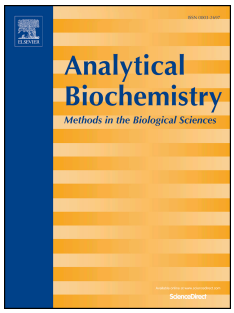
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5′-DMT-PROTECTED double-stranded DNA: Synthesis and competence to enzymatic reactions

Veronika V. Shchur, Yuliya P. Burankova, Andrei I. Zhauniarovich, Yaraslau V. Dzichenka, Sergei A. Usanov, Aleksei V. Yantsevich

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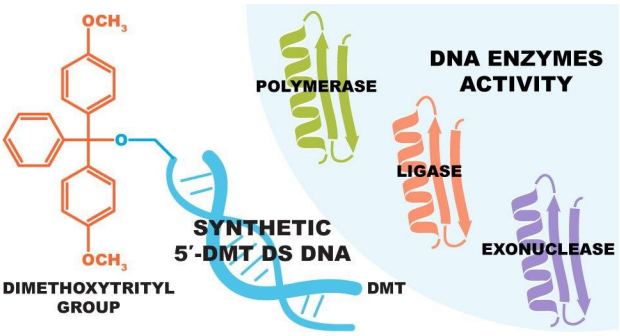
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Conceptualization	Ideas; formulation or evolution of overarching research goals and aims	Veronika Shchur Aleksei Yantsevich
Methodology	Development or design of methodology; creation of models	Veronika Shchur, Yuliya Burankova, Andrei Zhauniarovich
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data	Yaraslau Dzichenka
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection	Veronika Shchur, Yuliya Burankova, Andrei Zhauniarovich
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Writing - Review & Editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages	Veronika Shchur Aleksei Yantsevich
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5′-DMT-PROTECTED DOUBLE-STRANDED DNA: SYNTHESIS AND COMPETENCE TO ENZYMATIC REACTIONS

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The functionalization of 5′-OH group in nucleic acids is of significant value for molecular biology. In the current work we discovered that acid-labile 4,4′-dimethoxytrityl protecting group (DMT) of oligonucleotides (ONs) is stable under PCR conditions and does not interfere with activity of DNA polymerases. So application of 5′-DMT-protected ONs could allow producing both symmetric and asymmetric 5′-DMT-blocked double-stranded DNA (dsDNA) fragments. We demonstrated that the presence of thiol compounds (mercaptoethanol and dithiothreitol) in PCR mixture is undesirable for the stability of DMT-group. DMT-ONs can be successfully used during polymerase chain assembly of synthetic genes. We tested 5′-DMT dsDNA in blunt-end DNA ligation reaction by T4 DNA ligase and found that it could not be ligated with 5′-phosphorylated DNA fragments, namely linearized plasmid vector pJET1.2/blunt. Possible reason for this is steric hindrance created by bulky and rigid DMT-group, that prevents entering enzyme active site. We also demonstrated that 5′-DMT modification of dsDNA does not affect activity of T5 5′,3′-exonuclease towards both ssDNA and dsDNA. Further screening of the exonucleases, sensitive to 5′-DMT-modification or search of ways to separate long 5′-DMT-ssDNA and 5′-OH-ssDNA could allow finding application of 5′-DMT-modified oligo- and polynucleotides.

KEYWORDS: oligonucleotide, 4,4′-dimethoxytrityl protection group, blunt-end ligation, synthetic gene, cloning.

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Introduction

Functionalization of a 5′-hydroxyl group of nucleic acids founds many applications in DNA technology [1], because it allows following and detecting labeled molecules and to control specificity of the enzymes.

For example, stability, uptake and immunogenicity of therapeutic siRNA and mRNA could be improved by structural modification of the 5′-end [2]. Phosphorylation by T4 polynucleotide kinase is used for tagging of oligonucleotides (ONs) before sequencing, PCR and ligation [3]. In the latter case, selective ligation of phosphorylated DNA fragments into the dephosphorylated vector happens (Fig. 1-1) [4].

Asymmetric labeling of 5′-end of DNA is of particular importance for the generation of "gene-size" single-stranded DNA (ssDNA) [5]. Currently, ssDNA is used as a donor template for knocking experiments requiring DNA fragments with length more than 500 bases [6]. ssDNA repair templates have recently been shown to have advantages over dsDNA. Among them are a very low tendency to random genome integration [7], low cytotoxic response and absence of expression from nonintegrated templates, that facilitates correct identification of genome edited clones. At the same time, the usefulness of long ssDNA templates is limited due to the difficulty and cost of producing them.

One solution to generate ssDNA templates is to convert a dsDNA PCR product into ssDNA by selectively digesting either the sense or the antisense strand. Selective enzymatic degradation of one strand of DNA could be achieved by labeling of 5′-OH (for example, by phosphorylation), as shown on Fig.1-2. Such an idea was realized using a strandase enzyme-based approach from Takara Biosciences. Purely enzymatic synthesis to generate ssDNA greater than 15 kb using asymmetric PCR was reported [5].

Modifications of DNA 5′-ends was considered as a way to terminate non-template activity of DNA and RNA polymerases [8].

So, investigation of 5′-OH modification influence on enzymes interacting DNA ends could be of great interest to molecular biology.

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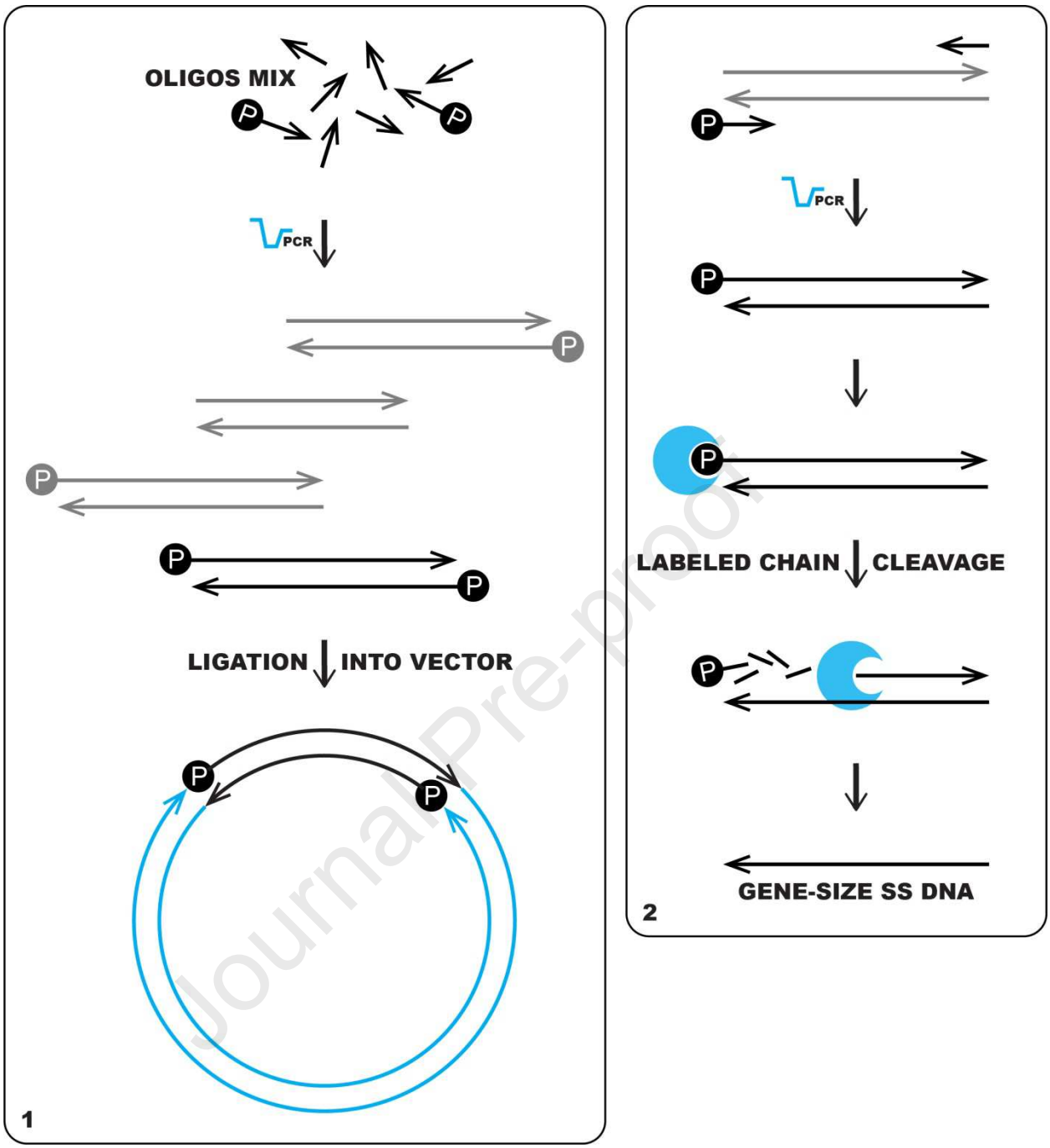


Figure 1. Exploring 5′-OH modifications of nucleic acids in molecular biology. 1. Selective ligation of full-length sequence from a mixture of possible synthetic gene assembly products. Only flanking ONs are phosphorylated, vector does not have 5′-phosphate and could not be self-ligated. 2. The general way to obtain gene-size ssDNA by asymmetrically labeled ONs. Currently used phosphorylating and splitting kit used to destroy phosphorylated DNA strand.

