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A methodological approach for the thermal stability and stress exposure studies of a model antibody

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ABSTRACT

The anti-horseradish peroxidase (HRP) antibody is conventionally used in immunohistochemistry. More recently, it has been used as the key element in a gold standard method to evaluate the functionality of antibody-based materials. However, few information are available about its melting temperature and its stability after exposition to laboratory stress conditions including freeze-drying and freeze-thawing cycles. The aim of this study was to evaluate the effects of these environmental constraints on the anti-HRP antibody in order to further use it as a reference in quality control and in the development of new antibody-based materials.

In the developed method, the anti-HRP antibody is covalently immobilized onto a solid surface. After the direct recognition of its antigen HRP, the signal is proportional to the number of antibody active binding sites. The method was successfully utilized to accurately evaluate the anti-HRP antibody melting temperature (Tm was 73.5 ± 0.2 °C). The method is a rapid and reliable tool with minimal cost for studying the anti-HRP antibody stability to solvent stress, freeze-thawing cycles, and freeze-drying process. The obtained information may be useful for routine analysis or in the development of antibody-based materials. This can be also proposed as an easy way to control antibody freeze-drying process.

Introduction

The use of biotherapeutics, such as monoclonal antibodies (mAb), has markedly been developed in recent years in modern medicine (formulation for cancer treatment, tissue typing, and drug delivery systems). Antibodies (Ab) have also tremendous applications in the field of prevention, in the follow-up in the treatment of diseases, and are fundamentals in the understanding of human physiology. In research lab, Ab are used in 1/immunohistochemistry (IHC) to localize a target compound in tissue, and in 2/liquid and solid phase ligand binding assays (LBA) such as enzyme linked immunosorbent assay (ELISA) to analyze Ab, protein antigens (Ag) and even low molecular weight drugs. However, Ab technologies are often limited by 1/the ability to consistently produce Ab with appropriate affinity and specificity, 2/the propensity of the Ab to self-assemble in solution in dimers, trimers, or higher aggregates. This can have important effects on drug potency with, for instance, the generation of undesirable immunological effects during patient therapy [1]. Aggregation of Ab is favored at high Ab concentrations, at alkaline pH under oxidizing conditions, by freeze–thawing, temperature variation, and other stress factors during development, production and even storage [2–4]. The accuracy and performance of LBA and IHC protocols depend on the quality of key reagents, *e.g* specific analyte and binding reagents (such as mAb). Indeed, mAb are classified by the Global Bioanalysis Consortium (GBC) and the European Medicines Agency (EMEA) as critical reagents [5–8]. Utilizing uncontrolled critical reagents solution could lead to inaccurate conclusions and delays in diagnostics and drug development process.

Defining formulations that enhance protein stability is a widely pursued goal. The basic determination of *in vitro* protein stability, particularly thermodynamic stability, involves biophysical techniques such as Differential Scanning Calorimetry (DSC) and Differential Scanning Fluorimetry (DSF). However, DSC requires expensive instrumentation and is limited to a small number of samples [9]. DSF is a more convenient and high-throughput technique utilizing a quantitative polymerase chain reaction (qPCR) thermocycler to monitor thermal unfolding of a protein in the presence of a hydrophobic fluorescent dye, which binds preferentially to the hydrophobic parts of the unfolded protein. The intensity of the fluorescent dye is plotted as a function of the temperature. The resulting sigmoidal curve (the melting curve)

