PRODUCTION OF UvrD HELICASE AND MutL RECOMBINANT PROTEINS FOR HELICASE-DEPENDENT ISOTHERMAL AMPLIFICATION

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAA^+	ATPases associated with various cellular activities
APS	ammonium persulfate
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAI	codon adaptation index
*ccd*B	control of cell death
cHDA	circular helicase-dependent amplification
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E. coli	*E. coli*
EDTA	ethylenediaminetetraacetic acid
EXPASY	expert protein analysis system
g	gram
HDA	helicase-dependent amplification
HF	high fidelity
HPLC	high performance liquid chromatography
IDA	iminodiacetic acid
IDT	Integrated DNA Technologies
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-D-thiogalacto pyranoside
kb	kilo base
KCl	potassium chloride
kDa	kilo Dalton
KH_2PO_4	potassium dihydrogen phosphate
LAMP	loop-mediated isothermal amplification of DNA
LB	Luria-Bertani
LBP	modified Luria-Bertani
M	molar
MALDI-TOF/TOF	matrix assisted laser desorption ionization-time of flight/time of flight
MCM	mini chromosome maintenance
$MgCl_2$	magnesium chloride
mHDA	mesophilic helicase-dependent amplification
mL	milli litre
mRNA	messenger RNA
µM	micro molar
µL	micro litre
µg	micro gram
NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
NASBA	nucleic acid sequence-based amplification
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs

ng	nano gram
$NiSO_4$	nickel sulphate
nm	nano metre
NMWL	nominal molecular weight limit
NTA	nitrilotriacetic acid
NTC	non-template control
OD	optical density
OE-PCR	overlap-extension PCR
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMF	peptide mass fingerprint
P. mirabilis	*P. mirabilis*
PVDF	polyvinylidene fluoride
RCA	rolling circle amplification
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
SDA	strand displacement amplification
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SF	superfamily
SMART	signal mediated amplification of RNA technology
SSB	single strand binding protein
ssDNA	single-stranded deoxyribonucleic acid
TB	terrific broth
TBE	tris-borate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
tHDA	thermophilic helicase-dependent amplification
TMA	transcription mediated amplification
U	unit
UTIs	urinary tract infections
UV	ultra violet
V	volt

LIST OF PRESENTATIONS

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Amplification, expression and purification of synthetic UvrD helicase gene

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ABSTRACT

Introduction: Helicase-dependant amplification (HDA) method uses a DNA helicase to unwind double-stranded DNA into two single strands. This enables primers hybridization and subsequent extension of the DNA strand. This method is an isothermal amplification method. Hence, various temperatures provided through the conventional PCR can be omitted. In this study, we applied assembly PCR method to produce synthetic UvrD helicase gene.

Objective: To construct and express the synthetic UvrD helicase gene.

Methods: Sixty pairs of oligonucleotides (oligos), representing both positive and negative strand were designed based on *Escherichia coli* UvrD helicase nucleotide sequence retrieved from NCBI website. The oligos were assembled and assembled product was used as template to synthesize the full length helicase gene. The synthetic gene was ligated into pET28a vector and expressed in BL21 (DE3) expression host. Expressed protein was purified by nickel charged affinity column.

Results & Discussion: Full length synthetic UvrD helicase gene with ~2.1 kb nucleotides was successfully amplified, and cloned into pET28a vector. The recombinant protein was expressed with the induction of 1.0 mM IPTG for 3 hours at 37°C. The nickel Immuno metal-affinity chromatography was performed to purify the Histidine-tag UvrD protein. The protein was detected through western blotting and showed a single specific band at size ~75 kDa.

Conclusion: Synthetic UvrD helicase gene was successfully amplified, expressed and purified to be used as an enzyme in HDA method.

PRODUKSI UvrD HELICASE DAN PROTIN MutL UNTUK AMPLIFIKASI ISOTERMA BERGANTUNG KEPADA HELICASE

ABSTRAK

Teknik amplifikasi isoterma bergantung kepada helicase merupakan sebuah teknik alternatif untuk amplifikasi gen, selain daripada penyahaslian haba oleh PCR. Teknik ini menggunakan helicase DNA untuk meleraikan bebenang DNA berkembar kepada bebenang tunggal secara enzimatik. Teknik ini dapat dilaksanakan dengan satu suhu yang tetap, jesteru itu mengabaikan kegunaan mesin PCR. Dua helicase DNA daripada *Escherichia coli* dan *Proteus mirabilis* telah dihasilkan melalui kajian ini untuk kegunaan dalam teknik isoterma tersebut, khususnya pada suhu bilik bagi memastikan teknik tersebut tidak melibatkan sebarang mesin menjana suhu. Gen *uvrD* helicase daripada *E. coli* telah dihasilkan melalui teknik PCR himpunan. Sebanyak 120 fragmen DNA telah direka cipta berdasarkan jujukan gen *uvrD E. coli* strain K-12 yang diperoleh dari National Center for Biotechnology Information, USA. Fragmen-fragmen DNA tersebut dibahagikan kepada tiga bahagian dan dihimpunkan asing. Produk himpunan kemudian digunakan sebagai templat untuk diamplifikasikan kepada blok 1, blok 2 dan blok 3 dengan menggunakan fragment DNA terluar blok masing-masing sebagai primer. Blok-blok tersebut telah diklonkan ke dalam vektor pCR^{TM}-Blunt sebelum dihantar ke analisis jujukan DNA. Mutasi dalam setiap blok telah diperbetulkan dan blok-blok tersebut dicantumkan bagi menghasilkan gen *E.coli uvrD* yang lengkap dan diklonkan balik dalam vector pengekspresan pET28a (+). Sebaliknya, gen *uvrD* helicase *P. mirabilis* telah diamplifikasikan dengan menggunakan DNA genom bakteria tersebut. Gen tersebut juga diklonkan dalam vektor pCR^{TM}-Blunt, jujukan

