Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Biologia Cellulare Molecolare e Industriale

Ciclo XXIII

**Settore scientifico-disciplinare di afferenza: CHIM/06**

# DEVELOPMENT OF BIONANOTECHNOLOGICAL STRATEGIES FOR SIGNAL ENHANCEMENT IN NUCLEIC ACIDS BIOSENSORS

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**Esame finale anno 2011**

*Per Luca e*

*la mia Famiglia*

# ABSTRACT

**Key-words**: nucleic acids, amplification, signal enhancement, isothermal reaction, DNA nanotechnology

Nucleic acid biosensors represent a powerful tool for clinical and environmental pathogens detection. For applications such as point-of-care biosensing, it is fundamental to develop sensors that should be automatic, inexpensive, portable and require a professional skill of the user that should be as low as possible. With the goal of determining the presence of pathogens when present in very small amount, such as for the screening of pathogens in drinking water, an amplification step must be implemented. Often this type of determinations should be performed with simple, automatic and inexpensive hardware: the use of a chemical (or nanotechnological) isothermal solution would be desirable.

My Ph.D. project focused on the study and on the testing of four isothermal reactions which can be used to amplify the nucleic acid analyte before the binding event on the surface sensor or to amplify the signal after that the hybridization event with the probe.

Recombinase polymerase amplification (RPA) and ligation-mediated rolling circle amplification (L-RCA) were investigated as methods for DNA and RNA amplification.

RPA mimics the replication machinery of the phage T4 using recombination reaction as alternative mechanism to melt the DNA double helix. It is mediated by the coordinated activity of four proteins (UvsX, UvsY, Gp32 and Bsu Pol I) which work in concert to amplify the target. RPA was found to be a very promising method for point-of-care biosensing application: as low as 100 CFU were amplified and detected in 15 minutes at physiological temperature. The feasibility of RPA as amplification and labeling technique was demonstrated by coupling the reaction with a commercial microarray detection platform.

Ligation-based rolling circle amplification (L-RCA) uses a linear DNA probe in which both ends are juxtaposed by the specific hybridization to a target DNA or RNA sequence. The two ends of the DNA probe are joined by a DNA ligase, and the resulting DNA ring serves as the template for an RCA reaction with a suitable DNA polymerase. In this work, L-RCA was directed for RNA detection. The results obtained showed that

the ligation step was the limiting factor for the strategy: long reaction time and low reaction yield penalize eventual point-of-care applications.

Both reactions were tested using *E. coli* as a model system, targeting the gene for 16S rRNA (in the case of RPA) or directly the transcript. For the investigation of RPA, our tests were extended also to the detection of the sexually transmitted pathogens *N. gonorrhoeae* and *N elongata*. Optimizations of the reaction conditions, sensitivity and specificity were evaluated using standard molecular biology technique and a colorimetric/chemiluminescence probe-based assay.

Hybridization chain reaction (HCR) and Terminal deoxynucleotidil transferase-mediated amplification were investigated as strategies to achieve the enhancement of the signal after the surface hybridization event between target and probe.

HCR is an interesting enzyme-free reaction based on DNA nano-assembly. It is founded on the storage of potential energy in the two hairpin species which are stable in this conformation. The binding event between probe and target triggers a cascade of hybridization event and leads to the accumulation of mass which can be easily detected. Sequences belonging to different waterborne pathogens organism as *C. parvum*, *G. lamblia* and HEV were chosen as target and in all the cases the reaction was successfully tested.

Terminal deoxynucleotidil transferase (TdT) exhibits the unique ability to incorporate nucleotides in a template-independent manner using only single-stranded DNA as nucleic acid substrate. In this work, it has been demonstrated that terminal transferase can catalyze the polymerization of deoxynucleotides starting from the free-3'-OH end of a DNA target molecule hybridized to a surface probe.

This work provides a proof-of-concept that both HCR and TdT-mediated amplification can occur in different experimental setups. The results on the surface implementation were studied by measuring the fluorescence emission of fluorescently labeled target DNA and hairpin oligonucleotides. For both reactions, we estimated amplification ratios which unfortunately do not appear suitable for biosensing application.

In conclusion, it can be said that only a small subset of the biochemical strategies that are proved to work in solution towards the amplification of nucleic acids does truly work in the context of amplifying the signal of a detection system for pathogens. Amongst those tested during my Ph.D. activity, recombinase polymerase amplification seems the best candidate for a useful implementation in diagnostic or environmental applications.

# RIASSUNTO

**Concetti chiave**: acidi nucleici, amplificazione, amplificazione del segnale, reazioni isotermiche, nanotecnologie basate sul DNA

I biosensori per acidi nucleici sono degli strumenti molto potenti per la rilevazione di organismi patogeni in ambito clinico ed ambientale. Per poter essere utilizzati *in situ,* al di fuori del laboratorio, è necessario sviluppare dei sensori che siano economici, portatili e automatici tali che possano essere adoperati facilmente da un operatore non specializzato. In caso di rilevazione di patogeni presenti in piccolissime quantità, come può succedere nell'analisi delle acque potabili, potrebbe essere necessario aggiungere un passaggio di amplificazione. Considerando che spesso questo tipo di analisi è condotta con degli strumenti molto semplici dal punto di vista *hardware,* l'utilizzo di reazioni chimiche (o strategie nanotecnologiche) isotermiche per aumentare la sensibilità dello strumento potrebbero rappresentare una soluzione.

Il mio progetto di ricerca è stato incentrato su lo studio e sul test di quattro reazioni isotermiche che possono essere utilizzate per amplificare l'acido nucleico analita prima dell'ibridizzazione con la sonda oppure per amplificare il segnale dopo che il legame con la sonda ha avuto luogo.

La *recombinase polymerase amplification* (RPA) e la *ligation-mediated rolling circle amplification* (L-RCA) sono due reazioni che in questo studio sono state testate come metodi per amplificare rispettivamente DNA e RNA.

La RPA si basa sull'apparato replicativo del batteriofago T4 e usa la ricombinazione fra filamenti omologhi come meccanismo alternativo per separare la doppia elica del DNA. La reazione è mediata dall'attività di quattro proteine (UvsX, UvsY, Gp 32 and Bsu Pol I) che agiscono in modo coordinato per amplificare il DNA bersaglio. In questo lavoro è stato dimostrato che la RPA è un metodo molto promettente per le applicazioni biosensoristiche: una quantità di DNA batterico pari a 100 CFU è stato amplificato in meno di 15 minuti a temperatura fisiologica. La possibilità di utilizzare la RPA come metodo per amplificazione e marcatura è stata inoltre dimostrata accoppiando la reazione con la rilevazione su una piattaforma commerciale di DNA *microarray.*

La L-RCA usa una sonda lineare di DNA che viene circolarizzata solo a seguito del legame con una sequenza di DNA o RNA specifica. Le due estremità della sonda sono

unite dall'enzima DNA ligasi e ciò che ne risulta, una sonda circolare, è utilizzata come substrato dalla polimerasi che catalizza la RCA. In questo lavoro la L-RCA è stata utilizzata come reazione per la rilevare RNA. I risultati ottenuti mostrano che il passaggio di ligazione è il fattore limitante per l'impiego di questa strategia: tempi di reazione molto lunghi e resa di reazione bassa penalizzano la sua applicazione in un biosensore.

Entrambe le reazioni sono state testate utilizzando *E. coli* come organismo modello. Il gene che codifica per l'acido ribonucleico ribosomale 16S e il suo trascritto sono stati scelti come sequenze bersaglio per RPA e L-RCA. Nel caso della RPA la reazione è stata testata anche per la rilevazione dei batteri *N. gonorrhoeae* e *N elongata*. Le condizioni di reazione, la sensibilità e la specificità delle reazioni sono state valutate tramite tecniche di biologia molecolare standard e con metodo colorimetrico e chemiluminescente.

La Hybridization chain reaction (HCR) e la Terminal deoxynucleotidil transferase (TdT)-mediated amplification sono state testate come strategie per amplificare il segnale dell'evento di ibridizzazione fra sonda e analita avvenuto sulla superficie del sensore.

HCR è un'interessante reazione non enzimatica basata sul nano-assemblaggio di DNA. La reazione si basa sul fatto che due filamenti di DNA ripiegati in una conformazione "a forcina" mantengono stabilmente questa struttura fino a quando un altro filamento di DNA, chiamato "iniziatore", è inserito in soluzione. In questo lavoro il filamento iniziatore corrispondeva all'analita. Il legame dell'analita alla sonda innescava una cascata di eventi di ibridizzazione che potevano essere facilmente rilevati. Come bersaglio sono state scelte le sequenze di diversi organismi patogeni quali *C. parvum*, *G. lamblia* il virus dell'epatite E.

L'enzima terminal transferasi (TdT), catalizza l'incorporazione di nucleotidi all'estremità 3'- di una molecola di DNA. A differenza della maggior parte delle polimerasi del DNA non richiede un filamento modello. In questo lavoro di tesi è stato dimostrato che la terminal transferasi catalizza la reazione a un DNA bersaglio ibridizzato sulla superficie di un sensore.

È stato dimostrato che sia la HCR e l'amplificazione mediata dalla TdT funzionano in diverse condizioni sperimentali. L'implementazione su superficie è stata valutata tramite misure di fluorescenza. Purtroppo, per entrambe le reazioni, è stato calcolato un fattore di amplificazione non sufficiente per poter utilizzare i metodi come strategie per l'amplificazione del segnale su biosensori.

Per concludere può essere detto che in generale, non tutte le reazioni di amplificazione che si dimostrano efficaci in soluzione, sono adatte all'applicazione in biosensori per la rilevazione di patogeni. Fra le reazioni testare in questo lavoro di tesi, solo la RPA ha dimostrato di poter essere utilizzata efficacemente per questo scopo.

# CONTENTS

# TABLE OF ABBREVIATIONS

AGE: agarose gel electrophoresis

ATP: adenosine triphosphate

AuNPs: gold nanoparticles

bp: base pairs

CCD: charge-coupled device

CdSe-Zns: Cadmium Selenide/Zinc Sulfide

CFU: colony forming unit

c-NDA: circular nicking endonuclease dependent amplification

$CoCl_2$: Cobalt(II) chloride

Cy3-Cy5: cyanine3-cyanine5

DNA: deoxyribonucleic Acid

dNTP: deoxyribonucleotide triphosphate

dsDNA: double-stranded deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

EtBr: ethidium bromide

FAM: carboxyfluorescein

HCR: hybridization chain reaction

HRP: horseradish peroxidase

Hz: hertz

LAMP: loop mediated isothermal amplification

L-RCA: ligation-mediated rolling circle amplification

miRNA: micro ribonucleic acid

mRNA: messenger ribonucleic acid

$Na_2HPO_4$: Disodium hydrogen phosphate

NaCl: sodium chloride

nm: nanometers

nt: nucleotides

NTP: nucleotide triphosphate

PAGE: polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

pg: picograms

POC: point-of-care

QCM: quartz crystal microbalance

QD: quantum dots

RCA: rolling circle amplification

RDR: recombination-dependent DNA replication

RNA: ribonucleic acid

RPA: recombinase polymerase amplification

rRNA: ribosomal ribonucleic acid

SA: streptavidin

SDA: strand displacement amplification

SIEP: surface initiated enzymatic polymerization

SIP: surface initiated polymerization

SPR: surface plasmon resonance

ssDNA: single strand deoxyribonucleic acid

TAMRA: tetramethylrhodamine

TBE: Tris/Borate/EDTA

TdT: terminal deoxynucleotidil transferase

TMA: transcription mediated amplification

TMB: 3,3',5,5'-Tetramethylbenzidine

Tris-HCl: Tris (hydroxymethyl) aminomethane hydrochloride

tRNA: transfer RNA ribonucleic acid

TSG: template stripped gold

# CHAPTER 1

## INTRODUCTION

### NUCLEIC ACID DETECTION IN PATHOGEN DIAGNOSTICS

The topic of rapid and reliable detection of pathogenic agents has come to the fore in many different research fields and industries like food and agriculture, healthcare, environmental monitoring, and bio-defense. For example, the development of a fast and sensitive platform for the detection of pathogens in human blood and waste samples is required in order to implement a quick and effective response to an outbreak. The recent pandemic alarms for SARS, avian and swine influenza, motivated academia and industry to move towards this research field[1]. In the environmental monitoring area, great attention is given to the evaluation of microbial cells in water and environment quality control[2,3]. In the area of biodefense, in the last 10 years, research projects finalize to the development of alarm systems against bioterrorism has been largely promoted and founded by European Governments[4,5]. In this setting, diagnostic systems based on nucleic acids represent an attractive tool for pathogenic detection thanks to the intrinsic specificity of nucleic acid interaction. In addition, mechanical and chemical properties of DNA and RNA make these molecules suitable for detection at very low concentration even to the single molecule level.

For applications such as point-of-care biosensing, it is fundamental to develop sensors that should be automatic, inexpensive, reliable and require a professional skill of the user that should be as low as possible. The need of producing a high number of preferably disposable measuring chips, to be read by a relatively inexpensive electronic apparatus implicitly points toward the direction of not employing sophisticated laboratory techniques. Instrumentations such as probe microscopy, fluorescence microscopy or spectroscopy should not be used for the read-out of the biosensors: despite their sensitivity, single-molecule techniques are difficult and expensive even when performed on the scale of the research laboratory, and they require a great deal of professional skill and time for each measurement. This practical need pushes back the limit for the detection of analyte molecules by attainable biosensors by many orders of magnitude with respect to research-level sensitivities. In the case of the detection of nucleic acids in

a solution (being for diagnostic or environmental testing purposes), this problem has been classically tackled by exposing the biosensors or other sensing elements to the result of analyte amplification, usually by means of techniques such as the polymerase chain reaction (PCR). This can be implemented in solution prior to introduction of the specimen in the sensing device or directly in the biosensor through a number of presented on-chip implementations of the PCR protocol[6-8].

## PCR VS. ISOTHERMAL AMPLIFICATION METHODS

Since the mid 80's, nucleic acid amplification test based (NAAT) on polymerase chain reaction revolutionized the field of pathogen detection. It was possible to detect virtually at single cell level every bacterial or virus pathogen through the design of specific probe sequences, typically on the order of 10 to 30 base pairs in length. Despite its high sensitivity, some drawbacks limit the use of PCR as amplification strategy in DNA biosensor and move researchers towards looking for some alternatives both for analyte and signal amplification.

Despite it is a well-known mechanism, hugely diffused in genomics, genetics and diagnostics, PCR applications in point-of-care devices are limited in a number of ways. The first limitation of PCR is that it relies on multiple cycles of thermal melting (denaturing) at high temperatures followed by hybridization and elongation at reduced temperatures. To maximize efficiency and minimize noise, complex temperature control of multiple reactions is required. The reaction requires the use of a thermocycler controllable rapid heating/cooling block, or an automatic mechanical device to move samples between temperature-controlled zones. Because of the high temperature required to melt DNA in physiological salt conditions, PCR technology requires the use of thermostable enzymes or to add fresh polymerase for cycle (a practice soon abandoned). In addition, due to its extreme sensitivity, contamination from non-template PCR is a serious problem. As a matter of facts, sample preparation is a key step in PCR amplification and extreme clean environment, dedicated labware and good manual skills are some basic requirements. A surface-integrated PCR could be helpful to reduce sample handling and contamination. Several attempts have been done in this direction[9-11].

The use of isothermal methods to achieve strong signal from analyte sample at very low concentration could potentially solve some of the difficulties related to the PCR. Two alternative strategies could be the following:

1. Target amplification with isothermal, non-PCR strategies
2. Signal amplification after the binding of the target with the capture probe.

In both cases the results is the same: we can detect theoretically single molecule.

In the next section, a number of isothermal amplification strategies and signal amplification methods which has been coupled with nucleic acid biosensors will be described. For each of them, detection limit, amplification factor (if evaluated) and reaction time will be reported in order to give to the reader a more precise idea about the state of the art.

## ISOTHERMAL STRATEGIES FOR SAMPLE AMPLIFICATION

To amplify a nucleic acid implies the use of a polymerase, RNA or DNA dependent, depending from the type of substrate. Several isothermal methods alternative to PCR have been proposed: they can mimic natural replication machineries, with cloned components which reproduced the system *in vitro*, or they can be artificial. In the case that they are targeted to DNA amplification they exhibit a mechanism to melt the double strand in alternative to heating.

### *TRANSCRIPTION MEDIATED AMPLIFICATION*

Transcription mediated amplification (TMA) is a Gen-Probe proprietary invention[12]. It is used to amplify RNA (mRNA or rRNA) thanks to the combined activity of two enzymes: RNA polymerase and reverse transcriptase. For the reaction, two primers are required and one of them includes the promoter sequence for the RNA polymerase. The entire reaction takes place at 41°C, after an initial step at 60°C for primer annealing. A scheme of the complete reaction is reported in Fig. 1.1. In the first reaction step, the forward primer, which contains the promoter sequence for the RNA polymerase, hybridizes to the target. The RNA strand in the resulting cDNA-RNA hybrid is degraded by the ribonuclease activity of the reverse transcriptase and the reverse primer can thus hybridizes to the cDNA strand. Reverse transcriptase can then elongate reverse primers leading to the synthesis of a double strand DNA molecule containing the promoter sequence for the RNA polymerase. Starting from this sequence RNA polymerase catalyzes the synthesis of 100-1000 copies of RNA which are used as template for reverse transcriptase. For this reason, it is reported than 10 billions copies of amplicon can be synthesized in one hour[13]. A series of commercial products is available from Gen-Probe for the detection of several pathogens (see Table 1.1). Several recent articles have reported the efficient use of TMA for pathogens diagnosis in water and biological fluids[14-16]. TMA is similar to nucleic acid sequence based amplification (NASBA) method which is object of this dissertation (see Chapter 5). TMA appears as a really effective amplification strategy of a RNA target but its application of surface appears difficult since it involves the use of more than one enzyme.

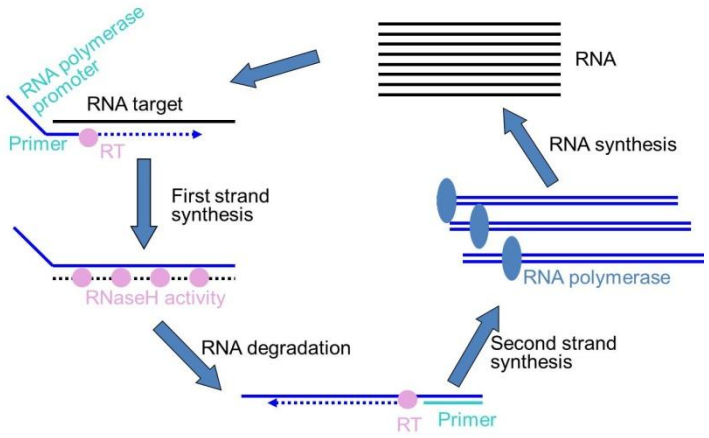


**Fig. 1.1:** Scheme of transcription mediated amplification (TMA). Pivture modified from the original published in http://pathology2.jhu.edu/molec/techniques_main.cfm.

### *STRAND DISPLACEMENT AMPLIFICATION*

Strand displacement amplification (SDA) is an isothermal DNA amplification method proposed in 1992 by Walker and coworkers[17,18]. It is a rather complex mechanism based on consecutive events of restriction and amplification. The use of four primers is requested (two amplification primers and two restriction primers) and an initial heating step for template denaturation is necessary. The overall reaction can be divided in two phases: target generation phase and exponential phase (see Fig. 1.2). In the first phase, during the initial melting step, the four primers are annealed to the template. They are elongated simultaneously by the exonuclease-deficient large fragment of *E. coli* DNA polymerase at 37°C. As result of this phase fragments containing the restriction site for HincII nicking enzyme are generated. During the second phase, the coordinate activity of restriction enzyme and DNA polymerase triggers a cascade of strand displacement events which substitute the denaturation step for template generation.

SDA has been largely used for pathogen detection in solution[14,] and some of articles reported the use of commercial platform for SDA real time monitoring (Table 1.1). Recently SDA has been coupled with piezoelectric detection for the real time monitoring of human cytomegalovirus[20]. Quartz crystals have been modified with a specific DNA capture probe: during the strand displacement amplification, RNA products accumulate on the crystal surface changing its resonance frequency. The limit of detection proposed is 1 ng/ml (Fig. 1.3).

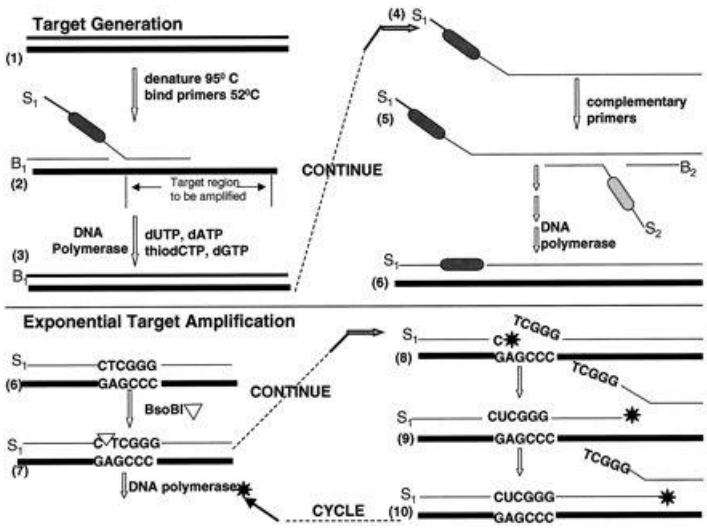


**Fig. 1.2**. Scheme of strand displacement amplification. The bold line in S1 and S2 primers indicates the position of the restriction site for HincII. Figure reproduced from Little *et al.*[19].

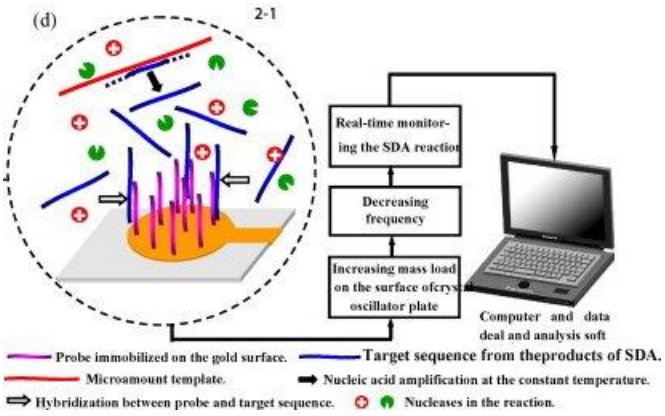


**Fig. 1.3**. Strand displacement amplification can be combined with label free detection of pathogens. Chen and coworkers combined a quartz crystal microbalance (QCM) biosensor and SDA. Picture reproduced from Chen *et al.*[20].

Despite its seeming complexity has been demonstrated that SDA can efficiently amplify a target molecule present at low concentration both in solution and on surface. The use in combination with a QCM biosensor open the door towards SDA use in lab-on-a chip device and render this technique really promising in DNA diagnostic.

### *LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)*

It is a method proposed ten years ago by Notomi and colleagues[21]. The LAMP mechanism is largely inspired to SDA and like SDA it is based on cycling strand displacement activity of DNA polymerase. For the reaction a set of four specific primers is necessary. Primer design is a key point of this complex mechanism: as a matter of fact the four primers hybridize to six different specific regions on the template DNA. For

primer annealing an initial heating step is necessary. Primers simultaneously hybridize to DNA target, generating a stem-loop structure for the next amplification cycle (Fig. 1.4).

LAMP is reported to be highly sensitive and fast: it can detect as few as six copies of hepatitis B virus DNA target in solution in 45 minutes[21]. If combined with a reverse transcription step LAMP can be used for RNA amplification (RT-LAMP)[21].

LAMP has been confirmed as a sensitive method for the detection of waterborne pathogens in solution as on a surface. A specific RT-LAMP assay for *Cryptosporidium parvum* detection has been developed by Inomata and colleagues[22]. Using 18S RNA as specific target, they detected a quantity of DNA equivalent to one *Cryptosporidium* oocysts genome in 4 ml of solution. Yang *et al.*, have designed specific primers for the detection of *Salmonella enteriditis*[23]. The reaction has been performed at 65°C for 20 min and a limit of detection of 4 CFU/µl has been calculated. A first implementation of LAMP on solid support has been proposed by Maruyama and colleagues for the specific amplification of gene *stx2* of *E. coli* O157:H7 cells[24].

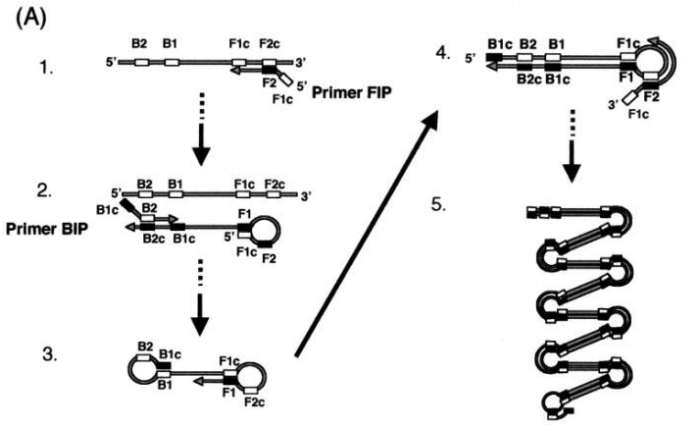


**Fig. 1.4**. Scheme of LAMP mechanism. Figure taken from Maruyama *et al.*[24].

LAMP combines the effectiveness of enzymatic-based amplification with a widely use nanotechnological approach like strand displacement. Base-pair complementarity allows the design of a multitude of engineered and controllable system which can be successfully employed to mechanically unwind the double helix. LAMP and SDA represents two fine example of how DNA nanotechnology can be combined with molecular biology.

### *Isothermal DNA amplification using the T4 replisome*

A new entry in the family of non-PCR based amplification techniques has been proposed by Schaerli and colleagues[25]. The authors reformed *in vitro* the bacteriophage T4 replisome amplifying 1100-fold in 1 hour a whole plasmid of 4.7 kb[25]. Combining the activity of the replisome with a nicking endonuclease (circular nicking endonuclease

dependent amplification, c-NDA) they have been able to synthesize linear copies of plasmids avoiding the generation of high molecular weight concatamers (Fig. 1.5). The reaction is totally isothermal: thermal denaturation of the duplex is replaced by helicase activity of some replisome components and the single strand parental strands are stabilized by a single strand binding protein present in solution. In addition, just one primer is required: the nick generated by the endonuclease serves to initiate amplification by the replisome and one synthetic oligonucleotide is required to prime the synthesis of the double strand amplification product (Fig. 1.5). Despite the complexity of the system (the complete T4 replication machinery is formed by 8 proteins) this method is presented by the authors as reliable and quite sensitive. In the same article, they demonstrated that T4 replisome can be used for the whole genome amplification, showing a detection limit of 10 pg of human genomic DNA in solution[25]. So far no diagnostic application for this method has been proposed. Nevertheless, the authors suggest that the c-DNA could be used in several molecular biology applications like DNA amplification for *in vitro* transcription and translation protein expression assays or bacterial colonies screening[25].

Respect to the aforementioned strategies, the T4 replisome-based amplification is really innovative because it is completely based on a cellular replication machinery. The replisome is perfectly reproduced *in vitro* and the authors have demonstrated to be able to keep constant the enzymatic efficiency and the reaction yield. Another very interesting isothermal amplification strategy which mimics a natural system is named recombinase polymerase amplification and it is object of this thesis work.

**Table 1.1 Isothermal methods for target amplification**

| Method | Detection limit or amplification power | Enzymes | Suitable for | Available commercial kit |
|---|---|---|---|---|
| **TMA** | ≤ 50 copies/ml[26] | Reverse transcriptase and RNA polymerase | RNA | TMA Component System (Siemens) APTIMA® assay kit (Gen-probe) |
| **SDA** | 100 copies/reaction[27] (in solution) and 1ng/ml[20] (if coupled to mass sensor) | DNA polymerase | DNA | BDProbeTec™ ET System (Becton. Dickinson) |
| **LAMP** | 6 copies/reaction (25 µl)[21] | Bst DNA polymerase large fragment | DNA and RNA | Loopamp® (Eiken Chemical Co.) |
| **c-NDA** | 1100-fold amplification/hour[25] | Endonuclease, DNA polymerase | DNA | none |

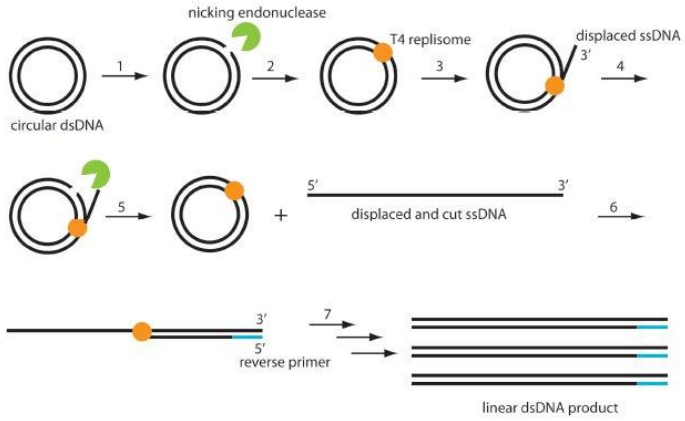


**Fig. 1.5**. Scheme of circular nicking endonuclease dependent amplification (c-NDA) used for plasmids amplification. The isothermal reaction combines the activity of the bacteriophage T4 replisome and the activity of a nicking endonuclease. The reaction takes place for 1 hour at 37°C and leads to the synthesis of more than 1000 linear copies of a whole plasmids. Figure reproduced from [25].

## ISOTHERMAL STRATEGIES FOR SIGNAL AMPLIFICATION

Signal enhancement strategies take place after analyte recognition by means of the biosensor probes, thus on the biosensor surface and in an independent stage from a target amplification step. A wide literature is present on the topic and some significant example will be described in this paragraph. For simplicity they will be divided in two categories: enzymatic-based strategies or not-enzymatic strategies. For both categories the final goal is to obtain the enhancement factor as high as possible maintaining, if possible, specificity in the detection (even though false positives are generally less harmful than false negatives in pathogen detection). The choice of one method with respect to another one must be done in order to be compatible with the detection technology that is used for the biosensor.

### *ENZYMATIC STRATEGIES*

Some of the most widespread (and commercially available) methods for the enhancement of the hybridization of nucleic acids are based on the presence of enzymes. A single recognition event typically leads to the immobilization of a single enzyme molecule close to the sensing area. There, the enzyme catalyses the conversion of many substrate molecules into an easily detectable product, thus leading to signal enhancement. If the enzyme is a polymerase and the substrates are dNTPs, the event triggers the accumulation of nucleic acids.

