



DEVELOPMENT OF DNA DETECTION BIOASSAYS BASED ON DNA BINDING PROTEINS

Ankur Ruhela

ADVERTIMENT. L'accés als continguts d'aquesta tesi doctoral i la seva utilització ha de respectar els drets de la persona autora. Pot ser utilitzada per a consulta o estudi personal, així com en activitats o materials d'investigació i docència en els termes establerts a l'art. 32 del Text Refós de la Llei de Propietat Intel·lectual (RDL 1/1996). Per altres utilitzacions es requereix l'autorització prèvia i expressa de la persona autora. En qualsevol cas, en la utilització dels seus continguts caldrà indicar de forma clara el nom i cognoms de la persona autora i el títol de la tesi doctoral. No s'autoritza la seva reproducció o altres formes d'explotació efectuades amb finalitats de lucre ni la seva comunicació pública des d'un lloc aliè al servei TDX. Tampoc s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX (framing). Aquesta reserva de drets afecta tant als continguts de la tesi com als seus resums i índexs.

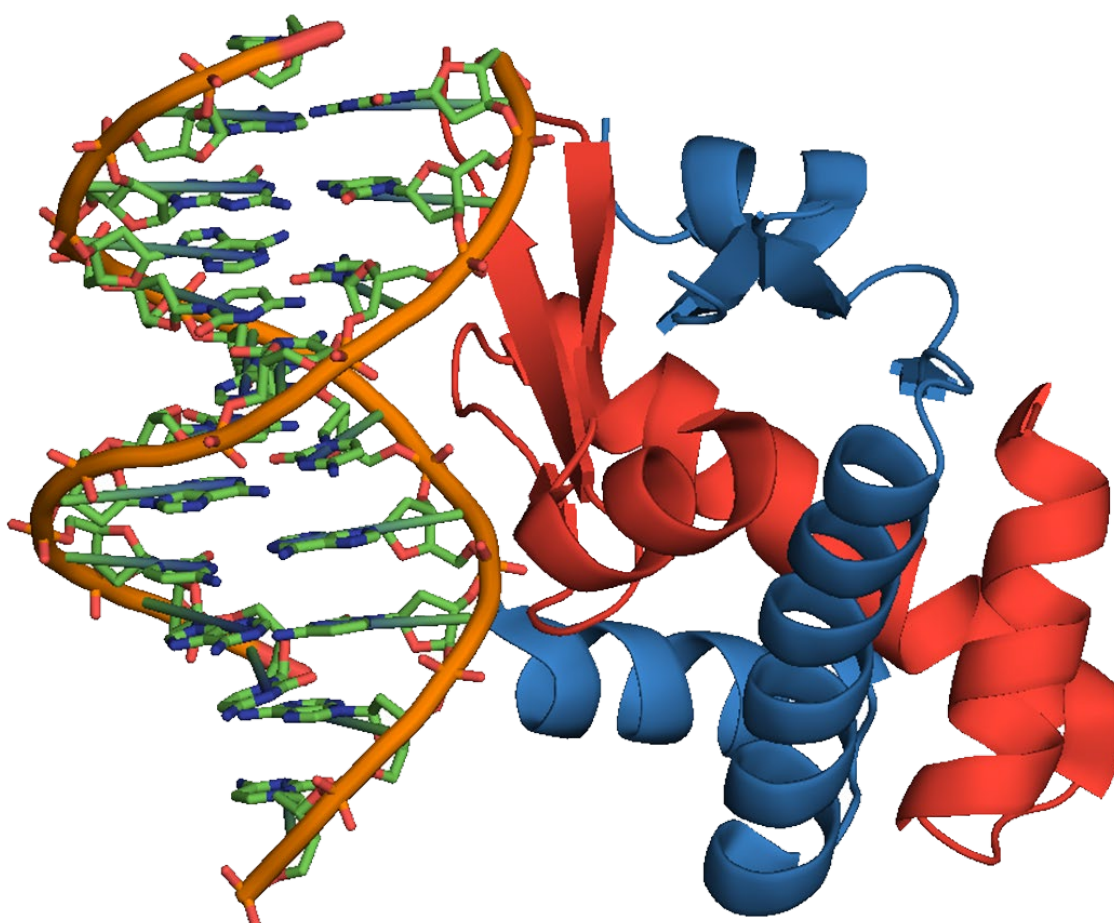
ADVERTENCIA. El acceso a los contenidos de esta tesis doctoral y su utilización debe respetar los derechos de la persona autora. Puede ser utilizada para consulta o estudio personal, así como en actividades o materiales de investigación y docencia en los términos establecidos en el art. 32 del Texto Refundido de la Ley de Propiedad Intelectual (RDL 1/1996). Para otros usos se requiere la autorización previa y expresa de la persona autora. En cualquier caso, en la utilización de sus contenidos se deberá indicar de forma clara el nombre y apellidos de la persona autora y el título de la tesis doctoral. No se autoriza su reproducción u otras formas de explotación efectuadas con fines lucrativos ni su comunicación pública desde un sitio ajeno al servicio TDR. Tampoco se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR (framing). Esta reserva de derechos afecta tanto al contenido de la tesis como a sus resúmenes e índices.

WARNING. Access to the contents of this doctoral thesis and its use must respect the rights of the author. It can be used for reference or private study, as well as research and learning activities or materials in the terms established by the 32nd article of the Spanish Consolidated Copyright Act (RDL 1/1996). Express and previous authorization of the author is required for any other uses. In any case, when using its content, full name of the author and title of the thesis must be clearly indicated. Reproduction or other forms of for profit use or public communication from outside TDX service is not allowed. Presentation of its content in a window or frame external to TDX (framing) is not authorized either. These rights affect both the content of the thesis and its abstracts and indexes.



Development of DNA Detection Bioassays Based on DNA Binding Proteins

ANKUR RUHELA



DOCTORAL THESIS

2023

Ankur Ruhela

Development of DNA Detection Bioassays Based on DNA Binding Proteins

DOCTORAL THESIS

Supervised by: Prof. Lluís Masip Vernis

Co-supervised by: Prof. Ioanis Katakis

Departament d'Enginyeria Química



UNIVERSITAT ROVIRA I VIRGILI

Tarragona 2023



I STATE that the present study, entitled “**Development of DNA detection bioassays based on DNA binding proteins**”, presented by **Ankur Ruhela** for the award of the degree of Doctor, has been carried out under my supervision at the Department Chemical Engineering of this university, and that it fulfils all the requirements to be eligible for the Doctorate Award.

Tarragona, September 1st, 2023

Doctoral Thesis Supervisors

Prof. Lluís Masip

Prof. Ioanis Katakis

Acknowledgements

This doctoral journey has been an incredible chapter of growth and discovery, and it wouldn't have been possible without the unyielding support and guidance of the remarkable individuals and entities who have played pivotal roles along the way.

Firstly, I would like to thank Dr. Vasso Skouridou, I am immensely grateful for your mentorship and unwavering guidance throughout my time in the University. Your influence has been truly transformative. The lessons I have learned from you, both in terms of technique and approach, have played a crucial role in shaping the outcomes of my research. Your unwavering belief in my potential has fueled my determination to excel and push the boundaries of my capabilities.

I am profoundly grateful to my thesis supervisor, Prof. Lluís Masip, whose weekly feedback and unwavering guidance have been the cornerstones of my PhD journey. Your insights, constructive critiques, and visionary perspective have consistently steered me towards the path of excellence. Your mentorship has been an invaluable asset. Your patient explanations and insightful feedback have been invaluable in helping me navigate through complex challenges.

To my dedicated co-supervisor Prof. Ioanis Katakis, your presence and contributions have added a unique dimension to this journey, and I acknowledge your role in this collaborative effort. Your insights and collaborative spirit have enriched my research experience.

The support of the Interfibio group have been the bedrock of my research experience. I am thankful to all the members of Interfibio group. Though our challenges might not have been shared directly, the resonance of our determination has woven a knot of unity. The exchange of ideas, while perhaps not in the formal settings of a group, has transcended boundaries, sparking a creative fire that fuels my own reflections.

This work has been made possible through the financial support of the CIGUASENSING project, funded by the Ministerio de Ciencia e Innovación (MICINN), the Agencia Estatal de Investigación (AEI), and the Fondo Europeo de Desarrollo Regional (FEDER). I extend my gratitude to these agencies for their invaluable contributions to advancing scientific knowledge.

In the realm beyond academia, the support of friends can never be overstated. Akash, Shuja, and Deepanshu, your presence in my life has been a source of immense strength. Your unwavering emotional support, uplifting conversations, and moments of shared laughter have

kept my spirits high during challenging times. So, here's to the kind of friendships that are as essential to life as oxygen is to breath.

Finally, I dedicate this thesis to the memory of my late Grandfather. His wisdom, values, and love continue to inspire me. This achievement stands as a tribute to his legacy.

With deepest gratitude,

Ankur Ruhela

TABLE OF CONTENTS

Acknowledgements	vi
SUMMARY	xiv
LIST OF PUBLICATIONS	xviii
LIST OF ABBREVIATIONS	xx
LIST OF FIGURES	xxii
LIST OF TABLES.....	xxviii
Chapter 1: General Introduction.....	1
1.1 Introduction	3
1.2 Basic components of biosensors	4
1.2.1 Biorecognition element.....	4
1.2.2 Transducer	5
1.2.3 Output units	5
1.3 Characteristics of biosensors	5
1.3.1 Sensitivity	5
1.3.2 Selectivity	6
1.3.3 Reproducibility	6
1.3.4 Stability.....	6
1.3.5 Characteristics of POC biosensors	7
1.4 Immobilization strategies in biosensors	7
1.4.1 Physical immobilization	8
1.4.2 Chemical immobilization	9
1.4.3 Affinity-based immobilization.....	10
1.5 DNA biosensors.....	11
1.5.1 Types of DNA biosensors	12
1.5.2 Optical DNA biosensors	12
1.5.3 Electrochemical DNA biosensors	14
1.5.4 Piezoelectric DNA biosensors	16
1.6 Applications of DNA biosensors	17
1.7 Generation/sources of target DNA for biosensors	18
1.8 DNA binding proteins	19
1.8.1 Sequence-specific DNA binding proteins.....	20
1.8.2 Non-sequence specific DNA binding proteins	22

1.8.3 Use of DNA binding proteins in biosensors	24
1.9 Thesis objectives	27
1.10 References	29
Chapter 2: Nucleic acid lateral flow dipstick assay for the duplex detection of <i>Gambierdiscus australes</i> and <i>Gambierdiscus excentricus</i>	45
2.1 Abstract	47
2.2 Introduction	47
2.3 Materials and methods	49
2.3.1 Materials	49
2.3.2 Expression and purification of scCro DNA binding protein	50
2.3.3 Genomic DNA from microalgae.....	50
2.3.5 Enzyme Linked Oligonucleotide Assay (ELONA) for colorimetric detection of PCR amplicons.....	53
2.3.6 Preparation of the LFA dipsticks	53
2.3.7 Detection of the PCR amplicons with the LFA dipsticks	54
2.3.8 Direct PCR amplification and LFD detection using <i>G. australes</i> and <i>G. excentricus</i> cells.....	54
2.4 Results	55
2.4.1 Preparation of the recombinant scCro DNA binding protein	55
2.4.2 Specificity of the primers and PCR amplicon detection by ELONA	55
2.4.3 Detection of <i>G. australes</i> and <i>G. excentricus</i> PCR amplicons by dipstick LFA	59
2.4.4 Sensitivity of the dipsticks for <i>G. australes</i> and <i>G. excentricus</i>	61
2.4.5 Specificity of the PCR-LFA.....	62
2.4.6 Detection of genomic DNA from <i>G. australes</i> and <i>G. excentricus</i> in the presence of non-target <i>Gambierdiscus</i> or <i>Fukuyoa</i> species	63
2.4.7 Detection of <i>G. australes</i> and <i>G. excentricus</i> cells.....	64
2.5 Discussion	65
2.6 Conclusions	70
2.7 Acknowledgments.....	70
2.8 References	72
Chapter 3: Capture, detection and purification of dsDNA amplicons using a DNA binding protein on magnetic beads.....	81
3.1 Abstract	83
3.2 Introduction	83
3.3 Materials and methods	85
3.3.1 Materials	85

3.3.2 Preparation of scCro DNA binding protein-magnetic beads (scCro-MB)	87
3.3.3 Determination of the binding capacity of the protein-magnetic beads.....	88
3.3.4 Determination of the specific activity of the scCro-HRP and SA-HRP enzyme conjugates	88
3.3.5 PCR amplicon detection using protein-magnetic beads (scCro-MB or SA-MB) ...	89
3.3.6 Effect of PCR primers concentration on the efficiency of PCR amplicon detection	89
3.3.7 Sensitivity and precision of the assay for different concentrations of target DNA .	90
3.3.8 Capture and release of PCR amplicons using scCro-MB.....	90
3.4 Results and discussion.....	91
3.4.1 Principle of the assay	91
3.4.2 Preparation and characterization of scCro-magnetic beads.....	91
3.4.3 Effect of excess primers on the efficiency of PCR amplicon detection with the protein-MB	94
3.4.4 Sensitivity and precision of the assay for target DNA detection	97
3.4.5 Purification of PCR amplicons using scCro-MB	100
3.5 Conclusions	101
3.6 Acknowledgments.....	102
3.7 References	102
Chapter 4: Universal and label-free detection of dsDNA using the HU DNA binding protein	107
4.1 Abstract	109
4.2 Introduction	109
4.3 Materials and methods	112
4.3.1 Materials	112
4.3.2 Preparation of the scHupBA expression plasmid	113
4.3.3 Expression and purification of the scHupBA DNA binding protein	114
4.3.4 Preparation of the scHupBA-HRP conjugate	115
4.3.5 Immobilization of scHupBA on magnetic beads.....	116
4.3.6 Preparation of dsDNA and ssDNA probes for binding studies	117
4.3.7 Characterization of the binding properties of scHupBA by ELONA.....	118
4.3.8 Detection of dsDNA on microtiter plates using scHupBA and scHupBA-HRP ...	118
4.3.9 Detection of dsDNA on magnetic beads using scHupBA and scHupBA-HRP.....	119
4.3.10 scHupBA-based lateral flow dispstick assay for dsDNA detection	119
4.4 Results and discussion.....	121
4.4.1 Recombinant expression of the scHupBA single-chain DNA binding protein	121

4.4.2 Evaluation of the DNA binding properties of scHupBA.....	124
4.4.3 Preparation of the scHupBA-HRP conjugate for use in DNA detection assays....	125
4.4.4 Microplate-based colorimetric detection of dsDNA using scHupBA and scHupBA-HRP	127
4.4.5 Colorimetric detection of dsDNA using scHupBA-magnetic beads and scHupBA-HRP	130
4.4.6 Detection of dsDNA with lateral flow dipstick assay.....	133
4.4.7 Direct analysis of PCR reactions by ELONA using microplates, magnetic beads and dipstick lateral flow assays	136
4.5 Conclusions	140
4.6 Acknowledgements	141
4.7 References	141
Chapter 5: General conclusions.....	149
5.1 General conclusions.....	151

SUMMARY

Nucleic acids are ideal target molecule identification biomarkers for molecular tests. Over the past decades, the detection of specific DNA sequences has become paramount for timely and accurate diagnosis of diseases and genetic disorders, as well as for environmental and food safety surveillance. Improving or maintaining the performance of these DNA bioassays and biosensors while reducing their cost and complexity could facilitate their utilization in low-resource settings in emerging and developing countries. Since DNA amplification, by thermal polymerase chain reaction (PCR) or isothermal techniques, is essential to achieve the required sensitivity and specificity, a broad repertoire of tools for facile, rapid and low-cost detection of the generated double stranded DNA (dsDNA) is necessary. The ability of DNA binding proteins (DBPs) to recognize and bind tightly to DNA in a sequence-specific or non-sequence specific manner makes them ideal candidates as DNA biorecognition and reporter elements. This thesis sought to explore different applications for these proteins in DNA detection bioassays and highlight their great promise, especially as an alternative to the well-established strept(avidin)-biotin-based strategies.

Two DBPs with different properties were evaluated. The first one was the sequence-specific Cro DBP from bacteriophage lambda. This protein recognizes and binds as a highly flexible homodimer to a specific 19 base pair (bp) dsDNA sequence with very high affinity (affinity dissociation constant K_D in the low nanomolar range). The second DBP was the HU histone-like protein from *Escherichia coli*. It is a nucleoid-associated protein participating in many DNA-based cell processes and in DNA folding and topological organization. HU recognizes DNA structures and not specific sequences, and its predominant heterodimeric form binds to DNA fragments regardless of their sequence at a stoichiometry of one heterodimer per approximately 9 bp. Its binding affinity varies depending on the specific conditions (low nanomolar to low micromolar range K_D values) but exhibits overall higher binding affinity under low salt conditions. For use in DNA bioassays, these two proteins were engineered into single-chain dimeric forms and produced recombinantly in *E. coli*. The expression construct for Cro was based on a previous work, but a new construct was prepared in the frame of this thesis for improved protein production. A novel single-chain HU construct was also prepared, similarly to Cro. Both proteins were purified to near homogeneity by a combination of affinity chromatography and ion exchange chromatography. To develop the bioassays for DNA capture and detection, synthetic and natural materials were used to immobilize the DBPs: carbon

nanoparticles (CNPs), magnetic beads (MB), the horseradish peroxidase (HRP) enzyme, microtiter plates, and nitrocellulose membrane (NC).

In Chapter 1, a general introduction was provided with regards to the general characteristics of biosensors with special focus on DNA detection and their applications, biomolecule immobilization strategies employed for their development, and the state-of-the-art in the use of DBPs for DNA detection.

Lateral flow dipstick assays were developed in Chapter 2, using a Cro-carbon nanoparticles conjugate (Cro-CNPs) for the simultaneous detection of multiple PCR products. Hybrid PCR amplicons were generated using modified primers with ssDNA tails (barcodes) on one end for target-specific capture via hybridization and the Cro-specific dsDNA sequence on the other one for detection. The assay was used to detect toxin-producing harmful microalgae without interference from other marine species, highlighting the utility of the sequence-specific DBP as a universal reporter molecule compatible with lateral flow assays and different sample types (genomic DNA and cells). The new construct for improved production of the engineered Cro was described in this chapter.

In Chapter 3, Cro was immobilized on magnetic beads (MB) to capture, detect and purify dsDNA PCR amplicons eliminating the need for PCR reaction purification with the commonly used spin columns. The use of modified primers resulted in the generation of PCR amplicons with the Cro-specific DNA binding sequence on one end and biotin on the other one. The Cro-MB were used to specifically capture the amplicons whereas colorimetric detection was provided by streptavidin-HRP (SA-HRP). The efficiency of Cro-MB was compared to the well-established SA-MB used in biosensing and purification applications, and the Cro-MB exhibited higher binding capacity and sensitivity compared to the SA-MB when synthetic or genomic DNA were used as PCR target templates. Most importantly, it was demonstrated that the presence of free ssDNA primers in the amplification reactions did not interfere with detection when the Cro-MB were used, as opposed to the SA-MB whose performance was significantly affected by free primers. A proof-of-concept for PCR amplicon purification using the Cro-MB under mild elution conditions using salt was also provided.

In Chapter 4, the application of a novel single-chain heterodimeric DBP without sequence specificity was reported for universal dsDNA PCR amplicon capture and detection. The design and preparation of the expression construct, as well as the purification of the HU DBP was detailed in this chapter. A HU-HRP conjugate was designed as a reporter and its preparation

and purification process were described. A study on the DNA binding properties of HU on microtiter plates was performed which revealed higher binding affinity for longer dsDNA fragments (K_D values in the low nanomolar range), and considerably lower affinity for ssDNA. A HU/HU-HRP sandwich system was then established for the detection of purified dsDNA after PCR amplification on microtiter plates and magnetic beads, without interference from ssDNA and with higher sensitivity under low salt conditions. A proof-of-concept for the use of a HU/HU-CNPs sandwich system with lateral flow assays was also provided for the detection of only the dsDNA fragment with no detectable signals for ssDNA. The compatibility of all three assay formats (microtiter plates, magnetic beads and lateral flow strips) with unpurified PCR reactions for the direct detection of the PCR amplicons was finally demonstrated. By simply diluting the PCR reactions, it was possible to directly detect the PCR amplicons in reactions performed even with low target template amounts with no background signals. The lateral flow dipstick format is particularly useful for fast and simple analysis of any PCR reaction since it can provide visual confirmation of the presence of amplicons in one step and within less than 20 min. This work exemplifies the use of non-sequence specific dsDNA binding proteins for universal DNA biosensing which has not been reported previously in the literature, circumventing the need for labelling or use of expensive reagents and materials.

Final conclusions were provided in Chapter 5, regarding the versatility of DBP and their wide variety of applications in DNA detection bioassays. The examples provided of a sequence-specific and non-sequence specific DBPs for dsDNA recognition and binding, exploiting different surfaces for DBP immobilization, demonstrate the potential of this type of proteins in biosensing. Depending on the specific target, sample or system requirements, a sequence-specific DBP like Cro could be used by incorporating its specific DNA binding sequence in one of the primers used for amplification. On the other hand, a non-sequence specific DBP like HU could be used to detect any amplified or non-amplified DNA fragment since it recognizes DNA structures and not specific sequences. This DPB could be used as a universal DNA bioreceptor and reporter as well. Overall, various DBP-based platforms were developed in this thesis with different sensitivities and characteristics. Future work will focus on further improvement of their performance by extensive optimization of operational conditions, incorporating novel nanomaterials, exploiting different signal transduction methods or other signal amplification techniques.

LIST OF PUBLICATIONS

1. Ankur Ruhela, Vasso Skouridou, Lluís Masip
“Capture, detection and purification of dsDNA amplicons using a DNA binding protein on magnetic beads”
Analytical Biochemistry (2022) Volume 658, 114923
ISSN 0003-2697
<https://doi.org/10.1016/j.ab.2022.114923>.
2. Iris Ginés, Greta Gaiani, Ankur Ruhela, Vasso Skouridou, Mònica Campàs, Lluís Masip
“Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*”
Harmful Algae (2021) Volume 110, 102135
ISSN 1568-9883
<https://doi.org/10.1016/j.hal.2021.102135>.
3. Ankur Ruhela, Vasso Skouridou, Lluís Masip
“Universal and label-free detection of dsDNA using the non-sequence specific HU DNA binding protein”
In preparation

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CNPs	Carbon nanoparticles
Da	Dalton; 1 Da = 1 g/mol
DNA	Deoxyribonucleic acid
DBP	DNA binding protein
dsDNA	Double stranded DNA
DTT	1,4-Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ELONA	Enzyme-linked oligonucleotide assay
HRP	Horseradish peroxidase
HU	Histone-like protein
K_d	Equilibrium dissociation constant
LFA	Lateral flow assay
LOD	Limit of detection
MB	Magnetic beads
MW	Molecular weight
NALFA	Nucleic acid lateral flow assay
NC	Nitrocellulose
PBS	Phosphate-buffered saline
PBST	PBS with 0.1% (v/v) Tween-20
PDB	Protein Data Bank

PCR	Polymerase chain reaction
pI	Isoelectric point
POC	Point of care
RT	Room temperature
SA	Streptavidin
SA-HRP	Streptavidin-horseradish peroxidase conjugate
SA-pHRP	Streptavidin-poly horseradish peroxidase conjugate
scCro	Single-chain dimeric bacteriophage Cro repressor protein
scHupBA	Single-chain dimeric Histone like protein
SDS	Sodium dodecyl sulfate
ssDNA	Single stranded DNA
TCEP	Tris (2-carboxyethyl) phosphine
Tm	Melting temperature
TMB	3,3',5,5'-Tetramethylbenzidine

LIST OF FIGURES

CHAPTER 1: General Introduction

Figure 1.1 Commonly used POC biosensors. (A) Amperometric biosensor for glucose detection (5). (B) Lateral flow test for pregnancy (6).....	3
Figure 1.2 Schematic diagram of a biosensor.	4
Figure 1.3 Common types of biorecognition elements in biosensors (9).	4
Figure 1.4 Physical immobilization by adsorption.	8
Figure 1.5 Chemical/covalent immobilization.....	9
Figure 1.6 Immobilization by crosslinking.	10
Figure 1.7 Affinity-based immobilization.....	10
Figure 1.8 A general schematic diagram of DNA biosensor (75).	12
Figure 1.9 Schematic representation of a nanoparticle-based colorimetric detection of hepatitis C virus (HCV) RNA after amplification (82).	13
Figure 1.10 Optical DNA biosensor (87).	14
Figure 1.11 Basic configuration of electrochemical DNA biosensors (92).	15
Figure 1.12 Piezoelectric DNA biosensors (97).....	16
Figure 1.13 scCro DNA binding protein with helix-turn-helix motif (146).	21
Figure 1.14 Zinc finger protein (150).	22

CHAPTER 2: Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*

Figure 2.1 Strategy for the detection of the target <i>Gambierdiscus</i> species with the PCR-LFA dipstick. (A) PCR amplification of <i>G. australes</i> and <i>G. excentricus</i> genomic DNA using modified primers. (B) PCR amplicon detection by LFA. Capture of the ssDNA-dsDNA hybrid amplicons on the dipsticks by complementary probes and detection with scCro/CNPs conjugate. (C) Design of the dipsticks. CL: control line; TL1: test line 1 for <i>G. australes</i> ; TL2: test line 2 for <i>G. excentricus</i>	52
Figure 2.2 Expression of scCro from BL21(DE3)/pLATE31 and purification by IMAC and IEC.	55

Figure 2.3 Amplification of genomic DNA from various <i>G. australes</i> and <i>G. excentricus</i> strains using the primers reported in this work.	56
Figure 2.4 PCR amplification of <i>G. australes</i> (GA) and <i>G. excentricus</i> (GE) genomic DNA (gDNA) and detection by ELONA. (A) Simplex PCR using primers for each species and (B) duplex PCR using primers for both strains. (C) Format of the ELONA for the detection of the two <i>Gambierdiscus</i> species. (D) Specificity of the duplex PCR-ELONA for the detection of the amplicons.	57
Figure 2.5 Optimization of the ELONA conditions for the detection of amplicons after PCR amplification of genomic DNA (gDNA) from <i>G. australes</i> and <i>G. excentricus</i> . (A) Capture probe concentration varied from 50 – 200 nM, while the HRP-scCro conjugate was used at 5 nM. (B) The HRP-scCro conjugate was used in the range of 2.5 – 10 nM; the capture probes for both species were used at 50 nM. (C) Dilution of the PCR reaction. (D) Number of PCR cycles. For panels C and D, the capture probes were used at 50 nM and the HRP-scCro conjugate at 15 nM. For panels A, B and D the PCR reactions were diluted 1/50. ntc: no template control.	58
Figure 2.6 Sensitivity of the PCR-ELONA for the detection of <i>G. australes</i> (GA) and <i>G. excentricus</i> (GE) genomic DNA (gDNA). (A) Representative images after agarose gel electrophoresis analysis of PCR reactions performed using serial two-fold dilutions of genomic DNA (gDNA) (4000 down to 16 pg/μL) from <i>G. australes</i> and <i>G. excentricus</i> . (B) Calibration curves for the detection of genomic DNA from <i>G. australes</i> and <i>G. excentricus</i> by ELONA. ntc: no template control.	59
Figure 2.7 Optimization of the volume of PCR reaction used for detection of the amplicons with the LFA dipsticks. Simplex PCR reactions were performed separately using 100 pg/μL of genomic DNA from each species. The desired volume of PCR reaction (0 – 10 μL) was mixed with 1 μL of 0.2% (w/v) scCro/CNPs conjugate and diluted to 50 μL with PBS containing 1% (w/v) skim milk and 0.1% (v/v) Tween-20. The asterisks (*) correspond to PCR reactions performed with genomic DNA from the non-target species, <i>G. excentricus</i> for the left panel and <i>G. australes</i> for the right panel.	60
Figure 2.8 LFA dipsticks for the detection of genomic DNA (gDNA) from <i>G. australes</i> (GA) and <i>G. excentricus</i> (GA) after duplex PCR amplification of genomic DNA (100 pg/μL) from each species. CL: control line; TL1 GA: test line 1 for <i>G. australes</i> ; TL2 GE: test line 2 for <i>G. excentricus</i>	61
Figure 2.9 Sensitivity of the PCR-LFA for each <i>Gambierdiscus</i> species. Serially two-fold diluted genomic DNA (gDNA) from each strain (400 to 1.6 pg/μL) was used in each PCR	

reaction containing primers for both species and added to the dipsticks for visual detection. CL: control line; TL1 GA: test line 1 for <i>G. australes</i> ; TL2 GE: test line 2 for <i>G. excentricus</i>	62
Figure 2.10 Evaluation of the specificity of the PCR-LFA using <i>G. australes</i> and <i>G. excentricus</i> primers. (A) Agarose gel electrophoresis and (B) dipstick LFA. PCR reactions were performed using 100 pg/μL of genomic DNA (gDNA) from various microalgae species and genera. ntc: no template control.	63
Figure 2.11 Specificity of the (A) PCR and (B) dipstick LFA in the presence of non-target <i>Gambierdiscus</i> and <i>Fukuyoa</i> species. Each combination used for PCR amplification contained 400 pg/μL genomic DNA from each strain. ntc: PCR no template control; CL: control line; TL1 GA: test line 1 for <i>G. australes</i> ; TL2 GE: test line 2 for <i>G. excentricus</i>	64
Figure 2.12 Detection of <i>G. australes</i> (GA) and <i>G. excentricus</i> (GE) cells. (A) PCR and (B) dipstick LFA. Each PCR reaction contained 4 cells/μL while 100 pg/μL of purified gDNA (gDNA) from each species was used in parallel as a control. ntc: no template control; CL: control line; TL1 GA: test line 1 for <i>G. australes</i> ; TL2 GE: test line 2 for <i>G. excentricus</i>	65

CHAPTER 3: Capture, detection and purification of dsDNA amplicons using a DNA binding protein on magnetic beads

Figure 3.1 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.	85
Figure 3.2 Confirmation of the immobilization of scCro on magnetic beads by bead-ELISA.	92
Figure 3.3 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).	94
Figure 3.4 Amplification of target DNA (500 pg/μL) using 25 – 800 nM of each primer. The bands corresponding to the PCR amplicons were semi-quantified with the ImageJ software and the “Analyze gels” option.	95

Figure 3.5 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. ...	96
Figure 3.6 Possible structures adopted by the primer containing the scCro binding site contributing to undesirable binding to the scCro-MB.	97
Figure 3.7 Analysis of PCR reactions performed with (A) synthetic DNA and (B) genomic DNA templates in combination with primers containing the scCro DNA binding protein and biotin by agarose gel electrophoresis.	97
Figure 3.8 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.	98
Figure 3.9 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.	100

CHAPTER 4: Universal and label-free detection of dsDNA using the HU DNA binding protein

Figure 4.1 Schematic representation of ELONA for dsDNA fragment detection using scHupBA and scHupBA-HRP and (A) microtiter plates, (B) magnetic beads and (C) paper-based lateral flow dipstick assay.	112
Figure 4.2 (A) Structure of <i>E. coli</i> HU heterodimeric DNA binding protein with bound dsDNA (4yew.pdb) (49). Monomer α is in red and monomer β in blue. The N and C terminals of each monomer and the 16 aa linker fusing the two genes are indicated. (B) pLATE31 expression system and design of the fusion gene for single-chain scHupBA protein production.	122
Figure 4.3 Recombinant expression and purification of scHupBA in <i>E. coli</i> monitored by SDS-PAGE and Western blot. (A) Whole W and soluble S cell lysates from uninduced and IPTG-induced cells. The expected scHupBA is indicated with an asterisk (*). (B) IMAC and IEC purification steps. Fractions FT flowthrough (unbound); W1, W2, W3: wash; E1, E2: elution; BX: buffer exchanged IMAC elution fractions E1+E2. (C) Final purified scHupBA. (D) Western blot analysis of IEC fractions using an anti-His tag antibody.	123

- Figure 4.4** (A) Schematic representation of the ELONA assay. (B) ELISA to evaluate the concentration of scHupBA required for coating the microtiter plate wells. An anti-His tag antibody was used for protein detection. (C) Binding curves for dsDNA and ssDNA fragments of varying length (25, 47 and 100 nucleotides). 125
- Figure 4.5** Preparation of the scHupBA-HRP conjugate. (A) Conjugation strategy. (B) Lane 1: SATA-modified scHupBA; lane 2: maleimide-activated HRP; lane 3: conjugation reaction. (C) IMAC purification. Lane 4: conjugation reaction; lane 5: flowthrough (unbound); lanes 6-8: wash fractions; lanes 9-10: elution fractions. (D) ConA purification. Lane 11: flowthrough (unbound); lane 12: wash fraction; lane 13: elution fraction. (E) Final purified scHupBA-HRP conjugate. 126
- Figure 4.6** (A) Detection of dsDNA and ssDNA of varying length (47, 100, 176 bases) using scHupBA for capture and scHupBA-HRP (5 µg/mL) for detection. (B) Effect of the concentration of scHupBA-HRP and (C) of the composition of buffer used for DNA binding on signal intensity for the detection of the 176 bp PCR amplicon. 128
- Figure 4.7** Detection of a 176 bp PCR amplicon using scHupBA (10 µg/mL) for capture and scHupBA-HRP (5 µg/mL) for detection by ELONA. The calibration curves were constructed using the target DNA in phosphate buffer with varying salt (NaCl) concentrations. The span corresponds to signal range of the fitted curves designated as the absorbance at highest concentration minus the absorbance at the lowest concentration. 129
- Figure 4.8** (A) Bead-ELISA to verify the immobilization of scHupBA on the magnetic beads. (B) Effect of the composition of the magnetic beads blocking solution on signal intensity for detection of the 176 bp PCR amplicon using scHupBA-MB and scHupBA-HRP. 131
- Figure 4.9** Detection of a 176 bp PCR amplicon using scHupBA-MB for capture and scHupBA-HRP (5 µg/mL) for detection by bead-ELONA. The calibration curves were constructed using the target DNA under different buffer conditions. 132
- Figure 4. 10** Lateral flow dipstick assays in the absence and presence of PCR amplicon. (A) Screening of membrane blocking (2 % w/v skim milk in 10 mM carbonate-bicarbonate pH 9.4 with 0.1 % v/v Tween-20 or Empigen BB) and running (10 mM Tris-HCl pH 7.5, PBS pH 7.4 or water with 0.05 % Tween-20 and 0.5 – 2 % w/v skim milk) buffers. (B) Detection of DNA (176 bp) using optimal blocking (2 % w/v skim milk and 0.1 % v/v Empigen BB in 10 mM carbonate-bicarbonate pH 9.4) and running (0.5 % w/v skim milk and 0.1 % Tween-20 in water) buffers. (C) Screening of scHupBA concentrations used for conjugation to 0.2 % w/v CNPs in the absence of DNA using optimal blocking and running buffers. DNA and scHupBA-CNPs

were pre-incubated in running buffer prior to analysis. The CNPs conjugate was prepared with 0.35 mg/mL scHupBA for (A) and (B). 134

Figure 4.11 Detection of the PCR amplicon in two steps, first the DNA sample and then the scHupBA-CNPs. The scHupBA-CNPs conjugate was prepared using 0.5 mg/mL scHupBA and optimal blocking and running buffer compositions were used. 135

Figure 4.12 Evaluation of the sensitivity and specificity of the lateral flow dipstick assay using dsDNA PCR amplicon and ssDNA PCR fragment. The membranes were blocked with 2 % w/v skim milk and 0.1 % v/v Empigen BB in 10 mM carbonate-bicarbonate pH 9.4 and the running buffer was 0.5 % w/v skim milk with 0.1 % Tween-20 in water. DNA and scHupBA-CNPs (prepared using 0.5 mg/mL scHupBA) were pre-incubated in running buffer prior to analysis. 136

Figure 4.13 Detection of PCR amplicons directly in the PCR reactions without prior purification by colorimetric ELONA. Analysis of PCR reactions performed using 0 – 25 pg/ μ L of target DNA by (A) agarose gel electrophoresis, (B) Plate-ELONA and (C) magnetic bead-based ELONA. The insets show the calibration curves constructed using logarithmic models. 137

Figure 4.14 Analysis of PCR reactions with (+ve) and without (-ve) target DNA template with dipstick lateral flow assays. The reporter carbon nanoparticles were prepared using 0.5, 0.75 or 1 mg/mL of scHupBA. The +ve reaction was performed using 25 pg/ μ L of target dsDNA. 138

Figure 4.15 Detection of PCR amplicons directly in PCR reactions without prior purification. The scHupBA-CNPs conjugate was prepared using 1 mg/mL of the DNA binding protein. The strips were prepared using (A) 200 ng, (B) 250 ng, (C) 300 ng or (D) 350 ng of scHupBA per 4 mm strip. Diluted PCR reactions and scHupBA-CNPs were pre-incubated in running buffer prior to analysis. The running buffer contained 0.5 % w/v skim milk with 0.1 % Tween-20 in water. 139

LIST OF TABLES

CHAPTER 2: Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*

Table 2.1 Oligonucleotides used in this work. The scCro DNA binding site is in bold and the DNA tails are in italics. The asterisk (*) denotes a biotin-TEG modification at the 3' end and the (#) a biotin modification at the 5' end.	50
Table 2.2 Microalgae strains used in this work.	51
Table 2.3 Nucleic acid LFAs developed for the detection of harmful algae based on different amplification methods.	71

CHAPTER 3: Capture, detection and purification of dsDNA amplicons using a DNA binding protein on magnetic beads

Table 3.1 Oligonucleotides used in this work. The binding sites for the scCro DNA binding protein are underlined.	86
Table 3.2 Specific activity of the scCro-HRP and SA-HRP enzyme conjugates using ABTS as the substrate.	93
Table 3.3 Precision of the assay for DNA detection when high or low concentrations of template DNA were used for PCR amplification prior to capture on the protein-magnetic beads.	99

CHAPTER 4: Universal and label-free detection of dsDNA using the HU DNA binding protein

Table 4.1 Oligonucleotides used in this work. The asterisk (*) denotes oligonucleotides labeled with a 5' biotin.	113
---	-----

Chapter 1: General Introduction

1.1 Introduction

The development of biosensors has revolutionized the field of bioanalysis by enabling the direct measurement of target analytes and converting biological responses into measurable signals. In 1962, Clark and Lyons pioneered the field by inventing the first biosensor, where they attached the glucose oxidase enzyme to an amperometric oxygen electrode surface, leading to the measurement of glucose concentration (1). Since then, biosensors have gained significant interest and have found applications in various areas such as medical diagnosis, food analysis, bioterrorism detection, clinical research, and agriculture. Biosensors provide a means for continuous monitoring of biological responses and offer advantages such as easy handling and reduced sample preparation, making them highly suitable for point-of-care (POC) testing. POC tests are simple and low-cost tests performed near or at the point of patient care and can provide rapid results, thus facilitating early disease diagnosis, monitoring and implementation of appropriate treatment. This is especially important for developing countries with limited laboratory infrastructure and trained personnel and where early diagnosis can greatly improve healthcare outcomes. The two types of POC biosensors holding the largest market share globally are glucose monitoring and fertility/pregnancy testing devices (see Figure 1.1) (2-4).

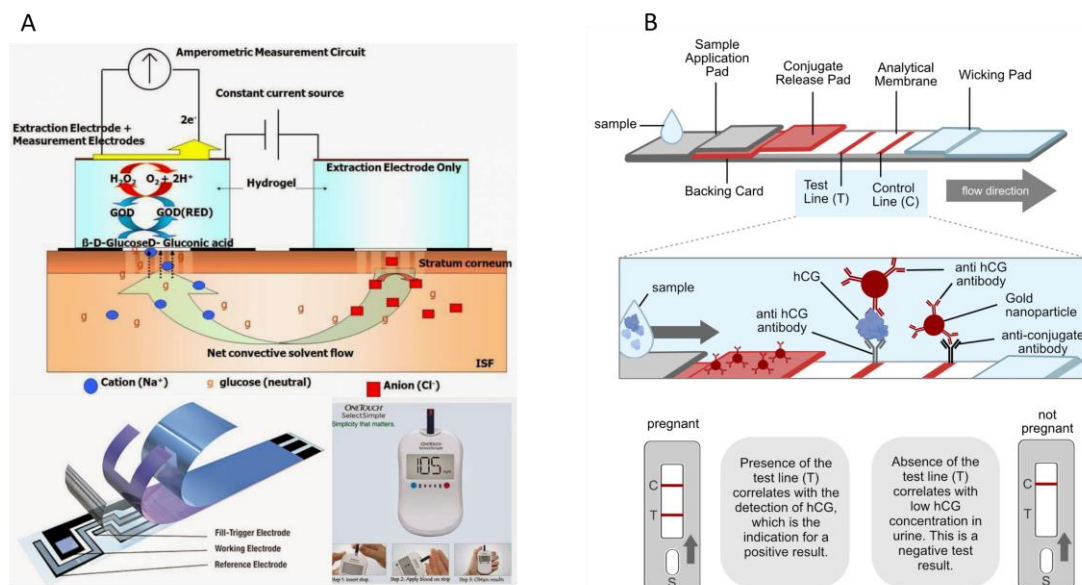


Figure 1.1 Commonly used POC biosensors. (A) Amperometric biosensor for glucose detection (5). (B) Lateral flow test for pregnancy (6).

1.2 Basic components of biosensors

Biosensors consist of three major components: the biorecognition element, the transducer, and the output units as illustrated in Figure 1.2 (7). These components collectively contribute to the development of an ideal biosensor for bioanalytical detection. Combination of these components can give important functionalities with enhanced properties or characteristics to a biosensor.

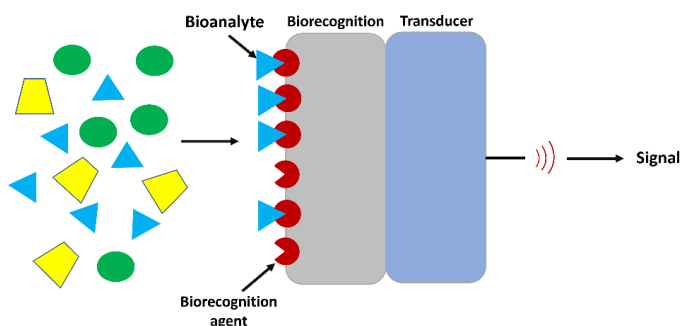


Figure 1.2 Schematic diagram of a biosensor.

1.2.1 Biorecognition element

The biorecognition element is a molecule that recognizes the target of interest. Examples of biorecognition elements include antibodies, enzymes, DNA, cells, and tissue probes (Figure 1.3). These biorecognition elements provide the interaction with the target, leading to a biorecognition event (8). Thus, the efficiency of biosensors relies on the ability of the biorecognition element to distinguish the target analyte from potentially interfering molecules.

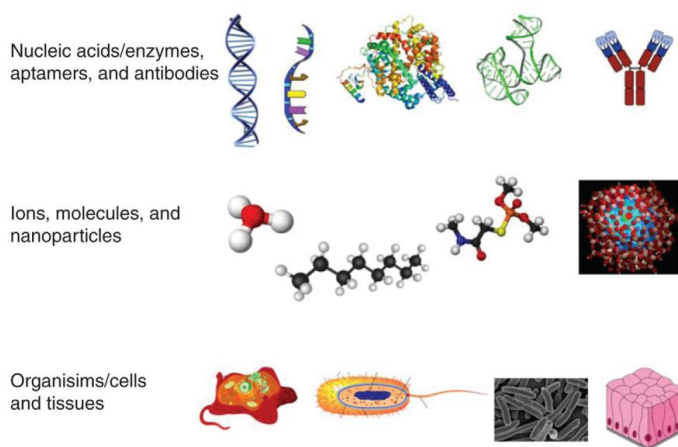


Figure 1.3 Common types of biorecognition elements in biosensors (9).

1.2.2 Transducer

It converts the signals generated from the biorecognition event into measurable responses. This conversion allows for signal generation and interpretation using output units (10).

1.2.3 Output units

They represent the measurement units for presenting results. These units are adaptable based on data type and biosensor application. Output information may encompass numeric data, graphical plots, tabulated data, or visual images, tailored to suit the preferences and requirements of the user (7, 11).

