffers from some shortcomings that should be addressed. In this paper, we develop a covalent-bonding tube-based proximity ligation assay (TB-PLA). The limit of detection of TB-PLA for 0.001 pM, and the method exhibited a broad dynamic range of up to seven orders of magnitude. Furthermore, we coupled the conformation-specific antibody PAb240 of p53 mutants to PCR tubes for TB-PLA. The assay was capable of detecting an approximately 500-fold lower concentration of mutant p53 in serum compared with sandwich ELISA. Thus, we demonstrate TB-PLA to be a highly sensitive and effective approach that is suitable for the early clinical diagnosis of cancer using the conformation-specific antibodies of protein mutants.

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1. Introduction

Quantitative detection of protein biomarkers from clinical samples can promote effective diagnosis and early prognosis of many diseases such as cancer [1]. However, detection of many important disease-related biomarkers in serum is often challenging because of their trace levels and the molecular complexity of plasma [2,3].

Proximity ligation assay (PLA), first described by Fredriksson et al. in 2002, is an immunoassay technique for the detection of protein molecules via DNA ligation and amplification, and it offers high specificity and sensitivity [4]. PLA has recently been developed into many forms, such as homogeneous-phase assay, in which the antigen is recognized by DNA aptamers or antibodies in solution prior to ligation [4,5], and solid-phase proximity ligation assay (SP-PLA), in which the antibodies are immobilized directly on the walls of PCR tubes or by immobilizing biotinylated antibodies on the surfaces of streptavidin-coated tubes or on streptavidin-coated magnetic beads [6–11]. The PLA technique has been implemented for a wide variety of applications. Unfortunately, PLA has some drawbacks that prevent it from being widely used. In a homogeneous-phase assay, the process does not include a washing step, and therefore, complex components in the sample may affect the activity of T4 ligase and Taq polymerase. In the case of SP-PLA, polycarbonate PCR tubes or streptavidin-coated tubes are costly and not generally available for real-time PCR instruments. We have previously demonstrated that SP-PLA performed very well using magnetic beads as solid supports for the capture and separation of the target molecules [10,12,13]. However, the beads are expensive and a cumbersome washing step is required during the magnetic separation, which limits the utility of the procedure. In addition, magnetic beads cause some interference in the fluorescence signal for real-time PCR.

We now present a simple and efficient assay format for a covalent-bonding tube-based proximity ligation assay (TB-PLA) (Fig. 1), which is the development of a generally useful SP-PLA protocol for highly sensitive and specific protein detection in limited samples using a single polyclonal antibody or a combination of monoclonal and polyclonal antibodies. The TB-PLA method exhibits very low limits of detection and broad working dynamic ranges, and furthermore, the entire procedure is completed in only one ordinary PCR tube. The assay performance is also far superior to that of assays performed on other solid supports. When a PCR tube treated with glutaraldehyde is used, the assay exhibits very good reproducibility and requires very few antibodies coated on the tubes.

In this report, we illustrate TB-PLA by detecting three different proteins: prostate-specific antigen (PSA), wild-type p53 protein, and mutant p53 protein. PSA is a widely used biomarker for prostate cancer screening, and the ability to measure lower concentrations of PSA protein could even provide useful prognostic information that may assist with breast cancer treatment [14–17]. Tumor suppressor protein p53, a nuclear transcription factor, plays an essential role in cell-cycle regulation and is frequently mutated or inactivated in many cancers [18–20]. Mutant p53 has been found to be accumulated at extremely high levels in more than 50% of human tumors [21]. By contrast, wild-type p53 is often maintained at low levels in normal tissues [22]. The determination of wild-type and mutant p53 with high specificity and sensitivity may provide prognostic and predictive information and offer novel clinical possibilities for both diagnosis and treatment [23,24]. Therefore, we achieved good results for TB-PLA using the conformation-specific antibody PAb240, which recognizes most of the p53 mutants but does not recognize wild-type p53 [25].

2. Materials and methods

2.1. Materials and equipment

PSA was purchased from the Shuangliu Zhenglong Laboratory of Biochemical Products (Chengdu, Sichuan, China). A recombinant p53 (human) sample was obtained from Boston Biochem (Cambridge, MA, USA). A biotinylated anti-PSA polyclonal antibody and a biotinylated anti-p53 polyclonal antibody were purchased from R&D Systems (Minneapolis, MN, USA). The anti-p53 monoclonal antibody (PAb240) was purchased from Abcam (Cambridge, MA, USA). Plasmid pGEX-p53 R175H (Addgene plasmid 39,480) was obtained from Addgen (Cambridge, MA, USA) [26]. A p53 pan ELISA kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All oligonucleotides (Table 1) used in this study were synthesized and purified via HPLC at Sangon (Shanghai, China). Streptavidin–horseradish peroxidase (STV-HRP) was obtained from the Beyotime Institute of Biotechnology (Shanghai, China), and 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from CWBIO (Beijing, China). T4 DNA ligase was purchased from Fermentas (Shenzhen, China), and SYBR Premix Ex Taq II was purchased from TaKaRa (Dalian, China). All other chemicals were of extra-pure analytical grade and were used without further purification. All aqueous solutions were prepared using high-purity deionized water (18 MΩ cm), which was obtained from a Milli-Q water purification system.

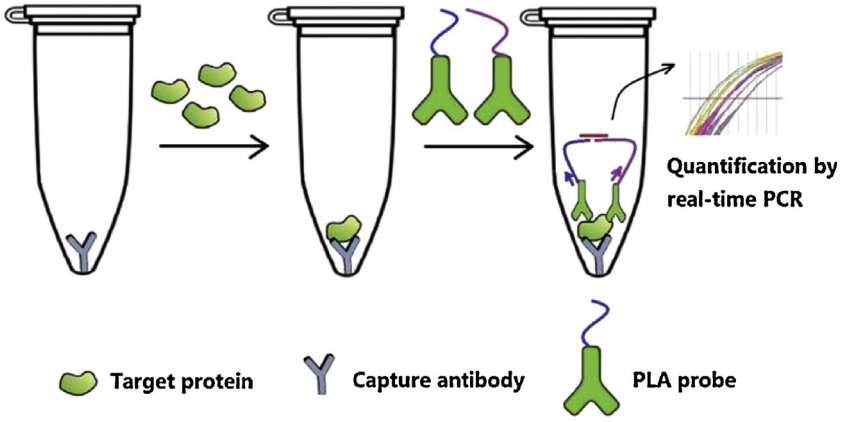

Fig. 1. Schematic description of tube-based PLA. Firstly, pretreated tubes are incubated with the sample. Next, tubes are washed and incubated with PLA probes. Finally, upon proximal binding, the oligonucleotides are ligated by a connector oligonucleotide and then detected with real-time PCR.