## Signal enhancement strategies based on the use of peroxidase and alkaline phosphatase

Horseradish peroxidase and alkaline phosphatase are the two of the most used enzymes in nucleic acid biosensor signal enhancement. A number of methods in which the enzyme is finally co-immobilized with the target on the sensing spot have been proposed [28]. Usually the enzyme converts an appropriate substrate present into a to-be-sensed product that is thus easily detected in several ways.

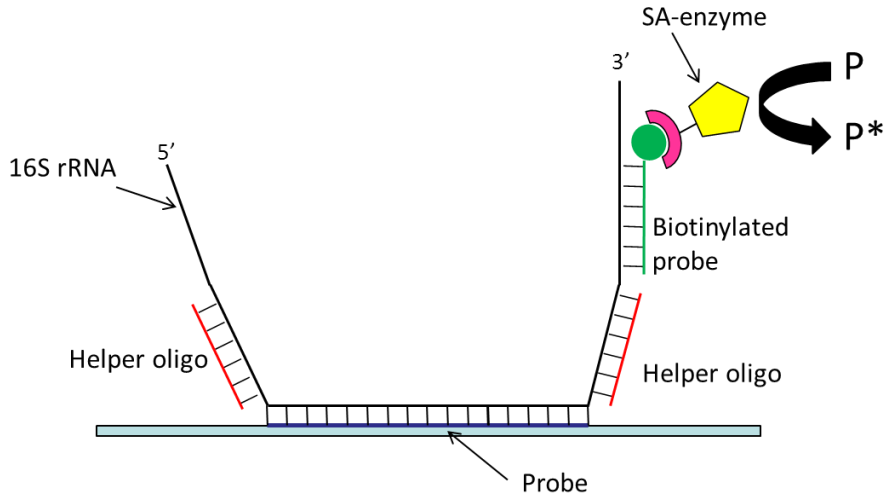


**Fig. 1.6**. Implementation scheme of the detection of RNA based on alkaline phosphatase. Helper oligonucleotides can enhance binding by reducing RNA secondary structure and/or increase signal enhancement factor if they can bind the enzyme as well.

Enzymes have been largely used in 16S RNA based-biosensor. In sandwich-type detection, a specific adsorbed oligonucleotide probe hybridizes with the analyte RNA. Other regions of known base sequence of the target can be exploited to bind one to several secondary biotinylated oligonucleotides. These can then bind streptavidin-conjugated alkaline phosphatase molecules. In the presence of alkaline phosphatase, p-aminophenyl phosphate is converted to p-aminophenol, which thus accumulates over time in the case (and in the location) of RNA recognition and binding. p-Aminophenol can be oxidized to the corresponding quinoimide compound. The electric current derived from redox-cycling of this compound can be measured (implementing the chemoelectronic sensor as a series of interdigitated electrodes). The presence of the bound enzyme will make the redox current grow with time due to the progressive accumulation of the electroactive molecule, thus leading to signal enhancement.

## Signal enhancement strategies based on the use of DNA polymerase

Phi 29 polymerase has been largely used in signal amplification thanks to its processivity and stability[29,30]. Like several other types of polymerase which share the same properties, Phi29 catalyzes the so called rolling circle amplification, an isothermal reaction which is extensively described in Chapter 6 of this dissertation. Chengde Mao and co-workers proposed a dual amplification strategy towards the amplification of the hybridization signal between complementary DNA molecules coupling RCA and peroxidase signal enhancement[31]. The analyte sequence hybridizes on a circular template, and perfect complementarity at the 3' end is necessary. The analyte sequence is thus used as a primer for rolling circle amplification by a Phi29 DNA polymerase so that a long piece of single-stranded DNA is produced as a consequence of recognition. This RCA represents the first amplification strategy of this detection method, as the long DNA fragment is easy to detect. On a non-probing section of the circular template strand, additional information content is included, to yield a second amplification step. A sequence is included that will be duplicated many times in case of DNA recognition. This sequence will fold into a G-quadruplex structure with binding capability for hemin, an iron-containing porphyrin that will then work as a peroxidase. The many copies of such DNA peroxidases can catalyse the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS). The reaction product ABTSC+ is blue-green (maximum absorption wavelength, $\lambda_{max}$=415 nm) and can serve as a convenient, colorimetric output signal. This reaction has multiple turnovers; each enzyme can generate multiple copies of products. This constitutes the second amplification step. The authors report the signal dependence of the colorimetric detection stating that they can clearly distinguish the presence of the analyte strand down to 1 pM. The output of this method depends on the careful tuning of the two amplification steps, as they cannot be optimized (or pushed) independently: too long a RCA amplification will create a highly entangled DNA molecule that will inhibit the diffusion of reactants for the second amplification step.

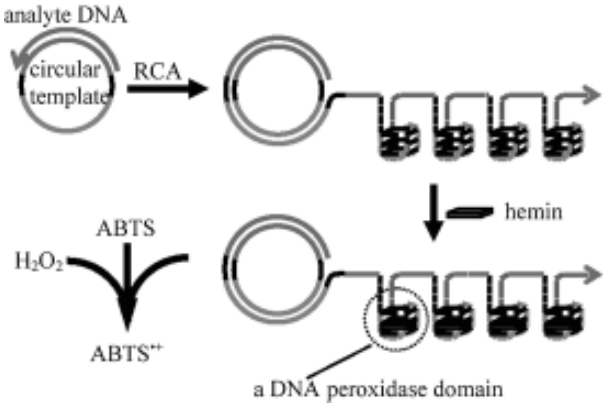


**Fig. 1.7**. Mao et al.'s strategy. Reproduced from Tian *et al.*[31].

## Signal enhancement of fluorescence through the use of a nickase

When a fluorescent probe is used for detecting a target (for example through molecular beacon approach), the cleavage of the probe in case of hybridization can release the target in solution for subsequent binding events. In an implementation of this strategy using a nickase, Zheleznaya and co-workers used molecular beacons and a site specific nickase to obtain an enhancement factor of 100[32]. Very interestingly, researchers also noted some interference in the assay in case of the presence of extraneous DNA that leads to a decrease in the signal (rather than to an expected increase due to non specific binding) probably due to the binding of the nickase to the extraneous DNA.

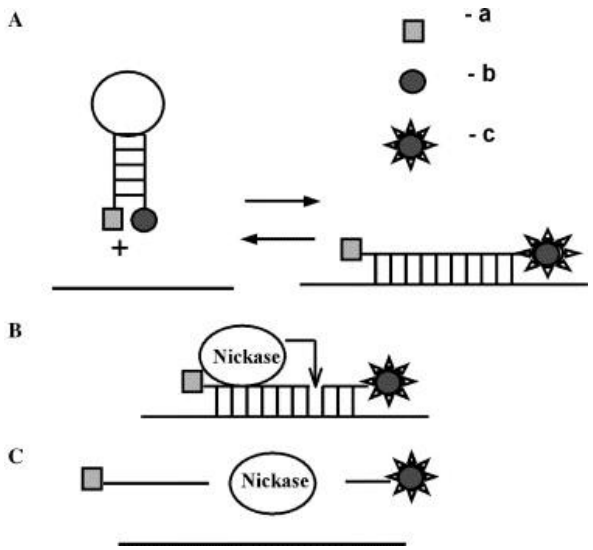


**Fig. 1.8**. Scheme of the nickase amplification. Picture taken from Zheleznaya *et al.*[32].

In a recent application, a nicking enzyme sensing assay was coupled with CdSe/ZnS quantum dots amplification for cymbidium mosaic virus detection[33]. A thiolated hairpin DNA probe labelled with biotin was immobilized on gold electrode via S-Au bond. The double strand loop of the hairpin contained the restriction site for the endonuclease BfuCI, the nicking enzyme. In absence of the target, the hairpin bounded on the surface was closed and the restriction enzyme could digest the loop. As a consequence, the oligo end labelled with biotin was released in solution and the avidin-QD conjugate could not bind. On the contrary, the presence of a target molecule in solution opened the hairpin, blocking the enzymatic digestion and leading to the binding of avidin-quantum dots conjugate to the biotinylated probe (Fig. 1.10). The excess of QD was removed and the electrochemical detection was performed after a treatment with acid solution to dissolve quantum dots. Stripping voltammetric measurements of the $Cd^{2+}$ ions were performed using an *in situ* plated mercury film on a glassy carbon electrode. The sensitivity of the DNA biosensor was calculated to be $\approx 1.0 \times 10^{-12}$ M.

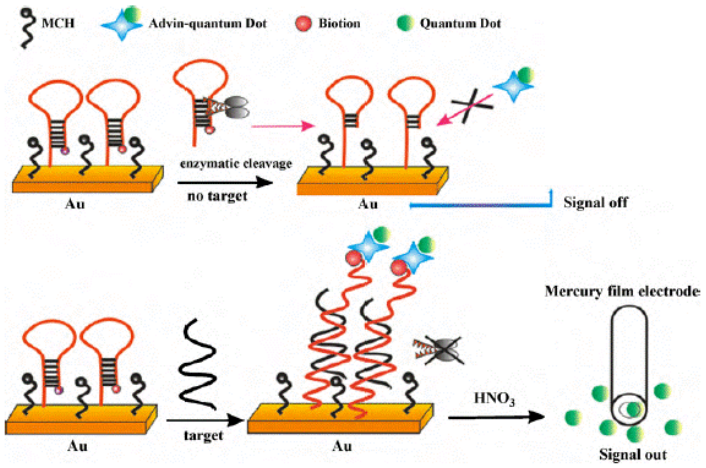


**Fig. 1.10**. Quantum dots mediated signal amplification and endonuclease cleavage can be coupled in a specific assay for DNA and RNA detection. Figure taken from Chen *et al.*[33].

## RNase H as a target recycling operator for RNA-based sensors

An alternative strategy that doesn't bring to the accumulation of new created material on the biosensor surface is that proposed by Goodrich and coworkers[34]. They implemented a method to recycle (and not to accumulate) the target DNA molecules by preparing a sensing surface with RNA oligonucleotide probes. After binding with the target (and thus forming a RNA-DNA hybrid) such double strand can be the substrate for RNase H present in solution. Its action leads to the hydrolysis of the bound RNA oligo and to freeing the target in solution again, so that it can bind to another surface-immobilized oligo. Over time, all the specific RNA oligonucleotide probes are digested by RNase, thus leading to signal enhancement (for instance if read through surface plasmon resonance or other techniques). The reported detection limit is 10 fM in a 13 µl sample volume.

Among the different enzymatic methods introduced, those based on the accumulation of easy to detect reaction products look as the most effective. Independently from the type of substrate used this strategy lead to the higher level of amplification. However the use of fluorescent reporter molecules requires the devices as CCD-cameras of fluorescence scanners which cannot be easily integrated in portable devices. In alternative the use of colorimetric substrate appears as an alternative also if less sensitive. RCA-mediated signal amplification lead to the accumulation on the sensor surface of big quantity of DNA that could be coupled to label-free transduction methods like potentiometric or mass sensitive biosensors.

In the final section of the Introduction are described the principles of the main transduction methods.

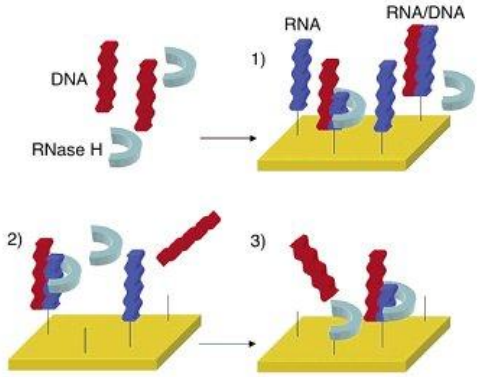


**Fig. 1.11.** Scheme of the amplification strategy proposed by Goodrich and coworkers. DNA target is recognized by RNA probes which, after the hybridization event, are digested by RNase H. Target is thus free to bind with another RNA probes, leading to multiple detectable hybridization events. Picture reproduced from Goodrich *et al.*[34].

### *NOT ENZYMATIC AND NANOTECHNOLOGICAL APPROACHES*

Several amplification strategies are based on the use of organic and nanocomposite labels. They work in three principal ways: (i) they can be used as carriers, targeting specifically and accumulating at the hybridization site multiple labels which can be easily detected; (ii) they can act as labels themselves, exploiting their chemical and physical protein or (iii) they can work as catalyst, enhancing the chemical reaction that could take place in the hybridization site.

## Liposomes as multiple labels carriers

Liposomes can be quite versatile vessels for the transportation of many types of molecules. They are vesicles made by a double layer of phospholipids that can covalently bind molecules on their surface or that can include them in the layer[35]. They can be used as labels for the detection of nucleic acids in analytical assays[36].

Esche *et al.*[37] used liposomes filled with carboxyfluorescein in a sandwich assay. The binding is revealed by fluorescence microscopy with a sensitivity of 0.4 fmoles/µl. Similar results have been obtained in other experimental efforts on different target nucleic acids.

Baeumner and coworkers published several interesting articles focused on detection of *E. coli*, *B. anthracis and C. parvum* mRNA transcripts in drinkable water after NASBA amplification (see Chapter 5), using liposome-based sandwich assay[38-40].

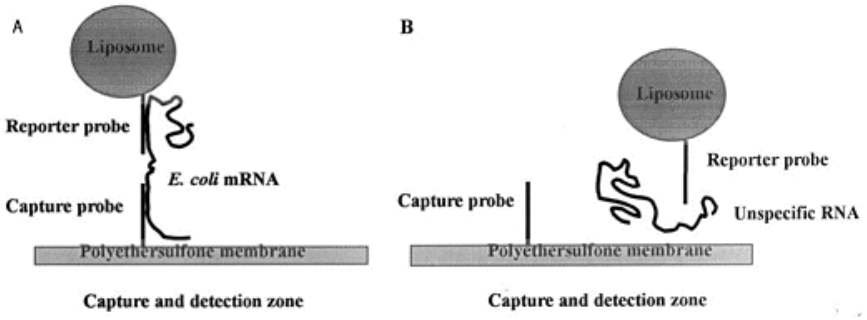


**Fig. 1.12.** Scheme for liposome based-assay proposed by Baeumner *et al.*[38]. A DNA capture probe is immobilized on a polyethersulfone membrane. A DNA reporter probe is coupled to the surface of a liposome. When a specific *E. coli* amplicon from NASBA reaction is present (Figure A), a sandwich is formed between capture probe, RNA and reporter probe. Thus, liposomes are captured in the capture/detection zone. The number of liposomes is directly proportional to the amount of *E. coli* RNA present. In Figure B it is shown that liposomes are not captured in the detection zone, when a nonspecific RNA molecule. Picture taken from Baeumner *et al.*[38].

## Metal nanoparticle and quantum dots as amplification labels

Metal nanoparticles have been used extensively as labelling agents for the detection of DNA hybridization. They are characterized by some interesting and chemical and physical properties which make these compounds suitable for biosensing applications[41-44]. Particular interests have excited their optical properties: the enhancement effect is due to their stability and to the easy detectability of the plasmonic resonance shift[45,46]. In addition metal nanoparticle can be easily functionalized[47,48] and on them nucleic acid hybridization takes place with very high specificity because of the increased sharpness in the DNA melting transition[49].

Storhoff and coworkers[50] could avoid the use of PCR target amplification in their detection of genomic DNA. They measured the plasmonic red-shift due to DNA-induced aggregation of nanoparticles[41]. This method was implemented by using two different nanoparticle-conjugated oligonucleotide probes that are complementary to different portions of the target DNA molecule. The binding event can be evidenced with optical methods. 2 x $10^5$ molecules/µl can be detected (333 zmoles).

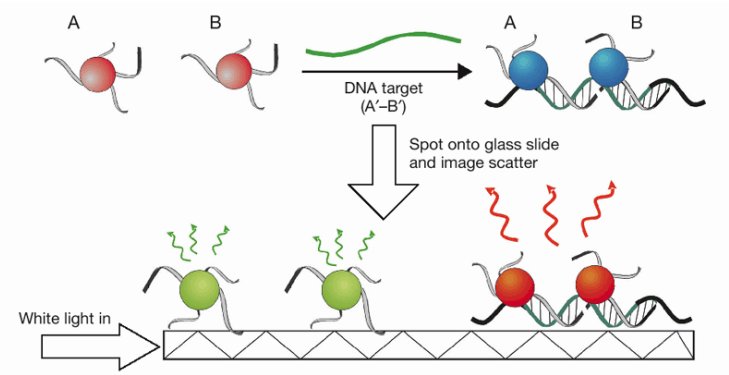


**Fig. 1.13**. Scheme of the use of nanoparticle-conjugated probes for the detection of DNA. Figure taken from Storhoff *et al.*[50].

AuNPs were used by Joung and co-workers to enhance 5500-fold the signal originated from 16s rRNA[51]. The target was detected by means of a specific PNA capture probe immobilized on a surface plasmon resonance (SPR) sensor. As PNA is characterized by a neutral backbone structure, the hybridization with the 16S RNA led to a change in the ionic charge from neutral to negative. Cationic AuNPs were synthetized and used to amplify locally the signal generated by the target binding. This method was applied on *E. coli* total RNA extraction showing a sensitivity of 58.2 ± 1.37 pg/ml RNA. For *S. aureus* detection the method was applied without preliminary nucleic acid extraction. In this case the achieved sensitivity was $7\times10^5$ CFU/ml.

As nanoparticle, alternative nanostructures as nanorods are characterized by interesting physical and chemical properties that make them suitable for biosensing and signal amplification[52]. Recently, Parab and colleagues demonstrated the use gold nanorods (GNR) for the optical detection of a *Chlamydia trachomatis* DNA in solution. Monitoring the absorption spectra of GNR-capture probe they reliably detected target DNA in the range of 250-50 nM in 100 µl sample[53].

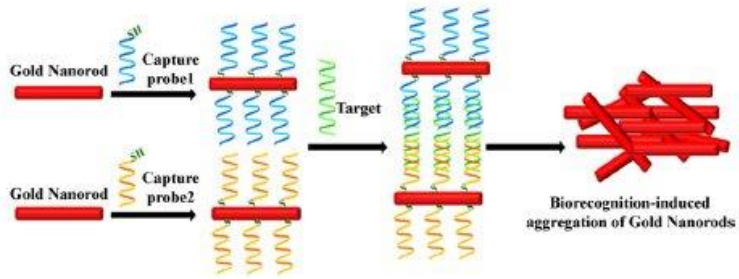


**Fig. 1.14**. Scheme of gold-nanorods based biosensor for *C. trachomatis* detection. Figure taken from Parab *et al.* [53].

In the last years quantum dots have found large application in DNA, RNA, protein microarray labeling and more frequent is also their use in DNA biosensors[54-56]. Zhang and colleagues reported a nanosensor based on fluorescence resonance energy transfer (FRET) coupling the use of CdSe–ZnS core–shell nanocrystals as donor and Cy5 dye as acceptor[57]. In solution, single strand target DNA bound to a biotinylated capture probe and to a reporter probe labeled with Cy5 forming a hybrid sandwich structure. Several hybrids are captured by streptavidin-QD hybrids, accumulating target and Cy5 dyes on it. In this configuration, acceptors dyes were in close proximity to the donor and were able to emit fluorescence by means of FRET after QDs excitation. As a result, fluorescent detection at the emission wavelength of Cy5, indicated the presence of target. The method has reported to be highly sensitive: as the unbound QD produced near-zero background fluorescence while a very clear FRET signal is generated after binding, up to ~ 50 copies of target DNA can be detected.

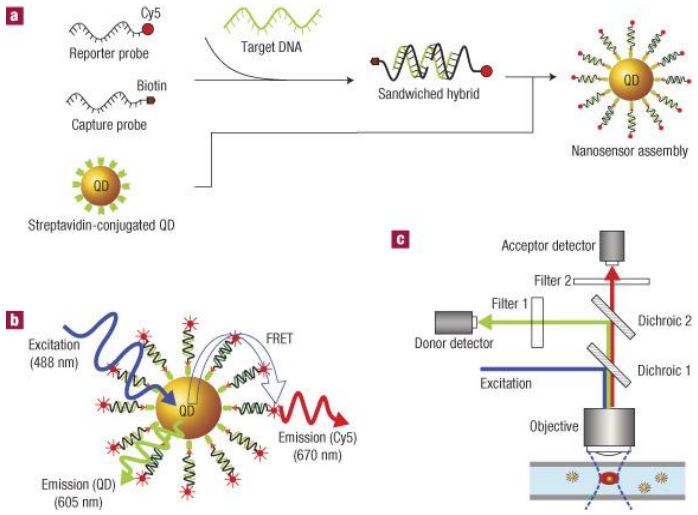


**Fig. 1.15**. Scheme of optical biosensor based on FRET. CdSe-ZnS quantum dots and Cy5 dye are locally concentrated after target detection. In this configuration, acceptors dyes were in close proximity to the donor and were able to emit fluorescence by means of FRET after QDs excitation. Picture taken from Zhang *et al.*[57].

Chad Mirkin and co-workers moreover employed another enhancement step in the nanoparticle-mediated detection of DNA. As their method leads to DNA signals that can be read with a standard flatbed optical scanner, they termed their method "scanometric detection"[58]. In their method, ultra-low levels of DNA labelled with oligonucleotide-functionalized nanoparticles are detected by inducing silver reduction on the surface of the nanoparticles. In a sandwich assay, the surface immobilized nanoparticles, bridged by the target DNA, can then be turned into silver microparticles that are detectable even to the naked eye. The authors claim that nanoparticle labelling leads to signals that are 3-4

orders of magnitude more intense than fluorophores. $6 \times 10^6$ molecules (200 fM in 50 µl) can be detected[50].

Besides their optical properties, metal nanoparticles can be used as catalysts, and thus permit the implementation of electrochemical signal enhancements when these are bound to reported oligonucleotide probes. In an example reported by Polsky and co-workers, a sandwich is obtained on the electrode surface by immobilizing the target and a reported oligonucleotide that is labelled with a platinum nanoparticle[59]. Such nanoparticles can catalyse the conversion of hydrogen peroxide to water at a controlled electrochemical potential, enabling the chronoamperometric measurement of the hybridization of 10 pM DNA.

## THE CHOICE OF AMPLIFICATION STRATEGY: WORKING WITH DIFFERENT TRANSDUCTION METHODS

The choice of one amplification method with respect to another must be motivated by the type of biosensor in which it will be integrated. The hybridization event between target and probe is converted in a measurable signal by a transducer which is intimately coupled with the sensing element. The use of fluorescent or redox labels with respect to the use of enzymes or of any other amplification strategy depends on what type of transduction method is used for the signal. In this paragraph, the different types of nucleic acid biosensors will be classified according to their transduction method, illustrating the general principles for each of them.

**Table 2: classification of biosensor according to transduction method**

| **Type of transducer** | **Measured property** |
|---|---|
| **Electrochemical** | Potentiometric<br>Amperometric<br>Voltametric |
| **Electrical** | Surface conductivity<br>Electrolyte conductivity |
| **Optical** | Fluorescence<br>Surface plasmon resonance |
| **Mass sensitive** | Resonance frequency |

### *OPTICAL BIOSENSOR*

Optical transducers are probably the most common in nucleic acids detection thanks to their sensitivity and specificity. They are usually coupled with signal amplification methods which enhance fluorescence, with methods based on optical properties of nanoparticles or with methods that generate a visible color.

## Fluorescence detection

Fluorescence detection works with the monitoring of the emission of light by a compound which goes back from an excited state to the ground energy state. The molecule is excited by the absorption of high energy light (shorter wavelength). When the molecule returns to the original ground state, part of the energy is re-emitted in light at lower energy (longer wavelength) while a part is lost as heat. In order to maximize the signal, it is desirable that the excitation wavelength and the emission wavelength be as far as possible from each other. The use of this type of biosensor implies the use of labels and the use of an excitation source, usually an ultraviolet laser, which must be integrated into the device or that more frequently is part of a scanner. For these reasons, despite their sensitivity, fluorescent detection is not easily integrable in portable devices.

## Surface plasmon resonance detection

Surface plasmon resonance (SPR) biosensors are based on the variations in the charge density waves at the surface of metallic structures. Electrons within the conduction band are free to move at the interface between the metal and the external medium. They oscillate collectively, generating surface waves of charge which propagate along the surface. The charge density wave is associated to an electromagnetic wave, which energy is confined mainly to the surface. In SPR biosensors the variations of the reflected light intensity are measured when the surface is illuminated with light beam: the presence of a biological element on the surface like an oligo probe and the consequent accumulation of material after the detection event, modifies the refractive index of the medium, changing the reflection angle. SPR biosensors are quite sensitive: they can detect nanograms of analyte on the surface but their application is limited by the size of the searched molecule. The main drawbacks of this powerful technique lay in its complexity (specialized staff is required), high cost of equipment and large size of most currently available instruments. On the other hand, SPR are prone to be use as label-free detection method for nucleic acid.

## Colorimetric biosensors

Colorimetric biosensors are based on the properties of some enzymes to convert a colorless substrate in a reaction product that can be easily detect with a standard photo scanner or even at naked eye. The most typical example is the use of horseradish peroxidase (HRP) which in presence of hydrogen peroxide catalyzes the conversion of suitable substrate, like –tetramethybenzidine (TMB), in a colored product (A blue precipitate in the case of TMB). If the enzyme is localized on the surface in

correspondence of the hybridization event between probe and target, the color will be accumulated on the probe feature.

### *ELECTROCHEMICAL BIOSENSORS*

These devices are mainly based on the observation of current or potential changes due to interactions occurring at the sensor-sample matrix interface. Techniques are generally classified according to the observed parameter: current (amperometric), potential (potentiometric) or impedance (impedimetric). Compared to optical methods, electrochemical biosensor allows the user to work with turbid samples, and the cost of equipment is cheaper. In addition electrochemical biosensors are suitable to perform label-free detection (they can work without the use of a label). On the contrary, they exhibit a slightly lower selectivity and sensitivity than their optical counterparts. Some suggested reviews are those recently proposed by Mir *et al*.[60], Bonanni and del Valle[61].

#### Amperometric biosensors

In amperometric biosensors a potential is applied between two electrodes. They measure the resulting current in oxidation or reduction processes of electro active species present in solution, generally using enzymes as biomarker molecules. The read-out of the enzyme needs the interaction with a specific substrate which is converted in a different molecule, usually a redox molecule that can be detected. In addition, for each catalytic site more than one redox molecule is produced, leading to signal enhancement.

#### Potentiometric biosensors

DNA potentiometric detectors are based on the use of transistors. They measure the current generated as consequence of the charge accumulation effect at the gate electrode after the specific binding of DNA molecules. Potentiometric DNA biosensors are particularly attractive since they can be fabricated in very small sizes and massively integrated in lab-on-a-chip devices.

#### Impedimetric biosensors

Impedimetric transduction is based on sensing the electrical resistance and reactance of a medium in an electrochemical cell, applying an alternating current potential. It provides electric information in the frequency domain. Among the different electrochemical transduction methods, electrochemical impedance spectroscopy is particularly suitable for label-free detection of DNA.

## *MASS SENSITIVE BIOSENSORS*

In mass-transducers, a mass change on the surface sensor due to the binding of the analyte to the probe determines a variation of the resonance frequency of the device.

### Cantilevers

Cantilevers are nanomechanical biosensors. The cantilever surface is usually functionalized with suitable DNA or RNA probes and interactions with the nucleic acid analyte take place only on where probes are present. The bind between probe and analyte bend the cantilever and such bending is usually monitored by electrical readout, since the lever is integrated in a piezoresistor. The binding of the cantilever in presence of the analyte provides information both on analyte concentration than on the kinetics of the interactions because the interactions can be monitored in real time. Cantilevers biosensors work both in air and in liquid environment.

### Quartz Crystal Microbalance

Quartz Crystal Microbalance (QCM) is ultra-sensitive weighing device based on the mechanical resonance of piezoelectric single-crystalline quartz. If a voltage is applied to the crystal quartz and the frequency of the applied voltage corresponds to the resonance frequency of the crystal, resonance condition occurs. The presence of mass on the crystal surface changes the frequency of the total oscillating mass. In addition, the presence of water, ions and some type of biomolecules/polymers (like DNA) determine energy dissipation of the oscillating system. Since QCM is sensitive also to the binding of water and ions, it has been found to be more sensitive than optical and electrochemical biosensors.

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# AIM OF THESIS AND OUTLINE

In the last ten years, nucleic acid biosensors have gained a huge interest, both in research and commercial applications. The intrinsic physical and chemical stability and suitability to discriminate different organism strains have made them attractive for point-of-care sensing applications. The challenge for the academia and the industry is to reach the highest sensitivity in miniaturized lab-on-a-chip devices which could include the complete pipeline for the analysis of the sample.

In this contest the study of biochemical strategies and of nanotechnological approaches that can be used to solve the problem of signal amplification, is a relevant topic.

The aim of this Ph.D. work was to investigate four biochemical reactions as methods for the amplification of the sample or of the signal in nucleic acids biosensors. Recombinase polymerase amplification and rolling circle amplification were used to amplify a DNA and a RNA target. Hybridization chain reaction and terminal transferase reaction were tested as strategies to achieve signal enhancement.

This thesis has been divided in two parts. In the first part, the results obtained for the sample amplification have been described. The second part has been reserved for the signal amplification methods. For each method, a general introduction, the descriptions of the experimental procedures and the detailed results have been reported. Finally the last chapter has been devoted to the general conclusions of the work.

# Section I:
# sample amplification strategies

# CHAPTER 2

## RECOMBINASE POLYMERASE AMPLIFICATION

### INTRODUCTION

The main limitation factor to polymerase chain reaction application in portable DNA biosensors is temperature cycling. A real alternative to it should be completely isothermal, thus the initial step of target melting for primer annealing should be avoided. The most effective isothermal mechanisms of dsDNA melting are catalyzed in nature by helicases and recombinases enzyme. Helicases unwind the double helix mechanically at physiological temperature using ATP hydrolysis[1]. They are involved in several mechanisms, like the separation the complementary strands of double-stranded nucleic acids during replication and removal of nucleic acid-associated proteins. The helicase-mediated mechanism of duplex separation has been used by Vincent and coworkers to develop a completely isothermal strategy to amplify DNA. The helicase-dependent isothermal DNA amplification (HDA) mimics the natural system, creating single strand templates for primer hybridization and the successive elongation[2]. HDA reaction is a proper example of how nature inspires researchers to create new biochemical tools. As helicases also recombinases and their biological role have prompted researchers to use them for *in vitro* nucleic acid amplification. Recombinase polymerase amplification (RPA) is an isothermal strategy for DNA amplification entirely inspired by the recombination-dependent DNA replication activity of the T4 bacteriophage[3].