Owing to the low reactivity of the 5′-hydroxyl, its modification with the required functional group is not easily achieved without side reactions at the nitrogen base level. So in order to functionalize 5′-end of dsDNA such modification should be introduced on the stage of ON synthesis [1] . Subsequent PCR will give 5′-functionalized dsDNA fragment.

During ON synthesis orthogonal protection systems must be used, most of which are based on acid-labile 4,4′-dimethoxytrityl (DMT) protective group. Since its introduction as acid-labile protecting group for deoxyribonucleotide chemistry, DMT becomes standard for the

protection of the 5′-OH group [9]. Usually deblocking is realized by 2–3% trichloroacetic acid solution in toluene or dichloromethane. After last base coupling, DMT could be removed ("DMT-OFF" synthesis) or left ("DMT-ON" synthesis) on the ON.

Hydrophobic properties of DMT allows blocked ON to be retained on reverse-phase sorbents (presumably C18) in the presence of trimethylamine as an ion-pairing reagent. So fast and effective procedures for ON purification based on reverse-phase solid-phase extraction cartridges were developed [10]. During purification, DMT is removed by washing with 2% trifluoroacetic acid. Earlier we demonstrated the ability of ZipTip microextractors to purify and dose ON pools for subsequent gene assembly. [11]

So DMT could be considered as intrinsic modification arising from phosphoamidite synthesis and its relevance to different enzymatic reactions could be of interest in some cases. This is expecially true for enzymes interacting with DNA ends. Among such enzymes should be listed 5′, 3′-exonucleases, DNA-ligases, DNA-polymerases and so on.

Search of 5′-end modification that could inhibit non-template nucleotide addition (NTA) by DNA polymerases in primer extension [8b] demonstrated that 5′-DMT modification of template ON showed no significant termination effect on enzymatic DNA synthesis performed by different DNA polymerases during primer extension. But nevertheless the modification had some interesting but unexplained effects: the NTA activity of KOD(exo-) and Deep Vent (exo-) increased with the DMT-modified template. In all cases primer extension with DMT-modified template furnished also incompletely extended products as well, indicating that this modification hampers the polymerase to reach the end of the template strand [8b].

The application of DMT-ONs in standard PCR (dsDNA template and 2 primers) and during gene assembly was not reported earlier. Thus effectiveness of DMT-ONs for PCR amplification or for gene assembly remains unknown as well as the fate of the protective group after PCR.

Further investigations of influence of bulky, lipophilic DMT-group on activity and specificity of DNA-enzymes, especially 5′, 3′-exonucleases, will allow outlining the prospects for practical application of 5′-DMT dsDNA.

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Materials and methods

Materials

5′-DMT-dA^{Bz}-, 5′-DMT-dT-, 5′-DMT-dC^{Ac}-, and 5′-DMT-dG^{iBu}-CPG with a pore size of 1000 Å and loading capacity of 42–45 μmol/g were from BioAutomation (Irving, TX). DMT-dA^{Bz}, DMT-dG^{iBu}, DMT-dC^{Bz}, and DMT-dT phosphoramidites; dichloromethane; acetic anhydride; lutidine; 1-methylimidazole; trifluoroacetic acid; and methanol were from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile with low water content (0.002%) was from Fisher Chemical (Waltham, MA). 5-(Ethylthio)-1H-tetrazole (Berlin, Germany). CloneJET PCR Cloning Kit, 10x DreamTaq buffer (for DreamTaq Polymerase) and dNTP mix were from Thermo Scientific (Waltham, MA). Q5 High-Fidelity DNA polymerase, 100 bp and 1 kb Plus DNA ladder were purchased from New England Biolabs (Ipswich, MA). 32% ammonia solution was from Roth (Karlsruhe, Germany). Triethylamine was from Alpha Aesar (Kandel, Germany).

General Methods

A_{260} and electronic spectra for ON and dsDNA solutions were recorded with NanoDrop UV-Vis spectrophotometer from Thermo Scientific (Waltham, MA).

PCR based gene assembly, ON annealing and heating before HPLC of ON were performed in thermocycler DNA Engine (Bio Rad Laboratories, Hercules, CA).

Agarose gel electrophoreses were performed in Model H4 Horizontal Gel Electrophoresis Apparatus (Gibco BRL, Life Technologies, Carlsbad, CA). Visualization of gel electrophoresis was made using Azure Biosystems c280 gel documentation system (Azure Biosystems, Dublin, CA).

ON preparation

Common ONs were synthesized, deprotected and purificated as described in [12]. DMT-protected ONs were purified by the same procedure, except the detritylation step with trifluoroacetic acid: after washing with 11% ACN in 0.1 M TEAAc (pH 8.0) buffer ONs were eluted in 20% ACN and vacuum evaporated. In order to minimize detritylation, 50 μl of concentrated aqueous ammonia was added to eluate before evaporation.

HPLC-MS analysis

Analytical HPLC analysis with a diode array detector (DAD) and MS detector was performed on an Agilent 1290 HPLC system according to the application note “Characterization of Synthetic Oligonucleotides Using Agilent LC/MS Systems” from Agilent (section “LC/MS Parameters for Agilent 6520 Accurate Mass QTOF Platform”).

Here we present distinctions of our method from Agilent’s one. We used column Poroshell 120 EC-C18 (2.1 x 100 mm, sorbent particle diameter – 2.7 μm). Phase A was 200 mM hexafluoroisopropanol in 8.6 mM TEA(pH 7.7). Concentration of ON solution was 0.2 mg/ml. The detection wavelength of DAD detector 1260 DAD VL (Agilent) was set to 260 nm. High-resolution mass spectra were registered with a Q-TOF 6550 high-resolution mass spectrometer (Agilent) in the ESI$^-$ mode. Mass spectra of ONs were deconvoluted by BioConfirm module of MassHunter software.