gen dianalisis dan gen itu diklonkan balik dalam vector pengekspresan pET28a (+). Protin MutL dari *E. coli*, sebuah protin tambahan yang diperlukan dalam teknik isoterma bergantung kepada helicase juga diekpres dalam kajian ini menggunakan gen *mutL* yang dibeli. Ketiga-tiga protin tersebut diekspres melalui BL21 (DE3) pada suhu 25°C, 200 rpm selama 16 jam tanpa IPTG. Protin-protin tersebut yang mempunyai his-tag dipurifikasi dengan teknik purifikasi IMAC yang menggunakan ion-ion Nikel. Protin-protin yang telah dipurifikasi ditukar buffer dan dikenalpasti dengan gel SDS-PAGE dan pemblotan Western. Kehadiran jalur pada pemblotan Western dengan berat molekul lebih kurang 82 kDa menunjukkan pengekspresan protein UvrD manakala 68 kDa menunjukkan pengekspresan protein MutL. Pengenalpastian yang lebih lanjut telah dilakukan dengan analisis MALDI-TOF/TOF dan mata-mata yang diperolehi padan dengan protin-protin target. Teknik amplifikasi isoterma bergantung kepada helicase menunjukkan UvrD helicase dari *E. coli* telah berjaya mengamplifikasi produk DNA bersaiz 120 bp pada suhu 30.0^{o}C, 37.0^{o}C dan 42.0^{o}C manakala UvrD helicase dari *P. mirabilis* berfungsi dengan baik pada suhu 37.0^{o}C dan 42.0^{o}C. Amplifikasi pada 25.0^{o}C ataupun suhu bilik telah diubahsuai dengan meningkatkan konsentrasi kedua-dua helicase. Sebanyak 3.0 µg/mL UvrD helicase *E. coli* dan sebanyak 10.0 µg/mL UvrD helicase *P. mirabilis* telah diperlukan untuk mengamplifikasi produk DNA bersaiz 120 bp pada suhu bilik. Pada keseluruhan, dua helicase DNA yang berfungsi baik dalam teknik isoterma bergantung kepada helicase telah berjaya dihasilkan melalui kajian ini.

PRODUCTION OF UvrD HELICASE AND MutL RECOMBINANT PROTEINS FOR HELICASE-DEPENDENT ISOTHERMAL AMPLIFICATION

ABSTRACT

Helicase-dependent amplification (HDA) method is an alternative gene amplification method besides PCR. This method uses a DNA helicase to unwind double-stranded DNA into two single strands. HDA reaction can be performed at one constant temperature, eliminating the need to use a thermal cycler. In this study, two mesophilic UvrD helicases from *Escherichia coli* and *Proteus mirabilis* were produced for HDA reaction to be performed at standard room temperature. *E. coli uvrD* helicase gene was produced synthetically through assembly PCR method. A total of 120 oligonucleotides were designed based on *E. coli* strain K-12 gene sequence obtained from National Center for Biotechnology Information, USA. The oligonucleotides were divided into three parts and assembled separately. Assembled products were used as templates and amplified into three separate DNA fragments known as block 1, block 2 and block 3, using outermost oligonucleotides of each block as primers. The blocks were cloned into pCR^{TM}-Blunt cloning vector before sending for DNA sequencing analysis. Mutations in each block were corrected and the error free blocks were combined into a full-length *E. coli uvrD* gene and subcloned into pET28a (+) expression vector. On the other hand, *P. mirabilis uvrD* helicase gene was amplified out from its genomic DNA. The gene was also cloned into pCR^{TM}-Blunt cloning vector, sequenced and subcloned into pET28a (+) expression vector. *E. coli* MutL protein, an accessory protein that is required for HDA reaction was also expressed from a gene that was purchased. All the three proteins were expressed through BL21 (DE3) at 25°C, 200 rpm for 16 hours without

IPTG induction. IMAC purification with Nickel ions was performed to purify the Histidine-tagged proteins. Purified proteins were buffer exchanged and were detected through SDS-PAGE and western blot analysis, showing a specific band at sizes ~82 kDa for UvrD helicases and ~68 kDa for MutL protein. Further confirmation was done through MALDI-TOF/TOF analysis and the scores significantly matched the proteins. HDA reaction showed that *E. coli* UvrD helicase had successfully amplified a 120 bp product at 30.0^{o}C, 37.0^{o}C and 42.0^{o}C while *P. mirabilis* UvrD helicase worked best at 37.0^{o}C and 42.0^{o}C. Amplification at 25.0^{o}C was enhanced by increasing the concentration of helicases. Around 3.0 µg/mL of *E. coli* UvrD helicase and 10.0 µg/mL of *P. mirabilis* UvrD helicase were required to amplify the 120 bp product at the standard room temperature. Overall, two DNA helicases that function well in HDA reaction were successfully produced in this study.

CHAPTER ONE: INTRODUCTION

In this current situation of fast moving era, the necessity for things that has to be done promptly have gained interest tremendously. Therefore, the requisite for rapid and fast detection of diseases becomes vital with current technologies being directed towards developing rapid diagnostic kits. Isothermal amplification methods were devised to serve this purpose and one such promising method is identified as helicase-dependent amplification. This method observes the unwinding potentiality of helicase in targeting the respective region for amplification. The features of helicases and isothermal amplification processes, chiefly the helicase-dependent amplification were focused and elucidated throughout this chapter.

1.1 Helicases

Helicase was first discovered in 1976 from *Escherichia coli* (*E. coli*) (Abdel-Monem and Hoffmann-Berling, 1976). Helicases play essential roles in many cellular processes such as genome replication, repair, recombination and transcription (Matson and Kaiser-Rogers, 1990; Kornberg and Baker, 1992; Lohman, 1992; Patel and Picha, 2000; Patel and Donmez, 2006). Approximately 1% of open reading frames in eukaryotes are involved in the production of helicases or helicase-like proteins (Wu, 2012). Apart from eukaryotes, many viruses are also known to code for helicases (Kadare and Haenni, 1997). The main function of a helicase is to catalyze the unwinding of energetically stable duplex DNA molecules into single DNA strands. It uses the energy derived from the hydrolysis of nucleotide triphosphate (for example ATP) to break the hydrogen bonds that link the two strands together in duplex DNA (Kornberg and Baker, 1992). All helicases share at least three common properties: a) nucleic acid binding; b) NTP/dNTP

binding and hydrolysis; and c) NTP/dNTP hydrolysis-dependent unwinding of duplex nucleic acids in the 3' to 5'or 5' to 3'direction (Hall and Matson, 1999). Helicases are classified into numerous categories based on their directionality (5' to 3' or 3' to 5'), substrate specificity (RNA vs DNA) (Gorbalenya and Koonin, 1993; Gwack *et al*., 1997) and oligomerization (Patel and Picha, 2000; Patel and Donmez, 2006). Helicases can function as a monomer, dimer or hexamer to enhance their activity (Nanduri *et al*., 2002; Xu *et al*., 2003; Levin *et al*., 2004; Byrd and Raney, 2005; Tackett *et al*., 2005; Jennings *et al*., 2009).

The renowned oligomerization state is a ring-shaped hexamer and interestingly, the monomers of hexameric helicases do not exhibit any NTP hydrolysis and nucleic acid unwinding activity. The presence of NTP, Mg^{2+} or nucleic acids usually stabilizes the formation of the hexamer (Picha and Patel, 1998; Patel and Picha, 2000; Picha *et al*., 2000; Donmez and Patel, 2006; Patel and Donmez, 2006). The enzyme commission number of this DNA helicase is 3.6.4.12 (3-hydrolases; 6-acting on acid anhydrides; 4-acting on ATP; 12-DNA helicases). A good understanding about the characteristics and function of helicases has enabled researchers to apply helicases in various fields such as targets for developing antiviral compounds (Borowski *et al*., 2001; Borowski *et al*., 2002; Hickman and Dyda, 2005; Kwong *et al*., 2005; Borowski *et al*., 2007) and helicase based isothermal nucleic acid amplification (Vincent *et al*., 2004; An *et al*., 2005a).