The mechanism of recombinase polymerase amplification (RPA) was first proposed by Piepenburg and coworkers[4]. It is mediated by the coordinated activity of four proteins: T4 UvsX, T4 UvsY, T4 Gp32 and *Bacillus subtilis* (Bsu) Pol I. Briefly, the UvsX recombinase forms a complex with single strand DNA primers and scans the template DNA looking for homologous sequences. When the homologous sequence is found, primers hybridize to the target sequence through a strand exchange mechanism displacing the parental strand. Proteins UvsY and Gp32 assist in the reaction. Primers are extended by Bsu Pol I in both directions, leading to the exponential amplification of DNA target sequence as schematized in Fig. 2.1.

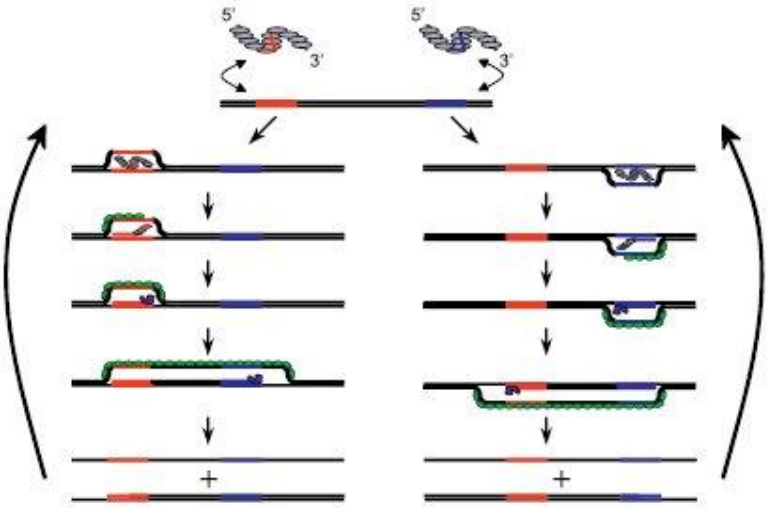


**Fig 2.1**. Scheme of recombinase polymerase amplification mechanism [4]. The reaction starts when a complex is formed by primers and UvsX protein scanning the target DNA in both directions. When a region of homology between the primer and target region is found, UvsX catalyzes the strand exchange reaction between primer and the homologous template strand.

## *TAKING A LOOK INSIDE THE MOLECULAR MECHANISM OF RPA.*

Recombinase polymerase amplification is based on the genetic recombination mechanism between homologous DNA filaments. Homologous recombination is an ubiquitous process shared by all living organisms[5] because it plays a key role in genome repair mechanisms and in the maintenance of genomic diversity in a population. During meiosis, homologous recombination is essential for a correct chromosome segregation[6] and it is known to initiate replication in virus and bacteria in the so called recombination-dependent DNA replication (RDR)[7].

For years, Bacteriophage T4 has been a model for the biochemical and genetic studies of recombination and replication proteins. The mechanism of recombination-dependent DNA replication is crucial for the life of this phage because the origin-dependent replication takes place only in the early phases of *E. coli* infection. The RPA method proposed by Piepenburg and co-workers is based on the activity of four proteins which are involved in one of the several RDR pathways. The RDR mechanism in phage T4 has been exhaustively reviewed by Kreuzer and Mozig[7,8]. In this chapter, we will take into account only the reaction features necessary to introduce the RPA mechanism.

Different molecular pathways have been proposed for T4 recombination dependent replication. In addition to recombinase and polymerase, several accessory proteins are involved in each phase of the process. Their role is to mediate and to coordinate the binding of the enzymes to DNA. Among mediator proteins, UvsY and Gp32 play a

critical role. The former is necessary for the proper assembly of UvsX recombinase on ssDNA[9] and the latter is a ssDNA binding protein[10]. Finally, other proteins like nucleases and helicases contribute to generate the single strand DNA sequences necessary for priming the recombination[3].

UvsX protein is a RecA protein homologue which binds cooperatively ssDNA molecules in an ATP dependent fashion[11]. Crystal structure of the large fragment of UvsX has been recently proposed by Gajewski and co-workers[12] demonstrating its close structural and functional similarities to RecA. UvsX forms a pre-synaptic filament with the recombination primer usually consisting of a single strand DNA tail formed during the origin-dependent replication of the phage genome[13]. The pre-synaptic filament aids the invasion and the scanning of a double strand DNA sequence looking for regions of homology. When homologous sequences are located, UvsX catalyzes a strand invasion reaction and the pairing of the primer with the homologous strand of target DNA. The original pairing strand is displaced by strand invasion leaving a bubble of single strand DNA in the region (D-loop structure) in which a considerable mismatch percentage is tolerated[14].

UvsY has an important role in the assembly of UvsX-ssDNA pre-synaptic filament[15]. During the early phase of T4 infection, ssDNA target is present only temporarily and it is bound by the single strand DNA binding protein Gp32 that competes with the recombinase for the binding site[3]. The role of UvsY is to stimulate the recombination event in two ways: displacing Gp32 protein from ssDNA and interacting with UvsX to stabilize its interaction with the primers[9] (Fig. 2.2). In the T4 RDR in vitro system, UvsY lowers the critical concentration of UvsX protein required for initiating the leading strand synthesis[16].

Gp32 is a protein expressed at high levels in the early and late phase of the T4 infection. It shows strong binding affinity for ssDNA and rapidly saturates all the filaments generated during T4 replication[17]. Gp32 is displaced from DNA by UvsY protein leading to the formation of the pre-synaptic filament[9,15]. After strand invasion, a D-loop structure is formed on the duplex and the DNA polymerase can start the synthesis of the leading strand (Fig. 2.3). As the parental strand is displaced during synthesis, Gp32 binds to it and stabilizes the replication bubble.

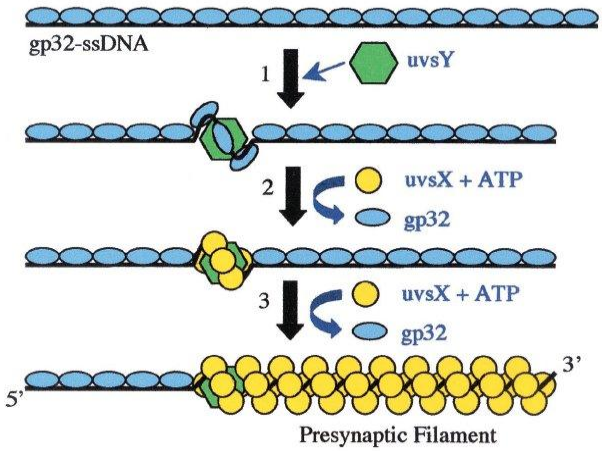


**Fig. 2.2**. Proposed model for UvsY mediated assembly of the presynaptic filament[3]. Step 1: Hexameric UvsY protein binds to Gp32-ssDNA complex destabilizing Gp32-ssDNA interactions. Step 2: UvsX recombinase is recruited to the UvsY-Gp32-ssDNA intermediate and locally displaces Gp32 to nucleate a filament. Step 3: UvsX-ssDNA filament assembly propagates in the 5'→3' direction while displacing Gp32. Picture reproduced from Bleuit *et al.*[3].

The large fragment of the *B.subtilis PolI* is the DNA polymerase chosen by the authors for DNA synthesis. It is a highly processive polymerase which retains the 5′→ 3' polymerase activity of the holoenzyme but lacks the 5′→ 3′ exonuclease domain. On the same time it naturally lacks 3′→ 5′ exonuclease activity.

At the base of the recombinase polymerase amplification mechanism, there is the evidence that *in vitro*, under physiological conditions and in presence of T4 UvsY and Gp32 proteins[18], T4 UvsX catalyzes the invasion of the ssDNA 3'end into an homologous duplex using a synthetic sequence of ssDNA. The characterization of the complex UvsX, UvsY and Gp32 has been proposed by Hashimoto and coworkers in 1991[15].

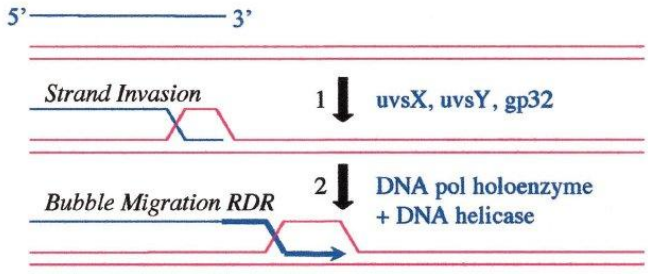


**Fig. 2.3.** Particular of the T4 in vitro system for RDR. Step 1: in reaction catalyzed by UvsX recombinase, the 3' end of linear ssDNA primer (blue) invades homologous dsDNA template (red) stimulated by UvsY and Gp32. Step 2: DNA polymerase holoenzyme uses primer terminus in D loop to initiate leading strand synthesis. Branch migration of the cross junction displaces the daughter strand from the template, leading to conservative DNA synthesis. Picture taken from Bleuit *et al.*[3].

In summary, RPA reaction can be schematized in four steps:

1. *Targeting of the sequence and recombination*: RPA starts when UvsX recombinase binds to synthetic primers in presence of ATP. Nucleoprotein filaments scan the template looking for the homologous sequence on DNA. When the sequence is found, UvsX catalyzes a strand exchange reaction and the D-loop structure is formed. In order to achieve exponential amplification, two primers must be used in the same reaction mix.

2. *Initiation of DNA synthesis*: Bsu DNA PolI starts DNA elongation adding dNTPs to the 3'-OH end of the primers. The release of UvsX from the hybrid is a necessary condition to start the polymerase reaction and it is accomplished by ATP hydrolysis.

3. *DNA elongation by means of strand displacement*: during elongation, Bsu DNA PolI displaces the parental strand which is stabilized by the Gp32 protein binding. In the ideal configuration two reaction start simultaneously on the same target sequence.

4. *End of the synthesis and re-invasion*: once the template strands are separated, Bsu PolI completes the extension to the end of the template and the newly synthetized sites are available to targeting by UvsX-primers nucleoprotein filaments.

The RPA mechanism is more complex than other well known amplification system, as described in Chapter 1. For instance, while PCR and other enzymatic amplification strategies are based on the activity of only one enzyme (usually a polymerase), RPA is characterized by a fine equilibrium between four proteins which interact in a coordinate way. Despite its complexity, it has been demonstrated that RPA can amplify DNA successfully and that it can be coupled to DNA biosensor detection.

### *LITERATURE OVERVIEW*

Piepenburg and co-workers first demonstrated that RPA can successfully be employed as isothermal amplification methods for DNA sequences[4]. They reported that the reaction takes place in a range of temperature comprised between 37 and 41°C and demonstrated that less than 30 minutes are sufficient to achieve exponential target amplification. A low unique reaction temperature and fast reaction time represent the main advantages of the use of RPA as potential PCR alternative. The authors used RPA for the amplification of up to 10 copies of both human and bacterial markers template in solution. The also reported that the RPA reaction carried out in the absence of template or

at low template concentrations eventually can generate a non-specific signal due to primer-dependent artifacts. Since such artifact could invalidate the results of analysis, they implemented a probe-based detection system which introduced a second level of specificity to the reaction. The proposed strategy was rather complicated and coupled enzymatic cleavage of a reporter probe with FRET signal (Fig. 2.4).

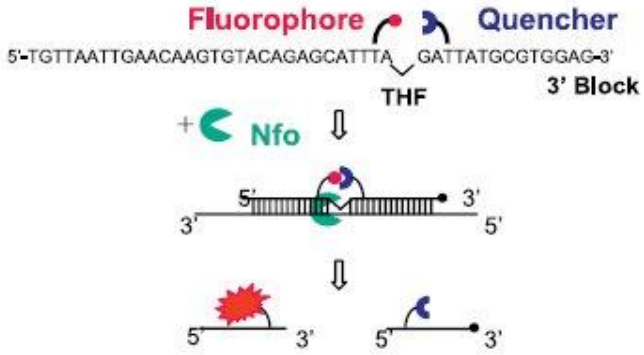


**Fig. 2.4.** Scheme of the probe-based detection system proposed by Piepenburg *et al.*[4]. A probe containing a tetrahydrofuran abasic–site mimic (THF) flanked by nucleotides modified with a fluorescent and a quencher dyes was used. When the construct was intact the fluorescence was low. The probe was also modified in 3'-end in order to prevent the oligonucleotide from acting as an amplification primer. Pairing of the probe to complementary DNA enabled the recognition of the THF by the double-strand–specific *E. coli* endonuclease IV (Nfo). The need for formation of a stable DNA duplex acted as an additional control step. The cutting of the probe separated the fluorophore/quencher complex leading to a measurable increase in fluorescence. The cleavage reaction generated a free 3'-OH end on the 5' tail of the digested probe. This oligomer could then be elongated by Bsu polymerase, thus serving as an amplification primer. Picture taken from Piepenburg *et al.*[4].

Several patent applications have been submitted on the RPA and a kit is currently available on the market. With this kit, we performed all the RPA experiments described in this chapter. Recently Lutz and colleagues[19] developed a system in which RPA was integrated into a microfluidic lab-on-a-chip system. The proposed microfluidic unit was a centrifugal foil cartridge which comprised chambers for reconstitution of reagents, for sample mixing and for sample distribution into test cavities (Fig. 2.5). The authors demonstrated the successfully detection and amplification of less than 10 copies of DNA coding for the antibiotic resistance gene *mecA* gene from *Staphylococcus aureus*. The work of Lutz and coworkers is the first application of RPA in DNA biosensors, demonstrating that RPA is potentially a powerful technique which can be employed on lab-on-chip devices.

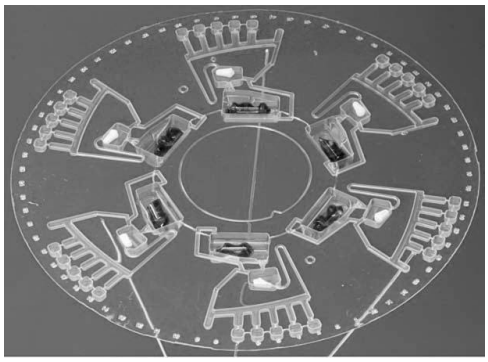

**Fig. 2.5.** Picture of the microfluidic system proposed by Lutz and colleagues. It integrates all the components necessary to perform RPA amplification of gene mecA of *S. aureus.* Picture modified from Lutz et al.[19].

### *CONSIDERATIONS ON RPA REACTION: ADVANTAGES AND LIMITATIONS*

RPA reaction conditions can be optimized in several ways. The reaction product yield is influenced by varying parameters like temperature, magnesium concentration, reaction time and reaction mixing rate. In this section the principal requirements of RPA and some drawbacks related to it will be described. Particular regard has been devoted to primer design which represent a key point for the implementation of the RPA system.

## Primer design considerations

Although Piepenburg and colleagues have suggested some basic rules for RPA primer design, the selection process of effective primer pairs is mainly trial and error. Primer length and primer sequence are the most important variable that should be taken into consideration for the development of a RPA-based assay.

RPA requires primers longer than those used in PCR. For RDR *in vitro* system the use of primer as long as even 69 nt has been recommended[15]. Piepenburg and colleagues recommended to design primers with a length between 30 and 35 nt. They reported that the recombination efficiency is decreased with the use of shorter primer but on the other hand lengthening the oligonucleotide beyond that interval might not necessarily improve the amplification performance. As a matter of fact, for long sequences the probability to form secondary-structures is higher, potentially leading to primer noise (artifacts)[4,20]. Moreover the use of longer primers could promote non-specific amplification. Although in PCR even a single mismatch at the 3'-end could guarantee specificity[21], the same or even greater number of mismatches might not prevent the formation of an unspecific primer-target complex in RPA since mismatches can be compensated by neighboring perfectly matched base-pairs interaction. In addition, the low reaction temperature of

RPA (37°C-41°C) and the involvement of proteins with high DNA binding affinity, could favor the formation of a stable primer-template complex and the trigger of the reaction.

Rules for primer design suggest that long tracks of guanines at the 5' end (first 3-5 nucleotides) should be avoided, while the presence of pyrimidines may be beneficial. In addition guanines and cytidines at the 3' end of the primer (last 3 nucleotides) improve recombination efficiency. Like for PCR, also for RPA, it has been suggested to avoid 'unusual' sequence elements within the primer, such as long tracks of one particular nucleotide or a large number of small repeats. Excessively high (>70%) or low (<30%) GC content is not recommended too. In addition it has been reported that base-pairing interactions both within and between primers could contribute to artifacts generation (primer dimers etc.). For this reason oligonucleotides that contain sequence elements that promote secondary structures and primer-primer interactions or hairpins should be discarded[22].

Although Piepenburg and co-workers reported the formation of a 1.5 kb amplicon[4], the kit currently available is optimized to facilitate rapid target amplification instead of maximizing the reaction product length. For this reason, amplicons longer than 500 bp should be avoided while it has been suggested that the ideal length should be between 100 and 200 bp.

## Primer artifacts formation

As mentioned previously, the choice of the best primers for a specific target is made mainly through trial and error. In addition to promoting specific amplification events, it is reported that the RPA reaction environment could allow undesired primer interactions to occur[23]. This phenomenon has not been investigated in literature until now but some hypothesis about its origin have been proposed by the authors[23]. Primer interactions could be intramolecular (hairpins, etc.) or result from primer dimer formation (both homodimeric and heterodimeric). These structures might provide a substrate for extension by DNA polymerase and some of the artifacts could template further recombination or extension events triggering an exponential non-specific amplification (Fig. 2.6). Primer artifacts are in competition (for primers, nucleotides, polymerase recruitment) with the real amplification process, and could eventually inhibit the latter. For this reason the selection of sequences which minimize the competitive production of primer artifacts is important. The authors suggested two solutions to solve primer artifacts problem: design of "blocked" primer to minimize any possible not specific interaction and the use of heparin (a molecules that in literature is reported to inhibit PCR).

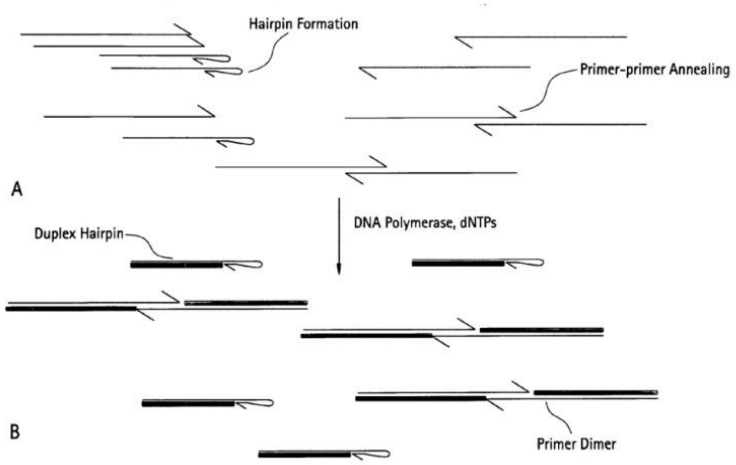


**Fig. 2.6.** Model for primers artifact formation in the RPA reaction[23]. Primers might form hairpins, (panel A), or could hybridize to a second primer (panel B). If the polymerase extends such primer structures, dsDNA molecules with significant length could be formed. Such molecules might become targets for the other recombinase loaded filaments and enter in the amplification mechanism.

## The role of magnesium

The recommended standard magnesium concentration is higher than that used in PCR. Magnesium is reported to be an essential cofactor for the reaction mechanism. As a matter of fact, the addition of magnesium acetate to the reaction mix triggers the amplification. In order to compare different reactions, magnesium should be added only in the last step, few seconds before starting the incubation since recombination starts immediately after its addition.

## Reaction time and temperature

RPA takes place in a range of temperatures between 37°C and 41°C. The big advantage of RPA with respect to PCR is that it can operate at the same single temperature without an additional pre-heating step necessary for primer-target annealing.

RPA is reported to be a really fast reaction. The suggested reaction times to achieve highest product yield are between 20 and 40 minutes, shorter than a PCR.

## Reaction mixing

In the manual of the commercial kit, it is reported that that the yield of the reaction is increased by sample agitation. To achieve highest sensitivity mixing should be carried out after four minutes of incubation and a longer agitation time should improve reaction

products formation. The reasons at the base of this suggestion are not known. In our opinion agitation could be a limitation for the user and might represent a source of variability of the reaction. For this reasons, mixing is one of the parameters investigated in this work.

### *REASONS FOR THE CHOICE OF THIS STRATEGY*

Recombinase polymerase amplification is a recent proposed mechanism and the articles about its diagnostics applications are limited. We chose to investigate RPA as biosensors coupled amplification strategy because of its interesting properties. Fast reaction time and sensitivity were two of the most attractive features.

## MATERIALS AND METHODS

### *MATERIALS*

Oligonucleotide primers and probes were purchased from Microsynth AG (Switzerland). The RPA kit was supplied by TwistDX (Cambridge, UK). For chemiluminescence detection, all reagents were purchased from Sigma, with the exception of chemiluminescent substrate supplied by GE Healthcare (USA).

### *BACTERIAL CULTURES AND DNA EXTRACTION*

*E. coli* cultures were grown and used as template DNA source for RPA amplification. Briefly, 20 µl of *E. coli* glycerol stock were pipetted and transferred in 4 ml of fresh LB medium (Sigma-Aldrich). Cells were grown overnight at 37°C under constant agitation (200 rpm). Bacteria concentration was estimated measuring optical density at 600 nm using the Eppendorf Biophotometer and single-use plastic cuvettes (Uvette®, Eppendorf). DNA was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's specification and starting from 10 µl of $1x10^9$ CFU/ml fresh cells culture which were diluted in 1 ml of fresh LB medium. DNA quality was assessed by means of electrophoresis in1% agarose gel in TBE 0.5X. Gel was run at 80 V for 30 min, stained with ethidium bromide and visualized under UV.

### *RECOMBINASE POLYMERASE AMPLIFICATION PROTOCOL*

RPA reactions were performed using the TwistAmp ™ Basic Kit (TwistDx, Cambridge-UK), according to the manufacturer's instructions or as indicated. Briefly, 29.5 µl of rehydration solution were mixed with 2.4 µl of each primer (final concentration 600 nM). Target solution and DEPC water were added to a final reaction volume of 47.5 µl. The entire reaction volume was used to resuspend the enzyme pellet and the reaction was started adding 2.5 µl of 280 mM magnesium acetate. Reactions tube were incubated for 4 min at 41°C, vortexed for 4 min and then incubated again at 41°C for a total reaction time of 60 min.

### *PURIFICATION OF REACTION PRODUCTS*

Purification of RPA reaction product was achieved using the ethanol precipitation method. Briefly, 25 µl of reaction solution were mixed thoroughly with 50 µl of 95% ethanol and 2.5 µl of 3 M sodium acetate. Samples was kept at –20°C for 30 min and then centrifuged at 14000 rpm for 20 min. The supernatant was discarded and the pellet was rinsed with 150 µl of 70% ethanol. The sample was spun for 5 min at 14000 rpm and the supernatant was aspirated. After drying, the pellet was resuspended in 25 µl of DEPC water (Sigma).

### *AGAROSE GEL ELECTROPHORESIS*

Electrophoresis end point detection of RPA reaction was performed using a 1.5% agarose gel in 0.5X TBE. 5 µl of purified RPA product were loaded on the gel and run for 40 min at 10 V/cm. Gel was stained with ethidium bromide and visualized under UV.

### *COLORIMETRIC AND CHEMILUMINESCENCE ASSAYS*

Probe for *E. coli* and *N. gonorrhoeae* targets were printed on plastic slides according to a proprietary process of Greiner-Bio-One. An aliquot of crude RPA product was mixed 1:10with guanidinium thiocyanate 3 M and incubated for 1 min at 95°C. 20µl were pipetted on the printed slide and incubated for 2 min. 30 µl of horseradish peroxidase solution (HRP) were added, incubated for 2 min and then washed with 40 µl of washing solution. Finally, 30 µl of 3,3′,5,5′-tetramethylbenzidine (TMB, STD Reagents, Germany) were added and incubated for 4 min or until blue color was visible. Chemilumiscence assay was performed using the same protocol with some variations as follow. An aliquot of crude RPA product was mixed with a 3 M guanidinium thiocyanate solution with a ratio 1:10 and incubated for 5 min at 95°C. 20 µl were pipetted on the printed slide and incubated for 10 min. 40 µl of HRP-Conjugated solution (Thermo Scientific) were added, incubated for 2 min and then washed with 60 µl of washing solution, 10°µl for time and incubating . Finally, 10 µl of chemiluminescent substrate (ECL™ Western Blotting System, GE) and the slide was scanned immediately.

### *REAL TIME MONITORING*

RPA reactions were performed in presence of Sybr Green I (10000X in DMSO, Sigma Aldrich) in a 96-well thin wall PCR plate (Bio-Rad). Samples were incubated in thermocycler coupled to a CCD camera (My iQ Single-Color Real-Time PCR Detection System, Bio-Rad) and measurements were taken every 15 seconds minute or as indicated. Fluorescence data were elaborated using the iCycler iQ™ Detection System software (Bio-Rad).

### *CHARACTERIZATION OF AMPLICONS BY MEANS OF ENZYMATIC DIGESTION*

XagI, Cfr10I and PdMI restriction enzymes (Fermentas) were used to digest purified samples and negative controls of RPA reaction. Briefly, 10 µl of RPA reaction product, 16 µl of water, 2 µl of suitable 10X buffer and 20 U of restriction enzyme were mixed together in a final reaction volumes of 30 µl. Reaction were carried separately for each enzyme. Sample were incubated for 1 h at 37°C in a thermoblock (Thermomixer Comfort, Eppendorf).

### *LABELING PROTOCOL FOR MICROARRAY DETECTION OF RPA PRODUCTS.*

RPA products were hybridized on PelvoCheck slides (Greiner Bio-One) according to the manufacturer's instructions. Briefly, 10 µl of amplified DNA were mixed with 30 µl of PelvoCheck hybridization buffer. In the same time 12 samples could be tested on a single array. 30 µl of each sample were pipetted in each compartment of the slide. Samples were incubated for 30 min at room temperature in humid chamber. The slides was then washed vigorously using suitable buffers provided by the vendor. The slides were dried by centrifugation and each hybridization site was incubated with 30 µl SA-polyHRP stabilizer solution (STD-Reagents, Germany) for 15 min . The excess of solution was removed and the slides were incubated with 1:100 diluted solution of Streptavidin, Alexa Fluor-647conjugate (Invitrogen) in SA-polyHRP stabilizer solution (STD-Reagents, Germany). The final concentration of labeled streptavidin was 10 µg/ml. The excess of solution was removed and the slide was washed twice with the same volume of fresh stabilization solution. Finally the slide was dried by centrifugation and stored until scanning. Slide was scanned using CheckScanner™ system(Greiner Bio-One) and the output images were analyzed using Gene Pix Acquisition and Analysis Microarray Software (Molecular Devices).

# RESULTS AND DISCUSSION

## *RPA PRIMER DESIGN*

Primer design has been reported to have a key role in recombinase polymerase amplification. Specificity and reduction of primer artifacts formation has been indicated as the two main challenges to achieve in order to successfully implement the reaction. We designed and tested different series of primer pairs looking for the most suitable.

First generation primers were designed on *Escherichia coli*. We chose this organism as model for several reasons. In addition to be one of the best studied prokaryotic cells, it could be grown easily in laboratory conditions, obtaining in one night a more than sufficient CFU number from which template DNA can be extracted. In addition, it represented a good model of pathogenic organism towards the development of a DNA biosensor. For instance, despite the majority of *E. coli* strains are harmless, some of them, like the serotype O157:H7, are responsible of serious food and waterborne diseases. For this reason, the development of a portable device for *E. coli* detection is a relevant topic, since its presence in water and in the external environment is a good indicator for fecal contamination. Primers of a length comprised between 32 and 37 nt were designed to amplify a region of the rrnB operon codifying for 16S rRNA, 5S, 23S and Glu-tRNA-2. 16S rRNA gene is approx. 1450 nt long and probably is the most sequenced one among bacteria. It is universally present in all bacteria and shows highly conserved regions, which are separated by variables regions. Therefore it has been used to establish phylogenetic relations between species. Primer pairs for *E. coli* 16S rRNA gene were created taking in consideration the general guidelines reported on the original paper and on the manual of the kit. To design them we used BioEdit, a free-software for sequence alignment which includes several useful features like the generation of restriction map (http://www.mbio.ncsu.edu/bioedit/bioedit.html). For every primer we calculated the possibility of homo- or hetero-dimer formation using dedicated bioinformatics tools like OligoCalc [1] and OligoAnalyzer [2], choosing those with low likelihood to secondary structure formation. Primer sequences are reported in Table 2.1. All the primers target the same template region while they differ only in their relative position, since it is reported that the efficacy of the recombination could change depending from the sequence. A biotin moiety was added to the 5'-end of reverse primer in order to detect the amplification product on a probe-based colorimetric assay as schematized in Fig. 2.7a.

[1] OligoCalc website: http://www.basic.northwestern.edu/biotools/oligocalc.html
[2] OligoAnalyzer website: http://eu.idtdna.com/analyzer/applications/oligoanalyzer/

For RPA products generated using *E. coli* primers, we measured a strong signal from the negative samples. Agarose gel electrophoresis experiments confirmed results for colorimetric detection (data not shown). To exclude the possibility of a contamination, we performed several control experiments and we found that the enzyme pellets exhibited a high *E. coli* DNA contamination (data not shown). Since the expression cloning of protein in *E. coli* is a common industrial practice, we realized that this organism could not be the best model to test such a sensitive amplification technique like RPA. In addition, on our query, the vendor confirmed that a quite high level of residual DNA could be present in the reaction tube containing the enzymes. For this reason, we had to switch to another model system.