Design and assembly of synthetic DNA fragment

As a model synthetic gene we used 312 bp sequence:

```
  1 CGCCATATGTTCCCGGACGGTGAATTCACCATGCAGGGTTGCCCGGAATGCAAACTGAAA
 61 GAAAACAAATACTTCTCTAAACCGGACGCTCCGATCTACCAGTGCATGGGTTGCTGCTTC
121 TCTCGTGCTTACCCGACCCCGGCTCGTTCTAAAAAAACCATGCTGGTTCCGAAAAACATC
181 ACCTCTGAAGCTACCTGCTGCGTTGCTAAAGCTTTCACCAAAGCTACCGTTATGGGTAAC
241 GTTCGTGTTGAAAACCACACCGAATGCCACTGCTCTACCTGCTACTACCACAAATCTTAA
301 TAAGGATCCGCG
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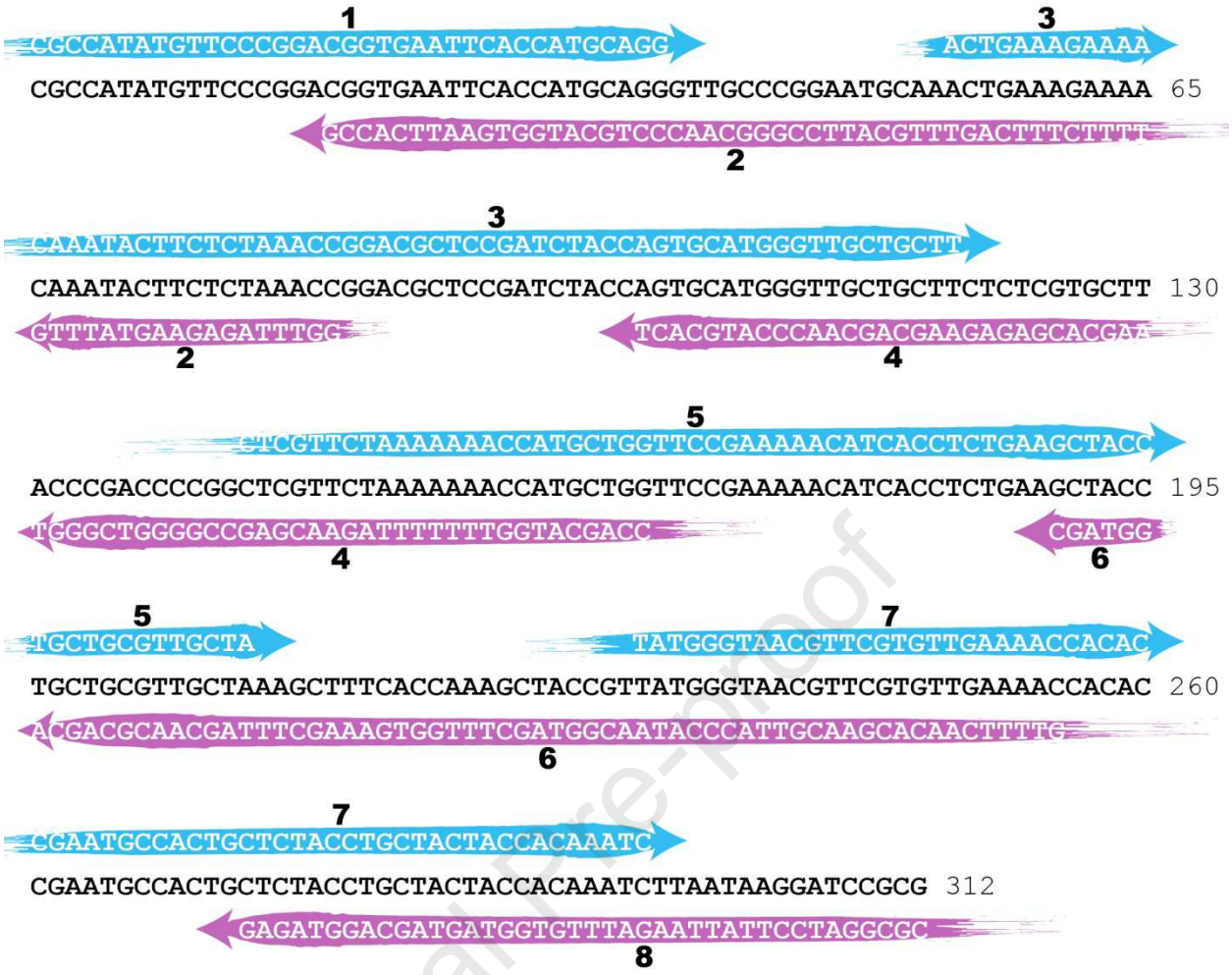


Figure 2. 312 bp DNA sequence was obtained from eight ONs by PCA. Design was generated by DNAWorks software [13]. Parameters for DNAWorks: ON Size – 66 nt, Annealing Temp – 65 °C.

Table 1. List of ONs used for enzymatic assembly of synthetic DNA fragment.

№	ON	Length	Position in sequence
1	CGCCATATGTTCCCGGACGGTGAATTCACCATGCAGG	37	1-37
2	GGTTTAGAGAAGTATTTGTTTTCTTTCAGTTTGCATTCCGGGCAACCCTGCATGGTGAATTCACCG	66	18-83
3	ACTGAAAGAAAACAAATACTTCTCTAAACCGGACGCTCCGATCTACCAGTGCATGGGTTGCTGCTT	66	54-119
4	CCAGCATGGTTTTTTTAGAACGAGCCGGGGTCGGGTAAGCACGAGAGAAGCAGCAACCCATGCACT	66	101-166
5	CTCGTTCTAAAAAAACCATGCTGGTTCCGAAAAACATCACCTCTGAAGCTACCTGCTGCGTTGCTA	66	143-208
6	GTTTTCAACACGAACGTTACCCATAACGGTAGCTTTGGTGAAAGCTTTAGCAACGCAGCAGGTAGC	66	190-255
7	TATGGGTAACGTTCGTGTTGAAAACCACACCGAATGCCACTGCTCTACCTGCTACTACCACAAATC	66	231-296
8	CGCGGATCCTTATTAAGATTTGTGGTAGTAGCAGGTAGAG	40	273-312

ON for gene assembly were purified individually as was described earlier, every ON was divided into 2 parts, one of them was purified to save DMT-group, another – to get rid of it. ON's mixes was made before PCR assembly. The concentrations of ONs in assembly mixture were 20 nM for ONs 2–7 and 150 nM for flanking ONs 1 and 8. Q5 High-Fidelity DNA polymerase and PCR cycle based on the suppliers recommendations (annealing temperature – 60 °C) were used for assembly. To control synthetic fragments formation we used electrophoresis in 1% agarose gel. We planned to insert 312 bp fragment into cloning vector. In order to easily control efficient insertion by restriction analysis before cloning we amplified

fragment with primers (with and without DMT), designed to introduce two flanking EcoRV sites (bold type) after insertion into vector:
5′-**ATC**CGCCATATGTTCCCGGACGGT-3′,
5′-**ATC**CGCGGATCCTTATTAAGATTTGTGGTA-3′.

Generation of short DMT-protected dsDNA fragment by direct ON annealing

In order to obtain short DMT-blocked dsDNA fragment (65 bp), we synthesized two complementary ONs (Table 2).

Table 2. List of ONs used for annealing in order to get a short DMT-protected dsDNA fragment.

Name	Sequence (5′–3′)
DNA65_1 sense	**ATC**TTCTCAATTGACCTTGCGAATTCCCCCCCATGGTAGCTCGAAGCTTATCACCTGCTTTC**GAT**
DNA65_2 antisense	**ATC**GAAAGCAGGTGATAAGCTTCGAGCTACCATGGGGGGGAATTCGCAAGGTCAATTGAGAA**GAT**

ONs were designed to introduce two flanking EcoRV restriction sites (bold type in table 2) after insertion to pJET1.2/blunt in order to allow easy control of fragment insertion by restriction analysis.

ONs were ammonia deprotected and purified in “DMT-ON” mode as stated above. Purified ON solutions were aliquoted and vacuum evaporated for storage. 50 µl of aqueous ammonia were added to the ON solution before evaporation in order to prevent the loss of DMT-group.

For dsDNA preparation, we used 100 pmol/µl solutions of DMT-ONs in deionized water.

For annealing of ONs we used the following program: 3 min at 95 °C then cooling to 20 °C at a rate of 0.1 °C per second.

For 5′-OH dsDNA 15 µl of water, 3.75 µl of each DMT-ON 100 pmol/µl solution were mixed in PCR tube. PCR tube was placed in thermocycler and heated and cooled according to the annealing program described above. At this stage DMT-ONs loss DMT group. Then 2.5 µl of 10× DreamTaq buffer was added, mixed and repeatedly annealed.

In order to get 5′-DMT dsDNA 15 µl of water, 3.75 µl of each DMT-ON solution and 2.5 µl of 10× DreamTaq buffer were mixed in PCR tube. The annealing program was repeated twice in order to ensure equal conditions with the previously described procedure for 5′-OH dsDNA generation.

Concentration of each ON after mixing with water and buffer was 15 pmol/µl. So after annealing of ONs we had 5′-OH and 5′-DMT dsDNA with concentration 15 pmol ends/µl. Before ligation into pJET1.2/blunt (0.05 pmol ends/µl) solutions of 5′-OH and 5′-DMT dsDNA was 100x diluted. So equal volumes of dsDNA and vector could be mixed to get molar ratio 3:1.

To control duplex formation we used electrophoresis in 4% agarose gel.

Blunt-end ligation into plasmid vector and transformation

