METHODS OF SINGLE STANDARD DNA SYNTHESIS AND ITS APPLICATIONS: A REVIEW

K. Vivehananthan*, E.D.C. Lilani, I. Abeygunawardena

Department of Basic Sciences, The Open University of Sri Lanka, Sri Lanka

INTRODUCTION

DNA, or deoxyribonucleic acid, is the genetic substance that allows humans and almost all other species to grow, survive, and reproduce. The cell nucleus contains the majority of DNA (nuclear DNA), but mitochondria also contain a small amount (mitochondrial DNA). Adenine (A) and thymine (T), guanine (G) and cytosine (C) are the four nucleotides that are used to store information in DNA. DNA is divided into two types: single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). Double stranded DNA is made up of two strands of DNA that coil around each other in double helixes (Watson & Crick, 1953). Double stranded DNA has a more stable structure than ssDNA due to hydrogen bonding between the base pairs of the two strands. However, ssDNA is exposed during several biological activities such as replication, transcription, and recombination. To avoid mutations, exposed ssDNA is effectively shielded from chemical attack and nucleolytic breakdown. It is acquired by binding of single-stranded DNA-binding proteins (SSBs) which prevents the formation of secondary structures in the ssDNA and inhibits polymerase activity. Single stranded DNAs are also found as genomes in animal viruses belonging to the parvoviridae family and a bacteriophage family called microviridae. Synthetic oligonucleotide sequences, such as synthetic genes and whole chromosomes are used in the majority of current biological and bioengineering research. There are several methods for manufacturing ssDNA including *in vitro* and *in vivo*; Chemical, Enzymatic and Bacteria based ssDNA synthesis. Synthetic oligonucleotide manufacture is crucial due to the increased need for long-length ssDNA. Therefore, the focus of the review is on current and emerging technologies for ssDNA synthesis and some applications of ssDNA.

DIFFERENT METHODS USED FOR THE SINGLE STRAND SYNTHESIS

The information was gathered based on different methods for synthesis of single stranded DNA namely Chemical, Enzymatic and Bacteria based methods for synthesis of single stranded DNA using approximately 50 Science Citation Index journal papers as references.

Chemical synthesis methods

This method can only produce ssDNA oligonucleotides with lengths of up to 200 bases (Veneziano *et al.,* 2018). It uses traditional column based or microarray based synthesizers to create oligonucleotides (Hughes & Ellington., 2017).

Column-based method

This method is often used to synthesize 96 to 384 oligos with an error rate of less than 0.5%. It uses phosphoramidite synthesis chemistry consisting with four-step chain elongation cycle of column-based method. Phosphoramidite synthesis chemistry is a standard method for DNA synthesis that has been used in the industry for almost 35 years. During the first phase, a dimethoxytrityl (DMT)-protected nucleoside phosphoramidite is attached to a solid support, which is de-protected by the addition of trichloroacetic acid. The addition of trichloroacetic acid allows the support for attached phosphoramidite to be activated for chain extension with the next phosphoramidite monomer. Then it forms a phosphite triester connection with the 5′-

hydroxyl group of the preceding nucleoside phosphoramidite in the sequence after adding the next base in the sequence. Any unreacted 5′-hydroxyl groups are capped by acylation, rendering any un-extended sequences inactive in subsequent rounds of the chain elongation cycle. The phosphite triester coupling between the monomers is changed to a phosphate linkage in the fourth step by oxidation with an iodine solution, resulting in a cyanoethyl-protected phosphate backbone. After removing the 5'-terminal DMT protective group, the synthesis cycle is repeated for the next base in the sequence. The oligonucleotide is chemically cleaved from the solid synthesis support and the protecting groups on the bases and backbone are removed when the desired sequence has been produced from 3' to 5' (Hughes & Ellington., 2017).

