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# Biobarcode assay for the oral anticoagulant acenocoumarol

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ABSTRACT

A novel approach for therapeutic drug monitoring of oral anticoagulants (OA) in clinical samples is reported, based on a *NP-based biobarcode* assay. The proposed strategy uses specific antibodies for acenocumarol (ACL) covalently bound to magnetic particles (pAb236-MP) and a bioconjugate competitor (hACL-BSA) linked to encoded polystyrene probes (hACL-BSA-*e*PSP) on a classical competitive immunochemical format. By using this scheme ACL can be detected in low nM range (LOD, 0.96 ± 0.26, N = 3, in buffer) even in complex samples such as serum or plasma (LOD 4 ± 1). The assay shows a high reproducibility (%CV 1.1 day-to-day) and is robust, as it is demonstrated by the fact that ACL can be quantified in complex biological samples with a very good accuracy (slope = 0.97 and $R^2$ = 0.91, of the linear regression obtained when analyzing spiked *vs* measured values). Moreover, we have demonstrated that the biobarcode approach has the potential to overcome one of the main challenges of the multiplexed diagnostic, which is the possibility to measure in a single run biomarker targets present at different concentration ranges. Thus, it has been proven that the signal and the detectability can be modulated by just modifying the oligonucleotide load of the encoded probes. This fact opens the door for combining in the same assay encoded probes with the necessary oligonucleotide load to achieve the detectability required for each biomarker target.

## 1. Introduction

*Biobarcode* assays emerged some years ago as a new generation of innovative approaches for assay signal amplification. *Biobarcode* refers to biomolecules that have specific features that made them suitable to become a code. Thus, the characteristic structure of DNA molecules based on four bases, allows designing an extraordinary wide number of different oligonucleotide sequences and, therefore, their use as *biobarcodes* [1]. The nucleotide bases are complementary two-by-two, which allows the specific binding to complementary sequence. These properties, combined with DNA versatile synthesis, permits the introduction of diverse functionalities and make designed DNA an exceptional option for *biobarcode* assays, particularly immunoassays [2]. Oligonucleotide sequences were first introduced in immunoassays as signaling readout, for amplification purposes, developing immuno-PCR [3]. Antibodies were labeled with oligonucleotide sequences instead of enzymes, providing increased detectability. However, this approach has not been routinely applied due to the requirement of highly standardized protocols, special instruments and qualified personnel.

The *biobarcode assay* concept, as we understand it today, was reported for the first time in 2002 by Mirkin's group (Nam et al. [4]), demonstrating that PSA (prostate-specific antigen) could be detected down to 30 aM concentration, six orders of magnitude more sensitive than the clinically accepted assay methods. The principle behind *biobarcode assays* is the use of bioreceptors attached to nanoparticles (NP) that contains a large number of specific nucleotide sequences (encoded probes). Every different bioreceptor is attached to encoded probes with a unique oligonucleotide sequence and, in this manner, the binding of a particular analyte is encoded. Since every NP is loaded with a large number of nucleotides (i.e. 360 on a 30 nm particle [5]) the signal for the detection of a particular target analyte is amplified. After the binding event, the bound oligonucleotides are released and subsequently detected through different means. This PCR-less technique, has the ability to achieve countless distinctive labels and signal amplification due the large surface/volume ratio of nanoparticles. Moreover, these *NP-based biobarcode assay*, usually employ biofunctionalized magnetic particles as capture probes that allow capturing/concentrating the target analytes present in the sample [10,11]. Both, the magnetic particle and the DNA encoded probes form a complex via sandwich with the target analyte, usually a protein or a particular DNA

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or RNA sequence, that can be isolated by the application of a magnetic field. This strategy has been found to increase sensitivity, reduce background signal (since oligonucleotide quantification is matrix free) and potential for high multiplexing [6,7]. Otherwise, oligonucleotide detection/quantification strategies include DNA-microarray based on silver enhancement of gold nanoparticles [8], oligonucleotide digestion coupled to electrochemical detection of purine bases [9], fluorophore functionalization of coding sequences [10], colorimetric particle aggregation [11], immunoPCR [12], capillary electrophoresis [13] and Raman or SERS [14] technologies among others. Each of them provide different advantages and drawbacks, while microarray remains the best strategy for simultaneous detection of a large number of sequences and is ideal for high-throughput analysis, conversely, fluorescence spectroscopy can allow time reduction and in situ detection.

The PCR-less *NP-based biobarcode assay* approach has been used for proteins [15], DNA [15], pathogens [16], cells [17] and miRNA [18] detection, but their application to the detection and quantification of small molecules is scarce [19]. Thus, some attempts have pursued determination of small molecules such as dopamine [20] or norepinephrine [14] using *biobarcodes*, either on a sandwich immunochemical format based on antibody competition [20] or either based on antigen competition [21,22]. However, both having one of the immunoreagents coated in a well-plate, limiting its applicability for multiplexed purposes. It has not been until very recently, that *NP-based biobarcode* formats, performed as described above, have been used for determination of small molecules. Hence, 17β-estradiol *NP-based biobarcode* was the first assay reported using this format. A LOD of 6.37 fg $mL^{-1}$ was reached using an immunochemical format with two antibodies competing for the target analyte [19], instead of employing a bioconjugate competitor as it is usual in the immunochemical field. A bioconjugate competitor immunochemical format for triazophos detection has been lately reported by the same authors but require real-time PCR [23], to reach a LOD of 0.02 ng $mL^{-1}$.