1.3 Characteristics of biosensors

Biosensors possess several important characteristics that define their performance and suitability for specific applications. The important characteristics of biosensors are briefly described below.

1.3.1 Sensitivity

It is the most critical property of biosensors and it refers to their ability to detect minute amounts or concentrations of the target analyte. Higher sensitivity allows for the detection of lower analyte concentrations. For example, an electrochemical DNA biosensor developed for hepatitis B virus (HBV) exploiting novel nanomaterial demonstrated high sensitivity with a limit of detection (LOD of 5.2 fM), surpassing the sensitivity of other reported electrochemical HBV DNA biosensors while also allowing direct detection in human serum and urine (12). Another example is a paper-based colorimetric lateral flow immunosensor targeting the human immunodeficiency virus (HIV) p24 biomarker. This biosensor facilitated naked-eye detection of p24 in serum samples in the low femtomolar range which is within the clinically relevant range for detection during the acute phase infection which greatly benefits from early antiretroviral therapy (13).

1.3.2 Selectivity

It concerns the capacity of the biosensor to specifically recognize the target analyte in complex environments such as biological fluids (e.g. blood, saliva, urine), environmental samples and food samples (14). This is critical for the generation of true positive responses as opposed to false positives when the biosensor generates signal for non-target molecules. For example, a colorimetric peptide probe biosensor developed for the detection of the pathogenic bacterium *Staphylococcus aureus* in food and environmental samples exhibited no interference from food-related or other kinds of pathogens (*Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*) (15). Another example is an electrochemical antibody-based biosensor used to detect the carcinoembryonic antigen (CEA) tumor marker in clinical serum samples consistent with reference values obtained with standard laboratory analysis (16).

1.3.3 Reproducibility

Reproducibility is essential for biosensors, as it ensures identical and consistent responses for repeated experiments. Precision and accuracy play significant roles in achieving reproducibility, contributing to the robustness and credibility of the biosensor (17). Reproducibility is usually expressed with the coefficients of variation (CV) which are relative statistical measures of variability around the mean value. According to the guidelines set by the Clinical and Laboratory Standards Institute (CLSI; EP05-A3, EP24-A2, EP25-A), it is necessary to achieve a CV value of less than 10 % for reproducibility purposes (18). For example, the reproducibility of a DNA electrochemical biosensor for the detection of genetically modified soybean was evaluated using five different electrodes and two distinct DNA concentrations and no significant discrepancy was found in the measurements (3.6 % CV), thus verifying the high reproducibility of the biosensor (19).

1.3.4 Stability

This feature refers to the effects of ambient changes on the different elements of a biosensor. These effects can be with regards to the biorecognition elements or the transducer and they can lead to erroneous measurements, thus affecting the precision and accuracy of the biosensor. Some biomolecules for example may require specific stabilization techniques to maintain their biological activity, improve their stability, and prolong the shelf-life of the biosensor (20).

1.3.5 Characteristics of POC biosensors

Researchers are motivated to explore the development of POC biosensors due to the rising demand for diagnostic tools with enhanced sensitivity, selectivity, and short response time. POC diagnostic devices offer valuable capabilities for detecting, diagnosing, and monitoring diseases at their early stages (21). The World Health Organization (WHO) has introduced a set of criteria known as ASSURED, which outline the characteristics of an ideal biosensor which are: affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users (22).

1.4 Immobilization strategies in biosensors

Immobilization methods are critical in biosensors due to their significant impact on the incorporation of biorecognition elements into the biosensing system and their subsequent functionality in recognizing the analyte accurately (23-25). Immobilization is defined as the technique of tethering the target biomolecules on the sensing surface resulting in lower or complete loss of mobility. Immobilization is not only important for the bioreceptor but also for the reporter and for other elements of a biosensor. With regards to the immobilization of the biorecognition element, this can be performed directly on a transducer or on a support system-based transducer for signal generation. Immobilization can be achieved with a variety of biomolecules, including DNA, aptamers, enzymes, and proteins (26), using various methods such as physical adsorption (27), chemical immobilization (28) or affinity-based immobilization (29). Reporting is performed with several types of methods like colorimetric detection (30), fluorescent detection (31) or electrochemical detection (32, 33). The main objective of immobilization in biosensors is to maintain the function of biorecognition molecules without losing their bioactivity and stability. Denaturation of biorecognition elements immobilized on support materials often leads to a significant loss of function. There are several factors that are vital for maintaining stability and bioactivity of biosensors such as shape, working area, polarity, presence of functional groups and storage conditions. Any changes in the transducer features also affect the bioreceptor immobilization (34).

Sensitive biorecognition elements such as DNA probes, antibodies and proteins can be immobilized onto support materials. These biomolecules have the capability of recognizing specific target analytes, including enzyme substrates, complementary DNA (35). DNA immobilization is simple, rapid, and inexpensive. The detection principle is based on the

recognition and detection of a specific sequence of DNA through hybridization. In contrast to enzyme and antibody elements, the biorecognition layers of nucleic acids can be generated and renewed (36). Proteins also provide specific recognition for analytes, but immobilization is a sensitive process as proteins can lose their biological activity during immobilization by conformational changes and structural modification upon immobilization. To overcome these challenges, the choice of a suitable immobilization strategy is important and should take into account the physical and chemical properties of protein bioreceptors and reporters to make a biosensor stable and specific. Significant differences in activity can arise due to different immobilization scheme, e.g. a 60000-fold increase in stability has been reported for chymotrypsin to aldehyde-agarose gels (37).

Various immobilization methods have been established to enable the attachment of biorecognition elements on surfaces. Each method offers unique advantages and considerations, making the selection of an appropriate immobilization strategy a crucial step in the biosensor design.

1.4.1 Physical immobilization

Physical immobilization involves the adsorption of biorecognition molecules onto support materials through weak non-specific forces such as van der Waals, hydrophobic interactions, and hydrogen bonds. Physical adsorption provides a straightforward method for immobilization; however, it may result in random orientation of the biomolecules (38). Common support materials include magnetic beads (39), cellulose (40), silica gel (41), cellulose (40), collagen (42), conducting polymers like polyaniline (43), polyethylenimine (44), polypyrrole (45) and polystyrene (46) and some biopolymers like L-lysine (47) and polyethylene glycol (48).



Figure 1.4 Physical immobilization by adsorption.

1.4.2 Chemical immobilization

It relies on the formation of strong covalent bonds between biomolecules and support materials. This method, also known as covalent immobilization, ensures robust attachment (38, 49). Covalent immobilization occurs due to the formation of chemical bonds between functional groups of biomolecules and the support materials. Nucleophilic functional groups such as amino (-NH₂), carboxyl (-COOH), hydroxyl (-OH), and thiol (-SH) play a crucial role in the binding of proteins, DNA and other biomolecules to support materials (50, 51).

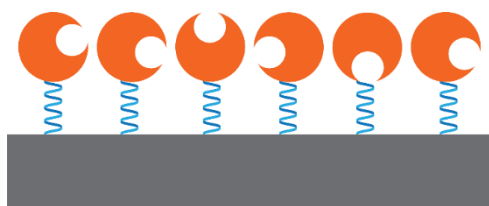


Figure 1.5 Chemical/covalent immobilization

Chemical crosslinking reagents are utilized to establish covalent bridges between the target molecule and the desired surface. They find extensive applications in nucleic acid modifications, functionalization of nanoparticles, and immobilization of proteins or enzymes. They do not exhibit site specificity, resulting in the random orientation of immobilized biomolecules, depending on the localization of the functional groups on the solvent-exposed regions of the biorecognition molecules. In contrast, site-specific immobilization methods offer enhanced stability and sensitivity for biosensors. Several examples of site-specific binding reported like immobilization by using sortase for site-specific protein immobilization involves the conjugation of adhesion proteins to fluorescent microsphere beads (52), modification of liposomes with green fluorescent protein (GFP) (53), immobilization of a bait protein to agarose beads for application in affinity purification mass spectrometry (54) and antibodies (55). One of the most common crosslinking reagents is glutaraldehyde because it is abundant and inexpensive and readily available in higher quantity. Glutaraldehyde has been in use since many years for crosslinking proteins (56). Immobilization using crosslinking is also possible with other reagents also, which have bifunctional reactive groups like hexamethylenediamine (57), glyoxal (58, 59) or other more expensive commercially available reagents with a wide variety of functional groups and lengths. Crosslinking mediated by a functionally inert protein

like bovine serum albumin is also possible. This method can form a very strong covalent attachment and is simple to perform (60).

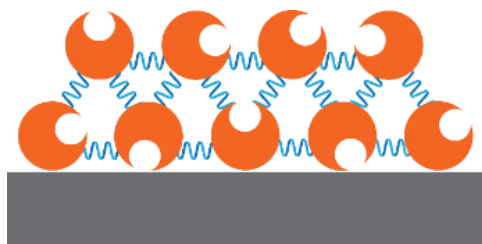


Figure 1.6 Immobilization by crosslinking.

1.4.3 Affinity-based immobilization

Affinity-based strategies can provide well-oriented, highly selective and homogenous immobilization of biomolecules. However, the procedure often requires the covalent binding of a costly affinity ligand (e.g., antibody or lectin) to the matrix (61). This strategy facilitates the bonding between different activated supports such as biotin-binding proteins (avidin, streptavidin or neutravidin), lectins, metal chelates with a specific affinity group incorporated to proteins or DNA, such as biotin, carbohydrate residue, or histidine (62). Affinity-based immobilization helps in controlling the orientation of the biomolecules and also prevents loss of biomolecule activity.

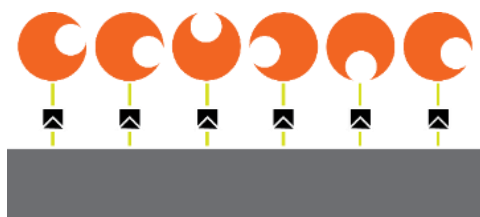


Figure 1.7 Affinity-based immobilization.

The strept(avidin)-biotin interaction is extensively used to mediate the immobilization of biomolecules. It provides very high specificity and a very strong non-covalent binding with a dissociation constant of K_D of 10^{-15} M (63). Strept(avidin) is stable at wide range of temperatures and pH values and can bind up to 4 biotin molecules. The binding is fast and can be maintained in harsh conditions without losing any biomolecule activity. Strept(avidin) is a glycoprotein with a tetrameric structure with affinity towards biotin (64). Neutravidin is a

deglycosylated form of avidin, which can lead to decreased non-specific interactions with negatively charged components such as cell surface or DNA/RNA, while maintaining its high binding activity for biotin (65). Different forms of biotinylation reagents are available which do not affect the binding affinity of biotin with the biotin-binding proteins, since only its bicyclic ring is necessary for binding while its carboxyl side chain can be used for attaching to proteins, DNA or other biorecognition elements. Biotin is small in size and therefore does not affect the biorecognition properties of the biomolecules after incorporation. Biotinylation can be achieved by several approaches using diverse functional groups. Most commonly used approach for biotinylation is through NHS ester to target amine reaction groups (66). Avidin immobilization is possible by both physical and chemical immobilization. In literature, chemical binding of neutravidin on a carboxyl-modified surface provided enhanced coating density compared to the strept(avidin)-biotin method (67). Another approach reported used a biotinylated aldo/keto reductase to immobilize on streptavidin-coated templates (68). Biotinylated reductase provided oriented and site-specific immobilization with 60 to 300-fold greater activity than randomly immobilized reductase enzymes and with improved stability (69). DNA binding proteins on the other hand offer a viable and versatile solution for DNA immobilization. This is due to their robust and specific affinity for DNA in general or for certain DNA binding sites on double-stranded DNA (dsDNA). By incorporating these binding sites directly into the target DNA molecule during synthesis or PCR, the requirement for post-synthesis chemical modifications is eliminated, resulting in cost savings (70).

1.5 DNA biosensors

They are used to detect and quantify specific DNA sequences or mutations within them (71) or employ DNA as biorecognition molecules (72). These biosensors rely on the immobilization of sensitive biomolecules, such as enzymes, DNA probes, or antibodies on transducer or several types of support materials (73). The most basic configuration of a DNA biosensor relies on the hybridization of the target DNA sequence with a complementary one immobilized on an electrode surface as shown in Figure 1.8. One of the notable features of DNA biosensors is their high sensitivity in detecting DNA molecules, making them suitable for a wide range of applications as summarized in the following section (74).

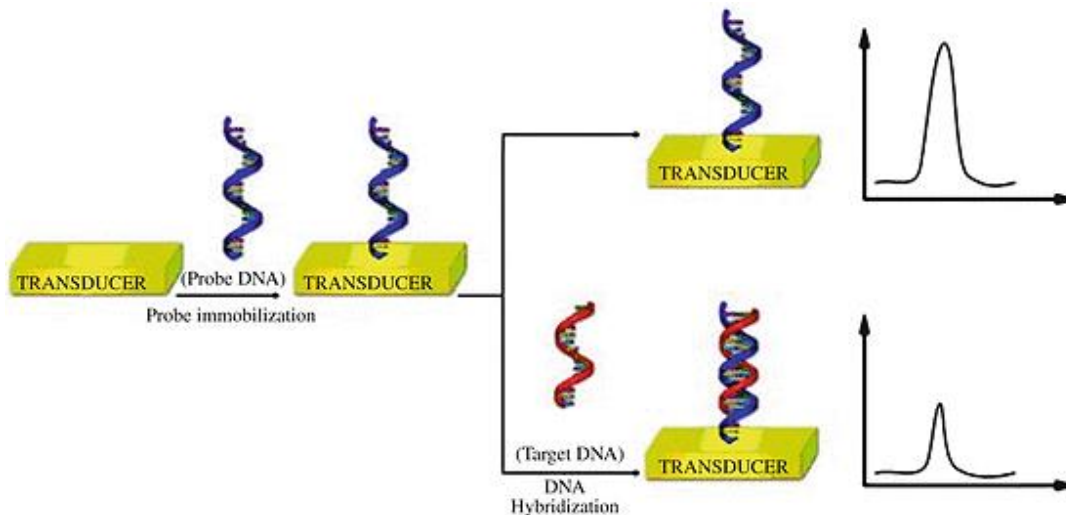


Figure 1.8 A general schematic diagram of DNA biosensor (75).

1.5.1 Types of DNA biosensors

There are several types of DNA biosensors, each designed for different applications and sensitivity levels. They can be classified according to the transducer element used. Some common types are discussed below.

1.5.2 Optical DNA biosensors

They are used to detect specific DNA sequences or biomolecules by measuring changes in the optical signals in the presence of the analyte, such as absorption, reflectance, emission, or interferometric pattern, which can be recorded by a photodetector (76). These biosensors are widely used in various fields, including medical diagnostics, environmental monitoring, and food safety testing. The main principle behind optical DNA biosensors is the interaction between the target DNA sequence and a complementary probe, which generates a detectable optical signal. Signal generation in optical DNA biosensors can be based on colorimetry, fluorescence or surface plasmon resonance (SPR) among others.

Colorimetric biosensors: They rely on color changes to indicate the presence of the target DNA (77). For example, dsDNA fragments in amplification reactions can be detected visually by naked eye using various dyes like the nucleic acid staining dye SYBR Green I, resulting in a color change from orange to green (78), or hydroxynaphthol blue (HNB) with a violet-to-blue color change in the presence of dsDNA (79). Another example is the use of HRP-mimicking

G-quadruplex-hemin DNAzymes incorporated into DNA detection probes which catalyze the oxidation of 2,2-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) by hydrogen peroxide (H_2O_2) upon hybridization with target DNA which is previously captured by a capture DNA probe. The change from colorless to green can be monitored by the absorbance changes at 418 nm (80). Another type of colorimetric biosensors are the gold or carbon nanoparticle-based lateral flow assays which have gained increased interest in diagnostics due to their ability to provide simple, fast and low-cost detection of target analytes (81). An example of gold nanoparticle-based colorimetric biosensor is provided in Figure 1.9.

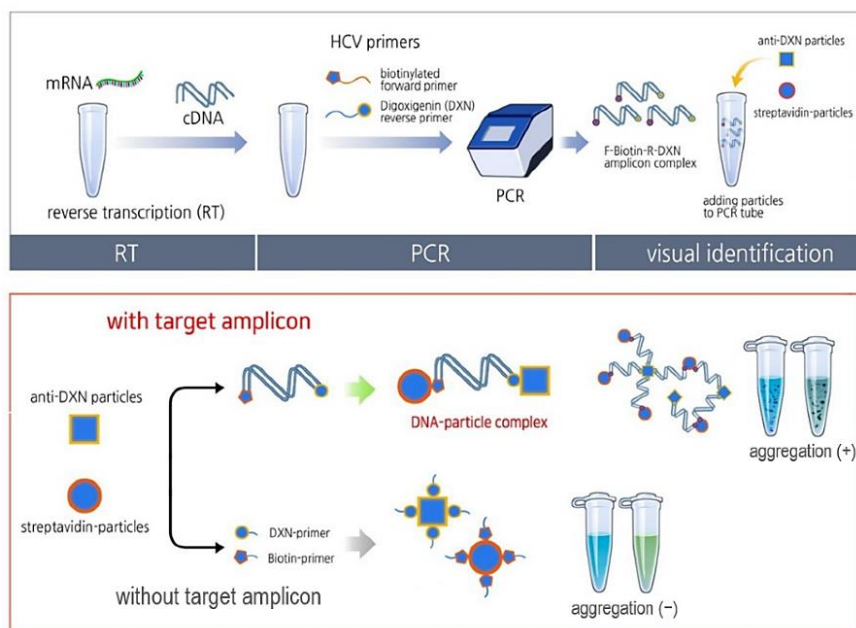


Figure 1.9 Schematic representation of a nanoparticle-based colorimetric detection of hepatitis C virus (HCV) RNA after amplification (82).

Fluorescence-based biosensors: These biosensors utilize the fluorescence emitted by a fluorophore-labeled probe upon binding or other reporter elements which are associated with the target DNA. The fluorescence signal intensity is proportional to the target DNA concentration, enabling quantitative analysis (83). There exists a great variety of configurations exploiting fluorescence signals in DNA biosensors, including directly labeled DNA probes (84) complementary to target sequences, fluorescently labeled nucleic acids (85), or molecular beacons (86). An example of a fluorescence DNA biosensor is provided in Figure 1.10.

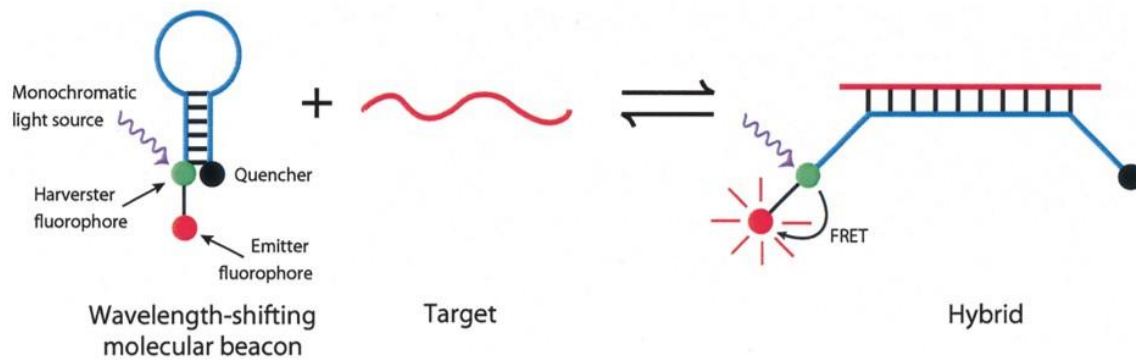


Figure 1.10 Optical DNA biosensor (87).

Surface Plasmon Resonance (SPR) biosensors: They detect changes in the refractive index at the sensor surface when the target DNA binds to the immobilized probe. This change in refractive index causes a shift in the resonance angle, which can be measured and correlated to the target DNA concentration (88, 89). Guan *et al.* proposed a highly sensitive optical fiber DNA sensor based on the micro-fiber Sagnac interferometer, with a detection range of 100 pM to 1 μ M and a detection limit of 75 pM. Zhang *et al.* presented an SPR biosensor in which DNA hybridization is detected by altering a graphene/Au film on a D-shape plastic optical fiber (90).

1.5.3 Electrochemical DNA biosensors

Electrochemical DNA biosensors are designed to detect specific DNA sequences by measuring changes in electrical properties. These biosensors are comprised of an electrode, a transducer, and a bioreceptor that binds to the target DNA sequence. The underlying principle of electrochemical DNA biosensors is the interaction between the target DNA and the bioreceptor, leading to an electrical signal that can be measured as depicted in Figure 1.11 (91, 92). Different types of electrochemical DNA biosensors are discussed briefly below.

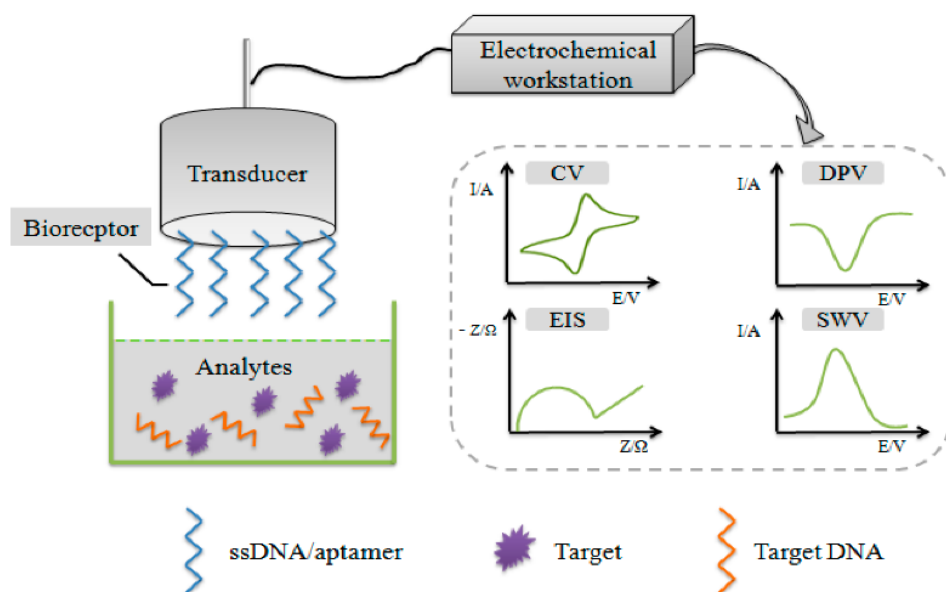


Figure 1.11 Basic configuration of electrochemical DNA biosensors (92).

Amperometric DNA biosensors: These biosensors measure the current produced by the oxidation or reduction of an electrochemically active label associated with the target DNA at the electrode surface. The current is directly proportional to the concentration of the target DNA, allowing for quantitative analysis (93). For example, combination of novel nanomaterial (flower-like carbon nanotubes-polyaniline hybrids) with furullene nanoparticles led to sensitive amperometric detection of *Mycobacterium tuberculosis* DNA upon recognition and binding to a capture DNA probe immobilized on a glassy carbon electrode, thus enabling detection in clinical samples (94).

Potentiometric DNA biosensors: They measure the potential difference between two electrodes, one of which is coated with the bioreceptor. The potential difference is directly related to the concentration of the target DNA, enabling quantitative analysis (95). A nucleic acid potentiometric biosensor relied on a sandwich DNA hybridization to capture a secondary oligonucleotide with CdS-nanocrystal tags. The assay utilized a miniaturized solid contact Cd-ISE and achieved a low LOD of 10^{-10} and 10^{-9} M Cd^{2+} in 100 mL and 200 L samples, respectively (96).

Impedimetric DNA biosensors: They measure the change in impedance when the target DNA binds to the bioreceptor. The change in impedance is proportional to the concentration of the

target DNA, allowing for quantitative analysis (97). An example is the use of aminated DNA probes mounted on hollow silica microspheres functionalized with 3-aminopropyltriethoxysilane. These probes were placed onto a carbon paste screen-printed electrode with supported gold nanoparticles. The biosensor exhibited enhanced specificity and sensitivity and achieved an LOD of $1.95 \times 10^{-15} \mu\text{M}$ in the absence of a redox mediator (41).

1.5.4 Piezoelectric DNA biosensors

Piezoelectric DNA biosensors exploit the piezoelectric effect to detect the presence of DNA. They operate based on the principle of affinity interaction recording, utilizing a piezoelectric platform or crystal as a sensor component that responds to changes in mechanical stress (98). By evaluating variations induced in the mechanical properties of the piezoelectric material, such as frequency, amplitude, and phase, these biosensors can detect biological interactions (99). Piezoelectric DNA biosensors can be used for the detection of various genetic markers (100), environmental pollutants (101), and for other applications. They employ various biological recognition elements like enzymes, antibodies, DNA, and other biomolecules (102). Piezoelectric DNA biosensors employ a variety of piezoelectric materials, including lead zirconate-lead titanate glass, quartz crystal microbalance (103), and surface acoustic wave devices (103).

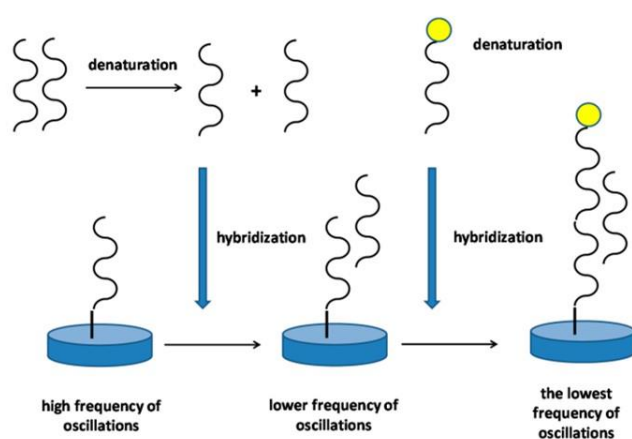


Figure 1.12 Piezoelectric DNA biosensors (97).

1.6 Applications of DNA biosensors

Disease diagnosis: They can be used to identify specific DNA sequences associated with diseases like cancer, viral infections, and genetic disorders, enabling early diagnosis and timely treatment (104). An ion-channel electrochemical biosensor employing PPV single stranded DNA (ssDNA) probes on a glassy carbon electrode surface was developed for the detection of Plum Pox Virus (PPV) and it exhibited a detection limit of 12.8 pg of PPV ssDNA/mL (105). A multiplex colorimetric paper-based analytical device was developed based on silver nanoparticles (AgNPs) to simultaneously detect DNA from viral and bacterial infectious diseases, including Middle East Respiratory Syndrome coronavirus (MERS-CoV), *Mycobacterium tuberculosis* bacteria (MTB), and human papillomavirus (HPV). It achieved limits of detection of 1 – 1.5 nM for each pathogen (106).

Genetic testing: DNA biosensors can be utilized for prenatal and paternity testing, and for identifying genetic predispositions to certain diseases. This information can help individuals and families to make informed decisions regarding their health and well-being (107).

Environmental monitoring: DNA biosensors play a vital role in detecting specific contaminants/pollutants, microorganisms or genetic material in environmental samples like water, soil, and air. This enables monitoring of pollution levels, identification of harmful pathogens, and assessment of ecosystem health (108). For example, the water-borne microbe *Vibrio cholera* can be detected by selectively binding its O1 OmpW gene with two DNA probes, one of which contains a fluorescent label which generates the signal to indicate the presence of the microbe (109). Xiang *et al.* developed an aptamer-based enzyme-linked oligonucleotide assay biosensor to detect the organophosphorus pesticide isocarbophos with the sensitivity required for environmental monitoring (110).

Food safety testing: DNA biosensors can detect foodborne pathogens, allergens, and genetically modified organisms in food samples. This ensures the safety and quality of the food supply (92). A report was conducted regarding the use of the isothermal rolling circle amplification (RCA) method in a solution and implemented a cleavage reaction to generate small fragments of ssDNA. These fragments readily hybridized with the capture probe

immobilized on gold nanoparticles (AuNPs) assembled onto the SPR chip surface and this approach enabled real-time monitoring of *Mycobacterium tuberculosis* and *M. avium* complexes. *M. tuberculosis* is transmitted through the air, whereas *M. avium* is found in dust, water, and soil, with infections occurring through inhalation or ingestion. The optical biosensor achieved LODs of 5 pg/ μ L and 2 pg/ μ L of genomic DNA for *M. tuberculosis* and *M. avium*, respectively (111, 112).

Forensic analysis: In forensic analysis, DNA biosensors are used to identify individuals based on their unique DNA profiles (113), aiding in solving criminal cases. They can also use DNA biorecognition elements for toxicological forensic analysis and detection chemical and biological weapons (114).

1.7 Generation/sources of target DNA for biosensors

Target DNA contained in a sample can be detected using a DNA biosensor either directly or after enzyme-mediated amplification for increased sensitivity, especially in the case of low-abundance targets. The most common approach is the use of amplified DNA resulting from either thermal cycling or isothermal methods as detailed below. However, non-amplified DNA can also be detected, directly or after a signal amplification strategy to increase sensitivity.

Amplified target DNA: The gold standard in DNA amplification is polymerase chain reaction (PCR) which was firstly reported in 1987 (115). It relies on the use of two short synthetic DNA fragments (primers) which specifically recognize and bind (anneal) to the 3' ends of the sense and anti-sense strands of the target DNA, and a thermostable DNA polymerase. Under thermal cycling conditions (50 – 95°C), the two strands of the target DNA are separated (denaturation), then the primers hybridize to their complementary strands (annealing) and finally the polymerase extends the primers using the deoxynucleotide triphosphates (dNTPs) present in the reaction mixture (extension). After several rounds of thermal cycling, millions to billions of copies of the target sequence are generated which can then be detected employing a DNA biosensor. Over the last two decades, novel isothermal amplification techniques have been developed to circumvent the need for thermal cycling and facilitate the integration of amplification steps in devices, especially for POC diagnosis (116). Among them, Nucleic Acid Sequence-based Amplification (NASBA), Loop-mediated Isothermal Amplification (LAMP),

Strand Displacement Amplification (SDA), Recombinase Polymerase Amplification (RPA) and Rolling Circle Amplification (RCA) are the most representative methods. NASBA is used for amplification of short (< 150 bases) RNA targets by combining reverse transcription with amplification employing a cocktail of enzymes, with operating temperature of 40 – 55°C and resulting in dsDNA products (117). LAMP is the most commonly used isothermal amplification method and it employs a strand displacing polymerase and four to six pairs of primers for amplification of DNA targets (< 250 bases) at 65°C resulting in long, branched dsDNA molecules which can be detected with high sensitivity (118). For SDA, two to four primers pairs, an endonuclease and a polymerase are required to amplify short target DNA sequences (50 – 120 bases) at approximately 37°C after a brief initial denaturation step (119). On the other hand, RPA operates at constant temperature of 37 – 42°C and it employs one pair of primers which are bound by a recombinase enzyme to scan the target DNA template and facilitate annealing at homologous sites by the formation of a D-loop, stabilized in turn by ssDNA binding protein (SSB) (120). A strand displacing polymerase then extends the primers resulting in long dsDNA amplicons (< 1000 bp). Finally, RCA is based on the use of a single primer and a strand displacing polymerase for the amplification of circular DNA templates (or ligated linear templates) at constant temperature (20 – 37°C) (121).

Non-enzyme amplified target DNA: Genomic DNA extracted from cells, tissues, biological fluids, environmental or food samples can also be detected with biosensors. However, different types of signal amplification strategies, usually by incorporating nanomaterials, are required to improve sensitivity (122). Hybridization chain reaction (HCR) is an alternative method to generate DNA without employing an enzyme-based amplification technique for subsequent detection. It relies on a cascade reaction of hybridization events between two partially complementary hairpin probes and the target DNA which acts as the initiator strand, yielding a long and nicked dsDNA molecule. HCR has been employed to construct various simple, sensitive and economic biosensors for detecting nucleic acids (123).

1.8 DNA binding proteins

DNA binding proteins are proteins which have specific or general affinity for ssDNA or dsDNA molecules and these interactions are vital for cellular and molecular functions, such as gene transcriptional regulation, DNA recombination, replication and repair, as well as DNA packing

and modification (124-126). The discovery of the preference of lysine-containing polypeptides for A-T rich DNA and arginine amino acid residues for G-C rich DNA provided a better understanding into how a protein interacts with DNA (127). Based on these findings, it was later suggested that these specific interactions were more likely to occur in the DNA major groove (128), and this was experimentally proven with the first crystal structure of a DNA-protein complex using the *Escherichia coli* catabolite gene activator protein (CAP) (129). Even though there are indeed some preferred interactions between amino acids and DNA bases, DNA binding protein families interact with DNA in different ways (130), and depending on their binding motifs, they can be grouped into sequence-specific and non-sequence specific. Sequence-specific DNA binding proteins recognize and bind to particular DNA sequences, while non-specific DNA binding proteins can bind to any DNA sequence. Based on their DNA binding properties, these proteins have potential applications in monitoring DNA-protein interactions (131, 132), and in gene (133) and antiviral therapies (134).

1.8.1 Sequence-specific DNA binding proteins

These are proteins which recognize and bind to specific DNA sequences with great affinity and specificity. They employ distinct structural motifs to achieve this, such as helix-turn-helix, zinc fingers, and leucine zippers, through hydrogen bonds, electrostatic interactions and non-polar interactions (135). Some types of sequence-specific DNA binding proteins are discussed below.

Transcription factors regulate the transcription of target genes by binding to specific DNA sequences in gene promoter regions (136). By recruiting other proteins to the transcriptional apparatus, they can activate or inhibit gene expression (137). The p53 tumor suppressor protein, which binds to specific DNA sequences to regulate genes involved in cell cycle control and apoptosis, is one example (138). Another is the estrogen receptor, which binds to specific DNA sequences to regulate genes involved in estrogen signaling (139). Cro DNA binding protein is a repressor protein from bacteriophage lambda and it plays a significant role in bacteriophage gene control during lytic growth by inhibiting early gene transcription (140). Cro is a homodimeric DNA binding protein with a high affinity and specificity for its operator DNA (141) and the interaction between Cro protein and the nucleic acid occurs through the helix-turn-helix (HTH) motif (Figure 1.13). Its three-dimensional structure reveals that upon Cro binding, the DNA undergoes a bending of approximately 40° over 19 base pairs (142). The recognition and binding of the Cro helix-turn-helix units and its operator involve hydrogen-

bonding and van der Waals contacts within the major groove of the DNA (143). The wild-type Cro repressor of bacteriophage lambda binds to a 17-bp operator dsDNA as a dimer composed of two identical 66-residue subunits (144,145). However, a single-chain version, called scCro, was developed by linking two Cro monomers through a flexible linker of either 8 or 16 residues (146). These versions of the Cro homodimer exhibited increased affinity for the operator dsDNA. Notably, the scCro with the 16-amino acid linker (single-chain Cro, scCro16) demonstrated a dissociation constant in the picomolar range (4×10^{-12} M) for the 5'-TATCACCGCAAGTGATA-3' dsDNA sequence (146).

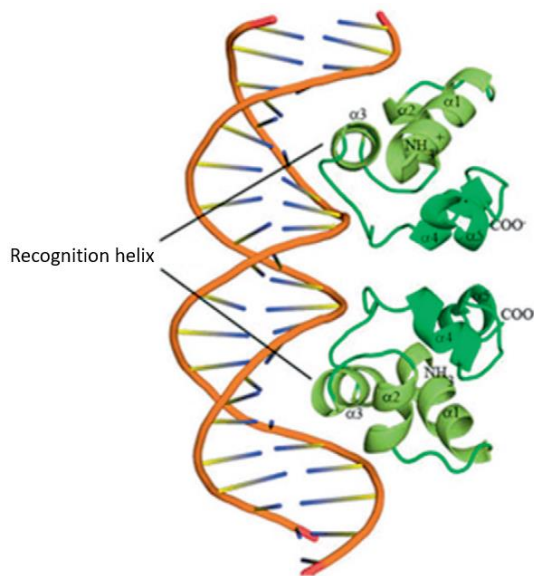


Figure 1.13 scCro DNA binding protein with helix-turn-helix motif (146).

Zinc finger proteins (ZFPs) are characterized by the presence of a zinc finger domain, a common structural motif found in many proteins involved in DNA interactions. These are short peptide sequences which adopt specific secondary structures, typically an alpha helix, stabilized by the binding of a zinc ion (Figure 1.14). The zinc ion is coordinated by two cysteine residues and two histidine residues within the zinc finger motif, forming coordination bonds that contribute to the stability and structure of the zinc finger domain (147, 148). This binding mode is simple and natural, allowing engineered ZFPs to be fused with various functional domains, enabling diverse applications such as gene regulation (147) and DNA diagnostics (149).

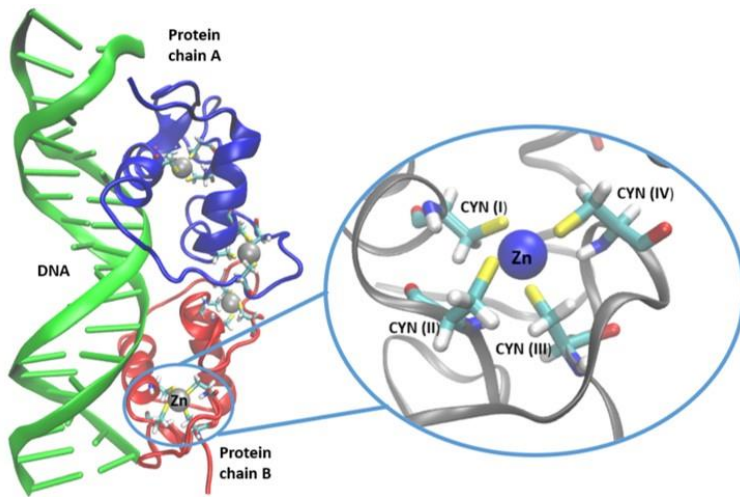


Figure 1.14 Zinc finger protein (150).

Homeodomain proteins belong to a class of transcription factors characterized by a conserved homeodomain DNA binding motif. This is a helix-turn-helix structure that detects and binds to specific DNA sequences often involved in developmental and cell differentiation processes (150). Hox genes for example encode transcription factors that control the development and patterning of body parts in animals (151).

Forkhead box (FOX) proteins are transcription factors which possess a DNA binding domain called a winged helix. This domain exhibits a globular structure composed of three alpha helices and two or three beta strands. The winged helix/forkhead pattern enables FOX proteins to regulate gene expression in various processes, including development, cell cycle control, and metabolism, by interacting with specific DNA sequences (152).

1.8.2 Non-sequence specific DNA binding proteins

Non-sequence specific DNA binding proteins are a group of proteins that interact with DNA in a non-sequence specific manner, lacking high specificity for particular DNA sequences. Unlike sequence-specific DNA binding proteins that recognize and bind to specific DNA sequences, non-sequence specific DNA binding proteins exhibit a more general or non-sequence specific mode of interaction with DNA. Some examples of non-sequence specific DNA binding proteins are discussed below.

Histones are a class of proteins crucial for the organization and compaction of DNA into a condensed structure called chromatin. They bind to DNA in a non-sequence specific manner, contributing to the structural stability and packaging of DNA molecules. Histones are essential for maintaining chromosome shape and function, even though they lack sequence-specific DNA binding capabilities (153, 154).

Bacterial histone-like proteins (HLPs) are small (16 – 20 kDa) and basic proteins, present in the cell in high amounts and they are responsible for maintaining DNA architecture by performing certain topological modifications (twisting, bending and folding) (155). They are critical for the regulation of cell metabolism, and they also participate in recombination and DNA replication (156, 157). The best characterized HLPs are Fis (factor for inversion stimulation), H-NS (histone-like nucleoid structuring), HU (heat unstable) and IHF (integration host factor) (156). HU and IHF have similar amino acid sequences, while Fis and H-NS are different. HU family proteins are found in almost all eubacteria as opposed to the other HLPs and they are in general homodimers or in the case of *Escherichia coli* heterodimers composed of two highly homologous subunits (155, 158). HU proteins exhibit non-sequence specific binding to DNA, with a higher affinity for nicked, gapped, and cruciform DNA structures (159). Despite lacking sequence specificity, bacterial HU proteins play roles in DNA compaction through mechanisms such as DNA bending, DNA bridging, or DNA wrapping (160).

Single-stranded DNA binding proteins (SSBs) bind to ssDNA regions with high affinity. They protect ssDNA from degradation, prevent the formation of secondary structures, and serve as platforms for other proteins involved in DNA replication, recombination, and repair. SSBs bind to ssDNA irrespective of the specific DNA sequence (161).

HMG (High Mobility Group) proteins are a protein family that binds to DNA in a non-sequence specific manner. The HMG box, a DNA binding domain found in HMG proteins, interacts with DNA, often through interactions with the minor groove. HMG proteins are involved in chromatin structure, gene control, DNA repair, and transcriptional activation, among other biological processes (162).

1.8.3 Use of DNA binding proteins in biosensors

Despite their attractive properties and their great potential in bioanalysis, the use of DNA binding proteins in DNA and DNA-based biosensors has been quite limited, attested by the very few works reported in the literature until today. And even though there is a plethora of DNA binding proteins with different properties, only a handful has been used in bioanalysis and these are the non-sequence specific ssDNA binding protein from *Escherichia coli* (SSB), the human sequence-specific dsDNA binding protein 53 (p53 tumor repressor protein), the engineered single-chain Cro bacteriophage repressor protein (scCro), the engineered dimeric headpiece domain of the *Escherichia coli* LacI repressor protein (dHP) and the SpoIIID transcription factor from *Bacillus subtilis*. The works described below highlight the great potential these proteins hold in biosensing, for the detection of not only nucleic acids but also other targets when incorporated into DNA and DNA-based biosensing platforms.

The first report was in 1989 when the SSB was covalently linked to a signal generating enzyme (HRP or urease) and was used to detect with colorimetry as low as 10 pg of total ssDNA bound on a nylon or nitrocellulose membrane (163). The idea was then exploited for the development of an electrochemical biosensor for the detection of residual amounts of DNA in mammalian cell culture products. This work was based on recognition of total DNA by SSB immobilized on an electrode and detection with an anti-DNA monoclonal antibody-urease conjugate and it achieved an LOD of 2 pg (164). SSB was also proposed as a general analytical tool and its use for the detection of nucleic acids and proteins using DNA/RNA or aptamers as biorecognition elements, respectively was demonstrated with capillary electrophoresis (165). Finally, a fluorescent biosensor was reported for ssDNA detection based on SSB labeled with the fluorophore diethylaminocoumarin and it was used to monitor the progress of helicase action along dsDNA (166).

The tumor repressor p53 protein was also used for the development of an electrochemical biosensor for miRNA detection. The target was sandwiched between a ssDNA probe immobilized on magnetic beads and a ssDNA detection probe containing the specific DNA binding sequence of p53. This was then filled in by a T4 DNA polymerase to produce the required dsDNA binding site for the binding of a p53-HRP conjugate which generated the signal after substrate addition. The reported LOD was 22 fM (167). A similar approach was used to detect exosomal miRNA in lung cancer patient serum. By replacing the p53-HRP conjugate with a p53-gold nanoparticle-hydrazine conjugate, the authors were able to improve the sensitivity and reported an LOD of 92 aM (168).