**Table 2.1:** List of RPA primers designed for *E. coli* 16S rRNA

| Sequence | Type | Length (nt) |
|---|---|---|
| **F1**: CCTGTTTGCTCCCCACGCTTTCGCACCTGAGCG | Forward | 33 |
| **F2**: CTACCAGGGTATCTAATCCTGTTTGCTCCCCACG | Forward | 34 |
| **F3**: TATCTAATCCTGTTTGCTCCCCACGCTTTCGC | Forward | 33 |
| **R1**: CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG | Reverse | 34 |
| **R2**: ACAAGCGGWGGARCATGTGGTTTAATTCGATG | Reverse | 32 |
| **R3**: CACAAGCGGTGGAGCATGTGGTTTAATTCGATG | Reverse | 33 |
| **F4**: CATCGAATTAAACCACATGYTCCWCCGCTTGT | Forward | 32 |
| **F5**: CTTCGCGTTGCATCGAATTAAACCACATGCTCC | Forward | 33 |
| **R4**: GTCGTCAGCTCGTGTYGTGARATGTTGGGTTAAGTCC | Reverse | 37 |
| **R5**: CTGTCGTCAGCTCGTGTYGTGARATGTTGGGTTAAG | Reverse | 36 |
| **R6**: CCCGCAACGAGCGCAACCCTTATCCTTTGTTGCC | Reverse | 34 |

The second-generation primers were designed for the detection of the *Neisseria .gonorrohoeae* 16S rRNA gene. *N. gonorrhoeae* is the etiologic agent for a dangerous and highly infectious sexual disease. The development of point-of-care devices for outpatient diagnosis of this pathogen is a topic of great interest. We designed two forward and two reverse biotinylated primers following the general guidelines for RPA primers. Like for the previous primer set, we targeted the same template region differing only in their relative position. In addition, primers for NG were chosen that would differ at the 3'-end of the reverse primers with respect to the *E. coli* sequence (Table 2.2). For PCR, it is reported that even one mismatch at the 3'-end is sufficient to achieve a specific amplification, so we chose the NG primers in the same way to achieve specific amplification of *N. gonorrhoeae* in the presence of endogenous *E. coli* DNA in the enzyme pellets. Amplicons of the expected length were visible for each primer pairs, suggesting a good efficacy of the amplification method allowing the detection of as low as 10 CFU of genomic DNA. A pattern of bands at high molecular weight was visible,

indicating that primers probably also generated artifacts. Nevertheless, we found that even the second primer generation was probably not specific enough to avoid unspecific amplification of endogenous *E. coli* DNA (Fig. 2.8) since an amplicon of the proper length was visible in the negative controls (with different intensities for different primer pairs).

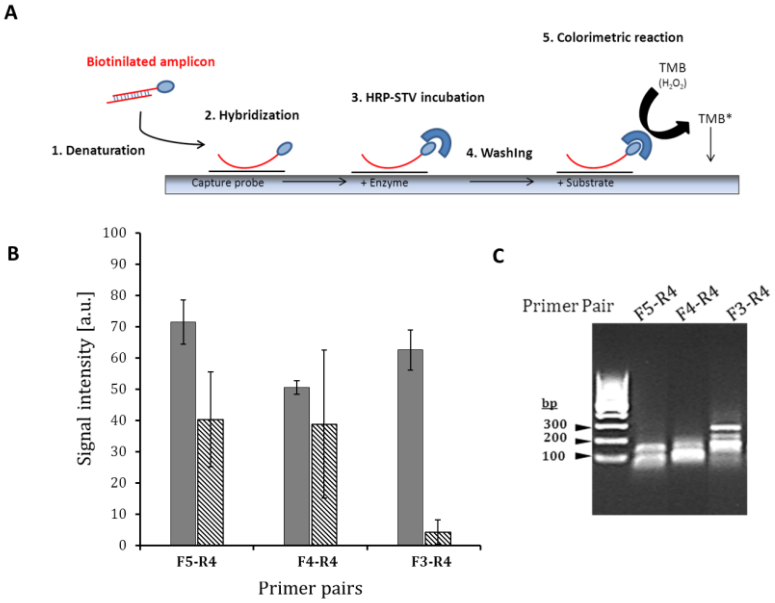


**Fig. 2.7**. (A) Scheme of the probe-based colorimetric assay. A probe specific for *E. coli* was printed on a solid support integrated in a microfluidic channel system. Biotinylated amplicon was first denatured and then hybridized on the slide. A solution containing STV-SA-conjugated horseradish peroxidase was incubated on the slide. The enzyme catalyzed the reduction of the tetramethylbenzidine (TMB) substrate, leading to the formation of a blue precipitate that could be visible also at the naked eye. A picture of the slide was collected using a flatbed photo-scanner and the intensity of the signal was calculated from the images using a Matlab-written software. (B) Colorimetric detection results for some of the possible amplicons generated by three different primer pairs showing different length. Both for template (filled grey bars) and for blank samples (patterned columns), a signal was visible. (C) We cold not observe any bands of the expected length for the amplicons. In details: amplicon F5-R4: 300 nt; amplicon F4-R4: 302 nt; amplicon F3-R4: 302 nt. 1% agarose gel in TBE 0,5X. Run for 40 min at 100 V.

**Table 2.2:** List of RPA primers designed for *N. gonorrhoeae* 16S rRNA

| **Sequence** | **Type** | **Length (nt)** |
|---|---|---|
| **NG_F1**: CCACACTGGGACTGAGACACGGCCCAGACTCCTACGG | Forward | 37 |
| **NG_F2**: CTCCTACGGGAGGCAGCAGTGGGGAATTTTGGAC | Forward | 34 |
| **NG_R1**: CGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTA | Reverse | 33 |
| **NG_R2**: GGCACGTAGTTAGCCGGTGCTTATTCTTCAGGTAC | Reverse | 35 |

| **Table 2.3:** *N. gonorrhoeae* primer pairs and amplicons | | |
|---|---|---|
| **Primer pair** | **Amplicon** | **Length (nt)** |
| **NG_F1-NG_R1** | A | 218 |
| **NG_F1- NG_R2** | B | 208 |
| **NG_F2- NG_R1** | C | 190 |
| **NG_F2- NG_R2** | D | 180 |

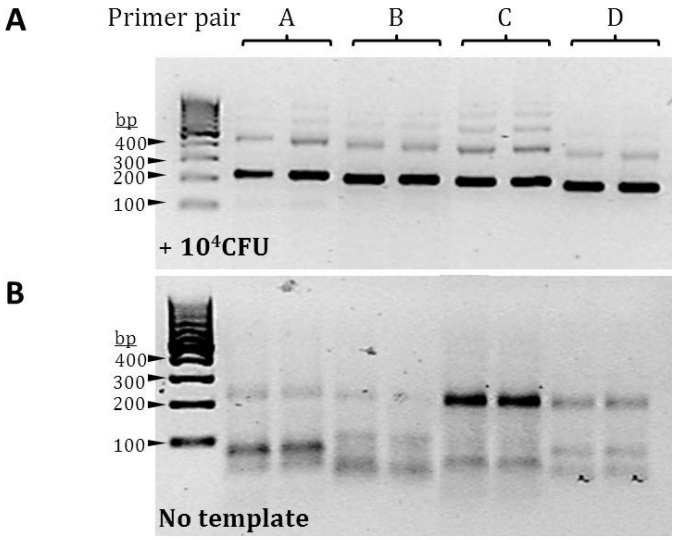


**Fig 2.8**. Gel electrophoresis results for RPA amplification of *N. gonorrhoeae* 16S rRNA gene. (A) Four primer pairs were tested in replicates to amplify *N. gonorrhoeae* DNA equivalent to $10^4$ CFU. Bands with a length corresponding to the expected amplicon were visible for each primer pair. (B) Negative samples were tested in replicates for each primer pair. A rather high non-specific amplification product was visible in every sample. Since we excluded *N. gonorrhoeae* amplification, we concluded that the designed primers were not sufficiently specific to avoid recombination with the endogenous *E. coli* DNA present in the enzyme preparation. Picture of 1.5% agarose gel in 0.5X TBE. Gel was run for 40 min at 100 V.

The origin of the reaction products in the negative control was investigated by means of enzymatic digestion. To exclude that the bands visible on the gel originate from amplification reaction artifacts from primers-enzymes interaction, we used restriction enzymes showing a unique restriction site on the primers and in the amplicon. PdmI enzyme cuts selectively the amplicon D in position 81, digesting the duplex in two fragments of 80 and 100 bp. As indicated in Fig. 2.9a, the same digestion pattern was visible for the negative control reaction products. From this, we concluded that the amplicon present in the negative control was not a simple primer artifact. Sequence comparison of *N. gonorrhoeae* and *E. coli* showed that the restriction enzyme site was

present also in *E. coli*. We obtained direct evidence that *N. gonorrhoeae* RPA primers amplified *E. coli* genomic DNA using the latter as reaction template (Fig. 2.9b).

Although the high level of aspecific amplification visible in the gel, we were able to efficiently discriminate between specific *N. gonorrhoeae* amplicon and the aspecific reaction product through the probe-based colorimetric assay (Fig. 2.10). The use of specific probes enabled the specific detection of *N. gonorrhoeae* in the presence of unspecific reaction products .

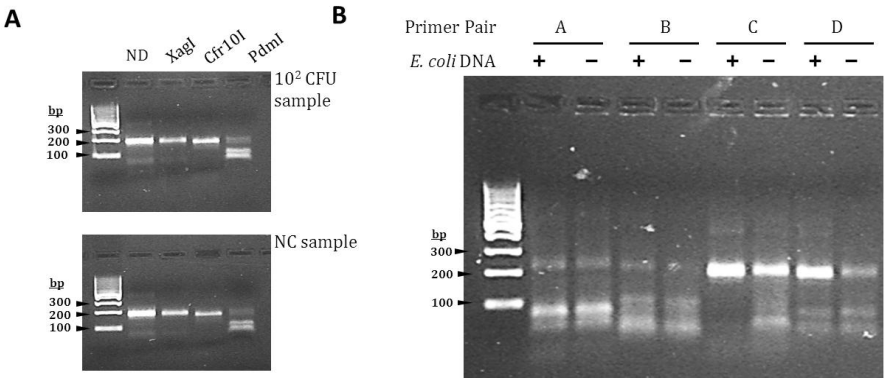


**Fig 2.9**. (A) Enzymatic digestion of RPA products. PdmI digested the amplicon in two fragments of 81 bp and 100 bp cutting one time in position 81. The same digestion pattern was visible both for specific sample and for negative control sample. Since the restriction site for PdmI was also present in the *E. coli* sequence, we concluded that *N. gonorrhoeae* primers amplified in a not specific way the endogenous *E. coli* DNA present in the pellet containing enzymes. Samples were loaded in 2% agarose gel prepared in 0.5 X TBE and run for 40 min at 100 V. Gel was stained with ethidium bromide and visualized under UV. ND: not digested; Xag1: restriction enzyme for forward primer; Cfr10I: restriction enzyme for reverse primer. (B) Gel electrophoresis results for RPA amplification of E. coli genomic DNA (1000 CFU/sample) using *N. gonorrhoeae* primers.

The third primer generation was designed to amplify *N. gonorrhoeae* and *N. elongata*. Primers positions were moved the less conserved region of the 16S gene where a significant sequence difference between the *E. coli* and the new target organisms could be found. We obtained satisfactory results: RPA detected specifically at least 100 CFU of *N. gonorrhoeae* or *N. elongata* genomic DNA (Fig. 2.11). We tested our primers using an excess of *E. coli* DNA as template, but we did not detect any amplification product (data not shown).

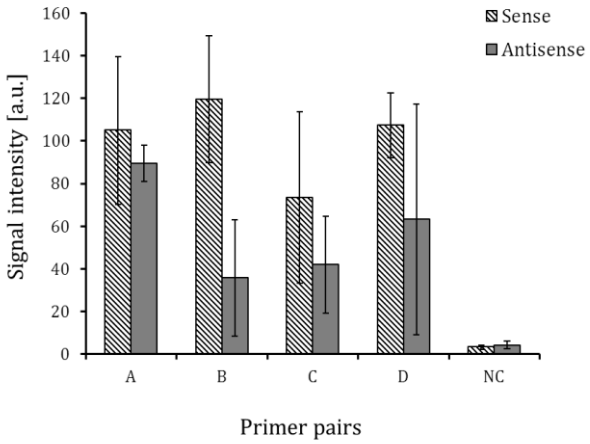


**Fig. 2.10**: The use of a probe-based end-point detection method of RPA product reveals the difference between specific and aspecific amplicons. We printed two specific probes for *N. gonorrhoeae* on the slide: one for the sense filament (patterned bars) and one for the anti-sense filament (grey bars). We found that the hybridization efficiency changed depending on the probe. High variability of the signal was due to trouble in the printing of the DNA probes on the slides. Compared to agarose gel electrophoresis probe-based detection discriminated between specific and aspecific signal.

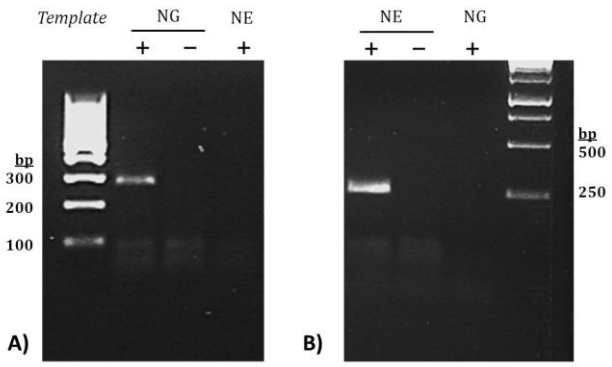


**Fig. 2.11**. RPA specific amplification of 16S RNA gene for *N. gonorrhoeae* (NG) and *N. elongata* (NE). For the reaction, 1000 CFU of each DNA species were used. 1.5% agarose gel in TBE 0.5 X. Gel was run for 40 min at 100 V, staining with ethidium bromide and visualized under UV.

### *OPTIMIZATION OF THE REACTIONS CONDITIONS*

The effect of magnesium concentration, primer concentration and reaction temperature were investigated. RPA reaction was performed using $1\times10^4$ CFU of *E. coli* DNA as template. Amplification was carried out for 40 min at two different temperatures, 37°C and 41°C, as suggested by the producer. In addition, two different primers concentration (100 nM and 150 nM) and the effect of magnesium acetate concentration (12 nM and 14 mM) were tested. Reaction yields were measured by means of the probe-based based colorimetric assay as depicted

(Fig. 2.12). Our results showed that the highest signal was obtained in correspondence to the highest reaction temperature, the highest magnesium acetate concentration and the highest primer concentration. In additional experiments the optimal primer concentration was established at 600 nM (data not shown).

*REACTION TIME INVESTIGATION*

In DNA biosensors and in medical diagnostic research fields, big efforts have made to achieve faster analysis time. Since RPA has shown to achieve sufficient amplification in 30 min,[4] we tried to decrease the reaction time. To measure the amplification of the reaction we used a probe-based chemiluminescence assay. Using chemiluminiscence detection instead of colorimetric detection, we were able to increase the sensitivity or the detection system (data not shown). We found that after 15 minutes of reaction, the signal was saturated indicating that 15 min of amplification were enough to obtain detectable quantities of DNA. Electrophoretic end-point detection confirmed these findings (Fig. 2.13). Our results were in agreement with those reported in the literature which demonstrated that RPA could amplify exponentially in≈ 10 minutes.

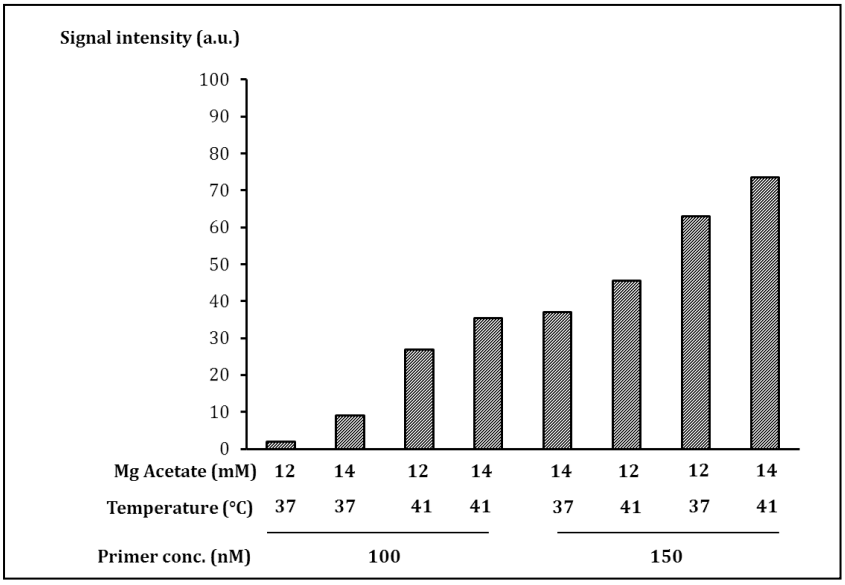


**Fig. 2.12**. Effect of Mg Acetate concentration, temperature and primer concentration on RPA product formation. HRP-based colorimetric detection of RPA product. Highest signal was obtained for higher primer temperature (41°C), higher Mg acetate concentration (14 mM) and higher primer concentration (150 nM).

We confirmed the results performing a real time RPA experiment, monitoring amplicon formation with Sybr Green I every 30 seconds (Fig. 2.14). We found that fluorescence reached a plateau after 15 minutes of RPA reaction and that the increase in fluorescence after this time was ca. 1% for minute. Since Sybr Green I fluorescence increased with the binding of the molecule to the dsDNA independently from the sequence, the presence of primer artifacts or the amplification of the endogenous E. coli DNA strongly biased the measurements and did not allow us to correlate the final fluorescence values with the starting amount of DNA in solution.

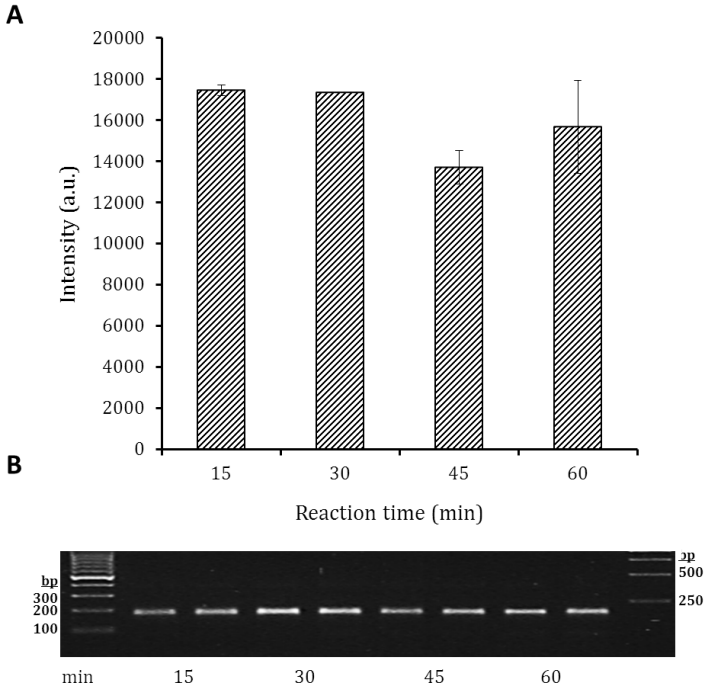


**Fig. 2.13.** RPA amplifies specific DNA region in 15 minutes. The experiment was performed using 100 CFU of *E coli* DNA. A) Chemiluminescence read-out of reaction product showed a saturated signal for all the sample independently from the reaction time. B) Electrophoresis detection: 1.5% agarose in TBE 0.5X. Gel was run for 40 min at 10 V/cm, stained with ethidium bromide and visualized under UV.

### *EFFECT OF AGITATION ON THE REACTION YIELD*

Shaking of RPA samples after four minutes of incubation was suggested by the vendor to be a good strategy to improve the product yield. In our opinion, sample agitation might represent a limitation for the RPA reaction in a number of ways. For example, mixing could be a quite big source of variability. Agitation forces the operator to follow the reaction minute by minute and to remove the sample from the incubator for shaking. We tested different agitation times and we investigated the possibility to remove

this step. We found that performing the RPA without the intermittent sample shaking as recommended in the vendor's protocol did not reduce the signal (Fig. 2.15).

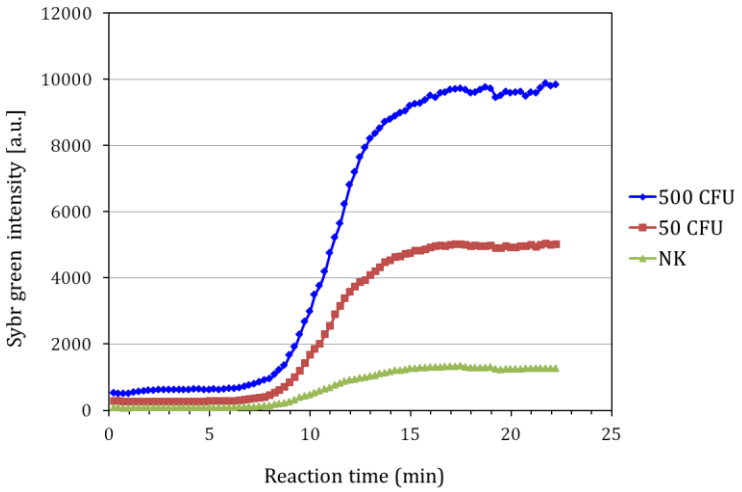


**Fig. 2.14**: Real Time RPA. The fluorescence signal increased starting from 8 min reaction and after 15 min it reached a plateau.

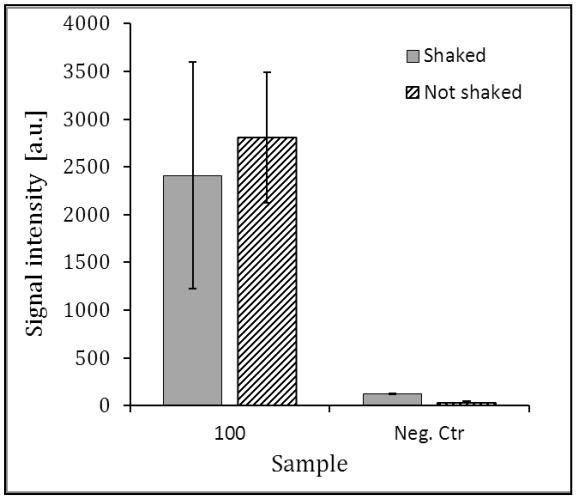


**Fig. 2.15**. Effect of the sample agitation on the RPA reaction yield. Using *N. gonorrhoeae* DNA as template (100 CFU of extracted DNA) we performed RPA in two different ways: incubating the sample for 4 min, shaking it for other 4 min and finally incubating for a total reaction time of 15 min (grey bars) or incubating for 15 min continuously without interruption (patterned bars). We found that without shaking signal was kept constant. Samples were tested in replicates.

### *RPA AS A SUITABLE AMPLIFICATION METHOD FOR MICROARRAY ANALYSIS.*

The development of a strategy for fast amplification and labeling of a nucleic acid sample is a relevant topic in the research field of microarrays. Considering that a molecule of target DNA can be amplified and labeled in ≈10 min by means of RPA, we

developed a protocol to combine the detection of a biotinylated RPA reaction product on a DNA microarray platform used for the simultaneous detection of sexually transmitted pathogens. We used Streptavidin-Alexa Fluor 647 conjugate to bind the *N. gonorrhoeae* amplicon marked with a biotin label after hybridization with the array probe. Our results showed that biotinylated target specifically bound on *N. gonorrhoeae* probes. Thus an additional working step of 1 hour including hybridization, labeling and slide scanning was sufficient to couple RPA with specific microarray detection (Fig. 16). We found also that the clean-up step of RPA product before the hybridization on the array was not necessary and the purification step even caused a loss of product. On the other hand the protocol has to be optimized since the labeling step and the increased number of washing steps reduced the signal and increased the variability. In addition, also the background signal increased. In alternative to biotin, fluorescent dyes could be used to mark RPA amplicon directly, thus avoiding the additional labeling step after hybridization which increased background signal and variability.

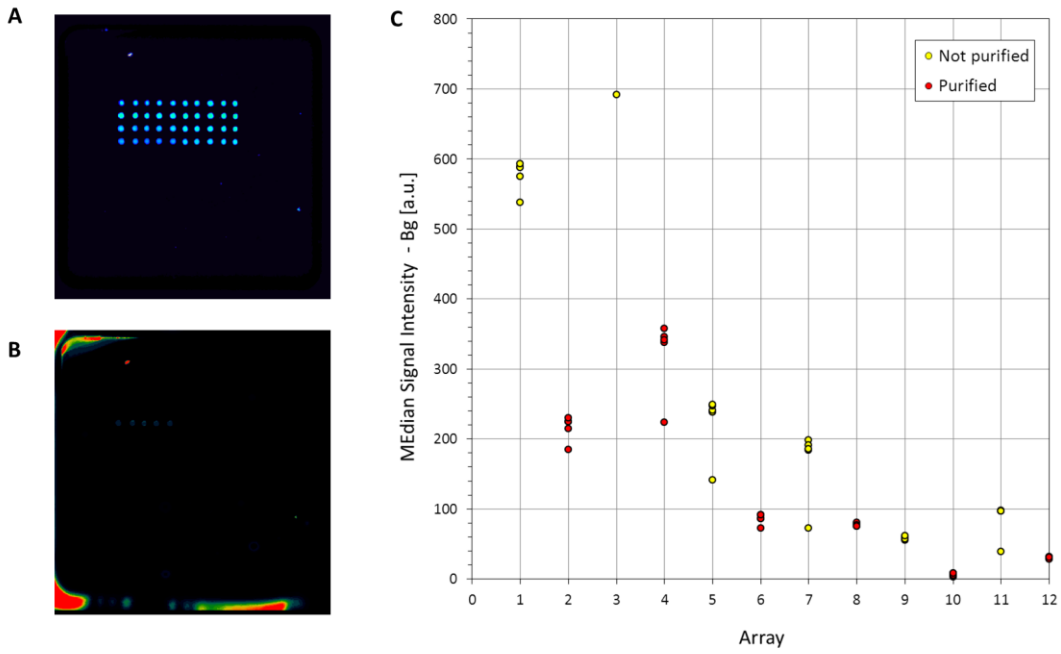


**Fig. 16**: RPA amplification can be coupled to microarray detection. We hybridize RPA samples on an array with specific probes for *N. gonorrhoeae* and we used the exposed biotin moiety to bind with Streptavidin-Alexa Fluor-647 conjugate. Panel A and B: slides scan images for control dye and sample dye, respectively. *N. gonorrhoeae* RPA amplicon was hybridized on the proper array features (5 spot for probes). Panel C: summary of signal intensity (median of intensity values-background).

## CONCLUSIONS AND FUTURE PERSPECTIVES

Albeit its complexity, the RPA mechanism represents a suitable method for nucleic acid amplification. Our results were in agreement with the literature: RPA was specifically amplified in less than 15 minutes starting from 100 CFU of target DNA. We demonstrated the versatility of the system by designing primers for the amplification of different pathogenic organisms and using different probe-based assays for the specific detection of amplicons. In addition, we found that the use of biotin-labeled primers did not interfere with the recombination process. The use of fluorescently-labeled selective primers in the reaction could represent a real major breakthrough for microarray application since amplification and, as a consequence, labeling could be achieved faster with respect to the current protocols.

In our opinion, RPA could find a number of applications in research and in clinical and environmental analytics. Since the process is completely isothermal and takes place at physiological temperature, it is well suited for point-of-care devices. The use of the probe-based colorimetric assay represented the first step towards this type of application, since the system could provide an on-off response without the use of complex instrumentation.

Experiments using RPA for multiplex applications are currently in progress.

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# CHAPTER 3

## ROLLING CIRCLE AMPLIFICATION OF RNA

### INTRODUCTION

Rolling circle amplification (RCA) is a widely used strategy for sample and signal amplification in nucleic acids detection. The success of this strategy can be justified with several attractive features of the reaction. First, RCA works at constant temperature. Polymerases catalyze DNA polymerization usually at 37°C helping to avoid the use sophisticated instrumentation for thermal cycling. Second, researchers have demonstrated that RCA can be implemented on numerous detection platforms (such DNA microarrays) and that the reaction is suitable for parallel or high-throughput analysis[3-5]. Finally, RCA-based assays offer both high sensitivity and high specificity. As a method for signal amplification (through either linear or exponential amplification), RCA typically provides a 1000 to 10000 fold increase in the intensity of the signal[6]. Moreover, single-molecule counting is possible, as the signal is localized on a single molecule (or as a single spot on the solid substrate). For the aforementioned reasons, extensive studies on the use of RCA as amplification tool for nucleic acid diagnostics have been conducted in the last decade with great success and RCA-based approaches have attracted the attention of diagnostics-oriented biotech companies and research centres which have included RCA technology in their products (See Table 6.1).

RCA is a powerful tool for DNA amplification, while its applications in RNA detection are more recent[1, 2].

In the next paragraphs, the molecular mechanism of RCA and the strategies at the basis of DNA and RNA detection will be described

| **Table 1**: commercial kits for DNA amplification based on RCA | | |
|---|---|---|
| **Product name** | **Producer** | **Description** |
| RCAT™ | Motorola - Molecular Staging Inc. | Technology developed for nucleic acids bioarray |
| Illustra™TempliPhi™ | GE Healthcare (UK) | Prepare micrograms of circular DNA from picogram input material |
| Illustra™GenomiPhi™ | GE Healthcare (UK) | Synthesis of High-quality DNA for PCR, restriction enzyme digestion, hybridization, cloning, array CGH high throughput genotyping, and DNA archival. |

## *MOLECULAR MECHANISM PRINCIPLES*

Since 1990s, it was discovered that some polymerases were able to elongate a short DNA strand annealed to a small circular ssDNA template[7-9]. The process was inspired by the rolling circle replication used by many plasmids and viruses to replicate their circular genome[10-13]. In nature, replication of these circular double strand molecules proceeds by the mechanism depicted in Fig. 1. Briefly, a nuclease cuts the DNA providing a free 3′-OH end to which nucleotides are added. As it can be seen from Fig. 3.1, during the elongation reaction, the template strand is displaced from the double-stranded circle and then copied. Because there is no termination point, synthesis often continues beyond a single circle unit, producing concatamers (a series of linked chains) of several circle lengths, which are then processed by recombination to yield normal-length circles[14].

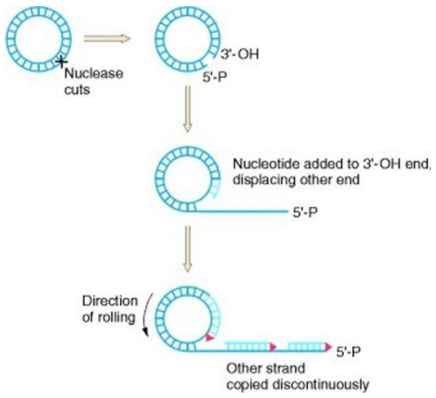


**Fig. 3.1.** Basic mechanism of rolling-circle replication in bacteria. Picture taken from Griffiths[14].

In the rolling circle amplification reaction, an oligonucleotide primer is extended by DNA polymerase after annealing to a single strand small circular template (typically less than 100 nt). Several DNA polymerases are effective towards the RCA reaction. Examples of such polymerases are Phi29 DNA polymerase[15, 16], Sequenase ™ (Ge Healthcare Life Science, UK)[17], Klenow, Vent™ exo-enzymes (New England Biolabs Inc., UK)[17, 18], and the large fragment of Bst DNA polymerase[18]. The RCA efficiency is

normally increased by the addition of proteins that bind single strand DNA like *E. coli* SSB protein[17]. Polymerases involved in RCA are highly processive and lead to the synthesis of kilobases of single-stranded DNA[19-22].