Microarray based method

This is a low-cost alternative method to column-based oligonucleotide synthesis. The cost of a nucleotide can range from $0.00001 to $0.0001 depending on the platform used for the synthesis, oligonucleotide length, and synthesis scale. It uses a microchip surface to generate oligonucleotides. Oligonucleotides are synthesized at femtomolar synthesis scales (Having a concentration of 10-15 moles per litre), typically two to four orders of magnitude lower than that used in column-based synthesis. As a result of the lowered synthesis scale; the number of chemicals needed for the synthesis is reduced. The use of fewer chemicals in the synthesis process results in lower costs and higher degrees of multiplexing, allowing for the synthesis of more unique oligonucleotide sequences in a single synthesis run. Microarray-based synthesis methods far outstrip the capacity of even the largest column-based equipment, with the ability to synthesize multiple chips at once and possible feature densities in the tens of thousands per chip (Hughes & Ellington., 2017).

Enzymatic synthesis methods

Asymmetric PCR (aPCR)

This is a technique for amplifying only one strand of DNA. This approach has been used to make ssDNA with lengths ranging from 100 to 1000 nt. It creates one of the strands via linear amplification, whereas symmetric PCR produces dsDNA. The primer concentration ratio used in the reaction determines the amount of double-stranded DNA in the final product. As a result, aPCR is advantageous when just one of the two complementary strands has to be amplified in sequencing, hybridization probing, and DNA-aptamer selection (SELEX-Systematic Evolution of Ligands by Exponential enrichment). In this method, polymerase chain reaction is carried out with unbalanced molar ratios of forward and reverse primers. Asymmetric PCR produces the desired ssDNA in two phases. The first phase involves dsDNA templates' exponential amplification, and in the second phase of linear amplification is used for producing ssDNA. It must be optimized by varying the primer ratios, number of cycles, and template concentrations in accordance with the study being conducted. As a result, a single protocol without optimization could not be possible to use (Hao *et al.*, 2020; Heiat *et al.*, 2017: Yoo *et al.*, 2021).

Terminal deoxynucleotidyl transferase (TDT)

TdT is a DNA polymerase X enzyme that inserts extra nucleotides into the gene sequences encoding T and B cell receptor sequences in order to enhance the number of epitopes recognized by the cells. As a result, long oligonucleotides can be made with it. TdT is a technique for adding deoxynucleoside triphosphates (dNTPs) to the 3' end of a single-stranded DNA (ssDNA) primer that lacks a template strand. "Reversible termination" is the strategy employed in this procedure to assure that a single nucleotide is added to each growing chain in a given reaction (Nick McElhinny *et al*., 2005; Barthel *et al.*, 2020). The TDT's template-independent polymerization and low substrate specificity for nucleotides make the TdT-based technique compatible with a wide range of modified nucleotides with simple purification processes because the template strand does not need to be removed. Other

than the above mentioned methods, Exonuclease III, lambda exonuclease, and T7 exonuclease are three enzymes that hydrolyze duplex DNA. The PCR product is digested with type II restriction enzymes to create four-base or longer 3′-protrusions because exonuclease III only hydrolyzes dsDNA in the 3′ to 5′ directions and is inactive on ssDNA. T7 exonuclease and exonuclease III may digest both strands of dsDNA at the same place, resulting in two shorter DNA fragments half the length of the dsDNA template. Lambda exonuclease preferentially hydrolyzes the 5′ phosphoryl strand of dsDNA while having very little activity for the 5′ hydroxyl strand of native and denatured (Hao *et al.,* 2020).

Alternative enzymatic methods

Rolling Circle Amplification (RCA)

RCA allows for the isothermal amplification of lengthy ssDNA utilizing a ssDNA template, a single primer, deoxynucleotide triphosphates (dNTPs), and a polymerase with strand displacement capabilities, such as Phi29, isolated from the *Bacillus subtilis* bacteriophage 29. Polymerase constantly adds nucleotides to the annealed primer, resulting in a lengthy ssDNA with a repeating sequence at a constant temperature, ranging from 30 to 37 °C (Dean *et al.,* 2001). As the polymerase returns to the template's origin, the old strand is replaced by the strand displacement activity, resulting in the formation of a new template for ongoing polymerization. After significant amplification, the desired sequence can be achieved by inserting a restriction site in the primer and performing enzyme digestion. Within 90 minutes, roughly 5 $x10^9$ copies of the 96 nt repetitions can be synthesized. RCA is a potent induction device that can achieve observable amplification of a single molecule as well as vast quantities of ssDNA on a micron scale (Yoo *et al.,* 2021).