The use of scCro DNA binding protein in bioanalysis was first reported by our group for the colorimetric detection of high-risk human papillomavirus DNA. The platform employed a ssDNA capture probe to recognize the target ssDNA which was in turn bound by a hybrid ssDNA-dsDNA detection probe containing one to three binding sites for Cro protein. An HRP-Cro conjugate was finally added to generate signal after substrate addition with an LOD of 2 – 7 pM (169). A similar approach was later adopted with the hybrid ssDNA-dsDNA detection probe containing the binding sites for both scCro and dHP DNA binding proteins. This probe was used in combination with the glucose oxidase (GOX)-dHP and HRP-Cro conjugates. Their specific binding to adjacent positions on the hybrid detection probe enabled the formation of an enzyme cascade. This resulted in signal generation due to the catalytic activity of GOX which generated one of the substrates required by HRP. The limits of detection were 21 – 46 pM ssDNA, depending on the stoichiometry of the binding sites of the DNA binding proteins on the detection probe (170). A colorimetric sandwich aptasensor for the detection of a protein (thrombin) was also developed exploiting the two DNA binding protein-enzyme conjugates GOX-dHP and HRP-Cro. The binding sites for each of the DNA binding proteins were incorporated in each of the aptamers during synthesis and signal was generated using the GOX-HRP enzyme cascade. The reported LOD was 0.92 nM (171). A lateral flow assay based on Cro-carbon nanoparticle (Cro/CNPs) conjugate was also demonstrated for the detection of dsDNA PCR amplicons. A fluorophore-labeled forward primer and a reverse primer extended with the binding sequence of Cro were used for amplification and the amplicon was captured on the strip by the fluorophore-specific antibody and detected with the Cro/CNPs or Cro-HRP/CNPs conjugate. The detection limits were 6.9 – 10.4 ng of PCR product depending on the conjugate used and the number of Cro binding sites in the amplicons (172). Finally, scCro and dHP were used for the development of microplate-based and lateral flow assays for the detection of a PCR amplicon. Both primers used during amplification were extended with each of the binding sites of scCro and dHP and the amplicon was detected with an HRP-Cro conjugate on plate or a Cro/CNPs conjugate on the lateral flow strips. The limits of detection achieved were 10.4 pM of target ssDNA, comparable with the one achieved with an equivalent signal-generation system based on the streptavidin/biotin interaction. The lateral flow assay was able to detect 33.5 ng of PCR amplicon (70).

Lastly, a recent report demonstrated the detection of PCR amplicons using SpoIII. In this case, the binding site for SpoIIID was incorporated in the primers by extension, while SpoIIID was individually fused to each of two fragments of split trehalase enzyme. Once bound to the

amplicon, the trehalase activity is restored and is used to generate signal in the presence of trehalose which is converted to glucose, glucose oxidase which uses glucose and generates hydrogen peroxide, and HRP which uses hydrogen peroxide to oxidize a substrate and produce colorimetric signal. The assay was used to discriminate gram-positive (*Staphylococcus devriesei*) from gram-negative (*Escherichia coli*) bacteria with potential use in pathogen detection (173).

1.9 Thesis objectives

The aim of this thesis is the development of DNA binding proteins and their application in bioanalysis, focusing on DNA biosensors. Specifically, we demonstrate the use of both sequence-specific and non-sequence specific DNA binding proteins as biorecognition elements for the detection of dsDNA generated by amplification techniques. Different materials are explored to immobilize these proteins and facilitate DNA capture and detection: nitrocellulose membranes, carbon nanoparticles, microtiter plates and magnetic beads. With this work, we seek to highlight the great potential of these proteins in DNA bioanalysis, especially as an alternative to the established strept(avidin)-biotin-based strategies, exploiting their ability to bind DNA in either a sequence specific or non-sequence specific manner.

To achieve this general goal, the following specific objectives were established:

Sequence-specific DNA binding proteins

- Enhance the recombinant production of scCro DNA binding protein.
- Develop a lateral flow dipstick assay based on scCro-carbon nanoparticle conjugate for the detection of multiplex PCR products generated using synthetic or genomic DNA targets.
- Immobilize scCro on magnetic beads and demonstrate its use as an attractive bioanalytic tool for the colorimetric detection of PCR amplicons.
- Demonstrate the use of scCro-magnetic beads for PCR reaction cleanup and amplicon purification.

Non-sequence-specific DNA binding proteins

- Engineer a single-chain heterodimeric scHupBA DNA binding protein and produce it recombinantly.
- Characterize the DNA binding properties of scHupBA on microtiter plates using dsDNA and ssDNA of varying length.
- Prepare and characterize a scHupBA-horseradish peroxidase (scHupBA-HRP) conjugate.
- Immobilize scHupBA on magnetic beads and carbon nanoparticles.
- Develop colorimetric assays exploiting microtiter plates and magnetic beads as immobilization supports for scHupBA and the scHupBA-HRP conjugate for label-free universal detection of PCR amplicons.

- Provide a proof-of-concept for the application of scHupBA to mediate universal capture and detection of DNA in lateral flow assays.

1.10 References

1. Lakshmipriya T, Gopinath SCB. 1 - An Introduction to Biosensors and Biomolecules. In: Gopinath SCB, Lakshmipriya TBT-N for BT, editors. Micro and Nano Technologies. Elsevier; 2019 Jan 01; p. 1–21.
2. Ensafi AA. Chapter 1 - An introduction to sensors and biosensors. In: Ensafi AABT-EB, editor. Elsevier; 2019. p. 1–10.
3. Ensafi AA, Karimi-Maleh H, Ghiaci M, Arshadi M. Characterization of Mn-nanoparticles decorated organo-functionalized SiO₂–Al₂O₃ mixed-oxide as a novel electrochemical sensor: application for the voltammetric determination of captopril. J Mater Chem. 2011;21(38):15022–30.
4. Vashist SK. Point-of-Care Diagnostics: Recent Advances and Trends. Vol. 7, Biosensors. Switzerland; 2017 Dec 13.
5. <https://technologyinscience.blogspot.com/2015/01/how-does-glucometer-or-glucose.html>
6. <https://www.milenia-biotec.com/en/universal-lateral-flow-development-tool/>
7. Bhalla N, Jolly P, Formisano N, Estrela P. Introduction to biosensors. Essays Biochem. 2016 Jun 30;60(1):1–8.
8. Mohanty SP, Kougiannos E. Biosensors: a tutorial review. IEEE Potentials. 2006;25(2):35–40.
9. Qazi S, Raza K. Chapter 4 - Smart biosensors for an efficient point of care (PoC) health management. In: Chaki J, Dey N, De DBT-SB in MC, editors. Advances in ubiquitous sensing applications for healthcare. Academic Press; 2020. p. 65–85.
10. GS S, CV A, Mathew BB. Biosensors: A Modern Day Achievement. J Instrum Technol. 2014 Dec 8;2(1):26–39.
11. Monošík R, Stred'anský M, Šturdík E. Biosensors - classification, characterization and new trends. Acta Chim Slovaca . 2012;5(1):109–20.
12. Lin X, Lian X, Luo B, Huang X-C. A highly sensitive and stable electrochemical HBV DNA biosensor based on ErGO-supported Cu-MOF. Inorg Chem Commun. 2020;119:108095.
13. Loynachan CN, Thomas MR, Gray ER, Richards DA, Kim J, Miller BS, et al. Platinum Nanocatalyst Amplification: Redefining the Gold Standard for Lateral Flow Immunoassays with Ultrabroad Dynamic Range. ACS Nano. 2018 Jan 23;12(1):279–88.

14. Li S, Zhang H, Zhu M, Kuang Z, Li X, Xu F, et al. Electrochemical Biosensors for Whole Blood Analysis: Recent Progress, Challenges, and Future Perspectives. *Chem Rev.* 2023 Jun 28;123(12):7953–8039.
15. Suaifan GARY, Alhogail S, Zourob M. Rapid and low-cost biosensor for the detection of *Staphylococcus aureus*. *Biosens Bioelectron.* 2017 Apr;90:230–7.
16. Liu Z, Ma Z. Fabrication of an ultrasensitive electrochemical immunosensor for CEA based on conducting long-chain polythiols. *Biosens Bioelectron.* 2013;46:1–7.
17. Morales MA, Halpern JM. Guide to Selecting a Biorecognition Element for Biosensors. *Bioconjug Chem.* 2018 Oct 17;29(10):3231–9.
18. Chen L-C, Wang E, Tai C-S, Chiu Y-C, Li C-W, Lin Y-R, et al. Improving the reproducibility, accuracy, and stability of an electrochemical biosensor platform for point-of-care use. *Biosens Bioelectron.* 2020;155:112111.
19. Raja Jamaluddin RZA, Tan LL, Chong KF, Heng LY. An electrochemical DNA biosensor fabricated from graphene decorated with graphitic nanospheres. *Nanotechnology.* 2020;31(48):485501.
20. Gibson T. Biosensors: The stabilité problem. *Analisis.* 1999 Sep 1;27:630–8.
21. Omidfar K, Ahmadi A, Syedmoradi L, Khoshfetrat SM, Larijani B. Point-of-care biosensors in medicine: a brief overview of our achievements in this field based on the conducted research in EMRI (endocrinology and metabolism research Institute of Tehran University of medical sciences) over the past fourteen years. *J Diabetes Metab Disord.* 2020;
22. Puiu M, Bala C. Microfluidics-integrated biosensing platforms as emergency tools for on-site field detection of foodborne pathogens. *TrAC Trends Anal Chem.* 2020;125:115831.
23. Brena B, González-Pombo P, Batista-Viera F. Immobilization of Enzymes: A Literature Survey BT - Immobilization of Enzymes and Cells: Third Edition. In: Guisan JM, editor. Totowa, NJ: Humana Press; 2013. p. 15–31.
24. Faccio G. From Protein Features to Sensing Surfaces. Vol. 18, *Sensors.* 2018.
25. Rashid JIA, Yusof NA. The strategies of DNA immobilization and hybridization detection mechanism in the construction of electrochemical DNA sensor: A review. *Sens Bio-Sensing Res.* 2017;16:19–31.
26. Smith S, Goodge K, Delaney M, Struzyk A, Tansey N, Frey M. A Comprehensive Review of the Covalent Immobilization of Biomolecules onto Electrospun Nanofibers. *Nanomater (Basel, Switzerland).* 2020 Oct;10(11).

27. Jesionowski T, Zdarta J, Krajewska B. Enzyme immobilization by adsorption: a review. *Adsorption*. 2014;20(5):801–21.
28. Silva RR, Avelino KYPS, Ribeiro KL, Franco OL, Oliveira MDL, Andrade CAS. Chemical immobilization of antimicrobial peptides on biomaterial surfaces. *FBS*. 2016;8(1):129–42.
29. Wang T-F, Lo H-F, Chi M-C, Lai K-L, Lin M-G, Lin L-L. Affinity Immobilization of a Bacterial Prolidase onto Metal-Ion-Chelated Magnetic Nanoparticles for the Hydrolysis of Organophosphorus Compounds. Vol. 20, *International Journal of Molecular Sciences*. 2019.
30. Materón EM, Gómez FR, Almeida MB, Shimizu FM, Wong A, Teodoro KBR, et al. Colorimetric Detection of SARS-CoV-2 Using Plasmonic Biosensors and Smartphones. *ACS Appl Mater Interfaces*. 2022 Dec 14;14(49):54527–38.
31. Liu Y, Li B. Label-free fluorescent detection of DNA sequence based on interaction of brilliant green with double-stranded DNA. *Microchim Acta*. 2010;171(3):349–54.
32. Santhanam M, Algov I, Alfonta L. DNA/RNA Electrochemical Biosensing Devices a Future Replacement of PCR Methods for a Fast Epidemic Containment. Vol. 20, *Sensors*. 2020.
33. Willner II, Katz E. Integration of Layered Redox Proteins and Conductive Supports for Bioelectronic Applications. *Angew Chem Int Ed Engl*. 2000 Apr;39(7):1180–218.
34. Naresh V, Lee N. A Review on Biosensors and Recent Development of Nanostructured Materials-Enabled Biosensors. *Sensors (Basel)*. 2021 Feb;21(4).
35. Asal M, Özen Ö, Şahinler M, Baysal HT, Polatoğlu İ. An overview of biomolecules, immobilization methods and support materials of biosensors. *Sens Rev*. 2019 Jan 1;39(3):377–86.
36. Habimana J de D, Ji J, Sun X. Minireview: Trends in Optical-Based Biosensors for Point-Of-Care Bacterial Pathogen Detection for Food Safety and Clinical Diagnostics. *Anal Lett*. 2018 Dec 12;51(18):2933–66.
37. Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme Microb Technol*. 2007;40(6):1451–63.
38. Mohamad NR, Marzuki NHC, Buang NA, Huyop F, Wahab RA. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnol Biotechnol Equip*. 2015 Mar;29(2):205–20.
39. Zhu D, Liu J, Tang Y, Xing D. A reusable DNA biosensor for the detection of genetically

- modified organism using magnetic bead-based electrochemiluminescence. *Sensors Actuators B Chem.* 2010;149(1):221–5.
40. Kamel S, A. Khattab T. Recent Advances in Cellulose-Based Biosensors for Medical Diagnosis. Vol. 10, *Biosensors*. 2020.
 41. Yuhana Ariffin E, Heng LY, Tan LL, Abd Karim NH, Hasbullah SA. A Highly Sensitive Impedimetric DNA Biosensor Based on Hollow Silica Microspheres for Label-Free Determination of *E. coli*. Vol. 20, *Sensors*. 2020.
 42. Bedi N, Srivastava DK, Srivastava A, Mahapatra S, Dkhar DS, Chandra P, et al. Marine biological macromolecules as matrix material for biosensor fabrication. *Biotechnol Bioeng.* 2022 Aug 1;119(8):2046–63.
 43. Dhand C, Das M, Datta M, Malhotra BD. Recent advances in polyaniline based biosensors. *Biosens Bioelectron.* 2011;26(6):2811–21.
 44. Lakard B, Herlem G, Lakard S, Antoniou A, Fahys B. Urea potentiometric biosensor based on modified electrodes with urease immobilized on polyethylenimine films. *Biosens Bioelectron.* 2004;19(12):1641–7.
 45. Ramanavičius A, Kaušaitė A, Ramanavičienė A. Polypyrrole-coated glucose oxidase nanoparticles for biosensor design. *Sensors Actuators B Chem.* 2005;111–112:532–9.
 46. Huang L, Liu S, Ren S, Zhang M, Wang T, Wang X, et al. Magnetic Relaxation Switch Biosensors Based on Self-Assembly of Polystyrene Microspheres and Magnetic Nanoparticles for Detection of Bisphenol A. *ACS Appl Nano Mater.* 2021 Jun 25;4(6):5963–71.
 47. Movilli J, Rozzi A, Ricciardi R, Corradini R, Huskens J. Control of Probe Density at DNA Biosensor Surfaces Using Poly(l-lysine) with Appended Reactive Groups. *Bioconjug Chem.* 2018 Dec 19;29(12):4110–8.
 48. Chen L, Liu X, Chen C. Impedimetric biosensor modified with hydrophilic material of tannic acid/polyethylene glycol and dopamine-assisted deposition for detection of breast cancer-related BRCA1 gene. *J Electroanal Chem.* 2017;791:204–10.
 49. Zucca P, Sanjust E. Inorganic materials as supports for covalent enzyme immobilization: methods and mechanisms. *Molecules.* 2014 Sep;19(9):14139–94.
 50. Nguyen HH, Kim and M. An Overview of Techniques in Enzyme Immobilization. *Appl Sci Converg Technol.* 2017 Nov;26(6):157–63.
 51. Hermanson GT. *Bioconjugate techniques*. Academic press; 2013.
 52. Wu S, Proft T. The use of sortase-mediated ligation for the immobilisation of bacterial adhesins onto fluorescence-labelled microspheres: a novel approach to analyse bacterial

- adhesion to host cells. *Biotechnol Lett.* 2010;32(11):1713–8.
53. Guo X, Wu Z, Guo Z. New Method for Site-Specific Modification of Liposomes with Proteins Using Sortase A-Mediated Transpeptidation. *Bioconj Chem.* 2012 Mar 21;23(3):650–5.
54. Kuropka B, Royla N, Freund C, Krause E. Sortase A mediated site-specific immobilization for identification of protein interactions in affinity purification–mass spectrometry experiments. *Proteomics.* 2015 Apr 1;15(7):1230–4.
55. Babacan S, Pivarnik P, Letcher S, Rand AG. Evaluation of antibody immobilization methods for piezoelectric biosensor application. *Biosens Bioelectron.* 2000;15(11):615–21.
56. Migneault I, Dartiguenave C, Bertrand MJ, Waldron KC. Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques.* 2004 Nov;37(5):790-796,798-802.
57. Lainioti GC, Savva P, Druvari D, Avramidis P, Panagiotaras D, Karellou EIE, et al. Cross-linking of antimicrobial polymers with hexamethylene diamine to prevent biofouling in marine applications. *Prog Org Coatings.* 2021;157:106336.
58. Xu J, Singh A, Amiji MM. Redox-responsive targeted gelatin nanoparticles for delivery of combination wt-p53 expressing plasmid DNA and gemcitabine in the treatment of pancreatic cancer. *BMC Cancer.* 2014;14(1):75.
59. Tian H, Cao L, Xu G, Liang Y, Yang H, Zhu J, et al. Investigation of Novel Thermosetting Copolymer-Based Monomethylolurea–Glyoxal for Wood Manufacturing. *ACS Omega.* 2022 Oct 4;7(39):35055–62.
60. Shah S, Sharma A, Gupta MN. Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. *Anal Biochem.* 2006 Apr;351(2):207–13.
61. Saleemuddin M. Bioaffinity Based Immobilization of Enzymes BT - Thermal Biosensors, Bioactivity, Bioaffinity. In: Bhatia PK, Danielsson B, Gemeiner P, Grabley S, Lammers F, Mukhopadhyay A, et al., editors. Berlin, Heidelberg: Springer Berlin Heidelberg; 1999. p. 203–26.
62. Sardar M, Mirza K. Chapter 12 - Affinity immobilization and affinity layers. In: Ferreira MLBT-BI, editor. Foundations and Frontiers in Enzymology. Academic Press; 2023. p. 269–90.
63. Delgadillo RF, Mueser TC, Zaleta-Rivera K, Carnes KA, González-Valdez J, Parkhurst LJ. Detailed characterization of the solution kinetics and thermodynamics of biotin, biocytin and HABA binding to avidin and streptavidin. *PLoS One.*

- 2019;14(2):e0204194.
64. Grunwald C. A Brief Introduction to the Streptavidin-Biotin System and its Usage in Modern Surface Based Assays. 2008;222(5–6):789–821.
 65. Sut TN, Park H, Koo DJ, Yoon BK, Jackman JA. Distinct Binding Properties of Neutravidin and Streptavidin Proteins to Biotinylated Supported Lipid Bilayers: Implications for Sensor Functionalization. Vol. 22, Sensors. 2022.
 66. Lee H, Rho J, Messersmith PB. Facile Conjugation of Biomolecules onto Surfaces via Mussel Adhesive Protein Inspired Coatings. *Adv Mater*. 2009 Jan 26;21(4):431–4.
 67. Rusmini F, Zhong Z, Feijen J. Protein Immobilization Strategies for Protein Biochips. *Biomacromolecules*. 2007 Jun 1;8(6):1775–89.
 68. Holland-Nell K, Beck-Sickinger AG. Specifically Immobilised Aldo/Keto Reductase AKR1A1 Shows a Dramatic Increase in Activity Relative to the Randomly Immobilised Enzyme. *ChemBioChem*. 2007 Jun 18;8(9):1071–6.
 69. Steen Redeker E, Ta DT, Cortens D, Billen B, Guedens W, Adriaenssens P. Protein Engineering For Directed Immobilization. *Bioconjug Chem*. 2013 Nov 20;24(11):1761–77.
 70. Aktas GB, Ribera A, Skouridou V, Masip L. DNA immobilization and detection using DNA binding proteins. *Anal Bioanal Chem*. 2021 Mar;413(7):1929–39.
 71. Babaei A, Pouremamali A, Rafiee N, Sohrabi H, Mokhtarzadeh A, de la Guardia M. Genosensors as an alternative diagnostic sensing approaches for specific detection of virus species: A review of common techniques and outcomes. *TrAC Trends Anal Chem*. 2022;155:116686.
 72. Hua Y, Ma J, Li D, Wang R. DNA-Based Biosensors for the Biochemical Analysis: A Review. *Biosensors*. 2022 Mar;12(3).
 73. Wang J. Electrochemical biosensors: towards point-of-care cancer diagnostics. *Biosens Bioelectron*. 2006 Apr;21(10):1887–92.
 74. Wang Q, Wang J, Huang Y, Du Y, Zhang Y, Cui Y, et al. Development of the DNA-based biosensors for high performance in detection of molecular biomarkers: More rapid, sensitive, and universal. *Biosens Bioelectron*. 2022 Feb;197:113739.
 75. Kuralay F, Erdem A. DNA Biosensors BT - Environmental Analysis by Electrochemical Sensors and Biosensors: Fundamentals. In: Moretto LM, Kalcher K, editors. New York, NY: Springer New York; 2014. p. 313–30.
 76. Peltomaa R, Glahn-Martínez B, Benito-Peña E, Moreno-Bondi MC. Optical Biosensors for Label-Free Detection of Small Molecules. *Sensors (Basel)*. 2018 Nov;18(12).

77. Zhao VXT, Wong TI, Zheng XT, Tan YN, Zhou X. Colorimetric biosensors for point-of-care virus detections. *Mater Sci energy Technol.* 2020;3:237–49.
78. Soliman H, El-Matbouli M. An inexpensive and rapid diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification. *Virol J.* 2005;2(1):83.
79. Wastling SL, Picozzi K, Kakembo ASL, Welburn SC. LAMP for human African trypanosomiasis: a comparative study of detection formats. *PLoS Negl Trop Dis.* 2010;4(11):e865.
80. Li Y, Liu S, Deng Q, Ling L. A sensitive colorimetric DNA biosensor for specific detection of the HBV gene based on silver-coated glass slide and G-quadruplex-hemin DNase. *J Med Virol.* 2018 Apr;90(4):699–705.
81. Porras JC, Bernuz M, Marfa J, Pallares-Rusiñol A, Martí M, Pividori MI. Comparative Study of Gold and Carbon Nanoparticles in Nucleic Acid Lateral Flow Assay. Vol. 11, *Nanomaterials.* 2021.
82. Kim S-K, Oh Y-H, Ko D-H, Sung H, Oh H-B, Hwang S-H. Nanoparticle-Based Visual Detection of Amplified DNA for Diagnosis of Hepatitis C Virus. Vol. 12, *Biosensors.* 2022.
83. Han J, Wu J, Du J. Fluorescent DNA Biosensor for Single-Base Mismatch Detection Assisted by Cationic Comb-Type Copolymer. *Molecules.* 2019 Feb;24(3).
84. Xu Y, Liu Y, Wu Y, Xia X, Liao Y, Li Q. Fluorescent Probe-Based Lateral Flow Assay for Multiplex Nucleic Acid Detection. *Anal Chem.* 2014 Jun 17;86(12):5611–4.
85. Podder A, Lee HJ, Kim BH. Fluorescent nucleic acid systems for biosensors. *Bull Chem Soc Jpn.* 2021;94(3):1010–35.
86. Kim Y, Sohn D, Tan W. Molecular beacons in biomedical detection and clinical diagnosis. *Int J Clin Exp Pathol.* 2008 Jan;1(2):105–16.
87. Mhlanga MM, Malmberg L. Using Molecular Beacons to Detect Single-Nucleotide Polymorphisms with Real-Time PCR. *Methods.* 2001;25(4):463–71.
88. Patching SG. Surface plasmon resonance spectroscopy for characterisation of membrane protein–ligand interactions and its potential for drug discovery. *Biochim Biophys Acta - Biomembr.* 2014;1838(1, Part A):43–55.
89. Poltronieri P, Mezzolla V, Primiceri E, Maruccio G. Biosensors for the Detection of Food Pathogens. Vol. 3, *Foods.* 2014. p. 511–26.
90. Gong W, Jiang S, Li Z, Li C, Xu J, Pan J, et al. Experimental and theoretical investigation for surface plasmon resonance biosensor based on graphene/Au film/D-POF. *Opt*

- Express. 2019;27(3):3483–95.
91. Liu A, Wang K, Weng S, Lei Y, Lin L, Chen W, et al. Development of electrochemical DNA biosensors. *TrAC Trends Anal Chem.* 2012;37:101–11.
 92. Wu Q, Zhang Y, Yang Q, Yuan N, Zhang W. Review of Electrochemical DNA Biosensors for Detecting Food Borne Pathogens. *Sensors (Basel).* 2019 Nov;19(22).
 93. Grieshaber D, MacKenzie R, Vörös J, Reimhult E. Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors (Basel).* 2008 Mar;8(3):1400–58.
 94. Chen Y, Guo S, Zhao M, Zhang P, Xin Z, Tao J, et al. Amperometric DNA biosensor for *Mycobacterium tuberculosis* detection using flower-like carbon nanotubes-polyaniline nanohybrid and enzyme-assisted signal amplification strategy. *Biosens Bioelectron.* 2018;119:215–20.
 95. Cosio MS, Scampicchio M, Benedetti S. Chapter 8 - Electronic Noses and Tongues. In: Picó YBT-CA of FT and A, editor. Boston: Academic Press; 2012. p. 219–47.
 96. Numnuam A, Chumbimuni-Torres KY, Xiang Y, Bash R, Thavarungkul P, Kanatharana P, et al. Potentiometric Detection of DNA Hybridization. *J Am Chem Soc.* 2008 Jan 1;130(2):410–1.
 97. Daniels JS, Pourmand N. Label-Free Impedance Biosensors: Opportunities and Challenges. *Electroanalysis.* 2007 Jun 1;19(12):1239–57.
 98. Pohanka M. Overview of Piezoelectric Biosensors, Immunosensors and DNA Sensors and Their Applications. *Mater (Basel, Switzerland).* 2018 Mar;11(3).
 99. Fathizadeh S, Behnia S. Control of a DNA Based Piezoelectric Biosensor. *J Phys Soc Japan.* 2020 Jan 29;89(2):24004.
 100. Minunni M, Tombelli S, Pratesi S, Mascini M, Piatti P, Bogani P, et al. A PIEZOELECTRIC AFFINITY BIOSENSOR FOR GENETICALLY MODIFIED ORGANISMS (GMOs) DETECTION. *Anal Lett.* 2001 Apr 30;34(6):825–40.
 101. Tombelli S, Mascini M, Sacco C, Turner APF. A DNA piezoelectric biosensor assay coupled with a polymerase chain reaction for bacterial toxicity determination in environmental samples. *Anal Chim Acta.* 2000;418(1):1–9.
 102. Lucarelli F, Tombelli S, Minunni M, Marrazza G, Mascini M. Electrochemical and piezoelectric DNA biosensors for hybridisation detection. *Anal Chim Acta.* 2008;609(2):139–59.
 103. Narita F, Wang Z, Kurita H, Li Z, Shi Y, Jia Y, et al. A Review of Piezoelectric and Magnetostrictive Biosensor Materials for Detection of COVID-19 and Other Viruses. *Adv Mater.* 2021 Jan 1;33(1):2005448.

104. Wang J. From DNA biosensors to gene chips. *Nucleic Acids Res.* 2000 Aug;28(16):3011–6.
105. Malecka K, Michalczuk L, Radecka H, Radecki J. Ion-Channel Genosensor for the Detection of Specific DNA Sequences Derived from Plum Pox Virus in Plant Extracts. Vol. 14, *Sensors*. 2014. p. 18611–24.
106. Teengam P, Siangproh W, Tuantranont A, Vilaivan T, Chailapakul O, Henry CS. Multiplex Paper-Based Colorimetric DNA Sensor Using Pyrrolidinyl Peptide Nucleic Acid-Induced AgNPs Aggregation for Detecting MERS-CoV, MTB, and HPV Oligonucleotides. *Anal Chem.* 2017 May 16;89(10):5428–35.
107. Giannico R, Forlani L, Andrioletti V, Cotroneo E, Termine A, Fabrizio C, et al. NIPAT as Non-Invasive Prenatal Paternity Testing Using a Panel of 861 SNVs. *Genes (Basel)*. 2023;14(2).
108. Ruppert KM, Kline RJ, Rahman MS. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Glob Ecol Conserv.* 2019;17:e00547.
109. Alahi MEE, Mukhopadhyay SC. Detection Methodologies for Pathogen and Toxins: A Review. *Sensors (Basel)*. 2017 Aug;17(8).
110. Xiang L, Wu H, Cui Z, Tang J. Indirect Competitive Aptamer-Based Enzyme-Linked Immunosorbent Assay (apt-ELISA) for the Specific and Sensitive Detection of Isocarbophos Residues. *Anal Lett.* 2019 Aug 13;52(12):1966–75.
111. Xiang Y, Zhu X, Huang Q, Zheng J, Fu W. Real-time monitoring of mycobacterium genomic DNA with target-primed rolling circle amplification by a Au nanoparticle-embedded SPR biosensor. *Biosens Bioelectron.* 2015;66:512–9.
112. Leonardo S, Toldrà A, Campàs M. Biosensors Based on Isothermal DNA Amplification for Bacterial Detection in Food Safety and Environmental Monitoring. Vol. 21, *Sensors*. 2021.
113. Bukyya JL, Tejasvi MLA, Avinash A, P CH, Talwade P, Afroz MM, et al. DNA Profiling in Forensic Science: A Review. *Glob Med Genet.* 2021 Dec;8(4):135–43.
114. Yáñez-Sedeño P, Agüí L, Campuzano S, Pingarrón JM. What Electrochemical Biosensors Can Do for Forensic Science? Unique Features and Applications. Vol. 9, *Biosensors*. 2019.
115. Mullis KB, Faloona FABT-M in E. [21] Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. In: *Recombinant DNA Part F*. Academic Press; 1987. p. 335–50.

116. Oliveira BB, Veigas B, Baptista PV. Isothermal amplification of nucleic acids: The race for the next “gold standard.” *Front Sensors*. 2021;2:752600.
117. Deiman B, van Aarle P, Sillekens P. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). *Mol Biotechnol*. 2002;20(2):163–79. Available from: <https://doi.org/10.1385/MB:20:2:163>
118. Soroka M, Wasowicz B, Rymaszewska A. Loop-Mediated Isothermal Amplification (LAMP): The Better Sibling of PCR? Vol. 10, *Cells*. 2021.
119. Hellyer TJ, Nadeau JG. Strand displacement amplification: a versatile tool for molecular diagnostics. *Expert Rev Mol Diagn*. 2004;4(2):251–61.
120. Lobato IM, O’Sullivan CK. Recombinase polymerase amplification: Basics, applications and recent advances. *TrAC Trends Anal Chem*. 2018;98:19–35.
121. Ali MM, Li F, Zhang Z, Zhang K, Kang D-K, Ankrum JA, et al. Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine. *Chem Soc Rev*. 2014;43(10):3324–41.
122. Marín S, Merkoçi A. Electrochemical Detection of DNA Using Nanomaterials Based Sensors. In: *Detection of Non-Amplified Genomic DNA*. Springer; 2012. p. 185–201.
123. Wu J, Lv J, Zheng X, Wu Z-S. Hybridization chain reaction and its applications in biosensing. *Talanta*. 2021;234:122637.
124. Cozzolino F, Iacobucci I, Monaco V, Monti M. Protein–DNA/RNA Interactions: An Overview of Investigation Methods in the -Omics Era. *J Proteome Res*. 2021 Jun 4;20(6):3018–30.
125. Ferraz RAC, Lopes ALG, da Silva JAF, Moreira DFV, Ferreira MJN, de Almeida Coimbra SV. DNA–protein interaction studies: a historical and comparative analysis. *Plant Methods*. 2021;17(1):1–21.
126. Siggers T, Gordân R. Protein–DNA binding: complexities and multi-protein codes. *Nucleic Acids Res*. 2014;42(4):2099–111.
127. Leng M, Felsenfeld G. The preferential interactions of polylysine and polyarginine with specific base sequences in DNA. *Proc Natl Acad Sci*. 1966;56(4):1325–32.
128. Seeman NC, Rosenberg JM, Rich A. Sequence-specific recognition of double helical nucleic acids by proteins. *Proc Natl Acad Sci*. 1976;73(3):804–8.
129. McKay DB, Steitz TA. Structure of catabolite gene activator protein at 2.9 Å resolution suggests binding to left-handed B-DNA. *Nature*. 1981;290(5809):744–9.
130. Hudson WH, Ortlund EA. The structure, function and evolution of proteins that bind DNA and RNA. *Nat Rev Mol cell Biol*. 2014;15(11):749–60.

131. Monico C, Capitanio M, Belcastro G, Vanzi F, Pavone FS. Optical Methods to Study Protein-DNA Interactions in Vitro and in Living Cells at the Single-Molecule Level. Vol. 14, International Journal of Molecular Sciences. 2013. p. 3961–92.
132. Segal DJ, Barbas CF. Design of novel sequence-specific DNA-binding proteins. *Curr Opin Chem Biol.* 2000;4(1):34–9.
133. Tachikawa K, Schröder O, Frey G, Briggs SP, Sera T. Regulation of the endogenous VEGF-A gene by exogenous designed regulatory proteins. *Proc Natl Acad Sci.* 2004;101(42):15225–30.
134. Mino T, Mori T, Aoyama Y, Sera T. Cell-permeable artificial zinc-finger proteins as potent antiviral drugs for human papillomaviruses. *Arch Virol.* 2008;153:1291–8.
135. Luscombe NM, Austin SE, Berman HM, Thornton JM. An overview of the structures of protein-DNA complexes. *Genome Biol.* 2000;1(1):REVIEWS001.
136. Maston GA, Evans SK, Green MR. Transcriptional Regulatory Elements in the Human Genome. *Annu Rev Genomics Hum Genet.* 2006 Sep 1;7(1):29–59.
137. Wang G, Wang F, Huang Q, Li Y, Liu Y, Wang Y. Understanding Transcription Factor Regulation by Integrating Gene Expression and DNase I Hypersensitive Sites. *Biomed Res Int.* 2015;2015:757530.
138. Chen J. The Cell-Cycle Arrest and Apoptotic Functions of p53 in Tumor Initiation and Progression. *Cold Spring Harb Perspect Med.* 2016 Mar;6(3):a026104.
139. Fuentes N, Silveyra P. Estrogen receptor signaling mechanisms. *Adv Protein Chem Struct Biol.* 2019;116:135–70.
140. Ohlendorf DH, Tronrud DE, Matthews BW. Refined structure of Cro repressor protein from bacteriophage lambda suggests both flexibility and plasticity. *J Mol Biol.* 1998 Jul;280(1):129–36.
141. Pabo CO, Sauer RT. PROTEIN-DNA RECOGNITION. *Annu Rev Biochem.* 1984 Jun 1;53(1):293–321.
142. Albright RA, Matthews BW. Crystal structure of lambda-Cro bound to a consensus operator at 3.0 Å resolution. *J Mol Biol.* 1998 Jul;280(1):137–51.
143. Jana R, Hazbun TR, Mollah AK, Mossing MC. A folded monomeric intermediate in the formation of lambda Cro dimer-DNA complexes. *J Mol Biol.* 1997 Oct;273(2):402–16.
144. Takeda Y, Sarai A, Rivera VM. Analysis of the sequence-specific interactions between Cro repressor and operator DNA by systematic base substitution experiments. *Proc Natl Acad Sci U S A.* 1989 Jan;86(2):439–43.
145. Brennan RG, Roderick SL, Takeda Y, Matthews BW. Protein-DNA conformational

- changes in the crystal structure of a lambda Cro-operator complex. *Proc Natl Acad Sci U S A*. 1990 Oct;87(20):8165–9.
146. Jana R, Hazbun TR, Fields JD, Mossing MC. Single-Chain Lambda Cro Repressors Confirm High Intrinsic Dimer–DNA Affinity. *Biochemistry*. 1998 May 1;37(18):6446–55.
 147. Camenisch TD, Brilliant MH, Segal DJ. Critical parameters for genome editing using zinc finger nucleases. *Mini Rev Med Chem*. 2008 Jun;8(7):669–76.
 148. Klug A, Schwabe JW. Protein motifs 5. Zinc fingers. *FASEB J Off Publ Fed Am Soc Exp Biol*. 1995 May;9(8):597–604.
 149. Kim M-S, Stybayeva G, Lee JY, Revzin A, Segal DJ. A zinc finger protein array for the visual detection of specific DNA sequences for diagnostic applications. *Nucleic Acids Res*. 2011 Mar 1;39(5):e29–e29.
 150. Son S-H, Son Y-E, Cho H-J, Chen W, Lee M-K, Kim L-H, et al. Homeobox proteins are essential for fungal differentiation and secondary metabolism in *Aspergillus nidulans*. *Sci Rep*. 2020;10(1):6094.
 151. Banerjee-Basu S, Baxevanis AD. Molecular evolution of the homeodomain family of transcription factors. *Nucleic Acids Res*. 2001 Aug;29(15):3258–69.
 152. Laissue P. The forkhead-box family of transcription factors: key molecular players in colorectal cancer pathogenesis. *Mol Cancer*. 2019;18(1):5.
 153. Mariño-Ramírez L, Kann MG, Shoemaker BA, Landsman D. Histone structure and nucleosome stability. *Expert Rev Proteomics*. 2005 Oct;2(5):719–29.
 154. Henikoff S, Smith MM. Histone variants and epigenetics. *Cold Spring Harb Perspect Biol*. 2015 Jan;7(1):a019364.
 155. Anuchin AM, Goncharenko A V, Demidenok OI, Kaprel'iants AS. [Histone-like proteins of bacteria (review)]. *Prikl Biokhim Mikrobiol*. 2011;47(6):635–41.
 156. Dorman CJ, Deighan P. Regulation of gene expression by histone-like proteins in bacteria. *Curr Opin Genet Dev*. 2003 Apr;13(2):179–84.
 157. Pettijohn DE. Histone-like proteins and bacterial chromosome structure. *J Biol Chem*. 1988;263(26):12793–6.
 158. Kamashev D, Rouviere-Yaniv J. The histone-like protein HU binds specifically to DNA recombination and repair intermediates. *EMBO J*. 2000;19(23):6527–35.
 159. Kamashev D, Balandina A, Rouviere-Yaniv J. The binding motif recognized by HU on both nicked and cruciform DNA. *EMBO J*. 1999 Oct;18(19):5434–44.
 160. Luijsterburg MS, Noom MC, Wuite GJL, Dame RT. The architectural role of nucleoid-

- associated proteins in the organization of bacterial chromatin: a molecular perspective. *J Struct Biol.* 2006 Nov;156(2):262–72.
161. Shlyakhtenko LS, Lushnikov AY, Miyagi A, Lyubchenko YL. Specificity of binding of single-stranded DNA-binding protein to its target. *Biochemistry.* 2012 Feb;51(7):1500–9.
 162. Antosch M, Mortensen SA, Grasser KD. Plant proteins containing high mobility group box DNA-binding domains modulate different nuclear processes. *Plant Physiol.* 2012 Jul;159(3):875–83.
 163. Sheldon EL, Nagainis PA, Kung VT. Detection of total DNA with single-stranded DNA binding protein conjugates. *Biochem Biophys Res Commun.* 1989;165(1):474–80.
 164. Kung VT, Panfili PR, Sheldon EL, King RS, Nagainis PA, Gomez B, et al. Picogram quantitation of total DNA using DNA-binding proteins in a silicon sensor-based system. *Anal Biochem.* 1990;187(2):220–7.
 165. Berezovski M, Krylov SN. Using DNA-Binding Proteins as an Analytical Tool. *J Am Chem Soc.* 2003 Nov 1;125(44):13451–4.
 166. Hedgethorpe K, Webb MR. Fluorescent SSB as a reagentless biosensor for single-stranded DNA. *Single-Stranded DNA Bind Proteins Methods Protoc.* 2012;219–33.
 167. Umer M, Aziz NB, Mahmudunnabi RG, Shim Y-B, Salomon C, Shiddiky MJA. A novel DNA binding protein-based platform for electrochemical detection of miRNA. *Analyst.* 2021;146(18):5496–501.
 168. Mahmudunnabi RG, Umer M, Seo K-D, Park D-S, Chung JH, Shiddiky MJA, et al. Exosomal microRNAs array sensor with a bioconjugate composed of p53 protein and hydrazine for the specific lung cancer detection. *Biosens Bioelectron.* 2022;207:114149.
 169. Aktas GB, Skouridou V, Masip L. Novel signal amplification approach for HRP-based colorimetric genosensors using DNA binding protein tags. *Biosens Bioelectron.* 2015;74:1005–10.
 170. Aktas GB, Skouridou V, Masip L. Nucleic acid sensing with enzyme-DNA binding protein conjugates cascade and simple DNA nanostructures. *Anal Bioanal Chem.* 2017;409:3623–32.
 171. Aktas GB, Skouridou V, Masip L. Sandwich-type aptasensor employing modified aptamers and enzyme-DNA binding protein conjugates. *Anal Bioanal Chem.* 2019;411:3581–9.
 172. Aktas GB, Wichers JH, Skouridou V, van Amerongen A, Masip L. Nucleic acid lateral flow assays using a conjugate of a DNA binding protein and carbon nanoparticles.

Mikrochim Acta. 2019 Jun;186(7):426.

173. Miao Z, De Buck J. Biosensor for PCR amplicons by combining split trehalase and DNA-binding proteins: A proof of concept study. J Microbiol Methods. 2023;211:106780.

Chapter 2: Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*

Harmful Algae, Volume 110, December 2021, 102135

2.1 Abstract

The proliferation of harmful microalgae endangers aquatic ecosystems and can have serious economic implications on a global level. Harmful microalgae and their associated toxins also pose a threat to human health since they can cause seafood-borne diseases such as ciguatera. Implementation of DNA-based molecular methods together with appropriate detection strategies in monitoring programs can support the efforts for effective prevention of potential outbreaks. A PCR-lateral flow assay (PCR-LFA) in dipstick format was developed in this work for the detection of two *Gambierdiscus* species, *G. australes* and *G. excentricus*, which are known to produce highly potent neurotoxins known as ciguatoxins and have been associated with ciguatera outbreaks. Duplex PCR amplification of genomic DNA from strains of these species utilizing species-specific ssDNA tailed primers and a common primer containing the binding sequence of scCro DNA binding protein resulted in the generation of hybrid ssDNA-dsDNA amplicons. These were captured on the dipsticks via hybridization with complementary probes and detected with a scCro/carbon nanoparticle (scCro/CNPs) conjugate. The two different test zones on the dipsticks allowed the discrimination of the two species and the assay exhibited high sensitivity, 6.3 pg/ μ L of genomic DNA from both *G. australes* and *G. excentricus*. The specificity of the approach was also demonstrated using genomic DNA from non-target *Gambierdiscus* species and other microalgae genera which did not produce any signals. The possibility to use cells directly for amplification instead of purified genomic DNA suggested the compatibility of the approach with field sample testing. Future work is required to further explore the potential use of the strategy for on-site analysis and its applicability to other toxic species.

2.2 Introduction

Ciguatera fish poisoning (CFP) is a highly common seafood-borne disease (1) caused by the potent marine microalgal neurotoxins named ciguatoxins (CTXs) (2). Ciguatera has become a global health concern due to the severe symptoms elicited after intoxication, including cardiovascular, gastrointestinal and neurological alterations which might last a few days but can also persist for longer periods of time (3). Several species of the marine dinoflagellate genus named *Gambierdiscus* have been confirmed to produce CTXs (4-6). Ingestion of CTX-producing microalgae by herbivorous fish and subsequent bioaccumulation of CTXs facilitates their introduction into the food chain, which ultimately may reach humans. This genus of

microalgae is epibenthic and endemic of tropical and subtropical waters such as the Pacific and Indian Ocean or the Caribbean Sea (1, 5, 7). However, their presence has expanded to non-endemic areas. Recently, Tester *et al.* reviewed the global distribution of the genus during the last decade or so (2009 – 2018), underlying zones in which the amount and diversity of *Gambierdiscus* species is higher, such as the French Polynesia, Caribbean coasts and Canary Islands (Spain) (8). In fact, several *Gambierdiscus* species have been reported during the last years in the Canary Islands (9, 10), the Madeira archipelago (Portugal) (11) and the Mediterranean Sea (12, 13). *Gambierdiscus australes* and *G. excentricus* strains exhibiting high CTX-like toxicities have been recently found in the Canary Islands and are considered the dominant species associated with ciguatera outbreaks in this area (14).

Monitoring the presence of these microalgae and the toxins they produce in the marine environment is essential for implementing appropriate risk prevention strategies. A battery of methods has been developed for the direct detection of CTXs in fish and have been extensively reviewed elsewhere (15, 16). These include animal and cell-based toxicity assays, biochemical assays employing receptors and antibodies, as well as liquid chromatography coupled with mass spectrometry. However, the complex chemical structures and scarce availability of pure CTXs, the low concentration at which they are encountered, and the complexity of fish matrices make the development of highly sensitive and specific assays for CTXs detection very challenging (15, 16). Biosensors on the other hand, extensively used for the detection of various foodborne pathogens (17, 18), can offer the required specificity and sensitivity when combined with highly specific biorecognition molecules. The recent generation of CTX-specific monoclonal antibodies using synthetic CTX fragments (19) has indeed allowed the development of sensitive sandwich-type immunoassays and biosensors for CTX detection in fish (20) and microalgal samples (21, 22).