1.2 UvrD helicase

E. coli UvrD helicase is one of the well-studied DNA helicase and has the ability to unwind blunt-ended DNA as well as nicked circular DNA molecules (Runyon and Lohman, 1989). Moreover, UvrD also able to unwind 3' tailed duplex

DNA molecules and it unwinds DNA in a 3' to 5' direction (Matson, 1986). UvrD is classified under superfamily 1 (SF1) and mainly involved in the DNA metabolic processes such as methyl-directed mismatch repair, UvrABC-mediated nucleotide excision repair of DNA, replication restart, and rolling circle replication of plasmids (Matson, 1986; Lahue and Matson, 1988; Runyon and Lohman, 1989). 'Uvr' stands for 'ultraviolet repair' and there are several identified Uvr protiens, such as; UvrA, UvrB, UvrC and UvrD (Papachristodoulou *et al*., 2014). UvrD participates in the DNA mismatch pathway by unwinding the duplex DNA containing mutation for corrections to be made (Modrich, 1989). The active form of UvrD helicase in vitro is a dimer. However, monomers of UvrD are shown to bind to the ssDNA but unable to unwind dsDNA (Maluf *et al*., 2003; Lee *et al*., 2013).

1.3 Helicase superfamilies

Previously, helicases were classified into three superfamilies (SF) and two families of putative helicases based on their primary structure analysis (Gorbalenya and Koonin, 1993). Later, some of the putative helicases were grouped into a new superfamily due to the dissimilarity in their functions compared to other helicases (Singleton *et al.*, 2007). Now, there are six superfamilies known as SF1, SF2, SF3, SF4, SF5 and SF6 (Singleton *et al.*, 2007).

SF1 can be further divided into SF1A and SF1B, based on their directional polarity (Singleton *et al.*, 2007). SF1A helicases translocate along with their nucleic acid substrates with a 3′–5′ polarity and those that translocate in 5′–3′ direction are grouped into SF1B enzymes (Saikrishnan *et al*., 2008). The well-studied enzymes of SF1A are, Rep and UvrD helicases, from gram-negative bacteria and PcrA helicase from gram-positive bacteria. Crystal structure analysis of PcrA and Rep helicases elucidate a common fold between them (Subramanya *et al.*, 1996; Korolev

et al., 1997). They are encompassed of two domains known as N- and C-core domains and each domain is divided into two sub-domains. The ATP-binding site is located between these two sub-domains, in a cleft, that is lined by several SF1 motifs. SF1B on the other hand comprises of RecD helicase from *E.coli*, Dda helicase from bacteriophage T4, while less studied Pif1 and Rrm3 represent eukaryotes (Ivessa *et al*., 2002). The only structural information about this subdivision comes from the RecD subunit of the RecBCD complex (Singleton *et al.*, 2004). The structure of RecD resembles SF1A enzymes, but there is an additional N-terminal domain that forms the interface with the RecC subunit. However, this structure is not conserved in Dda. SF1A helicases are extensively studied both from structural and biochemical perspectives while the mechanism of SF1B enzymes remains unclear (Saikrishnan *et al*., 2008).

SF2 is considered to be the largest superfamily among all. The subfamilies of SF2 consist of DEAD-box RNA helicases (Cordin *et al.*, 2006), the RecQ-like family (Bennett and Keck, 2004) and the Snf2-like enzymes (Flaus and Owen-Hughes, 2004; Flaus *et al.*, 2006), all of which are extensively studied. Some of the SF2 members are RecG, PriA and HCV NS3 helicases (Tuteja and Tuteja, 2004). The nonstructural protein 3 (NS3) of hepatitis C virus is perhaps the best-characterized helicase among them (Singleton *et al.*, 2007). It has four domains namely protease, N-core, C-core and Domain 3. The N-terminal protease domain is responsible for processing viral polyprotein while the C-terminal domain helps in protein-protein interactions (Mackintosh *et al.*, 2006).

SF3 helicases are encoded by small DNA and RNA viruses (Gorbalenya *et al.*, 1990). They are related with multiple enzymatic activities, such as origin recognition and unwinding (Hickman and Dyda, 2005). SF3 proteins share four

conserved motifs generally known as A, B, B', and C. The conserved arginine finger is located after motif C. They also have an ori DNA-binding domain preceding the conserved domain. Helicase domain has a modified AAA^+ (ATPases Associated with various cellular Activities) core containing the nucleotide-binding motifs in the central β-strands and a set of inserted loops mostly involved in DNA binding or protein-protein interactions. A small, poorly conserved, helical domain prior to the AAA^+ core speculated to be involved in stabilizing the hexamer (Singleton *et al.*, 2007). One of the well-studied member of SF3 is Papilloma virus E1 helicase (Enemark and Joshua-Tor, 2006).

SF4 members are mostly replicative helicases. In bacteria, the replicative helicase associates with a primase for DNA replication and the two proteins exist as separate polypeptides. Interestingly, in some bacteriophages, both proteins and their activities reside within a single polypeptide (Singleton *et al.*, 2007). All characterized SF4 helicases so far have 5'-3' (type B) translocation directionality. There are five sequence motifs in SF4 members: H1, H1a, H2, H3, and H4 (Ilyina *et al.*, 1992). Motifs H1 and H2 are more similar to the Walker A (phosphate binding loop or P-loop) and Walker B (Mg^{2+} binding aspartic acid) motifs. However, motifs H1a, H3, and H4 have no obvious counterparts in any other helicase family, although motif H3 contains a conserved glutamine (occasionally histidine), which is the functional equivalent of motif 3 of SF1. One of the best studied SF4 helicase is the gene 4 protein (gp4) from bacteriophage T7. This enzyme has both primase and helicase domains connected by a flexible linker (Toth *et al.*, 2003). Another example is DnaB enzyme from bacteria which exist as separate entity from its primase counterpart (Bailey *et al.*, 2007).

SF5 consist of Rho helicases that involves in the transcription termination in bacteria by binding to a specific sequence on the nascent RNA and then unwinding the DNA/RNA hybrid. These helicases were grouped in separate family on the basis of its sequence, though they are much closely related to SF4 (Singleton *et al.*, 2007).

