Analytical Biochemistry 658 (2022) 114923

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Capture, detection and purification of dsDNA amplicons using a DNA binding protein on magnetic beads

Ankur Ruhela, Vasso Skouridou, Lluis Masip *

Departament d'Enginyeria Química, Universitat Rovira i Virgili, 26 Països Catalans, 43007, Tarragona, Spain

ARTICLE INFO

Keywords:
scCro dsDNA binding protein
DNA sequence-specific binding
Magnetic beads
PCR amplicon detection
dsDNA amplicon purification

ABSTRACT

Magnetic separation has been widely exploited for capture and detection of nucleic acids, including amplicons. Streptavidin-magnetic beads (SA-MB) are typically employed for this purpose, as well as in biosensing applications. However, remaining biotinylated primer in the amplification reaction can compete with labeled amplicon for binding to the beads. Also, the harsh conditions needed for elution of bound amplicons restrict their use for purification purposes. Herein we show that a sequence-specific DNA binding protein immobilized on magnetic beads can serve as an alternative to SA-MB for these applications. This is enabled by the high binding affinity of scCro DNA binding protein for its specific sequence and its ability to bind dsDNA but not ssDNA. This specific sequence is easily incorporated in the amplicon during amplification with an extended primer. The scCro-MB exhibited higher amplicon binding capacity and detection sensitivity compared to SA-MB when both synthetic and genomic DNA were used as templates for PCR. This resulted not only from increased protein load on the beads but also from minimized interference of excess labeled primer remaining in the unpurified amplification reactions. Finally, a proof-of-concept was provided for the use of the scCro-MB for PCR amplicon purification under mild elution conditions using salt.

1. Introduction

Molecular diagnostics assays are essential for fast, sensitive and specific detection of nucleic acid sequences [1]. These are typically based on amplification tests and they find applications in numerous areas including genetics, infectious diseases, oncology, food safety and forensics among others [2]. Polymerase chain reaction (PCR) and real-time PCR have been the gold standards in nucleic acid amplification. The technological advances achieved over the last few decades though have led to the implementation of more effective alternatives like digital PCR [3], whereas isothermal amplification techniques [4] have also been developed as non-PCR alternatives more compatible with point-of-care applications [5]. Generic, end-point detection of the generated amplicons can be carried out using ultraviolet absorbance and fluorescence staining (in solution or in a gel matrix) which generate bulk signals. For specific detection of a particular sequence, hybridization assays employing oligonucleotides complementary to the target sequence are performed producing optical or electrochemical readouts [6]. Typically, amplification reactions are analyzed by agarose gel electrophoresis, which does not only allow visualization and semi-quantification, but also discrimination of dsDNA amplicons according to their sizes [7]. It is however instrument-dependent, relatively time-consuming especially when purifying the amplicons and provides limited sensitivity.

Magnetic beads (MB) have been extensively used for the separation and detection of different target molecules including nucleic acids [8,9]. Their applications have expanded further into biosensor development, due to the several advantages they offer such as fast and easy magnetic separation, high surface-to-volume ratio and availability of a plethora of functional groups for conjugation of different molecules [10,11]. MB can be used to mediate the capture and detection of nucleic acid targets (especially of low abundance ones) before or after amplification. When the MB are used prior to amplification, complementary ssDNA probes are tethered to the surface of beads with different surface functional groups to capture the target sequence, followed by PCR amplification and detection [12,13]. When the beads are used post-amplification, the generated amplicons can be detected after hybridization with complementary ssDNA probes immobilized on the beads [14,15] or via binding to streptavidin-coated MB (SA-MB) when a biotinylated primer is used during amplification [16–19]. SA-MB have been extensively used for

* Corresponding author.
E-mail address: lluis.masip@urv.cat (L. Masip).

https://doi.org/10.1016/j.ab.2022.114923
Received 10 July 2022; Received in revised form 15 September 2022; Accepted 18 September 2022
Available online 24 September 2022
0003-2697/© 2022 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

capturing biotinylated nucleic acids [20,21], and they can also serve as a solid surface for subsequent detection of bead-bound amplicons. When the amplification reactions are directly incubated with the SA-MB without prior purification though, unreacted biotinylated primers can compete with biotinylated amplicons for binding to the beads, thus reducing the actual amplicon binding efficiency of the beads. On the other hand, harsh conditions are required to elute captured amplicons for downstream applications due to the extremely strong interaction between streptavidin and biotin (affinity dissociation constant K_D of ~1 fM) [22]. High temperature and alkaline treatment can be employed for the elution of biotinylated molecules captured on the beads, however this can lead to detachment of streptavidin monomers from the surface of the beads [23,24].

In this work we sought to evaluate the use of a sequence-specific double stranded DNA binding protein immobilized on MB as an alternative to the SA-MB for the specific capture and detection of dsDNA amplicons. The specific binding of the DNA binding protein to dsDNA was expected to eliminate any potential interference of unreacted ssDNA primers with dsDNA amplicon detection. In addition, milder conditions could be used to elute bead-bound amplicons compared to the harsh ones required for the SA-MB. To this end, a single-chain homodimeric variant of the bacteriophage Cro DNA binding protein (scCro) was used, which binds to a specific 20-bp long dsDNA sequence with high affinity ($K_D \approx 4$ pM) [25]. We have previously demonstrated the detection of PCR amplicons on microtiter plates and on lateral flow strips using scCro conjugated to horseradish peroxidase (HRP) or carbon nanoparticles [26–28]. Herein, the target sequence was amplified using a forward primer extended with the specific Cro binding sequence and a biotinylated reverse primer (Fig. 1A), followed by capture of the double-tagged amplicon on the scCro-MB and detection with SA-HRP enzyme conjugate. The performance of the scCro-MB and SA-MB was compared in terms of binding capacity, effect of the presence of unreacted primers and sensitivity. A synthetic DNA target sequence (high-risk human papillomavirus HPV type 16) was initially utilized, whereas the compatibility of the strategy with other types of PCR templates was demonstrated with the use of genomic DNA extracted from chicken meat and primers targeting its cytochrome *b* gene. Finally, the scCro-MB were used for the binding and subsequent elution of PCR amplicon under mild conditions to demonstrate their potential utility in PCR amplicon purification.