## *RCA–BASED APPROACHES FOR DNA DETECTION*

Shortly after this discovery, RCA gained considerable attention as a novel DNA amplification tool and was confirmed as an important technique for sensitive DNA detection. The basic strategy for RCA-based DNA detection and amplification is simple: a primer (a target or a secondary probe) is hybridized to a small circular DNA (primary probe) and it is repeatedly replicated by a suitable polymerase. As a result a long DNA repeats of the circular DNA, easily detectable, will be formed.

According to Demidov[23], priming approaches for RCA in DNA diagnostics can be grouped in two categories: *single-primed* or *double-primed*. The single-primed, or linear, RCA (Fig. 3.2), results in the continuous rolling out of long, repeated DNA sequences of different length that gradually accumulate. These products cannot be resolved by gel electrophoresis but figure like a broad smear of high molecular weight DNA filaments. RCA products are mostly represented by single strand DNA molecules typically consisting of $10^2$–$10^3$ repeats of the complementary sequence to circular probe. The single-stranded products can be easily manipulated for successive applications or for their detection.

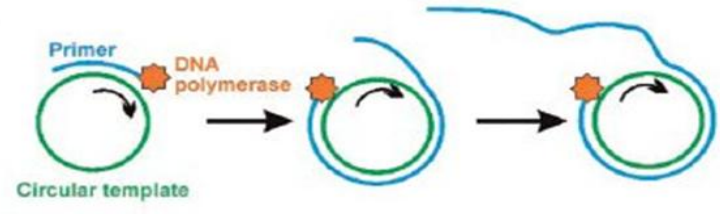


**Fig. 3.2**. Scheme of basic RCA mechanism. A DNA primer binds to a small single-stranded DNA circular template. A suitable polymerase catalyzes primer elongation adding dNTPs to the 3'-OH end. Figure taken from[24].

In double-primed, or hyper-branched, RCA an additional oligonucleotide serves as primer for the rolling-circle amplified nucleic acid, so that a new duplication reaction is started on the same molecule for each binding event of the second primer. The result is a geometric amplification of the circular probe. This strategy greatly enhances the amplification factor because a big amount of DNA is produced for each positive binding event (Fig. 3.3). With respect to linear RCA, in hyper-branched RCA a discrete set of dsDNA concatamers is formed. As a consequence of the replication, on a gel, a discrete

pattern of filaments will be visible. In terms of the degree of amplification, the geometric RCA is much more potent, if compared with its linear alternative, yielding $10^9$ or more copies of a circular sequence in 1 h.

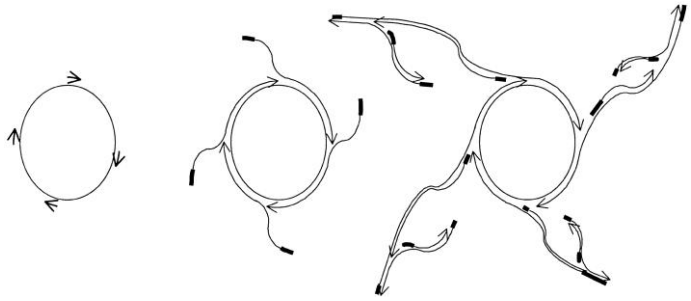

**Fig. 3.3**. Scheme for hyperbranched rolling circle amplification (HRCA). Picture taken from Dean *et al.*[6].

In both approaches, the RCA product represents the first amplification step of this detection method, as the long DNA fragment is easy to detect. For example, in the circular template strand, in addition to the probing section, another sequence box containing information could be included to achieve a second amplification step. A really efficient approach has been suggested by Mao and co-workers[16]. They introduced in the circular probe the sequence folding for G-quadruplex structures. A G-quadruplex structure shows a binding capability for hemin, an iron-containing porphyrin that mimics a peroxidase. The many copies of such DNA peroxidases can catalyse the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS). The reaction product $ABTSC^+$ is blue-green (maximum absorption wavelength, $\lambda_{max}$=415 nm) and can serve as a convenient, colorimetric output signal. This reaction has multiple turnovers; each enzyme can generate multiple copies of products. Thanks to this second amplification step they reported a limit of detection of 1 pM.

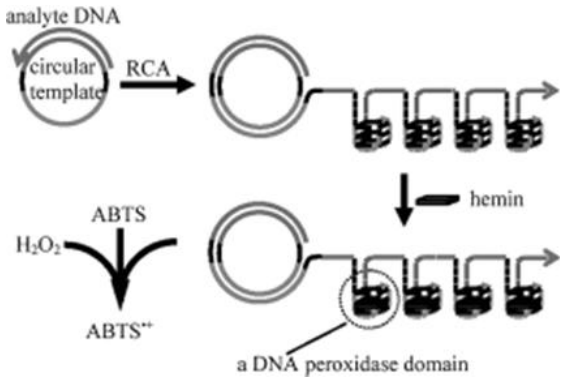


**Fig. 3.4**. Mao et al.'s approach for a second amplification step in RCA detection. The long single strand RCA product contains multiple copies of the G-quadruplex sequence. The folded G-quadruplex contains a DNA peroxidase domain which can catalyses a colorimetric reaction. Figure taken from Tian *et al.*[16]

Using the same strategy, Cheglakov and co-workers in Itamar Willner's lab implemented the same amplification strategy towards a biosensor-type application[25]. They reported a detection limit of $10^{-14}$ M (10 fM) for a long viral DNA. For a scheme of the proposed system, see Fig. 3.5.

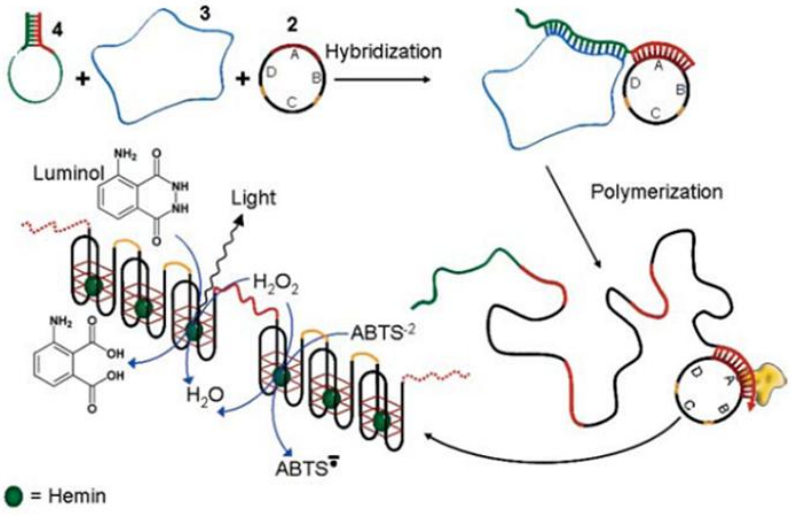


**Fig. 3.5**: Cheglakov et al. implementation of the rolling circle amplification enhancement of detection with colorimetric or luminescence detection. Figure taken from Cheglakov and co-workers[25].

A milestone work in RCA research field is that of Paul Lizardi and co-workers, who first proposed a surface–based implementation of the reaction[26]. A probe is bound to the solid surface via its 3'end, in order to expose a 5'phosphate end which cannot serve as primer for RCA. Upon binding of the target, a second oligo complementary to the target can also bind and this and the probe oligo can be ligated in case of perfect match with the target. The second type of oligonucleotide (that can be used to test for sequence variations, such as polymorphisms) is a special oligo that contains a reversed backbone and thus has two 3' ends. After ligation and binding, the presence of this second oligo (and thus the positive binding event) is detected by running a rolling circle amplification extension of it, using a soluble circular template (step 5 and 6 in the scheme below). After rolling circle amplification, the presence of the amplified DNA is detected by binding several copies of fluorescently labelled oligonucleotides complementary to a tandemly repeated section on the amplified DNA.

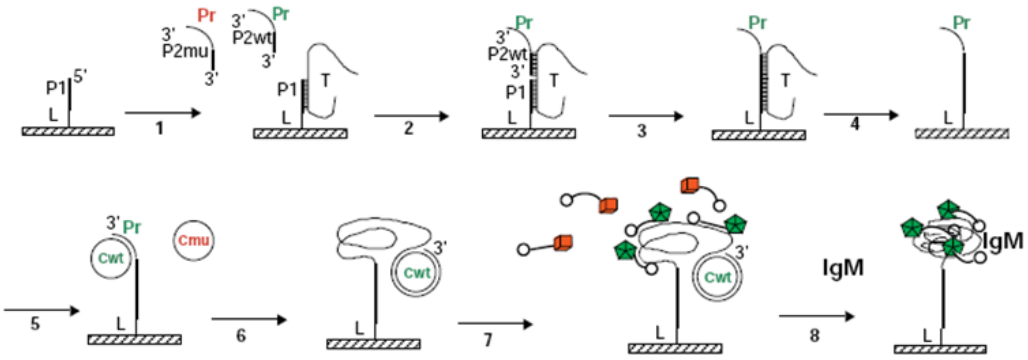


**Fig. 3.6**. Lizardi's use of RCA to enhance the readout signal, to the point of being able to count molecules (with fluorescence microscopy read-out). Figure reproduced from Lizardi *et al.*[26].

## *RCA-BASED APPROACHES IN RNA DIAGNOSTIC*

The most used strategy in RCA-based RNA diagnostic is the association with ligation assay. The ligation-mediated rolling circle amplification (L-RCA) makes use of the so-called padlock probes and it has been extensively tested for polymorphism detection in DNA[4, 27, 28]. The method uses a linear DNA probe in which both ends are juxtaposed by the specific hybridization to a target DNA sequence. The two ends of the DNA probe are joined by a DNA ligase, (usually the T4 DNA ligase) and the resulting DNA ring serves as the template for an RCA reaction with a suitable DNA polymerase. Ligation reaction is specific and a combination of ligation-mediated single nucleotide polymorphism detection with RCA or hyper-branched RCA, represents a powerful method single nucleotide polymorphism(SNP) detection.

L-RCA reaction conditions have been deeply investigated and confirmed on DNA. On the contrary, publications dedicated to RNA detection based on RCA are limited but promising. The major drawback for RNA-templated DNA ligation is the intrinsic lower efficiency with respect to the DNA-templated reaction[29]. Several parameters influence the catalytic efficiency of the T4 DNA ligase. For example ATP concentration must be kept low, a molar excess of ligase over substrate must be used and the reaction should be performed for an extended time[30]. Nilsson's group pioneered this type of studies using padlock-probes to discriminate a single nucleotide DNA probe-RNA target mismatch[31](Fig. 3.7).

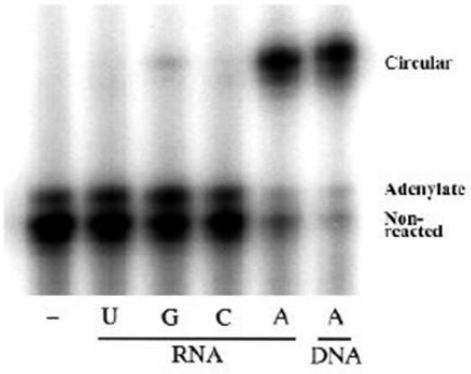


**Fig. 3.7**. Nilsson and colleagues demonstrated that T4 DNA ligase catalyzes the circularization of a DNA padlock probe on RNA template. Ligase mediated circularization is sensitive to single nucleotide mismatches. Picture reproduced from Nillson *et al.*[30].

Lagunavicius and colleagues combined Phi29-mediated RCA and RNA-templated ligation in a specific assay for RNA transcript detection *in vitro* and *in situ*[1, 32].

L-RCA has been also used to detect and quantify micro RNAs (miRNAs) in few nanograms of total RNA. In this system, miRNA was used both as template for ligation and as a primer for RCA[33]. A scheme for the reaction is reported in Fig. 3.8.

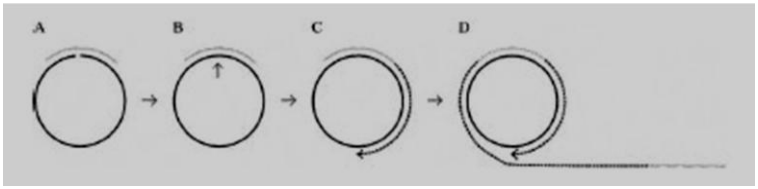


**Fig. 3.8**. Scheme of the strategy used by Jonstrup and colleagues to detect synthetic miRNA and natural miRNA extracted from cultured cells. Figure taken from Jonstrup *et al.*[33].

A very remarkable use of L-RCA for RNA detection has been recently reported by Chatarina Larsson in the group of Nilsson[2](Fig. 9). Here, RCA is used for RNA detection inside living cells..

We have not found any examples of RCA used to amplify the signal of a RNA target hybridized on the surface.

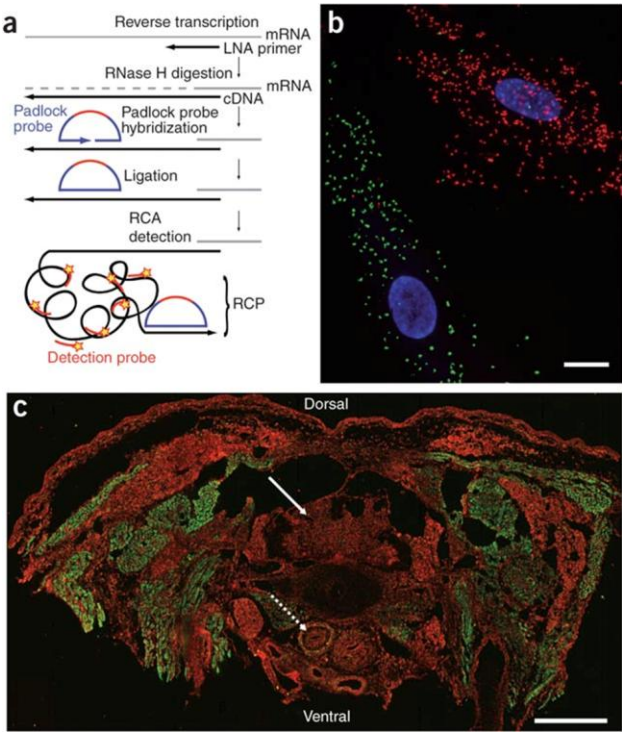


**Fig. 9**. *In situ* application of L-RCA for detection of mRNA in mouse fibroblast cells. (a) Schematic representation of the assay. Respect to the work of Lagunavicius[1] mRNA is not directly used as template for ligation. A previous cDNA transcription step is done. Considering the overall low efficiency of ligation of RNA, this step increased the probability to be successful in the reaction. (b) Effect of single mutation on two variants of the same target gene. Green: human β-actin sequence variant; red: mouse β-actin sequence variant. Picture taken from Larsson *et al.*[2].

### *REASONS FOR THE CHOICE OF THIS STRATEGY*

The detection of pathogenic organisms by means of RNA detection is an attractive tool for researchers. With respect to DNA, RNA is present in a high number of copies and, in addition to rRNAs and mRNAs, there exist several other types of ribonucleic acid molecules that can be targeted (for example miRNAs and tmRNAs).

Amongst nucleic acids detection techniques, rolling circle amplification is a sensitive method that can be used to physically amplify the target molecule or that can be implemented on the surface to enhance the signal derived by the hybridization event between the probe and the target.

For these reasons, we investigated the possibility to combine rolling circle amplification and RNA-templated ligation as strategy for *E. coli* 16S rRNA detection

## MATERIALS AND METHODS

### *MATERIALS*

Padlock probe and synthetic RNA oligonucleotides were purchase by Microsynth AG (Switzerland). T4 DNA ligase and Phi29 polymerases were supplied by Fermentas (USA). Chemical reagents were purchased from Sigma-Aldrich (USA) where not differently specified.

### *BACTERIAL CULTURE AND RNA EXTRACTION*

*E. coli* cultures were grown and used as template RNA source for RCA amplification. Briefly, 20 µl of *E. coli* glycerol stock were pipetted and transferred in 4 ml of fresh LB medium (Sigma-Aldrich). Cells were grown overnight at 37°C under constant agitation (200 rpm). Bacteria concentration was estimated measuring optical density at 600 nm using the Eppendorf Biophotometer and single-use plastic cuvettes (Uvette®, Eppendorf). RNA was extracted using the GenElute™ Bacterial Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer protocol's starting from 100µl of liquid culture in guanidinium thiocyanate 800 mM, laurylsarcosine 1% Tris-HCl 100 mM (pH 7.4 at 25°C). RNA quality was assessed performing optical density measurements by means of Biophotometer plus, UV/Vis Photometer (Eppendorf) and gel electrophoresis using 1% agarose in TBE 0.5X and ethidium bromide staining.

### *LIGATION ASSAY*

*Ligation on synthetic RNA template*. Reaction was performed in 10 µM ATP, 40 mM Tris-HCl, 10 mM $MgCl_2$–pH 7.5 in the presence of varying amounts of synthetic RNA target and 10 ng of padlock probe. Samples were heated to 65°C for 3 min, cooled down to 25°C and kept at this temperature for 10 min. Five, ten or forty units of T4 DNA ligase (Fermentas) were added in a final reaction volume of 6 µl. Samples were incubated for 2 h at 37°C. The reaction were stopped incubating at 65°C for 15 min. The ligation products were analyzed on denaturing urea 10% polyacrylamide gel in 1X TBE using (100 mM Tris Borate, 2 mM EDTA, pH 8). Sample were run for 1 h and 30 min at 180 V Gels were stained with Sybr Gold (Invitrogen) and visualized under UV light.

*Ligation on total RNA*. 0.2 µg of template and 10 ng of padlock probe were mixed in ligation buffer (40 mM Tris-HCl, 10 mM $MgCl_2$–pH 7.5) testing different concentrations of ATP in solution (10, 5 and 2.5 µM). Samples were heated to 65°C for 3 min, cooled down to 25°C and kept at this temperature for 10 min. Five or ten units of T4 DNA ligase (Fermentas) were added in a final reaction volume of 20 µl. Samples were incubated for 2 h at 37°C. Reaction were stopped incubating at 65°C for 15 min. The ligation products were analyzed on denaturing urea 10% polyacrilamide gel in 1X TBE using (100 mM

Tris Borate, 2 mM EDTA, pH 8). Samples were run for 1 h and 30 min at 180 V. Gels were stained with Sybr Gold (Invitrogen) and visualized under UV light.

### *ROLLING CIRCLE AMPLIFICATION PROTOCOL*

One microliter of ligation product was mixed with 100 pmoles RCA primer. Sample was incubated 30 sec at 95°C and cooled down to room temperature for 10 min. One microliter of annealed padlock probe-primer solution was incubated with 10 U of Phi29 DNA polymerase (Fermentas) in 1X Reaction Buffer (Fermentas) in the presence of 0.5 mM dNTPs. The final sample volume was 50 µl. Samples were incubated for 2 h or overnight at 37°C. The reactions were then incubated at 65°C for 10 min to inactivate the polymerase. The samples were immersed in an ultrasonic bath (VWR, USA) and sonicated for 15 min at 60 Hz. 10% of the reaction mix was loaded on 1% agarose gel and run for 30 min at 80V. Gel was stained with ethidium bromide and visualized under UV light.

### *COLORIMETRIC ASSAY*

Probes for *E. coli* targets were printed on plastic slides according to a proprietary process of Greiner-Bio-One. An aliquot of RCA product was mixed with 3 M guanidinium thiocynate solution with a ratio 1:10 and incubated for 1 min at 95°C. 20 µl were pipetted on the printed slide and incubated for 2 min. 30 µl of horseradish peroxidase solution (HRP) were added, incubated for 2 min and then washed with 40 µl of washing solution. Finally, 30 µl of 3,3′,5,5′-tetramethylbenzidine (TMB, STD Reagents, Germany) were added and incubated for 4 min or until a blue color was visible. The slides were scanned immediately with a flatbed photo scanner (CanoScan 5600F, Canon) and the signal was measured from the pixel intensity of the picture using a Matlab-written software.

## RESULTS AND DISCUSSION

### *CIRCULARIZATION OF THE DNA PROBE ON SYNTHETIC RNA*

The use of RCA to amplify a RNA target molecule after circularization of a padlock-probe is known to be limited by the lower efficiency of ligation on RNA with respect to DNA. The literature on this topic is rather limited. Nilsson and co-workers described the conditions where 80% of RNA target molecules can serve as a template for a diagnostic ligation reaction[30]. They investigated the ability of T4 DNA ligase to catalyse the reaction changing several parameters. For example, they reported that the ATP concentration in solution must be $\leq 14$ µM (the $K_m$ value for ATP binding) and that a molar excess of ligase over substrate should be adopted. In any case, the reaction takes place if sufficient time is spent for the incubation, typically longer than 2 hours[30]. Unusually, it has been reported that an excess of template RNA compared to the amount of circular probe should be used (equal or higher than 100-fold). This ratio is in contrast with that used in PCR and in other amplification techniques where a big excess of primers (in a defined range of concentrations) is used to amplify few molecules. The thermodynamic driving force for PCR is the molar excess of reagents with respect to the template, since the goal of this strategy is to push the reaction toward the formation of the reaction product (the amplicon) but without significant consumption of reagents. For RNA-templated ligation we assumed that the use of a large excess of template on the circular probe could have two meanings: to push the reaction toward the reaction product formation and to avoid self-circularization of the probe since the efficiency of ligation is higher on DNA than on RNA. Starting from this background knowledge, in our preliminary studies we tested RNA-templated RCA starting from a synthetic RNA template. We designed a 74 nt DNA padlock probe specific for a 20 nt RNA oligonucleotides with a sequence identical to a small region of 16S rRNA of *E. coli*. We assumed that testing the reaction using a synthetic template could facilitate the successive optimization of the system for cellular RNA. Sequences used for the experiments are listed in Table 3.1.

**Table 1**: sequences for RNA template, circular probe and primer

| | **Sequence** | **Type** |
|---|---|---|
| Circular probe | TCMTTTGAGTTTACATCGAATTAAACCACATGYTCCWCCGCT TGTAAAAAAAAAAAAAAAGGGCCCCCGTCAAT | DNA |
| Primer | TTTTTTTTTTTTTTTACAA | DNA |
| Template | AAACUCAAAUG*A*AUUGACGGGGGCCC | RNA |

We investigated the ligation of the circular probe on the synthetic RNA template working in strong excess of template (300 or 150 fold) and testing different ATP concentrations and different amounts of T4 DNA ligase. Our goal was to detect a difference in band migration of the non-ligated DNA probe (linear) respect to the ligated-probe (circular). Since only 10 ng of the probe were incubated with a strong excess of template RNA, 50% of the ligation mix were loaded on the gel in order to appreciate the relative bands. As it can be seen in Fig. 3.10, the excess of RNA template present in solution overloaded the gel, leading to a deep smear for each lane. A band of length comparable to a dimer of RNA was visible for each sample. We did not see clearly the expected band shift for the ligated or non-ligated probe. As a matter of fact, we could not appreciate a clear difference between the negative control sample (probe and enzymes in the reaction mix without template) and any other sample, independently from the reaction conditions used. Since the attempts done to isolate DNA from RNA did not help our interpretation (data not shown), we assumed that, in our working concentration, PAGE coupled to Sybr Gold detection were not sensitive enough to detect the ligation product.

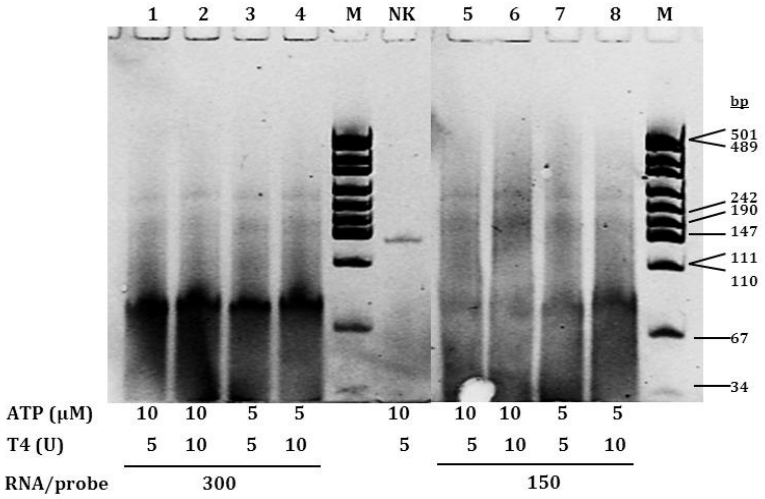


**Fig. 3.10**: Ligation of padlock probe on synthetic RNA target. 10 ng of linear probe were incubated with different amounts of RNA template in a reaction buffer containing 5 or 10 µM of ATP in presence of 5 or 10 units of T4 DNA ligase. Sample 1-4: template/probe ratio = 300. Sample 5-8: template/probe ratio = 150. Overloading was due to the excess RNA template used for ligation, since the attempts done to degrade RNA selectively in the reaction mix before the detection were negative. Sample *M*: pUC19/MspI DNA ladder (Fermentas). Ligation product of sample n°5 was used to template a RCA reaction which results are reported in Fig. 11.

### *ROLLING CIRCLE AMPLIFICATION OF LIGATION PRODUCTS*

Since our final goal was to detect RNA targets by means of RCA, we tested the reaction using one of our ligation products as template. Considering that RCA takes place

only if a circular template is present in solution, we assumed that the detection of the amplified product could give the indirect proof that the linear DNA probe was circularized on RNA. We conducted RCA incubating one aliquot of the ligation product (2 ng of DNA probe) for 2 h at 37°C with an excess of another short DNA sequence used to prime the reaction. In Fig. 3.11-A, the gel electrophoresis results of the reaction product are reported. We did not observe the broad smear of high molecular weight DNA molecule expected for the linear RCA reaction but only the formation of a really high molecular weight product that was retained in the well (sample 3, Fig.3 11A). RCA performed using the linear DNA probe did not produce the same pattern (sample 4, Fig. 3.11A). Sonication of the reaction product for different times (30 sec, 1, 5 and 10 min) produced an unusual effect: ultrasounds did not fragment the DNA in a wide range of fragment sizes as expected, but only produced a sharp band correspondent to a reduced size with respect to the RCA product. As a matter of fact, a band with high molecular weight was slightly visible and its intensity increased with the increasing of sonication time. Fragmentation of RCA product was also necessary for the successive detection step on a colorimetric probe-based assay. The results are shown in Fig. 11. We found that RCA exhibited a strong signal only when performed on the ligation products. A signal considerably lower was present for the RCA reaction implemented with the linear probe (negative control) and no signal was visible for the ligation product mixed with biotinylated capture probe (Control 1) nor for a solution containing padlock probe and RCA primer at the working concentration (Control 2). This result confirmed that RCA took place and we assumed that padlock probe was circularized using RNA as a template for the ligation.

We performed a number of attempts devoted to the optimization of the ligation step. In any case, we could not discriminate between the linear and the circular probe on the gel. Thus, no significant difference between the use of a buffer containing 5 µM or 10 µM ATP as well that 5U or 10U of T4 DNA ligase was visible. Despite the lack of direct evidences for ligation, we found that all the ligation samples could template RCA reaction leading to the same results previously described (data not shown).

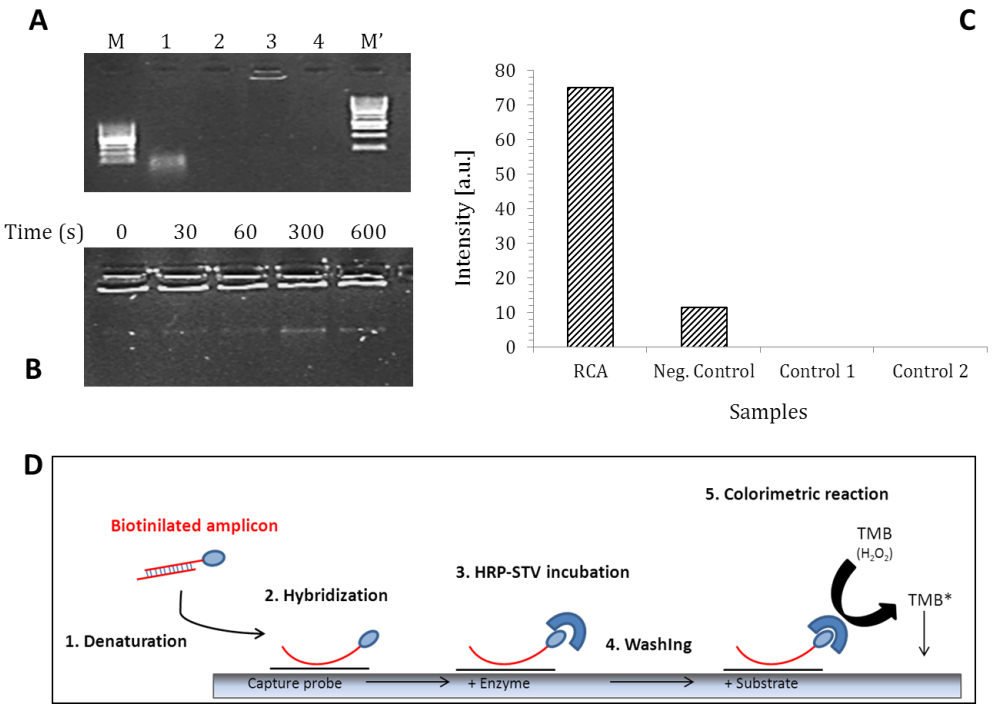


**Fig. 3.11**: Results of rolling circle amplification performed using a ligation product (sample n°5 of the ligation experiment summarized in Fig. 3.10) and a short DNA primer complementary to the circle. *(A)* Electrophoretic detection of RCA product. Reaction was conducted for 2 h at 37°C. *Sample 1*: ligation product. *Sample 2*: negative control of ligation reaction. *Sample 3*: RCA performed using circularized-probe. *Sample 4*: RCA performed using linear probe. AGE condition: 1% agarose gel in TBE 0.5X. Gel was run for 15 min at 80 V, stained with ethidium bromide and visualized under UV. *(B)* RCA sample was sonicated before detection on colorimetric probe-based assay as schematized in panel *(D)*. *(C)* We detected the RCA reaction using a colorimetric probe-based assay. A strong signal was visible for the RCA performed using the circularized probe (sample 3 in panel A). A lower signal was detectable for the control reaction. *(D)* Scheme of the probe-based colorimetric assay.

## *Effect of template/probe ratio on RCA*

Since the aim of the amplification is to detect a few molecules with a large excess of primers, we investigated the possibility to reduce the target/probe ratio that is usually kept high for RNA-templated ligation. We found that for ligation reaction performed with high RNA/probe ratio, two slightly visible bands corresponding to a single strand of 74 nt and 100 nt were visible. We presumed that a circular DNA molecule should exhibit a lower migration rate than the corresponding linear probe, thus we assigned the two bands as the non-ligated and ligated probe (sample 1 and 2 of Fig. 3.12A). The results of rolling circle amplification performed on ligation samples confirmed our interpretation showing that amplification took place only for high RNA/probe samples (Fig. 3.12B and 3.12C).