SF6 houses hexameric motor proteins containing the core AAA^+ fold that is not included in the SF3 classification (Singleton *et al.,* 2007). The mini chromosome maintenance (MCM) protein complex known as the main eukaryotic replicative helicase is categorized under this superfamily. There are six protein complex (MCM 2–7) involved in replication initiation and elongation (Labib *et al.*, 2000) but helicase activity has only been detected in the MCM 4, 6, 7 hetero-hexamer (Kaplan and O'Donnell, 2004). Structural information obtained from intact archaeal MCM shows a hexameric ring with a large central channel, wide enough to accommodate dsDNA (Pape *et al.,* 2003). As for prokaryotes, the RuvB protein, together with RuvA and RuvC is responsible for processing Holliday junctions, a universal intermediate during homologous recombination (West, 1996). RuvB migrates Holliday junctions by pumping dsDNA through the central channel of a hexameric ring in an ATP-dependent process. Crystal structures of the protein reveal a monomeric form with a bound nucleotide, suggesting that nucleotide binding drives a conformational change between the domains, thus helping in translocational activity of the enzyme (Putnam *et al.*, 2001).

1.4 Helicase motifs of superfamily I

Among the six superfamilies, SF1 and SF2 are the largest superfamilies. They are closely related and share at least seven conserved motifs, called as 'helicase signature motifs' (I, Ia, II–VI) (Gorbalenya and Koonin, 1993). New motifs, such as TxGx (Pause and Sonenberg, 1992), motif 4a (Korolev *et al.*, 1998),

Q-motif (Tanner *et al.*, 2003), and TRG (Mahdi *et al.*, 2003) were discovered later and added into the category. In contrast, Motif III differs between both superfamilies. It neither shares sequence similarity nor similar positon in the enzymes (Singleton *et al.*, 2007). The seven conserved motifs of SF1 and SF2 are grouped into a core region, a region of 200–700 amino acids and they are separated by stretches of low sequence but high length conservation (Tuteja and Tuteja, 2004). This core region forms tandem RecA-like folds (three-dimensional folds) either within the same polypeptide chain or between subunits. These folds are responsible for converting chemical to mechanical energy by coupling NTP binding and hydrolysis to protein conformational changes (Ye *et al.*, 2004). Two important features of the core domains are the involvement of conserved residues in the binding and hydrolysis of the NTP (Walker *et al.*, 1982) and an "arginine finger" that plays a key role in energy coupling (Scheffzek *et al.*, 1997).

On the other hand, a high degree of sequence and length variability was observed at the N-terminal and C-terminal regions of helicases. The helicase core is surrounded by these C and N-terminal domains, often exceeding the helicase core in size (Fairman *et al.*, 2010). These divergent regions are believed to be responsible for individual protein functions while the highly conserved domains are involved in ATP binding and hydrolysis or binding and unwinding of nucleic acids (Tuteja and Tuteja, 2004). These domains adopt defined folds to perform specific functions such as RNA or DNA binding domains (e.g., Zn-fingers, OB-folds, dsRBDs) (Bernstein and Keck, 2003; Zhang and Grosse, 2004 Cui *et al*., 2008; He *et al*, 2010) or engaging in protein-protein interactions (e.g., CARD-domains) (Yoneyama and Fujita, 2008). These domains are also involved in helicase oligomerization (Klostermeier and Rudolph, 2009). Thus, due to the distinctive characteristics, C-

and N-terminal accessory domains are usually not conserved within a family, though some degree of structural conservation of the C-terminal domains was observed in the Ski2-like and the DEAH/RHA families (Buttner *et al.,* 2007; He *et al.,* 2010).

Overall, the crystal structures of SF1 helicases have revealed that the helicase motifs are clustered together in two RecA like domains, forming an ATP-binding pocket between them and part of the nucleic acid binding site. The non-conserved regions may contain specific domains such as protein-protein interaction domains, cellular localization domains, and DNA-recognition domains specific to individual helicases (Raney *et al.,* 2013). Figure 1.1 highlights the conserved sequence motifs of the SF1 members (Lee and Yang, 2006). The functions of some selected motifs of superfamily I helicase, particularly in relation to *E. coli* UvrD helicase were described further in detail.

The Q motif is located upstream of motif I and therefore outside of what was previously considered to be the helicase core (Tanner *et al.,* 2003). The motif contains nine amino acid sequence, including the invariant glutamine (Q). The glutamine forms hydrogen bonds with the N6 and N7 positions of adenine and another conserved aromatic group within the Q motif stacks on the adenine base (Tanner, 2003). The Q of the Q motif is also conserved among all SF1 helicases (Fairman *et al.,* 2010). This motif however is less conserved among those helicase families which do not show specificity for ATP (Fairman *et al.,* 2010). In vivo analyses show that Q motif is required for yeast cell viability while in vitro analyses of purified protein indicate that it plays a role in ATP binding and hydrolysis (Tanner, 2003).