2. Materials and methods

2.1. Materials

Dynabeads M-270 Epoxy and Dynabeads M-270 Streptavidin magnetic beads (SA-MB, 10 mg/mL), phosphate buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4), horseradish peroxidase (HRP), maleimide activated HRP, skim milk powder, TransformAid Bacterial Transformation Kit, HisPure Ni-NTA agarose, SP Sepharose Fast Flow resin, DreamTaq DNA polymerase and dNTPs were purchased from Fisher Scientific (Spain). Streptavidin-HRP conjugate (SA-HRP), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide were obtained from Merck (Spain). The TMB substrate of HRP (BioFX TMB Super Sensitive One Component HRP Microwell Substrate) was from Surmodics (USA). The DNA oligonucleotides were purchased from Biomers.net (Germany) and their sequences are shown in Table 1. The sequences of the synthetic DNA target (high-risk human papillomavirus HPV16) and its corresponding primers were based on a previous work [29]. The design of the primers targeting the cytochrome *b* gene of chicken (*Gallus gallus*) were reported elsewhere [30]. For both targets, the binding site for the scCro DNA binding protein was incorporated on the 5′-end of the forward primers and the biotin on the 5′-end of the reverse primers. The scCro DNA binding protein was expressed and purified as described previously [28] and the scCro-HRP conjugate was prepared as detailed before [31]. All other reagents and material were obtained from Fisher Scientific (Spain), Merck (Spain) and Scharlau (Spain). Genomic DNA was extracted from chicken meat obtained from the local market using the GeneJET Genomic DNA Purification kit (Fisher Scientific, Spain) according to the manufacturer's instructions.

Table 1
Oligonucleotides used in this work. The binding sites for the scCro DNA binding protein are underlined.

Oligonucleotide	Sequence (5' – 3′)	Length (nt)
Hairpin probe	Biotin-TTTTTTTTTT GGTTGGTGTG GTTGGTTTTT TTATCACCGC AAGTGATATT TTTATCACTT GCGGTGATA	70
Synthetic DNA target: high-risk human papillomavirus HPV16		
Synthetic DNA template	GCCCATTAAC AGGTCTTCCA AAGTACGAAT GTCTACGTGT GTGCTTTGTA CGCACAACCG AAGCGTAGAG TCACACTTGC AACAAAAGGT TACAATATTG TAATGGGCTC TGTCCGGTTC TGCTTGTCCA GCTGGACCAT CTATTTCATC CTCCTCCTC	159
Forward primer	TATCACCGCA AGTGATAGCC CATTAACAGG TCTTCCAA	38
Reverse primer	Biotin-GAGGAGGAGG ATGAAATAGA TGGT	24
Genomic DNA target: chicken cytochrome *b* gene		
Forward primer	TATCACCGCA AGTGATAATT CCCTACATTG GACACA	36
Reverse primer	Biotin-TGATAGTAAT ACCTGCGATT GCA	23

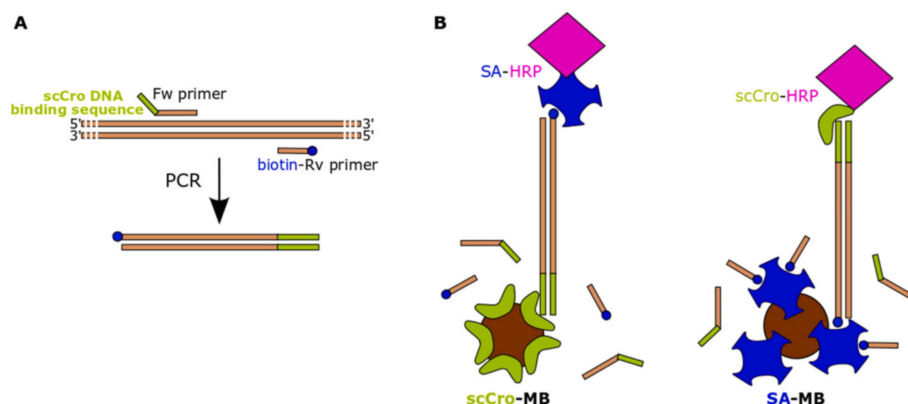

Fig. 1. Amplicon detection using scCro DNA binding protein-magnetic beads. (A) PCR amplification of target DNA with modified primers. (B) Magnetic bead-based colorimetric assay using scCro-MB and SA-HRP for detection. For comparison, SA-MB were used in parallel as shown on the right.