With this scenario, we report here for the first time a PCR-less *NP-based biobarcode* assay for acenocumarol (ACL), a frequently used oral anticoagulant (OA), using a bioconjugate competitor immunochemical format according to the scheme shown in (Fig. 1). ACL is a vitamin K antagonist (VKAs) that belong to coumarin derivatives group as also does warfarin and phenprocoumon. Although new OAs have appeared, vitamin K antagonists are still used by millions of patients worldwide for the primary and secondary prevention of venous and arterial thromboembolitic events. They are highly efficient drugs, however, their complex pharmacokinetics and pharmacodynamics together with the remarkable variability in the dose-response relationship, as well as, their narrow therapeutic index determine a high risk of bleeding complications (up to 10–17% of the patients treated with these drugs [24]) or recurrent thrombosis. Appropriate management of patients under VKAs treatment calls for efficient laboratory and clinical monitoring of these drugs. Chromatographic methods are the most frequently used tools for the detection of these drugs or their metabolites in plasma. LOD around 0.1–3 nM have been reported [25–27] for methods based on liquid chromatography coupled to either electrospray tandem mass spectrometry or ultraviolet detection. Nevertheless, the main drawback is the need of sample pretreatment (either solid phase extraction, sample derivatization or sample clean-up) which make them not suitable for routine pharmacokinetic and pharmacodynamics studies of many samples from each of the patients treated.

## 2. Materials and methods

### 2.1. Materials and instruments

Polystyrene particles (PSP) were carboxy modified Dyed Microspheres (K1 050 noir) were supplied by Estapor® (Millipore Corp., Billerica, MA, USA). The magnetic particles (MP), Dynabeads MyOne Tosylactivated, and the magnetic rack Magnarack™ were obtained from Invitrogen (Life technologies™, Paisley, UK). The pre-cleaned plain slides were purchased from Corning® (Corning Inc., New York, NY, USA). Functionalized slides were spotted with a BioOdissey Calligrapher MiniArrayer (Bio-Rad Laboratories, Inc. USA). Measurements were recorded on a ScanArray Gx PLUS (Perkin Elmer, USA) with a Cy3 optical filter with 5-μm resolution. Slide gaskets were purchased from ArrayIt Coporation (Sunnyvale, CA, USA). U-bottom polystyrene microplates were purchased from Nirco (Barberà del Vallès, Spain). IKA® MS 3 basic shaker with a MS 3.4 Microtiter attachment (IKA®, Staufen, Germany) was used to shake the microplates at 800 rpm. A thermos-shaker (TS-100C Biosan, Riga, Latvia) was used to shake Eppendorfs. Eppendrofs were centrifuged with a Legend Micro 21, 10 min, 17,500*g* (Thermo Fisher Scientific Inc.) and plates were centrifuged with a Centrifuge 5810 R, 10 min, 3220*g* (Eppendorf, Hamburg, Germany). The pH and the conductivity of all buffers and solutions were measured with a 540 GLP pH meter and an LF 340 conductometer (WTW, Weilheim, Germany), respectively. Absorbance and fluorescence were read on a Spectramax Plus and a Spectramax Gemini XPS (Molecular Devices, Sunnyvale, CA, USA), respectively. Microscope images were recorded with a Cytoviva (Auburn, AL, USA) coupled to an Olympus VX43 with a 100X Dage objective and software used was Exponent7 Dage MTI XL16 45.

Chemicals and biochemicals. The chemical reagents used were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) and from Sigma Chemical Co. (St. Louis, MO, USA). N2*down*-$NH_2$ oligonucleotide sequence, [AmC6F]CGGAGGTACATTCGACTTGA, was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and TAMRA-N2*up*-SS-$NH_2$,

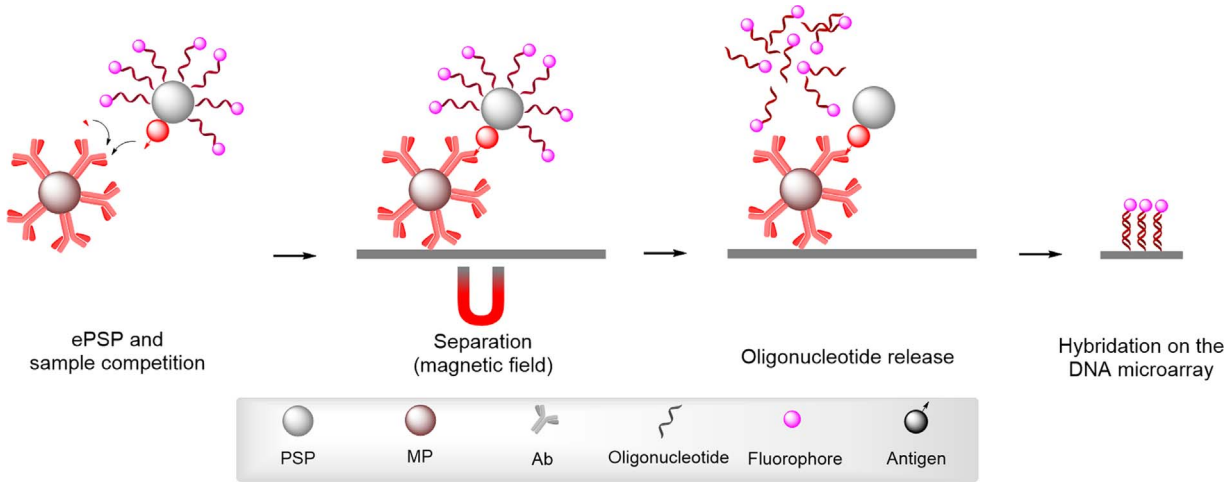


**Fig. 1.** Schematic diagram of the NP-based biobarcode strategy used in this study.