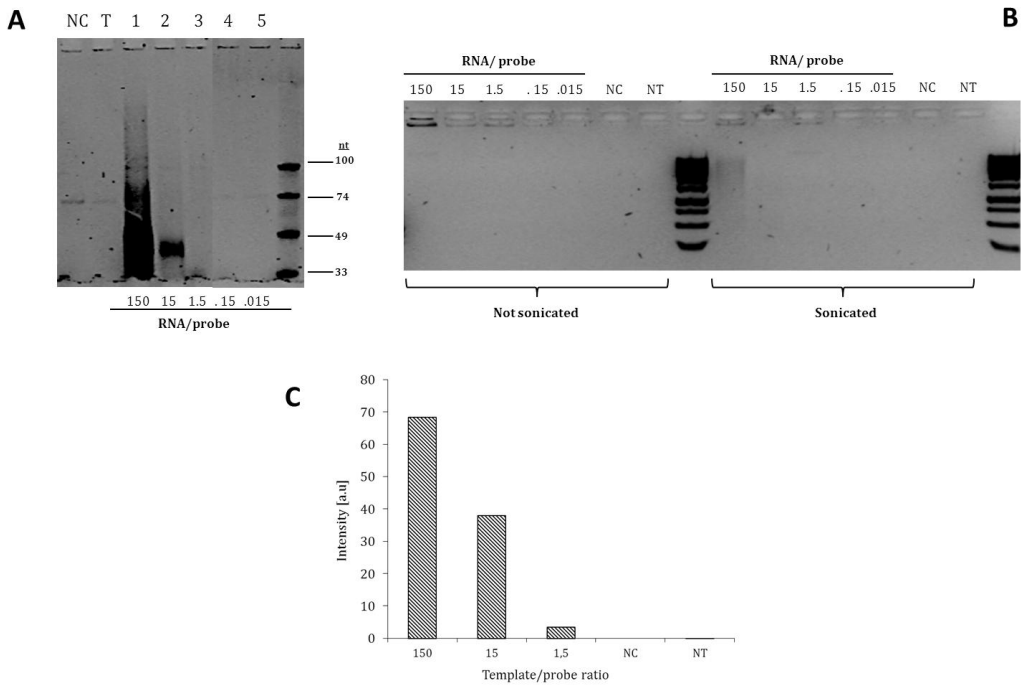


**Fig. 3.12**. RCA was performed various template-probe ratio. *(A)* 1% agarose gel in TBE 0.5X. Gel was run for 15 min at 80 V. RCA sample was sonicated before detection on colorimetric probe-based assay. *(C)* We detected RCA reaction using a colorimetric probe-based assay.

We performed the ligation reaction on total RNA working at the same condition tested before for synthetic RNA. The results were the same as expected: we could not evaluate the circularization of the probe on PAGE and no amplified product was visible for RCA performed on ligation samples (Fig. 3.13). Further studies and investigation for protocol optimization will be necessary to achieve the goal of RCA-based RNA amplification.

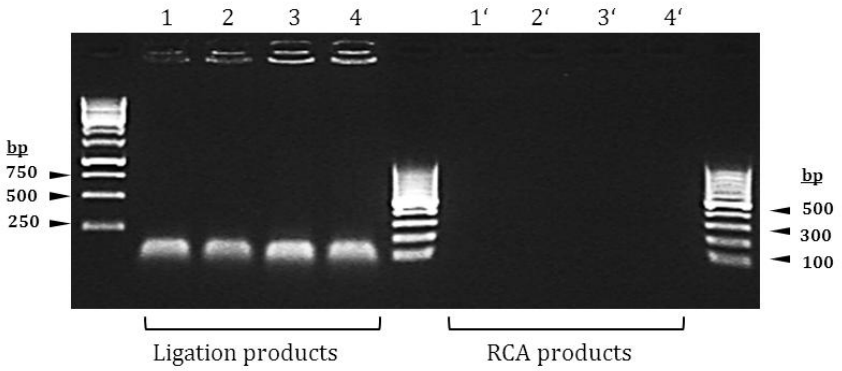


**Fig. 3.13**: Results for ligation of the padlock probe on total RNA (sample 1,2,3 and 4) and result for RCA performed on ligation products (samples 1', 2', 3' and 4'). 1% agarose gel in TBE 0.5X. Gel was run for 15 min at 80 V, stained with ethidium bromide and visualized under UV.

## CONCLUSIONS AND FUTURE PERSPECTIVES

We obtained the proof of principle that RCA can be used to amplify the presence of 16S rRNA molecules in solution. However our protocol must be optimized in several steps. First, we would optimize the ligation reaction on the RNA template, in order to maximize the efficiency of the reaction and isolate the padlock probe in circular form. Ligation time is another critical parameter that should be investigated in the future, towards the application of ligation-mediated rolling circle amplification in RNA biosensors and diagnostic kits. Since our work was focused on testing a strategy that can be applied in a DNA biosensor, we have not evaluated the yield of the ligation reaction with increasing the reaction time. As a matter of fact, in the literature, very long reaction times (up to 8 hours) are reported for this step, which make this strategy incompatible with diagnostic applications. Another parameter that can be considered as a limitation for diagnostic applications of the ligation-mediated rolling circle amplification, is the ratio between probe and template. The experimental evidences collected until now does not allow to exclude that this ratio could be decreased, making this strategy suitable for our purposes.

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# Section II:
# signal amplification strategies

# CHAPTER 4

## HYBRIDIZATION CHAIN REACTION

### INTRODUCTION

When complex laboratory methodologies, such as PCR, should be avoided, then the detection principle should find the sequence of interest amongst few, unlabelled molecules of variable size (the result of cell lysis and of a crude not amplified preparation). In this case, the detection principle should be sensitive to the presence of the analyte molecule itself, or to the presence of a label that is introduced at a second time, in a so-called 'sandwich' strategy. Here, a label (or a labeled oligonucleotide) binds (ideally in a specific way) to the target after it is immobilized on the interface thanks to the specific probe layer. This binding of the second type of molecule produces the signal, and it can *amplify* it, with a suitably designed strategy.

In Chapter 1, several successful examples of signal amplification methods based on the use of reporter molecules, like metal nanoparticles or organic tags, have been reported. DNA nanoassembly as a consequence of target-probe hybridization looks as an easy and versatile strategy that can be used to enhance the signal. If properly designed, a large number of oligonucleotide tiles can bind to the sensor surface playing on base-pairing complementarity. Synthetic DNAs are real nano-bricks which can be customized in several ways and whose assembly leads to the formation of easily detectable structures in two or three dimensions. Furthermore, a wide variety of other materials could be spatially patterned into such structures.

The hybridization chain reaction represents an interesting example of DNA nanoassembly guided by the strand-displacement mechanism. The key of reaction is the storage of potential energy in two hairpin species which are stable in this conformation until a single strand DNA initiator is added in solution.

#### *MOLECULAR MECHANISM*

The hybridization chain reaction (HCR), originally proposed by Dirks and Pierce in 2004[1], is an isothermal, enzyme-free process where a hybridization event triggers the polymerization of oligonucleotides into a long nicked double-strand DNA molecule. The

two kinds of co-monomer oligonucleotides are present in solution as closed hairpins, and are added to the growing polymer only after the initial triggering hybridization event (Fig. 4.1).

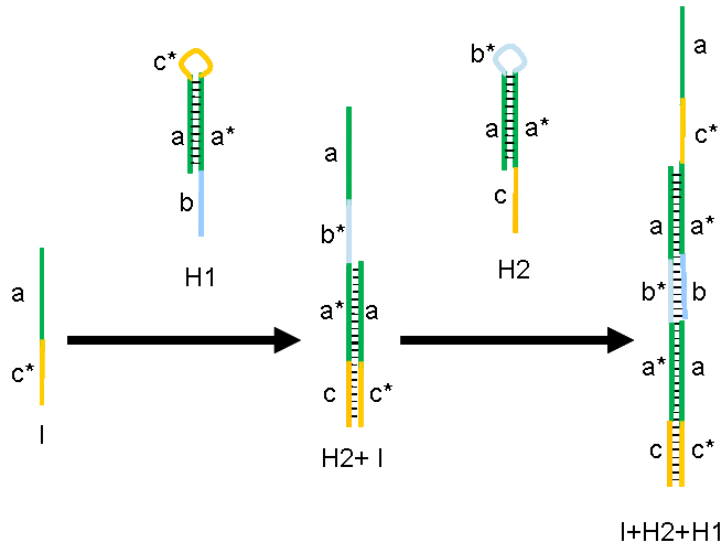


**Fig. 4.1.** Scheme for the first round of solution HCR. Hairpins with a protrusive end are added to an initiator (the target) and opened one after the other. A copolymer of nicked dsDNA with repetitive sequence results from the spontaneous repeated additions.

HCR is a very simple mechanism: it involves two species of DNA oligonucleotide which at room temperature are stable in hairpin conformation with short loop and long stem. The key point of the system is that in such conformation, hairpins are kinetically blocked until an initiator strand is added to the solution. Initiator DNA triggers a cascade of hybridization events during which the hairpins escape from their conformation. Several initiator strands in solution initiate multiple reactions leading to the formation of a set of nicked double helixes of different length. In this way, a few (or even one) initiating hybridization events are amplified thanks to the formation of long double strands molecules which are easy to detect.

### *POTENTIAL APPLICATIONS OF HCR IN BIO-SENSING*

The characteristics of HCR make it an attractive strategy for DNA sensing application. The chemical and physical requirements for the reactions are compatible with the set-up necessary for point-of-care detection. First, the reaction is isothermal. Second, as no enzymes are used, there would be no serious problems with the reagents shelf-life. In a detection assay for a target DNA or RNA, HCR could be used to amplify the results of its presence in solution or to enhance the binding event with a surface capture probe. In solution, HCR leads to the formation of several polymers of different lengths that can be easily visualized with standard end-point detection as gel electrophoresis[1]. In our opinion,

the most promising application for HCR is the use of this mechanism for signal amplification after the hybridization event (Fig. 4.2). When the nucleic acid that starts the polymerization reaction is bound on a surface, then all the grown polymer would co-localize on the surface, thus leading to accumulation of material at the site of hybridization. In a HCR-based sandwich assay, a portion of the target nucleic acid would be recognized by the probe oligonucleotide that would bind it on the surface, while another portion would trigger the opening and polymerization of the hairpins (Fig. 4.2). This reaction, if successful, would lead to accumulation of many oligonucleotides per each bound target molecule, thus to an accumulation of material on the recognition layer. Such accumulation of nucleic acids should lead to signal amplification for a detection method based on fluorescence or chemiluminescence. In these cases hairpins could be designed including a label, like a fluorophore, or a tag for enzyme binding. In alternative, in principle HCR could be used for label-free detection of DNA hybridization in methods measuring the electrical properties of the solid-liquid interface.

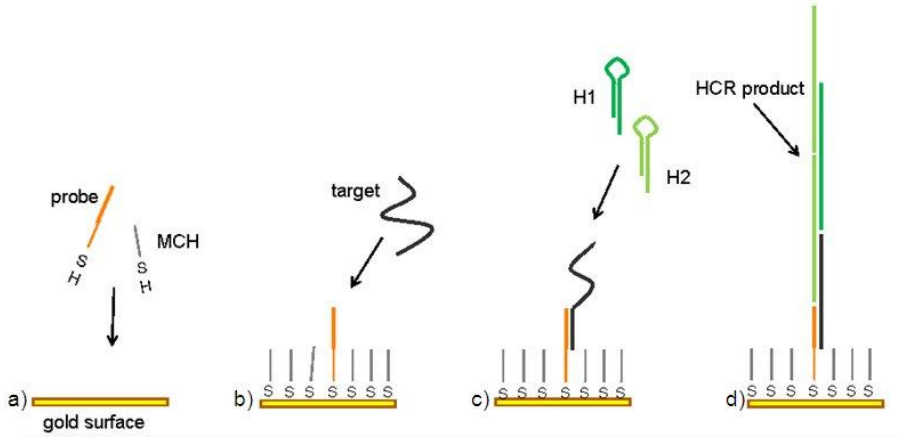


**Fig 2**. Scheme of HCR implementation on surface. After mixed SAM formation on a gold surface (a), target-probe hybridization can take place (b). The target triggers the polymerization of specific hairpins present in solution into a long nicked double strand molecule (c and d) that can be detected in different ways, for example with fluorescence and electrochemical detection.

In the original paper on HCR, it is highlighted that background spontaneous polymerization could occur [1]. This would not be a problem for its implementation in signal amplification, as background amplification would take place in solution and thus washed off during rinsing (or buffer flow in a continuous flow system).

### *LITERATURE OVERVIEW*

Despite the potential applications in nucleic acid detection, the use of hybridization chain reaction and similar mechanisms driven by base pairs complementarity has not

been deeply investigated. For example Niu and coworkers combined HCR with enzyme-enhancement system for DNA detection. In this assay, streptavidin-coated magnetic beads were conjugated with a biotinylated hairpin. The hybridization with the DNA target on the particles opened the structure and lead to the consecutive polymerization of complementary biotinylated hairpins present in solution (Fig. 4.3). Biotin moieties exposed on the formed dsDNA is used as anchor group for the binding of HRP-conjugated streptavidin. The target DNA was then quantified through the fluorescent detection of bi-p,p'-4 hydroxyphenylacetic acid (DBDA) generated from the substrate 4-hydroxyphenylacetic acid (p-HPA) under the catalysis of HRP[2]. The reported amplification factor for the system was ≈30-fold with a limit of detection of 0.8 fM.

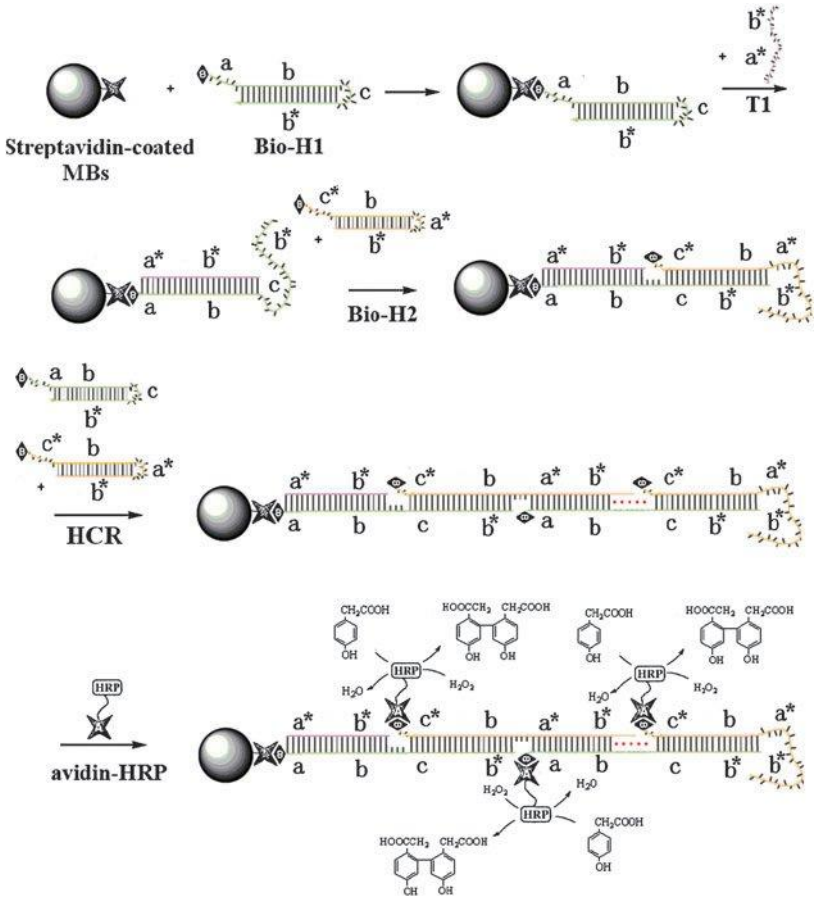


**Fig. 4.3.** Scheme of Niu's HCR system[2]. When the target (T1) is captured by the MBs, the c–b* tail is exposed, leading to the opening of Bio-H2. These two steps represent one cycle of the chain reaction where the target sequences a*–b* were regenerated. After the double helix growth was completed, avidin-HRP was added for the next enzyme-enhanced fluorescence detection.

Very recently, Zheng and coworkers employed another type of surface-initiated DNA polymerization as signal amplification strategy for electrochemical DNA sensors[3]. They used the assembly chain reaction developed in Turberfield's Lab at Cambridge[4] to

amplify ssDNA-binding on gold electrodes performing different types of electrochemical measurements (Fig. 4). Briefly, assembly chain reaction is based on the use of double stranded monomers (M1 and M2) and auxiliary "rubbish collector" strands (R1 and R2). Monomers are involved in a cascade of strands exchange reactions which lead to the formation of a long nicked double strand molecule. Respect to HCR, the resulting linear assemblies does not incorporate sequence which contain regions of self-complementarity (like the hairpins of HCR). The scheme proposed in the original article in showed in Fig. 5. In their article, Zheng and colleagues coupled the system with the use of a redox molecule and showed an amplification factor of 300-fold for target hybridization[3].

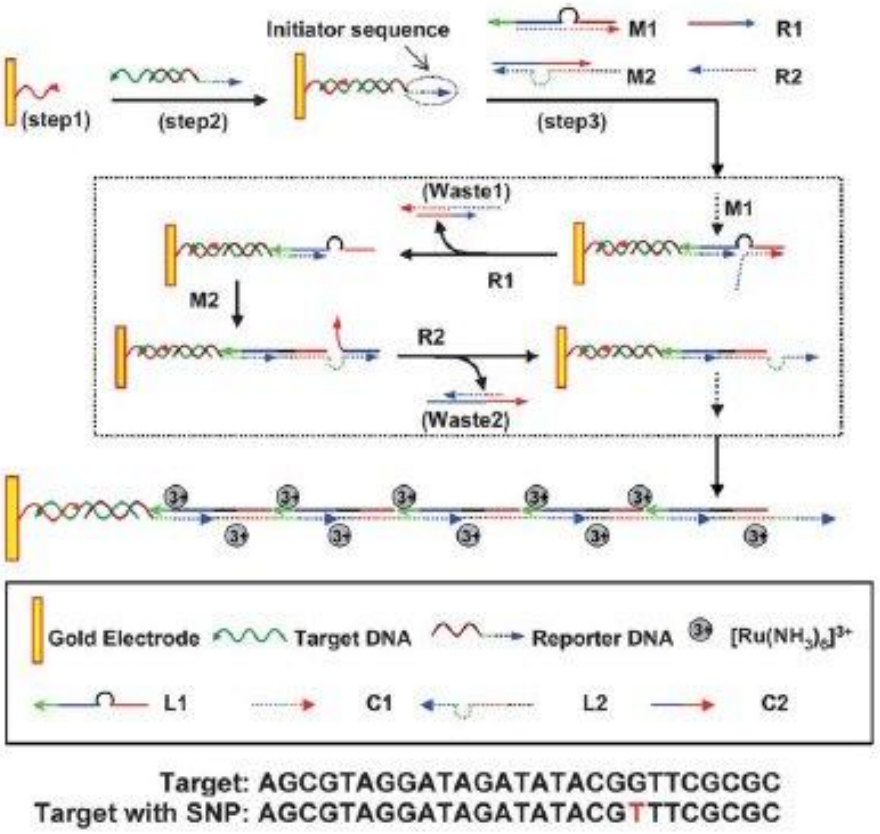


**Fig. 4.4**. DNA self-assembly on surface can be used as enzyme-free strategy for signal amplification[3]. The authors reported a detection limit of 5 pM under not fully optimized conditions. Picture reproduced from Zheng *et al.*[3].

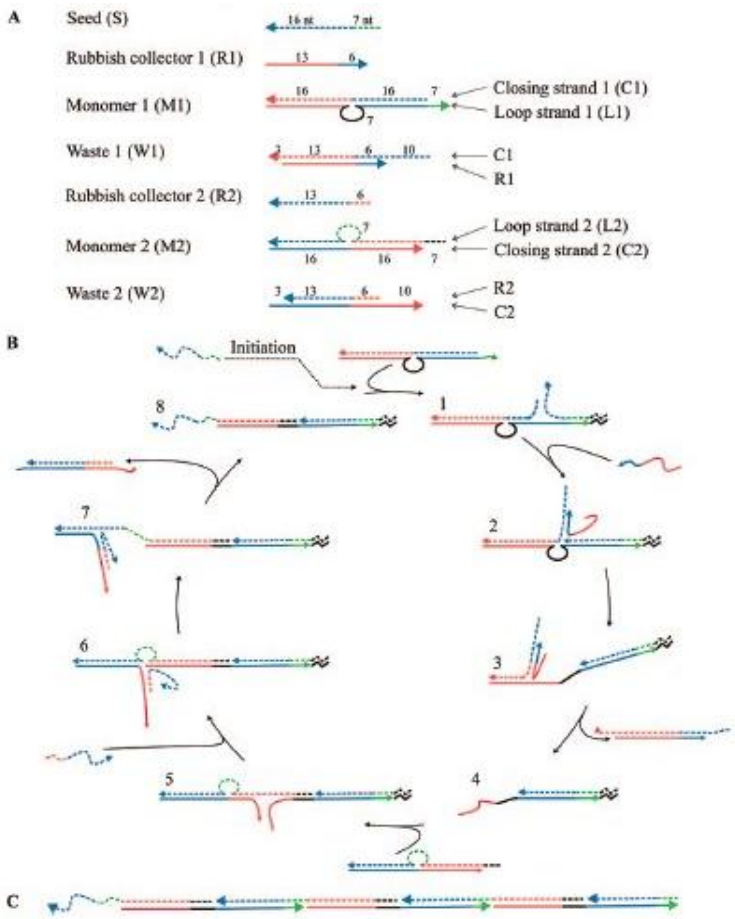


**Fig. 4.5**. Scheme of the assembly chain reaction. Picture taken from the original article of Lubrich *et al.* [4].

*REASONS FOR THE CHOICE OF THIS STRATEGY*

When the nucleic acid that starts the polymerization reaction (target) is bound on a surface, then all the grown polymer would co-localize on the surface, thus leading to accumulation of material at the site of hybridization. In a 'sandwich assay', a portion of the target nucleic acid would be recognized by the probe oligo nucleotide and bind it on the surface, while another portion would trigger the opening and polymerization of the hairpin co-monomers.

Hybridization chain reaction, if successful, would lead to accumulation of many oligonucleotides per each bound target molecule, thus to an accumulation of material on the recognition layer.

Hybridization chain reaction looks perfectly compatible with the set-up of portable biosensors: no strict temperature control should be needed; there would be no serious preservation problems, as DNA reagents can be stored for longer time than enzymes; no contamination should occur and the accumulation of nucleic acids should lead to signal amplification suitable for different detection methods, also label-free. Finally overall reaction cost should be kept down. For this reason, the hybridization chain reaction appears as a very promising candidate for signal amplification in nucleic acid biosensors.

## MATERIALS AND METHODS

### *MATERIALS*

Oligonucleotides sequences were purchased from Eurofins MWG Operon (Germany). They were resuspended in ultrapure water to 100 µM final concentration. All chemical reagents were purchased from Sigma-Aldrich (USA).

### *HYBRIDIZATION CHAIN REACTION IN SOLUTION*

For standard hybridization chain reaction conditions, hairpin and target-initiator oligonucleotides were diluted in NaCl 500 mM, $Na_2HPO_4$ 50 mM (pH 7.8 at 25°C) to 3 µM final concentration. Hairpins solution were incubated at 95°C for 2 min and cooled slowly at room temperature for 1 hour. 9 µl of each species (target, H1 and H2) were mixed together and incubated for 1 hour at room temperature. 10 µl of reaction volume were loaded on native 10% polyacrylamide gel in TBE 1X and run at 80 V for 1 h.

### *PREPARATION OF TEMPLATE STRIPPED GOLD SURFACES (TSG)*

A piece of freshly-cleaved muscovite mica of the area of approximately 2 $cm^2$ (RubyRed Mica Sheets, Electron Microscopy Sciences, USA) was used as substrate for thermal deposition of gold in a high vacuum pump according to the protocol proposed by Hegner and coworkers[5,6]. Briefly, gold (Electron Microscopy Sciences, 99.99 %) was deposited onto freshly cleaved mica substrates in a high-vacuum evaporator (Edwards, UK) at ~$10^{-6}$ Torr or less. Before deposition, the mica was preheated to 300°C for 2 hours by a heating stage mounted behind it to enhance the formation of terraced Au(111) domains[5,6]. The thickness of the gold films resulting was around 200 nm. These films were fixed to a glass substrate with EPO-TEK 377 (Epoxy Tech,Billerica, USA). They were then separated at the gold–mica interface by peeling immediately before functionalization with the mixed SAM. This procedure produced gold substrates with a flat surface morphology due to the templating effect of the atomically flat mica surface[5,6].

### *THIOL-MODIFIED PROBE REDUCTION*

Thiol-modified probes were supplied from Eurofins MWG Operon in the non-protected form. In this arrangement the probability to form oligo dimer trough the disulfide linkage was higher. Thiol-modified oligonucleotides were resuspended in 50 mM Tris(2-carboxyethyl)phosphine hydrochloride acqueous solution (TCEP, Sigma-Aldrich) and incubated at room temperature for 1 hour. 100 µl of the solution were loaded on ultrafiltration column for desalting (Microcon YM-100, Millipore). Purified oligo was then quantified measuring absorbance value at 260 nM with SmartSpec Plus spectrophotometer (Bio-Rad).

### *PREPARATION OF MIXED SELF-ASSEMBLED MONOLAYERS AND HYBRIDIZATION CHAIN SOLUTION ON SURFACE*

10 µl of 3 µM thiolated probe solution in 500 mM NaCl, 50 mM $Na_2HPO_4$ (pH 7.8 at 25°C) were deposited on a freshly prepared template stripped gold surface (TSG) and immediately covered with another one (a TSG "sandwich" was formed). Solution was incubated for more than 2 hour in humid chamber. TSG were then washed with 2 ml of fresh buffer and 10 µl of 1 mM of 6-mercapto-1-hexanol (Sigma-Aldrich) were incubated using the same "sandwich" strategy for 1 hour. Surfaces were washed again with 2 ml of fresh buffer and 300 µl of target-initiator oligo at different concentrations in 500 mM NaCl, 50 mM $Na_2HPO_4$, SDS 7 %. Surfaces were washed with 2 ml of fresh buffer to remove the excess of non-hybridized target. 5 µl of 10 µM fluorescent labeled hairpin H1 solution and 5 µl of 10 µM hairpin H2 solution were pipetted on functionalized TSG and covered with Hybri-slip (Sigma-Aldrich). Hairpin solutions were previously incubated at 95°C for 2 min and cooled slowly at room temperature for 1 hour. Hybridization chain reaction was performed for 1 hour on surface. The excess of not hybridized hairpins was removed by washing with 2 ml of fresh buffer. Detachment of HCR products from the surface was achieved incubating overnight surfaces with 20 µl of 50 mM 2-Mercaptoethanol (Sigma-Aldrich) in a humid chamber. Samples were collected, diluted in a final volume 2ml of NaCl 500 mM, $Na_2HPO_4$ 50 mM (pH 7.8 at 25°C) and heated for 2 min at 95°C before measuring fluorescence. Fluorescence measurements were performed using quartz cuvettes (Hellma) with LB-50 fluorescence spectrometer (Perkin Elmer).

# RESULTS AND DISCUSSION

## *SEQUENCE DESIGN*

Hairpin and sequence design are a critical point for hybridization chain reaction. The key of the system is the storage of potential energy in the two hairpin species which are stable in this conformation until a single strand DNA initiator (target) is added to the mixture. The initiator opens the hairpin of one species, exposing a new single strand region which opens the hairpin of the other species. This process, in turn, exposes a single-stranded region identical to the original initiator. Since the cascade of hybridization events can be triggered only if the first hairpin exhibits a single strand sequence complementary to the target, each hairpin is formed by a loop, a stem and a single strand tail, as depicted in Fig. 4.6.

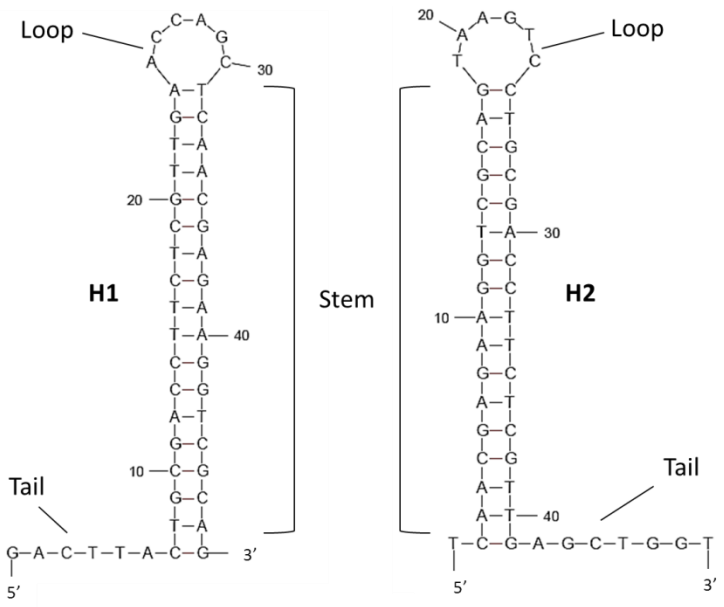


**Fig. 4.6**: Typical structure of HCR hairpins. A long stem keeps them stable in solution.

In a first attempt, we used the basic set of HCR sequences proposed by Dirks and Pierce to perform a series of reaction feasibility experiments. Then we selected a series of target sequences of interest and we designed hairpins specific for them. Since the rules for hybridization chain reaction sequence design are not available in literature, target length and hairpins structure were chosen using the sequences proposed in the original article as a model. The original initiator sequence was a short single strand DNA molecule of 21 nt. It was perfectly matched for its overall length to a portion of the first hairpin including the single strand tail and one filament of the double strand stem. In the HCR mechanism,

using the short tail as trigger, the initiator binds to the hairpin opening it through a strand-displacement mechanism. For our purposes, we chose targets longer than 21 nt adding a portion of variable length potentially able to hybridize with an immobilized DNA probe. *Criptosporidium parvum*, *Giardia lamblia* and Hepatitis E virus are three waterborne pathogen organisms which cause severe diseases. Since in case of suspect water contamination, it is important to achieve a fast response, the development of biosensors for the early diagnosis of these organisms is a relevant topic. The gene for the Cryptosporidium oocyst wall protein (COWP) was chosen as target for detection of *C. parvum*. The *cowp* gene was selected because it codes for a protein that is important in maintaining the integrity of the oocyst wall, allowing the parasite to withstand harsh environmental factors until it is ingested by a new host, and it is unique to the organism. The *β-giardin* gene of *G. lamblia* codes for a structural protein that is a component of the adhesive disk of the parasite and it is unique to the organism. Polyprotein gene of Hepatitis E virus (HEV) is approximately 5 kb long and encodes the four different proteins. This region of the genome is more conserved than other parts of the viral RNA, therefore can be a reliable target sequence of the RT-PCR reactions. Although HEV is positive sense RNA virus, we designed a DNA sequence for it, replacing U bases with T bases. In principle HCR might work also with RNA initiator, but this version has not been investigated in our work. For the selection of HCR targets, we started from the work done by two research group which were our partners in the European Project DINAMICS (Diagnostic Nanotech and Microtech Sensors, Sixth Framework Programs). The colleagues of Lambda GmbH (Rainbach, Austria) and the colleagues of MikroMikoMed Ltd (Budapest, Hungary) designed PCR primers ands probes for the amplification and detection of the aforementioned target sequences. Using a Matlab-written software, we selected a region of 40°nt within each amplicon which showed the lowest likelihood to form secondary structures, as the target must be in the linear form to prime the reaction. In Table 1, the target sequences are reported.