For blunt-end ligation we used commercially available CloneJET PCR Cloning Kit from Thermo Scientific. Ligation was performed according to supplier recommendation. The molar ratio of dsDNA fragment to vector was 3:1. We used 5 µl of ligation mix per 100 µl of chemically competent *E. coli* cells (strain DH5α).

Ligation was controlled by electrophoresis of ligation mixes in 1.5% agarose gel, by PCR of ligation mixes with vector’s primers to evaluate the presence of insert and its length, by transformation of competent cells. In PCR mix we used expected for complete ligation 1 ng of

vector with insert, 0.4 µM of each pJET sequencing primers and other reagents in concentrations optimal for DreamTaq polymerase, PCR cycle for colony PCR from pJET supplier.

For ligation we used short (65 bp) synthetic dsDNA obtained by ONs annealing and amplification product of a synthetic gene (312 bp) obtained by polymerase chain assembly as described above.

Sensitivity to 5′, 3′-exonuclease activity

In order to compare the sensitivity of 5′-OH-dsDNA, 5′-DMT dsDNA and 5′-DMT-ONs to T5 5′, 3′-exonuclease activity, we mixed 41.7 µl H_2O, 5 µl 10× buffer N4, 3 µl of DNA solution (to get 15 pmol/µl for each chain) and 0.3 µl T5 5′, 3′-exonuclease (10 U/µl) in PCR tube and incubate 30 min at 37 °C.

For the analysis, we used electrophoresis in 3% agarose gel.

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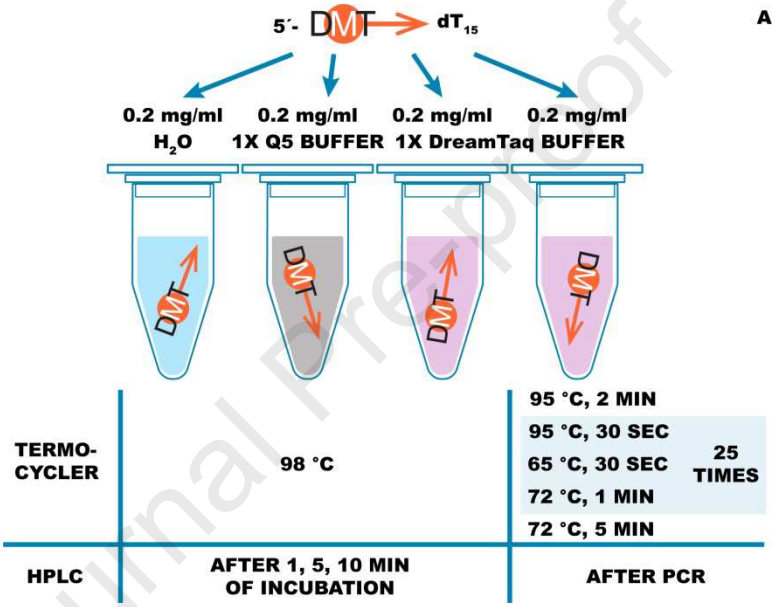
RESULTS AND DISCUSSION

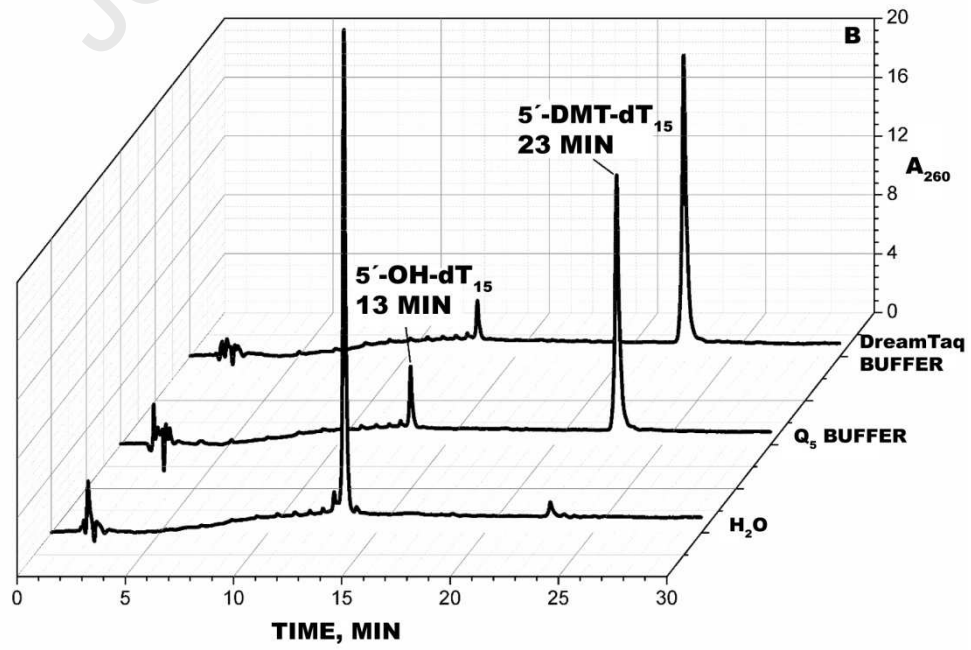
Heat-activated detritylation of ONs

In order to simplify stability investigation of DMT protecting group, we used 5′-DMT-dT_{15} ON as a model. 5′-DMT-ON could be easily separated from 5′-OH ON by ion-pairing reversed phase HPLC analysis, thus allowing to monitor detrytilation process. PCR cycle was simulated in the simplest case as heating at 98 °C as this is a maximum temperature used in PCR. In a more representative experiment PCR cycle recommended for DreamTaq polymerase was used (Fig. 3A).

HPLC analysis demonstrated that in deionized water more than 90% of ON converts to unprotected form during short 5 min heating at 98 °C (Fig. 3B–D, Table 2).

Commercially available PCR buffers tested by us demonstrated a significant effect in stabilizing DMT-ON.





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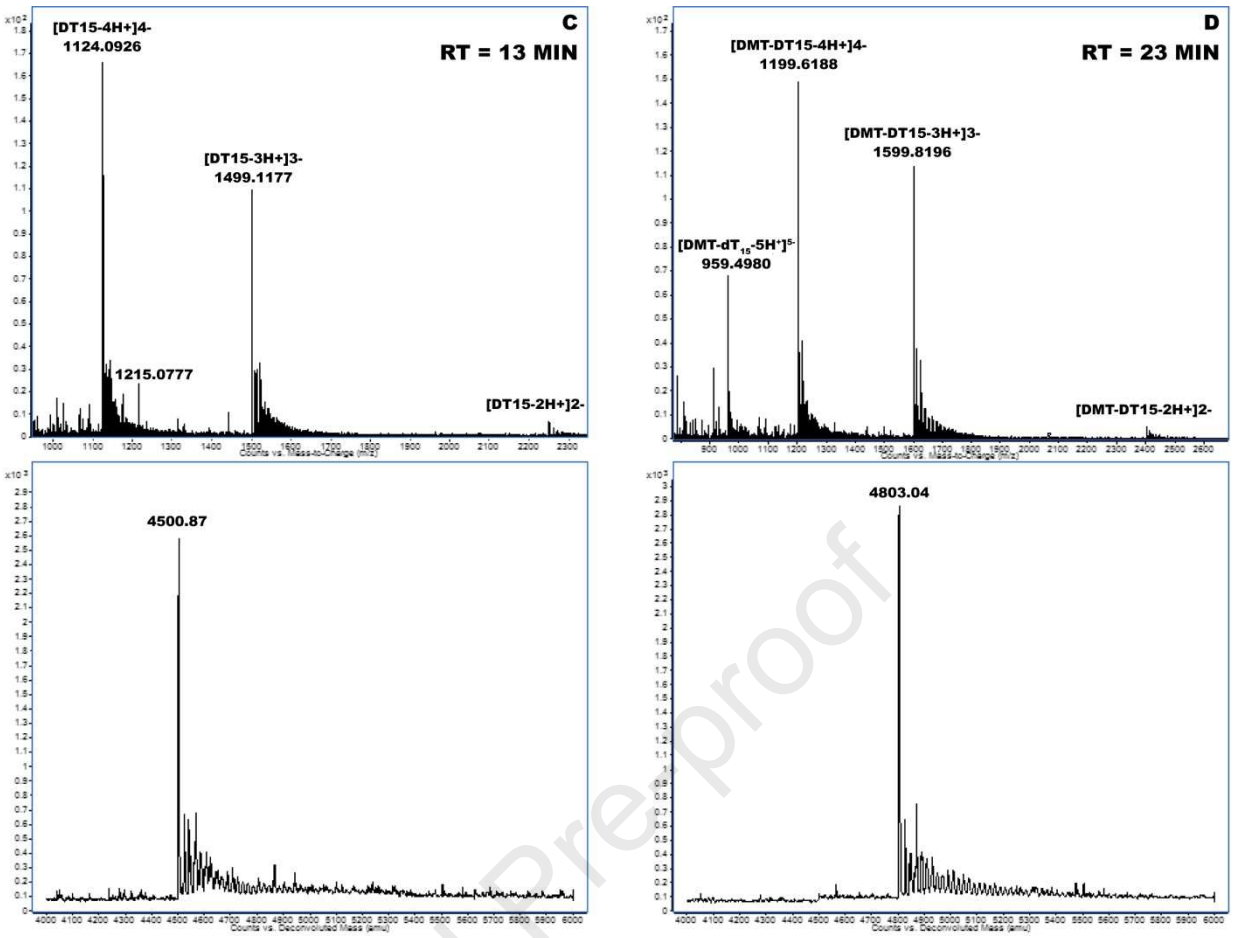


Figure 3. Experiment scheme to study of 5′-DMT-dT_{15} stability in PCR conditions (A). Heat-activated detritylation of DMT-dT_{15} ON in water, Q5 and DreamTaq DNA polymerase buffers after heating at 98 °C during 5 min as observed by HPLC with detection at 260 nm (B). Mass-spectra of peaks eluted near 13 and 23 min presented at C and D and correspond to 5′-OH-dT_{15} and 5′-DMT-dT_{15} ONs.

Even 15 min of heating at 98 °C in 1× DreamTaq polymerase buffer allows saving more than 83% DMT-protected dT_{15} (Table 2). Buffer for Q5 polymerase showed worse results – about 72% of DMT-protected dT_{15} (Table 2).

Analysis of PCR buffer composition (Table 3) demonstrated, that tested buffers differ in pH values: Q5 buffer (pH 9.3) has a higher pH than DreamTaq buffer (pH 8.3).

Table 2 – Content of 5′-DMT-dT_{15} ON in different solutions after heating at 98 °C.

Solvent	Content of 5′-DMT-dT_{15}		
	1 min	5 min	15 min
Water	44.5%	7.43%	0%
1× Q5 DNA polymerase buffer	-	88.4%	71.5%
1× DreamTaq DNA polymerase buffer	-	93.5%	83.69%
25 PCR cycles in 1× DreamTaq DNA polymerase buffer	79.5%		

Table 3 – Commercially available PCR buffers (supplier information).

DNA polymerase	pH	1× buffer composition	Supplier
Taq	pH 8.3 (25 °C)	10 mM Tris-HCl 50 mM KCl 1.5 mM $MgCl_2$ 0.01% gelatin	Sigma-Aldrich
Pfu	pH 8.8 (25 °C)	20 mM Tris-HCl 10 mM KCl 10 mM $(NH_4)_2SO_4$ 2 mM $MgSO_4$ 1.0% TritonX-100 1 mg/ml nuclease-free BSA	Promega
Pwo	pH 8.85 (20 °C)	10 mM Tris-HCl 25 mM KCl 5 mM $(NH_4)_2SO_4$ 2 mM $MgSO_4$	Roch
Tth	pH 8.9 (25 °C)	10 mM Tris-HCl 1.5 mM $MgCl_2$ 0.1 M KCl 500 μg/ml BSA 0.5% Tween 20	Sigma-Aldrich
Q5	pH 9.3 (25 °C)	25 mM TAPS-HCl 50 mM KCl, 2 mM $MgCl_2$ 1 mM β-mercaptoethanol	NEB
Phusion	pH 9.3 (25 °C)	25 mM TAPS-HCl, 50mM KCl 2 mM $MgCl_2$ 1 mM β-mercaptoethanol	NEB

Given the nature of the DMT group, elevated pH should further stabilize DMT-ON, but in the real experiment, we obtained the opposite result. This contradictory result could be explained by the presence of β-mercaptoethanol in the Q5 DNA polymerase buffer.