Detection of harmful microalgae like the CTX-producing *Gambierdiscus* species based on molecular methods has also been introduced over the last years to implement the monitoring and containment efforts of harmful algae blooms (23). These methods are considered as easier, faster and more specific alternatives to the traditional light microscopy. They rely on the amplification of genomic DNA from the target species using specific primers in combination with colorimetric, fluorescent or electrochemical detection techniques. Even though the gold standard for amplification is PCR, isothermal amplification has also been reported (23). Nevertheless, few reports can be found in the literature exploiting molecular methods for the identification of *Gambierdiscus* species (24-29).

In this work, a nucleic acid lateral flow assay (LFA) in a dipstick format was developed for the duplex detection of two toxin-producing *Gambierdiscus* species, *G. australes* and *G. excentricus*. This is the first report of an LFA employed for the identification of *Gambierdiscus* species. It relies on PCR amplification of genomic DNA using specifically modified primers followed by detection with dipsticks. Two reverse primers were designed, each one modified with a distinct single-stranded DNA (ssDNA) tail to allow discrimination of the two species. The amplicons were captured on two separate test lines of the dipsticks via hybridization with capture probes complementary to these ssDNA tails. On the other hand, the common forward primer contained the binding sequence of scCro DNA binding protein to facilitate detection with scCro/carbon nanoparticles (scCro/CNPs) conjugate. Enzyme Linked Oligonucleotide Assay (ELONA) was used to demonstrate correct amplification of both targets before transferring the assay to its final LFA format. The performance of the approach in terms of sensitivity and specificity was evaluated. Genomic DNA of several microalgae genera, different combinations of genomic DNA from five *Gambierdiscus* and one *Fukuyoa* species, and DNA extracted and amplified directly from cells of the two target species, were used to test the potential applicability of the strategy on field samples.

2.3 Materials and methods

2.3.1 Materials

Phosphate buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4), DreamTaq DNA polymerase, neutravidin and neutravidin-coated microplate strip plates and Tween-20 were obtained from Fisher Scientific (Spain). The BioFX TMB Super Sensitive One Component HRP Microwell Substrate was from Surmodics (USA), the FF170HP nitrocellulose membrane from Cytiva (Spain), the C083 cellulose fiber absorbent pad and Empigen BB from Merck (Spain). HRP-scCro and scCro/CNPs conjugates were prepared as previously described (30, 31). Primers and DNA probes were purchased from Biomers.net (Germany) and their sequences are shown in Table 2.1.

Table 2.1 Oligonucleotides used in this work. The scCro DNA binding site is in bold and the DNA tails are in italics. The asterisk (*) denotes a biotin-TEG modification at the 3' end and the (#) a biotin modification at the 5' end.

Oligonucleotide	Sequence (5' – 3')
<i>G. australes</i> & <i>G. excentricus</i> forward primer	TATCACTTGCGGTGATATGCTGCATGYGGAGATTCTTTYTKG
<i>G. australes</i> reverse primer	<i>GTTTTCCAGTCACGAC</i> -C3-ATGCATAACTCTTCATTGCCAGTAG
<i>G. excentricus</i> reverse primer	<i>TCTACAGGCTCGTATATGTA</i> -C3-GCTTGGGTCACAGTGCAACAGAG
<i>G. australes</i> capture probe *	GTCGTGACTGGGAAAACCTTTTTTTTTTTTTTTT
<i>G. excentricus</i> capture probe *	TACATATACGAGCCTGTAGATTTTTTTT TTTTTT
NALFA control line probe #	AGTCCGTGGTAGGGCAGGTTGGGGTGACTTTTTTTTT TATCACCG CAAGTGATATTTTATCACTTGCGGTGATA

2.3.2 Expression and purification of scCro DNA binding protein

The scCro DNA binding protein was expressed using the aLICator LIC Cloning & Expression System (Thermo Fisher Scientific). The gene of scCro was amplified by PCR from a pET-based expression vector described previously (30) using forward (5'-AGA AGG AGA TAT AAC TAT GTG TGAACAACG CAT AAC TTT AAA AGA TT-3') and reverse (5'-GTG GTG GTG ATG GTG ATG GCC TGC TGT TGT TTT TTT GTT ACT CG-3') primers designed for ligation independent cloning in the pLATE31 vector according to the instructions of the manufacturer. The new expression vector was verified by DNA sequencing. The protein was purified by immobilized metal affinity chromatography (IMAC) followed by ion exchange chromatography (IEC) as described previously (30).

2.3.3 Genomic DNA from microalgae

The microalgae strains used in this work are shown in Table 2.2. Maintenance of the cultures and genomic DNA extraction from each strain were performed as in previous works (29). Briefly, cultures were maintained under a photon flux of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a

12:12-h light/dark regime and at $24 \pm 1^\circ\text{C}$. For DNA extraction, cell pellets were resuspended in lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), 1:8 volumes of 10 % (w/v) DTAB and 1 volume of chloroform. Subsequently, cellular disruption was achieved by adding zirconium beads (0.5 mm diameter) to the mixture and using a Bead Beater-8 (BioSpec, USA). Then, DNA was extracted from the aqueous phase using standard phenol/chloroform method. Precipitation of the DNA was achieved by the addition of 2 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate (pH 8.0). Finally, DNA was rinsed with 70% (v/v) ethanol and then dissolved in 1:4 volumes of molecular DNase/RNase-free water. The concentration and purity of the extracted DNA were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Spain) and it was stored at -20°C until analysis. Each strain was analyzed individually or in mixtures.

Table 2.2 Microalgae strains used in this work.

Species	Strain	Origin	GenBank Accession
<i>Gambierdiscus australes</i>	IRTA-SMM-13-09	Hierro Island, Spain	KY564322
	IRTA-SMM-13-11	Selvagem Grande Island, Portugal	KY564324
	IRTA-SMM-16-286	Lanzarote Island, Spain	MT119197
<i>Gambierdiscus balechii</i>	VGO920	Manado, Indonesia	KX268469
<i>Gambierdiscus belizeanus</i>	IRTA-SMM-17-421	Hierro Island, Spain	MT379471
<i>Gambierdiscus caribaeus</i>	IRTA-SMM-17-03	Hierro Island, Spain	MT119203
<i>Gambierdiscus excentricus</i>	IRTA-SMM-17-407	Gomera Island, Spain	MT119200
	IRTA-SMM-17-428	Gomera Island, Spain	MT119201
	VGO791	Tenerife, Spain	JF303066; JF303075
<i>Fukuyoa paulensis</i>	VGO1185	Ubatuba, Brazil	KM886379
	IRTA-SMM-17-211	Menorca Island, Spain	MT119205
<i>Coolia monotis</i>	IRTA-SMM-16-285	Formentera Island, Spain	MW328563
<i>Ostreopsis c.f. ovata</i>	IRTA-SMM-16-133	Catalonia, Spain	MH790463
<i>Prorocentrum lima</i>	IRTA-SMM-17-47	Lanzarote Island, Spain	MW328564

2.3.4 PCR amplification of genomic DNA

The primers used for amplification of genomic DNA from *G. australes* and *G. excentricus* were designed within the D1-D3 region of the 28 S large subunit (LSU) ribosomal DNA (29) and are shown in Table 2.1. The strategy for duplex PCR amplification of the target *Gambierdiscus*

species with the modified primers is illustrated in Figure 2.1A. Each reverse primer was extended at its 5' end with a distinct ssDNA tail to allow the discrimination of the two species. The common forward primer for the two species was extended at its 5' end with the DNA binding site for the scCro DNA binding protein.

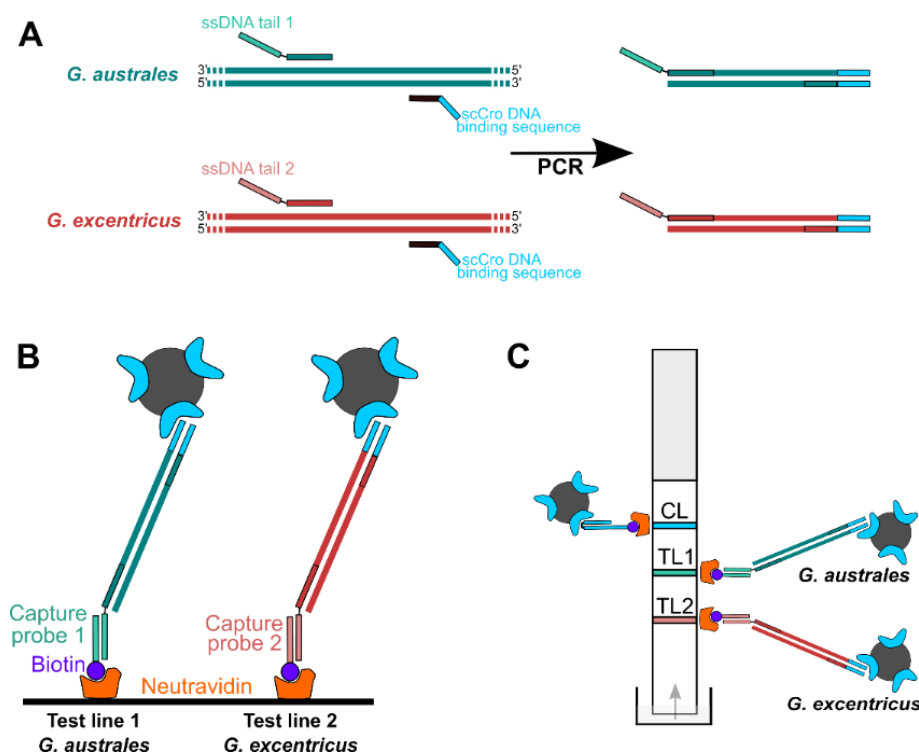


Figure 2.1 Strategy for the detection of the target *Gambierdiscus* species with the PCR-LFA dipstick. (A) PCR amplification of *G. australes* and *G. excentricus* genomic DNA using modified primers. (B) PCR amplicon detection by LFA. Capture of the ssDNA-dsDNA hybrid amplicons on the dipsticks by complementary probes and detection with scCro/CNPs conjugate. (C) Design of the dipsticks. CL: control line; TL1: test line 1 for *G. australes*; TL2: test line 2 for *G. excentricus*.

For simplex PCR, 200 nM of each primer were used whereas duplex PCR was performed using 200 nM of *G. australes* reverse primer, 300 nM of *G. excentricus* and 500 nM of the common forward primer for both *G. australes* and *G. excentricus*. Purified genomic DNA from each species was added to the PCR reactions at final concentrations of 400 pg/μL down to 400 fg/μL. PCR was performed using an initial denaturation step for 3 min at 95°C, 30 cycles of denaturation for 10 sec at 95°C, annealing for 10 sec at 58°C and extension of 10 sec at 72°C, and a final extension step for 5 min at 72°C. Strains *G. australes* IRTA-SMM-13-11 and *G.*

excentricus IRTA-SMM-17-407 were used in all experiments unless otherwise stated. The PCR reactions were analyzed by agarose gel (2.6 % w/v agarose in Tris/borate/EDTA buffer) electrophoresis.

2.3.5 Enzyme Linked Oligonucleotide Assay (ELONA) for colorimetric detection of PCR amplicons

A colorimetric ELONA was designed to verify the correct incorporation of the ssDNA tails and scCro DNA binding site to the *G. australes* and *G. excentricus* PCR amplicons. Several assay parameters were optimized in advance to achieve high sensitivity and specificity, and these included the concentrations of the biotinylated capture probes (50 – 200 nM) and the HRP-scCro conjugate (2.5 – 15 nM), the dilution factor of the PCR reactions (1/25 – 1/100) as well as the number of PCR cycles. The biotinylated capture probes (Table 2.1) specific for each species [50 µL of 50 nM in PBS with 0.05 % (v/v) Tween-20 (PBST)] were immobilized on separate wells of neutravidin-coated microplates for 15 min at room temperature. After washing (3 x 300 µL of PBST), 1 µL of PCR reaction was mixed with 49 µL of PBS and added to the wells for a 30-min incubation step. After washing, the HRP-scCro conjugate was added (50 µL of 15 nM in PBS) and the plate was incubated for another 30 min. After a final washing step, 50 µL of TMB ELISA substrate were added and signal generation was stopped after 10 min with the addition of equal volume of 1 M H₂SO₄. The absorbance was finally recorded at 450 nm. Target genomic DNA calibration curves were constructed using 1/50 diluted PCR reactions performed with serially two-fold diluted genomic DNA from each species (final concentrations of 8 pg/µL down to 8 fg/µL). The absorbance data was fitted to four-parameter logistic model using the GraphPad Prism software and the limits of detection (LOD) were interpolated from the curves as the blank signals (ntc) plus three times their standard deviation (ntc + 3xSD_{ntc}). Four replicates were prepared for each concentration examined.

2.3.6 Preparation of the LFA dipsticks

Nitrocellulose FF170HP membrane (height 3 cm) was used to prepare the dipsticks. The control line was constructed at 1.8 cm from the bottom of the membrane and separated from the two test lines by 0.4 cm and 0.8 cm. To facilitate ssDNA immobilization, each biotinylated probe was mixed with neutravidin in PBS at final concentrations of 35 µM and 8.3 µM, respectively. The mixtures were incubated for 15 min at ambient temperature and then

dispensed on the membrane using a Lateral Flow Reagent Dispenser (Gentaur, Belgium). *G. australes* and *G. excentricus* capture probes were used for the construction of the two test lines, whereas a hairpin probe forming the scCro dsDNA binding site was used for the control line (see Table 2.1 for sequences). The membranes were dried at room temperature for at least 2 h and then blocked for 30 min with 2% (w/v) skim milk and 0.1% (v/v) Empigen BB in 10 mM carbonate-bicarbonate buffer pH 9.4. The strips were assembled on backing cards with a 2 cm absorbent pad overlapping the nitrocellulose membrane by 2 mm to ensure correct wicking. Finally, the dipsticks were cut at a width of 4 mm using an Autokun Cutter (Hangzhou Autokun Technology, India), packaged in plastic pouches and stored at 4°C until use.

2.3.7 Detection of the PCR amplicons with the LFA dipsticks

The PCR amplicons were detected on the dipsticks using scCro/CNPs conjugate for carbon black signal (Figure 2.2B and 2.2C). The scCro/CNPs conjugate was prepared as previously described (31). The dipsticks were dipped vertically in the wells of a microtiter plate containing 10 µL of diluted PCR reaction, 1 µL of scCro/CNPs conjugate suspension (0.2% w/v) and 39 µL of PBS with 1% (w/v) skim milk and 0.1% (v/v) Tween-20. Black color signals were observed within 15 min and the dipsticks were dried and finally imaged by flatbed scanning. Each experiment was performed in triplicate for the construction of the calibration curves or at least in duplicate for other experiments. The volume of the PCR reaction used for analysis with the dipsticks was optimized. To this end, 0.5 – 10 µL of simplex PCR reaction was mixed with PBS to a final volume of 10 µL and used for analysis as detailed above.

2.3.8 Direct PCR amplification and LFD detection using *G. australes* and *G. excentricus* cells

The possibility of detecting the two *Gambierdiscus* species using directly cells instead of purified genomic DNA was also evaluated. Cell suspensions containing 40 cells/µL from each strain (*G. australes* IRTA-SMM-16-286 and *G. excentricus* VGO791) were prepared in milli-Q water and heated for 5 min at 95°C to promote lysis and release of genomic DNA. Crude cell extracts (2 µL) from each species were then added directly, individually or in a mixture, to PCR reactions containing primers for both strains to a final volume of 20 µL. Purified genomic DNA (100 pg/µL) was used for PCR in parallel as a control. PCR amplification was performed as

detailed in section 2.3 but with an extended initial denaturation step of 10 min at 95°C instead of 5 min. Amplicons were finally detected with the LFA dipsticks as described in section 2.6.

2.4 Results

2.4.1 Preparation of the recombinant scCro DNA binding protein

The scCro DNA binding protein with a C-terminal His-tag was expressed in *E. coli* BL21(DE3) cells using the new construct detailed in section 2.3.2. A representative SDS-PAGE gel of the two-step purification process (IMAC and then IEC) is shown in Figure 2.2. The use of the pLATE31 vector, which minimizes basal expression of the recombinant protein, resulted in the preparation of 12 mg purified protein per 1 L of bacterial culture, corresponding to a 6-fold improvement of protein yield compared to the pET-based expression system we reported previously.

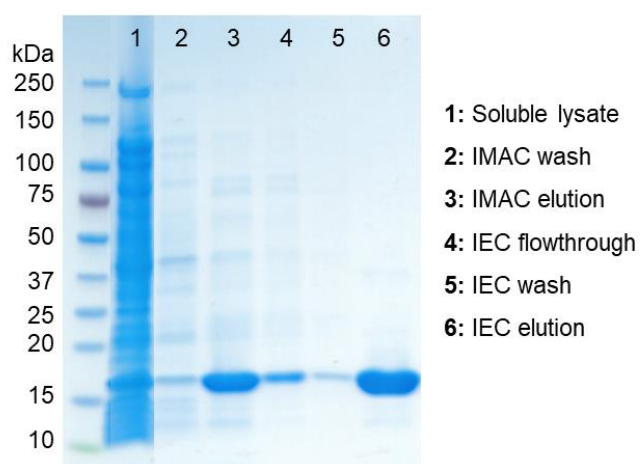


Figure 2.2 Expression of scCro from BL21(DE3)/pLATE31 and purification by IMAC and IEC.

2.4.2 Specificity of the primers and PCR amplicon detection by ELONA

Initially, the use of the designed primers for the amplification of various strains of *G. australes* and *G. excentricus* species was validated as shown in Figure 2.3. All the strains tested were successfully amplified using the modified primers.

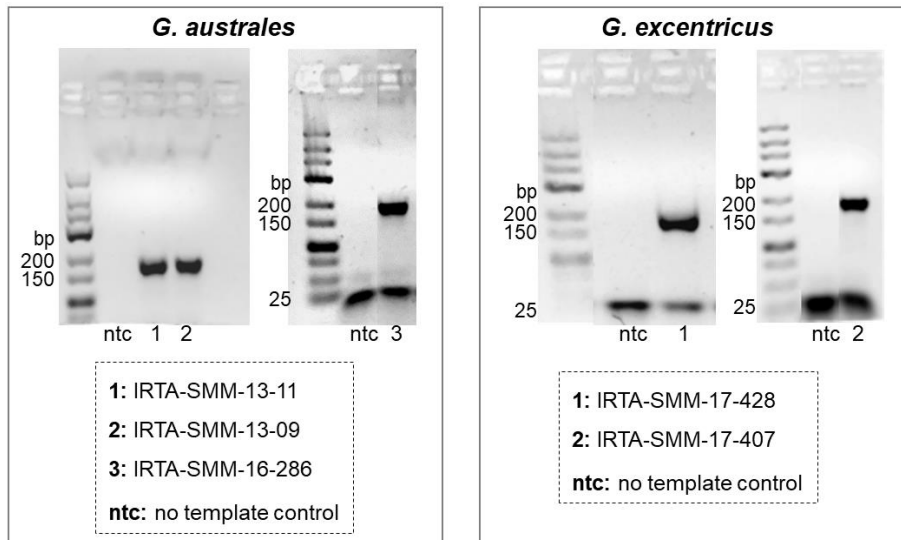


Figure 2.3 Amplification of genomic DNA from various *G. australes* and *G. excentricus* strains using the primers reported in this work.

Then, the specificity of the primers was evaluated with simplex PCR. DNA from each species was added to a PCR reaction containing only its corresponding primers, and after amplification, the reactions were analyzed by agarose gel electrophoresis. As shown in Figure 2.4A, only the specific genomic DNA was amplified with its corresponding primers whereas no amplification was observed for the other one.

Duplex PCR was then performed using a mixture of the two reverse primers and the common forward primer. Again, successful amplification for each strain was observed when only one of the targets was present (Figure 2.4B). Since the expected size of the two amplicons is very similar (approximately 150 bp), it is not possible to differentiate the two amplicons in the duplex PCR reaction solely by gel electrophoresis. Therefore, ELONA was performed using capture probes specific to each target, which were complementary to the ssDNA tails at one end of the generated PCR amplicons for each species. For colorimetric detection, the HRP-scCro conjugate was used to bind the dsDNA binding site of scCro formed at the other end of the amplicons after incorporation of the specific sequence in the common forward primer of the two species (Figure 2.4C). Correct amplification of each target genomic DNA in the duplex PCR reaction was verified when added individually or simultaneously whereas no signal was observed when the non-specific capture probe was used (Figure 2.4D).

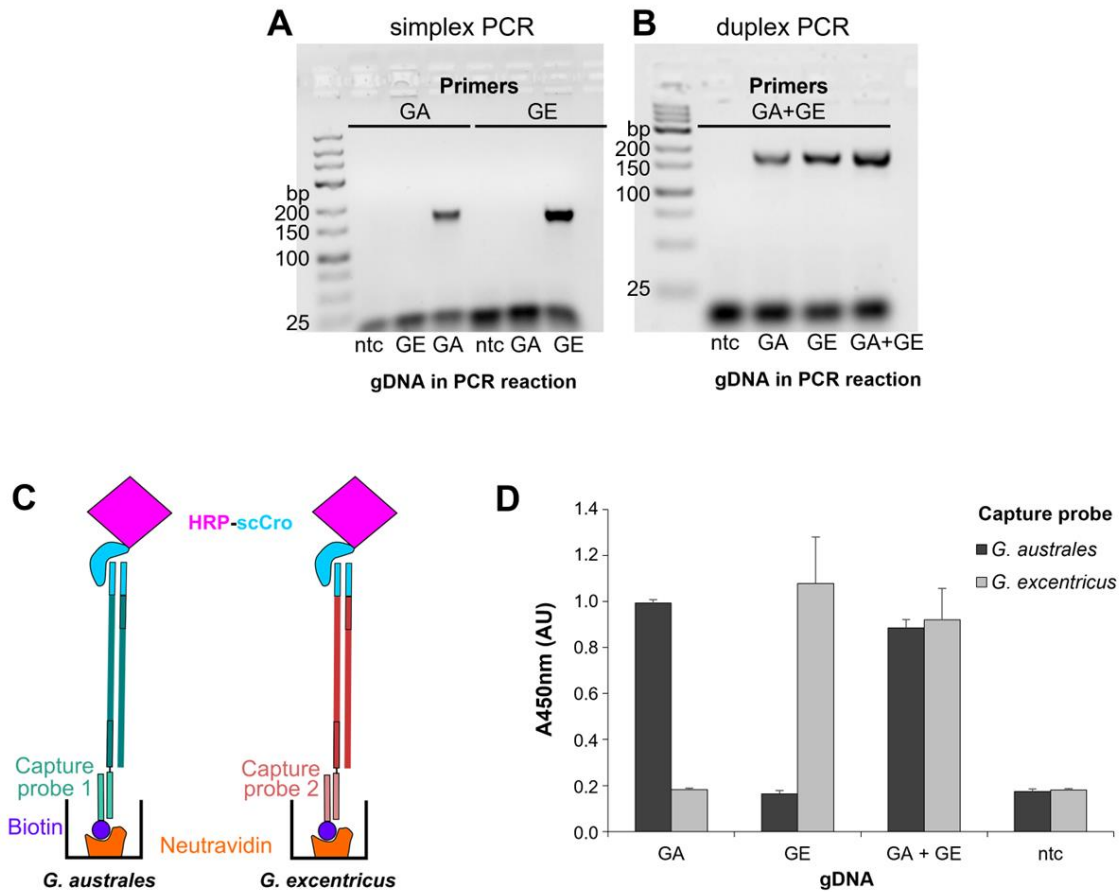


Figure 2.4 PCR amplification of *G. australes* (GA) and *G. excentricus* (GE) genomic DNA (gDNA) and detection by ELONA. (A) Simplex PCR using primers for each species and (B) duplex PCR using primers for both strains. (C) Format of the ELONA for the detection of the two *Gambierdiscus* species. (D) Specificity of the duplex PCR-ELONA for the detection of the amplicons.

Several parameters of the ELONA assay were then optimized before proceeding with the construction of calibration curves. A concentration of 50 nM of the capture probes (Figure 2.5A), in combination with 15 nM of HRP-scCro conjugate (Figure 2.5B) and a 1/50 dilution of the PCR reactions (Figure 2.5C) performed for 30 cycles (Figure 2.5D) were optimal for the detection of the gDNA from the two *Gambierdiscus* species.

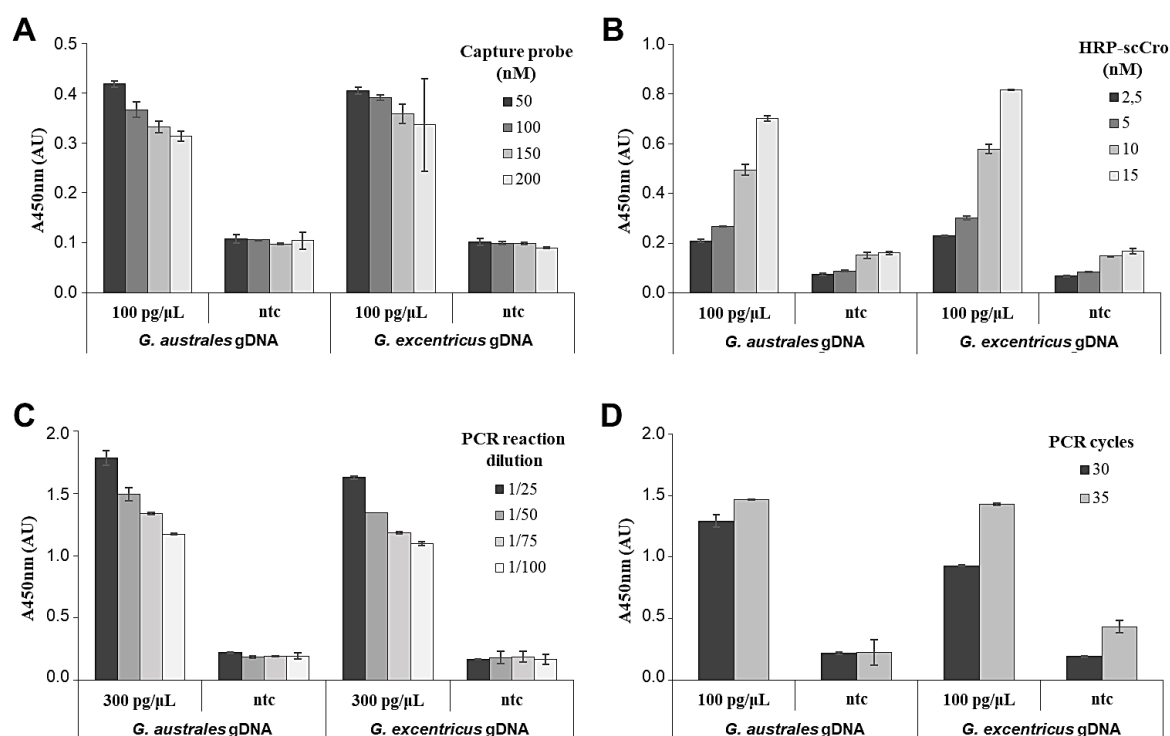


Figure 2.5 Optimization of the ELONA conditions for the detection of amplicons after PCR amplification of genomic DNA (gDNA) from *G. australes* and *G. excentricus*. (A) Capture probe concentration varied from 50 – 200 nM, while the HRP-scCro conjugate was used at 5 nM. (B) The HRP-scCro conjugate was used in the range of 2.5 – 10 nM; the capture probes for both species were used at 50 nM. (C) Dilution of the PCR reaction. (D) Number of PCR cycles. For panels C and D, the capture probes were used at 50 nM and the HRP-scCro conjugate at 15 nM. For panels A, B and D the PCR reactions were diluted 1/50. ntc: no template control.

The sensitivity of the ELONA was finally assessed using optimized conditions for PCR amplification and ELONA detection. Genomic DNA from each species was used for PCR amplification in a master mix containing primers for both species and representative agarose gels are shown in Figure 2.6A. The calibration curves are shown in Figure 2.6B and the LODs were calculated to be 22.8 fg/μL and 52.3 fg/μL of genomic DNA for *G. australes* and *G. excentricus*, respectively. These correspond to 1.1 pg of genomic DNA for *G. australes* and 2.6 pg for *G. excentricus*, considering the 50 μL sample volume used for analysis.

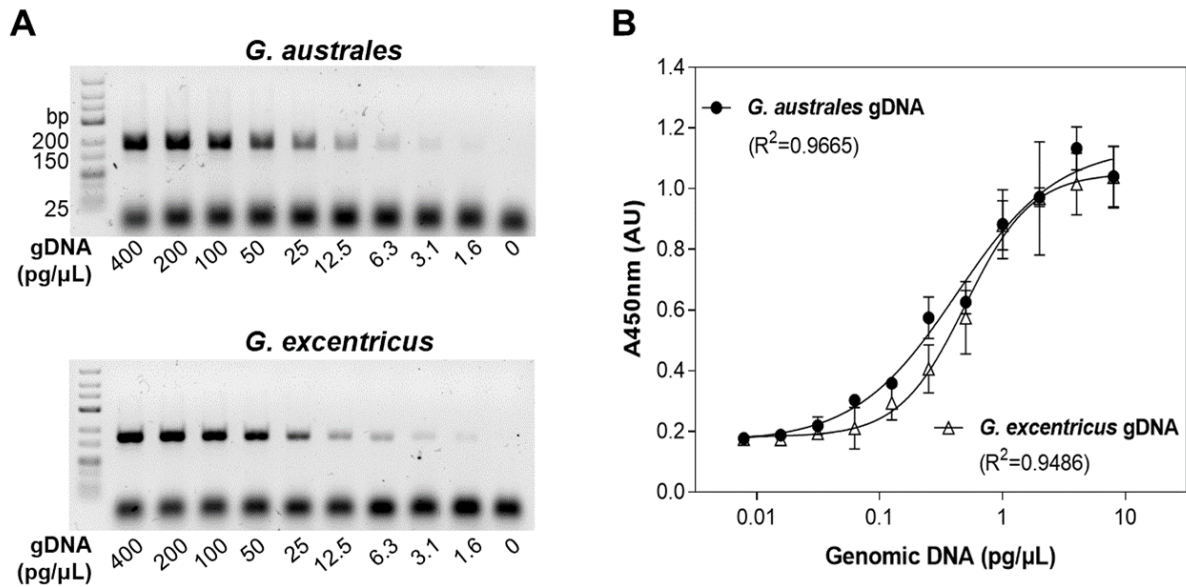


Figure 2.6 Sensitivity of the PCR-ELONA for the detection of *G. australes* (GA) and *G. excentricus* (GE) genomic DNA (gDNA). (A) Representative images after agarose gel electrophoresis analysis of PCR reactions performed using serial two-fold dilutions of genomic DNA (gDNA) (400 down to 16 pg/μL) from *G. australes* and *G. excentricus*. (B) Calibration curves for the detection of genomic DNA from *G. australes* and *G. excentricus* by ELONA. ntc: no template control.

2.4.3 Detection of *G. australes* and *G. excentricus* PCR amplicons by dipstick LFA

The approach used for the detection of the PCR amplicons by LFA and the design of the dipsticks, based on two test lines, one for each of the *Gambierdiscus* species, are illustrated in Figure 2.1B and 2.1C, respectively. Simplex PCR reactions were initially analyzed using 0.5 – 10 μL of PCR reaction diluted to a final volume of 10 μL in order to optimize the dilution factor of the PCR reaction for LFA. As shown in Figure 2.7, the use of 10 μL of PCR reaction resulted in the most intense signals in the respective test lines for each species, while control reactions performed with non-target species did not produce any non-specific signal.

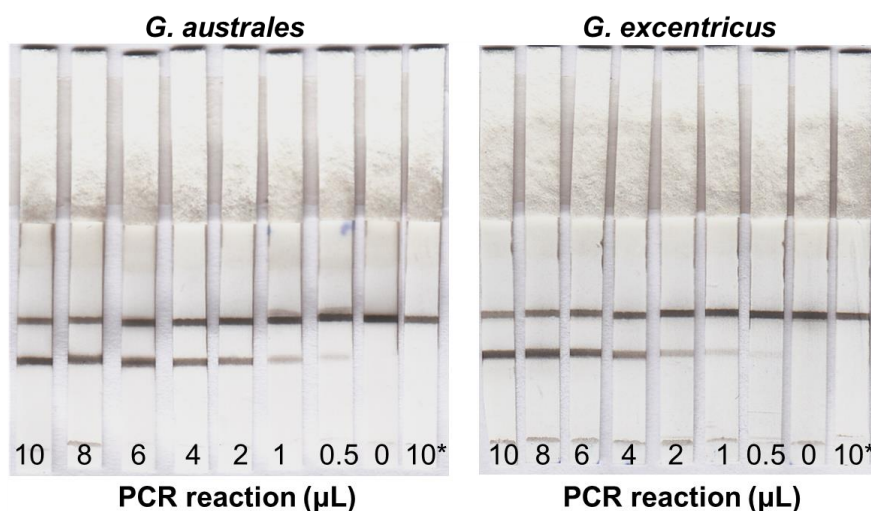


Figure 2.7 Optimization of the volume of PCR reaction used for detection of the amplicons with the LFA dipsticks. Simplex PCR reactions were performed separately using 100 pg/ μ L of genomic DNA from each species. The desired volume of PCR reaction (0 – 10 μ L) was mixed with 1 μ L of 0.2% (w/v) scCro/CNPs conjugate and diluted to 50 μ L with PBS containing 1% (w/v) skim milk and 0.1% (v/v) Tween-20. The asterisks (*) denote PCR reactions performed with genomic DNA from the non-target species, *G. excentricus* for the left panel and *G. australes* for the right panel.

Duplex PCR reactions were performed using 100 pg/ μ L of genomic DNA from each species, separately or in a mixture, and the amplicons were detected with the dipsticks as described in section 2.3.7 using 10 μ L of PCR reaction. As it can be seen in Figure 2.8, successful detection was observed when genomic DNA from each strain was added separately or simultaneously, while no signal was observed in the absence of both targets.

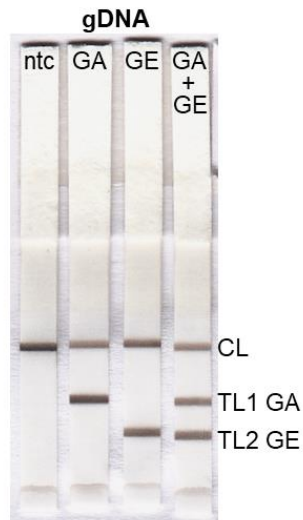


Figure 2.8 LFA dipsticks for the detection of genomic DNA (gDNA) from *G. australes* (GA) and *G. excentricus* (GA) after duplex PCR amplification of genomic DNA (100 pg/μL) from each species. CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

2.4.4 Sensitivity of the dipsticks for *G. australes* and *G. excentricus*

To assess the sensitivity of the LFA dipsticks, PCR reactions were performed using a series of genomic DNA concentrations from each species (1.6 – 400 pg/μL) and a mixture of the primers for the two species. After amplification, the PCR reactions were analyzed by agarose gel electrophoresis as shown in Figure 2.8 and then by dipstick LFA. Each PCR reaction was mixed with the scCro/CNPs conjugate and analyzed with the dipsticks. The visual LODs were determined as the minimum amounts of genomic DNA used for PCR amplification which resulted in visible test lines on the strips. As it can be seen in Figure 2.9, the visual LODs were 6.3 pg/μL of genomic DNA for both *G. australes* and *G. excentricus*, corresponding to 63 pg of genomic DNA for each species considering that 10 μL of PCR reactions were analyzed with the dipsticks.

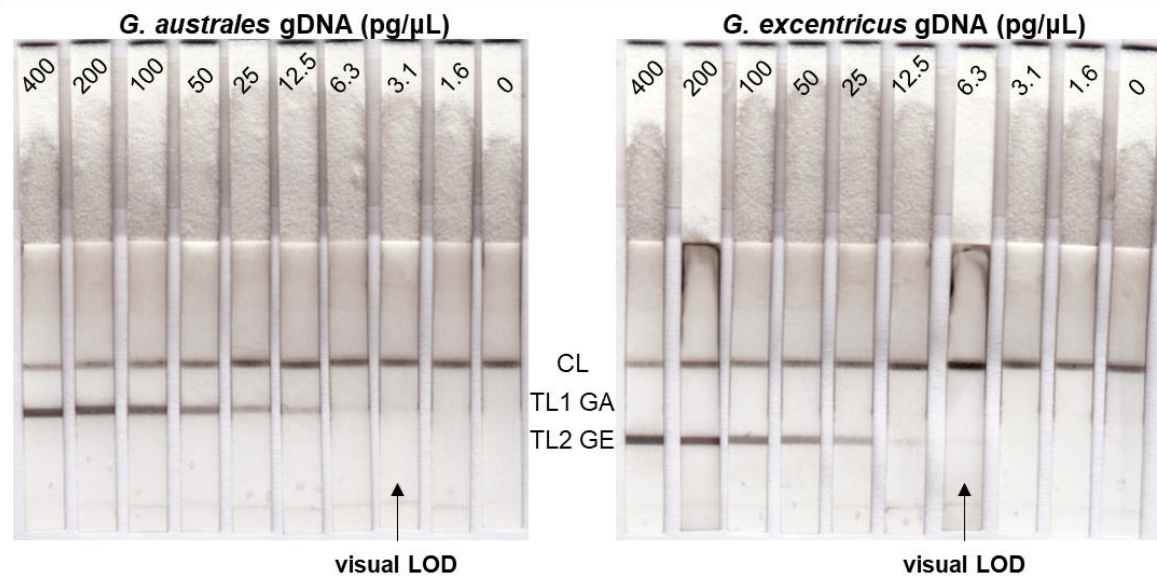


Figure 2.9 Sensitivity of the PCR-LFA for each *Gambierdiscus* species. Serially two-fold diluted genomic DNA (gDNA) from each strain (400 to 1.6 pg/μL) was used in each PCR reaction containing primers for both species and added to the dipsticks for visual detection. CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

2.4.5 Specificity of the PCR-LFA

The dipsticks were tested with PCR reactions performed with genomic DNA from different non-target microalgae species to evaluate the specificity of the developed approach. Each PCR reaction contained the three primers for *G. australes* and *G. excentricus* and 100 pg/μL of genomic DNA from each species. Only the PCR reactions containing genomic DNA from *G. australes* and *G. excentricus* resulted in amplification as seen after agarose gel electrophoresis (Figure 2.10A) and positive test lines signals on the dipsticks (Figure 2.10B). The genomic DNA from the other microalgae did not cross-react with the primers, the capture probes or the reporter scCro/CNPs conjugate used for detection, thus demonstrating the specificity of the approach.

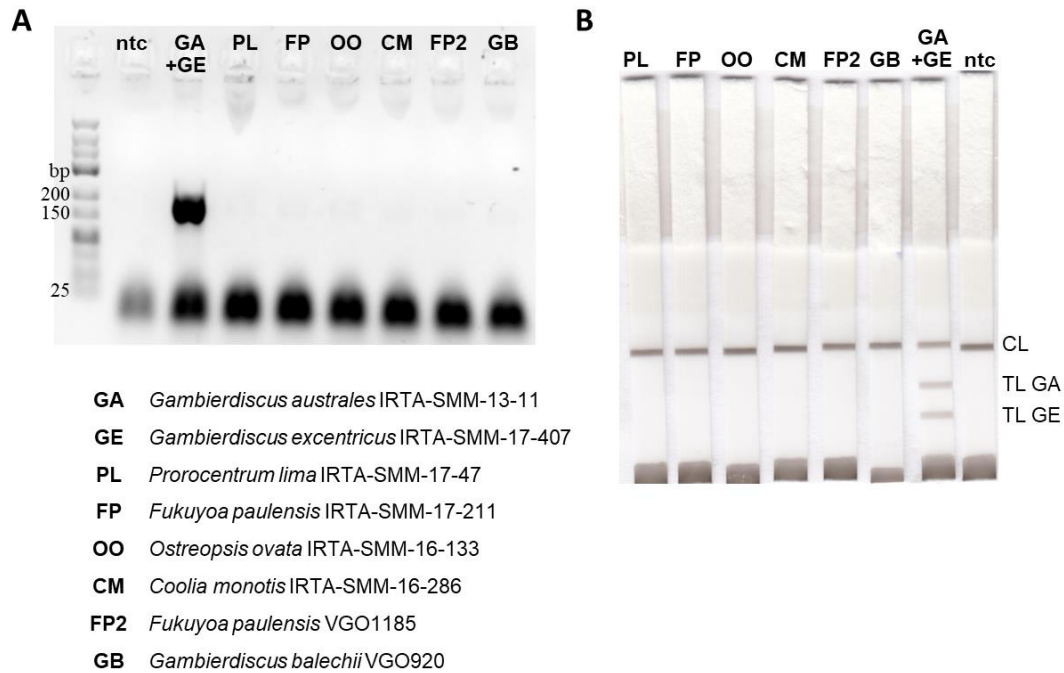


Figure 2.10 Evaluation of the specificity of the PCR-LFA using *G. australes* and *G. excentricus* primers. (A) Agarose gel electrophoresis and (B) dipstick LFA. PCR reactions were performed using 100 pg/μL of genomic DNA (gDNA) from various microalgae species and genera. ntc: no template control.

2.4.6 Detection of genomic DNA from *G. australes* and *G. excentricus* in the presence of non-target *Gambierdiscus* or *Fukuyoa* species

The effect of various non-target *Gambierdiscus* and a *Fukuyoa* species on the detection of genomic DNA from *G. australes* and *G. excentricus* was evaluated next. Mixtures containing equal concentrations of genomic DNA from each of the *Gambierdiscus* and *Fukuyoa* species were thus prepared and used for PCR amplification with the *G. australes* and *G. excentricus* primers. Analysis of the PCR reactions by agarose gel electrophoresis (Figure 2.11A) revealed successful amplification only in mixtures containing genomic DNA either from *G. australes* or *G. excentricus* or both. Likewise, amplicons were detected as black lines on the dipsticks when either one or both target *Gambierdiscus* species were present (Figure 2.11B). The presence of the non-target *Gambierdiscus* or the *Fukuyoa* species included in this study did not interfere with the detection of the two target species since the intensity of the test lines was similar in all cases regardless of the composition of the DNA mixtures.