According to the original work proposed by Dirks and Pierce[1], we designed hairpin sequences 48 nt long exhibiting a double helix stem of 17-18 bp, a hairpin loop of 6 nt and an external tail of 6-7 nt. The choice of long stem with a considerable GC-content percentage (≈ 50%) and short loop ensures stable hairpins. Hairpins for *C. parvum*, *G. lamblia* and HEV were generated starting from the target sequences using the software NANEV[7]. Sequences with the lowest probability to form homo- and heterodimers were finally selected. In Table 4.1, the complete list of designed sequences is reported. In black, target sequences and hairpin region with sequence identical to the target. In blue, target region complementary to the probe. In green hairpin sequences complementary to each others.

| **Table 4.1**: sequences for hybridization chain reaction system | | |
|---|---|---|
| **Basic sequences**[3] | | |
| 5'-CAAAGTAGTCTAGGATTCGGCGTG-3' | Initiator | 24 nt |
| 5'-CTACGCTCGACCCAAAGTAGTCTAGGATTCGGCGTG-3'[4] | Target | 36 nt |
| 3'-TCATCAGATCCTAAGCCGCACAATTGGGTGCGGCTTAGGATCTGA-5' | H1 | 45 nt |
| 5'-TTAACCCACGCCGAATCCTAGACTCAAAGTAGTCTAGGATTCGGC-3' | H2 | 45 nt |
| **Biological sequences** | | |
| *C. parvum* | | |
| 5'-ATTTCATTTTACAAGGCCTCCAATGTATACAAACTAGTTC-3' | Target_P | 40 nt |
| 3'-GGAGGTTACATATGTTTGATCAAGGCGGCACTTGATCAAACATATGTA-5' | H1_P | 48 nt |
| 5'-CGCCGTGAACTAGTTTGTATACATCCTCCAATGTATACAAACTAGTTC-3' | H2_P | 48 nt |
| *G. lamblia* | | |
| 5'-AGGAAGAAGATGTACGACCAGCTCAACGAGAAGGTCGCAG-3' | Target_G | 40 nt |
| 3'-TGGTCGAGTTGCTCTTCCAGCGTCCTGAATGAGCCTGGAAGAGCAACT-3' | H1_G | 48 nt |
| 5'-GACTTACTGCGACCTTCTCGTTGAACCAGCTCAACGAGAAGGTCGCAG-3' | H2_G | 48 nt |
| *Hepatitis E Virus* | | |
| 5'-GCGGCTGTGGCCGCAGCAAAGGCATCCATGGTGTTTGAGA-3' | Target_H | 40 nt |
| 3'-GTTTCCGTAGGTACCACAAACTCTCGCGCTAGAGTTTGTGGTACCTAC-5' | H1_H | 48 nt |
| 5'-GCGCGATCTCAAACACCATGGATGCAAAGGCATCCATGGTGTTTGAGA-3' | H2_H | 48 nt |

[3] Sequences originally proposed by Dirks and Pierce, 2004.
[4] Target sequence was designed modifying the initiator of the original article.

## Effect of initiator length on HCR

We investigated the feasibility to use a sequence longer than the original initiator sequence to trigger HCR. Our final goal was to implement HCR on the surface after the binding of the target to an immobilized probe, thus we would demonstrate that our longer target sequence could prompt the assembly of DNA in standard reaction conditions, as previously described (500mM NaCl/50 mM $Na_2HPO_4$, 1 h of incubation at room temperature)[1]. In a first attempt, we performed HCR using the basic set of sequences proposed by Dirks and Pierce[1] comparing the use of two initiator with different length (see Table 1). We found that the initiator sequence with a protruding tail (36 nt) triggered HCR effectively (Fig.4 7). The HCR reaction product was a set of nicked double-stranded molecules showing a discrete set of molecular weights (lane 7 and lane 9). Compared with the short initiator sequence of 21 nt (lane 8), the former prompted the formation of longer HCR products. Moreover we demonstrated that HCR took place only if both hairpin species were present in solution (Fig. 4.8).

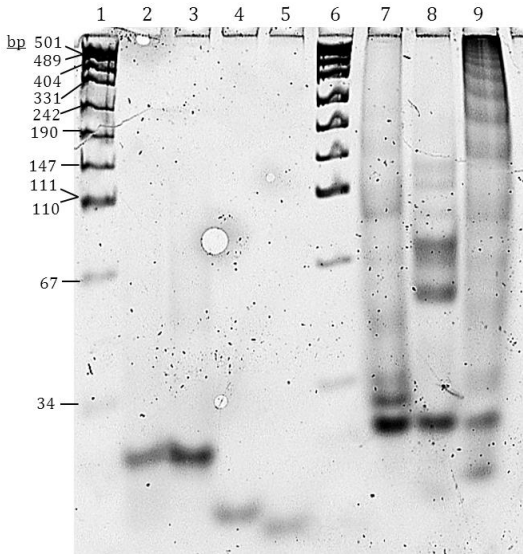


**Fig. 4.7**. Basic HCR system. The presence of a target in solution triggered a cascade of hybridization events leading to the formation of high molecular weight DNA molecules. Lane 1: pUC19/MspI DNA marker. Lane 2 and 3: H1 and H2 (48 nt). Lane 4: 36 nt initiator filaments. Lane 5: 24 nt initiator filament. Lane 6: pUC19/MspI DNA marker. Lane 7: HCR triggered with 36 nt initiator, H1_fluo. Lane 8: HCR triggered with 21 nt initiator. Lane 9: HCR triggered with 36 nt initiator. Concentration of each species in solution was 1 μM. 10% native page in TBE 1X. Gel was run at 8 V/cm for 1 hour in TBE 1X, stained with Sybr Gold and visualized under UV.

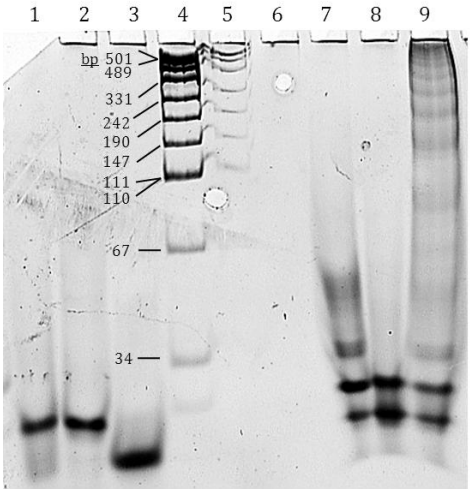


**Fig 4.8**. Basic HCR system. A complete polymerization reaction starts only when both hairpins are present in solution with the initiator. If only H1 was present in solution with target, the formation of a complex between target and hairpin was visible (lane 7). No interaction between H2 and the target were detectable (lane 8). Lane 9: in presence of both hairpin and target, high molecular weight products were produced. Lane 1: H1. Lane 2: H2. Lane 3: target. Lane 4: pUC19/MspI DNA marker. Lane 5 and 6: empty. 10% native PAGE in TBE 1X. Gel was run at 8 V/cm for 1 hour in TBE 1X, stained with Sybr Gold and visualized under UV.

## Effect of temperature and saline concentration on HCR

In the original work, it is reported that the hybridization chain reaction takes place at room temperature while in nucleic acid detection it might be convenient to work under high stringency conditions, in order to reduce unspecific interactions between probe and target. We performed HCR at three temperatures, 20°C, 37°C and 55°C, but we didn't detect any significant differences in the reaction products (Fig. 4.10). We assumed that the duplex was properly assembled as a consequence of the hybridization events cascade between target and hairpins.

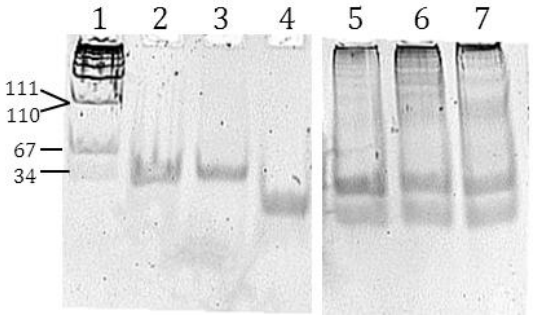


**Fig. 4.10**. Effect of temperature on HCR. Lane 1: pUC19/MspI DNA marker. Lane 2: H1. Lane 3: H2, Lane 4: target. Lane 5: 20°C HCR. Lane 6: 37°C HCR. Lane 7: 55°C HCR. Hairpin and target were heated to 95°C for 2 min and then allowed to cool to room temperature for 1 h before use. The concentration of each species in solution was 1 μM. 15% native PAGE in TBE 1X. Gel was run at 8 V/cm for 1 hour in TBE 1X, stained with Sybr Gold and visualized under UV.

Changing the salt concentration had a strong effect on HCR. HCR performed using different NaCl concentration revealed that a high salt concentration was necessary to stabilize the reaction product (Fig. 4.11).

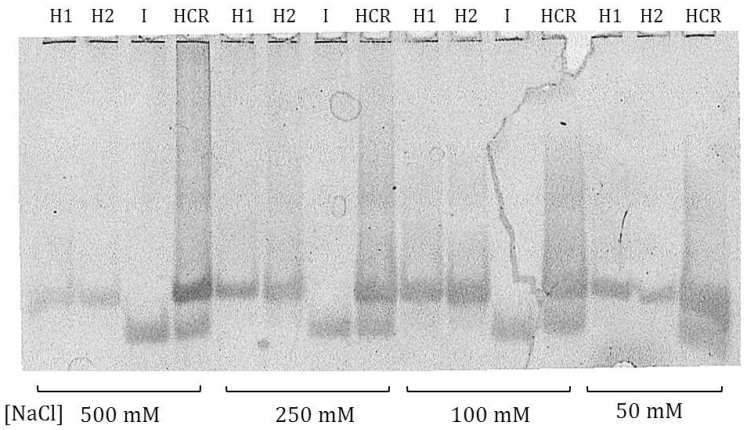


**Fig. 4.11.** Effect of saline concentration on HCR reaction. At high stringency, 50 mM NaCl in hybridization buffer, the formation of high molecular weigh products is inhibited. At higher saline concentrations, we observed a broad smear, due probably to the random formation of double strand molecules of different length. Hairpin and target were heated to 95°C for 2 min and then allowed to cool to room temperature for 1 h before use. The concentration of each species in solution was 1 μM. 10% native PAGE in TBE 1X. Gel was run at 8 V/cm for 1 hour in TBE 1X, stained with Sybr Gold and visualized under UV.

## HCR on sequences of biological interest

As previously described, we designed hairpins sequences for target of real pathogens as *G. lambia*, HEV and *C. parvum* (Fig. 4.12). Reaction was performed in standard HCR conditions as previously described[1]. The typical HCR pattern was visible for all the reaction. This demonstrated that HCR can work as a general amplification reaction since suitable hairpins can be designed for different target sequences.

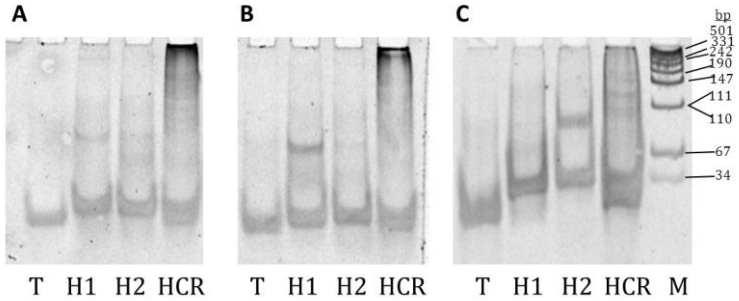


**Fig. 4.12**. HCR worked in solution using as trigger a real pathogen DNA sequence. (A) *G. lamblia*, (B) HEV, (C) *C. parvum*. Hairpins and target were heated to 95°C for 2 min and then allowed to cool to room temperature for 1 h before use. The concentration of each species in solution was 1 μM. 10% native PAGE in TBE 1X. Gel was run at 8 V/cm for 1 hour in TBE 1X, stained with Sybr Gold and visualized under UV.

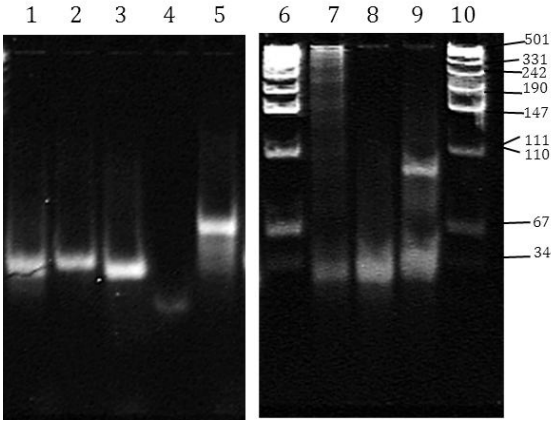


**Fig. 4.13**. Intermediates of hybridization chain reaction performed on *C. parvum* target. Lane 1: H1_P; lane 2: H2_P; lane 3: Target; lane 4: probe; lane 5: target+probe; lane 6: pUC19/MspI DNA marker; lane 7: HCR; lane 8: target+H2; lane 9: target+H1; lane 10: pUC19/MspI DNA marker. 10% native PAGE in TBE 1X. Gel was run at 8 V/cm for 1 hour in TBE 1X, stained with Sybr Gold and visualized under UV.

## Effect of target orientation on reaction triggering – Preliminary studies for sequence implementation

In the ideal implementation HCR would be used without pre-amplification step but directly on the raw cellular lysate (or on purified DNA). In both cases, the probability to detect the sense or the antisense filament is the same. For this reason we investigated the effect of target orientation on trigger efficiency of HCR We designed 2 set of hairpin sequences specific for the *Cryptosporidium parvum* oocyst wall protein (cowp) gene: one set for the amplification of the target hybridized with a 5'-end tiolated probe (Fig 4.14A) and one set for the amplification on the complementary sequence hybridized with a 3'-end tiolated probe (Fig. 14B). In both cases, HCR worked in solution with the same efficiency. We demonstrated also that HCR was triggered by a few amount of target in solution (lane 3 of the gels, hairpins/target=10:1).

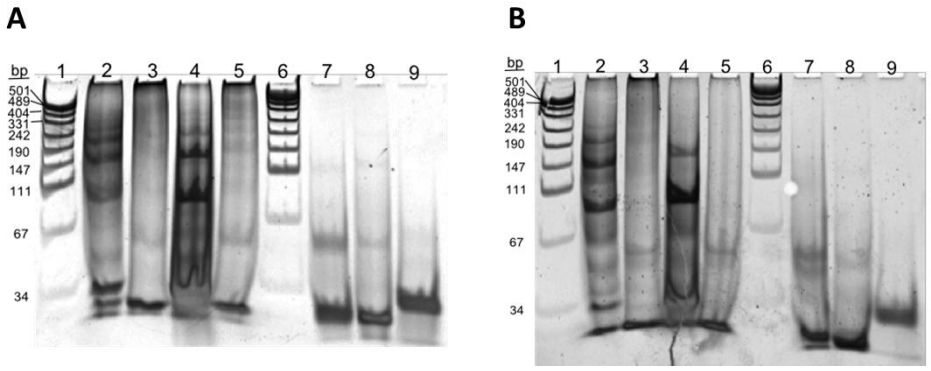


**Fig. 4.14**: For both panel: Lane 1: pUC19/MspI DNA marker. Lane 2. HCR, hairpins/target=1:1. Lane 3:HCR, hairpins/target=10:1. Lane 4: HCR, hairpin/target=1:10. Lane 5: H1+H2. Lane 6: pUC19/MspI DNA marker. Lane 7: H1_P. Lane 8: H2_P. Lane 9: target. Hairpins and target were heated to 95°C for 2 min and then allowed to cool to room temperature for 1 h before use. 10% native PAGE in TBE 1X. Gel was run at 8 V/cm for 1 hour in TBE 1X, stained with Sybr Gold and visualized under UV.

### *IMPLEMENTATION OF HCR ON THE SURFACE*

We implemented HCR on a self-assembled monolayer (SAM) of thiolated probes on gold. The probes were single-stranded DNA oligonucleotides with a thiol moiety at the 3'-end (or 5'-end in an alternative setup), a spacer sequence of poly-T and the sequence complementary to the target (HCR initiator). As described in the Sequence Design section of this chapter, the initiators sequences were designed adding a tail complementary to the probe to the sequence complementary to one hairpin. We prepare the SAM as suggested by Herne and Tarlov[8] with some modifications. In this procedure, the thiolated oligonucleotides (in a high salt buffer) is deposited on the surface and incubated for a sufficient time (in our experimental condition ≥2 hour) to adsorb to the metal surface through the thiol-group. Then another type of thiolated molecule is deposited in order to control the probe density and to make the layer suitable for hybridization. With this method, it has been estimated that the final oligonucleotide concentration on the surface is $\approx 1\times10^{12}$[8]. The density of the probe can strongly influence the hybridization event since it has been reported that high probe density can decrease hybridization efficiency[9]. Applying the describe functionalization protocol, Herne and Tarlov observed that a fluorescent target hybridized with high efficiency to the probe. Since our idea was to evaluate HCR amplification factor by means of fluorescence measurements, the use of this protocol seemed appropriate.

The scheme for HCR implementation on surface is reported in Fig. 4.15. A target labeled with 5-carboxytetramethylrhodamine fluorophore (5-TAMRA, ex. 546 nm, em. 574 nm) was hybridized on the mixed SAM. Then, we performed the amplification reaction exposing the bound target to a solution containing the hairpin mixture. In our

setup, the hairpin that was opened by the target was labeled with 5-carboxyfluorescein (5-FAM, ex. 495 nm, em. 520 nm). After the reaction, target and HCR reaction products were detached from the gold surface reducing the thiol-group by means of 2-Mercaptoethanol. The amplification factor was evaluated for different target concentration calculating the ratio between the number of fluorescent hairpin molecules/$cm^2$ and the number of target molecules/$cm^2$. Results are summarized in Fig. 4.15. We obtained the evidence that HCR properly worked on the surface and we calculated an amplification factor of 10-fold. Considering a density probe layer of $\approx 1\times10^{12}$ molecules/$cm^2$, we estimated a yield of hybridization of ≈15%. Even though the reaction conditions could be further optimized, the amplification ratios seem far from what would be needed for the detection of a small number of cells/viruses without preliminary PCR amplification. Fluorescence measurements gave information on the absolute number of molecules that were immobilized on the surface. As both the target and one of the hairpins were fluorescently labeled we could determine the number of immobilized target molecules per unit/surface area and compare them with the number of immobilized hairpin molecules. In none of the experimental conditions we tried, we could measure a number of immobilized hairpin molecules larger than 20 hairpin hairpin (10 fluorescent and 10 non fluorescent that should go together) for each target molecule. The HCR reactions were conducted for 1 hour, a reaction time considered as the longest that could be feasibly applied in a point-of-care sensor.

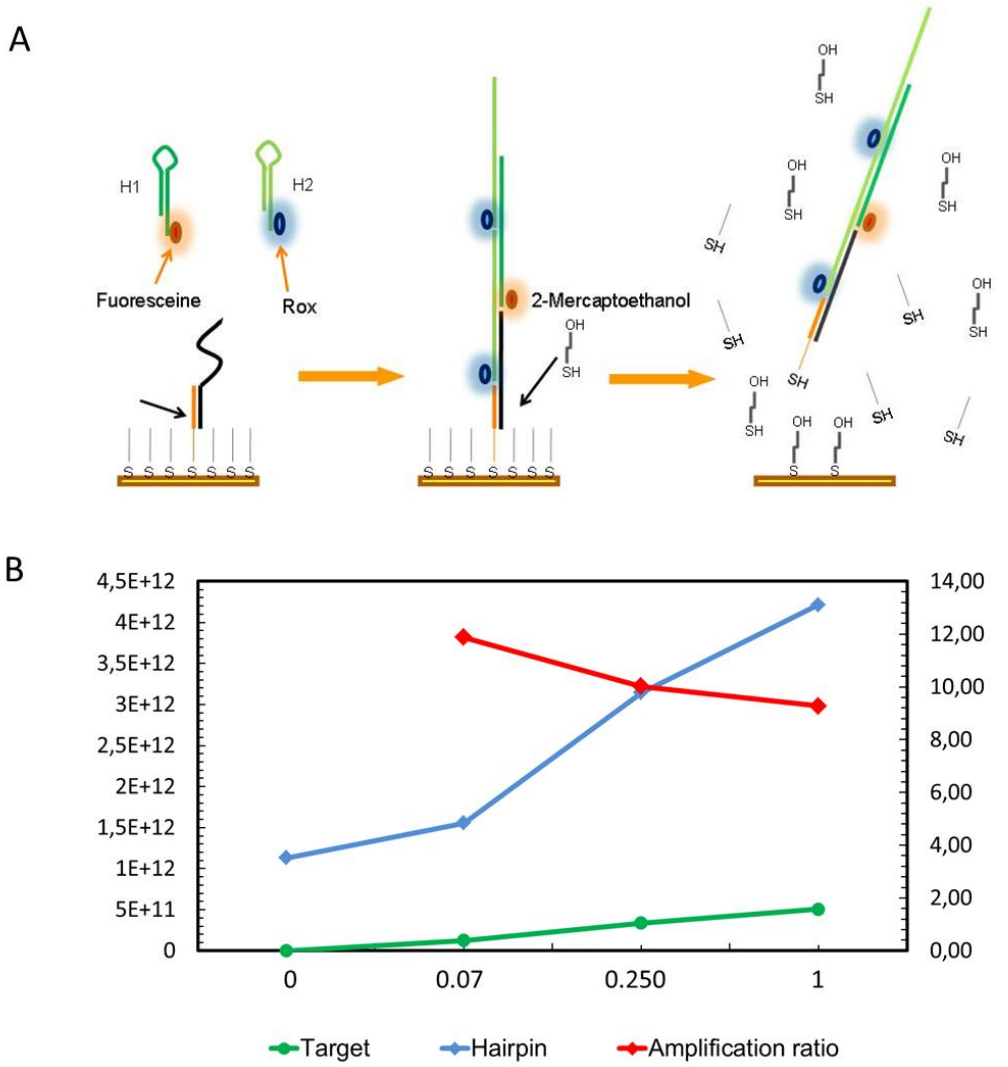


**Fig 4.15**. (A) Scheme of HCR implementation on surface. After mixed-SAM formation on a gold surface, target-probe hybridization takes place. The target triggers the polymerization of specific hairpins present in solution into a long nicked double strand that can be detected in different ways. In our laboratory we tested fluorescence detection. (B) The amplification ratio shows the improvement with respect the fluorescence reading of the labelled hybridized target.

## CONCLUSIONS AND FUTURE PERSPECTIVES

This work provides proof-of-concept that HCR can occur in different experimental setup. Experiments were performed in solution, to evidence that the HCR reaction was working as expected, and on surfaces. Target sequences belonging to different organism were chosen as target and in all the cases the reaction was successfully tested in solution. The results on surface implementation were studied by measuring the fluorescence emission of fluorescently labeled target DNA and hairpin oligonucleotides.

Interestingly, despite we found that HCR worked when the target/initiator was immobilized on a surface and that the conditions adopted in our experiments were effective, we calculated a maximum amplification ratio of 10, which looks far from what could be sufficient for biosensing applications.

Although hybridization chain reaction apparently looks simple, the full detection pipeline for a nucleic acid sensor is complex and includes several steps which can contributes to an overall decreased reaction efficiency. The main contribution is the exposure of the probe-target layer to successive hybridization events. This process could be quite complex since it includes several reagents and reactions which take place consecutively leading to the formation of long and weak structures. This implies that different thermodynamic and kinetic steps will rule each reaction, so that a less than quantitative yield can be expected for each step. As it is often the case, thermodynamics and kinetics can be two totally different situations. Even if each reaction can be set to take place with a favourable thermodynamic constant, it is often the rate of reaction that will make it efficient or not. As a solid-liquid interface is involved, steric hindrance might affect the reaction rates significantly. Furthermore, close-packing of oligonucleotides in a layer would definitely worsen the thermodynamics of the interaction as well: hybridization in a packed layer would mean a likely increase of the packing, so it is expected that a denser layer will provide with a lower hybridization yield. On the other hand, a less dense layer will probably immobilize a lower overall quantity of target molecules. Some complex optimization steps are surely necessary in order to obtain the maximum overall yield. A considerable increase in reaction time could improve the overall magnification factor but could render the strategy not compatible with the short detection time required by biosensing application. In this hypothesis, the coupling of the sensor surface with a microfluidic system looks as forced choice. As a matter of fact, the hairpins supply in solution could be depleted during reaction moving equilibrium towards the disassembly of the structure.

In each case, our results are not so far from the previously published results for signal amplification by means of HCR. For example Niu and colleagues showed an

amplification factor of 30, but they implemented HCR on nanoparticles, probably improving reagent diffusion rate and forcing less steric hindrance[2]. On the other hand, Lin and colleagues reported that, using HCR as signal amplification strategy, they improved sensitivity for a biological target (SARS viral DNA) by two order of magnitude compared with a detection performed without signal amplification[10].

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# CHAPTER 5

# TERMINAL DEOXYNUCLEOTIDIL TRANSFERASE MEDIATED SIGNAL AMPLIFICATION

## INTRODUCTION

In the research field and on the market of biosensors, the signal amplification strategies based on the use of enzymes are widely utilized. Their success is due to their effectiveness and large choice of versions. Amongst the class of enzymatic strategies only a limited number of them rely on the controlled growth of DNA after the target binding event. For example, Phi29 polymerase catalyzes rolling circle amplification directly at the surface, thus accumulating DNA mass that can be easily detect (see Chapter 1). This approach looks convenient since it can be coupled with label-free biosensors like mass sensors or electrochemical sensors (see Chapter 1). Like Phi29 polymerase, also terminal deoxynucleotidil transferase (TdT) is a DNA polymerase with interesting properties that could find space in nucleic acids detection.

### *TERMINAL DEOXYNUCLEOTIDIL TRANSFERASE REACTION AS ISOTHERMAL SIGNAL AMPLIFICATION STRATEGY*

Terminal deoxynucleotidil transferase (TdT), often simply called terminal transferase, is an unusual DNA polymerase which exhibits the unique ability to incorporate nucleotides in a template-independent manner using only single-stranded DNA as nucleic acid substrate. For this reason it does not require a primer to initiate the reaction. In nature, terminal transferase is expressed only in some specific type of mammalian cells like calf thymus cells[1], leukemic cells from children with acute lymphoblastic leukemia[2] and bone marrow cells[3]. The biological role of terminal transferase is the random addition of nucleotides to single-stranded DNA during V(D)J recombination, thus generating immunological diversity[4,5].

The biochemical and structural properties of terminal transferase are established[6,7]. It is a monomeric protein with a molecular weight of 60,000 Da[8] which is able to initiate elongation starting from at least a three-nucleotide long sequence with a free 5′-phosphate end and a free 3′-hydroxyl end for extension[9]. In addition terminal transferase exhibits the unique ability to perform *de novo* synthesis of polynucleotides ranging in size from 2- to 15-mers when provided with dNTPs in the absence of a primer[10].

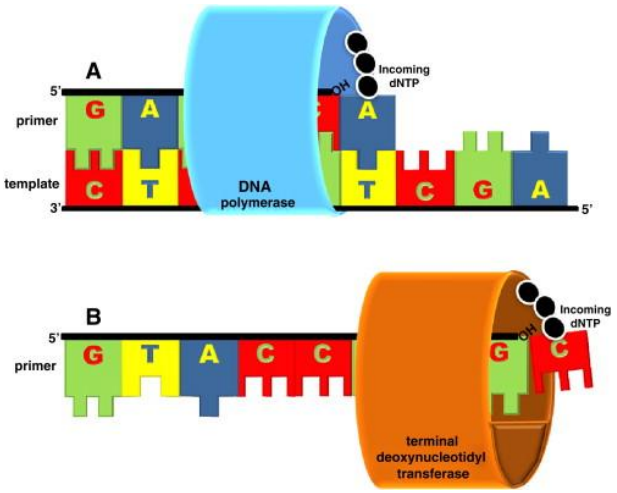


**Fig 5.1**. Scheme of the template-independent polymerization catalyzed from the terminal transferase (at the bottom). The differences with the standard primer-dependent polymerization catalyzed by the other DNA polymerases (at the top) are highlighted. Picture reproduced from Motea et al.[11].

Terminal transferase, as the others DNA polymerases, necessitates divalent metal ions for catalysis[12]. Therefore it can use a variety of divalent cations such as $Co^{2+}$, $Mn^{2+}$, $Zn^{2+}$and $Mg^{2+}$ and the efficacy to incorporate purines or pyrimidines is dependent from the type of metal cofactor present in the reaction mix[13]. For example, $Mg^{2+}$ facilitates the preferential utilization of dGTP and dATP whereas $Co^{2+}$ increases the catalytic polymerization efficiency of the pyrimidines, dCTP and dTTP[13].

## *TDT AS A BIOCHEMICAL TOOL*

Thanks to its interesting properties terminal transferase finds a number of applications in molecular biology. For example it is used for labeling the 3'- ends of DNA *in vivo* and *in vitro*. Usually the substrate for this reaction is a fragment of DNA generated by digestion with a restriction enzyme that leaves a 3' overhang, but oligodeoxynucleotides can also be used. When such DNA is incubated with tagged

nucleotides and terminal transferase, a string of the tagged nucleotides will be added to the 3' overhang or to the 3' end of the oligonucleotide. TdT can also utilize a broad variety of nucleotide analogs. TUNEL assay (*T*dT-mediated dUTP-biotin *n*ick *e*nd-*l*abeling) is a widely used method for the detection of cell apoptosis. It is based on the ability of TdT to efficiently incorporate biotinylated dUMP on to 3′-ends of single-stranded DNA that occur at the sites of DNA breaks. The incorporated biotin dUMP is easily visualized by fluorescently labeled avidin or streptavidin and allows for direct conformation and quantitative measurements of the number and location of DNA breaks. In alternative, radioactive nucleotide or a variety of fluorescent probes can be incorporated in a DNA filament by TdT catalytic activity. Another application of terminal transferase is the rapid amplification of cDNA ends (RACE), a technique used to investigate the sequence of a RNA transcript.