Unwanted retritylation, that can occur through the reversal of the equilibrium generated by nonaqueous detritylation, between the colored cationic species and the DMT-ON was reported earlier[14]. Allowing such equilibrium under heating, we could propose that such a strong nucleophile as a sulfur atom of mercaptoethanol could be a good acceptor of trityl-cation and could shift the balance to the side of the 5′-OH ON formation.

Synthetic DNA assembly from 5′-DMT-ONs

Superimposed structures of 5′-phosphorylated and 5′-DMT-modified dsDNA (Fig. 4) suggest that bulky, hydrophobic DMT group could change the specificity of enzymes that recognize DNA ends and catalyze DNA ends modification reactions. So in order to check some promising reactions, we proposed three ways of generating of 5′-DMT-modified dsDNA: annealing of 5′-DMT-ONs for short sequences (<100 bp), polymerase chain assembly by Stemmer approach [15] from 5′-DMT-ONs for middle size sequences (<500 bp), and amplification of target sequence with 5′-DMT-primers for DNA with length more than 300 bp.

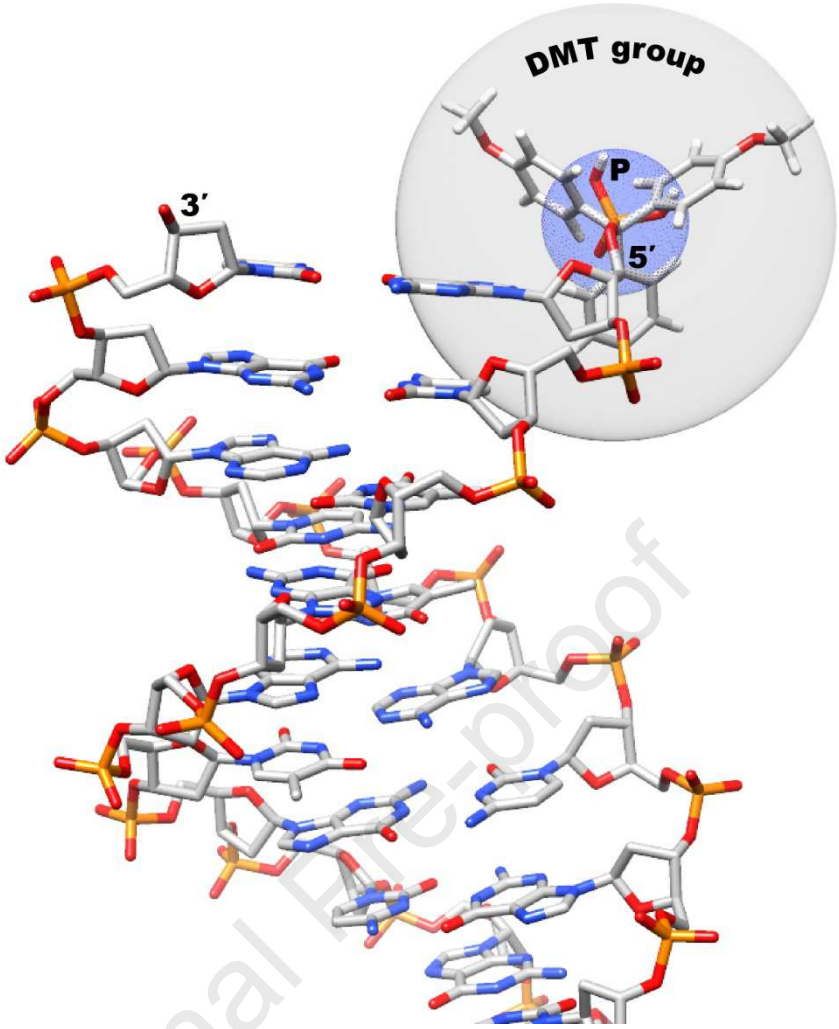


Figure 4. Superimposed structures of 5′-phosphorylated and 5′-DMT-modified dsDNA. General volumes occupied by phosphate and DMT are shown as blue and gray spheres respectively. Molecular graphics and analyses performed with UCSF Chimera [16].

The result of 312 bp synthetic DNA fragment assembly from 8 ONs is shown in Fig. 5. PCA from all DMT-ONs, common ONs, or from inner DMT-ONs and outer ONs showed no differences as observed in 1% agarose gel electrophoresis.

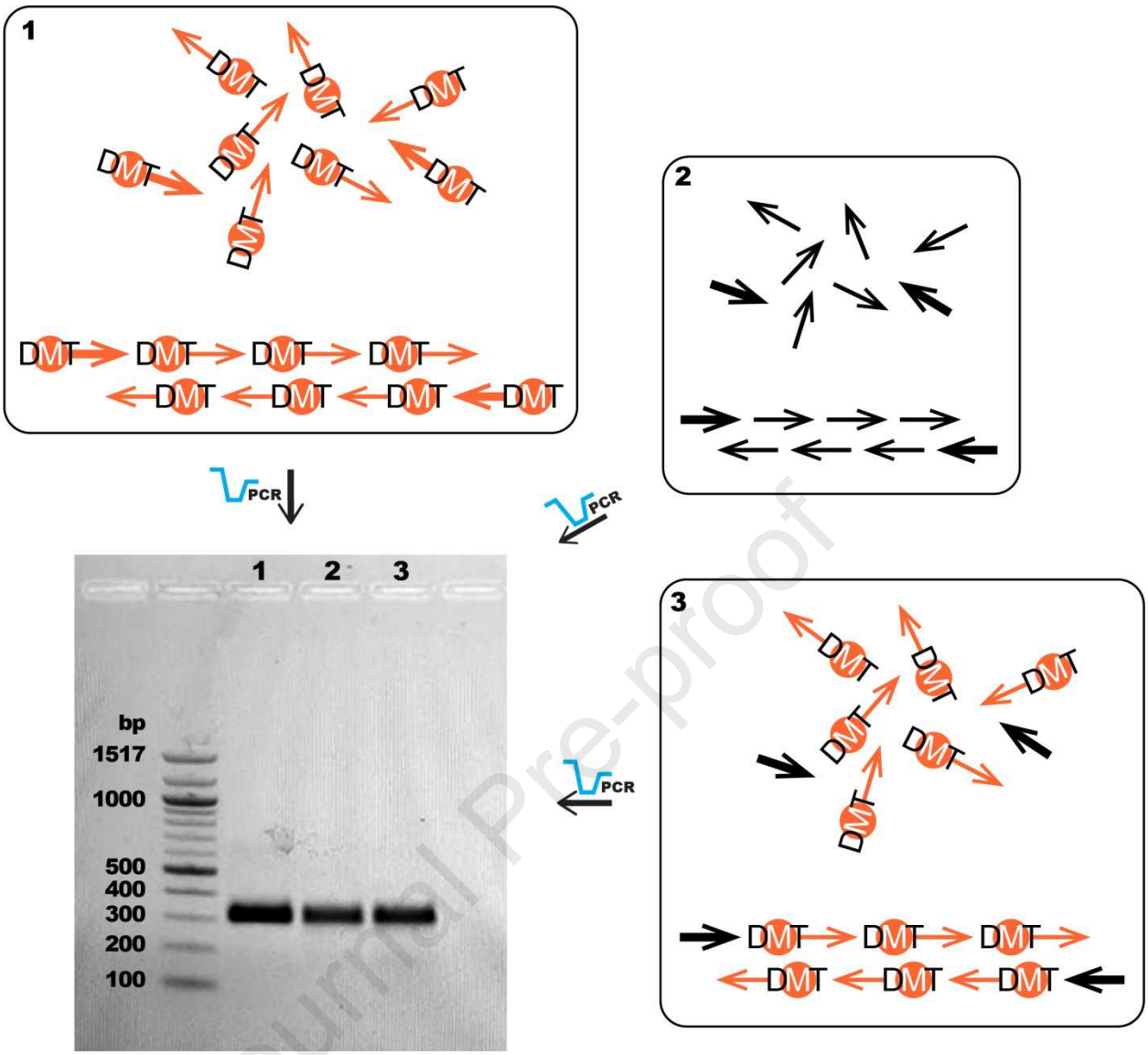


Figure 5. Agarose gel electrophoresis (1%) of "one-pot" polymerase chain assembly of 312 bp synthetic DNA fragment from 8 ONs. The concentrations of ONs in assembly mixture were 20 nM for ONs 2–7 and 150 nM for flanking ONs 1 and 8. 1. Gene assembly from DMT-ONs. 2. Assembly from deblocked ONs. 3. Assembly from inner DMT-ONs and flanking deblocked ONs.

Generation of short DMT-protected dsDNA fragment agarose electrophoresis in 4% gel showed that 5′-DMT dsDNA had lower electrophoretic mobility as compared to natural DNA (Fig. 6).

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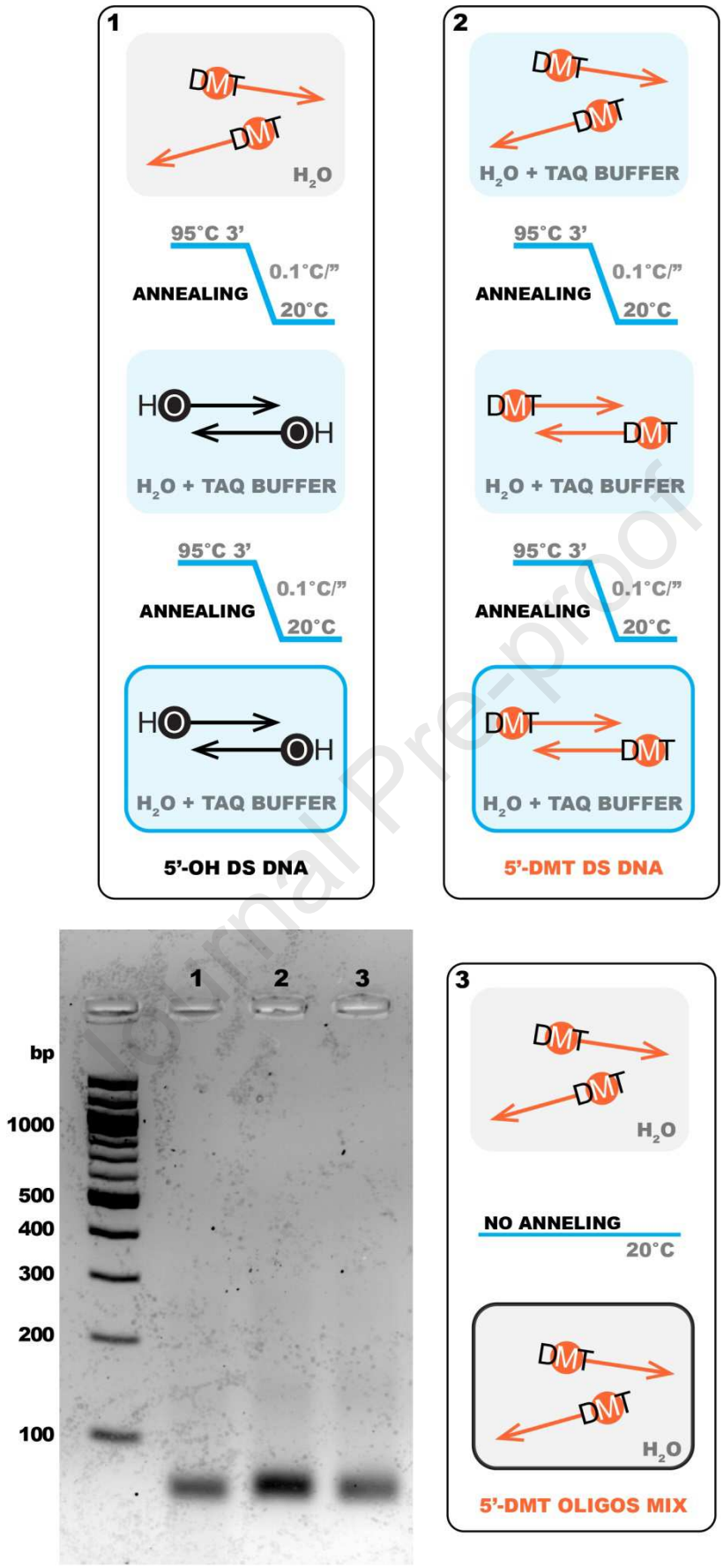


Figure 6. Agarose gel electrophoresis (4%) of dsDNA fragment obtained by ON annealing. 1. 5′-OH dsDNA. 2. 5′-DMT dsDNA. 3. ON mixture without annealing.

Blunt-end ligation of 5′-DMT dsDNA

Non-phosphorylated DNA fragment (PCR-product or synthetic fragment originated from 5′-OH-ONs) could be ligated only with phosphorylated DNA fragments. Such ligation product restores single-strand nicks but if phosphorylated DNA is a vector, such a product could be used for bacteria transformation. Bacterial cell enzymes repair single-strand nicks of ligation product.

To test the ability of T4 DNA ligase to perform blunt-end ligation of 5′-DMT dsDNA fragments to a vector we used 5′-DMT dsDNA, obtained in different ways:

1. short fragment of 65 bp obtained by annealing of ONs,
2. fragment of 312 bp obtained by PCR amplification of synthetic gene using DMT-blocked primers.

Both fragments were flanked by sequences “ATC” and “GAT”, which allows to restore EcoRV recognition sites on the sides of the insert. So after ligation DNA fragment could be cut out with mentioned enzyme.

As phosphorylated fragment we used commercially available vector for blunt-ended cloning pJET1.2/blunt. This vector is a linear blunt-end DNA molecule, that being self-ligated recover the toxic gene Eco47I/T7, product of which could kill the bacterial cells under expression. So only plasmid with insert could give viable colonies on LB-agar containing ampicillin. The reason for this choice is in ability to monitor ligation products not only by direct observation with electrophoresis or selective amplification but also by bacterial transformation. Bacterial transformation efficiency could be more sensitive indicator, so as we could detect single molecule as a colony in Petri dish.

Analysis of ligation mixtures of short 65 bp fragment by agarose gel electrophoresis (Fig. 7) allowed us to confirm that it is not possible at a first glance to distinguish electrophoretically blunt-end ligation of pJET1.2/blunt with 5′-OH dsDNA and 5′-DMT dsDNA.

But still electrophoresis of both insert-vector ligation mixture (lane 3 and 4) differ from self-ligation mixture on lane 2.

Considering excess of insert (vector/insert ration is 1:3) and partially deblocked molecules due to spontaneous detrytilation the variety of ligation ways could make the electrophoresis picture and its interpretation rather difficult.

