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Unified-amplifier based primer exchange reaction (UniAmPER) enabled detection of SARS-CoV-2 from clinical samples

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A B S T R A C T

Primer exchange reaction (PER) is an emergent method for non-templated synthesis of single stranded DNA molecules. PER has been shown to be effective in cell imaging systems and for detection of macromolecules. A particular application of PER is to detect a specific target nucleic acid. To this endeavor, two coupled DNA hairpins, a detector and an amplifier, play in accordance to extend a target nucleic acid with a concatemer DNA sequence. Here we introduced unified-amplifier based primer exchange reaction (UniAmPER) that beneficially extends the target by a unified-amplifier. The unified-amplifier operates as both detector and amplifier hairpins. The extension resulted in synthesis of concatemer G-rich sequences. The G-rich sequences were expected to form hairpins upon intercalation of thioflavin T (ThT). The presented unified-amplifier in this study facilitates application of PER systems for development of colorimetric or fluorogenic biosensors. As a proof of principle, the method has been applied for detection of reversely transcribed cDNAs from clinical SARS-CoV-2 samples.

1. Introduction

Single stranded DNA (ssDNA) synthesis is an emanating powerful approach that has been used in the field of biosensors [1,2], cell imaging [3,4], DNA-based data storage and DNA nanotechnology [5]. It has also been applied in combination with clustered regulatory interspaced short palindromic repeats (CRISPR) [6] to detect viral genomic contents and to synthesize drug delivery carriers [7]. Methods for enzymatic synthesis of ssDNAs include asymmetric polymerase chain reaction (aPCR) [8], rolling circle amplification (RCA) [9], strand-displacement DNA amplification (SDA) [10], polymerization and isomerization cyclic amplification (PICA) [11], utilization of terminal deoxynucleotidyl transferase (TdT). [12] and primer exchange reaction (PER) [13].

Primer exchange reaction (PER) is a particular type of ssDNA synthesis by primer extension. It has been developed to generate desired ssDNA sequences by consecutive extension reactions each of which with a separate DNA template [13]. Virtually it adjoins several templates with no ligating enzyme. PER is performed through a cyclic set of reactions consisting of four steps. In the first step, a primer binds to its complementary sequence in a specific hairpin structure. Second step is polymerization reaction with specific polymerases that lack the 5′ to 3′ exonuclease activity. Primer is extended by strand displacement activity of the polymerase up to the stopper. In the third step, polymerase stalls at the stopper and consequently branch migration causes a return of the stem to the complementary state and displaces the newly synthesized strand. Finally, the extended primer is released and may start a new cycle with the same or a different hairpin.

PER reactions were capable of being programmed for synthesis of blocks of ssDNAs that constitute deoxyribozymes, platforms of DNA origamis and tandem repeats of an arbitrary and G-rich DNA sequences [13]. Tandem DNA sequences that have been synthesized by PER have been utilized in technologies such as signal amplification by exchange reaction (SABER) [3]. SABER in combination with present technologies such as fluorescence in situ hybridization (FISH) made scaffold DNAs that bind fluorescently labeled “imagers” and produced highly elaborate cell images in multiplex formats [4]. In addition, concatemer DNA sequences that have been synthesized by PER have been recently used for detection of macromolecules [14–19] and as a therapeutic agent [20]. In a very recent paper, the genomic RNA of SARS-CoV-2 was detected by
combination of CRISPR-Cas9 technology with PER [16].