### *TDT AS NANOTECHNOLOGICAL TOOL*

Recently terminal transferase has found applications in the field of nanobiotechnology. Surface initiated polymerization (SIP) is a nanotechnological technique used to synthetize polymers directly on the surface. SIP is a technique of great interest for surface modifications as an alternative to conventional approaches in which polymers are grafted from the solution on the surface. As a matter of facts, several different mechanisms have been proposed for the controlled growth of polymers[14]. In surface initiated enzymatic polymerization (SIEP), a number of polymers can be synthesized by means of different enzymes [15-17]. Terminal deoxynucleotidyl transferase has been reported as catalyst for the controlled growth of DNA layers on gold[18,19]. Chows and coworkers demonstrated for the first time the ability of TdT to catalyze DNA polymerization on the surface starting from a layer of trigger oligos which exposed their 3'-OH ends at the surface/solution interface. They showed that TdT can work on the surface, generating a DNA layer whose thickness depends on the dNTP monomer used[19]. In addition, they combined TdT reaction with a well-known surface patterning technique based on microfluidic-channel in order to create spatially ordered DNA nano-features.

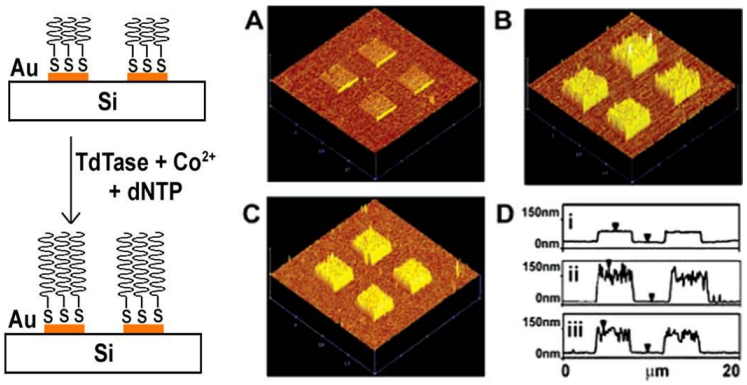


**Fig. 5.2**. Picture taken from two articles of Chow *et al.*[18,19]. On the left, a scheme of the approach used by the authors to growth DNA on a surface. A monolayer of thiolated oligos has been patterned on gold trough the S-Au bond. TdT, in presence of a metal cofactor and dNTPs, catalyze the polymeration of the monomers. On the right, AFM characterization of the formed layer. In order to regulate the length of the filaments and thus the height of the layer they introduced the use of DNase digestion (panel D)[18].

### *REASONS FOR THE CHOICE OF THIS STRATEGY*

For the above-mentioned reasons, terminal transferase appeared as a good candidate for signal amplification. As it grows oligonucleotides, it can be used to extend immobilized oligonucleotides on the surface, thus leading to the accumulation of material where the right free 3'-OH terminus is found. If the oligonucleotides probe layer only exposes a 5'-OH terminus, then growth is not possible until a target binds to the probes and gets immobilized. Once a polynucleotide with a free 3'-OH end gets immobilized, then TdT in solution, in presence of dNTPs and of the appropriate cofactors, can promote the growth of such molecule, accumulating more ssDNA close to the surface.

As mentioned in the previous paragraph, TdT incorporates dNTPs randomly with some preferences (also dependent on the cation cofactor). It is so possible to grow homopolymers or heteropolymers. A random copolymer grown on the surface will fold on itself upon formation, leading to a somewhat compact three-dimensional shape with also the possibility of binding intercalating labels (fluorescent of redox-active). A grown homopolymer (like poly-T) could be secondarily targeted by an oligonucleotides label (like fluorescently tagged oligo-A) to achieve a poly-labeled nanostructure with a more controlled average shape.

This chapter focuses on the proof of concept that TdT can be used as amplification strategy to enhance the binding event between a DNA target molecule and a DNA probe bound on surface.

## MATERIALS AND METHODS

### *MATERIALS*

Oligonucleotide sequences were purchased from Eurofin MWG Operon (Germany). Terminal deoxynucleotidil transferase from calf thymus 20 U/µl was supplied by Sigma-Aldrich (USA). All chemical reagents were purchased from Sigma-Aldrich (USA) with the exception of $CoCl_2$ supplied by Carlo Erba Reagenti (France). Muscovite mica and ultrapure gold wire were purchased respectively from Electron Microscopy Sciences (USA) and Alfa Aesar – Johnson Matthey (England). Sybr Gold was supplied by Molecular Probes® – Invitrogen (USA).

### *TDT MEDIATED POLYMERIZATION IN SOLUTION*

The reaction in solution took place as follow: 10 pmoles of 3'-OH free oligo and 1 µl of 100 mM dNTPs mix solution were combined in 20 mM Tris-Acetate, 50 mM potassium acetate and 10 mM magnesium acetate (pH 7.9 at 25°C). 20 U of TdT were added in a final volume of 50 µl. Sample was incubated for 1 h at 37°C and then for 10 min at 70°C to inactivate the enzyme. Reaction product was detected by electrophoresis on native 6% polyacrylamide gel in TAE $Mg^{2+}$ 1X and run for 50 min at 8 V/cm. Gel was stained with Sybr Gold and visualized under UV.

### *PREPARATION OF TEMPLATE STRIPPED GOLD SURFACES (TSG)*

A piece of freshly-cleaved muscovite mica of the area of approximately 2 $cm^2$ (RubyRed Mica Sheets, Electron Microscopy Sciences, USA) was used as substrate for thermal deposition of gold in a high vacuum pump according to the protocol proposed by Hegner and coworkers[20,21]. Briefly, gold (Alfa Aesar, 99.99 %) was deposited onto freshly cleaved mica substrates in a high-vacuum evaporator (Edwards, UK) at ~$10^{-6}$ Torr or less. Before deposition, the mica was preheated to 300 °C for 2 hours by a heating stage mounted behind it to enhance the formation of terraced Au(111) domains [20,21]. The thickness of the gold films resulting was around 200 nm. These films were fixed to a glass substrate with EPO-TEK 377 (Epoxy Tech, Billerica, USA). They were then separated at the gold–mica interface by peeling immediately before functionalization with the mixed SAM. This procedure produced gold substrates with a flat surface morphology due to the templating effect of the atomically flat mica surface [20,21].

### *THIOL-MODIFIED PROBE REDUCTION AND PREPARATION OF MIXED ASSEMBLED MONOLAYERS*

Thiol-modified probes were supplied from Eurofins MWG Operon in the non-protected form. In this arrangement, the probability to form oligo dimer through the

disulfide linkage was higher. For TdT reaction on surface 3'-end thiol-modified oligonucleotides were resuspended in 50 mM Tris(2-carboxyethyl) phosphine hydrochloride aqueous solution (TCEP, Sigma-Aldrich) and incubated at room temperature for 1 hour. 100 µl of the solution were loaded on ultrafiltration column for desalting (Microcon YM-100, Millipore). The purified oligo was then quantified measuring absorbance value at 260 nM with SmartSpec Plus spectrophotometer (Bio-Rad). 30 µl of 10 µM thiolated probe solution in 20 mM Tris-Acetate, 50 mM potassium acetate and 10 mM magnesium acetate buffer (pH 7.9 at 25°C) were deposited on a freshly prepared template stripped gold surface (TSG) and immediately covered with another one (a "sandwich" of TSG was formed). Solution was incubated overnight in humid chamber. TSG were then washed with 2 ml of fresh buffer and 10 µl of 1 mM of 6-mercapto-1-hexanol (Sigma-Aldrich) were incubated using the same "sandwich" strategy. Then 10 µl of 1 µM target solution in reaction buffer was incubated for 2 hours. The excess of target was removed by washing with fresh reaction buffer.

### *TdT MEDIATED POLYMERIZATION ON GOLD SURFACE*

60 U of TdT and 1 mM dTTP were mixed in 100 µl of 20 mM Tris-Acetate, 50 mM potassium acetate and 10 mM magnesium acetate and 0.4 mM $CoCl_2$ (pH 7.9 at 25°C). The entire volume was incubated for 2 hours at 37°C on the probe coated gold surface. Enzyme was removed by washing carefully with 2 ml of the same fresh buffer. Reaction product was evaluated after detachment from the surface by means of gel electrophoresis and fluorescence measurement. For detachment, 20 µl of 50 mM 2-Mercaptoethanol (Sigma-Aldrich) were incubated overnight on the surface covered by Hybri-slips foil (Sigma-Aldrich). 10% of reaction product was loaded on native 8% polyacrylamide gel in TAE $Mg^{2+}$ 1X and run for 45 min at 8 V/cm. The rest of the volume was mixed with 1 µl of 1:10000 Sybr Gold stock solution (Invitrogen) and diluted in a final volume 2 ml of 20 mM Tris-Acetate, 50 mM potassium acetate, 10 mM magnesium acetate and 0.4 mM $CoCl_2$. Fluorescence measurements were performed using quartz cuvette (Hellma) with LB-50 fluorescence spectrometer (Perkin Elmer).

## RESULTS AND DISCUSSIONS

### *TERMINAL TRANSFERASE REACTION OPTIMIZATION: TESTS IN SOLUTION.*

To investigate the best reaction conditions, some preliminary tests were performed in solution. Terminal transferase catalyzes the incorporation of dNTPs at the free 3′-hydroxyl end of ssDNA in presence of metal ions. It is known that the efficiency of polymerization and the bias toward purines and pyrimidines can be significantly affected by the identity of the metal ion bound to TdT. In particular, it is reported that purines are incorporated faster in the presence of $Mg^{2+}$, while pyrimidines are the favorite substrate in presence of $Co^{2+}$, as reported by Fowler and colleagues[6]. Since $Mg^{2+}$ ion was present in the enzyme reaction buffer, we evaluated the effect of the addition $Co^{2+}$ ions to the reaction mix. The presence of $Co^{2+}$ in solution caused the formation of longer DNA filaments, probably favoring the incorporation of dTTP and dCTP otherwise failed to be incorporated in presence of only $Mg^{2+}$ (See Fig. 5.3). It is interesting to know that while the incorporation rate of purines is the same both in the presence of $Mg^{2+}$ and $Co^{2+}$ ions, the incorporation rate of pyrimidine is increased by a factor 10 in the presence of the latter [6].

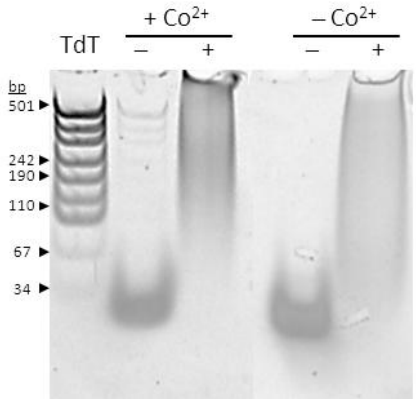


**Fig. 5.3**. The presence of $CoCl_2$ in the reaction mix favored the formation of long DNA filament. (1) DNA ladder (pUC19 DNA/MspI, Fermentas). (2) and (3) 3′-OH end ssDNA without and with terminal transferase addition in presence of $CoCl_2$. (4) and (5) 3′-OH end ssDNA without and with terminal transferase addition without $CoCl_2$ in solution. 6% polyacrylamide gel in TAE $Mg^{2+}$ 1X; run 8 V/cm for 50 min.

We tested the unique ability of terminal transferase to add dNTPs solely to the free 3'-OH end single strand DNA. Modified oligonucleotides with a thiol group at the 5'-end or 3'-end were used as alternative substrate for the reaction. The reaction took place only if the substrate showed the thiol group at the 5'-end, thus with the free 3'-OH end. (Fig. 5.4, lane 7 and 8). In addition, we noted that the efficiency of dNTPs incorporation was higher for substrate with free 3'-OH end and with a 5'-phophate than for template

with the 5'-end modified. As it can be seen in Fig. 5.4 (lane 9), high molecular weight reaction products were obtained when the enzyme was incubated with a suitable substrate not modified at the 5'-end. The sequences of DNA templates used for the experiments are listed in Table 5.1

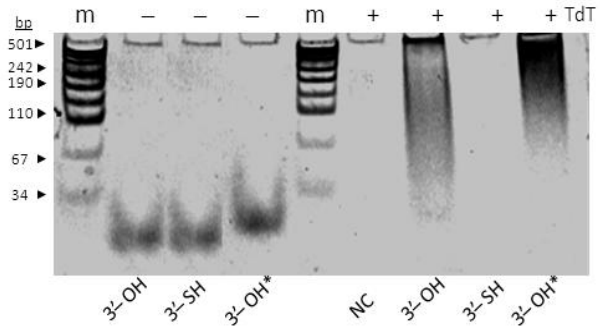


**Fig. 5.4**. Terminal transferase incorporates dNTPs only at free 3'-OH of a single strand. (1) DNA ladder, pUC19 DNA/MspI (Fermentas). (2) Template 1, free 3'-OH end, 5'-SH end. (3) Template 2, 3'-SH end. (4) Template 1, free 3'-OH end, 5'-phophate end. (5) DNA ladder, pUC19 DNA/MspI (Fermentas). (6) Negative control (NC), no template. (7) Terminal transferase reaction product on template 1. (8) Terminal transferase reaction product on template 2. (9) Terminal transferase reaction product on template 3. 6% polyacrylamide gel in TAE $Mg^{2+}$ 1X. Gel was run at 8 V/cm for 50 min, stained with Sybr Gold and visualized under UV.

| **Table 5.1: template sequences** | |
|---|---|
| **Sequence** | **Modification** |
| **T1:** TCAGGCTCGCGG | 5'-SH |
| **T2:** GGTCGAGCGTAG | 3'-SH |
| **T3:** CTACGCTCGACCCAAAGTAGTCTAGGATTCGGCGTG | none |
| **Target:** ATTTCATTTTACAAGGCCTCCAATGTATACAAACTAGTTC | none |
| **Probe:** CCTTGTAAAATGAAATTTTT | 3'-SH |

From the literature, it is known that terminal transferase can synthesize homopolymeric or heteropolymeric tails with different efficacy. This phenomenon depends not only on the type of metal cation present in solution, but also on the type of nucleotide which must be incorporated. For example, it is known that dATP and dTTP homopolymerization usually proceed well, leading to the formation of long molecules that can represent more that 90% of the total reaction product. On the contrary, dCTP polymerizations are somewhat limited due to the formation of double-strand interactions in the products. Finally, dGTP polymerization usually stops after about 25 nucleotides are added due to the aggregation of the product. We tested the efficacy of incorporation of

dTTP with respect to dNTPs mix. We found that in the former case, the total reaction product is enriched by a fraction of single-stranded oligo showing a long homopolymeric tail. As it can be seen in Fig. 5.5, the average molecular weight of the products resulting from the reaction conducted in the presence of dTTP was higher than that resulting from the dNTPs mix.

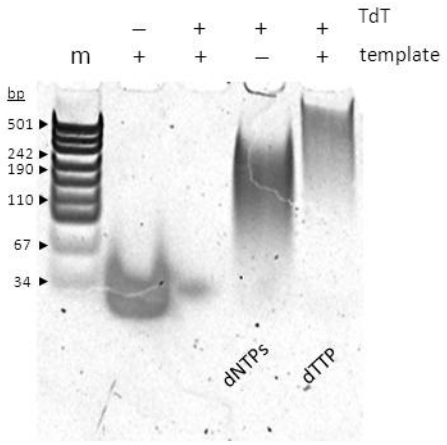


**Fig. 5.5**. TTP incorporation on single stranded DNA by means of terminal transferase led to the formation of long DNA filaments that can be easily visualized on a gel. Result of 6% polyacrylamide gel in TAE $Mg^{2+}$ 1X; run 8 V/cm for 50 min. Gel was stained with Sybr Gold and visualized under UV.

Since terminal transferase can incorporate different types of nucleotides, it could add also ribonucleotides to a DNA filament. In the literature, it is reported that the enzyme prefers deoxynucleotide substrates. Ribonucleotides, arabinonucleotides and 3'-deoxynucleotides are also used as substrates but often at considerably lower rates[22]. Towards a possible application of TdT as an amplification method for DNA and RNA biosensors, we investigated the versatility of the reaction verifying if TdT could incorporate with a comparable efficiency dATP or ATP. In Fig. 5.6, the results of the experiment are reported. For the reaction performed in presence of the ribonucleotide substrate, we couldn't observe the typical broad smear band on the gel resulting from the single strand DNA with different molecular weight. We concluded that in our experimental conditions the reaction did not take place in presence of ATP, as the reaction time needed to be limited. This is probably due to the notably slower incorporation rate of this type of nucleotides by means of TdT.

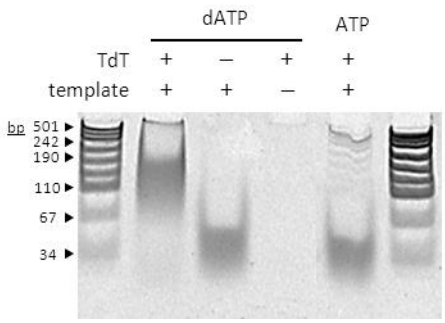


**Fig. 5.6.** Terminal transferase is not able to add NTP to the free 3'-OH end of a single strand DNA template. 6% polyacrylamide gel in TAE $Mg^{2+}$ 1X; run 8 V/cm for 50 min.

### *TERMINAL TRANSFERASE REACTION ON THE SURFACE*

A number of tests in solution showed that the best conditions for enzymatic reactions had been found. Solution simulations of the surface growth gave positive results: in these cases, oligonucleotides with either the 3'-OH or the 5'-OH chemically blocked towards growth showed a correct behavior when reacted with TdT and dNTPs in presence of $CoCl_2$. Our idea was to evaluate TdT reaction yield on surface and thus the amplification of the probe-analyte recognition event, monitoring the amount of fluorescent dye that can bind to the homopolymeric tails of TTP generated on the target. Since the gold surface was functionalized with a mixed assembled monolayer of 3'-SH DNA probes and 2-Mercaptoethanol (MCH), the hybridized target exposed to the liquid interface a free 3'-OH, triggering the surface initiated polymerization of TTP by means of the enzyme present in solution. A scheme of the proposed amplification strategy is depicted in Fig. 5 7.The utilization of terminal transferase implied temperature control in order to give optimal yield (as reaction time needed to be limited). The enzyme works ideally at 37°C. The inherent complexity of the reaction and the necessary cycles of washes made the study more difficult.

We choose the detachment of the final reaction product from the surface as end-point detection method for terminal transferase mediated amplification. Since the probe layer was formed by means of SH-Au bond, we used 2-Mercaptoethanol to reduce the thiol-group in order to remove from the surface the probe and everything that was bound to it. As proof of the concept of the method we performed an electrophoresis experiment in order to visualize on the gel the reaction product.

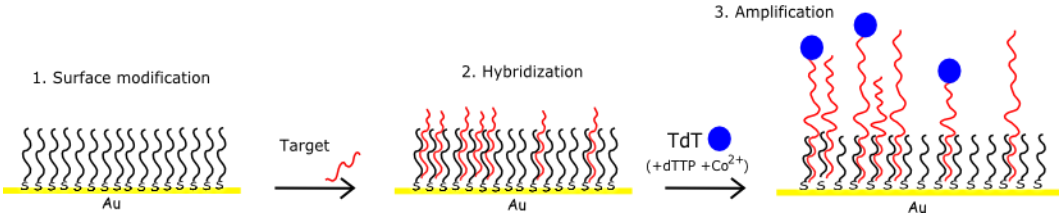


**Fig. 5.7**. Scheme of the proposed amplification strategy. After the hybridization with a probe bound to the Au surface via its 5'-SH end, the target exposes the free 3'-OH at the surface/solution interface. In presence of $Co^{2+}$ and dTTP the terminal transferase present in solution should catalyze the incorporation of the monomer leading to the formation of long polyT-tails and thus to the accumulation of material. Such poly-T tails are suitable to be detected in different ways, like single strand intercalating labels or secondary labeled oligonucleotide like fluorescently tagged oligo-A.

On the gel, a clearly visible broad smear is present for the detached reaction product and a band whose length was compatible with the double-stranded probe-target (Fig. 5.8). Nevertheless, this type of detection was not suitable to give a quantitative response. In addition, the electrophoretic run was biased both from the presence of MCH in solution which interfere with the migration both from the small quantity of DNA that was present on the surface without the TdT-amplification.

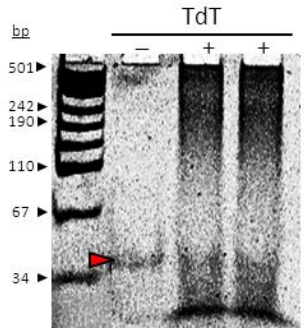


**Fig. 5.8**. Polyacrylamide gel electrophoresis detection of the TdT reaction product after detachment. Gel stained with Sybr Gold at the end of the run. The red arrow in lane 2 indicates the band corresponding to the duplex formed by the probe and the target. In lane 3 and 4 the reaction product of TdT amplification after detachment from the surface. Due to its large volume, the sample was split in two wells. DNA ladder in lane 1 is pUC19 DNA/MspI (Fermentas).

We used Sybr Gold as a fluorescent dye to label the ssDNA growth on the surface after detachment. Sybr Gold belongs to the cyanine dye family. Binding DNA (both ssDNA than dsDNA) it enhances its fluorescence 1000-fold. Comparisons of Sybr-gold emission efficiency on the target molecules before and after TdT reaction showed only a moderate increase of signal due to TdT mediated dTTP incorporation. No more than 5-fold binding was measured after TdT mediated amplification (Fig. 5.9).

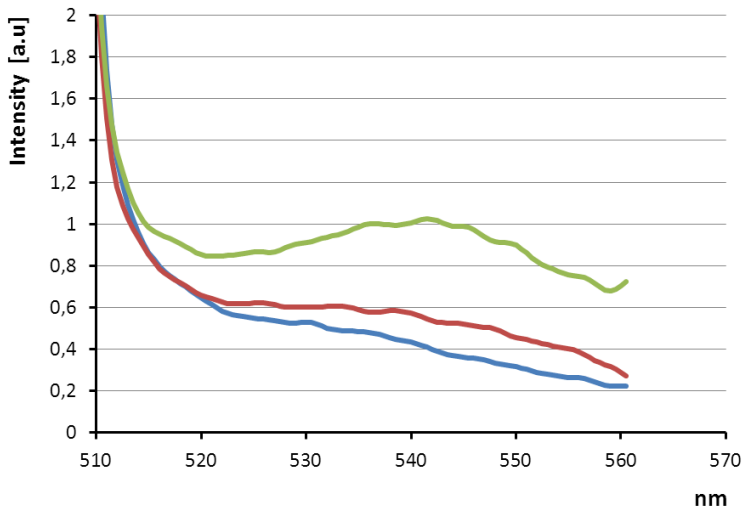


**Fig. 5.9**. Fluorescence spectra of Sybr Gold displaced from the surface of the electrodes after target hybridization prior (red) and after TdT amplification (green). The blue spectrum indicates a blank reading without target and TdT. The amplification factor estimated here (amplify – blank)/(no amplify – blank) is approximately 5. Excitation and emission peaks of Sybr Gold are respectively 495 nm and 537 nm.

A magnification factor of 5-fold could not be sufficient to confirm TdT-mediated elongation as a valuable strategy for signal amplification. However protocol optimization and the use of instrumentation devoted to fluorescence measurement on the surface, could improve the results of some order or magnitude.

In alternative to Sybr Gold, staining with fluorescent-polyA oligo labeling was attempted. Since the terminal transferase reaction product was constituted from long poly-T tails, the fluorescence emission of poly-A reporter should be proportional to the length of the tails. Despite the simple principle at the base, the results were different from those expected. The presence of a poly-T spacer in each probe increased strongly the background, and decreased the signal/noise ratio in an unexpected way. Probably the use of probe with an alternative spacer (like poly-A itself) combined with a more stringent reaction conditions could markedly improve the result.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Terminal deoxynucleotidil transferase is a versatile biochemical tool. Thanks to its unique property to initiate DNA polymerization without a primer, it finds a number of applications both in molecular biology and in nanobiotechnology. We demonstrated that terminal transferase can catalyze the polymerization of deoxynucleotides starting from the free-3'-OH end of a DNA target molecule hybridized to a surface probe. The reaction could potentially use as strategy for signal amplification, leading to the accumulation of DNA on the biosensor surface and thus facilitating the detection of the hybridization event. With our protocols we found that the signal after the enzymatic reaction was 5-higher than the not-amplified signal. The magnification factor could be enhanced coupling the method to a more sensitive and reliable detection technique like SPR. As a matter of fact the detachment of the reaction product before detection could bias the measurement because of a loss of material and its successive dilution in cuvette for the measure. Although the possible improvement, the amplification mediated by terminal transferase reaction is claimed to be an *aspecific* strategy for signal enhancement. Since the enzyme works randomly incorporating dNTPs to any free 3'-OH end available on the surface sensor, a not specific hybridization event should be amplified in the same manner. Coupling of TdT mediated elongation with another step able to lend specificity to the reaction (like the use of blocked-probe and a ligation reaction) could motivate the use of this strategy in nucleic acid detection.

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# CHAPTER 6

## CONCLUSIONS

High sensitivity is a challenge for the use of point-of–care devices in pathogens detection. The development of simple, portable and automatic sensors which can be also highly sensitive, is a relevant research topic.

In the case of nucleic acids biosensors, the problem is classically tackled with a pre-amplification step of the DNA (or RNA) sample by means of PCR. Although PCR is an unquestionably powerful and versatile tool, its integration in portable biosensors exhibits some disadvantages. The main difficulty is the integration of the apparatus for thermal cycling in a miniaturized device.

The use of isothermal reactions for amplifying the analyte or for the amplification of the binding event between analyte and probe, represents a solution to overcome the above-mentioned trouble.

The goal of this work was to evaluate the effectiveness and the possible integration of four isothermal strategies in point-of-care devices for DNA (and RNA) detection.

An isothermal reaction for DNA amplification must exhibit an alternative mechanism to thermal denaturation for double helix melting or an alternative route to the accumulation of the target amplicon. The recombinase polymerase amplification (RPA) and the rolling circle amplification (RCA) are two isothermal processes characterized by interesting properties.

RPA is based on the recombination between homologous DNA filaments. It mimics the replication machinery of T4 bacteriophage on which recombinase and polymerase act as catalysers. In RPA, the double helix is mechanically open by a strand displacement reaction which takes place at the same temperature of primer elongation. The overall process occurs between 37°C and 41°C, no heating is necessary even for primer annealing. The use of lower reaction temperatures and longer primer than those used in PCR focus the attention on the topic of reaction specificity. As a matter of fact, in the literature it is reported that UvsX catalyzes a strand invasion reaction where a considerable mismatch percentage is tolerated{Birkenkamp, 1996 #13} and that as few as two mismatches out of 30 possible base-pairs significantly reduces the kinetics of the strand displacement phase{Lee, 2006 #188}. In this work, it has been demonstrated that the introduction of some mismatches at the 3'-end of primers were not

sufficient to discriminate between two sequences with a high level of homology. It can be concluded that for RPA the choice of unique target sequences and the design of perfectly matched primers are important. It has been also demonstrated that RPA is an extremely fast reaction since it has been found that 100 CFU of target DNA are amplified in less than 15 minutes. The RPA reaction product has been detected on a point-of-care colorimetric platform which will be commercialized by Greiner-Bio-One in the next future.

RCA has been tested in this work as strategy for RNA amplification. Compared to PCR and RPA, the reaction product is not an amplicon of the target, but a long tandem repeat of a circular probe complementary to the analyte. In this work, the probe has been circularized on a RNA target by means of T4 DNA ligase and then used by Phi29 polymerase as template starting from a DNA primer. On the whole, despite ligation and elongation take place at constant, physiological temperature, two heating step for primer annealing are required. The main advantage of ligation-mediated RCA is the high specificity of the reaction, since ligation is sensitive to single nucleotide polymorphism. However, the use of L-RCA for RNA amplification is limited by the low ligation efficiency on this type of template. In this work, it has been found that the entire process needs no less than 3 hours to be completed, a time too long for a possible integration in point-of-care devices.

Signal amplification takes place in a step successive to the binding event between target and probe. In principle, this type of amplification should be easily integrated in point-of-care devices since the sample handling is reduced and fewer reagents are needed.

The key point of hybridization chain reaction (HCR) and terminal deoxynucleotidil transferase(TdT)-mediated amplification as signal amplification strategies is the localized accumulation of DNA as a consequence of the binding event between probe and target. This type of amplification is suitable for a label-free type of detection, which in turn results particularly appropriate for the use in point-of-care devices.

HCR is an enzyme-free reaction based on the guided assembly of DNA bricks and prompted by the hybridization between target and probe. In this work, it has been demonstrated that HCR can be triggered by target DNA sequences of pathogenic organisms and that it can be implemented on the surface. Although its promising features, HCR has not showed a satisfactory yield.

TdT reaction is an isothermal and versatile reaction. In this work it has been demonstrated that TdT can elongate a target bound to a probe and exposing the 3'-

OH end to the solution. As for HCR, also for TdT-mediated amplification, the estimated magnification factor appears quite far from that needed for the exploitation of the strategy. The real limitation of the strategy lies in the lack of specificity of the reaction, since TdT elongate 3'-OH without selectivity between sequences.

In conclusion, amongst the four strategies investigated so far, only RPA looks as a promising strategy to be implemented in point-of-care devices.

## Ringraziamenti

Desidero ringraziare innanzitutto il Professor Bruno Samorì per avermi dato l'opportunità di lavorare nel suo gruppo. Un grazie speciale va al Dott. Giampaolo Zuccheri per l'affetto e la stima incondizionata e per avermi seguito durante tutto il lavoro di ricerca.

I have to switch in English to **acknowledge** the enthusiastic supervision of Dott. Christian Mittermyar during the months of working in Lambda GmbH.

Grazie a tutti i ragazzi del lab per il supporto e il tempo passato assieme: Daniele, Rosita, Simone, Manuele, Marco, Dhruv e Daniel.

Grazie alla mia famiglia, sempre vicina anche se lontana.

Grazie a Luca perché se non ci fosse stato sarebbe stato tutto più difficile.

This research has been supported by a PhD fellowship from the Italian Government and by the EU project DINAMICS FP6 IP 026804-2.