2.2. Preparation of scCro DNA binding protein-magnetic beads (scCro-MB)

The magnetic beads (MB) chosen for the immobilization of scCro were the Dynabeads M-270 Epoxy. They have a diameter of 2.8 μm and a hydrophilic surface according to the manufacturer exhibiting low non-specific binding. The epoxy functional group on the beads readily reacts with thiol groups at physiological pH values (7.5–8.5), whereas amines require moderate alkaline pH (≥9) [32]. The native homodimeric Cro DNA binding protein contains 8 lysine residues per monomer but no cysteines. To facilitate its oriented conjugation on the beads and avoid any possible negative impact to its DNA binding properties, a unique cysteine residue was added to its N-terminal during the cloning step. This would result to the coupling of the protein on the epoxy-functionalized beads via a single tethering point. To perform the immobilization, the MB (100 μL of 30 mg/mL in dimethylformamide) were initially washed three times with 100 μL of 0.1 M sodium phosphate buffer pH 7.4. Then, they were resuspended in 60 μL of 1.25 mg/mL scCro in PBS and then 60 μL of each 0.1 M sodium phosphate buffer pH 7.4 and 3 M ammonium sulfate were added. The mixture was left to incubate overnight at 18 °C under mild rotation followed by the blocking of unreacted epoxy groups with 200 μL of 1 M of Tris-HCl pH 9 for 2 h at 22 °C under rotation. The scCro-MB conjugate was finally washed four times with 300 μL of PBS containing 0.05% (v/v) Tween-20 (PBST), resuspended in 100 μL of PBS (final concentration of 30 mg/mL) and stored at 4 °C until use. The presence of scCro on the magnetic beads was confirmed with a bead-ELISA using an anti-His-tag antibody specifically recognizing the C-terminal His-tag added to the protein during cloning [28] as detailed in the Supplementary Data.

2.3. Determination of the binding capacity of the protein-magnetic beads

Two different assays were designed to evaluate the DNA binding capacity of the protein-magnetic beads. For the first assay, 30 μg of magnetic beads (1 μL of 30 mg/mL for scCro-MB or 3 μL of 10 mg/mL for SA-MB) were incubated with 50 μL of 20 nM (∼22 ng/μL) of a hairpin probe containing the binding site of scCro on one end and a biotin on the other (sequence shown in Table 1). After incubation for 30 min at 22 °C under rotation, the supernatant was recovered, and the amount of DNA (unbound) was quantified with SimpliNANO spectrophotometer. Three replicates were performed for each bead type. The amount of DNA bound on each bead type was calculated from the difference between starting solution and unbound fraction. For the second assay, the same biotinylated hairpin probe (50 μL of 20 nM in PBS) was incubated with 30 μg of each bead type (scCro-MB or SA-MB) for 30 min at 22 °C under rotation. After washing three times with 100 μL of PBST, the beads were resuspended in 50 μL of enzyme conjugate (SA-HRP for the scCro-beads and scCro-HRP for the SA-beads) at varying concentrations (62–500 pM in PBS) and incubated further for 30 min. Finally, the beads were washed three times with 200 μL of PBST, resuspended in 50 μL of TMB substrate and color development proceeded for 2 min. Sulfuric acid (50 μL of 1 M) was then added to stop the enzymatic reaction, the supernatants were separated with a magnet and transferred to the wells of a 96-well plate for measuring the absorbance at 450 nm. The average signals were calculated from triplicate measurements and three independent experiments.

2.4. PCR amplicon detection using protein-magnetic beads (scCro-MB or SA-MB)

The DNA target templates were amplified with specific forward and reverse primers (Table 1) to generate double-tagged dsDNA amplicons, with the double stranded DNA binding sequence for scCro on one end and a biotin on the other end. Each PCR reaction (50 μL) contained equal concentrations of the two primers ranging from 25 to 1600 nM, 200 μM of dNTPs, 1x DreamTaq polymerase buffer, 1 U of DreamTaq DNA polymerase and up to 500 pg/μL of the synthetic or the genomic DNA templates. Amplification of the synthetic DNA target was performed by heating initially for 3 min at 95 °C, followed by 20 cycles of 10 s at 95 °C, 10 s at 56 °C and 10 s at 72 °C, and a final extension step for 5 min at 72 °C. For the amplification of genomic DNA, the annealing temperature was set at 60 °C and a total of 25 cycles were performed for 15 s each step. The expected size of the resulting amplicons was 176 bp and 144 bp for the synthetic and the genomic DNA templates, respectively. For amplicon detection using the protein-magnetic beads, 30 μg of each type (1 μL of 30 mg/mL for scCro-MB or 3 μL of 10 mg/mL for SA-MB) were blocked with 100 μL of 5% (w/v) milk in PBST for 30 min at 22 °C under rotation, followed by washing (3 × 100 μL of PBST). The beads were resuspended in 50 μL of 1/10 diluted PCR reaction in PBS and incubated for another 30 min. After washing (3 × 100 μL of PBST), the magnetic beads were resuspended in 50 μL of 500 pM of either SA-HRP (for the scCro-MB) or scCro-HRP (for the SA-MB) and incubated for 30 min. After a final washing step (3 × 100 μL of PBST), 50 μL of TMB substrate were added and color development proceeded for 2 min. Then, 50 μL of 1 M sulfuric acid was added to stop the reaction, the yellow-colored solution was recovered through magnetic separation, transferred to the wells of a 96-well plate and the absorbance at 450 nm was measured.

2.5. Effect of PCR primers concentration on the efficiency of PCR amplicon detection

The potential interference of primers on the efficiency of direct detection of amplicons without prior removal via purification of the PCR reactions was evaluated using two different approaches. In the first one, the synthetic DNA target (500 pg/μL) was amplified as detailed in section 2.4 but using 25–800 nM of each primer (instead of 200 nM). The detection of the PCR amplicon using scCro-MB or SA-MB was performed as detailed in the same section. In the second approach, the same amount of synthetic DNA target was used for amplification (500 pg/μL) and 200 nM of each primer, but after PCR, the reactions were diluted 1/10 and extra primers were added (200, 600 or 1400 nM), followed by detection on the magnetic beads as explained above.