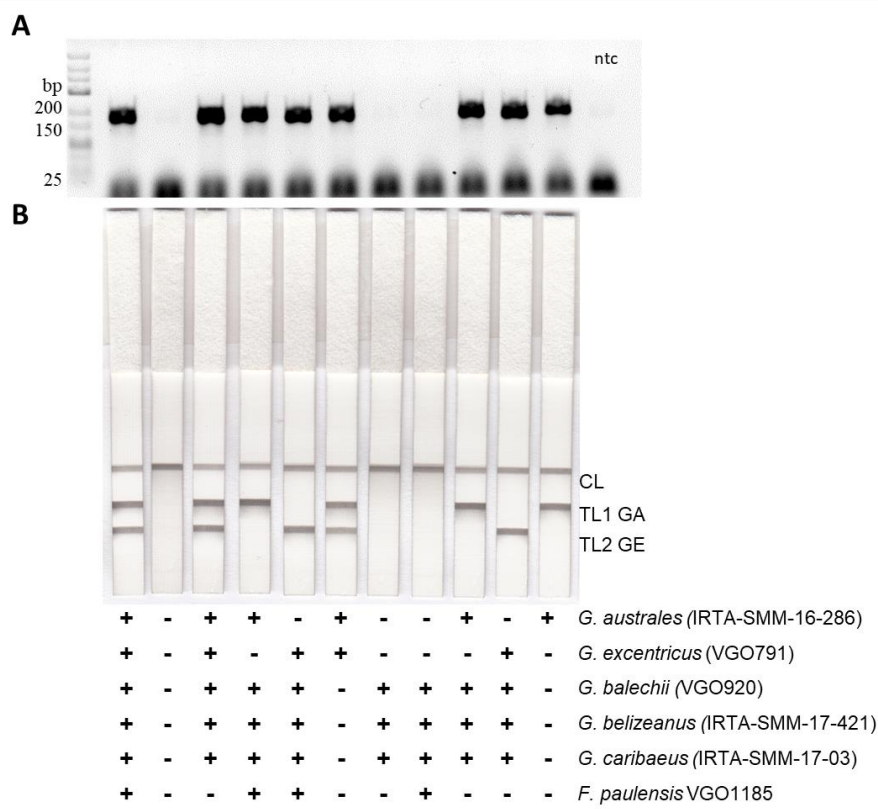


Figure 2.11 Specificity of the (A) PCR and (B) dipstick LFA in the presence of non-target *Gambierdiscus* and *Fukuyoa* species. Each combination used for PCR amplification contained 400 pg/μL genomic DNA from each strain. ntc: PCR no template control; CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

2.4.7 Detection of *G. australes* and *G. excentricus* cells

The possibility to detect the two target *Gambierdiscus* species with the PCR-LFA approach developed in this work using directly cells was finally evaluated. To this end, cell suspensions from each species were heated briefly and the crude cell lysates were used directly for PCR amplification instead of purified genomic DNA. PCR amplicons were detected by agarose gel electrophoresis for both species when used individually or in a mixture (Figure 2.12A). Similar amplification efficiency of the duplex reaction was observed when using cells or 100 pg/μL of purified genomic DNA from each species. The amplicons were finally detected with the LFA dipsticks (Figure 2.12B). However, the intensity of the test lines of the duplex reactions using crude cell extracts was slightly lower compared to when purified genomic DNA from each strain was used for amplification.

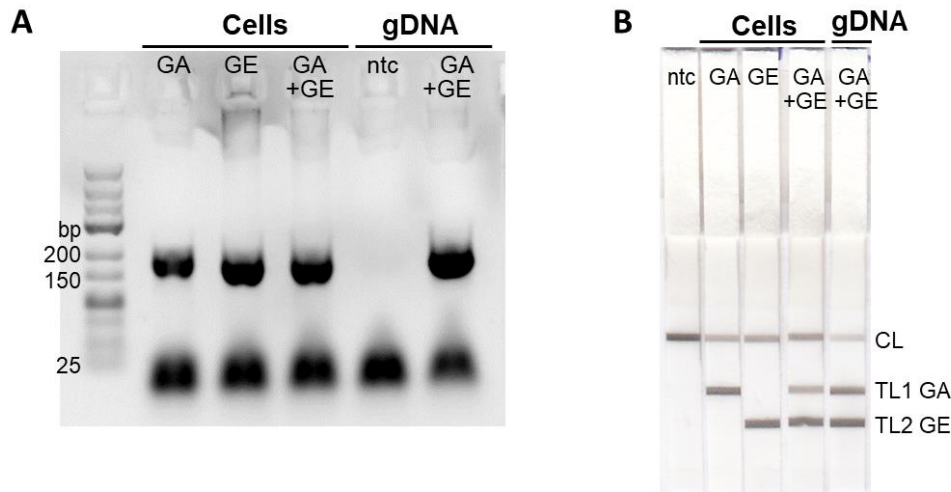


Figure 2.12 Detection of *G. australes* (GA) and *G. excentricus* (GE) cells. (A) PCR and (B) dipstick LFA. Each PCR reaction contained 4 cells/ μ L while 100 pg/ μ L of purified gDNA (gDNA) from each species was used in parallel as a control. ntc: no template control; CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

2.5 Discussion

The main objective of this work was to detect two toxin-producing *Gambierdiscus* species, *G. australes* and *G. excentricus*, associated with ciguatera outbreaks using PCR combined with lateral flow dipsticks. The spread of ciguatoxin-producing microalgae of the *Gambierdiscus* and *Fukuyoa* genera in non-endemic areas has increased the demand for fast and reliable detection methods to monitor these microalgae and prevent future outbreaks. Hence, a lot of effort has been invested over the last years into moving from laboratory-based techniques, which are inherently slow and equipment-dependent, to decentralized, user-friendly platforms such as biosensors which are suitable for on-site detection (32). The sensitivity and specificity provided by nucleic acid amplification tests has allowed the implementation of molecular methods as promising tools for the detection of microalgae (33, 34). There is a plethora of reports on the detection of microalgae nucleic acids using both PCR and isothermal amplification (23).

Studies on the detection of ciguatoxin-producing microalgae based on molecular methods are scarce though. The first one reported the development of a qPCR assay for the simultaneous detection of several *Gambierdiscus* species with an LOD of 10 cells (24). In another qPCR-based study, identification and enumeration of four *Gambierdiscus* species was demonstrated with LODs of 10 gene copies (25). Alternatively, restriction fragment length polymorphism

(RFLP) was used to identify *Gambierdiscus* and *Fukuyoa* species in field samples (26, 27). In addition to these methods, species-specific fluorescence *in situ* hybridization (FISH) probes were also designed for the multiplex detection of several *Gambierdiscus* species in another report (28). Finally, isothermal recombinase polymerase amplification in combination with a sandwich hybridization assay was recently developed for the detection of single cells from *Gambierdiscus* and *Fukuyoa* strains (29). Most of these studies targeted the detection of *G. australes* strains (26-29), among other species, however only two of them focused also on *G. excentricus* (26, 29). The combination of molecular methods for target DNA amplification with a sensitive detection strategy that is also simple, cost-effective and compatible with field testing would be ideal for monitoring the presence of toxic algae species in the marine environment. LFAs can potentially serve this purpose. The low cost and easy operation of these simple devices have encouraged their use for the detection of a wide variety of target analytes such as proteins, nucleic acids, toxins, drugs and others even in complex samples, and are especially suitable for on-site testing (35). For nucleic acid targets, detection of DNA amplicons generated by PCR or isothermal amplification is generally achieved using different labels (35, 36). Gold nanoparticles have been the mainstay in nucleic acid LFAs (37-38), mainly for qualitative or semiquantitative detection. Other labels have also been reported, such as CNPs (31, 39, 40), fluorescent nanoparticles (41), colored latex beads (42), enzymes (31) or enzymes combined with nanoparticles (31, 43). Label materials such as quantum dots, upconversion nanoparticles, NIR dye-doped beads, SERS tags and semiconductor polymer dots were further developed to improve sensitivity and achieve quantitative analysis when combined with specific reader devices (44, 45). Magnetic nanoparticles are also extremely useful as labels because of their dual magnetic and optical properties (45, 46). On the other hand, CNPs are particularly attractive because of their low cost, high stability, easy modification and high signal-to-noise ratio (36, 47). CNPs were previously shown to provide enhanced sensitivity in LFAs when compared to gold nanoparticles, silver-enhanced gold nanoparticles and blue latex beads (48). Generic lateral flow immunoassay strips based on CNPs are in fact commercially available by Abingdon Health (UK) for the detection of double-labeled (biotin/fluorophore) DNA amplicons. CNPs were chosen as the colorimetric label for this work as well. Even though CNPs are not typically used for quantitative analysis, the main advantage they provide compared to the more sophisticated labels mentioned above is the simplicity of use and facile interpretation of the visual results without the need of a specific reader when a yes/no response is appropriate for the target analyte.

The primers used for PCR amplification of DNA from the two target toxic microalgal *Gambierdiscus* species were based on a previous work (29). They were designed within the large subunit of ribosomal DNA (rDNA) which provides the necessary sequence variability to allow specific amplification of the target strain and high copy number for increased sensitivity. The same region of rDNA was also targeted in previous studies with regards to the detection of several *Gambierdiscus* species including *G. australes* and *G. excentricus* (26-28). To facilitate capture of the amplicons on the dipsticks, each reverse primer was modified at its 5' end with a ssDNA sequence (tail) separated from the rest of the sequence with a carbon-based spacer. For detection, the common forward primer for both species was extended with the specific DNA sequence recognized by scCro DNA binding protein. In this way, the generated hybrid ssDNA-dsDNA amplicons were tagged at one end with distinct ssDNA tails to allow discrimination of the two species on the two individual test lines comprised of complementary ssDNA probes. The other end of the amplicons contained the dsDNA binding site for scCro DNA binding protein to facilitate detection with the scCro/CNPs conjugate. The performance of HRP-scCro, scCro/CNPs and HRP-scCro/CNPs conjugates regarding the detection of *Escherichia coli* bacterial DNA by PCR-LFA was compared in a previous report (31). Even though all three approaches exhibited very similar sensitivity, the use of scCro/CNPs provided the fastest (less than 20 min) and more reliable detection with no false positives, and this conjugate was chosen for this work as well. The use of tailed primers for amplification combined with LFAs for detection via hybridization with complementary probes has also been reported previously (38, 49). Isothermal recombinase polymerase amplification (RPA) was used in these reports for amplification of target DNA from the bacterial biowarfare agents *Yersinia pestis* and *Francisella tularensis*, whereas gold nanoparticles served as the colorimetric reporter achieving high sensitivity (< 1 pg of genomic DNA). However, purified amplicons were used in these studies for detection, resulting in extended time-to-result and requirement of additional material or infrastructure. In the current work, the assay was simplified by solely diluting the PCR reactions prior to analysis. Since scCro specifically binds only to dsDNA, no interference from unreacted primers was expected thus eliminating the need for amplicon purification.

For the initial validation of the primer design and to ensure correct incorporation of the tags at the two ends of the generated amplicons (ssDNA tail and dsDNA binding site for scCro), an ELONA was developed. Since the expected length of the two amplicons was similar, it was not possible to discriminate them by agarose gel electrophoresis. Therefore, the same capture

probes later used for the preparation of the LFA dipsticks were employed for the ELONA which allowed the validation of the specificity of the primers and of the overall strategy for amplification and detection. Similar enzyme-based DNA hybridization assays have been developed before for other toxic algae like *Karlodinium* (50) and *Ostreopsis* (51). In these studies, ssDNA tails were used for capturing the amplicons with complementary ssDNA probes whereas a ssDNA-HRP conjugate was utilized for detection. The LODs reported for example for *Ostreopsis* (50 – 70 pg of gDNA) were more than 10-fold higher compared to the ones achieved in this work for the two *Gambierdiscus* species (1.1 – 2.6 pg). However, it is important to note that these LODs are expressed in genomic DNA amount. In *Gambierdiscus* species, the rDNA copy number per cell has been reported to be as high as 4,560–21,500 (24) or even up to 3,197,000 (25), probably due to the large cell size and high amount of genomic DNA. This means that the genomic DNA amount detected in this work for *Gambierdiscus* species may have a higher number of copies than that of *Ostreopsis* species. Additionally, the rDNA copy number can vary between genus, species, strains, geographic origins, and even cell growth phases and thus sample harvesting times (29). Also, in this work, detection of the amplicons was achieved using an HRP-scCro conjugate. Because of the dimeric nature of scCro and the imperfect dyad symmetry of its specific DNA binding sequence, stoichiometries of scCro/DNA binding sites can range from 1:1 to 2:1, potentially allowing more than one HRP-scCro conjugate to associate with each amplicon and resulting in increased sensitivity. This target-independent HRP-scCro conjugate can be very useful for colorimetric assays and it could be potentially used in any assay provided that the dsDNA binding site for scCro is incorporated in the target amplicon.

As mentioned earlier, there are very few reports in the literature on the detection of harmful algae using DNA amplification combined with visual LFAs and they are summarized in Table 2.3. In these reports, amplification of the target microalgae species was performed with PCR, loop-mediated isothermal amplification (LAMP), RPA and rolling circle amplification (RCA), exploiting one or two labeled primers. Commercially available generic dipsticks were used for detection of the generated amplicons via hybridization or by sandwich formation with capture/reporter probes in almost all the reports. In the case of hybridization, only one labeled (biotinylated) primer was required for amplification, and the biotinylated amplicon was hybridized with a ssDNA probe labeled with a fluorophore, followed by capture on the test line using a biotin-binding biomolecule and detection with an AuNPs/anti-fluorophore IgG conjugate. On the other hand, for sandwich detection, two labeled primers were used and the

biotin-fluorophore double-labeled amplicon was captured on the test line and detected again with an AuNPs/anti-fluorophore IgG conjugate. The generic LFA dipsticks used were obtained from two different companies (Ustar Biotech Ltd, China and Milenia Biotec GmbH, Germany) and were used directly for the detection of the amplicons. The reported LODs were in the range of 0.34 pg/ μ L – 10 ng/ μ L, with few exceptions reporting ≤ 1 fg/ μ L when variations of RCA were employed for the amplification step. The sensitivity achieved in this work for the two *Gambierdiscus* species with the PCR-LFA dipsticks (6.3 pg/ μ L) was in line with the previous reports on the other microalgal species where an isothermal amplification method was used. Two reports were found in the literature showing the combination of PCR with LFAs for the detection of toxic microalgae. In these reports, ssDNA tagged primers were used for PCR amplification and the detection of the ssDNA double-tagged DNA amplicons was performed with LFA dipsticks via hybridization with complementary DNA probes (52, 53). In one of these works, several *Alexandrium* species were detected using commercially available universal dipsticks from Kaneka Co. (Japan) and detection limits of $< 0.1 - 10$ pg were achieved (52). In the second work, home-made dipsticks were prepared for the detection of *Karlodinium veneficum* exhibiting a sensitivity of 91.3 pg/ μ L (53) in comparison to the 63 pg (6.3 pg/ μ L) of genomic DNA from the two *Gambierdiscus* species demonstrated in this work also exploiting home-made LFA dipsticks.

The PCR-LFA strategy developed in this work was not only sensitive but also very specific. DNA from other microalgae genera potentially present in the same habitat as the two target *Gambierdiscus* species like *Prorocentrum lima*, *Ostreopsis ovata* and *Coolia monotis* as well as non-target *Gambierdiscus* (*G. balechii*, *G. belizeanus*, *G. caribaeus*) or *Fukuyoa* (*F. paulensis*) species did not produce positive signals on the dipsticks. This is not only due to the high specificity of the primers but also the specific DNA binding properties of the scCro reporter protein. The analysis of mixtures containing equal concentrations of genomic DNA from non-target *Gambierdiscus* species further demonstrated the specificity of the approach since positive signals on the dipsticks were obtained only when the target species were present. These samples were analyzed in an effort to mimic field samples potentially containing more than one microalgae species. These findings further highlight the importance of the strategy shown herein which is highly specific and does not produce any false positive signals. Research efforts should focus on the validation of the PCR-LFA system for the analysis of field samples and compared to light microscopy. Nevertheless, the results obtained in this work demonstrate that the strategy should be implementable in *in situ* monitoring and research activities, since

portable PCR devices can be brought to the field, whereas LFA is easy and fast to perform, and results are easy to interpret by simple visual inspection.

2.6 Conclusions

In this work, a PCR-LFA dipstick was developed for the simultaneous detection of the toxin-producing *G. australes* and *G. excentricus* species. Duplex PCR amplification of genomic DNA from these species with specifically modified primers allowed facile visual detection with the dipsticks employing a scCro/CNPs conjugate generating black colored line signals. The strategy was highly sensitive whereas the presence of non-target *Gambierdiscus* species or other microalgae genera potentially co-habiting in the same waters did not interfere with the assay. The approach combines the specificity and sensitivity provided by PCR amplification with the simplicity, low cost and on-site testing compatibility of LFAs. Preliminary results demonstrating DNA amplification directly from cells instead of purified genomic DNA and subsequent detection with the LFA dipsticks suggests that the approach could be applied to field sample testing, further highlighting its potential to be implemented in monitoring programs.

2.7 Acknowledgments

This work was supported financially by the CIGUASENSING (BIO2017-87946-C2-1-R and BIO2017-87946-C2-2-R) project funded by the Ministerio de Ciencia e Innovación (MICINN), the Agencia Estatal de Investigación (AEI) and the Fondo Europeo de Desarrollo Regional (FEDER). The authors thank Dr. Aart van Amerongen (Wageningen University and Research, The Netherlands) for the carbon nanoparticles.

Table 2.3 Nucleic acid LFAs developed for the detection of harmful algae based on different amplification methods.

Method	Target	NALFA design	Reporter probe	LOD	Ref.
RPA	<i>Karlodinium veneficum</i>	Biotin-FITC double labeled amplicon ^a	AuNPs/anti-FITC IgG	10 ng/μL	(54)
	<i>Prorocentrum minimum</i>	Biotin-FAM double labeled amplicon ^a	AuNPs/anti-FAM IgG	8.1 pg/μL	(55)
	<i>Heterosigma akashiwo</i>	Biotin-FAM double labeled amplicon ^a	AuNPs/anti-FAM IgG	0.34 pg/μL	(56)
RCA	<i>Karlodinium veneficum</i>	Biotinylated amplicon/FAM-DNA probe hybridization ^a	AuNPs/anti-FAM IgG	80 ag/μL	(57)
	<i>Chattonella marina</i>	Biotinylated amplicon/FAM-DNA probe hybridization ^a	AuNPs/anti-FAM IgG	8.3 pg/μL	(58)
	<i>Karenia mikimotoi</i>	Biotinylated amplicon/FAM-DNA probe hybridization ^a	AuNPs/anti-FAM IgG	1 fg/μL	(59)
LAMP	<i>Karlodinium veneficum</i>	Biotin-FITC double labeled amplicon ^b	AuNPs/anti-FITC IgG	7.4 pg/μL	(60)
	<i>Prymnesium parvum</i>	Biotin-FITC double labeled amplicon ^b	AuNPs/anti-FITC IgG	30 pg/μL	(61)
	<i>Alexandrium catenella</i>	Biotinylated amplicon/FITC-DNA probe hybridization ^a	AuNPs/anti-FITC IgG	0.46 pg/μL	(62)
	<i>Chattonella marina</i>	Biotinylated amplicon/FAM-DNA probe hybridization ^b	AuNPs/anti-FAM IgG	0.34 pg/μL	(63)
	<i>Karenia mikimotoi</i>	Biotinylated amplicon/FITC-DNA probe hybridization ^a	AuNPs/anti-FITC IgG	72 pg/μL	(64)
	<i>Amphidinium carterae</i>	Biotinylated amplicon/FITC-DNA probe hybridization ^c	AuNPs/anti-FITC IgG	0.83 pg/μL	(65)
	<i>Skeletonema costatum</i>	Biotin-FITC double labeled amplicons ^c	AuNPs/anti-FITC IgG	0.94 pg/μL	(66)
PCR	<i>Alexandrium species</i>	ssDNA double-tagged amplicons ^d	AuNPs/ssDNA probe	0.05 - 5 pg/μL	(52)
	<i>Karlodinium veneficum</i>	ssDNA double-tagged amplicon ^e	AuNPs/ssDNA probe	91.3 pg/μL	(53)
	<i>Gambierdiscus australes</i>	ssDNA-scCro dsDNA binding site double tagged amplicons ^e	CNPs/scCro DNA binding protein	6.3 pg/μL	This work
	<i>Gambierdiscus excentricus</i>				

RPA: recombinase polymerase amplification; RCA: rolling circle amplification; LAMP: loop-mediated isothermal amplification; PCR: polymerase chain reaction; ^a: commercial strips by Ustar Biotech Ltd (China); ^b: commercial strips by Milenia Biotec GmbH (Germany); ^c: commercial strips from undisclosed source; ^d: commercial strips by Kaneka Co. (Japan); ^e: home-made strips; AuNPs: gold nanoparticles; CNPs: carbon nanoparticles.

2.8 References

1. Lewis RJ. The changing face of ciguatera. *Toxicon*. 2001 Jan;39(1):97–106.
2. YASUMOTO T. Chemistry, etiology, and food chain dynamics of marine toxins. *Proc Japan Acad Ser B*. 2005;81(2):43–51.
3. Friedman MA, Fernandez M, Backer LC, Dickey RW, Bernstein J, Schrank K, et al. An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. *Mar Drugs*. 2017 Mar;15(3).
4. Caillaud A, de la Iglesia P, Barber E, Eixarch H, Mohammad-Noor N, Yasumoto T, et al. Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: Application to *Gambierdiscus pacificus* in culture. *Harmful Algae*. 2011;10(5):433–46.
5. Litaker RW, Holland WC, Hardison DR, Pisapia F, Hess P, Kibler SR, et al. Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. *PLoS One*. 2017;12(10):e0185776.
6. Reverté L, Toldrà A, Andree KB, Fraga S, de Falco G, Campàs M, et al. Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. *J Appl Phycol*. 2018;30(4):2447–61.
7. Hamilton B, Hurbungs M, Vernoux J-P, Jones A, Lewis RJ. Isolation and characterisation of Indian Ocean ciguatoxin. *Toxicon*. 2002 Jun;40(6):685–93.
8. Tester P, Wickliffe L, Jossart J, Rhodes L, Enevoldsen H, Adachi M, et al. Global distribution of the genera *Gambierdiscus* and *Fukuyoa*. 2020.
9. Fraga S, Rodríguez F, Caillaud A, Diogène J, Raho N, Zapata M. *Gambierdiscus excentricus* sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). *Harmful Algae*. 2011 Nov 1;11:10–22.
10. Rodríguez F, Fraga S, Ramilo I, Rial P, Figueroa RI, Riobó P, et al. “Canary Islands (NE Atlantic) as a biodiversity ‘hotspot’ of *Gambierdiscus*: Implications for future trends of ciguatera in the area”. *Harmful Algae*. 2017 Jul;67:131–43.
11. Kaufmann M, Böhm-Beck M. *Gambierdiscus* and related benthic dinoflagellates from Madeira archipelago (NE Atlantic). *Harmful Algae News*. 2013;47:18–9.
12. Aligizaki K, Nikolaidis G. Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. *J Biol Res*. 2008 Jun 1;9:75–82.
13. Tudó À, Toldrà A, Andree K, Rey Varela M, Fernández M, Campàs M, et al. First report

- of Gambierdiscus in the Western Mediterranean Sea (Balearic Islands). 2018 Feb 1;
14. Rossignoli AE, Tudó A, Bravo I, Díaz PA, Diogène J, Riobó P. Toxicity Characterisation of Gambierdiscus Species from the Canary Islands. Vol. 12, Toxins. 2020.
15. Pasinszki T, Lako J, Dennis TE. Advances in Detecting Ciguatoxins in Fish. Toxins (Basel). 2020 Jul;12(8).
16. Reverté L, Soliño L, Carnicer O, Diogène J, Campàs M. Alternative Methods for the Detection of Emerging Marine Toxins: Biosensors, Biochemical Assays and Cell-Based Assays. Vol. 12, Marine Drugs. 2014. p. 5719–63.
17. Lazcka O, Del Campo FJ, Muñoz FX. Pathogen detection: a perspective of traditional methods and biosensors. Biosens Bioelectron. 2007 Feb;22(7):1205–17.
18. Velusamy V, Arshak K, Korostynska O, Oliwa K, Adley C. An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnol Adv. 2010;28(2):232–54.
19. Tsumuraya T, Fujii I, Hiramama M. Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: sandwich ELISA detection of ciguatoxins. J AOAC Int. 2014;97(2):373–9.
20. Leonardo S, Gaiani G, Tsumuraya T, Hiramama M, Turquet J, Sagristà N, et al. Addressing the Analytical Challenges for the Detection of Ciguatoxins Using an Electrochemical Biosensor. Anal Chem. 2020 Apr 7;92(7):4858–65.
21. Gaiani G, Leonardo S, Tudó À, Toldrà A, Rey M, Andree KB, et al. Rapid detection of ciguatoxins in Gambierdiscus and Fukuyoa with immunosensing tools. Ecotoxicol Environ Saf. 2020 Nov;204:111004.
22. Tudó À, Gaiani G, Rey Varela M, Tsumuraya T, Andree KB, Fernández-Tejedor M, et al. Further Advance of Gambierdiscus Species in the Canary Islands, with the First Report of Gambierdiscus belizeanus. Vol. 12, Toxins. 2020.
23. Toldrà A, O’Sullivan CK, Diogène J, Campàs M. Detecting harmful algal blooms with nucleic acid amplification-based biotechnological tools. Sci Total Environ. 2020 Dec;749:141605.
24. Vandersea MW, Kibler SR, Holland WC, Tester PA, Schultz TF, Faust MA, et al. DEVELOPMENT OF SEMI-QUANTITATIVE PCR ASSAYS FOR THE DETECTION AND ENUMERATION OF GAMBIERDISCUS SPECIES (GONYAULACALES, DINOPHYCEAE)(1). J Phycol. 2012 Aug;48(4):902–15.
25. Nishimura T, Hariganeya N, Tawong W, Sakanari H, Yamaguchi H, Adachi M. Quantitative PCR assay for detection and enumeration of ciguatera-causing

- dinoflagellate *Gambierdiscus* spp. (Gonyaulacales) in coastal areas of Japan. *Harmful Algae*. 2016 Feb;52:11–22.
26. Lyu Y, Richlen ML, Sehein TR, Chinain M, Adachi M, Nishimura T, et al. LSU rDNA based RFLP assays for the routine identification of *Gambierdiscus* species. *Harmful Algae*. 2017 Jun;66:20–8.
 27. Lozano-Duque Y, Richlen ML, Smith TB, Anderson DM, Erdner DL. Development and Validation of PCR-RFLP Assay for Identification of *Gambierdiscus* species in the Greater Caribbean region. *J Appl Phycol*. 2018 Dec;30(6):3529–40.
 28. Pitz KJ, Richlen ML, Fachon E, Smith TB, Parsons ML, Anderson DM. Development of fluorescence in situ hybridization (FISH) probes to detect and enumerate *Gambierdiscus* species. *Harmful Algae*. 2021 Jan;101:101914.
 29. Gaiani G, Toldrà A, Andree KB, Rey M, Diogène J, Alcaraz C, et al. Detection of *Gambierdiscus* and *Fukuyoa* single cells using recombinase polymerase amplification combined with a sandwich hybridization assay. *J Appl Phycol*. 2021;33(4):2273–82.
 30. Aktas GB, Skouridou V, Masip L. Novel signal amplification approach for HRP-based colorimetric genosensors using DNA binding protein tags. *Biosens Bioelectron*. 2015;74:1005–10.
 31. Aktas GB, Wichers JH, Skouridou V, van Amerongen A, Masip L. Nucleic acid lateral flow assays using a conjugate of a DNA binding protein and carbon nanoparticles. *Mikrochim Acta*. 2019 Jun;186(7):426.
 32. McPartlin DA, Loftus JH, Crawley AS, Silke J, Murphy CS, O’Kennedy RJ. Biosensors for the monitoring of harmful algal blooms. *Curr Opin Biotechnol*. 2017 Jun;45:164–9.
 33. Ebenezer V, Medlin LK, Ki J-S. Molecular detection, quantification, and diversity evaluation of microalgae. *Mar Biotechnol (NY)*. 2012 Apr;14(2):129–42.
 34. Medlin LK, Orozco J. Molecular Techniques for the Detection of Organisms in Aquatic Environments, with Emphasis on Harmful Algal Bloom Species. Vol. 17, *Sensors*. 2017.
 35. Bahadır EB, Sezgintürk MK. Lateral flow assays: Principles, designs and labels. *TrAC Trends Anal Chem*. 2016;82:286–306.
 36. Zheng C, Wang K, Zheng W, Cheng Y, Li T, Cao B, et al. Rapid developments in lateral flow immunoassay for nucleic acid detection. *Analyst*. 2021;146(5):1514–28.
 37. Aveyard J, Mehrabi M, Cossins A, Braven H, Wilson R. One step visual detection of PCR products with gold nanoparticles and a nucleic acid lateral flow (NALF) device. *Chem Commun*. 2007;(41):4251–3.
 38. Jauset-Rubio M, Svobodová M, Mairal T, McNeil C, Keegan N, Saeed A, et al.

- Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay. *Sci Rep*. 2016 Nov;6:37732.
39. Noguera P, Posthuma-Trumpie GA, van Tuil M, van der Wal FJ, de Boer A, Moers APHA, et al. Carbon nanoparticles in lateral flow methods to detect genes encoding virulence factors of Shiga toxin-producing *Escherichia coli*. *Anal Bioanal Chem*. 2011 Jan;399(2):831–8.
 40. El-Tholoth M, Branavan M, Naveenathayalan A, Balachandran W. Recombinase polymerase amplification-nucleic acid lateral flow immunoassays for Newcastle disease virus and infectious bronchitis virus detection. *Mol Biol Rep*. 2019 Dec;46(6):6391–7.
 41. Takalkar S, Baryeh K, Liu G. Fluorescent carbon nanoparticle-based lateral flow biosensor for ultrasensitive detection of DNA. *Biosens Bioelectron*. 2017 Dec;98:147–54.
 42. Mao X, Wang W, Du TE. Dry-reagent nucleic acid biosensor based on blue dye doped latex beads and lateral flow strip. *Talanta*. 2013 Sep;114:248–53.
 43. He Y, Zhang S, Zhang X, Baloda M, Gurung AS, Xu H, et al. Ultrasensitive nucleic acid biosensor based on enzyme-gold nanoparticle dual label and lateral flow strip biosensor. *Biosens Bioelectron*. 2011 Jan;26(5):2018–24.
 44. Gui C, Wang K, Li C, Dai X, Cui D. A CCD-based reader combined with CdS quantum dot-labeled lateral flow strips for ultrasensitive quantitative detection of CagA. *Nanoscale Res Lett*. 2014 Feb;9(1):57.
 45. Liu X, Wu W, Cui D, Chen X, Li W. Functional Micro-/Nanomaterials for Multiplexed Biodetection. *Adv Mater*. 2021 Jul 1;33(30):2004734.
 46. Yan W, Wang K, Xu H, Huo X, Jin Q, Cui D. Machine Learning Approach to Enhance the Performance of MNP-Labeled Lateral Flow Immunoassay. *Nano-Micro Lett*. 2019;11(1):7.
 47. Posthuma-Trumpie GA, Wichers JH, Koets M, Berendsen LBJM, van Amerongen A. Amorphous carbon nanoparticles: a versatile label for rapid diagnostic (immuno)assays. *Anal Bioanal Chem*. 2012 Jan;402(2):593–600.
 48. Linares EM, Kubota LT, Michaelis J, Thalhammer S. Enhancement of the detection limit for lateral flow immunoassays: evaluation and comparison of bioconjugates. *J Immunol Methods*. 2012 Jan;375(1–2):264–70.
 49. Jauset-Rubio M, Tomaso H, El-Shahawi MS, Bashammakh AS, Al-Youbi AO, O’Sullivan CK. Duplex Lateral Flow Assay for the Simultaneous Detection of *Yersinia*

- pestis and *Francisella tularensis*. *Anal Chem*. 2018 Nov;90(21):12745–51.
50. Toldrà A, Jauset-Rubio M, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, et al. Detection and quantification of the toxic marine microalgae *Karlodinium veneficum* and *Karlodinium armiger* using recombinase polymerase amplification and enzyme-linked oligonucleotide assay. *Anal Chim Acta*. 2018 Dec;1039:140–8.
 51. Toldrà A, Alcaraz C, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, et al. Colorimetric DNA-based assay for the specific detection and quantification of *Ostreopsis cf. ovata* and *Ostreopsis cf. siamensis* in the marine environment. *Harmful Algae*. 2019 Apr;84:27–35.
 52. Nagai S, Miyamoto S, Ino K, Tajimi S, Nishi H, Tomono J. Easy detection of multiple *Alexandrium* species using DNA chromatography chip. *Harmful Algae*. 2016 Jan;51:97–106.
 53. Chen Q, Zhang C, Liu F, Ma H, Wang Y, Chen G. Easy detection of *karlodium veneficum* using PCR-based dot chromatography strip. *Harmful Algae*. 2020 Nov;99:101908.
 54. Fu M, Chen G, Zhang C, Wang Y, Sun R, Zhou J. Rapid and sensitive detection method for *Karlodinium veneficum* by recombinase polymerase amplification coupled with lateral flow dipstick. *Harmful Algae*. 2019 Apr;84:1–9.
 55. Fu M, Yang Y, Zhang C, Chen G, Wang Y. Recombinase polymerase amplification combined with lateral-flow dipstick for rapid detection of *Prorocentrum minimum*. *J Appl Phycol*. 2020;32(3):1837–50.
 56. Wang B, Zhang C, Liu F, Li R, Wang Y, Chen G. Development of a recombinase polymerase amplification combined with lateral flow dipstick assay for rapid and sensitive detection of *Heterosigma akashiwo*. *J Appl Phycol*. 2021 Oct 1;33:1–14.
 57. Liu F, Chen G, Zhang C, Wang Y, Zhou J. Exponential rolling circle amplification coupled with lateral flow dipstick strips as a rapid and sensitive method for the field detection of *Karlodinium veneficum*. *J Appl Phycol*. 2019 Aug 1;31.
 58. Qin Y, Zhang C, Liu F, Chen Q, Yang Y, Wang Y, et al. Establishment of double probes rolling circle amplification combined with lateral flow dipstick for rapid detection of *Chattonella marina*. *Harmful Algae*. 2020 Jul;97:101857.
 59. Zhang C, Chen G, Wang Y, Zhou J, Li C. Establishment and application of hyperbranched rolling circle amplification coupled with lateral flow dipstick for the sensitive detection of *Karenia mikimotoi*. *Harmful Algae*. 2019 Apr;84:151–60.
 60. Huang H-L, Zhu P, Zhou C-X, He S, Yan X-J. The development of loop-mediated

- isothermal amplification combined with lateral flow dipstick for detection of *Karlodinium veneficum*. *Harmful Algae*. 2017 Feb;62:20–9.
61. Zhu P, Huang H-L, Zhou C-X, Xu J, Qiao L-L, Dang C-Y, et al. Sensitive and rapid detection of *Prymnesium parvum* (Haptophyceae) by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Aquaculture*. 2019;505:199–205.
 62. Wang L, Chen G, Zhang C, Wang Y, Sun R. Application of loop-mediated isothermal amplification combined with lateral flow dipstick to rapid and sensitive detection of *Alexandrium catenella*. *Environ Sci Pollut Res Int*. 2020 Feb;27(4):4246–57.
 63. Qin Y, Chen G, Zhang C, Wang Y, Zhou J. Development of loop-mediated isothermal amplification combined with a chromatographic lateral-flow dipstick for rapid detection of *Chattonella marina*. *Harmful Algae*. 2019 Nov;89:101666.
 64. Huang H-L, Gao W-F, Zhu P, Zhou C-X, Qiao L-L, Dang C-Y, et al. Molecular method for rapid detection of the red tide dinoflagellate *Karenia mikimotoi* in the coastal region of Xiangshan Bay, China. *J Microbiol Methods*. 2020 Jan;168:105801.
 65. Wang L, Chen G-F, Zhang C-Y, Wang Y-Y, Sun R. Rapid and sensitive detection of *Amphidinium carterae* by loop-mediated isothermal amplification combined with a chromatographic lateral-flow dipstick. *Mol Cell Probes*. 2019 Feb;43:72–9.
 66. Huang H-L, Zhu P, Zhou C-X, Yan X-J, Zou Y-X, Lv P-W. Detection of *Skeletonema costatum* based on loop-mediated isothermal amplification combined with lateral flow dipstick. *Mol Cell Probes*. 2017 Dec;36:36–42.

Chapter 3: Capture, detection and purification of dsDNA amplicons using a DNA binding protein on magnetic beads

Analytical Biochemistry, Volume 658, 1 December 2022, 114923

3.1 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 primers 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.

3.2 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 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 (Figure 3.1A), 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.

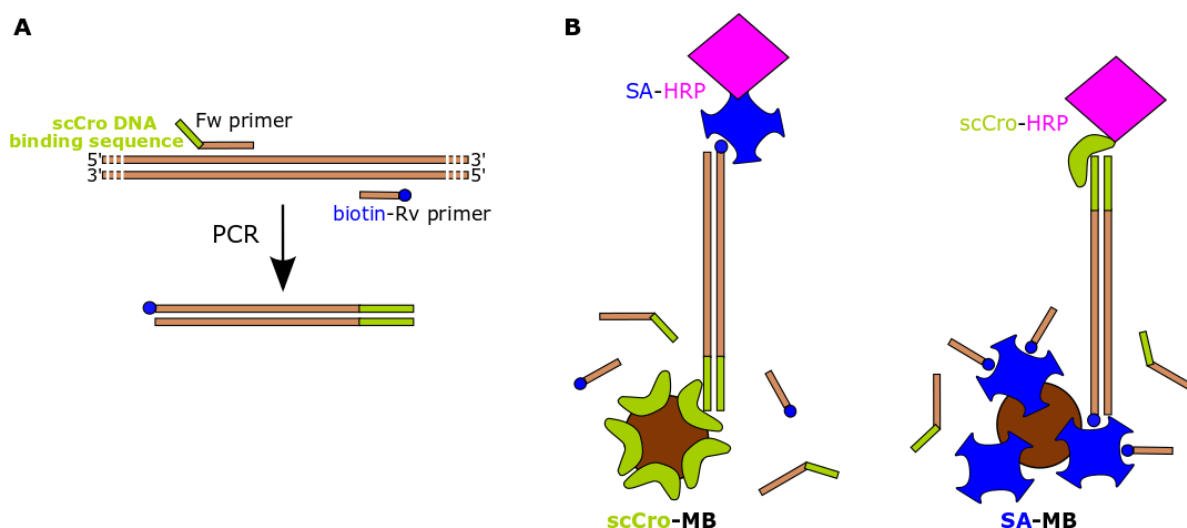


Figure 3.1 Amplicon detection using scCro DNA binding protein-magnetic beads. (A) PCR amplification of target DNA with modified primers. (B) Magnetic bead-based colorimetric assay using scCro-MB and SA-HRP for detection. For comparison, SA-MB were used in parallel as shown on the right.

3.3 Materials and methods

3.3.1 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 3.1. 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 3.1 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- <u>TTTTTTTTTT</u> GGTG GGTGTG GTTGGTTTTT <u>TTATCACCGC AAGTGATATT TTTATCACTT GCGGTGATA</u>	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 CTATTTTCATC CTCCTCCTC	159
Forward primer	<u>TATCACCGCA AGTGATAGCC</u> CATTAACAGG TCTTCCAA	38
Reverse primer	Biotin-GAGGAGGAGG ATGAAATAGA TGGT	24
Genomic DNA target: chicken cytochrome b gene		
Forward primer	<u>TATCACCGCA AGTGATAAATT</u> CCCTACATTG GACACA	36

3.3.2 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. The scCro-magnetic beads (2 μL of 30 mg/mL) were blocked for 30 min with 100 μL of 5 % (w/v) non-fat dry milk in PBST at 22°C under rotation, followed by incubation with a mouse monoclonal anti-His tag antibody diluted 1/3000 in 1 % (w/v) milk in PBST for 30 min. After washing three times with 100 μL of PBST, the beads were resuspended in 100 μL of rabbit anti-mouse IgG-HRP (diluted 1/10,000 in PBST) followed by another 30 min incubation. Finally, after washing three times with 100 μL of PBST, the beads were resuspended in 100 μL of TMB substrate and color development was stopped after 5 min with the addition of an equal volume of 1 M of sulfuric acid. The supernatant was transferred to the wells of a 96-well plate and the absorbance was recorded at 450 nm. Control magnetic beads (control-MB, without scCro) prepared simultaneously with scCro-MB were used as the negative control of the bead-ELISA.

3.3.3 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 3.1). 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.

3.3.4 Determination of the specific activity of the scCro-HRP and SA-HRP enzyme conjugates

The enzymatic activity of the HRP enzyme conjugates was determined using ABTS as the substrate. The substrate solution containing 0.4 mM of ABTS and 0.025 % (v/v) of hydrogen peroxide in 10 mM potassium phosphate pH 6 was added to the wells of a microtiter plate (118 µL per well) and 2 µL of 500 nM each enzyme conjugate (scCro-HRP or SA-HRP) were added. The absorbance at 415 nm was recorded every 15 sec for a total of 5 min. One Unit was defined as the amount of enzyme required to oxidize 1 µmole of ABTS per min at pH 6 at 22°C. The specific activities were thus calculated from the slopes of absorbance versus time for each enzyme/enzyme conjugate using the extinction coefficient of the colored product of the

enzymatic reaction (oxidized ABTS). Triplicate measurements were acquired to calculate the specific activities of the conjugates.

3.3.5 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 3.1) 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 μ L) contained equal concentrations of the two primers ranging from 25 to 1600 nM, 200 μ M of dNTPs, 1x DreamTaq polymerase buffer, 1 U of DreamTaq DNA polymerase and up to 500 pg/ μ L 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 sec at 95°C, 10 sec at 56°C and 10 sec 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 sec 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 μ g of each type (1 μ L of 30 mg/mL for scCro-MB or 3 μ L of 10 mg/mL for SA-MB) were blocked with 100 μ L of 5 % (w/v) milk in PBST for 30 min at 22°C under rotation, followed by washing (3 x 100 μ L of PBST). The beads were resuspended in 50 μ L of 1/10 diluted PCR reaction in PBS and incubated for another 30 min. After washing (3 x 100 μ L of PBST), the magnetic beads were resuspended in 50 μ L 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 x 100 μ L of PBST), 50 μ L of TMB substrate were added and color development proceeded for 2 min. Then, 50 μ L 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.

3.3.6 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/ μ L) was amplified as detailed in section 3.3.5 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/ μ L) 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.

3.3.7 Sensitivity and precision 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 – 500 pg/ μ L for the synthetic DNA and 10 – 500 pg/ μ L for genomic DNA. Each template was amplified using its respective modified primers (Table 3.1). After amplification, the PCR reactions were diluted 10-fold and detection was performed as described in section 3.3.5. 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}). The reproducibility and repeatability of the assay were also assessed when high or low concentration of template DNA was used for PCR amplification prior to amplicon detection. For the reproducibility, the intra-assay coefficients of variation (% CV) were determined by analyzing triplicate samples. For repeatability, the inter-assay % CVs were calculated for triplicate samples analyzed on four independent experiments.

3.3.8 Capture and release of PCR amplicons using scCro-MB

PCR was performed as detailed in section 3.3.5 using 20 pg/ μ L of the synthetic DNA target. Then, the PCR reaction was diluted in PBS to a final volume of 50 μ L (dilution 1/25) and was incubated with 5 μ L of scCro-MB for 30 min at 22°C under rotation. The unbound fraction was recovered and then 50 μ L 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.

3.4 Results and discussion

3.4.1 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 (Figure 3.1A). 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 (Figure 3.1B). 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 (Figure 3.1B), therefore reducing the efficiency of amplicon detection. A purification step would thus be necessary to remove excess primers and ensure high sensitivity of detection.

3.4.2 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 3.3.2 and the presence of the protein on the surface of the MB was evaluated with a bead-ELISA. As shown in Figure 3.2, scCro was successfully detected on the surface of the magnetic beads using an anti-His tag antibody, thus verifying the successful immobilization.

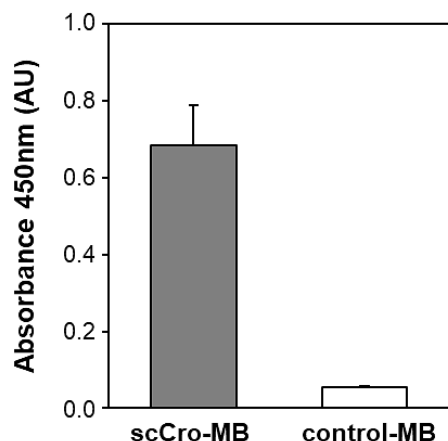


Figure 3.2 Confirmation of the immobilization of scCro on magnetic beads by bead-ELISA.

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 Figure 3.3A, the scCro-MB exhibited a binding capacity of 181 ± 36 pmol per 1 mg beads, two-fold higher than the SA-MB ones which were able to bind approximately 88 ± 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 section 3.2.4 and the SA-HRP conjugate was found to exhibit approximately 90 % of the activity of the scCro-HRP one (Table 3.2).

Table 3.2 Specific activity of the scCro-HRP and SA-HRP enzyme conjugates using ABTS as the substrate.

Enzyme conjugate	Specific activity (U/mL)
scCro-HRP	9.5 ± 0.4
SA-HRP	8.5 ± 0.3

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 Figure 3.3B, 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.