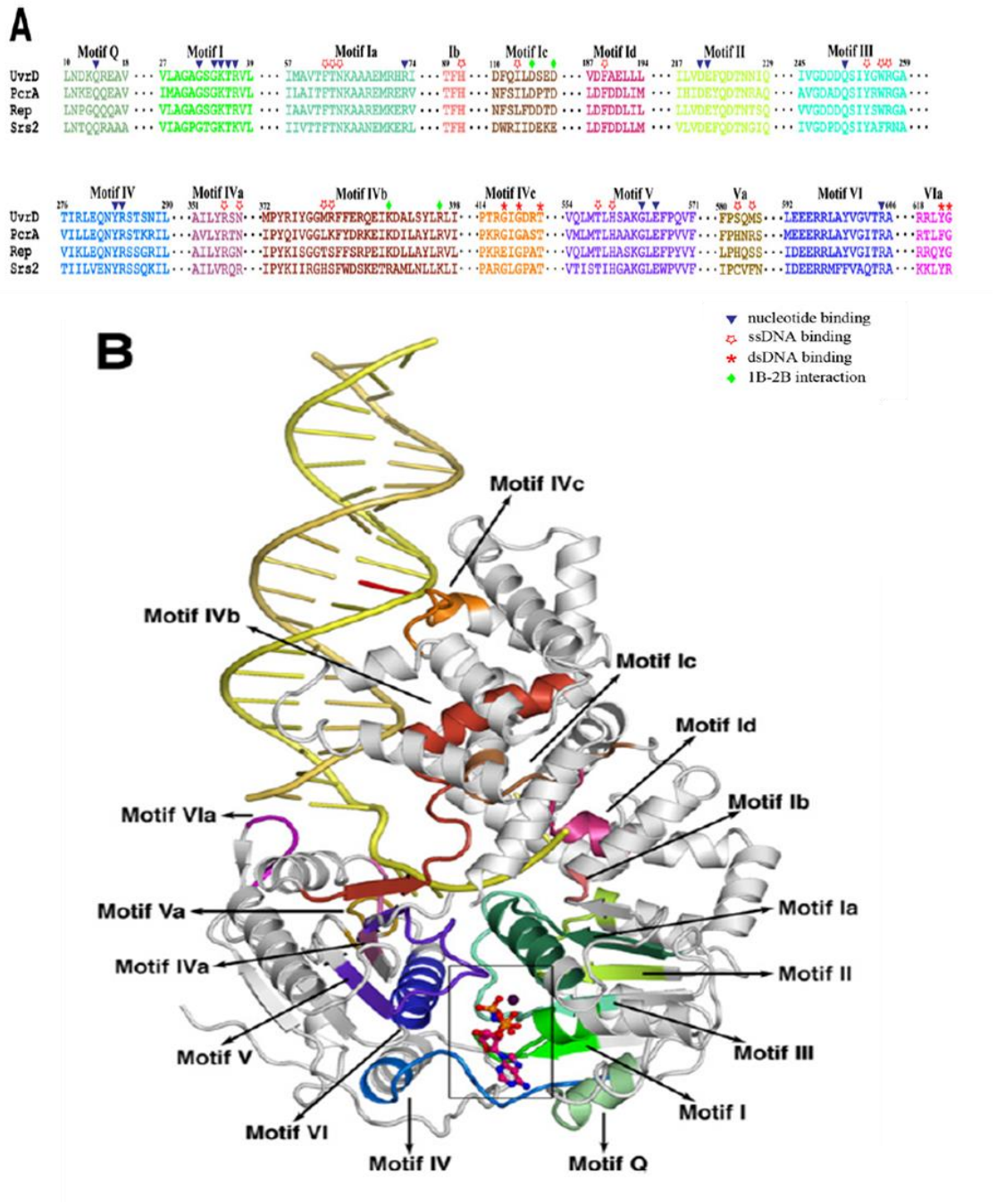

Figure 1.1: Schematic representation of the Conserved Sequence Motifs among UvrD, PcrA, Rep, and Srs2. (A) Sequence alignment of the eight ATPase motifs (in different shades of green [domain 1A] and blue and purple [2A]) and eight newly found DNA-binding and domain interaction motifs (in brown to red colours). (B) The 16 motifs are mapped onto the UvrD-DNA-AMPPNP (ATP analog) structure using the same colour scheme as in (A). (Lee and Yang, 2006).

Motif I was previously identified as ‘A’ motif of ATPase or Walker A motif (Walker *et al.,* 1982). This motif is responsible for ATP-binding in the SF1 and SF2 helicases, including the UvrD protein. The function was studied by creating mutations in that particular motif which in turn result in significant reduction of ATPase activity and various levels of reduced helicase activities (Washburn and Kushner, 1993; George *et al.,* 1994). The consensus sequence for motif I of UvrD is AxxGxGKT (Gorbalenya *et al.,* 1989). Studies conducted on DEAD family of RNA helicases shown that the lysine (K) that found in motif I binds to β and γ phosphates of the ATP molecule, and the mutation of K to the uncharged amino acid asparagine abolished the binding of ATP to the motif (Pause and Sonenberg, 1992). An ATPase-deficient mutant also lacked helicase activity, suggesting that ATP hydrolysis was required for the unwinding of duplex nucleic acid molecules (Hall and Matson, 1999). Furthermore, the conserved G in GKT helps to maintain the flexible loop conformation of that site (Gorbalenya *et al.,* 1989).

Motif Ia mostly involves in ssDNA binding. A study was conducted on motif Ia of UL9 (a SF2 helicase) of HSV-1, by introducing specific mutations and these mutants were analyzed for their inherent properties (Marintcheva and Weller, 2003). The study shows that mutant proteins with residues predicted to be involved in ssDNA binding (R112A and R113A/F115A) exhibited defects in ssDNA-stimulated ATPase activity and ssDNA binding. Moreover, they lacked helicase activity but were able to dimerize and bind the HSV-1 origin of replication, similar to wild-type UL9. Hence, it was concluded that residues from the Ia motif contribute to the ssDNA-binding and helicase activities of UL9 and eventually for viral growth (Marintcheva and Weller, 2003). The residues from motif Ia of *E. coli* Rep helicase (a SFI helicase), are also shown to participate in ssDNA binding (Korolev *et al.,*

1997). The FTN residues from the motif Ia (T**FTN**KAAREM) together with other residues from other motifs were known to participate in the ssDNA binding.

Motif II was initially known as Walker B motif (Walker *et al.,* 1982). The proteins containing this motif are famously called as the DEAD-box proteins (Linder *et al.,* 1989). Other variations in motif II are termed as DEAH and DEXH box proteins. The D and E amino acids of the DEAD sequence are highly conserved and play a significant role in DNA and RNA replication (Gorbalenya *et al.,* 1989). The residue D interacts with Mg^{2+}, which is required for ATP binding. Mutation in D was proven to affect the ATPase and helicase activities (Pause and Sonenberg, 1992). The functional significance of motif II in *E. coli* UvrD was also studied extensively (Brosh and Matson, 1995). Two specific mutations were introduced in the amino acid sequence of UvrD motif II (NILV**DE**FQNTNNIQYAW); one is the single substitution replacing the highly conserved glutamic acid (E) at position 221 with a glutamine (Q) (E221Q) and another was double substitution, replacing the highly conserved aspartic and glutamic acids, with an asparagine and glutamine, at positions 220 and 221, respectively (D220N and E221Q). The results indicate that E221Q mutant protein did not exhibit any unwinding of either partial duplex or blunt duplex DNA substrates. Surprisingly, D220NE221Q mutant do exhibit unwinding activity but at greatly reduced rate compared with that of the wild-type enzyme. Further analysis shows that the mutants retained ATP and DNA binding capability as in wild-type helicase II. Therefore, highly conserved acidic residues of motif II believed to involve in ATP hydrolysis reaction.

Motif III acts as an interface between the ATP binding and DNA binding domains of a helicase (Brosh and Matson, 1996). The sequence of Motif III in *E. coli* UvrD is (VMIV**GD**D**Q**SIY). Among them, residues G, D and Q were highly

conserved in members of SFI. The function of this motif in *E. coli* UvrD was verified by substituting the negatively charged conserved aspartic acid at position 248 to neutral asparagine (D248N). The result shows that the mutant UvrD failed to form stable binary complexes with either DNA or ATP. It is proposed that the aspartic acid stabilizes the interaction between ATP and the helicase, not likely to be directly involved in ATP binding. Moreover, the DNA-stimulated ATPase activity was reduced when compared to wild-type UvrD. The mutant protein also demonstrated a reduced DNA unwinding activity. The requirement for high concentration of mutant proteins to achieve unwinding of long duplex substrates reflects the reduced stability of various binary and ternary complexes that must exist for a proper helicase reaction. Another example will be the mutation in herpes simplex virus UL5 protein from highly conserved glycine in motif III to a serine resulted in the failure of the mutant protein to function in viral DNA replication (Zhu and Weller, 1992).