2.6. Sensitivity of the assay for different concentrations of target DNA

The magnetic bead-based assay was used for the detection of PCR amplicons when different types and concentrations of target DNA templates were used for amplification. PCR was performed using 200 nM of each primer whereas the concentration of the template varied from 2 to 500 pg/μL for the synthetic DNA and 10–500 pg/μL for genomic DNA. Each template were amplified using its respective modified primers (Table 1). After amplification, the PCR reactions were diluted 10-fold and detection was performed as described in section 2.4. Each PCR reaction was analyzed in triplicate, whereas four independent experiments were performed in the case of the synthetic DNA target. The concentration of template DNA used for PCR was plotted against the absorbance recorded after colorimetric detection on the magnetic beads. The data was fitted to logarithmic regression models to establish the sensitivity and to calculate the limit of detection (LOD) after interpolation of the average blank signals plus three times the standard deviation of the blanks (blank + $3xSD_{blank}$).

2.7. Capture and release of PCR amplicons using scCro-MB

PCR was performed as detailed in section 2.4 using 20 pg/μL of the synthetic DNA target. Then, the PCR reaction was diluted in PBS to a final volume of 50 μL (dilution 1/25) and was incubated with 5 μL of scCro-MB for 30 min at 22 °C under rotation. The unbound fraction was recovered and then 50 μL of 1 M NaCl were added to elute the bound PCR amplicon. After a 5 min incubation with the NaCl solution, the elution fraction was collected. All samples (diluted PCR reaction, unbound and elution fractions) were analyzed by agarose gel

electrophoresis, while the DNA concentration in each fraction was quantified with the SimpliNANO spectrophotometer. The experiment was repeated three times.

3. Results and discussion

3.1. Principle of the assay

The strategy used for amplicon capture on magnetic beads was based on the use of specifically modified primers for PCR amplification of the target DNA sequence and magnetic beads functionalized with scCro DNA binding protein. During the amplification step, a forward primer extended with the specific site of scCro was used in combination with a biotinylated reverse primer. The resulting amplicon contained the dsDNA binding site for scCro on one end and a biotin on the other one (Fig. 1A). The unique cysteine residue added to the N-terminal of the scCro protein during the cloning step [28] prevents any direct alteration of the DNA binding site of the protein due to its immobilization on the epoxy-functionalized magnetic beads, thus minimizing negative effects on PCR amplicon binding. After amplification, the PCR reaction was added directly to the scCro-MB utilizing the biotin on the other end of the amplicon and SA-HRP enzyme conjugate for detection (Fig. 1B). No prior purification was needed since remaining primers not consumed during PCR, especially in the case of low-abundance targets, were not expected to interact with the scCro-MB. The scCro binds specifically dsDNA and not ssDNA. On the other hand, in the case of SA-MB, if the PCR reaction is added directly to the beads for detection, the biotinylated amplicon will be captured but remaining biotinylated reverse primer can block SA binding sites on the beads (Fig. 1B), therefore reducing the efficiency of amplicon detection. A purification step would thus be necessary to remove excess primers and ensure high sensitivity of detection.

3.2. Preparation and characterization of scCro-magnetic beads

Oriented immobilization of the scCro DNA binding protein on magnetic beads was achieved via a unique tethering point as detailed in section 2.2 and the presence of the protein on the surface of the MB was verified with a bead-ELISA (Supplementary Data Fig. S1). The performance of protein-magnetic beads for any given application greatly depends on their binding capacity for the intended target. And it is important to use the same method for determining binding capacity of different types of magnetic beads for comparison purposes [33]. Herein, to facilitate a direct comparison of the scCro-MB and the SA-MB for PCR amplicon detection, their nucleic acid binding capacity was evaluated in parallel using the same methods. The Dynabeads M-270 Streptavidin SA-MB were thus used which have similar characteristics as the ones used for the preparation of the scCro-MB: same diameter (2.8 μm) and surface properties (hydrophilic due to the carboxylic acid modification used to tether SA). A common DNA probe was used for both types of beads, with a biotin modification on one end and the dsDNA binding site for scCro in the form of a hairpin on the other one. The first assay was based on the measurement of the amount of tagged DNA before and after incubation with the magnetic beads. The difference between the measurements was considered as the amount of bound DNA. As shown in Fig. 2A, the scCro-MB exhibited a binding capacity of 181 $\pm$ 36 pmol per 1 mg beads, two-fold higher than the SA-MB ones which were able to bind approximately 88 $\pm$ 7 pmol per 1 mg beads. According to the manufacturer, the specific type of SA-MB used in this work have a binding capacity of approximately 200 pmol of biotinylated ssDNA per 1 mg of beads. Since no information is provided on the length of the ssDNA or the type of method used for this determination and taking into consideration that oligonucleotides with increased length can lead to reduced binding capacity, it is not unreasonable that this difference was observed.

To further explore the binding properties of the two protein-bead types, a second assay was performed exploiting the same biotinylated oligonucleotide with the dsDNA hairpin binding site for scCro. For this assay though, the amount bound on the beads was detected colorimetrically using an enzyme conjugate. A SA-HRP conjugate was used for the scCro-MB, and a scCro-HRP conjugate previously described [31] for the SA-MB ones. The enzymatic activity of the two conjugates was determined as detailed in the Supplementary Data, and the SA-HRP conjugate was found to exhibit approximately 90% of the activity of the scCro-HRP one (Table S1). For the binding capacity determination, the oligonucleotide probe was used at a constant concentration (20 nM), whereas different concentrations of the two HRP conjugates were used (62.5–500 pM). As it can be observed in Fig. 2B, higher signals were measured for the scCro-MB, approximately 1.8-fold higher than the ones achieved with the SA-MB regardless of the concentration of the enzyme conjugate used for detection. Overall, both assays suggest that the scCro-MB have almost two-fold higher binding capacity than the SA-MB ones. Considering that SA is almost four times bigger than scCro (60 kDa versus 17.5 kDa), and that it can bind up to four biotins whereas scCro can bind one probe, it is reasonable to assume that the higher binding capacity of the scCro-MB compared to the SA-MB ones might be due to higher protein load on the beads.