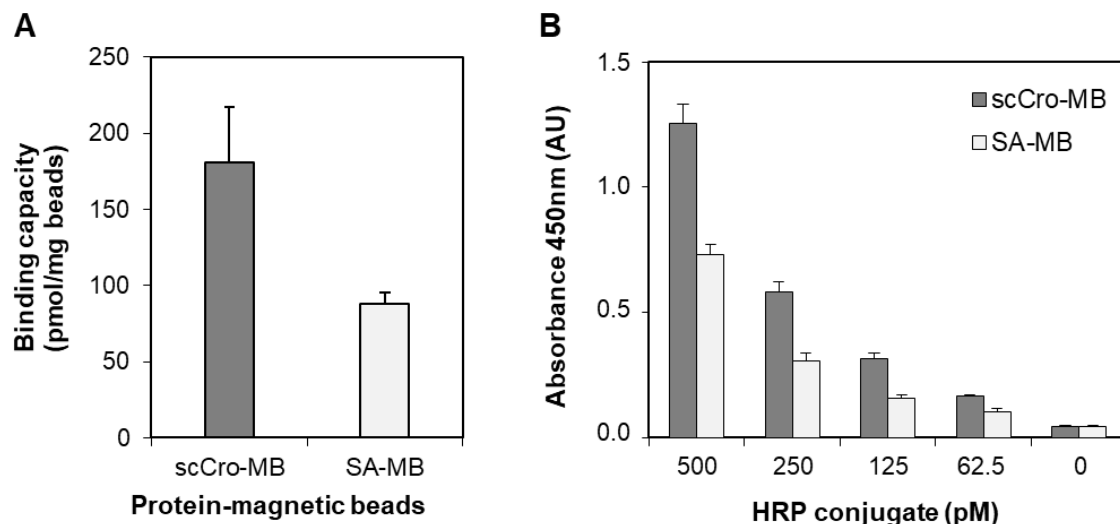


Figure 3.3 Evaluation of the binding capacity of the protein-magnetic beads using a biotinylated hairpin DNA probe containing the dsDNA binding site for scCro. (A) Determination of the amount of the probe binding directly to the beads [input(ng DNA) - unbound(ng DNA)]. (B) Detection of the probe binding to the beads using HRP conjugates (SA-HRP for the scCro-MB and scCro-HRP for the SA-MB).

3.4.3 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 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). The reactions were analyzed by agarose gel electrophoresis as shown in Figure 3.4 and the intensity of the bands corresponding to the amplicons was semi-quantified using the ImageJ software. As expected, the amount of amplicon increased with increased primer concentration.

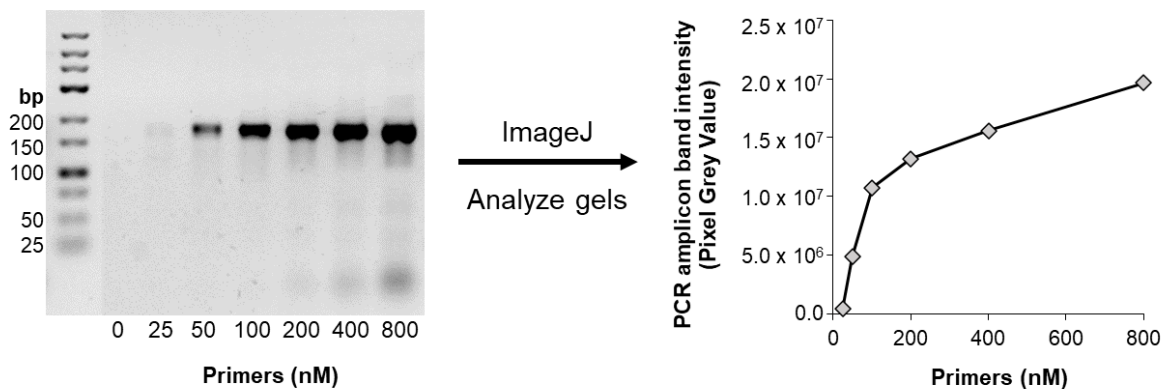


Figure 3.4 Amplification of target DNA (500 pg/ μ L) using 25 – 800 nM of each primer. The bands corresponding to the PCR amplicons were semi-quantified with the ImageJ software and the “Analyze gels” option.

Then, 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 Figure 3.5A, 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 (Figure 3.4), 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.

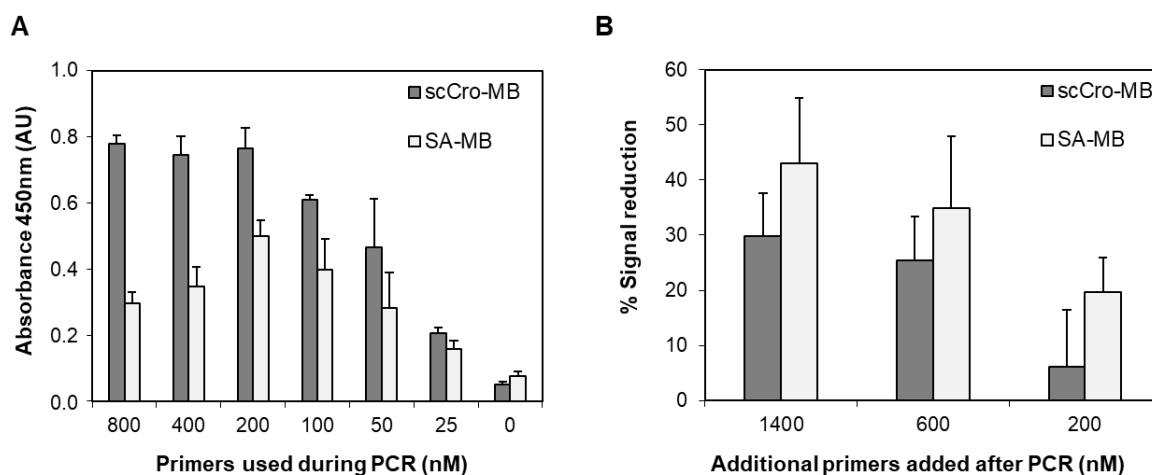
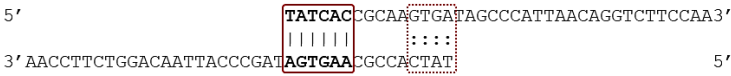


Figure 3.5 Effect of residual primers on the efficiency of PCR amplicon detection using protein-magnetic beads. (A) PCR was performed using 25 – 800 nM primers. (B) Post-PCR addition of primers (200, 600 or 1400 nM) to the diluted PCR reaction prior to detection.

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 Figure 3.5B, 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 (Figure 3.6), 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.

Primer self-dimer

($\Delta G = -7.31$ kcal/mole)



Primer hairpin

($\Delta G = -5.14$ kcal/mole)

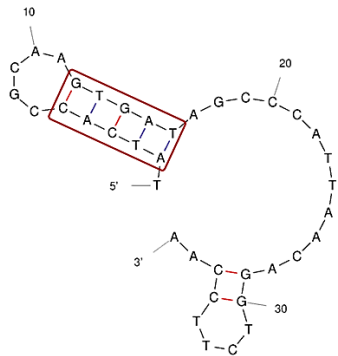


Figure 3.6 Possible structures adopted by the primer containing the scCro binding site contributing to undesirable binding to the scCro-MB.

3.4.4 Sensitivity and precision of the assay for target DNA detection

The analytical sensitivity of the assay for DNA capture and subsequent detection was evaluated. PCR reactions were performed using different concentrations of synthetic or genomic DNA for amplification (2 – 500 pg/ μ L, equivalent to 0.04 – 9.69 fmol/ μ L) as shown in Figure 3.7 after agarose gel electrophoresis.

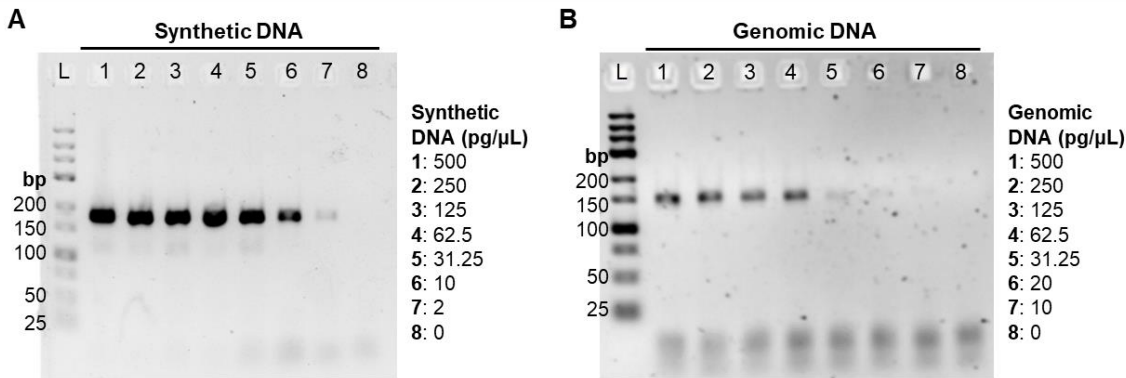


Figure 3.7 Analysis of PCR reactions performed with (A) synthetic DNA and (B) genomic DNA templates in combination with primers containing the scCro DNA binding protein and biotin by agarose gel electrophoresis.

Colorimetric detection of the amplicons generated using the synthetic DNA target for PCR amplification employing the protein-magnetic beads and reporter HRP enzyme conjugates was then performed and the calibration curves constructed are shown in Figure 3.8A. Higher

sensitivity was achieved with the scCro-MB, and the limit of detection calculated was 0.30 pg/ μ L (6.1 pM). This was 9-fold lower than the one achieved with the SA-MB (2.81 pg/ μ L; 57.2 pM).

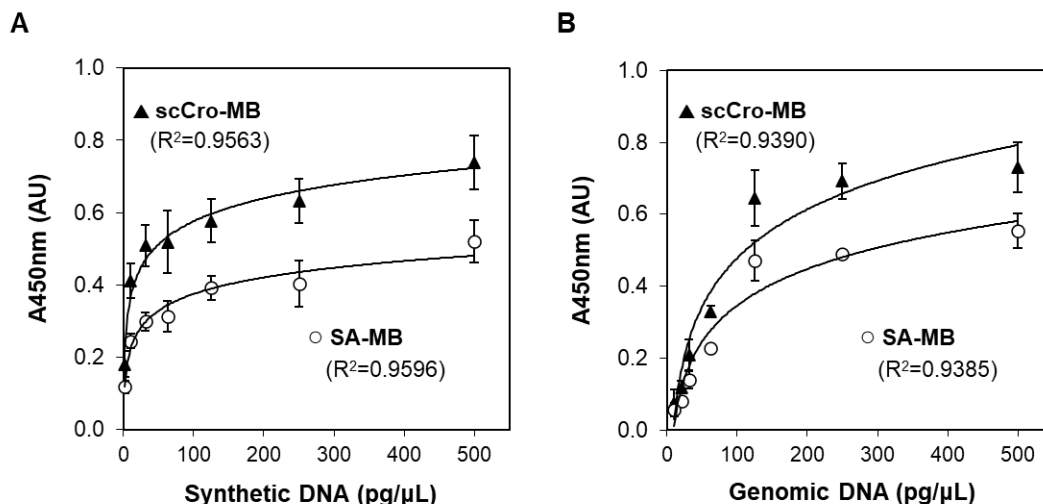


Figure 3.8 Detection of PCR amplicons by a magnetic bead-based colorimetric assay using scCro DNA binding protein or SA. (A) Synthetic DNA and (B) genomic DNA were used as templates for PCR amplification. The error bars correspond to the standard deviation of at least triplicate samples.

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 3.3, 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.

Table 3.3 Precision of the assay for DNA detection when high or low concentrations of template DNA were used for PCR amplification prior to capture on the protein-magnetic beads.

Sample	Reproducibility (intra-assay % CV, $n=3$)		Repeatability (inter-assay % CV, $n=4$)	
	scCro-MB	SA-MB	scCro-MB	SA-MB
High [DNA]	7.7	5.8	10.1	12.4
Low [DNA]	8.9	1.1	11.7	8.6

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 (Figure 3.7B) 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 (Figure 3.8B). 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/ μ L genomic DNA when the scCro-MB were used for detection and 14.6 pg/ μ L for the SA-MB. These correspond to 56.2 and 73.1 pg of genomic DNA template respectively considering that only 5 μ L 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, demonstrating the versatility of the proposed assay and its potential use for amplicon detection regardless of the nature of the template.

3.4.5 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 Figure 3.9. 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.

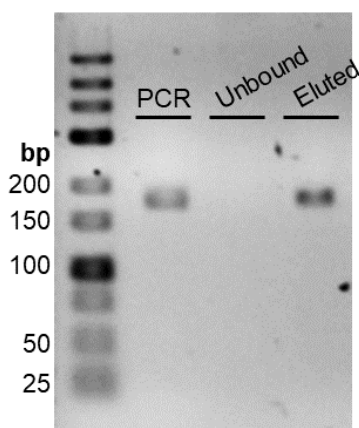


Figure 3.9 Purification of PCR amplicon using scCro-MB. PCR: PCR reaction diluted with PBS before addition to the beads; Unbound: unbound fraction after incubation with the beads; Eluted: fraction eluted with 1 M NaCl after incubation with the beads.

3.5 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.

3.6 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.

3.7 References

1. Lee SH, Park S, Kim BN, Kwon OS, Rho W-Y, Jun B-H. Emerging ultrafast nucleic acid amplification technologies for next-generation molecular diagnostics. *Biosens Bioelectron.* 2019;141:111448.
2. Ballard Z, Ozcan A. Nucleic acid quantification in the field. *Nat Biomed Eng.* 2018;2(9):629–30.
3. Chiu RWK, Lo YMD, Wittwer CT. *Molecular diagnostics: a revolution in progress.* Oxford University Press; 2015.
4. Oliveira BB, Veigas B, Baptista PV. Isothermal amplification of nucleic acids: The race for the next “gold standard.” *Front Sensors.* 2021;2:752600.
5. Mauk MG, Song J, Liu C, Bau HH. Simple approaches to minimally-instrumented, microfluidic-based point-of-care nucleic acid amplification tests. *Biosensors.* 2018;8(1):17.
6. Wittmeier P, Hummel S. Agarose gel electrophoresis to assess PCR product yield: comparison with spectrophotometry, fluorometry and qPCR. *Biotechniques.* 2022;72(4):155–8.
7. Rifai N, Horvath AR, Wittwer CT, Park J. *Principles and applications of molecular diagnostics.* Elsevier; 2018.
8. Ma Y, Chen T, Iqbal MZ, Yang F, Hampp N, Wu A, et al. Applications of magnetic materials separation in biological nanomedicine. *Electrophoresis.* 2019;40(16–17):2011–28.
9. Tang C, He Z, Liu H, Xu Y, Huang H, Yang G, et al. Application of magnetic nanoparticles in nucleic acid detection. *J Nanobiotechnology.* 2020;18(1):1–19.
10. Kim S-E, Tieu M Van, Hwang SY, Lee M-H. *Magnetic particles: Their applications from*

- sample preparations to biosensing platforms. *Micromachines*. 2020;11(3):302.
11. Chen Y-T, Kolhatkar AG, Zenasni O, Xu S, Lee TR. Biosensing using magnetic particle detection techniques. *Sensors*. 2017;17(10):2300.
 12. Parham NJ, Picard FJ, Peytavi R, Gagnon M, Seyrig G, Gagné PA, et al. 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*. 2007;53(9):1570–6.
 13. Archer MJ, Long N, Lin B. Effect of probe characteristics on the subtractive hybridization efficiency of human genomic DNA. *BMC Res Notes*. 2010;3(1):1–5.
 14. Low K-F, Rijiravanich P, Singh KKB, Surareungchai W, Yean CY. An electrochemical genosensing assay based on magnetic beads and gold nanoparticle-loaded latex microspheres for vibrio cholerae detection. *J Biomed Nanotechnol*. 2015;11(4):702–10.
 15. Toldrà A, Alcaraz C, Diogène J, O’Sullivan CK, Campàs M. Detection of *Ostreopsis cf. ovata* in environmental samples using an electrochemical DNA-based biosensor. *Sci Total Environ*. 2019;689:655–61.
 16. Chen J, Johnson R, Griffiths M. Detection of verotoxigenic *Escherichia coli* by magnetic capture-hybridization PCR. *Appl Environ Microbiol*. 1998;64(1):147–52.
 17. Lermo A, Liébana S, Campoy S, Fabiano S, García MI, Soutullo A, et al. A novel strategy for screening-out raw milk contaminated with *Mycobacterium bovis* on dairy farms by double-tagging PCR and electrochemical genosensing. *Int microbiol*. 2010;13(2):91–7.
 18. Chang D, Tram K, Li B, Feng Q, Shen Z, Lee CH, et al. Detection of DNA Amplicons of Polymerase Chain Reaction Using Litmus Test. *Sci Rep*. 2017;7(1):3110.
 19. Kyaw SP, Hanthamrongwit J, Jangpatarapongsa K, Khaenam P, Leepiyasakulchai C. Sensitive detection of the IS 6110 sequence of *Mycobacterium tuberculosis* complex based on PCR-magnetic bead ELISA. *RSC Adv*. 2018;8(59):33674–80.
 20. Smith CL, Milea JS, Nguyen GH. Immobilization of nucleic acids using biotin-strept (avidin) systems. *Immobil DNA Chips II*. 2005;63–90.
 21. Sassolas A, Leca-Bouvier BD, Blum LJ. DNA biosensors and microarrays. *Chem Rev*. 2008;108(1):109–39.

22. Haun M, Wasi S. Biotinylated antibodies bound to streptavidin beads: a versatile solid matrix for immunoassays. *Anal Biochem.* 1990;191(2):337–42.
23. Paul A, Avci-Adali M, Ziemer G, Wendel HP. Streptavidin-coated magnetic beads for DNA strand separation implicate a multitude of problems during cell-SELEX. *Oligonucleotides.* 2009;19(3):243–54.
24. Wilson R. Preparation of single-stranded DNA from PCR products with streptavidin magnetic beads. *Nucleic Acid Ther.* 2011;21(6):437–40.
25. Jana R, Hazbun TR, Fields JD, Mossing MC. Single-Chain Lambda Cro Repressors Confirm High Intrinsic Dimer-DNA Affinity. *Biochemistry.* 1998;37(18):6446–55.
26. Aktas GB, Wichers JH, Skouridou V, van Amerongen A, Masip L. Nucleic acid lateral flow assays using a conjugate of a DNA binding protein and carbon nanoparticles. *Mikrochim Acta.* 2019 Jun;186(7):426.
27. Aktas GB, Ribera A, Skouridou V, Masip L. DNA immobilization and detection using DNA binding proteins. *Anal Bioanal Chem.* 2021;413(7):1929–39.
28. Ginés I, Gaiani G, Ruhela A, Skouridou V, Campàs M, Masip L. Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*. *Harmful Algae.* 2021;110:102135.
29. Civit L, Fragoso A, Hölters S, Dürst M, O’Sullivan CK. Electrochemical genosensor array for the simultaneous detection of multiple high-risk human papillomavirus sequences in clinical samples. *Anal Chim Acta.* 2012;715:93–8.
30. Skouridou V, Tomaso H, Rau J, Bashammakh AS, El-Shahawi MS, Alyoubi AO, et al. Duplex PCR-ELONA for the detection of pork adulteration in meat products. *Food Chem.* 2019;287:354–62.
31. Aktas GB, Skouridou V, Masip L. Novel signal amplification approach for HRP-based colorimetric genosensors using DNA binding protein tags. *Biosens Bioelectron.* 2015;74:1005–10.
32. Hermanson GT. *Bioconjugate techniques.* Academic press; 2013.
33. Berg Luecke L, Gundry RL. Assessment of streptavidin bead binding capacity to improve quality of streptavidin-based enrichment studies. *J Proteome Res.* 2020;20(2):1153–64.

Chapter 4: Universal and label-free detection of dsDNA using the HU DNA binding protein

4.1 Abstract

A novel single-chain heterodimeric format of the HU DNA binding protein with no sequence specificity is demonstrated for the universal capture and detection of double-stranded DNA (dsDNA) PCR amplicons. A comprehensive account of the design and preparation of the expression construct is provided, as well as the purification process. To enhance the detection capabilities of scHupBA-based assays for dsDNA detection, a horseradish peroxidase (HRP) conjugate was developed as a reporter molecule and the design, preparation, and purification of this conjugate is also provided. The binding properties of scHupBA were evaluated on microtiter plates, which demonstrated the high binding affinity of this protein for dsDNA, especially for longer dsDNA fragments (> 50 bp), with affinity dissociation constant values in the low nanomolar range. Its affinity for ssDNA was considerably lower, suggesting its suitability for dsDNA detection, without the potential interference from ssDNA. Based on these observations, dsDNA sandwich detection assays were designed exploiting microtiter plates and magnetic beads for immobilization of scHupBA to capture a purified dsDNA PCR amplicon, while the scHupBA-HRP conjugate was used for colorimetric detection. Higher sensitivity was observed under low salt conditions, consistent with the salt-dependent binding of this protein to dsDNA, and with low interference from ssDNA. Furthermore, the compatibility of a scHupBA/scHupBA sandwich system with lateral flow assays was demonstrated using a scHupBA-carbon nanoparticles conjugate for detection. Finally, all three assays were validated for the detection of dsDNA amplicons directly in PCR reactions without prior purification, which validated the absence of interference from remaining ssDNA primers. Especially the dipstick lateral flow assay was able to detect specifically dsDNA amplicon in a PCR reaction performed with as low as 25 fg/ μ L of target template in a simple (1-step) and fast (< 20 min) way. To the best of our knowledge, this is the first report of utilization of non-sequence specific dsDNA binding protein for universal DNA biosensing. This approach eliminates the need for labeling or the use of expensive reagents and materials, making it a cost-effective and efficient method for DNA detection.

4.2 Introduction

Molecular diagnostics have enabled rapid and accurate detection of diseases and genetic disorders and also facilitate environmental and food safety surveillance. It is a rapidly evolving field that involves the detection and quantification of biological molecules, such as DNA,

RNA, and proteins (1-3). In particular, the detection of double-stranded DNA (dsDNA) has found various applications in clinical diagnostics (4, 5), environmental monitoring (6), and food safety (7, 8). These biosensors utilize the principles of molecular recognition and signal transduction to provide sensitive and specific detection of target DNA molecules (9). They are typically used to detect DNA after amplification (10) but also non-amplified DNA (11).

Initially, DNA biosensing of amplification products was based exclusively on the detection of polymerase chain reaction (PCR) amplicons since PCR was the gold standard in DNA amplification (12). This changed in the early 2000's when alternative isothermal amplification techniques emerged (13). While PCR requires a dedicated instrument (thermal cycler) equipped with a Peltier module to rapidly heat and cool at different temperatures in a repetitive manner, isothermal amplification can be performed at a constant temperature utilizing simple heating elements. The most representative methods of isothermal amplification are nucleic acid sequence-based amplification (NASBA) (14), loop-mediated isothermal amplification (LAMP) (15), strand displacement amplification (SDA) (16), recombinase polymerase amplification (RPA) (17) and rolling circle amplification (RCA) (18), each offering advantages while exhibiting certain drawbacks as well. Direct detection of non-amplified DNA is also attractive since it circumvents amplification and potentially labeling as well, thus lowering the overall cost of detection. Indeed, detection of non-amplified genomic DNA has been documented, even though to a lesser extent compared to amplified DNA, and novel nanomaterial and signal amplification strategies are typically employed to achieve the required sensitivity without amplification (19, 20). Hybridization chain reaction, an isothermal amplification-free method, can be alternatively used for highly sensitive detection of DNA. It employs two metastable hairpin DNA probes which hybridize between them only in the presence of the target sequence which acts as the initiator of a hybridization cascade reaction between the two probes. This results in the generation of nicked double stranded DNA molecule with many repeating units (21).

DNA amplicons and genomic DNA can be detected using UV-Vis spectroscopy and DNA stains (22) but neither approach can provide information on specific DNA sequences. This is achieved using biosensors and bioanalytical assays which incorporate target-specific biorecognition elements. Many types of DNA biosensors have been developed for this purpose, and the most common ones are optical and electrochemical ones (23-26). Nucleic acid lateral flow tests (27, 28) are another type of biosensors used for colorimetric or fluorescence detection of DNA (29)

and are considered especially suitable for point-of-care (POC) testing as they fulfil the REASSURED criteria for ideal POC tests (30).

DNA binding proteins are proteins which recognize and bind single stranded (ssDNA) or double stranded DNA (dsDNA) in a general (non-sequence specific) (31, 32) or sequence-specific (33) manner, and they play crucial roles in various cellular processes, including transcription, replication, repair, and recombination. Despite the existence of a variety of DNA binding proteins with different properties, only a handful have been exploited in DNA biosensors as biorecognition elements to detect DNA. Specifically, the *Escherichia coli* single stranded DNA binding protein SSB has been used for total ssDNA detection (34-36), whereas the sequence-specific dsDNA binding proteins p53, the SpoIIID transcription factor from *Bacillus subtilis*, the Cro bacteriophage lambda repressor and the *E. coli* LacI repressor protein headpiece domain (dHP) have been utilized for specific DNA/RNA target detection (36-38). The latter two sequence-specific DNA binding proteins have been used as engineered single chain variants by our group for the colorimetric detection of ssDNA and dsDNA. For those studies, it was necessary to incorporate the specific DNA binding sites for these proteins in either detection probes or in primers for ssDNA or PCR amplicon detection, respectively. Specifically, scCro and dHP were conjugated to the signal-generating enzymes horseradish peroxidase (HRP) and glucose oxidase (GOX), respectively and were used to detect a target ssDNA sequence through their binding to hybrid ssDNA-dsDNA detection probes containing the target-specific ssDNA sequence and the dsDNA sites for the DNA binding proteins (39). Alternatively, the binding sites of scCro and dHP were incorporated in PCR primers, allowing the direct amplicon capture and detection using DNA binding protein enzyme or nanoparticle conjugates (38, 40).

In this work, we aim to demonstrate the use of a non-sequence specific dsDNA binding protein for universal and label-free detection of DNA, which is expected to be compatible with amplicons generated with thermal or isothermal methods. Specifically, herein we report the engineering of a single-chain heterodimeric variant of the histone-like (Hup) protein from *Escherichia coli* by fusing its α and β monomers through a flexible amino acid linker. The resulting scHupBA protein was prepared recombinantly in *E. coli* and its DNA binding properties were characterized by an enzyme-linked oligonucleotide assay (ELONA). Finally, scHupBA was immobilized to different surfaces (microtiter plates, magnetic beads, nitrocellulose membrane, carbon nanoparticles) and an enzyme (HRP) and different assays were developed for target DNA capture and detection as illustrated graphically in Figure 4.1.

Overall, with this study we seek to highlight the great potential of non-sequence specific dsDNA binding proteins as universal anchors and reporters for label-free detection of DNA amplicons.

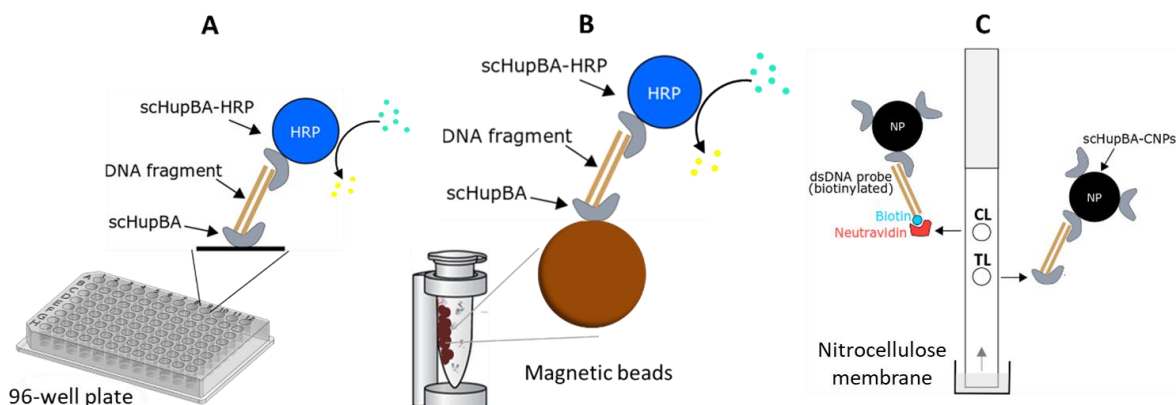


Figure 4.1 Schematic representation of ELONA for dsDNA fragment detection using scHupBA and scHupBA-HRP and (A) microtiter plates, (B) magnetic beads and (C) paper-based lateral flow dipstick assay.

4.3 Materials and methods

4.3.1 Materials

Phosphate buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4), the TransformAid Bacterial Transformation Kit, the GeneJET Plasmid Miniprep Kit, PD-10 desalting columns, PES protein concentrators 3 K MWCO (2 – 6 mL), Zeba Micro Spin desalting column 7 K MWCO, the Micro BCA Protein Assay Kit, HisPure Ni-NTA agarose, SP Sepharose Fast Flow resin, Con A Sepharose 4B resin, horseradish peroxidase (HRP), maleimide activated HRP, skim milk powder, the Dynabeads M-270 Epoxy and Dynabeads MyOne Streptavidin T1 magnetic beads (SA-MB), DreamTaq DNA polymerase, dNTPs and N-succinimidyl S-acetylthioacetate (SATA) were purchased from Fisher Scientific (Spain). Streptavidin-polyHRP80 conjugate (SA-pHRP) was obtained from SDT (Germany), the Zymo Research DNA Clean & Concentrator-5 kit from Ecogen (Spain), the BioFX TMB Super Sensitive One Component HRP Microwell Substrate and BioFX TMB One Component HRP Membrane Substrate from Surmodics (USA). The DNA oligonucleotides were purchased from Biomers.net (Germany) and their sequences are shown in Table 4.1. All other reagents and material were obtained from Fisher Scientific (Spain), Scharlab (Spain) and Merck (Spain).

Table 4.1 Oligonucleotides used in this work. The asterisk (*) denotes oligonucleotides labeled with a 5' biotin.

Oligonucleotide	Sequence (5' – 3')	Length (nt)
hupB.LIC.Fw	AGAAGGAGATATAACTATGTGTAATAAATCTCAATTG ATCGACAAG	46
hupA.LIC.Rv	GTGGTGGTGATGGTGATGGCCCTTAACTGCGTCTTTCA GTGC	42
LIC.Fw	TAATACGACTCACTATAGGG	20
LIC.Rv	GAGCGGATAACAATTCACACAGG	24
Probe 25 *	CTCTCTCTCTCTCTCTCTCTCTCTC	25
Probe 25- complement	GAGAGAGAGAGAGAGAGAGAGAGAGAG	25
Probe 47 *	GTATATTATCACCGCAAGTGATACGACATGAATTGTGA GCGCTCACAATT	47
Probe 47- complement	AATTGTGAGCGCTCACAATTCATGTCGTATCACTTGCG GTGATAATA	47
Probe 100 *	TGACGAATATCAGGGCAAAGTACCGTTGCAAACTG AACATCGATCAAAACCCTGGCACTGCGCCGAAATATG GCATCCGTGGTATCCCGACTCTGCTG	100
Probe 100- complement	CAGCAGAGTCGGGATACCACGGATGCCATATTTTCGGC GCAGTGCCAGGGTTTTGATCGATGTTTCAGTTTTGCAAC GGTCAGTTTGCCCTGATATTCGTCA	100
Model target DNA (HPV16)	GCCCATTAACAGGTCTTCCAAAGTACGAATGTCTACGT GTGTGCTTTGTACGCACAACCGAAGCGTAGAGTCACAC TTGCAACAAAAGGTTACAATATTGTAATGGGCTCTGTC CGGTTCTGCTTGTCCAGCTGGACCATCTATTTTCATCCTC CTCCTC	159
HPV.Fw	TATCACCGCAAGTGATAGCCCATTAACAGGTCTTCCAA	38
HPV.Rv-bt *	GAGGAGGAGGATGAAATAGATGGT	24

4.3.2 Preparation of the scHupBA expression plasmid

The sequence of the schupBA gene was based on the UniProt accession IDs P0ACF0 (α monomer) and P0ACF4 (β monomer). The two genes (270 base pairs each) were fused using a 48 base pair linker which will result in a 16 amino acid linker (AAGGTGGGSGGGTGGS) linking the C-terminal of the β monomer with the N-terminal of the α monomer. The resulting 588 bp gene was synthesized by Eurofins Genomics (Germany). The gene was inserted into the pLATE31 vector using the primers hupB.LIC.Fw and hupA.LIC.Rv for amplification and the aLICator ligation independent cloning and expression system according to the instructions of the manufacturer. The resulting annealed mixture (pLATE31/schupBA) was transformed in

E. coli DH5a cells using the TransformAid bacterial transformation kit. Clones grown on LB/ampicillin agar plates were screened by colony PCR using the LIC.Fw/LIC.Rv primer pair and agarose gel electrophoresis for the presence of the expected 800 bp amplicon. Selected clones were grown in Luria-Bertani (LB) medium with ampicillin (100 µg/mL) and plasmids were extracted using the GeneJET Plasmid Miniprep Kit to verify the sequence by Sanger sequencing using primers LIC.Fw and LIC.Rv, which was performed by the Servei de Genòmica of the Universitat Autònoma de Barcelona.

4.3.3 Expression and purification of the scHupBA DNA binding protein

The pLATE31-schupBA expression vector was transformed in *E. coli* BL21(DE3) cells using the TransformAid bacterial transformation kit. One-two colonies were inoculated in Terrific broth (TB) medium with ampicillin (100 µg/mL), the culture was grown overnight at 37°C and 200 rpm and used to subculture 1 L of TB/ampicillin at an optical density (OD₆₀₀) of 0.05 in a 2 L baffled Erlenmeyer flask. The culture was grown at 37°C and 200 rpm until OD₆₀₀ of 1, at which point isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.25 mM to induce protein expression. The culture was incubated post-induction for 3 hours at 30°C and 200 rpm followed by cell harvesting using centrifugation for 10 min at 7000 rpm and 4°C. For lysis, the cell pellets were resuspended in ice-cold lysis buffer composed of modified PBS with 0.3 M NaCl (PBS*), 1 % v/v Triton X-100, 5 % v/v glycerol and 5 mM imidazole at 5 mL per gram of wet cell weight. The suspension was incubated for 30 min on ice with gentle agitation followed by sonication for 10 min on ice (60 % amplitude, cycles of 5 sec ON and 5 sec OFF). The lysate was centrifuged at 10,000 rpm for 10 minutes at 4°C, the soluble fraction (supernatant) was collected and finally filtered through a 0.45 µm PES filter. Batch purification of scHupBA was performed with gravity columns by a combination of immobilized metal affinity chromatography (IMAC) and ion exchange chromatography (IEC). For IMAC, 0.5 mL of HisPur Ni-NTA agarose resin was used per 1 L of bacterial culture. The resin was washed with 10 column volumes (CV) of milli-Q water and then equilibrated with 10 CV of PBS*/5 mM imidazole. The filtered soluble fraction containing scHupBA was added to the column and incubated for 30 min at 22°C with gentle rotation. The unbound fraction (flowthrough) was collected, and the column was successively washed with 20 CV of PBS with 1 M NaCl (wash 1) and 2 x 5 CV of PBS*/25 mM imidazole (wash 2 & wash 3). Finally, scHupBA was eluted with 2 x 5 CV of PBS*/500 mM imidazole. The IMAC elution fractions were combined, and the buffer was exchanged to IEC equilibration buffer (50

mM potassium phosphate pH 7, 1 mM EDTA, 5 mM DTT) using a PD-10 desalting column. For IEC purification, 0.5 mL of SP Sepharose FF resin was used per 1 L of bacterial culture. The column was initially washed with 10 CV of milli-Q water and then equilibrated with 10 CV of IEC equilibration buffer. The IMAC elution fraction in IEC equilibration buffer was loaded to the column and incubated for 30 min at 22°C with gentle agitation. The unbound fraction (flowthrough) was collected, the column was washed with 5 CV of IEC equilibration buffer containing 0.2 M NaCl and then eluted with 2 x 5 CV of IEC equilibration buffer containing 1 M NaCl. The IEC fractions containing highly pure scHupBA protein were combined, the buffer was exchanged to PBS using a PD-10 column and finally concentrated using a 3 K MWCO PES centrifuge filter. The purification process was monitored by SDS-PAGE and Western blot. For SDS-PAGE, 4-15% Mini-PROTEAN TGX precast protein gels were used and a PVDF membrane for Western blot. After SDS-PAGE, proteins were transferred from the gel to the membrane using the TransBlot Turbo transfer system (Biorad, Spain), which was then blocked with 5 % w/v skim milk in PBS with 0.05 % v/v Tween-20 (PBST), and successively probed with a mouse anti-His tag monoclonal antibody and a rabbit anti-mouse-HRP polyclonal antibody conjugate in 1 % w/v skim milk/PBST. The membrane was stained with the TMB One Component HRP Membrane Substrate solution for 5 min to reveal dark blue precipitates. For quantification of scHupBA, the Micro BCA Protein Assay Kit was used following product instructions employing BSA to construct the protein calibration curve. The expected size of scHupBA is approximately 21 kDa.

4.3.4 Preparation of the scHupBA-HRP conjugate

The conjugate was prepared using thiol-modified scHupBA and maleimide-activated HRP. To add thiol groups to scHupBA, the protein was dissolved in PBS at 1.25 mg/mL, SATA was added to a final concentration of 0.34 mg/mL (SATA/scHupBA molar ratio of 25:1) and the mixture was incubated for 30 min at 22°C. For deacetylation and deprotection of the SATA-modified (acetylated) scHupBA, hydroxylamine hydrochloride was added to a final concentration of 4.5 mg/mL in 0.1 M sodium phosphate pH 7.2, 10 mM EDTA and the mixture was incubated for 2 hours at 22°C. A PD MidiTrap G-25 desalting column was used to purify and elute the SATA-modified (deprotected) scHupBA with PBS, which was then concentrated with a 3K MWCO PES centrifuge filter. It was then mixed with maleimide-activated HRP (prepared in PBS) at a 1:1 molar ratio and the mixture was incubated overnight at 4°C under gentle rotation. The conjugate was purified by IMAC (removal of unconjugated HRP) and

concanavalin A affinity chromatography (ConA; removal of unconjugated scHupBA). For IMAC, 0.16 mL CV of HisPur Ni-NTA agarose resin was used for a conjugation reaction containing 1 mg of SATA-modified scHupBA and 2 mg of maleimide-activated HRP. The resin was initially washed with 10 CV of milli-Q water and 10 CV of IMAC equilibration buffer (modified PBS with 0.3 M NaCl), the conjugation mixture was added and incubated with the resin for 1 h at 22°C under rotation. The unbound fraction (flowthrough) was collected, and the column was washed with 10 CV of IMAC equilibration buffer containing 25 mM imidazole. Proteins were finally eluted with 15 CV of IMAC equilibration buffer containing 250 mM imidazole. The buffer of the solution was exchanged to ConA equilibration/wash buffer (20 mM Tris-HCl pH 7, 0.5 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂) using a Zeba Micro Spin desalting column (7 K MWCO) and was applied to a gravity column containing 0.32 mL CV of Con A Sepharose 4B resin washed with 10 CV of milli-Q water and 10 CV of ConA equilibration buffer. After 1 h at 22°C under mild rotation, the unbound fraction (flowthrough) was collected, the column was washed with 5 CV of ConA wash buffer (20 mM Tris-HCl pH 7, 0.5 M NaCl) and the conjugate was eluted with 20 CV of ConA elution buffer (20 mM Tris-HCl pH 7, 0.5 M NaCl, 0.38 M methyl- α -D-mannopyranoside). The entire process was monitored by SDS-PAGE and the scHupBA-HRP conjugate was quantified using the molar extinction coefficient of HRP at 403 nm ($\epsilon^{403\text{nm}}=102000 \text{ M}^{-1}\text{cm}^{-1}$). The expected size of a scHupBA-HRP conjugate at a 1:1 stoichiometry is approximately 64 kDa.

4.3.5 Immobilization of scHupBA on magnetic beads

Dynabeads M-270 Epoxy magnetic beads were used to immobilize scHupBA. To this end, 100 μL of the beads (30 mg/mL in dimethylformamide) were washed with 100 mM sodium phosphate buffer at pH 7.4 (3 x 100 μL) and were resuspended in 60 μL of 1.25 mg/mL protein in PBS. Then, 60 μL of 100 mM sodium phosphate buffer pH 7.4 and 60 μL of 3 M ammonium sulfate were added, followed by overnight incubation at 18°C under rotation. Afterwards, the supernatant was removed using a magnetic separator, and the beads were resuspended in 200 μL of 1 M Tris-HCl at pH 9 for blocking any remaining epoxy groups. The beads were incubated for 2 h at 18°C under rotation, washed with PBST (4 x 300 μL) and finally resuspended in 100 μL of PBS. In parallel, control beads were prepared following the same steps but by replacing the protein solution with PBS. The scHupBA-MB beads (30 mg/mL in PBS) were stored at 4°C until use. A colorimetric bead-ELISA was performed to verify the immobilization of scHupBA on the beads. Specifically, 2 μL of each bead type (with and

without protein) were first blocked with 100 μ L of 5 % w/v skim milk in PBST for 30 min under rotation, followed by washing with PBST (3 x 100 μ L). Then, the beads were incubated with 100 μ L of a mouse anti-His tag monoclonal antibody solution (1 μ g/mL in 1 % w/v skim milk/PBST) for 30 min under rotation, washed again with PBST (3 x 100 μ L) and resuspended with 100 μ L of rabbit anti-mouse-HRP conjugate (1 μ g/mL in 1 % w/v skim milk/PBST). After an incubation of 30 min under rotation, the beads were washed with PBST (3 x 100 μ L) and resuspended with 100 μ L of TMB microwell substrate. Color development was stopped after 5 min by adding 100 μ L of 1 M sulfuric acid. Using the magnetic separator, the colored solution was separated from the beads and transferred to the wells of a 96-well plate to measure the absorbance at 450nm. The amount of scHupBA on the magnetic beads was estimated by comparing the signal obtained from the binding of an anti-His tag antibody to different amounts of the protein immobilized on microtiter plate wells to the signal on the magnetic beads.

4.3.6 Preparation of dsDNA and ssDNA probes for binding studies

The dsDNA probes used for the experiments were prepared by hybridization of two complementary ssDNA probes with a length of 25, 47 and 100 nucleotides. Specifically, each ssDNA probe was mixed with its complementary strand at a 1:1 molar ratio in PBS (e.g. Probe 25 with Probe 25-complement), incubated for 5 min at 95°C and then gradually cooled to room temperature. Hybridization was verified by agarose gel electrophoresis. A model PCR amplicon was used to evaluate the assays. For the amplicon preparation, the model target (high risk human papillomavirus 16; HPV16) with a length of 159 nt was used at 25 pg/mL and amplified by PCR using HPV.Fw/HPV.Rv-bt primers and the following program: 3 min at 95°C, followed by 30 cycles of 10 sec at 95°C, 10 sec at 58°C and 10 sec at 72°C and a final step of 5 min at 72°C. The PCR amplicon was purified using the DNA Clean & Concentrator-5 kit. To prepare ssDNA, the purified PCR amplicon was diluted with PBS to a final concentration of 40 ng/ μ L and it was mixed with streptavidin-magnetic beads (20 μ L of 10 mg/mL per 50 μ L of diluted PCR amplicon) which were previously washed with PBS (3 x 100 μ L). The mixture was incubated for 30 min at 22°C under rotation, the supernatant was decanted, the beads were washed with PBS (3 x 100 μ L) and the non-biotinylated ssDNA strand was finally eluted with 20 μ L of 30 mM NaOH after a 10 min incubation. When PCR reactions were analyzed directly without prior amplicon purification, the dsDNA target template (HPV16) was used at 2.5 fg/mL to 25 pg/mL.