But the ability to produce viable clones of *E. coli* on LB medium with ampicillin is the property of only circular molecules with insert (self-ligated vector does not produce clones due to assembly of lethal gene). So transformation efficiency data obtained under the same conditions could be very sensitive criterion of insert ligation.

Another sensitive approach to detect correct assembly of vector-insert could be PCR-amplification of ligation product with vector’s primers (pJET1.2/blunt sequencing primers from cloning kit).

By the way amplification of two ligation mixtures give completely different pictures (Fig. 7). In lane 3 (insert without DMT) there is a band about 184 bp, which corresponds to circular vector with insert. In lane 4 (insert with DMT) there is no such band, there is only a band about 119 bp, which is seen in all 3 lanes and corresponds to self-ligated vector. So 5’-DMT on insert ends prevent its blunt-end ligation into cloning vector.

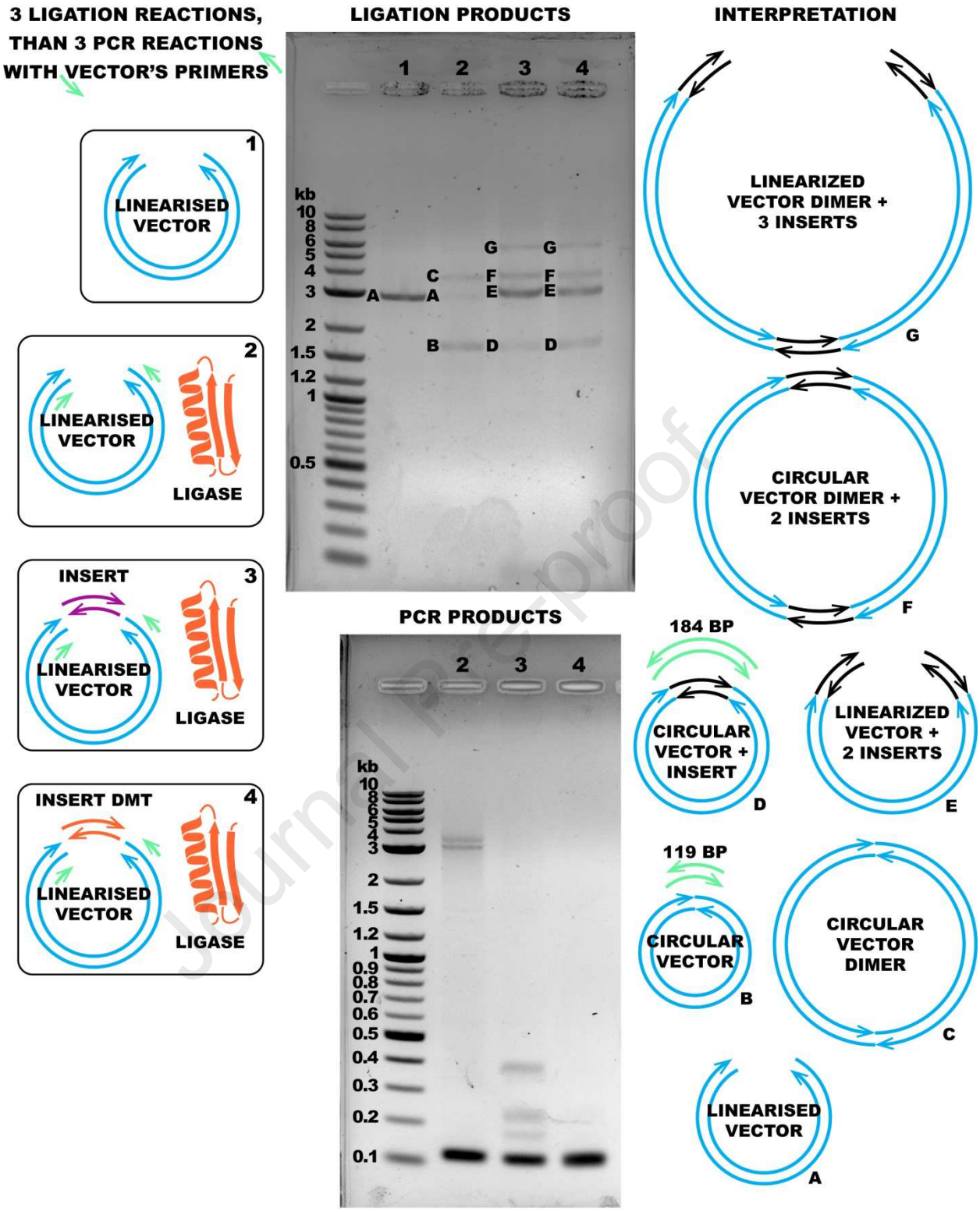


Figure 7. Ligation of test dsDNA fragment (65 bp) into pJET1.2/blunt cloning vector (upper electrophoresis) and PCR of ligation products with vector's primers (lower electrophoresis) 1. pJET1.2/blunt; 2. pJET1.2/blunt + T4 DNA ligase; 3. pJET1.2/blunt + 5′-OH-dsDNA + T4 DNA ligase; 4. pJET1.2/blunt + 5′-DMT dsDNA + T4 DNA ligase. Due to minor difference in length (65 bp) it is difficult to separate self-ligated vector and vector containing insert. But bacterial transformation and selective amplification of insert region can be a reliable evidence of the presence of vector, containing insert.

Methods for directed fusion of DNA-fragments is the base of modern synthetic biology. Currently methodology realized mainly by BioBrick Assembly Standard [17] allow to design and assemble large synthetic biological circuits from individual parts (BioBricks) with defined functions. All current BioBricks standards rely on restriction-enzyme assembly standard [18] and require usage of restriction enzymes, generating sticky ends that could be ligated by DNA-ligase.

The ability of DMT-modification to prevent blunt-end ligation creates background for directed linking of blunt-end DNA fragments, which could be realized step-by-step in cycle manner.

From this point of view lability of DMT becomes an advantage, because it could be easily removed in a cycle by acid treatment (like in ON synthesis) or heating under neutral pH.

So, based on mentioned above, we propose a hypothetical scheme for directed ligation of DNA fragments that could be realized on solid-support (Fig. 8).

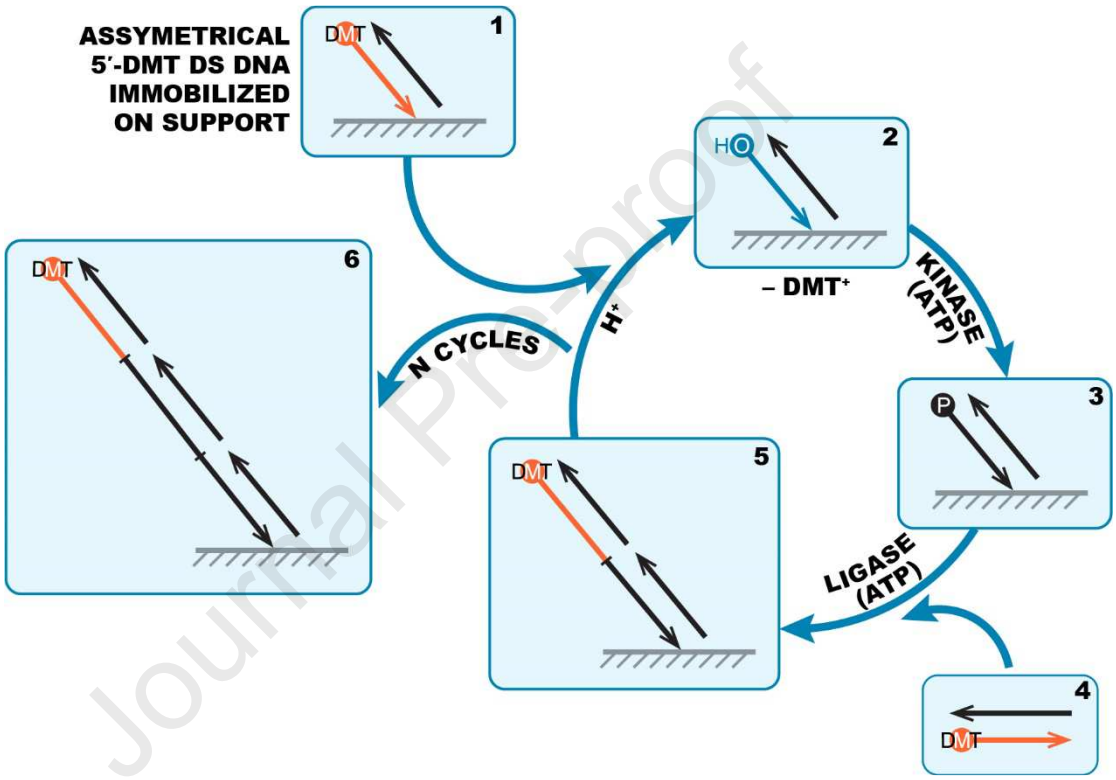


Figure 8. Hypothetical scheme for blunt-end solid-phase dsDNA ligation directed by asymmetrical 5′-DMT-labeling

E. coli transformation by ligation mixture

Transformation of competent *E. coli* cells (DH5α) by ligation mixtures showed positive results for 5′-DMT dsDNA and 5′-OH-dsDNA on both cases of synthetic gene and annealed ONs. Subsequent isolation of the corresponding plasmid from night culture and restriction analysis by EcoRV enzyme confirmed insert correctness. Transformation efficiency for 5′-DMT dsDNA-containing ligation mixture was about 10x lower than for 5′-OH-dsDNA (about 40 000 cfu per µg vector DNA for 5′-OH-dsDNA with the molar ratio of insert to vector equal to 3). The data obtained are in agreement with selective amplification of the incorporated region from ligation mixture (Fig. 7) and reliably confirm the impossibility to ligate 5′-DMT and 5′-phosphate DNA blunt-ends. Positive clones in case of 5′-DMT dsDNA insert could be explained by small population of 5′-OH-dsDNA, that arise from temperature induced detrytilation.

Exposure to 5′, 3′-exonuclease activity

The ability of 5′,3′-exonuclease to differentiate modification of 5′-end of DNA underlies “The Guide-it Long ssDNA Strandase Kit” that is designed to produce a long single-

stranded DNA (ssDNA) ON of up to 5 kb length for use as a repair template in knocking experiments using CRISPR/Cas9 or other genome editing tools. So discovery of new pairs of "modifier-exonuclease", which allow selective degradation of one strand preserving the other, could open alternative ways of ssDNA synthesis.

T5 exonuclease degrades DNA in the 5′ to 3′ direction and is able to initiate nucleotide removal from the 5′-termini or at gaps and nicks of linear or circular dsDNA. As follows from the Fig. 9, T5 exonuclease did not show specificity to structure of 5′-termini: 5′-DMT dsDNA is degraded by T5 exonuclease as well as 5′-OH dsDNA and 5′-DMT ONs.