3.3. Effect of excess primers on the efficiency of PCR amplicon detection with the protein-MB

The main advantage of the scCro-MB is their ability to bind dsDNA

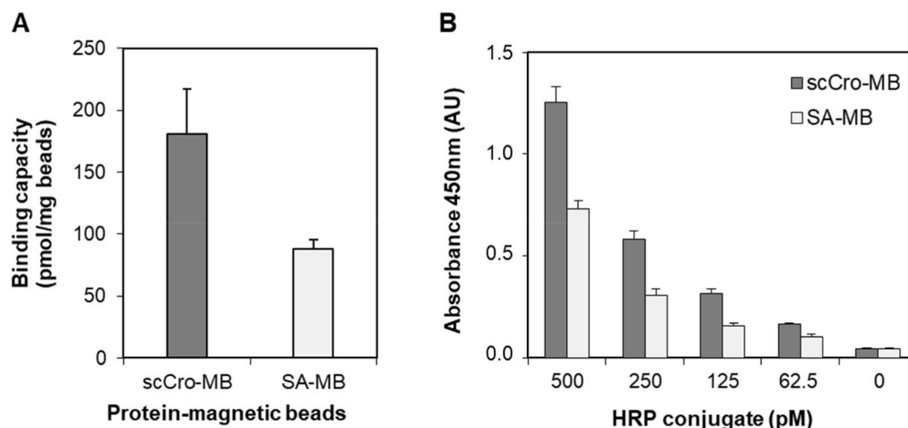

Fig. 2. Evaluation of the binding capacity of the protein-magnetic beads using a biotinylated hairpin DNA probe containing the dsDNA binding site for scCro. (A) Determination of the amount of the probe binding directly to the beads [input(ng DNA) - unbound(ng DNA)]. (B) Detection of the probe binding to the beads using HRP conjugates (SA-HRP for the scCro-MB and scCro-HRP for the SA-MB).

PCR products and not free ssDNA primers. Therefore, residual primers in the PCR reaction, especially in the case of the amplification of low abundance targets (low amount of template DNA), are not expected to affect the efficiency of PCR amplicon detection. This is not the case for the SA-MB. Free biotinylated primers bind to the beads as efficiently at least as the biotinylated PCR amplicons. Therefore, they can block binding sites on the beads and are thus expected to decrease the sensitivity of PCR amplicon detection. To confirm this hypothesis, PCR was performed using a constant concentration of the synthetic DNA target and different concentrations of primers (25–800 nM each). After amplification, the PCR reactions were diluted 10-fold and the presence of the amplicon was verified using the respective HRP conjugate as detailed earlier. As shown in Fig. 3A, increasing the concentration of the primers up to 200 nM resulted in elevated signal regardless of the type of magnetic beads used for detection. The signals achieved with the scCro-MB though were around 1.5-fold higher than the ones recorded for the SA-MB. On the other hand, when more than 200 nM of primers were used, signals obtained with the scCro-MB were not affected, while a significant decrease was observed in the case of the SA-MB. Although the increase of primer concentration up to 800 nM improved the efficiency of PCR (Fig. S2), this was not reflected when the magnetic beads were used for detection. In the case of the scCro-MB, the beads were apparently saturated with amplicon generated using 200 nM of primers since further increase of primers concentration did not improve the detection signals. However, when the SA-MB were used, up to 40% signal decrease was observed in the case of using 800 nM primers compared to 200 nM. Apparently, residual primers in the unpurified PCR reactions can bind efficiently to the beads and limit the available binding sites for amplicon capture and detection. This is even more evident when additional primers were added to the diluted PCR reaction (performed with 25 ng target DNA and 200 nM primers) prior to detection. As shown in Fig. 3B, addition of even 200 nM primers resulted in 20% signal decrease when using the SA-MB but only 6% in the case of the scCro-MB. Further increase of added primers had an even more negative impact on amplicon detection using either type of beads: signals decreased by 35–43% for the SA-MB and 25–30% for the scCro-MB ones. Biotinylated excess primers appear to preferentially bind to the SA-MB as commented above. In the case of the scCro-MB, residual amount of primer containing the scCro binding site (especially at such high concentrations as ≥ 600 nM) may encourage the formation of half-sites in secondary structures like self-dimers and hairpins (Fig. S3), to which scCro may bind but with lower affinity. Nevertheless, such high concentrations of free primers are not typically present in normal PCR conditions, so no interference is expected when using the scCro-MB for detection.

3.4. Sensitivity and precision of the assay for target DNA detection

The analytical sensitivity of the assay for DNA capture and subsequent detection was evaluated. Initially the assay was performed using different concentrations of the synthetic DNA target for PCR amplification (2–500 pg/μL, equivalent to 0.04–9.69 fmol/μL) in combination with its corresponding modified primers (Fig. S4A), followed by colorimetric detection on protein-magnetic beads using reporter HRP enzyme conjugates. The calibration curves constructed are shown in Fig. 4A. Higher sensitivity was achieved with the scCro-MB, and the limit of detection calculated was 0.30 pg/μL (6.1 pM). This was 9-fold lower than the one achieved with the SA-MB (2.81 pg/μL; 57.2 pM). The precision of the assays employing the two types of protein-magnetic beads was also assessed. The intra-assay and inter-assay coefficients of variation (% CV) were calculated for the analysis of a sample for which high concentration of target DNA template was used during PCR amplification and one for low concentration. As shown in Table S2, the intra-assay CVs were <9% for scCro-MB and <6% for SA-MB, while the inter-assay CVs were <13% for both types of protein magnetic beads. Considering that for the acceptable CV values for bioanalytical assays are ≤20%, both the reproducibility and repeatability of the assays employing either type of protein-magnetic beads were considered satisfactory.