4.3.7 Characterization of the binding properties of scHupBA by ELONA

To evaluate the concentration of scHupBA required to coat the microtiter plate wells, an ELISA assay was initially performed. Specifically, the protein was used at 0.6 – 40 $\mu\text{g/mL}$ (50 μL in 5 mM carbonate-bicarbonate buffer pH 9.4) and it was incubated on Nunc MaxiSorp immuno 96-well plates for 1 h at 22°C. The wells were washed (3 x 200 μL of PBST), blocked with 200 μL of 5 % w/v skim milk/PBST for 30 min under mild agitation and the immobilized protein was detected with a mouse anti-His tag antibody (1 $\mu\text{g/mL}$ in 1 % w/v skim milk/PBST) in combination with a rabbit anti-mouse-HRP conjugate (1 $\mu\text{g/mL}$ in 1 % w/v skim milk/PBST) as described in section 4.2.5 for the bead-ELISA. To assess the DNA binding properties of scHupBA by ELONA, the protein was used at 10 $\mu\text{g/mL}$ to coat the wells followed by washing and blocking as described above. Then, biotinylated dsDNA or ssDNA probes of different lengths (25, 47 or 100 bases) were added to the wells (50 μL of 0 – 4000 nM in PBS) and incubated for 30 min under mild agitation. After incubation, the wells were washed again with PBST (3 x 200 μL) and 50 μL of SA-pHRP (1 $\mu\text{g/mL}$ in PBST) were added for another 30-min incubation. Finally, the wells were washed (5 x 200 μL of PBST), 50 μL of TMB microwell substrate were added and 5 min afterwards, the reaction was stopped with 50 μL of 1 M sulfuric acid and the absorbance was recorded at 450 nm. Duplicate values from three independent experiments were used for the calculation of the affinity dissociation constants (K_D values). Background-subtracted absorbance values were plotted against DNA concentration and the data was fitted to the “One site specific binding” model using GraphPad Prism for K_D determination.

4.3.8 Detection of dsDNA on microtiter plates using scHupBA and scHupBA-HRP

scHupBA was immobilized on Nunc MaxiSorp immuno 96-well plates for 1 h at 22°C using 50 μL of 10 $\mu\text{g/mL}$ of the protein in 5 mM carbonate-bicarbonate buffer pH 9.4. The wells were blocked with 200 μL of 5 % w/v skim milk/PBST for 30 min and washed with PBST (3 x 200 μL). Then, dsDNA or ssDNA fragments of different lengths (47, 100 and 176 bases) was added to the wells (50 μL in PBS) and incubated for 30 min. The plate was washed again with PBST and 50 μL of 5 $\mu\text{g/mL}$ scHupBA-HRP (in PBS) were added, followed by another 30 min incubation. Finally, the plate was washed (5 x 200 μL of PBST), 50 μL of TMB microwell substrate were added and signal was stopped after 15 min by adding 50 μL of 1 M sulfuric acid. Absorbance was recorded at 450 nm. When DNA fragments of different lengths and type (dsDNA or ssDNA) were used, the concentration of DNA was constant at 500 nM (~ 57 ng/ μL).

The effects of buffer composition (water, 0.1xPBS and PBS) and scHupBA-HRP concentration (0 – 20 µg/mL) on signal intensity were studied. To evaluate the sensitivity of the assay, the 176 bp model PCR amplicon was used at a concentration range of 0 – 1000 nM, equivalent to 0 – 115 ng/µL in phosphate buffer containing 30 – 240 mM of NaCl. The data were fitted to a logarithmic model and the limit of detection (LOD) was interpolated as the blank plus three times its standard deviation ($\text{blank} + 3 \times \text{SD}_{\text{blank}}$). All steps were performed at 22°C under mild agitation. Duplicate values from at least two independent experiments were used for these studies. In the case of direct analysis of unpurified PCR reactions, 10 µL of each PCR reaction performed using different amounts of target template (2.5 fg/mL – 25 pg/mL) were diluted with 40 µL of water and then added to the microplate wells containing scHupBA as detailed above.

4.3.9 Detection of dsDNA on magnetic beads using scHupBA and scHupBA-HRP

scHupBA-MB (1 µL of 30 mg/mL in PBS) were blocked with 100 µL of 5 % w/v skim milk/PBST for 30 min and then washed with PBST (3 x 100 µL). The beads were resuspended with 50 µL of PCR amplicon (176 bp) at a concentration range of 0 – 1000 nM in PBS (equivalent to 0 – 115 ng/µL) and incubated for 30 min. Subsequently, the beads were washed with PBS (3 x 100 µL) and scHupBA-HRP was added (50 µL of 5 µg/mL in PBS) for another 30 min incubation. Finally, the beads were washed (5 x 100 µL of PBS) and 50 µL of TMB microwell substrate were added. Signal generation was stopped after 5 min by the addition of 50 µL of 1 M sulfuric acid. The supernatants were separated from the magnetic beads and transferred to the wells of a 96-well plate to measure the absorbance at 450 nm. The effects of the type of blocking solution for the magnetic beads (5 % w/v skim milk or 1 % w/v BSA in PBST) and the buffer used for the incubations on signal intensity were evaluated. All incubation steps were performed at 22°C under mild rotation. Duplicate values from at least two independent experiments were used for these studies. When PCR reactions were analyzed directly without purification, these (5 µL) were diluted with 45 µL of water prior to incubation with the magnetic beads.

4.3.10 scHupBA-based lateral flow dipstick assay for dsDNA detection

In-house lateral flow dipstick strips were prepared using nitrocellulose membrane FF170HP (height 2.5 cm and width 0.4 cm). Initially, the control and test zones were constructed by manual deposition of the reagents on the nitrocellulose membrane using micropipette tips. scHupBA (0.5 µL of 0.5 mg/mL in PBS) were used to construct the test zone (height of 1 cm).

Neutravidin (0.1 mg/mL) was incubated for 30 min at 22°C with the biotinylated 100 bp-long dsDNA probe (35 μ M) in PBS and 0.5 μ L was spotted on the control zone (height 1.5 cm). When the ALFRD lateral flow reagent dispenser (ClaremontBio) was used for automatic dispensing, a flow rate of 0.25 mL/min was used at a speed of 50 mm/sec. For the control line, 0.76 mg/mL neutravidin was pre-incubated with 36 μ M of biotinylated dsDNA fragment of 100 bp length in PBS for 30 min prior to dispensing. For the test line, four different concentrations of HU protein solutions were used in the range of 0.61 - 1.1 mg/mL in PBS. All the strips were dried for 1 h at 37°C after dispensing, blocked for 15 min with 2 % skim milk and 0.1 % v/v Empigen BB in 10 mM carbonate buffer pH 9.4 and then dried again 1 h at 37°C. Finally, the strips were assembled on a backing card with an absorbent pad for wicking and stored at 4°C until use. The scHupBA-carbon nanoparticle conjugate employed for detection was prepared as described previously with some modifications (40). Briefly, a 100 μ L suspension of 0.2 % w/v CNPs suspension in 5 mM sodium borate pH 8.8 was sonicated for 5 min and scHupBA was added to a final concentration of 0.5 – 1 mg/mL. The mixtures were incubated overnight at 18°C under rotation and then BSA was added to a final 1 % w/v for blocking. The suspensions were incubated for 30 min at 22°C under rotation and then centrifuged for 30 min at 10,000 rpm at 6°C. The supernatants were decanted, the pellets were resuspended with 100 μ L of washing buffer (5 mM sodium borate pH 8.8, 1 % w/v BSA) and then centrifuged again. The washing step was repeated for two more times, the conjugates were finally resuspended in 100 μ L of storage buffer (100 mM sodium borate pH 8.8, 1 % w/v BSA) to 0.2 % w/v CNPs and stored at 4°C until use. For detection, the strips were first dipped in a well of a microtiter plate with 25 μ L of the DNA sample in running buffer. When all the solution was wicked through the strips, they were transferred to another well containing 1 μ L of scHupBA-CNPs and 24 μ L of running buffer. Alternatively, the DNA sample (25 μ L) was pre-incubated for 10 min at 22°C with the scHupBA-CNPs (1 μ L) in running buffer (24 μ L) before dipping the strips for analysis. For direct analysis of PCR reactions without prior purification, 5 μ L of the PCR reaction were pre-incubated for 10 min with 1 μ L of scHupBA-CNPs and 44 μ L of running buffer (0.1 % v/v Tween-20 and 0.5 % w/v skim milk in water) prior to dipping the strips. Carbon black signals were observed within 20 min of CNPs application. Different compositions of blocking and running buffers were evaluated, as well as concentrations of scHupBA used for immobilization on CNPs (0.35, 0.5, 0.75 and 1 mg/mL). A 176 nt-long DNA fragment (dsDNA or ssDNA) used for all experiments.

4.4 Results and discussion

4.4.1 Recombinant expression of the scHupBA single-chain DNA binding protein

Histone-like proteins (HU) can be found in a variety of organisms, including bacteria (41) like *E. coli*, archaea (42), and eukaryotes (32) and they a role in DNA compaction, gene regulation, and other cellular processes (43). HU are basic, heat-stable and low molecular weight proteins (approximately 16–20 kDa), well-preserved and highly abundant in prokaryotes (ca. 30,000 dimers per cell in enterobacteria like *E. coli*) (32). The *E. coli* HU is a heterodimer composed of two highly homologous subunits α and β of ~ 9.5 kDa each with almost 70 % amino acid sequence identity (44). Even though it can be naturally found in the HU α_2 and HU β_2 homodimeric forms, the HU $\alpha\beta$ heterodimer is the predominant native form in *E. coli* (45). HU proteins bind to DNA fragments in a sequence-independent manner and exhibits higher affinity in lower salt concentrations (46). Each dimer binds to a fragment of approximately 9 base pairs (9 bp), causing slight bending to DNA once several HU dimers are bound (47). The universal DNA binding properties of these proteins make them particularly attractive as biorecognition molecules for universal DNA bioanalysis. In this work, we sought to engineer a recombinant HU $\alpha\beta$ heterodimer for universal detection of DNA. To facilitate recombinant expression in *E. coli* and purification, a single-chain version of the protein was designed. Examining the three-dimensional structure of the *E. coli* HU bound to a 9 bp DNA fragment (Figure 4.2A), it was assumed that the most appropriate manner in joining the two monomers would be through the C-terminal of the β monomer and the N-terminal of the α monomer since these are further away from the protein-DNA interaction surface and are less likely to interfere with the DNA binding properties of the protein. A flexible glycine-rich 16 amino acid linker (AAGGTGGGSGGGTGGS) was chosen to link these monomers, based on a previous report on the engineering of a single-chain Cro bacteriophage lambda DNA binding protein homodimer (48). This peptide is mainly neutral, thus not expected to affect protein-DNA binding, and its approximate length of 58 Å (3.6 Å inter-residue distance in fully extended polypeptides) was considered appropriate to link the two HU monomers with a 28 Å approximate distance between their chosen ends. The fused gene was synthesized commercially and was inserted in the pLATE31 expression vector as detailed in the experimental part which will result in the expression of the protein with a C-terminal polyhistidine tag (6xHis) to facilitate purification and detection (Figure 4.2B). The pLATE bacterial expression vectors are based on the strong bacteriophage T7 promoter and additional elements of these vectors ensure tight regulation of gene expression, leading to high levels of

protein expression with minimized basal (uninduced) expression. The expressed scHupBA has a predicted size of 21 kDa and a theoretical isoelectric point (pI) of 9.65 (calculated with the Expasy ProtParam tool using its expected amino acid sequence).

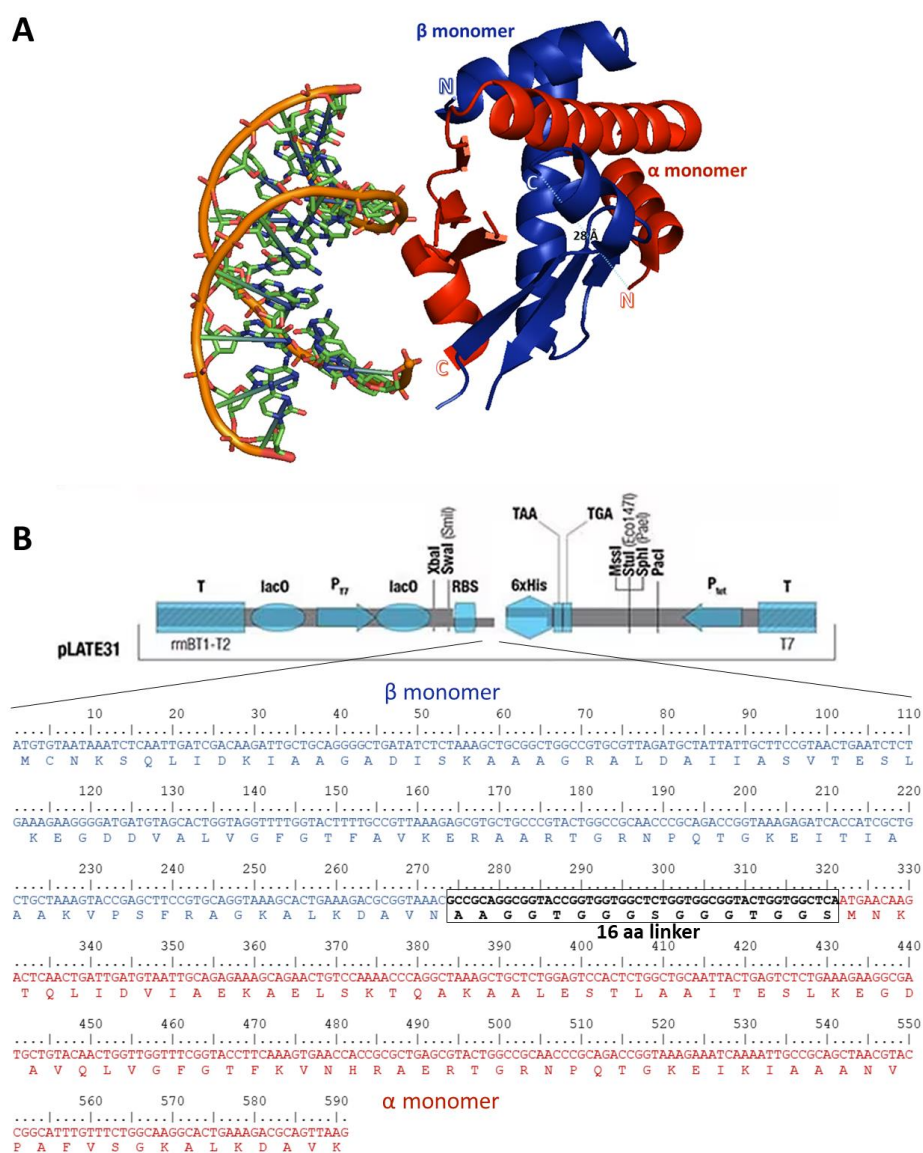


Figure 4.2 (A) Structure of *E. coli* HU heterodimeric DNA binding protein with bound dsDNA (4yew.pdb) (49). Monomer α is in red and monomer β in blue. The N and C terminals of each monomer and the 16 aa linker fusing the two genes are indicated. (B) pLATE31 expression system and design of the fusion gene for single-chain scHupBA protein production.

The expression of scHupBA protein in *E. coli* BL21(DE3) cells was induced with IPTG for 3 h at 30°C to promote soluble protein production and avoid insoluble aggregates (50). As it can

be seen in Figure 4.3A, no over-expressed protein was observed in uninduced cells whereas a band corresponding to the scHupBA protein was visible in both whole cell lysate and more importantly in the soluble cell lysate, confirming the production of soluble protein. The cells were disrupted by a combination of chemical (detergent-based lysis buffer) and physical (sonication) lysis methods to release the cytoplasmic protein. The whole lysate was cleared by centrifugation and filtration (0.45 μ m) to finally obtain the lysate containing the scHupBA in a soluble form. The protein was purified with a two-step process (Figure 4.3B).

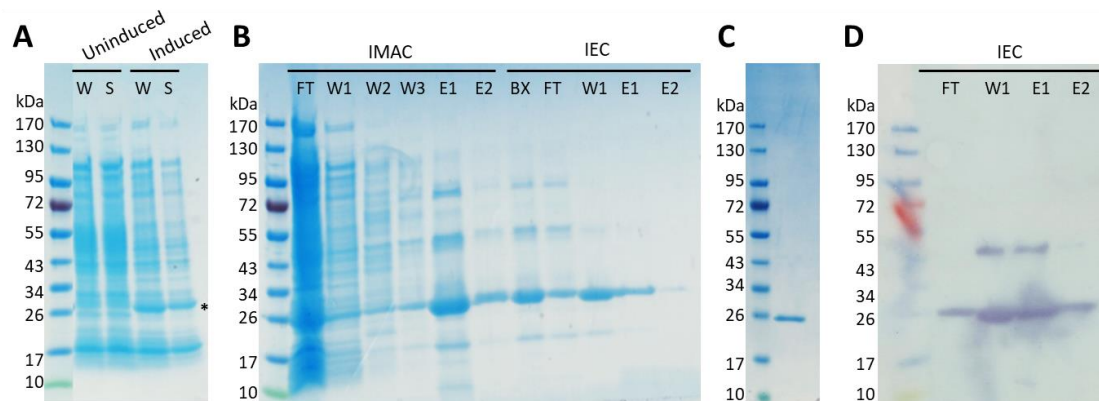


Figure 4.3 Recombinant expression and purification of scHupBA in *E. coli* monitored by SDS-PAGE and Western blot. (A) Whole W and soluble S cell lysates from uninduced and IPTG-induced cells. The expected scHupBA is indicated with an asterisk (*). (B) IMAC and IEC purification steps. Fractions FT flowthrough (unbound); W1, W2, W3: wash; E1, E2: elution; BX: buffer exchanged IMAC elution fractions E1+E2. (C) Final purified scHupBA. (D) Western blot analysis of IEC fractions using an anti-His tag antibody.

First, Immobilized Metal Affinity Chromatography (IMAC) was performed exploiting the affinity of the Ni-NTA resin for the C-terminal 6xHis tag of the protein. As expected, this step led to protein with moderate purity (70 – 80 %) since some *E. coli* proteins bind non-specifically to the IMAC resin. To remove these contaminants, a second purification step was performed. Based on the positive charge of the scHupBA protein and the acidic/neutral nature of most *E. coli* proteins, Ion Exchange Chromatography (IEC) purification was employed using the strong cation exchange chromatography resin SP Sepharose FF. Indeed, contaminants were eliminated with IEC and the final purified scHupBA exhibited high purity of > 95 % (Figure 4.3C). Western blot analysis of IEC fractions verified the protein size with the C-terminal His-tag detected by the anti-His tag antibody employed to probe the membrane. It was not possible to use UV-Vis spectroscopy to quantify the purified scHupBA, since it does not contain any tryptophan or tyrosine residues which absorb at 280 nm. A colorimetric assay for total protein

quantification was employed alternatively, based on bicinchoninic acid (BCA) and BSA standard solutions for the construction of a calibration curve. The final yield of scHupBA was approximately 6 mg of purified protein per 1 L of bacterial culture.

4.4.2 Evaluation of the DNA binding properties of scHupBA

The DNA binding properties of the expressed scHupBA protein were assessed on microtiter plates using an enzyme-linked oligonucleotide assay (ELONA) illustrated in Figure 4.4A. Prior to that though, an ELISA was performed to determine the maximum amount of protein which can be coated on the wells of the plate. Using a range of scHupBA concentrations of up to 40 $\mu\text{g/mL}$ and an anti-His tag antibody to detect the immobilized protein, a linear increase of colorimetric signal was observed when 1.25 – 10 $\mu\text{g/mL}$ of the protein were used, whereas further increase did not improve the signal significantly (Figure 4.4B). Therefore, the protein was used at 10 $\mu\text{g/mL}$ to coat the wells and study its DNA binding proteins. A range of concentrations of biotinylated DNA probes of varying length (25, 47 or 100 bases, Table 4.1), both dsDNA and ssDNA, were prepared as detailed in section 4.2.6 and were used for binding the protein. Bound DNA was detected with a streptavidin-HRP conjugate and a colorimetric substrate. The DNA binding properties of the protein were indeed verified, as demonstrated by the concentration-dependent binding curves obtained for all three fragments as shown in Figure 4.4C. The affinity dissociation constants (K_D values) decreased with the increase of dsDNA fragment length, confirming the higher binding affinity of scHupBA for longer DNA fragments since it can accommodate more protein molecules, considering that four HU dimers can bind to each 42 bp segment (46). The K_D values calculated were 1937 nM, 55 nM and 13 nM for the dsDNA fragments of 25, 47 and 100 bp length, respectively. The protein was also able to bind ssDNA but with markedly lower affinity compared to dsDNA. The K_D values were 4160 nM, 2008 nM and 67 nM for ssDNA of 25, 47 and 100 bases, respectively. The strong binding affinity of scHupBA for dsDNA fragments of ≥ 50 bp in combination with its evidently lower affinity for ssDNA are very important characteristics for a potential biorecognition element to be used in bioanalytical assays for the detection of dsDNA, with specific interest in amplification products.

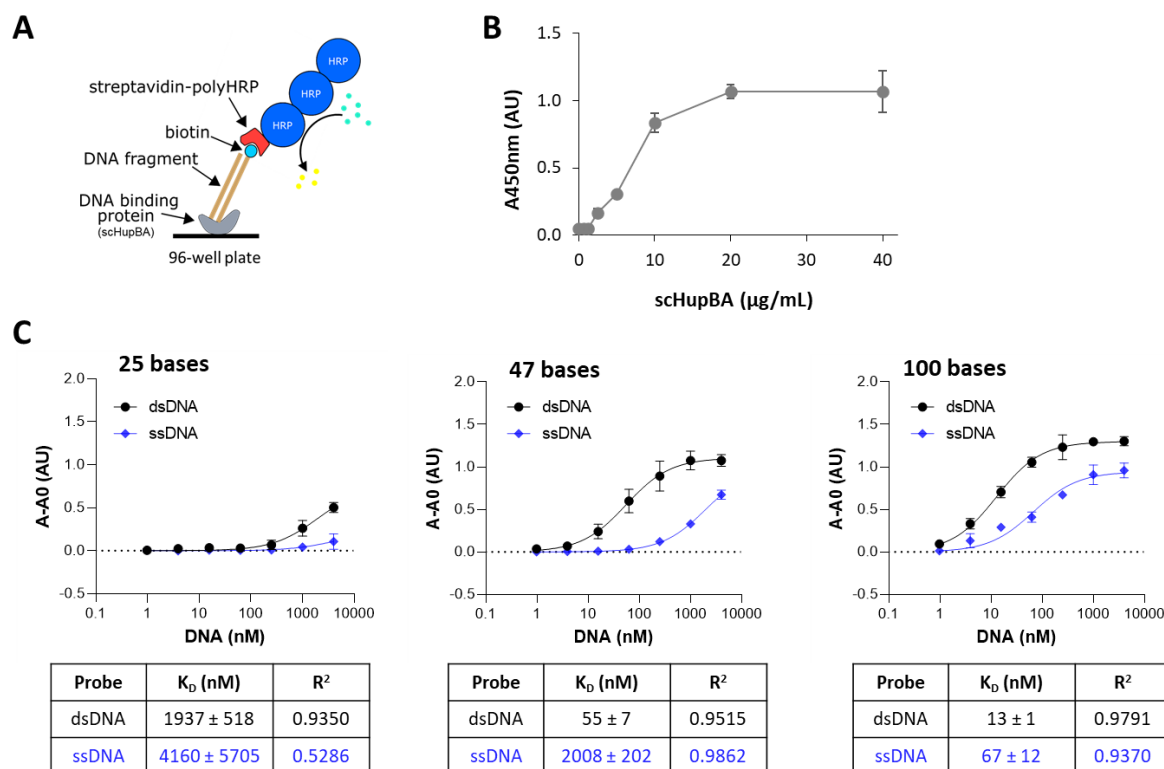


Figure 4.4 (A) Schematic representation of the ELONA assay. (B) ELISA to evaluate the concentration of scHupBA required for coating the microtiter plate wells. An anti-His tag antibody was used for protein detection. (C) Binding curves for dsDNA and ssDNA fragments of varying length (25, 47 and 100 nucleotides).

4.4.3 Preparation of the scHupBA-HRP conjugate for use in DNA detection assays

Several approaches were evaluated to conjugate scHupBA to HRP. Initially, the protein with an engineered N-terminal cysteine residue, was incubated directly with maleimide-activated HRP, to react the thiol group of the protein with the maleimide groups of HRP. Even though this strategy was successfully used in the past to prepare another DNA binding protein-HRP conjugate (51), in this case the conjugation efficiency was very low, presumably due to insufficient solvent exposure of the cysteine residue (data not shown). Other strategies assessed were glutaraldehyde- and carbodiimide (EDC/NHS)-mediated conjugation of scHupBA with HRP, but they also proved unsuccessful (data not shown). Finally, sulfhydryl groups (-SH) were added to the solvent-exposed lysine residues of scHupBA using SATA and this SATA-activated scHupBA was successfully conjugated to maleimide-activated HRP (Figure 4.5A and 4.5B).

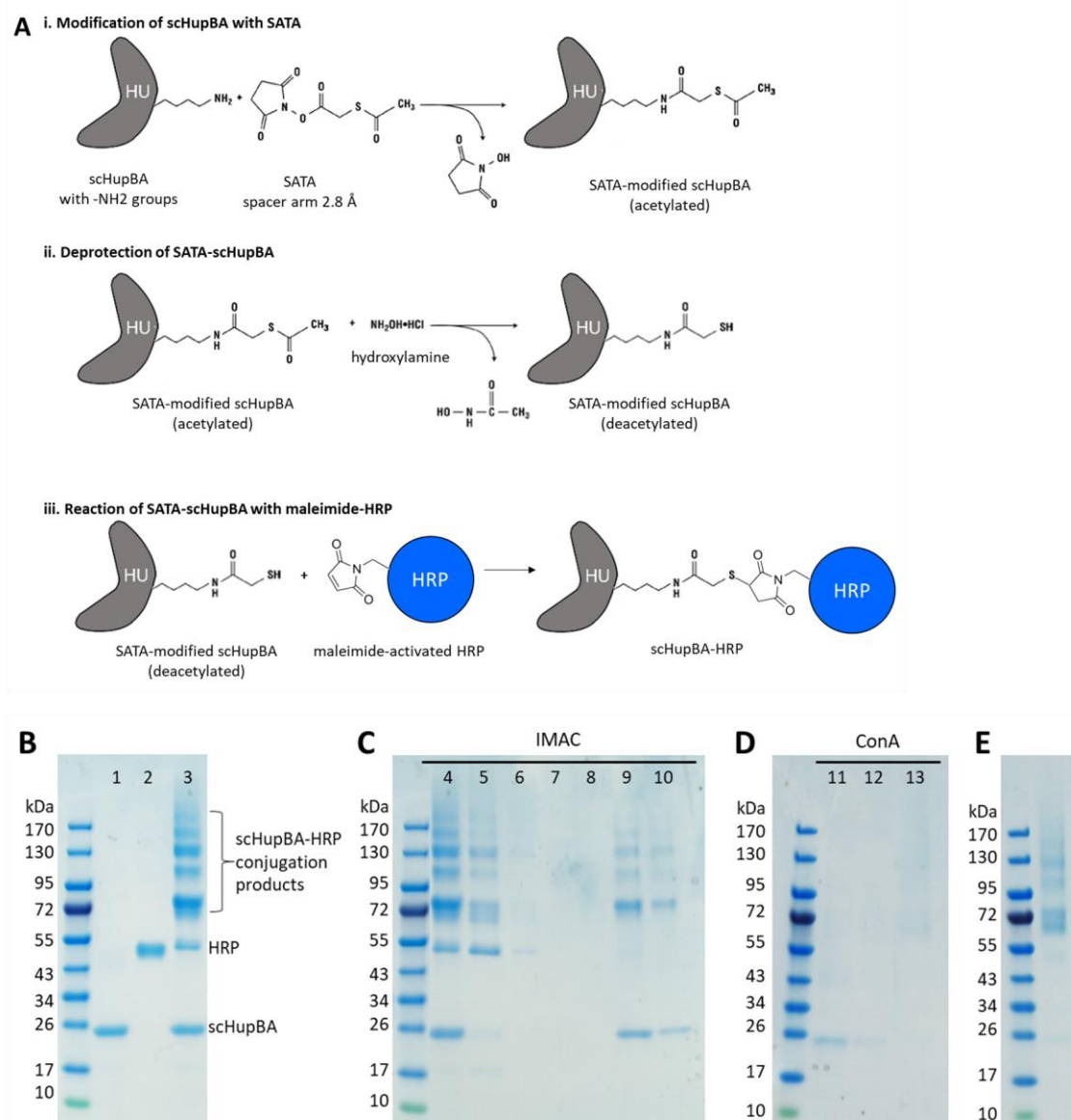


Figure 4.5 Preparation of the scHupBA-HRP conjugate. (A) Conjugation strategy. (B) Lane 1: SATA-modified scHupBA; lane 2: maleimide-activated HRP; lane 3: conjugation reaction. (C) IMAC purification. Lane 4: conjugation reaction; lane 5: flowthrough (unbound); lanes 6-8: wash fractions; lanes 9-10: elution fractions. (D) ConA purification. Lane 11: flowthrough (unbound); lane 12: wash fraction; lane 13: elution fraction. (E) Final purified scHupBA-HRP conjugate.

The main conjugation product had an apparent size of 72 kDa (Figure 4.5B, lane 3). This is consistent with a conjugate at a 1:1 stoichiometry, considering the apparent sizes of ~ 26 kDa for scHupBA and 45-50 kDa for HRP according to SDS-PAGE analysis (Figure 4.5B, lanes 1 and 2, respectively). The conjugation reaction was not complete since it contained unreacted

proteins, which could have negative effects during its application for DNA detection. Unreacted scHupBA could bind DNA but it will not generate signal, while unreacted HRP could bind non-specifically to surfaces and biomolecules and lead to signal generation in the presence of its chromogenic substrate. A two-step purification process was designed to remove these unconjugated proteins. The first was based on IMAC to eliminate unreacted HRP which does not bind to the IMAC. Indeed, remaining HRP was observed mainly in the IMAC unbound fraction and partially in the wash fraction (Figure 4.5C lane 6) while it was not in the IMAC elution fractions (Figure 4.5C lanes 9-10). After eliminating the unreacted HRP, a second purification step was performed to remove the unreacted scHupBA. This involved the use of Concanavalin A affinity resin. ConA is a tetrameric metalloprotein lectin binding to polysaccharides and glycolipids and for this reason it is widely exploited for the affinity purification of glycosylated proteins such as HRP. As expected, unconjugated scHupBA did not bind to the ConA resin (Figure 4.5D lane 11) and the final purified and concentrated conjugate did not contain any unconjugated proteins (Figure 4.5E). Overall, the described method demonstrates the successful preparation and purification of the scHupBA-HRP conjugate which can be used for as a reporter for DNA detection.

4.4.4 Microplate-based colorimetric detection of dsDNA using scHupBA and scHupBA-HRP

To develop a scHupBA-based bioanalytical assay for dsDNA detection, the protein was physically absorbed on microtiter plates at saturating concentration (Figure 4.4B) as the biorecognition molecule for capture, whereas the scHupBA-HRP conjugate (5 $\mu\text{g/mL}$) was used for detection (reporter), as illustrated in Figure 4.1A. By using these two proteins in a sandwich format, dsDNA molecules can be detected regardless of their specific sequence. And if these DNA molecules are amplification products from PCR or an isothermal amplification method, unmodified primers can be used. Initially, the detection of three DNA fragments with varying length was evaluated, using both dsDNA and ssDNA prepared in PBS under physiological salt conditions (137 mM NaCl and 2.7 mM KCl). As can be observed in Figure 4.6A, only the 176 bp fragment was detected whereas the dsDNA fragments of ≤ 100 bp were not. These results suggest that since capture and detection molecules share the same binding sites, a minimum DNA length is required to allow for both to simultaneously bind and produce signal. On the other hand, ssDNA did not produce any signal thus demonstrating the specificity

of the detection strategy for dsDNA fragments with a length of > 100 bp, consistent with the typical size of amplification products.

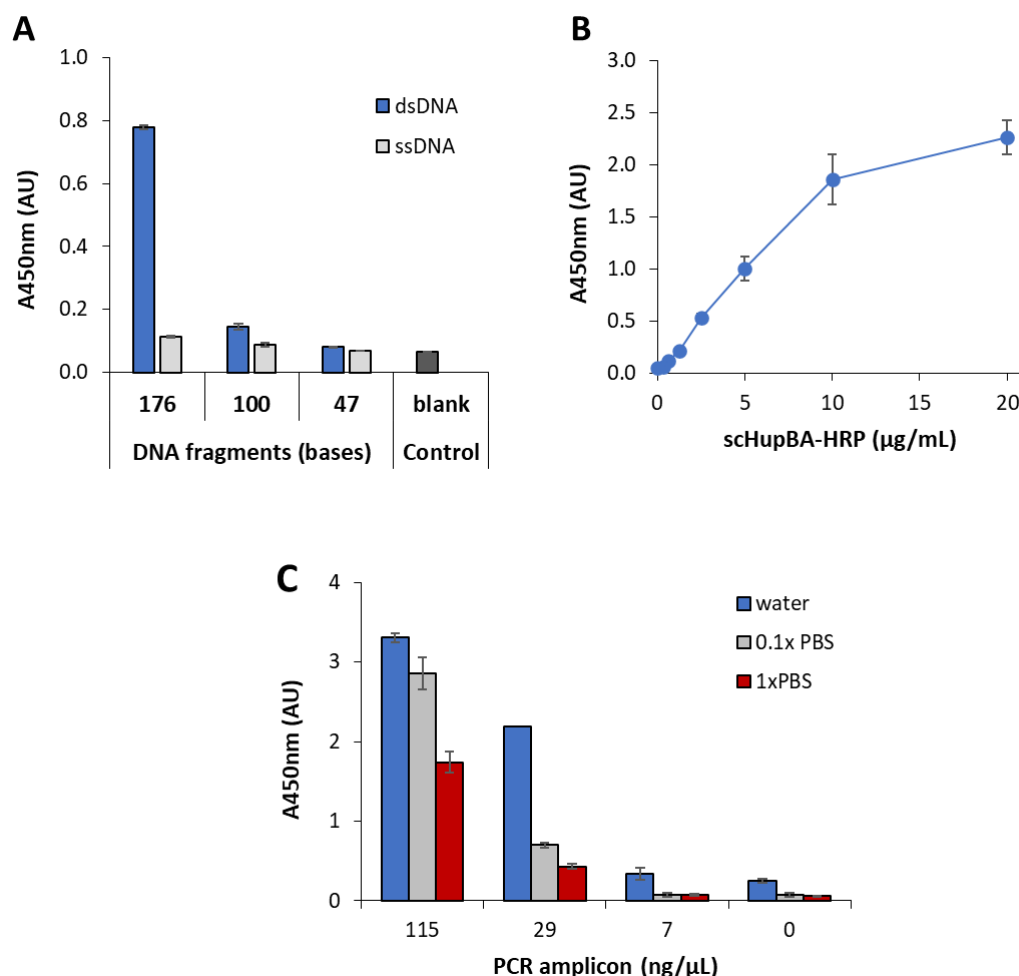


Figure 4.6 (A) Detection of dsDNA and ssDNA of varying length (47, 100, 176 bases) using scHupBA for capture and scHupBA-HRP (5 µg/mL) for detection. (B) Effect of the concentration of scHupBA-HRP and (C) of the composition of buffer used for DNA binding on signal intensity for the detection of the 176 bp PCR amplicon.

To evaluate the effect of the scHupBA-HRP conjugate concentration on signal intensity, the 176 bp PCR amplicon (used at a constant concentration of ~ 57 ng/µL in PBS) was captured on the plate by scHupBA and different concentrations of the conjugate were employed for detection, ranging from 0.3 – 20 µg/mL. A linear increase of colorimetric signal was observed for scHupBA-HRP concentrations up to 10 µg/mL, achieving twice as much signal when using double amount of conjugate. Further increase of the conjugate concentration did not significantly improve detection (Figure 4.6B). These results suggest that since capture and detection molecules compete for binding sites on the target dsDNA, increased concentration of

the detection conjugate might be needed to improve colorimetric signals. Nevertheless, for more cost-effective assays considering that the conjugate is the costliest component of the bioassay, a constant concentration of 5 $\mu\text{g/mL}$ scHupBA-HRP was used for following experiments.

As it was commented earlier, binding of native HupBA heterodimer to DNA is salt-dependent, with increased affinity at sub-physiological conditions (≤ 100 mM NaCl) and complete dissociation from DNA for > 300 mM NaCl (52). To evaluate the properties of the engineered scHupBA protein, DNA capture and detection were performed at different buffer conditions using the model target PCR amplicon of 176 bp. The studies were performed in water, 0.1x PBS (13.7 mM NaCl and 0.27 mM KCl) and 1x PBS (137 mM NaCl and 2.7 mM KCl) and as it can be observed in Figure 4.6C, higher signals were achieved in water, followed by 0.1x PBS while considerably lower ones were recorded in 1x PBS. However, when the assay was performed in water, higher background non-specific signals were observed, which were eliminated when salt was present, even at low concentrations (14 mM). Based on these observations, we sought to evaluate the sensitivity for DNA detection under different NaCl concentrations, ranging from 30 – 240 mM in a phosphate buffer.

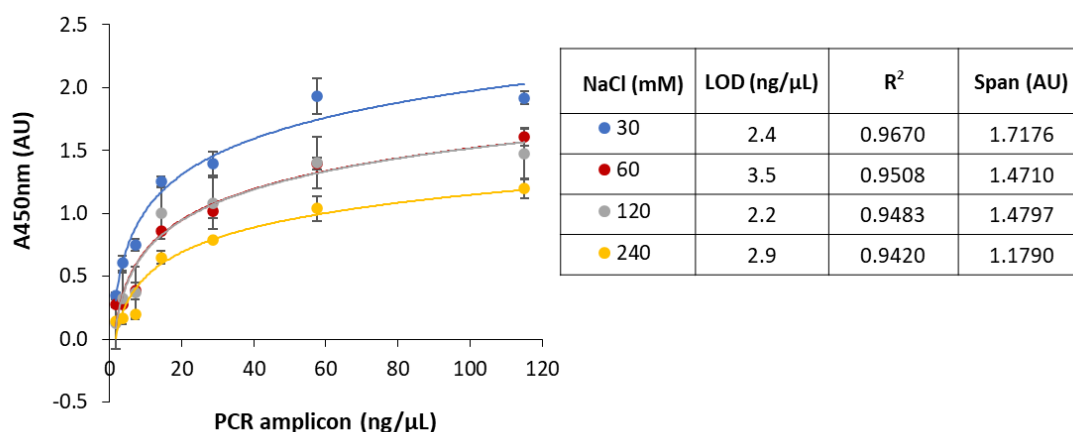


Figure 4.7 Detection of a 176 bp PCR amplicon using scHupBA (10 $\mu\text{g/mL}$) for capture and scHupBA-HRP (5 $\mu\text{g/mL}$) for detection by ELONA. The calibration curves were constructed using the target DNA in phosphate buffer with varying salt (NaCl) concentrations. The span corresponds to signal range of the fitted curves designated as the absorbance at highest concentration minus the absorbance at the lowest concentration.

As shown in Figure 4.7, the limit of detection (LOD) was calculated at 2.2 – 3.5 ng/ μL PCR amplicon and it was not affected by the NaCl concentration. The sensitivity though was

increased at lower NaCl concentrations since the signal span was approximately 1.5 times higher at 30 mM NaCl compared to 240 mM (1.7176 AU vs 1.1790 AU). These findings confirm that the binding of the engineered scHupBA is salt-dependent and lower concentrations favor binding for DNA detection.

4.4.5 Colorimetric detection of dsDNA using scHupBA-magnetic beads and scHupBA-HRP

After establishing the use of microtiter plates as a platform for DNA detection using scHupBA, magnetic beads were evaluated. Magnetic beads are an extremely useful tool for bioanalysis because of their attractive properties, such as high binding capacity for different biomolecules, facile and quick magnetic separation as well as the possibility of pre-concentration of dilute samples for improved sensitivity especially for nucleic acid detection (53). scHupBA was immobilized on epoxy-activated magnetic beads via its amine groups since when the same functional groups were targeted to prepare the scHupBA-HRP conjugate, the protein maintained its DNA binding properties. The presence of the protein on the magnetic beads was confirmed with a bead-ELISA using an anti-His tag antibody targeting the C-terminal His-tag of the engineered protein (Figure 4.8A). Next, the composition of the blocking solution was evaluated. Skim milk and BSA in a detergent-supplemented physiological buffer (PBST) are typically used for blocking the plastic surface of polystyrene microtiter plates and these were also used for blocking the scHupBA-MB before capturing the 176 bp model target PCR amplicon and detection with the scHupBA-HRP conjugate. As can be seen in Figure 4.8B, skim milk in PBST was appropriate to block the beads and obtain specific signal in the presence of DNA, whereas BSA was not. Therefore, 5 % w/v skim milk in PBST was used as the blocking solution for following experiments.

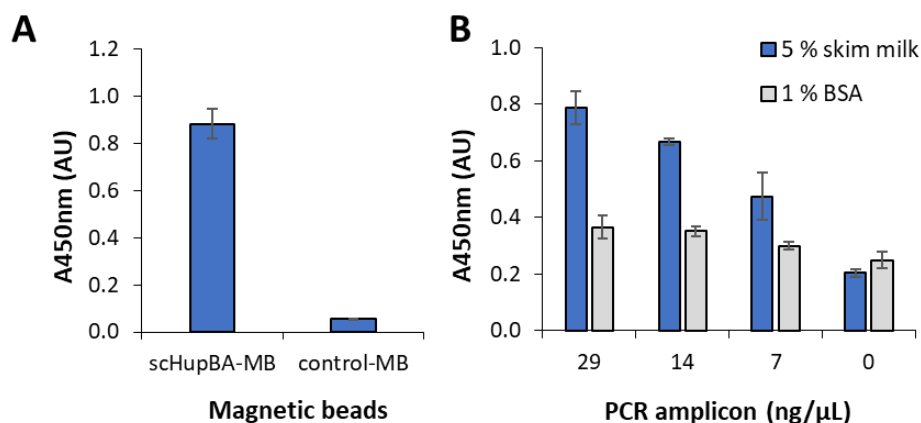


Figure 4.8 (A) Bead-ELISA to verify the immobilization of scHupBA on the magnetic beads. (B) Effect of the composition of the magnetic beads blocking solution on signal intensity for detection of the 176 bp PCR amplicon using scHupBA-MB and scHupBA-HRP.

With the microtiter plate assay, it was observed that lower salt concentrations were beneficial for specific detection of dsDNA. Therefore, a study was conducted to evaluate the sensitivity of the magnetic beads-based platform for the detection of the model PCR amplicon using different buffer conditions which were 0.1xPBS and 1xPBS. Water was not used since previous results suggested that it promoted non-specific signals (Figure 4.6C). Surprisingly, the sensitivity on the magnetic beads was slightly lower compared to the microtiter plate-based assay. The LOD values were 16.5 ng/μL and 8.9 ng/μL in 0.1xPBS and 1xPBS, respectively (Figure 4.9) as compared to 2.2 – 3.5 ng/μL achieved on microtiter plates (Figure 4.7). And even though the buffer strength, therefore salt concentration, did not have significant effect on the LOD, it did affect the sensitivity of the assay since almost two-fold higher signal span was observed in 0.1xPBS with lower NaCl concentration compared to 1xPBS.