Motif IV functions as ATP binding site during unwinding reaction (Hall and Matson, 1997). The consensus sequence of Motif IV in *E. coli* UvrD is (QNY**R**STSNI). The function of this motif was confirmed by replacing the highly conserved arginine at position 284 with alanine to construct mutant protein UvrD-R284A. A large decrease in ATP binding by the mutant protein was confirmed using a nitrocellulose filter binding assay. Moreover, UvrD-R284A also failed to unwind a 92-base pair duplex region and less than ten percent unwinding was detected for a shorter (20 base pair) duplex region, as a result of lack of affinity towards ATP. There were no significant difference reported for DNA binding of both mutant and wild-type UvrD proteins, suggesting that motif IV highly involved in ATP binding activity.

Motif V interacts with the sugar-phosphate backbone of the DNA. Highly conserved glycine at position 815 in Motif V of UL5 from HSV-1 was replaced with an alanine. The mutant proteins exhibited reduced affinity for ssDNA and reduced rates of ATP hydrolysis (Graves-Woodward and Weller, 1996).

Motif VI generally involves conformational changes related to the coupling of ATPase and DNA binding activities. Two point mutations were introduced to create two mutant proteins to study the functional significance of motif VI in *E. coli* UvrD. The consensus sequence of Motif VI in *E. coli* UvrD is (YVGV**TR**AMQ) (Gorbalenya and Koonin, 1993). The Threonine (T) and arginine (R) residues are invariant in most helicases. Threonine at position 604 and arginine at position 605, were replaced with alanine, generating the mutant alleles UvrD-T604A and UvrD-R605A. Results indicate that UvrD-T604A has a serious defect in ssDNA binding in the absence of nucleotide. However, in the presence of a non-hydrolysable ATP analog, DNA binding was only slightly compromised. Limited proteolysis experiments show that UvrD-T604A had a "looser" conformation and could not undergo necessary conformational changes that needed for ATP binding/hydrolysis and DNA binding. Another mutant protein, UvrD-R605A exhibited nearly normal DNA binding but had a severe defect in ATP hydrolysis. In another study, mutations of the conserved threonine and arginine residues in motif VI of the yeast superfamily I RNA/DNA helicase Upf1p severely affected RNA binding, RNA-stimulated ATP hydrolysis, and RNA unwinding (Weng *et al*., 1996).

1.5 DNA unwinding mechanism by helicase

DNA unwinding mechanism can be classified as either active or passive (Lohman, 1992; Amaratunga and Lohman, 1993; Lohman and Bjornson, 1996; von

Hippel and Delagoutte, 2001; Betterton and Julicher, 2005). In a passive mechanism, the helicase interacts with ssDNA that resulted from momentary base-pair opening at the junction between ssDNA and dsDNA that occurs due to thermal fluctuations. The helicase uses its directional ssDNA-translocase activity to move onto the ssDNA and thus stabilizing it, without directly interacting with the dsDNA. On the contrary, the helicase interacts directly with the double-stranded region and facilitates duplex destabilization in an active mechanism. Studies of DNA unwinding by the Rep and UvrD helicases (Amaratunga and Lohman, 1993), PcrA helicase (Soultanas *et al.*, 2000) and the SF2 NS3 RNA helicase (Cheng, 2007) have shown that these enzymes use an active mechanism of unwinding. The mechanism for DNA or RNA unwinding remains highly controversial and debatable despite extensive biochemical and structural characterization of SF1 and other helicases have been elucidated. The main reason is that to unwind a double helix, contacts between the helicase and the duplex region of DNA and a rotational movement are likely required in addition to ssDNA translocation, but so far no DNA rotation has been detected in any unwinding activities studied (Lee and Yang, 2006). Nevertheless, two popular models exist in regards of active unwinding mechanism; the 'active rolling' and 'inchworm' models (Yarranton and Gefter, 1979; Lohman and Bjornson, 1996; Velankar *et al.*, 1999).

In the active rolling model, initially both the subunits of a helicase dimer are bound to the ssDNA. As ATP binding occurs, one of the subunits releases the ssDNA and binds to the duplex region at the fork. This leads to helix destabilization and the release of one of the DNA strands, accompanied by hydrolysis of ATP (Lohman and Bjornson, 1996). In this model, translocation along ssDNA is coupled to ATP binding, whereas ATP hydrolysis drives the unwinding of multiple DNA

base pairs for each catalytic event. The Rep protein exists as a stable monomer in the absence of DNA. Once the Rep monomer binds to DNA, conformational changes happen and it forms a homodimer, becoming functionally active in translocation and unwinding (Delagoutte and von Hippel, 2002). It is proposed that ATP hydrolysis alternates between one subunit and the other as an integral part of the translocation and unwinding reactions (Hsieh *et al.,* 1999).

For the inchworm model, helicase is bounded to the ssDNA only and then translocates along the DNA strand to the fork region, probably on binding ATP. As the ATP gets hydrolysed, the helix destabilizes and eventually one of the ssDNA strand is released (Yarranton and Gefter, 1979). A detailed model was proposed based on the crystal structure of the PcrA protein (Velankar *et al.,* 1999). At first, ssDNA is bound to both domains 1A and 2A of the PcrA protein. After ATP binds to the protein-ssDNA complex, conformational changes happen that the cleft between domains 1A and 2A closes prompting domain 1A to release its grip on ssDNA and slides along it, while domain 2A maintains a tight grip on ssDNA. Once ATP gets hydrolysed, the protein returns to its initial conformation, the inter-domain cleft opens and domain 2A now translocates along the ssDNA.

1.6 Non-PCR based methods

Polymerase chain reaction (PCR) is an in-vitro technique used for nucleic acid amplification. This method was developed by Kary Mullis in 1980s (Mullis and Faloona, 1987). This inspiring technique has been widely used in molecular biology, forensic fields and detection methods especially in the field of clinical microbiology for detection and identification of many pathogens (Duggan, 1988; Bernet *et al.,* 1989; Brisson *et al.,* 1989; Holland *et al.,* 1990; Hoshina, 1990; Holodniy, 1991;

Victor, 1991; Lisby and Dessau, 1994; Moore, 2005). The beauty of PCR that amplifies from even scarce amounts of nucleic acid in vitro, has helped in establishing all the above mentioned fields. However, the requirement for expensive and power consuming thermocycler in conventional PCR can be a major drawback in some situations, especially when requiring rapid pathogen detection at point-of-care diagnosis (Vincent *et al*., 2004). Due to this, several isothermal nucleic acid amplification techniques have been developed. They are the nucleic acid sequence-based amplification (NASBA) (Compton, 1991), signal mediated amplification of RNA technology (SMART) (Wharam *et al.,* 2001), strand displacement amplification (SDA) (Walker *et al.,* 1992), rolling circle amplification (RCA) (Fire and Xu, 1995), loop-mediated isothermal amplification of DNA (LAMP) (Notomi *et al.,* 2000) and helicase-dependent amplification (HDA) (Vincent *et al.,* 2004). One of the major difference between PCR and isothermal amplification is the temperature requirement for the reactions. Various temperatures are required in PCR techniques, for template denaturation, primer annealing and extension of the separated template. Conversely, only a single optimal reaction temperature is needed for the entire amplification process for most of the isothermal reactions (Chang *et al.,* 2012). Table 1.1 highlights the reaction mechanisms involved in selected isothermal assays and PCR (Gill and Ghaemi, 2008)

Table 1.1: Properties of various isothermal amplification methods and PCR (Gill and Ghaemi, 2008).