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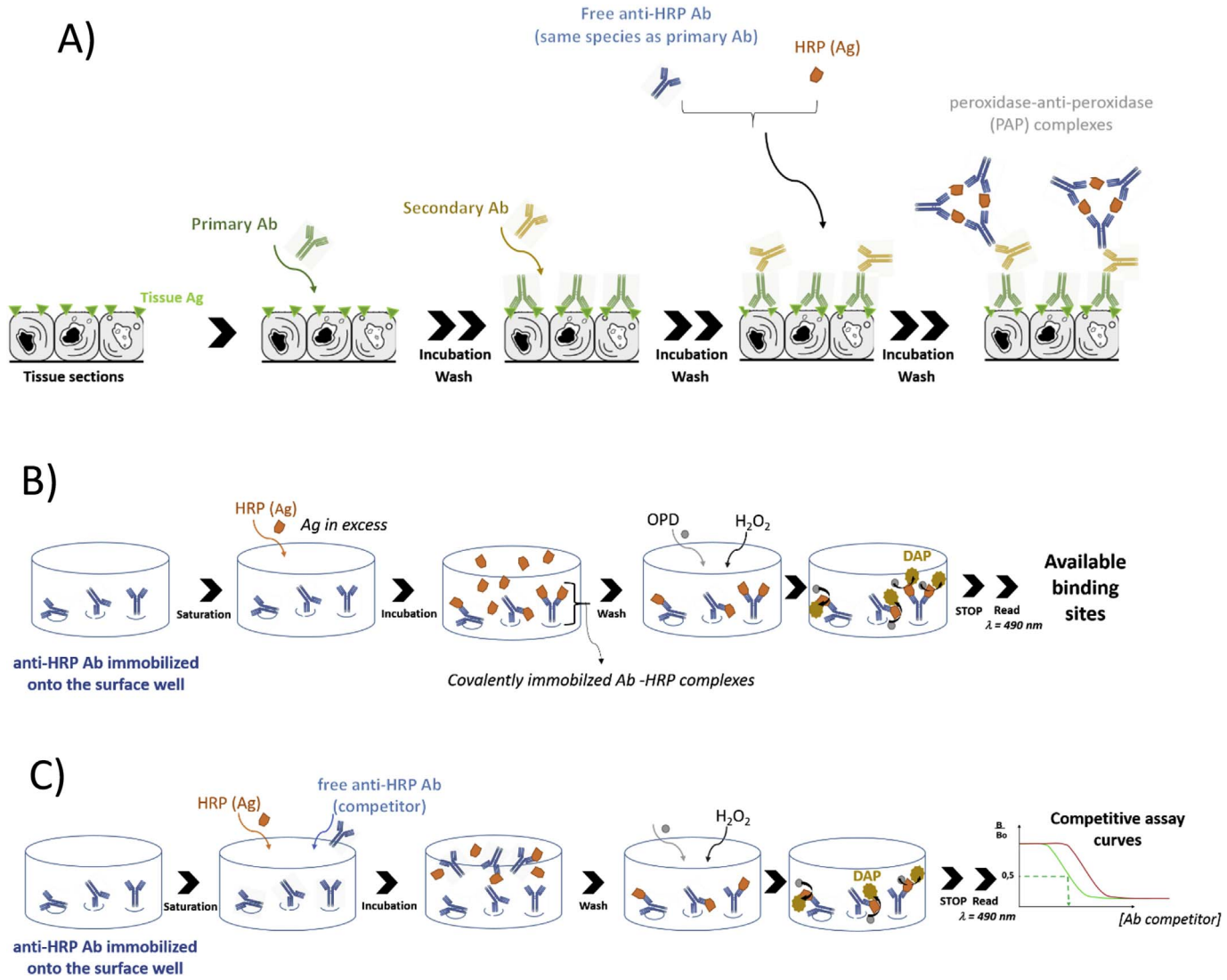

Fig. 1. IHC assays and LBA using anti-HRP Ab reported in literature.
(A) The PAP principles and its use in staining tissues to detect antigen. (B) The validated A2HRP principles and its use to determine active binding sites of anti-HRP Ab covalently immobilized onto the surface of the wells, and consequently if Ab surface was (partially or totally) affected or not after undergoing laboratory stress conditions. Results are expressed in % of active surface (C) The competitive inhibition method using a covalent grafting step for anti-HRP Ab, and the competitor are the anti-HRP Ab that had been subjected to laboratory stress conditions. The binding inhibition expressed in B/B_0 varies with the amount of added competitor. Comparison of the values obtained at the mid-point ($B/B_0 = 0.5$) of the competitive inhibition curves permits to calculate the percent remaining Ab activity (see experimental part).

permits to determine, at the point of inflection, the apparent melting temperature (Tm) of the protein. Tm is an indicator of the global thermal stability of the protein [10,11]. DSF was also used for the quantitation of protein–protein interactions, the identification of novel ligands, and in the early phase of vaccine formulation development [12]. However, in the presence of stabilizing Ab formulation reagents, nonspecific background signals or interfering protein–dye interactions have been reported, leading to a bad determination of Tm [13,14].

Horseradish Peroxidase (HRP), HRP conjugates, and HRP-Ab complexes are commonly used in IHC and LBA. For instance, HRP can be used in free form in the peroxidase-antiperoxidase (PAP) approach in IHC. The PAP approach utilizing the HRP-anti-HRP Ab was the pioneer of IHC methods in paraffin-embedded tissues [15]. This approach indirectly detects the amount of primary Ab, which interacts with Ag tissue. The formation of PAP soluble immune complexes, with a molar ratio of two molecules of anti-HRP Ab bound to three molecules of HRP, allows for amplification of the signal (Fig. 1A). The sensitivity of this unlabeled Ab method was reported to be equal to or even a factor of two or three higher than conventional labeled ones since HRP is not chemically linked to the Ab but immunologically bound, and thus does not loss any of its enzyme activity [16]. In LBA, HRP is conventionally used as a label in classical ELISA systems. Recently, our group proposed and validated a direct and label-free method using HRP as Ag [17,18]. The assay called “Antibody Anti-HorseRadish Peroxidase (A2HRP)” is based on the direct detection of HRP by an anti-HRP Ab immobilized onto the solid surface (Fig. 1B). This A2HRP protocol has been validated according to guidelines for bioanalytical method validation [8,18–20]. The optimized A2HRP method was demonstrated to be accurate and gave excellent inter-day variability (< 12.1%). The A2HRP method was successfully applied to evaluate coatings of Ab surfaces (relative to density and activity of immobilized anti-HRP Ab). The Lower Limit of Quantification (LLOQ) corresponds to 1.40 ± 0.18% of the active surface [17,18]. Despite wide applications of the anti-HRP Ab in IHC assays and its more recent uses in determining functionality of Ab surfaces, information on its thermal and solvent stabilities have not yet been reported. In addition, as usual, anti-HRP Ab performances decrease over time and cannot be safely stored in low concentrated ready-to-use dilutions. To prevent the loss of activity and to maintain