In the special case of application of PER for detection of nucleic acid molecules, the molecular setup of the reactions consisted of three DNA elements; a primer strand, the detector hairpin and the amplifier hairpin [13]. The detector was also named as translator [16]. In such cases, the detection cycle was distinct from amplification cycles. The detector hairpin was responsible for extending the primer in the first PER cycle with a defined DNA sequence (only once). The detector hairpin was uniquely designed for each target (primer) (Fig. 1A steps 1–4). The next PER cycles started and continued with the amplifier hairpin. Amplifier had a universal sequence and hybridized to the product of the detector hairpin, or its own products, and catalyzed the extension of the DNA with repeats of the same DNA sequences (Fig. 1 A, steps 5–8). Such a coupled-hairpin strategy was advantageous since it allowed applying a universal amplifier for detection of various DNA strands and could accommodate multiplexing. Applying a universal amplifier could reduce costs and experimental variations. However, the strategy requires an answer to the question that what should be the molar ratio of the target: detector: amplifier. The answer to this question gets intricate when counteracting molecular players are acknowledged. In another words, presence of the coupled hairpins increases complexity of the design, in terms of thermodynamic competitions among extended and in-extended primers for binding to the detector hairpin and also, competition of the detector and the amplifier hairpins for binding to the extended form of the primer. Studies are required to assure highest yield of DNA concatemerization when a new set of primer and detector are going to be used for a given substrate. To avoid such complications, here we introduce a unified-hairpin strategy. The unified-amplifier based PER reactions (UniAmPER) is expected to be simpler in terms of thermodynamic characterization of the design.

The detection strategies that are based on synthesis of a concatemer DNA sequence, may produce either electrochemical, colorimetric or fluorescent signals [17–23]. In a common strategy, concatemer G quadruplex (GQ) sequences are synthesized and presence of the GQs are monitored [18,24]. In this study, GQs were selected to produce colorimetric and fluorogenic readout for PER reactions. GQs are DNA sequences with at least four repeat of guanine nucleotides, i.e. G-runs, that are capable of folding into G-tetrad structures. Formation of at least two layers of G-tetrads lead to formation of G-quadruplexes (GQs) [25–27]. Synthetic GQs have been shown to be useful in many diagnostics and pharmaceutical applications [28,29]. In many cases, GQs were signal generating part of biosensors either by intrinsic [30] or extrinsic fluorescence [31]. Extrinsic fluorescence of GQs were generated upon intercalation of specific dyes such as malachite green (MG) [32], crystal

![Fig. 1. Comparison of the coupled-hairpin based PER and UniAmPER for detection of single stranded nucleic acid targets. A) The coupled-hairpin strategy of PER for detection of single stranded nucleic acids. Primer (a) binds acceptor (a*) (step 1) and get extended by displacing the flexible arm of the detector hairpin (b) (step 2). A stopper sequence stalls the polymerase at the junction of the flexible and sturdy region of the stem. The flexible arm returns to the close state by branch migration (step 3) and the extended primer is released (step 4). The extended primer (ab) binds the amplifier hairpin at b* (step 5), gets extended by polymerase (step 6) and displaced by the flexible binding arm b (step 7). Finally, it is detached from the hairpin (step 8) and may start the cycle again at step 5. B) The UniAmPER strategy for extension of a target single stranded nucleic acid. The unified amplifier provides both detection (steps 1–4) and amplification (steps 5–8) reactions. C) An example of the sequence content of a typical unified amplifier. The product of the amplifier is a polymeric GQ that affords analysis upon TMB oxidation or ThT intercalation.](image-url)
violet (CV), cyanovinyl-pyridinium triphenylene (CPT), N-Methyl mesoporphyrin (NMM) and Protoporphyrin IX (PPIX) and thioflavin T (ThT) [31–33].

A second approach that implicates utility of GQs in biosensors is to analyze the peroxidase activity of the synthesized GQs. In 1998, Travascio et al. reported that guanine rich sequences could act as a hemin aptamer and elevate the low intrinsic peroxidase activity of hemin [34]. In this respect, GQs are classified as DNA catalysts, that are also named R. Tavakoli-Koopaei et al. reported that guanine rich sequences could act as a hemin accelerator region (1 and 2) and finally the blocker (supplementary Fig. SI). In this study, the blocker was a dT7 and the loop was dT4 as described earlier [13]. The sturdy arm was also kept with the same sequence as reported, utilizing a dC3 as the stopper, as the “three letter code” strategy was applied here. See supplementary info. SI for more explanation. The sequences of the primers, acceptors, flexible arms and their complements were altered to achieve the highest level of extension. The melting temperature of the sequences were calculated by Tm calculator of NEB (whenever long enough).