Property	**PCR**	**NASBA**	**SMART**	**SDA**	**RCA**	**LAMP**	**HDA**
DNA amplification	+	+	+	+	+	+	+
RNA amplification	+	+	+	+	+	+	+
Temperature (s) (°C)	94, 56-60, 72	37-42	41	37	37	60-65	Room*, 37, 60-65
Number of enzymes (s)	1	2-3	2-3	2	1	1	2
Primer design	Simple	Simple	Complex	Complex	Simple	Complex	Simple
Multiplex amplification	+	+	-	-	+	-	+
Product detection method	Gel electrophoresis, ELISA, Real-time	Gel electrophoresis, ELISA, Real-time, ECL	ELOSA, Real-time	Gel electrophoresis, Real-time	Gel electrophoresis, Real-time	Gel electrophoresis, turbidity, Real-time	Gel electrophoresis, ELISA, Real-time
Tolerance to biological components	-	-	-	-	-	+	+
Need to template denaturation	+	+	+	+	-	-	-
Denaturing agent (s)	Heat	RNase H	RNase H	Restriction enzymes, bumper primers	Strand displacement property of phi29 DNA polymerase	Betaine	Helicase

*Room: 22-24°C

ELISA: Enzyme-linked immunosorbent assay

ELOSA: Enzyme-linked oligosorbent assay

ECL: Electrochemiluminesce

Strand displacement amplification (SDA) utilizes four sequence-specific primers in the reaction. The first set of primers (S1 and S2) are designed to have single-stranded restriction enzyme recognition site overhangs, and the second set of the primers (B1 and B2) represent the bumper primers (Walker *et al.,* 1992). Template is first denatured by heat and each strand is allowed to hybridize with two primers. For instance, B1 primer anneals at 5' end of one strand and S1 primer too anneals at the same strand, but further downstream than B1 primer. Both the primers will be extended and B1 extended product displaces the extension from the S1 primer. Subsequently, B2 and S2 primers will now hybridize to S1 extension product and the cycle continues as such. Newly synthesized DNA that has been extended from the primers is cleaved by a corresponding restriction endonuclease, and the amplification is repeated by the polymerase, thus generating the newly synthesized strands. However, the drawbacks of this technique would be that it needed an initial higher temperature (95°C) for template denaturation, requires four primers to generate initial amplicons and modified deoxynucleotides to provide strand specific nicking (Walker, 1992; Walker *et al.,* 1992).

Rolling circle amplification (RCA) method is initiated by annealing a linear ssDNA primer to a specific circular ssDNA template, generated by joining two ends of the DNA using a DNA ligase (Fire and Xu, 1995; Demidov, 2002). A suitable DNA polymerase such as phi29 DNA polymerase extends the annealed primer, generating tandemly linked copies of the complementary sequence of the template. This polymerase is crucial for RCA reaction due to its excellent strand displacement activity enabling to continuously progress around the circular template, creating long ssDNA products (Dean *et al.,* 2001). This technique is simple and robust yet requires initial heat step at higher temperature.

Loop-mediated isothermal amplification (LAMP) uses *Bst* DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA (Notomi *et al.,* 2000). *Bst* polymerase was used as it possesses polymerize activity, 5'-3' exonuclease activity, and strand displacement ability. First, an inner primer containing sequences of the sense and antisense strands of the target DNA initiates the amplification. Strand displacement by *Bst* polymerase releases a single-stranded DNA. This serves as template for new DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates strand displacement, yielding the original stem–loop DNA and a new stem–loop DNA with a stem twice as long. LAMP reaction is highly specific for its use of four target primers and can amplify many folds of target DNA in shorter time. In addition, pyrophosphate ions are produced in large amounts and form white precipitates of magnesium pyrophosphate during the reaction. The presence or absence of this white precipitate allows for easy detection of the amplified product (Mori *et al*., 2001).

Helicase-dependent amplification (HDA) method was developed in 2004 (Vincent *et al*., 2004). HDA developed by Vincent's group known as mesophilic HDA (mHDA). In living organisms, a DNA helicase is used to separate two complementary DNA strands into single strands for DNA replication (Kornberg and Baker, 1992). The similar concept was applied to develop this isothermal gene amplification technique. HDA utilizes a DNA helicase to unwind a double-stranded DNA into two single strands, with the presence of ATP. This enables primers hybridization and subsequent extension of the DNA strands by DNA polymerase.

Hence, various temperatures provided through conventional PCR can be omitted. The HDA uses one temperature (37°C) throughout the reaction (Vincent *et al*., 2004). Besides *E. coli* UvrD helicase, three other major components involved in mHDA are *E. coli* MutL, *E. coli* exo- Klenow Fragment (DNA polymerase) and Phage T4 gene 32 protein, a single strand binding (SSB) protein.

MutL acts as the master coordinator of mismatch repair by forming a direct physical interaction with UvrD. MutL is responsible for loading the UvrD onto the DNA substrate (Hall *et al*., 1998; Mechanic *et al*., 2000). Study shows that, a dimer of MutL in the ATP-bound form, which may be attached to the end of blunt-ended DNA, recruits and loads multiple molecules of UvrD onto the ends of dsDNA substrates to initiate unwinding (Matson and Robertson, 2006). In mesophilic HDA, no amplification was observed in the absence of MutL, suggesting the presence of coordinated function between MutL and mesophilic UvrD (Vincent *et al*., 2004). Interestingly, amplification was the same in thermophilic HDA, with or without MutL, further suggesting the presence of MutL is specific for mesophilic HDA only (An *et al*., 2005a).

Exo- Klenow Fragment derived from DNA polymerase I of *E. coli* by truncating and mutating the polymerase to remove its exonuclease activities (5′→ 3′ and 3′→ 5′) while retaining its polymerase activity only. The polymerase activity of exo- Klenow Fragment is identical to that of the wild type polymerase (Derbyshire, 1988).

Phage T4 gene 32 protein, a SSB protein was included in the HDA reaction, without which no amplification product was obtained (Vincent *et al*., 2004). T4 gene 32 protein is a single-stranded DNA binding protein required for bacteriophage T4

replication and repair mechanism (Alberts and Frey, 1970; Kornberg and Baker, 1992). It binds to and stabilizes transiently formed regions of ssDNA and plays an important role during T4 phage replication (Bittner *et al*., 1979). The affinity of this protein to ssDNA is 10^4 or 10^5 times greater than for duplex DNA. Once bound to a ssDNA region, the affinity to bind to the next protein increases 10^3 fold. Therefore, each protein binds to the adjacent protein forming a continuous track of proteins (Lohman, 1984). In mHDA, *E. coli* SSB was substituted with phage protein as the former produces lower yield compared to the latter (Vincent *et al*., 2004). A simple reaction mechanism of HDA is shown in Figure 1.2 (Jeong *et al*., 2009). HDA was further improved and developed into circular HDA (cHDA) and termophilic HDA (tHDA).