To demonstrate the compatibility of the assay with different types of PCR templates, a second target was amplified from genomic DNA, again using a forward primer extended with the Cro DNA binding protein site and a biotinylated reverse primer. The PCR reactions were performed using different concentrations of genomic DNA and analysis by agarose gel electrophoresis (Fig. S4B) revealed only one amplification product of the expected size (144 bp). This finding suggested that the modification of the forward primer with the Cro binding site did not interfere with PCR specificity. Furthermore, as it was the case with the synthetic DNA target, higher signals were observed when the scCro-MB were used to detect the amplicons generated from the amplification of eukaryotic genomic DNA compared to the SA-MB (Fig. 4B). The limit of detection of the assay, based on the average blank signals plus three times their standard deviation, was calculated at 11.2 pg/μL genomic DNA when the scCro-MB were used for detection and 14.6 pg/μL for the SA-MB. These correspond to 56.2 and 73.1 pg of genomic DNA template respectively considering that only 5 μL of PCR reaction were required for the assay. The signals obtained for each genomic DNA template concentration with the scCro-MB were consistently 1.3–1.5 fold higher than the ones observed with the SA-MB, similar to when the synthetic DNA target was used (1.4–1.7 fold higher signals with the scCro-MB). The limits of detection achieved with the two different protein bead types did not differ significantly though in the case of the genomic DNA template. This could be potentially attributed to the relatively low concentration of primers used for amplification, considering the length and sequence complexity of genomic DNA. Nevertheless, the sensitivity of the scCro-MB-based assay was overall superior compared to the one using SA-MB even when a complex template was used for amplification,

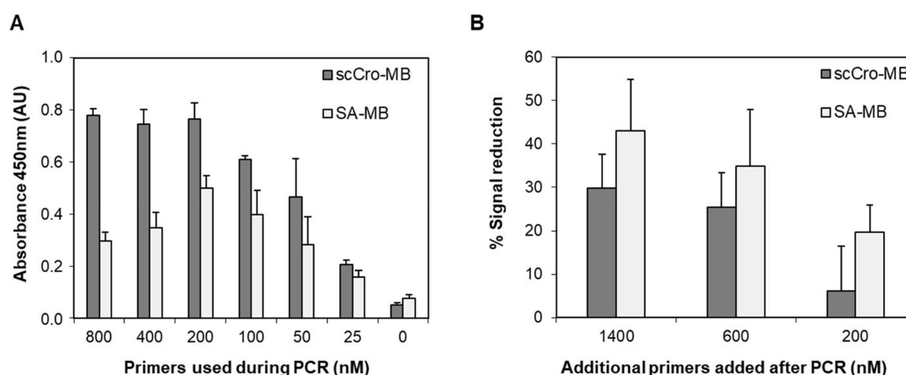

Fig. 3. Effect of residual primers on the efficiency of PCR amplicon detection using protein-magnetic beads. (A) PCR was performed using 25–800 nM primers. (B) Post-PCR addition of primers (200, 600 or 1400 nM) to the diluted PCR reaction prior to detection.

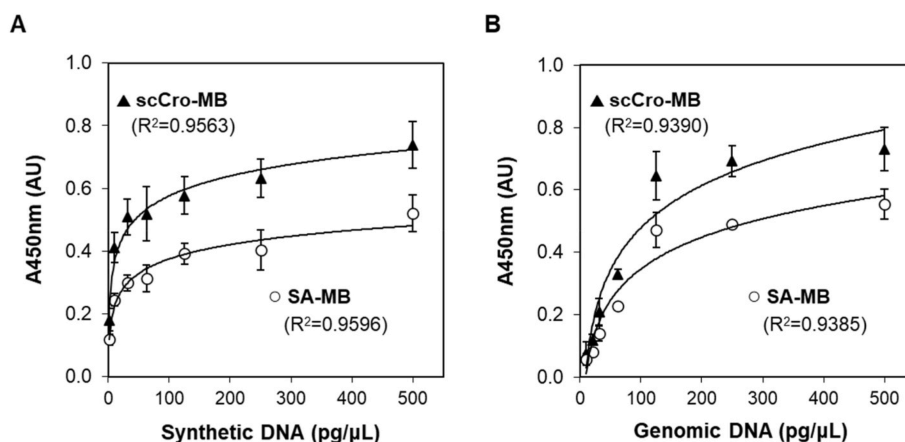

Fig. 4. Detection of PCR amplicons by a magnetic bead-based colorimetric assay using scCro DNA binding protein or SA. (A) Synthetic DNA and (B) genomic DNA were used as templates for PCR amplification. The error bars correspond to the standard deviation of at least triplicate samples.

demonstrating the versatility of the proposed assay and its potential use for amplicon detection regardless of the nature of the template.

3.5. Purification of PCR amplicons using scCro-MB

Finally, the scCro-MB were used for the capture and subsequent elution of PCR amplicons under mild conditions as a proof-of-concept for their potential use in PCR clean-up and amplicon purification. The synthetic DNA target was amplified as detailed in section 2.7 and the diluted PCR reaction was incubated with the scCro-MB and then eluted after a simple 5 min incubation with a salt solution. A representative agarose gel showing the analysis of the diluted PCR reaction before and after incubation with the scCro-MB, as well as of the elution fraction electrophoresis can be seen in Fig. 5. By measuring the concentration of the eluted DNA, it was calculated that 6.7 ± 0.9 μg of PCR amplicon can be eluted per mg of scCro-MB, while the recovery is 71.1 ± 9.4%. The simple process (bind-separate-elute), the mild elution conditions as well as the sole requirement of a magnet are advantages these beads offer over other PCR clean-up methods based on spin columns requiring multiple steps and a centrifuge or on specifically modified magnetic beads with lower binding capacities also requiring multiple steps. The mild elution conditions are specifically advantageous, since the harsh conditions required to disrupt the extremely strong streptavidin-biotin interaction promote the release of streptavidin monomers from the beads and prevent the use of SA-MB for elution of captured amplicons. Additionally, the beads could be potentially reused since the mild elution conditions are not expected to affect the binding properties of scCro.