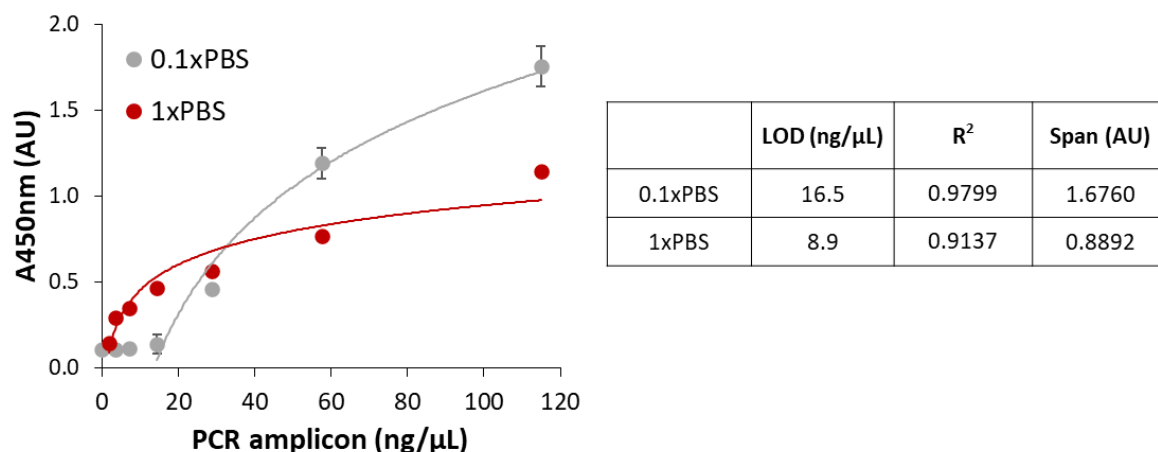


Figure 4.9 Detection of a 176 bp PCR amplicon using scHupBA-MB for capture and scHupBA-HRP (5 µg/mL) for detection by bead-ELONA. The calibration curves were constructed using the target DNA under different buffer conditions.

The lower sensitivity of the magnetic beads-based assay could be due to lower DNA binding capacity of the beads compared to the microtiter plates. A simple experiment was designed to assess this hypothesis. A dsDNA probe (47 bp) was prepared at a constant concentration and was incubated in parallel in a microtiter plate well and with magnetic beads with immobilized scHupBA. The supernatant was recovered, and the DNA concentration was determined by UV-Vis spectroscopy (absorbance at 260 nm). The amount of bound DNA to these surfaces (binding capacity) was calculated as the difference of starting concentration from supernatant (unbound fraction) concentration. This experiment showed that the binding capacity of these two surfaces was almost identical, being able to bind 330 – 388 ng of the 47 bp dsDNA, corresponding to 11 – 13 pmol. Therefore, the lower sensitivity observed with the magnetic beads does not seem to be a direct result of lower binding capacity. So even though the binding capacity is the same, the spatial distribution of scHupBA on the magnetic beads is expected to be different compared to the flat microtiter plate well. This could potentially increase the availability of binding sites on the DNA molecule. However, since the scHupBA-HRP conjugate will compete with the scHupBA on the magnetic beads for these sites, it is plausible that fewer conjugate molecules can bind to the captured DNA for detection and result in lower sensitivity. Further work is required to fully understand the distribution of scHupBA on the magnetic beads in conjunction with its DNA binding properties to improve the performance of the assay. Nevertheless, the sensitivity could be potentially improved by using higher sample volumes and perform sample pre-concentration which is one of the most attractive properties of magnetic beads.

4.4.6 Detection of dsDNA with lateral flow dipstick assay

A nucleic acid dipstick assay was also evaluated as a potential platform for easy and fast detection of dsDNA, employing scHupBA on the membrane to capture the target DNA and scHupBA immobilized on carbon nanoparticles to provide the colorimetric signal. Gold and carbon nanoparticles are widely exploited in lateral flow assays for the detection of proteins and nucleic acids. However, the black color of carbon nanoparticles offers better contrast on the white nitrocellulose membrane and can achieve higher sensitivities compared to gold nanoparticles as demonstrated previously (54). Moreover, our previous works on nucleic acid dipstick assays exploiting sequence-specific DNA binding proteins and carbon nanoparticles have demonstrated the compatibility of this nanomaterial for immobilization of DNA binding proteins and use for DNA capture and detection (38, 40).

The strips were prepared using scHupBA to immobilize on the test zone whereas a biotinylated dsDNA probe (100 bp) was immobilized on the control zone using neutravidin as a carrier. Initially, different combinations of membrane blocking solutions and running buffers were evaluated in the absence of DNA target to establish the conditions assuring good wicking of the scHupBA-CNPs conjugate on the membrane and absence of non-specific signals. The membranes were blocked with skim milk in carbonate buffer and were supplemented with either Tween-20 or Empigen BB detergents. The scHupBA-CNPs conjugate was added to different running buffers: 2 % w/v skim milk in 10 mM Tris-HCl pH 7.5 or PBST (with 0.05 % v/v Tween-20) when Tween-20 was included in the blocking buffer or 0.5 – 2 % w/v skim milk and 0.1 % Tween-20 in water when Empigen BB was in the blocking buffer. High non-specific signals were observed on the test zones when the blocking buffer was supplemented with Tween-20 regardless of the running buffer composition. At the same time, the wicking of the nanoparticles was not optimal, since aggregation was observed at the bottom of the strips. These results suggest that Tween-20 is not appropriate to use in the blocking buffer (Figure 4.10A, left). On the other hand, when Empigen BB was added to the blocking buffer and the water-based running buffer was used with 0.1 % v/v Tween-20 and < 1 % w/v skim milk, these background signals were decreased, while minimum nanoparticle aggregation was observed at the bottom of the strips (Figure 4.10A, right).

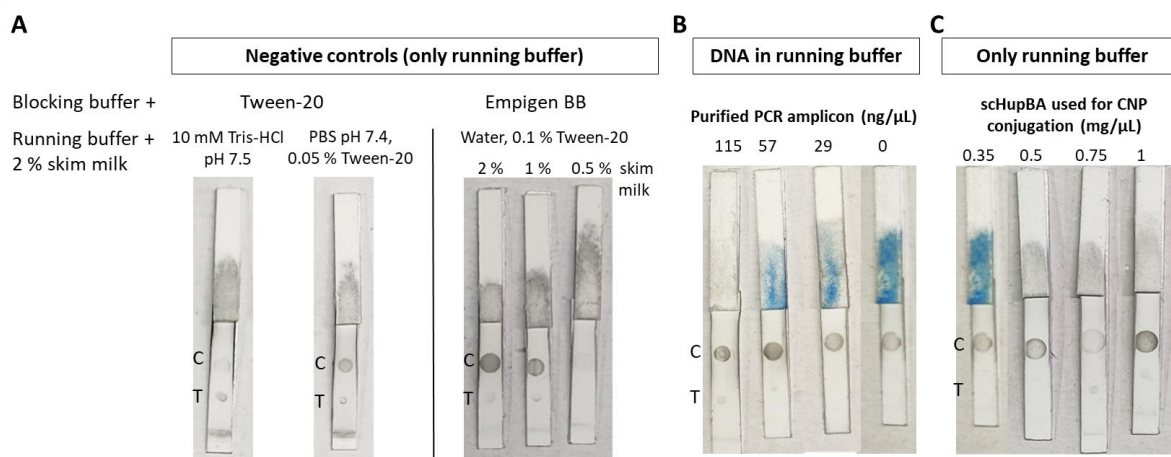


Figure 4. 10 Lateral flow dipstick assays in the absence and presence of PCR amplicon. (A) Screening of membrane blocking (2 % w/v skim milk in 10 mM carbonate-bicarbonate pH 9.4 with 0.1 % v/v Tween-20 or Empigen BB) and running (10 mM Tris-HCl pH 7.5, PBS pH 7.4 or water with 0.05 % Tween-20 and 0.5 – 2 % w/v skim milk) buffers. (B) Detection of DNA (176 bp) using optimal blocking (2 % w/v skim milk and 0.1 % v/v Empigen BB in 10 mM carbonate-bicarbonate pH 9.4) and running (0.5 % w/v skim milk and 0.1 % Tween-20 in water) buffers. (C) Screening of scHupBA concentrations used for conjugation to 0.2 % w/v CNPs in the absence of DNA using optimal blocking and running buffers. DNA and scHupBA-CNPs were pre-incubated in running buffer prior to analysis. The CNPs conjugate was prepared with 0.35 mg/mL scHupBA for (A) and (B).

The next step was to assess the detection of the model target PCR amplicon with the dipstick assay using 0.5 % w/v skim milk and 0.1 % v/v Tween-20 in water as the optimum running buffer. Indeed, black precipitates appeared at the test zone with an increasing intensity when using increased DNA concentration, while no background signal could be seen in the absence of DNA. These results demonstrate that the scHupBA/scHupBA-CNPs system can be used for DNA detection by a dipstick assay. The signals observed were faint though, and for this reason we explored the use of CNPs conjugates prepared with higher concentration of scHupBA. So far, 0.35 mg/mL of scHupBA were used to immobilize on 0.2 % w/v CNPs. Additional conjugates were prepared using 0.5 - 1 mg/mL and when they were applied to the strips in the absence of DNA, non-specific signals were observed on the test zones for ≥ 0.75 mg/mL scHupBA (Figure 4.10C), therefore following experiments were performed with 0.5 mg/mL scHupBA for CNPs conjugation.

The previous experiments were performed in one step by applying the DNA sample/scHupBA-CNPs mixture in running buffer which was briefly pre-incubated to make the assay faster and

simpler. However, since faint signals were obtained (Figure 4.10B), we considered performing the assay in two steps to increase the signals. Therefore, the strips were dipped first in the DNA sample and when these were completely wicked through the membrane, the scHupBA-CNPs were applied to provide the signal. It was hypothesized that longer contact time of DNA with the scHupBA on the membrane could potentially improve DNA capture capacity and potentially lead to increased signal. This however was not the case, since even lower signals were observed (Figure 4.11). It should be noted that higher scHupB concentration was used to prepare to CNPs conjugate (0.5 mg/mL). These results suggest that perhaps the extended contact time of DNA with scHupBA on the membrane leads to saturation of the available binding sites on the DNA, preventing the scHupBA-CNPs from binding to provide the signal.

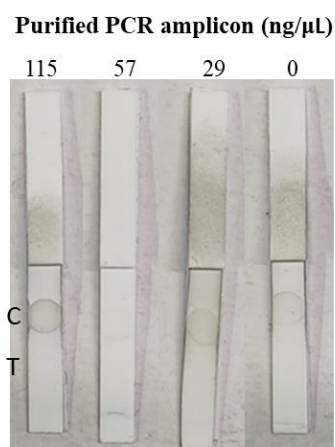


Figure 4.11 Detection of the PCR amplicon in two steps, first the DNA sample and then the scHupBA-CNPs. The scHupBA-CNPs conjugate was prepared using 0.5 mg/mL scHupBA and optimal blocking and running buffer compositions were used.

The sensitivity and specificity of the assay was then evaluated using different concentrations of dsDNA amplicon as well as a ssDNA fragment of the same length. Conditions optimized thus far were applied and it can be seen in Figure 4.12, dsDNA in all the solutions analyzed could be detected with higher signal intensities compared to previous experiments performed under sub-optimal conditions. The lowest detectable concentration was of dsDNA was 15 ng/μL. A very faint signal was observed when ssDNA was analyzed and only for the highest

concentration (115 ng/ μ L), suggesting that the presence of ssDNA in the solution is not expected to interfere with dsDNA detection.

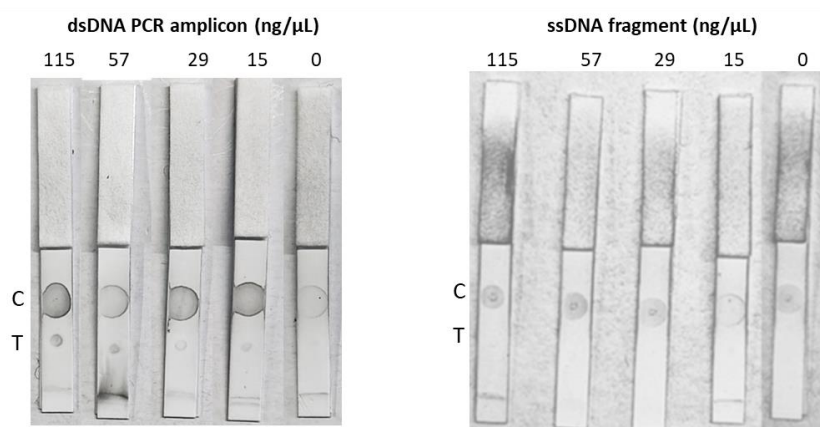


Figure 4.12 Evaluation of the sensitivity and specificity of the lateral flow dipstick assay using dsDNA PCR amplicon and ssDNA PCR fragment. The membranes were blocked with 2 % w/v skim milk and 0.1 % v/v Empigen BB in 10 mM carbonate-bicarbonate pH 9.4 and the running buffer was 0.5 % w/v skim milk with 0.1 % Tween-20 in water. DNA and scHupBA-CNPs (prepared using 0.5 mg/mL scHupBA) were pre-incubated in running buffer prior to analysis.

4.4.7 Direct analysis of PCR reactions by ELONA using microplates, magnetic beads and dipstick lateral flow assays

Having established the use of scHupBA for detection of purified dsDNA on microtiter plates, magnetic beads and dipstick lateral flow assays, the compatibility of the assays with direct analysis of PCR reactions without prior amplicon purification was finally tested. To this end, PCR reactions were performed using different concentrations of the target and analyzed by agarose gel electrophoresis as shown in Figure 13A. For analysis using the scHupBA-based assays, the reactions were only diluted five-fold and the amplicons were detected as detailed in the respective sections. The results obtained with the purified dsDNA amplicon suggested better performance in the presence of low salt concentrations, therefore water was chosen for this part as the diluent. All other conditions were identical to the ones summarized in sections 4.3.8 (microplate), 4.3.9 (magnetic beads) and 4.3.10 (dipstick lateral flow).

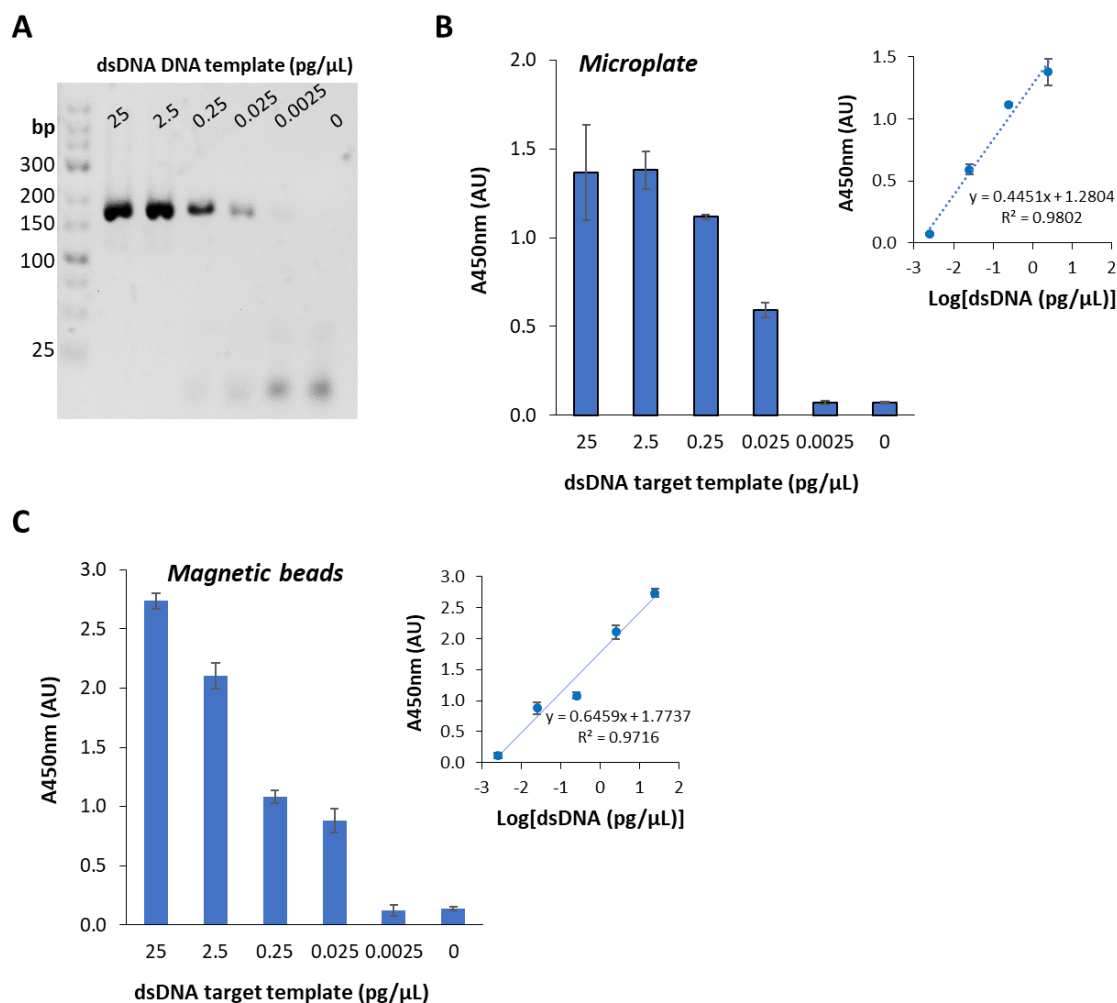


Figure 4.13 Detection of PCR amplicons directly in the PCR reactions without prior purification by colorimetric ELONA. Analysis of PCR reactions performed using 0 – 25 pg/μL of target DNA by (A) agarose gel electrophoresis, (B) Plate-ELONA and (C) magnetic bead-based ELONA. The insets show the calibration curves constructed using logarithmic models.

As can be seen in Figure 4.13B and 4.13C, the dsDNA amplicon was successfully detected by microplate and magnetic beads ELONA directly in the PCR reactions without prior purification when 0.025 – 25 pg/μL of target DNA was used for amplification. Background-level signals were obtained when 0.0025 ng/μL were used. The LODs were calculated using a logarithmic model and they were almost identical at 3.3 fg/μL and 3.5 fg/μL for the microplate and magnetic beads-based assays, respectively. These results confirm the suitability of the scHupBA-based assays for detecting amplicons in amplification reactions without the requirement of purification. The absence of signals from the control PCR reactions with no target but which contain the starting concentration of primers further confirm the very low binding affinity of

scHupBA for small ssDNA fragments. This is very important since they do not interfere with the detection of the dsDNA amplicons, especially the ones with a length of more than 100 bp, and their removal by purification is not required. Further optimization of assay conditions could potentially improve their sensitivity.

Finally, the same PCR reactions were analyzed by dipstick lateral flow dipsticks which is a much simpler and faster assay format compared to the microplates and magnetic beads. For these tests, the PCR reactions were diluted ten-fold using 0.5 % (w/v) skim milk and 0.1 % (v/v) Tween 20 in water as the diluent. Then they were mixed with 1 μ L of scHupBA-CNPs briefly for 10 min before dipping the strips. Three different concentrations of scHupBA were initially evaluated for the preparation of the carbon nanoparticle conjugate, 0.5, 0.75 and 1 mg/mL. As can be seen in Figure 4.14, clear carbon-black signal was obtained at the test line only when 1 mg/mL of the DNA binding protein was used for preparation of the reporter conjugate. Therefore, this concentration was chosen for further experiments.

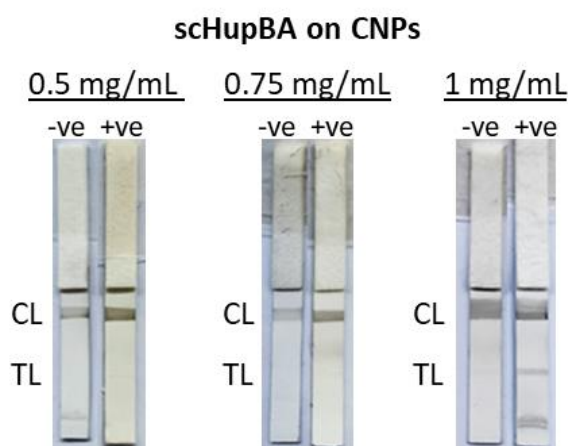


Figure 4.14 Analysis of PCR reactions with (+ve) and without (-ve) target DNA template with dipstick lateral flow assays. The reporter carbon nanoparticles were prepared using 0.5, 0.75 or 1 mg/mL of scHupBA. The +ve reaction was performed using 25 pg/ μ L of target dsDNA.

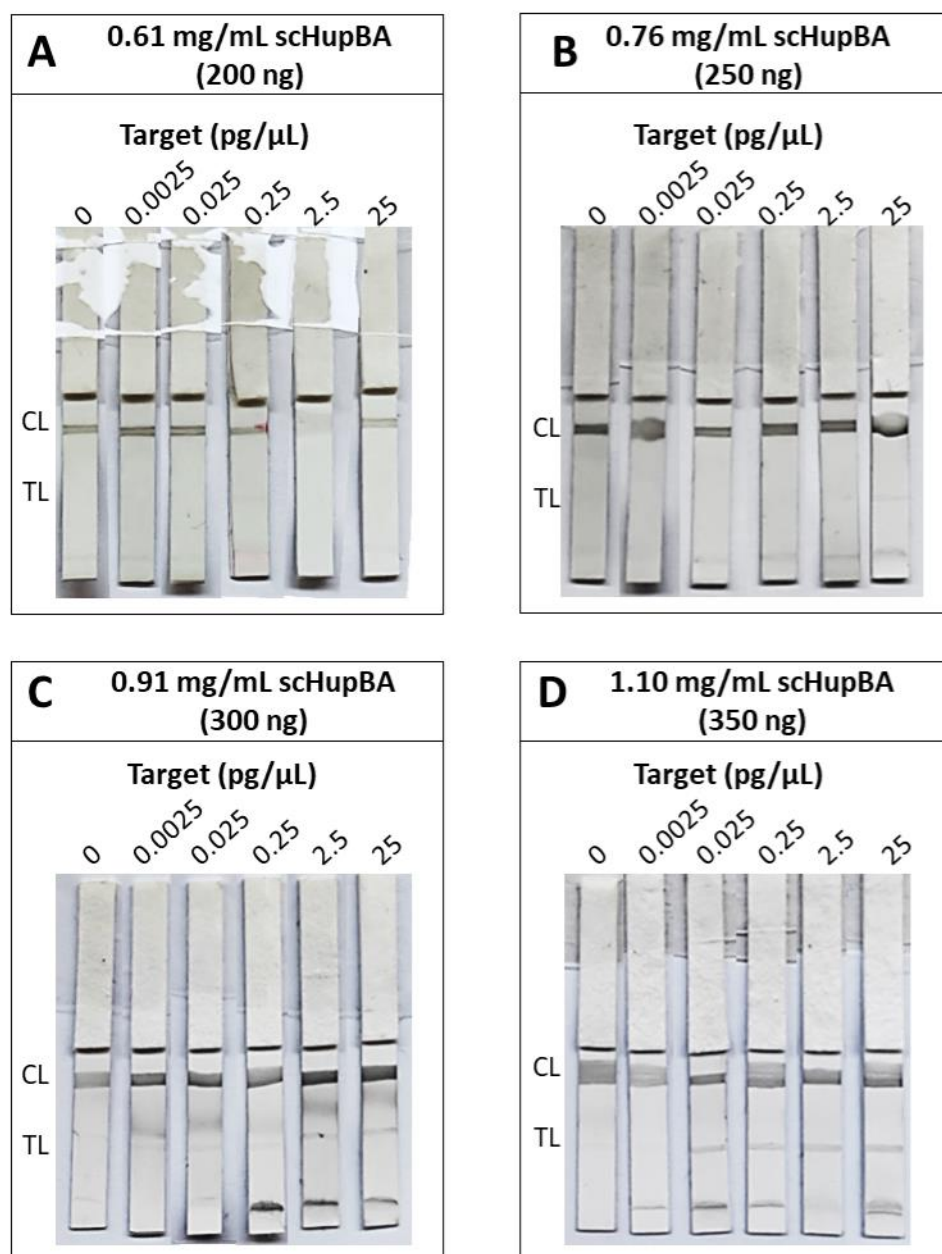


Figure 4.15 Detection of PCR amplicons directly in PCR reactions without prior purification. The scHupBA-CNPs conjugate was prepared using 1 mg/mL of the DNA binding protein. The strips were prepared using (A) 200 ng, (B) 250 ng, (C) 300 ng or (D) 350 ng of scHupBA per 4 mm strip. Diluted PCR reactions and scHupBA-CNPs were pre-incubated in running buffer prior to analysis. The running buffer contained 0.5 % w/v skim milk with 0.1 % Tween-20 in water.

Using 1 mg/mL to prepare the scHupBA-CNPs conjugate, the PCR reactions were finally analyzed with strips containing different amounts of scHupBA on the test line. To this end, the protein was diluted to 0.61 – 1.1 mg/mL and using automatic dispensing, 200 – 350 ng of the protein were dispensed per 4 mm strip. As can be observed in Figure 4.15A, the PCR amplicon

could not be detected with the strips prepared with 200 ng of scHupBA on the test line, whereas when 250 ng were used, black signals were observed at the test lines only for the PCR reactions performed using high concentrations of the target dsDNA (≥ 2.5 pg/ μ L) (Figure 4.15B). When ≥ 300 ng of scHupBA were deposited on the test lines, the amplicons could be successfully detected for target templates of ≥ 2.5 fg/ μ L, even though signals with higher intensity were observed when the highest amount of scHupBA tested was used (350 ng). Considering that scHupBA binds non-specifically to all dsDNA fragments and that it was used for both capture and detection of these fragments, the requirement of higher amounts of the protein is reasonable. Further work will focus on the improvement of sensitivity of this assay, since it is the preferable format for fast (< 20 min) and simple (1-step) detection of dsDNA amplicons in amplification reactions.

4.5 Conclusions

This study sought to explore different applications for the histone-like *E. coli* protein HU DNA binding protein in the field of DNA detection. A single-chain heterodimeric scHupBA protein was engineered by fusing the two highly homologous monomers with a flexible amino acid linker and the protein was expressed in *E. coli*. A two-step purification based on the C-terminal His-tag of the protein and its high surface charge was designed. The protein was obtained at high purity (> 95 %) and with satisfactory yields (6 mg per 1 L of bacterial culture). The DNA binding properties of this recombinant protein were confirmed by an enzyme-linked oligonucleotide assay (ELONA) on microtiter plates using biotinylated dsDNA and ssDNA probes of varying length. The studies showed that the affinity of the protein increased with increased length of dsDNA fragment (lower affinity dissociation constants), while it exhibited considerably lower affinity for ssDNA. The protein was conjugated to HRP, aiming at using a scHupBA/scHupBA-HRP sandwich system for universal capture and detection of dsDNA. The scHupBA-HRP enzyme conjugate was prepared by crosslinking thiol-activated scHupBA with maleimide-activated HRP. The conjugate was purified with a two-step process which successfully removed unconjugated proteins. The scHupBA/scHupBA-HRP sandwich system was then applied for the detection of a model target PCR amplicon using microtiter plates and magnetic beads for the immobilization of scHupBA to capture the target DNA. Studies performed under different buffer conditions suggested improved sensitivity at lower salt (NaCl) concentrations, consisted with literature findings of higher DNA binding affinity of the native

E. coli HU protein at moderate to low NaCl concentrations. Under current assay conditions, approximately 2 – 16 ng/μL dsDNA can be detected with these assays, whereas the presence of ssDNA does not produce non-specific signals. A demonstration of the utility of this protein for the development of POC tests was also provided by the detection of a PCR amplicon with a lateral flow dipstick assay. The tests were constructed using scHupBA immobilized on the strips and a scHupBA-carbon nanoparticle conjugate for colorimetric signal generation. The effect of different parameters on signal intensity was studied and under optimized conditions with low salt conditions it was possible to detect approximately 15 ng/μL of purified dsDNA PCR amplicon, while it did not detect ssDNA. The compatibility of the three assays with direct analysis of PCR reactions without prior purification was finally verified, where the amplicons were detected when the target template used was as low as 3.3 - 25 fg/μL. Overall, this novel engineered non-sequence specific DNA binding protein scHupBA appears to have a great potential for the detection of DNA thus circumventing the need of labelling or use of expensive reagents and materials. While in this work we provide some insights into the potential applications of this protein in DNA bioanalysis, further work is required to fully understand its functions and optimize assay parameters accordingly so that it can fully reach its potential as a universal dsDNA bioreceptor and reporter.

4.6 Acknowledgements

This work was supported financially by the CELLECTRA project (PID2020-112976RB-C22) 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.

4.7 References

1. Hua Y, Ma J, Li D, Wang R. DNA-Based Biosensors for the Biochemical Analysis: A Review. *Biosensors*. 2022;12(3).
2. Su Y, Hammond MC. RNA-based fluorescent biosensors for live cell imaging of small molecules and RNAs. *Curr Opin Biotechnol*. 2020;63:157–66.
3. Jackson C, Anderson A, Alexandrov K. The present and the future of protein biosensor engineering. *Curr Opin Struct Biol*. 2022;75:102424.
4. Li M, Yin F, Song L, Mao X, Li F, Fan C, et al. Nucleic Acid Tests for Clinical

- Translation. *Chem Rev.* 2021;121(17):10469–558.
5. Sokolenko AP, Imyanitov EN. Molecular diagnostics in clinical oncology. *Front Mol Biosci.* 2018;5:76.
 6. Palchetti I, Mascini M. Nucleic acid biosensors for environmental pollution monitoring. *Analyst.* 2008;133(7):846–54.
 7. Ceuppens S, Li D, Uyttendaele M, Renault P, Ross P, Ranst M Van, et al. Molecular methods in food safety microbiology: interpretation and implications of nucleic acid detection. *Compr Rev Food Sci Food Saf.* 2014;13(4):551–77.
 8. Law JW-F, Ab Mutalib N-S, Chan K-G, Lee L-H. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Front Microbiol.* 2015;5:770.
 9. Kim DC, Kang DJ. Molecular Recognition and Specific Interactions for Biosensing Applications. *Sensors (Basel).* 2008;8(10):6605–41.
 10. Chang D, Tram K, Li B, Feng Q, Shen Z, Lee CH, et al. Detection of DNA Amplicons of Polymerase Chain Reaction Using Litmus Test. *Sci Rep.* 2017;7(1):3110.
 11. Kaewphinit T, Santiwatanakul S, Promptmas C, Chansiri K. Detection of Non-Amplified Mycobacterium tuberculosis Genomic DNA Using Piezoelectric DNA-Based Biosensors. Vol. 10, *Sensors.* 2010. p. 1846–58.
 12. Bartlett JMS, Stirling D. A short history of the polymerase chain reaction. *PCR Protoc.* 2003;3–6.
 13. Oliveira BB, Veigas B, Baptista PV. Isothermal amplification of nucleic acids: The race for the next “gold standard.” *Front Sensors.* 2021;2:752600.
 14. Deiman B, van Aarle P, Sillekens P. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). *Mol Biotechnol.* 2002;20(2):163–79.
 15. Soroka M, Wasowicz B, Rymaszewska A. Loop-Mediated Isothermal Amplification (LAMP): The Better Sibling of PCR? Vol. 10, *Cells.* 2021.
 16. Hellyer TJ, Nadeau JG. Strand displacement amplification: a versatile tool for molecular diagnostics. *Expert Rev Mol Diagn.* 2004;4(2):251–61.
 17. Lobato IM, O’Sullivan CK. Recombinase polymerase amplification: Basics, applications and recent advances. *TrAC Trends Anal Chem.* 2018;98:19–35.
 18. Xu L, Duan J, Chen J, Ding S, Cheng W. Recent advances in rolling circle amplification-based biosensing strategies-A review. *Anal Chim Acta.* 2021;1148:238187.
 19. Li D, Fan C. Optical Detection of Non-amplified Genomic DNA. In: *Detection of Non-amplified Genomic DNA.* Springer; 2012. p. 153–83.

20. Marín S, Merkoçi A. Electrochemical Detection of DNA Using Nanomaterials Based Sensors. In: *Detection of Non-Amplified Genomic DNA*. Springer; 2012. p. 185–201.
21. Wu J, Lv J, Zheng X, Wu Z-S. Hybridization chain reaction and its applications in biosensing. *Talanta*. 2021;234:122637.
22. Kricka LJ. Stains, labels and detection strategies for nucleic acids assays. *Ann Clin Biochem*. 2002;39(2):114–29.
23. Sassolas A, Leca-Bouvier BD, Blum LJ. DNA biosensors and microarrays. *Chem Rev*. 2008;108(1):109–39.
24. VS AP, Joseph P, SCG KD, Lakshmanan S, Kinoshita T, Muthusamy S. Colorimetric sensors for rapid detection of various analytes. *Mater Sci Eng C*. 2017;78:1231–45.
25. Wu Q, Zhang Y, Yang Q, Yuan N, Zhang W. Review of Electrochemical DNA Biosensors for Detecting Food Borne Pathogens. *Sensors (Basel)*. 2019;19(22).
26. Leonardo S, Toldrà A, Campàs M. Biosensors Based on Isothermal DNA Amplification for Bacterial Detection in Food Safety and Environmental Monitoring. Vol. 21, *Sensors*. 2021.
27. Zheng C, Wang K, Zheng W, Cheng Y, Li T, Cao B, et al. Rapid developments in lateral flow immunoassay for nucleic acid detection. *Analyst*. 2021;146(5):1514–28.
28. García-Bernalt Diego J, Fernández-Soto P, Muro A. The future of point-of-care nucleic acid amplification diagnostics after COVID-19: time to walk the walk. *Int J Mol Sci*. 2022;23(22):14110.
29. Wang J, Jiang H, Pan L, Gu X, Xiao C, Liu P, et al. Rapid on-site nucleic acid testing: On-chip sample preparation, amplification, and detection, and their integration into all-in-one systems. *Front Bioeng Biotechnol*. 2023;11:1020430.
30. Land KJ, Boeras DI, Chen X-S, Ramsay AR, Peeling RW. REASSURED diagnostics to inform disease control strategies, strengthen health systems and improve patient outcomes. *Nat Microbiol*. 2019;4(1):46–54.
31. Mariño-Ramírez L, Kann MG, Shoemaker BA, Landsman D. Histone structure and nucleosome stability. *Expert Rev Proteomics*. 2005;2(5):719–29.
32. Anuchin AM, Goncharenko A V, Demidenok OI, Kaprel'iants AS. [Histone-like proteins of bacteria (review)]. *Prikl Biokhim Mikrobiol*. 2011;47(6):635–41.
33. Pabo CO, Sauer RT. PROTEIN-DNA RECOGNITION. *Annu Rev Biochem* . 1984;53(1):293–321.
34. Kung VT, Panfili PR, Sheldon EL, King RS, Nagainis PA, Gomez B, et al. Picogram quantitation of total DNA using DNA-binding proteins in a silicon sensor-based system.

- Anal Biochem. 1990;187(2):220–7.
35. Hedgethorne K, Webb MR. Fluorescent SSB as a reagentless biosensor for single-stranded DNA. *Single-Stranded DNA Bind Proteins Methods Protoc.* 2012;219–33.
 36. Umer M, Aziz NB, Mahmudunnabi RG, Shim Y-B, Salomon C, Shiddiky MJA. A novel DNA binding protein-based platform for electrochemical detection of miRNA. *Analyst.* 2021;146(18):5496–501.
 37. Mahmudunnabi RG, Umer M, Seo K-D, Park D-S, Chung JH, Shiddiky MJA, et al. Exosomal microRNAs array sensor with a bioconjugate composed of p53 protein and hydrazine for the specific lung cancer detection. *Biosens Bioelectron.* 2022;207:114149.
 38. Aktas GB, Ribera A, Skouridou V, Masip L. DNA immobilization and detection using DNA binding proteins. *Anal Bioanal Chem.* 2021;413(7):1929–39.
 39. Aktas GB, Skouridou V, Masip L. Nucleic acid sensing with enzyme-DNA binding protein conjugates cascade and simple DNA nanostructures. *Anal Bioanal Chem.* 2017;409:3623–32.
 40. Aktas GB, Wichers JH, Skouridou V, van Amerongen A, Masip L. Nucleic acid lateral flow assays using a conjugate of a DNA binding protein and carbon nanoparticles. *Mikrochim Acta.* 2019;186(7):426.
 41. Wang G, Maier RJ. Bacterial histone-like proteins: roles in stress resistance. *Curr Genet.* 2015;61(4):489–92.
 42. Henneman B, van Emmerik C, van Ingen H, Dame RT. Structure and function of archaeal histones. *PLoS Genet.* 2018;14(9):e1007582.
 43. Swinger KK, Rice PA. Structure-based analysis of HU-DNA binding. *J Mol Biol.* 2007;365(4):1005–16.
 44. Laine B, Kmiecik D, Sautiere P, Biserte G, Cohen-Solal M. Complete amino-acid sequences of DNA-binding proteins HU-1 and HU-2 from *Escherichia coli*. *Eur J Biochem.* 1980;103(3):447–61.
 45. Barlow VL, Tsai Y-H. Acetylation at Lysine 86 of *Escherichia coli* HU β Modulates the DNA-Binding Capability of the Protein. *Front Microbiol.* 2021;12:809030.
 46. Bonnefoy E, Rouvière-Yaniv J. HU and IHF, two homologous histone-like proteins of *Escherichia coli*, form different protein-DNA complexes with short DNA fragments. *EMBO J.* 1991;10(3):687–96.
 47. Kamashev D, Rouviere-Yaniv J. The histone-like protein HU binds specifically to DNA recombination and repair intermediates. *EMBO J.* 2000;19(23):6527–35.
 48. Jana R, Hazbun TR, Fields JD, Mossing MC. Single-Chain Lambda Cro Repressors

- Confirm High Intrinsic Dimer–DNA Affinity. *Biochemistry*. 1998;37(18):6446–55.
49. Hammel M, Amlanjyoti D, Reyes FE, Chen J-H, Parpana R, Tang HYH, et al. HU multimerization shift controls nucleoid compaction. *Sci Adv*. 2016;2(7):e1600650.
 50. Sørensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J Biotechnol*. 2005;115(2):113–28.
 51. Aktas GB, Skouridou V, Masip L. Novel signal amplification approach for HRP-based colorimetric genosensors using DNA binding protein tags. *Biosens Bioelectron*. 2015;74:1005–10.
 52. Xiao B, Johnson RC, Marko JF. Modulation of HU–DNA interactions by salt concentration and applied force. *Nucleic Acids Res*. 2010;38(18):6176–85.
 53. Tang C, He Z, Liu H, Xu Y, Huang H, Yang G, et al. Application of magnetic nanoparticles in nucleic acid detection. *J Nanobiotechnology*. 2020;18(1):1–19.
 54. Porras JC, Bernuz M, Marfa J, Pallares-Rusiñol A, Martí M, Pividori MI. Comparative Study of Gold and Carbon Nanoparticles in Nucleic Acid Lateral Flow Assay. Vol. 11, *Nanomaterials*. 2021.

Chapter 5: General conclusions

5.1 General conclusions

The overall objective of this thesis was to demonstrate the versatility of DNA binding proteins (DBP) by incorporating them in various bioanalytical assays exploiting different surfaces for the detection of double stranded DNA (dsDNA) in laboratory and field-compatible applications. Considering the increasing use of molecular diagnostics for detecting specific DNA sequences, sensitive, accurate and fast detection of dsDNA fragments resulting from amplification reactions is of paramount importance. The natural properties of certain proteins to recognize and selectively bind dsDNA in a sequence-specific or non-specific manner makes them ideal candidates as biological recognition elements for bioanalysis. In this thesis, we show the immobilization and conjugation of both sequence-specific and non-sequence specific DBPs on microtiter plates, magnetic beads, nitrocellulose membranes, carbon nanoparticles (CNPs) and horseradish peroxidase (HRP) enzyme for the development of microplate- and magnetic bead-based enzyme-linked oligonucleotide assays (ELONA), as well as dipstick lateral flow assays (LFA) for the detection of dsDNA amplicons in simple and multiplex PCR reactions. DBP-based capture and detection of dsDNA can therefore be regarded as an alternative to the well-established strept(avidin)-biotin-based strategies in bioanalytical applications.

The DBP utilized were the sequence-specific Cro from bacteriophage lambda and the non-sequence specific HU histone-like protein from *Escherichia coli*. Both proteins were engineered in single-chain (sc) dimeric formats to mimic their natural configurations and were expressed recombinantly in *E. coli* bacteria. The preparation of scCro was based on previous work but herein, a more robust and efficient expression system was demonstrated for increased protein production. On the other hand, to the best of our knowledge, this is the first report of the recombinant expression of HU as a single-chain heterodimer and its use as dsDNA biorecognition element in bioanalytical assays.

Initially we demonstrated the use of scCro conjugated to CNPs in dipstick lateral flow assays for the simultaneous detection of dsDNA amplicons generated in a multiplex PCR reaction. For this assay, the reverse primers used for the amplification steps were extended with single stranded DNA (ssDNA) tails, which were used as barcodes to facilitate differentiation of the two target DNA amplicons via hybridization with complementary probes. On the other hand, the common forward primer was extended with the specific dsDNA sequence recognized by Cro for detection using the scCro-CNPs conjugate. The assay was used to detect toxin-producing harmful microalgae without interference from other marine species, highlighting the

utility of the sequence-specific DBP as a universal reporter molecule compatible with lateral flow assays and different sample types (genomic DNA and cells).

We also demonstrated the compatibility of scCro with magnetic beads (MB) for dsDNA detection in PCR reactions performed with modified primers. The forward primer was extended with the scCro binding sequence for capture whereas the reverse primer was biotinylated for detection using streptavidin-HRP (SA-HRP). MB have been extensively used for the separation and detection of different target molecules including nucleic acids due to the several advantages they offer in terms of separation, surface area and functionalization. By immobilizing scCro on MB, we were able to specifically capture, detect and purify dsDNA amplicons directly in PCR reactions, eliminating the need for prior purification to remove remaining primers. The presence of residual biotinylated ssDNA primers in the amplification reactions did not interfere with detection when using the Cro-MB since scCro binds specifically only to dsDNA, as opposed to the well-established SA-MB, to which the Cro-MB system was compared, whose performance was significantly affected by the residual primers. Moreover, the Cro-MB showed higher dsDNA binding capacity and sensitivity compared to the SA-MB when synthetic or eukaryotic genomic DNA were used as PCR target templates. Finally, the Cro-MB did not only serve for dsDNA amplicon capture and detection but also demonstrated their potential for purification purposes. We provided a proof-of-concept for the purification of dsDNA under mild elution conditions, which could potentially allow for the reuse of the magnetic beads. This purification approach using Cro-MB eliminates the need for centrifuge spin column-based or other multiple-step magnetic bead-based PCR clean-up methods or harsh elution conditions as in the case of SA-MB.

All the previous applications were based on the use of modified primers for amplification required for incorporating the required binding sequence of the sequence-specific Cro DBP. Aiming at simplifying the assays and rendering them compatible with any amplification reaction, we envisioned the use of a non-sequence specific DBP for universal detection of dsDNA. The HU histone-like DBP from *E. coli* was engineered for the first time as a single-chain heterodimer (scHupBA), expressed recombinantly in *E. coli* and purified to near homogeneity. Microtiter plates were used to immobilize the protein and evaluate its binding properties using dsDNA fragments with a biotin on one end for detection of bound DNA with SA-HRP. It was demonstrated that scHupBA exhibited a length-dependent affinity for DNA, with preferential binding to longer and dsDNA fragments and significantly lower affinity for ssDNA. The scHupBA DBP was also conjugated to HRP to enable universal and label-free

capture and detection of a model PCR amplicon utilizing a scHupBA/scHupBA-HRP sandwich system on both microtiter plates and magnetic beads. Our studies under different buffer conditions indicated improved sensitivity at lower salt concentrations, aligning with previous findings on the higher DNA binding affinity of the native *E. coli* HU protein. Furthermore, the compatibility of a scHupBA/scHupBA sandwich system with lateral flow assays was demonstrated using a scHupBA-CNPs conjugate for detection of a purified dsDNA amplicon. Finally, all three assays based on microtiter plates, magnetic beads and lateral flow strips were validated for the detection of dsDNA amplicons directly in PCR reactions without prior purification, which validated the absence of interference from remaining ssDNA primers. The use of the dipstick lateral flow assay for universal and label-free detection of dsDNA amplicons directly in PCR reactions without prior purification is particularly important, considering the extremely simple (one-step) and fast (< 20 min) nature of the assay. Overall, we demonstrated that HU-based strategies for dsDNA detection eliminate the need for labeling or the use of expensive reagents and materials, making them cost-effective and efficient methods for DNA detection.



UNIVERSITAT
ROVIRA i VIRGILI