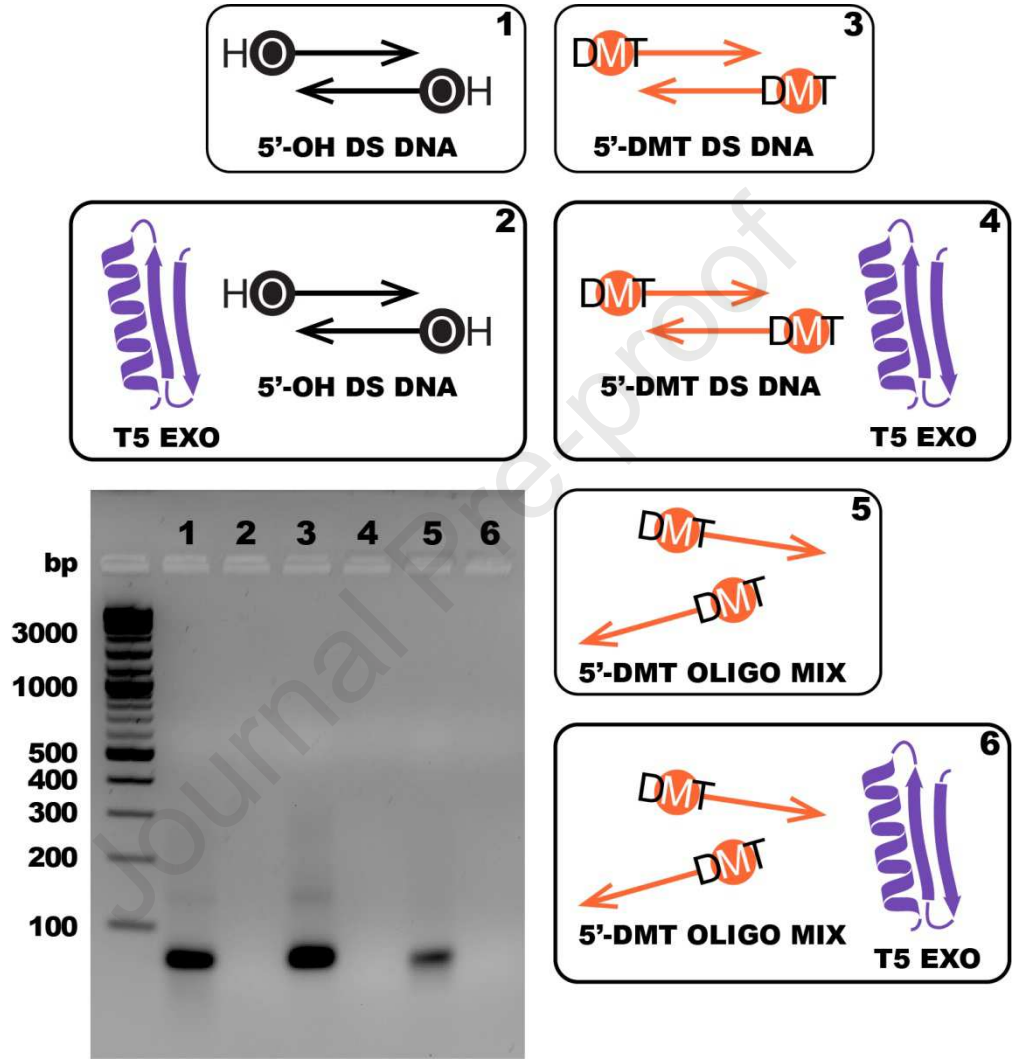


Figure 9. Exposure of 5′-OH dsDNA, 5′-DMT dsDNA and 5′-DMT ON mix to T5 5′, 3′-exonuclease. 1. 5′-OH dsDNA; 2. 5′-OH dsDNA + T5 exonuclease; 3. 5′-DMT dsDNA; 4. 5′-DMT dsDNA + T5 exonuclease; 5. 5′-DMT-ON mix; 6. 5′-DMT-ON mix + T5 exonuclease.

CONCLUSION

In the current study, we confirmed that the acid-labile DMT-protecting group primarily remains on ONs in PCR conditions, pH and content of PCR buffers being essential to preserve this modification.

We demonstrated that DMT-ONs could be efficiently used for PCR-amplification and as building blocks during gene assembly, allowing to generate symmetrically or asymmetrically 5′-DMT-protected dsDNA.

We showed that DMT-modification has little effect on DNA electrophoretic mobility in agarose gel, which could be observed only for short fragments.

We assumed that the bulky and rigid DMT protecting group could be a modifier of activity for enzymes that contact 5′-function of dsDNA, which could be useful for selective/directed ligation or generation of ssDNA.

In order to test functional competence of 5′-DMT dsDNA to the enzymatic reaction, we examined it in blunt-end ligation reaction catalyzed by T4 DNA ligase and in specific hydrolysis catalyzed by T5 exonuclease.

Despite our assumptions, we found that 5′-DMT-ONs and 5′-DMT-dsDNA is sensitive to enzymatic hydrolysis by T5 exonuclease.

At the same time 5′-DMT-modified DNA blunt ends could not be ligated with 5′-phosphorylated dsDNA blunt ends. Possible explanation of the fact is the steric hindrance that prevents 5′-DMT-modified DNA entering ligase active site. So usage of 5′-DMT-modified DNA fragments (symmetrically of asymmetrically labeled) allows for directed or selected DNA ligation and we proposed possible realization of such cycling scheme, that hypothetically could ligate dsDNA blunt-end fragments step by step.

Possible limitation of 5′-DMT-dsDNA application could be the low stability of this group, which leads to presence of unblocked molecules in population. Thus, precautions must be taken when working with 5′-DMT-modified molecules: it is recommended to work with freshly synthesized ONs and to avoid long storage or heating in solutions with acid or close to neutral pH.

Further work on the investigation of biological properties of 5′-DMT-dsDNA, including testing of other DNA-enzymes interacting with DNA ends, is required to find its possible application in molecular biology.

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REFERENCES

1. Miller, G. P.; Kool, E. T., Versatile 5'-functionalization of oligonucleotides on solid support: amines, azides, thiols, and thioethers via phosphorus chemistry. *The Journal of organic chemistry* **2004,** *69* (7), 2404-10.
2. (a) Issur, M.; Bougie, I.; Despins, S.; Bisaillon, M., Enzymatic synthesis of RNAs capped with nucleotide analogues reveals the molecular basis for substrate selectivity of RNA capping enzyme: impacts on RNA metabolism. *PloS one* **2013,** *8* (9), e75310; (b) Manoharan, M., RNA interference and chemically modified small interfering RNAs. *Current opinion in chemical biology* **2004,** *8* (6), 570-9; (c) Warminski, M.; Kowalska, J.; Buck, J.; Zuberek, J.; Lukaszewicz, M.; Nicola, C.; Kuhn, A. N.; Sahin, U.; Darzynkiewicz, E.; Jemielity, J., The synthesis of isopropylidene mRNA cap analogs modified with phosphorothioate moiety and their evaluation as promoters of mRNA translation. *Bioorganic & medicinal chemistry letters* **2013,** *23* (13), 3753-8; (d) Ziemniak, M.; Strenkowska, M.; Kowalska, J.; Jemielity, J., Potential therapeutic applications of RNA cap analogs. *Future medicinal chemistry* **2013,** *5* (10), 1141-72.
3. Wang, L. K.; Lima, C. D.; Shuman, S., Structure and mechanism of T4 polynucleotide kinase: an RNA repair enzyme. *The EMBO journal* **2002,** *21* (14), 3873-80.
4. Hoffman, L. M.; Jendrisak, J., Heat-labile phosphatase simplifies the preparation of dephosphorylated vector DNA. *Gene* **1990,** *88* (1), 97-9.
5. Veneziano, R.; Shepherd, T. R.; Ratanalert, S.; Bellou, L.; Tao, C.; Bathe, M., In vitro synthesis of gene-length single-stranded DNA. *Scientific reports* **2018,** *8* (1), 6548.