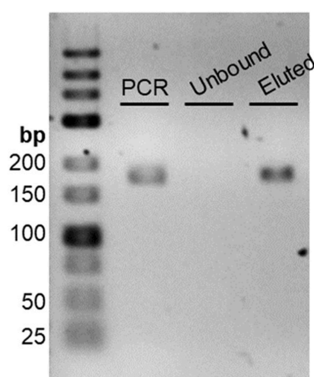

Fig. 5. Purification of PCR amplicon using scCro-MB. PCR: PCR reaction diluted with PBS before addition to the beads; Unbound: unbound fraction after incubation with the beads; Eluted: fraction eluted with 1 M NaCl after incubation with the beads.

4. Conclusions

SA-MB are commonly employed for enrichment, capture and detection of biotinylated nucleic acid amplicons resulting from different amplification techniques, thermal and isothermal. However, residual biotinylated ssDNA primers in the amplification reaction can interfere with the specific capture of the biotinylated dsDNA amplicons on the magnetic beads and their subsequent detection. Prior purification of the amplification reactions could eliminate this problem, but additional steps, materials and time would be needed. In this work we demonstrated that magnetic beads modified with the scCro sequence-specific dsDNA binding protein can serve as an alternative to the SA-MB for amplicon capture and detection. The magnetic beads with scCro covalently immobilized on their surface exhibited almost two-fold higher oligonucleotide binding capacity and approximately nine-fold higher sensitivity for PCR amplicon detection than commercial SA-MB when synthetic DNA target was used as a template. The detection assay on the surface of the scCro-MB beads also exhibited very good reproducibility and repeatability. The presence of free ssDNA primers in the amplification reactions did not interfere with the detection of dsDNA amplicon when the scCro-MB were used, as opposed to the SA-MB whose performance was significantly affected by free primers. Genomic DNA was also evaluated as a PCR template in combination with modified primers and amplification resulted in the generation of only one amplicon. The signals observed after capture of the generated amplicons by the scCro-MB were consistently higher compared to when the SA-MB were used as it was the case when synthetic DNA was used as a template. It can be thus concluded that the assay is not restricted to simple targets like synthetic DNA but is also compatible with more complex samples like eukaryotic genomic DNA. Moreover, it was demonstrated that the scCro-MB could not only serve for dsDNA amplicon capture and detection but also for purification purposes using a simple process and mild elution conditions which could potentially permit the reuse of the magnetic beads. Purification using the scCro-MB would also eliminate the need for centrifuge spin column-based or other multiple step magnetic bead-based PCR clean-up approaches. Finally, it is expected that the strategy presented herein for PCR amplicon capture/detection and purification could be applied to amplicons generated using different amplification techniques so long as one primer can be extended with the specific binding sequence for scCro.

CRediT authorship contribution statement

Ankur Ruhela: Methodology, Investigation, Data curation, Formal

analysis, Writing – original draft. **Vasso Skouridou:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Lluis Masip:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Data availability

No data was used for the research described in the article.

Acknowledgments

This work was supported financially by the CIGUASENSING project (BIO2017-87946-C2-1-R) funded by the Ministerio de Ciencia e Innovación (MICINN), the Agencia Estatal de Investigación (AEI) and the Fondo Europeo de Desarrollo Regional (FEDER). A.R. acknowledges the same agencies for his Ph.D. scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2022.114923.