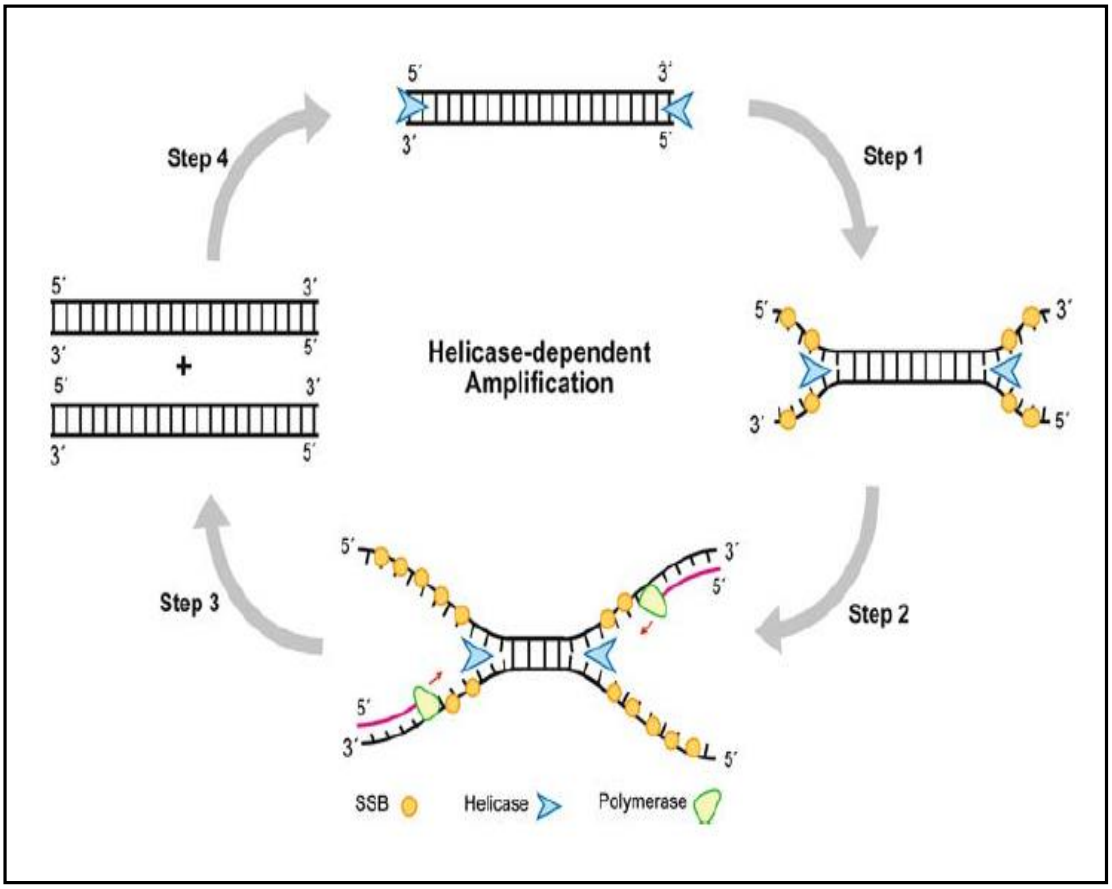

Figure 1.2: Schematic diagram of the helicase-dependent amplification. Helicases unwind dsDNA and SSB proteins bind to exposed ssDNA. Subsequently, DNA polymerases start synthesizing the complementary strand from the bound primers, and the cycles repeat continuously (Jeong *et al*., 2009).

As the name suggests, cHDA uses a circular DNA as the template to amplify a target sequence or the entire circular DNA template containing the target sequence (Xu *et al*., 2006). The idea was conceived based on the T7 replication machinery, which includes the processive T7 helicase, an exonuclease-deficient T7 DNA polymerase (T7 sequenase) and the T7 Gp2.5 single-stranded DNA binding (SSB) protein. First, the duplex DNA template is unwound by T7 helicase followed by specific primers annealing to the separated DNA strands. T7 sequenase extends the primers by a rolling circle mechanism to amplify the entire circular DNA continuously. The entire process can be carried out at one single temperature (25°C). Purified plasmid DNA or crude cell lysate can be used to amplify inserts as large as 10 kilo base pairs (Xu *et al*., 2006). However, cHDA only uses circular DNA as the template. No amplification was observed when linear template was used (Xu *et al*., 2006).

Alternatively, tHDA was developed with simplified reagents compared to mHDA (An *et al*., 2005a). It utilizes a thermostable UvrD helicase derived from *Thermoanaerobacter tengcongensis* and polymerase I large fragment from *Bacillus stearothermophilus*, both are thermophilic bacteria. These enzymes operate well at temperatures from 45°C to 65°C and does not require SSB and MutL proteins for successful amplification. The ends of duplex DNA may become single-stranded due to the thermal breathing at higher temperatures. This enables the UvrD to capture the transient ssDNA generated and initiate unwinding without MutL (An *et al*., 2005a). Furthermore, probably the slower rate of reannealing of separated DNA strands at higher temperatures render the use of SSB unnecessary (An *et al*., 2005a). Thermophilic HDA also can amplify longer sequences than mHDA (An *et al*.,

2005a). Currently, Biohelix has commercialized tHDA amplification kits (Biohelix, USA).

1.7 Advantages of HDA

HDA can be a cost saving technique since it does not require the usage of a thermocycler, which is quite expensive. This technique can be performed in a single test tube and incubated at one constant temperature for a given period of time (Vincent *et al*., 2004). Alternatively, cheaper instruments such as water bath, thermo-block or even incubators that already available in laboratory can be used to provide the needed constant temperature.

Compared to other isothermal assays, HDA has a simple reaction scheme, in which a target sequence can be amplified by two flanking primers, similar to PCR. LAMP and SDA methods require four to six primers with complicated design mechanisms (Vincent *et al*., 2004; An *et al*., 2005a).

HDA is truly isothermal that it does not require initial heat denaturation step, unlike SDA and RCA. The ability of UvrD helicase to unwind blunt ended as well as 3' tailed and nicked circular DNA molecules does not necessitates the use of initial heat, though it does help in increasing the amplification yield (Vincent *et al*., 2004).

HDA can be performed using various templates such as plasmid DNA, genomic DNA, crude samples (bacterial cells) and real samples (human blood). The presence of genomic DNA of a pathogen was successfully detected in a human blood sample, through HDA (Vincent *et al.*, 2004). This paves the way for HDA method to be advanced as a potential diagnostic tool.