6. (a) Chen, F.; Pruett-Miller, S. M.; Huang, Y.; Gjoka, M.; Duda, K.; Taunton, J.; Collingwood, T. N.; Frodin, M.; Davis, G. D., High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nature methods* **2011,** *8* (9), 753-5; (b) Cong, L.; Ran, F. A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P. D.; Wu, X.; Jiang, W.; Marraffini, L. A.; Zhang, F., Multiplex genome engineering using CRISPR/Cas systems. *Science* **2013,** *339* (6121), 819-23; (c) Davis, L.; Maizels, N., Homology-directed repair of DNA nicks via pathways distinct from canonical double-strand break repair. *Proceedings of the National Academy of Sciences of the United States of America* **2014,** *111* (10), E924-32; (d) Simon, J. R.; Moore, P. D., Homologous recombination between single-stranded DNA and chromosomal genes in Saccharomyces cerevisiae. *Molecular and cellular biology* **1987,** *7* (7), 2329-34.
7. (a) Takasu, Y.; Kobayashi, I.; Tamura, T.; Uchino, K.; Sezutsu, H.; Zurovec, M., Precise genome editing in the silkworm Bombyx mori using TALENs and ds- and ssDNA donors - A practical approach. *Insect biochemistry and molecular biology* **2016,** *78*, 29-38; (b) Wu, Z.; Chen, Z.; Gao, X.; Li, J.; Shang, G., Combination of ssDNA recombineering and CRISPR-Cas9 for Pseudomonas putida KT2440 genome editing. *Applied microbiology and biotechnology* **2019,** *103* (6), 2783-2795.
8. (a) Guixens-Gallardo, P.; Hocek, M.; Perlikova, P., Inhibition of non-templated nucleotide addition by DNA polymerases in primer extension using twisted intercalating nucleic acid modified templates. *Bioorganic & medicinal chemistry letters* **2016,** *26* (2), 288-291; (b) Moran, S.; Ren, R. X.; Sheils, C. J.; Rumney, S. t.; Kool, E. T., Non-hydrogen bonding

'terminator' nucleosides increase the 3'-end homogeneity of enzymatic RNA and DNA synthesis. *Nucleic acids research* **1996,** *24* (11), 2044-52.
9. Schaller, H.; Weimann, G.; Lerch, B.; Khorana, H. G., Studies on polynucleotides. XXIV. The stepwise synthesis of specific deoxyribopolynucleotides (4). Protected derivatives of deoxyribonucleosides and new syntheses of deoxyribonucleoside-3′-phosphates. *J. Am. Chem. Soc.* **1963,** *85*, 3821-3827.
10. Gilar, M.; Bouvier, E. S. P., Purification of crude DNA oligonucleotides by solid-phase extraction and reversed-phase high-performance liquid chromatography. *Journal of chromatography. A* **2000,** *890* (1), 167-77.
11. Yantsevich, A. V.; Shchur, V. V.; Usanov, S. A., Oligonucleotide Preparation Approach for Assembly of DNA Synthons. *SLAS technology* **2019,** *24* (6), 556-568.
12. Shchur, V. V.; Burankova, Y. P.; Shapira, M. A.; Klevzhits, D. V.; Usanov, S. A.; Yantsevich, A. V., Programmed assembly of long DNA synthons: design, mechanism, and online monitoring. *Applied microbiology and biotechnology* **2019,** *103* (21-22), 9103-9117.
13. (a) Hoover, D., Using DNAWorks in designing oligonucleotides for PCR-based gene synthesis. *Methods in molecular biology* **2012,** *852*, 215-23; (b) Hoover, D. M.; Lubkowski, J., DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis. *Nucleic acids research* **2002,** *30* (10), e43.
14. Dellinger, D. J., Wyrzkewicz, T.K., Betley, J.R., and; Caruthers, M. H. *Solid-phase synthesis of oligodeoxyribonucleotides using phosphoramidite synthons in a two step synthesis cycle.*; Poster 209.; XIIIth International Round Table: Nucleosides, Nucleotides and their Biological Applications, Montpellier., 1998.
15. Stemmer, W. P.; Crameri, A.; Ha, K. D.; Brennan, T. M.; Heyneker, H. L., Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* **1995,** *164* (1), 49-53.
16. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E., UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of computational chemistry* **2004,** *25* (13), 1605-12.
17. Sleight, S. C.; Bartley, B. A.; Lieviant, J. A.; Sauro, H. M., In-Fusion BioBrick assembly and re-engineering. *Nucleic acids research* **2010,** *38* (8), 2624-36.
18. (a) Rokke, G.; Korvald, E.; Pahr, J.; Oyas, O.; Lale, R., BioBrick assembly standards and techniques and associated software tools. *Methods in molecular biology* **2014,** *1116*, 1-24; (b) Shetty, R.; Lizarazo, M.; Rettberg, R.; Knight, T. F., Assembly of BioBrick standard biological parts using three antibiotic assembly. *Methods in enzymology* **2011,** *498*, 311-26.

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Manuscript highlights.

1. 5′-DMT group remains on oligonucleotides in PCR conditions and this allows enzymatic synthesis of 5′-DMT dsDNA.
2. DMT-oligonucleotides could be efficiently used for gene assembly by polymerase chain assembly approach (PCA).
3. Functional competence of 5′-DMT dsDNA to the enzymatic reaction was tested, namely, during blunt-ended ligation reaction catalyzed by T4 DNA ligase and specific hydrolysis catalyzed by T5 exonuclease.

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