References

[1] S.H. Lee, S.M. Park, B.N. Kim, O.S. Kwon, W.Y. Rho, B.H. Jun, Emerging ultrafast nucleic acid amplification technologies for next-generation molecular diagnostics, Biosens. Bioelectron. 141 (2019), 111448.
[2] Z. Ballard, A. Ozcan, Nucleic acid quantification in the field, Nat Biomed Eng 2 (2018) 629–630.
[3] R.W.K. Chiu, Y.M.D. Lo, C.T. Wittwer, Molecular diagnostics: a revolution in progress, Clin. Chem. 61 (2015) 1–3.
[4] B.B. Oliveira, B. Veigas, P.V. Baptista, Isothermal amplification of nucleic acids: the race for the next "gold standard", Front Sens 2 (2021), 752600.
[5] M.M. Mauk, J. Song, C. Liu, Changchun, H.H. Bau, Simple approaches to minimally-instrumented, microfluidic-based point-of-care nucleic acid amplification gests, Biosensors 8 (2018) 17.
[6] P. Wittmeier, S. Hummel, Agarose gel electrophoresis to assess PCR product yield: comparison with spectrophotometry, fluorometry and qPCR, Biotechniques 72 (2022) 155–158.
[7] C.T. Wittwer, G.M. Makrigiorgos, Nucleic acid techniques, in: N. Rifai, A. R. Horvath, C.T. Wittwer (Eds.), Principles and Applications of Molecular Diagnostics, Elsevier, 2018, pp. 47–86.
[8] Y. Ma, T. Chen, M.Z. Iqbal, F. Yang, N. Hampp, A. Wu, L. Luo, Applications of magnetic materials separation in biological nanomedicine, Electrophoresis 40 (2019) 2011–2028.
[9] C. Tang, Z. He, H. Liu, Y. Xu, H. Huang, G. Yang, Z. Xiao, S. Li, H. Liu, Y. Deng, Z. Chen, H. Chen, N. He, Application of magnetic nanoparticles in nucleic acid detection, J. Nanobiotechnol. 18 (2020) 62.
[10] S.E. Kim, M.V. Tieu, S.Y. Hwang, M.H. Lee, Magnetic particles: their applications from sample preparations to biosensing platforms, Micromachines 11 (2020) 302.
[11] Y.T. Chen, A.G. Kolhatkar, O. Zenasni, S. Xu, T.R. Lee, Biosensing using magnetic particle detection techniques, Sensors 17 (2017) 2300.
[12] N.J. Parham, F.J. Picard, R. Peytavi, M. Gagnon, G. Seyrig, P.A. Gagné, M. Boissinot, M.G. Bergeron, Specific magnetic bead based capture of genomic DNA from clinical samples: application to the detection of group B streptococci in vaginal/anal swabs, Clin. Chem. 53 (2007) 1570–1576.
[13] M.J. Archer, N. Long, B. Lin, Effect of probe characteristics on the subtractive hybridization efficiency of human genomic DNA, BMC Res. Notes 3 (2010) 109.
[14] K.F. Low, P. Rijiravanich, K.K. Singh, W. Surareungchai, C.Y. Yean, An electrochemical genosensing assay based on magnetic beads and gold nanoparticle-loaded latex microspheres for *Vibrio cholerae* detection, J. Biomed. Nanotechnol. 11 (2015) 702–710.
[15] A. Toldrà, C. Alcaraz, J. Diogène, C.K. O'Sullivan, M. Campàs, Detection of *Ostreopsis* cf. *ovata* in environmental samples using an electrochemical DNA-based biosensor, Sci. Total Environ. 689 (2019) 655–661.
[16] J. Chen, R. Johnson, M. Griffiths, Detection of verotoxigenic *Escherichia coli* by magnetic capture-hybridization PCR, Appl. Environ. Microbiol. 64 (1998) 147–152.
[17] A. Lermo, S. Liébana, S. Campoy, S. Fabiano, M.I. García, A. Soutullo, M. J. Zumárraga, S. Alegret, M.I. Pividori, A novel strategy for screening-out raw milk contaminated with *Mycobacterium bovis* on dairy farms by double-tagging PCR and electrochemical genosensing, Int. Microbiol. 13 (2010) 91–97.
[18] D. Chang, K. Tram, B. Li, Q. Feng, Z. Shen, C.H. Lee, B.J. Salena, Y. Li, Detection of DNA amplicons of polymerase chain reaction using litmus test, Sci. Rep. 7 (2017) 3110.
[19] S.P. Kyaw, J. Hanthamrongwit, K. Jangpatarapongsa, P. Khaenam, C. Leepiyasakulchai, Sensitive detection of the IS6110 sequence of *Mycobacterium tuberculosis* complex based on PCR-magnetic bead ELISA, RSC Adv. 8 (2018) 33674–33680.
[20] C.L. Smith, J.S. Milea, G.H. Nguyen, Immobilization of nucleic acids using biotin-strept(avidin) systems, in: C. Wittmann (Ed.), Immobilisation of DNA on Chips II. Topics in Current Chemistry, Springer, 2006, pp. 63–90.
[21] A. Sassolas, B.D. Leca-Bouvier, L.J. Blum, DNA biosensors and microarrays, Chem. Rev. 108 (2008) 109–139.
[22] M. Haun, S. Wasi, Biotinylated antibodies bound to streptavidin beads: a versatile solid matrix for immunoassays, Anal. Biochem. 191 (1990) 337–342.
[23] A. Paul, M. Avci-Adali, G. Ziemer, H.P. Wendel, Streptavidin-coated magnetic beads for DNA strand separation implicate a multitude of problems during cell-SELEX, Oligonucleotides 19 (2009) 243–254.
[24] R. Wilson, Preparation of single-stranded DNA from PCR products with streptavidin magnetic beads, Nucleic Acid Therapeut. 21 (2011) 437–440.
[25] R. Jana, T.R. Hazbun, J.D. Fields, M.C. Mossing, Single-chain lambda Cro repressors confirm high intrinsic dimer-DNA affinity, Biochemistry 37 (1998) 6446–6455.
[26] G.B. Aktas, J.H. Wichers, V. Skouridou, A. van Amerongen, L. Masip, Nucleic acid lateral flow assays using a conjugate of a DNA binding protein and carbon nanoparticles, Microchim. Acta 186 (2019) 426.
[27] G.B. Aktas, A. Ribera, V. Skouridou, L. Masip, DNA immobilization and detection using DNA binding proteins, Anal. Bioanal. Chem. 413 (2021) 1929–1939.
[28] I. Ginés, G. Gaiani, A. Ruhela, V. Skouridou, M. Campàs, L. Masip, Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*, Harmful Algae 110 (2021), 102135.
[29] L. Civit, A. Fragoso, S. Hölters, M. Dürst, C.K. O'Sullivan, Electrochemical genosensor array for the simultaneous detection of multiple high-risk human papillomavirus sequences in clinical samples, Anal. Chim. Acta 715 (2012) 93–98.
[30] V. Skouridou, H. Tomaso, J. Rau, A.S. Bashammakh, M.S. El-Shahawi, A. O. Alyoubi, C.K. O'Sullivan, Duplex PCR-ELONA for the detection of pork adulteration in meat products, Food Chem. 287 (2019) 354–362.
[31] G.B. Aktas, V. Skouridou, L. Masip, Novel signal amplification approach for HRP-based colorimetric genosensors using DNA binding protein tags, Biosens. Bioelectron. 74 (2015) 1005–1010.
[32] G.T. Hermanson, The chemistry of reactive groups, in: Bioconjugate Techniques, Elsevier, 2008, pp. 169–182.
[33] L.B. Luecke, R.L. Gundry, Assessment of streptavidin bead binding capacity to improve quality of streptavidin-based enrichment studies, J. Proteome Res. 20 (2021) 1153–1164.