Strand-displacement amplification (SDA)

This is another isothermal amplification method. Using a DNA polymerase activity with the *HincII* endonuclease it amplifies ssDNA via two steps. First, a pair of primers amplifies the dsDNA template and creates the *HincII* recognition site at the target sequence's 5’ terminal end. This can be resolved by applying a primer containing a restriction site to denatured ssDNA. *HincII* cuts the restriction site-containing strand of dsDNA, and DNA polymerase, such as a Klenow fragment, extends the primer strand. Polymerization takes place at the restriction site of 3’ end by nicking after the formation of a new strand, allowing for cyclic amplification (Shi C *et al.,* 2014).

Loop-mediated isothermal amplification (LAMP)

This method allows a particular DNA sequence to be amplified directly under isothermal conditions. LAMP amplifies DNA with excellent sensitivity, efficiency, and speed. To specifically identify six to eight distinct areas of the target gene, this approach uses DNA polymerase and a set of four to six primers, including inner and outer primers. All four primers are employed, however only the inner primers are used for strand displacement DNA synthesis later in the cycling process. The DNA polymerase used in this LAMP approach lacks exonuclease activity at the 5’-3’ position, resulting in the synthesis of ssDNA. LAMP can generate 109 copies of target DNA in less than an hour (Notomi *et al.,* 2000).

Bacteria based ssDNA synthesis

This synthesis is used for large-scale bacterial production of pure ssDNA using modified plasmids17 or helper M13-*Escherichia coli* strains in combination with phagemids (Veneziano *et al.,* 2018). Bacteriophages with ssDNA genomes, such as M13, fd, and f1, can replicate as plasmids within the cell, and phagemids can replicate as plasmids dependent on their backbones. It has been shown that plasmid rolling-cycle replication (RCR) generates circular ssDNA intermediates. For replication, these RCR plasmids only need three components: a gene encoding the initiator protein (Rep), a double-strand origin (dso), and a

single-strand origin (sso). In *Escherichia coli*, the RCR plasmid pC194 has been demonstrated to replicate successfully and produce circular ssDNA. The ability to generate ssDNA *in vivo* and use it for genome editing with reverse transcriptase has been discovered. However, this technology has only been used to synthesize short ssDNAs, with no reports of longer ssDNA synthesis or use (Farzadfard & Lu., 2014: Hao *et al.,* 2020).

APPLICATIONS OF SINGLE STRAND DNA

Sequencing, cloning, homology directed repair templating for gene editing, DNA-based digital information storage, aptamers, DNA robotics, and scaffolded DNA origami are a few of the applications for kilobase-length ssDNA in biological imaging, bio nanotechnology, and synthetic biology (Shepherd *et al.,* 2019: Nehdi *et al.,* 2020). Single-strand DNA is commonly used in nanostructure creation, and to make DNA origami nanoparticles. Short sequences of ssDNA, RNA, tiny peptides, or antibody fragments can be used as molecular recognition elements (MREs). Food safety, environmental monitoring, and health care are only some of the applications for portable ssDNA MRE-based biosensors (Shiratori *et al*, 2014). Single stranded DNA has resulted in therapies for previously untreatable hereditary illnesses ranging from neurological to muscular to metabolic, as well as the production of vaccines. For instance, Gapmer ASOs, Inotersen, Mipomersen, and Nusinersen, among others (Scharner & Aznarez., 2021).

CONCLUSIONS

This study detailed the typical techniques utilized for ssDNA synthesis, including chemical, enzymatic, and bacteria-based procedures. Chemical synthesis provides a powerful tool for the synthesis of custom ssDNA sequences without templates. Enzymatic methods produce ssDNA fragments ranging in size from several hundred base pairs to more than 10 kb. However, some enzymatic methods like aPCR require additional purification steps. Bacteria-based synthesis produces miligram-scale yields of ssDNA which could be boosted further using bioreactors for large scale productions. Each procedure and alteration has different benefits and downsides, but as technology advances, ssDNA will be synthesized more efficiently and used in a variety of applications, providing more benefits to humanity.

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