2.3. Primer exchange reaction

The reactions were performed in presence of standard reaction buffer that contained TPol’ buffer (20 mM Tris-HCl pH 8.8, 10 mM (NH4)2SO4, 12 mM MgSO4, 10 Mm KCl), 0.1 mM dDT, 0.1 µm primer and 1 µM amplifier hairpins. In experiments that detector hairpins (H1 and H3) were present, the concentration of the detector was one tenth of the concentration of the amplifier. The reactions were setup in 20 µl and were incubated at 37 °C in a thermocycler for 2 h. For polycrylamide gel electrophoresis (PAGE) analysis, 5 µl of each reaction mixture was mixed with 5 µl loading buffer (80% formamide, 1x TBE (89 mM tris-HCl, 89 mM boric acid and 2 mM EDTA pH 8.0), 0.025% each bromophenol blue and xylene cyanol). The samples were directly loaded on denaturing polyacrylamide gels. In the case of kinetic assays, the early time points were quenched and stored at −20 °C prior to be loaded on the gel. Extension of the primers were analyzed by 15% or 20% denaturing polyacrylamide gels. The bands were visualized by silver staining and scanned with a canon LiDE110 scanner [42]. Details of gel electrophoresis and the method of silver staining that have been applied here were explained in supplementary info. SI.

2.4. Peroxidase activity assay

The peroxidase activity of PER or UniAmPER products were investigated with TMB as the substrate. Peroxidase activities were measured in a 20 µl reaction that contained 15 µM of PER product in presence of 50 mM tris-HCl, 150 mM ammonium chloride, 0.5 µM hemin. Finally, 40 µl TMB solution and 0.5 µl 35% H2O2 were added. Tris-HCl and ammonium chloride were added at once. Hemin and TMB solutions were added sequentially with no incubation time in between. The reaction mixtures were incubated at room temperature for 5 min and were stopped by addition of 5 µl of H2SO4. The intensities of the yellow color were recorded by plate-reader at 450 nm. The experiments were performed in triplicate reactions.

2.5. Florescent analysis

The reaction tubes were prepared by addition of 20 µl ThT buffer (15 mM KCl, 100 mM tris-HCl and 50 µM ThT) to 20 µl of PER or UniAmPER product. Florescent spectra were recorded with Synergy HTX multimode-reader in a 96- well plate that was read from bottom. A tungsten lamp was used for excitation. The excitation and emission filters were 420/27 and 485/20 nm, as the QG bound ThT is usually excited at 425 nm and maximal emission is recorded at 490 nm. All data were collected with at least three different samples and averaged after normalization.

2.6. Data normalization

For peroxidase activity (PA) measurements, the raw data were treated with background subtraction. Background, PA hemin only samples, i.e. the peroxidase activity of hemin only samples for oxidation of TMB solutions were measured for 5 times and averaged. Hemin only samples contained 50 mM tris-HCl, 150 mM ammonium chloride, 0.5 µM hemin, 40 µl TMB solution and 1% H2O2. Then, the average background was subtracted from all data with TMB (Eq. 1). In addition,
Table 1
The DNA sequences used in this study. The sequences of hairpins and primer and the products of PER or UniAmPER reactions are shown up to 4 cycles of amplification.

| ID | Amplifiers and the respected Primers | Extended Primers |
|----|------------------------------------|------------------|
|    |                                     |                  |
| H1 | CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
| H2 | CAAAGGGAACCCCTTGGTCGA              | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H3 | CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
| H4 | CAAAGGGAACCCCTTGGTCGA              | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H5 | CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H6 | CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H7 | CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H8 | CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H9 | CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H10| CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H11| CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H12| CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H13| CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
| H13| CAAAGGGAACCCCTTGGTCGA              | 5' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 2' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 3' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     | 4' CCCTAAATGCTTGTTGAAAAGTTT |
|    |                                     |                  |
to have a better comparison for synthesis of GQs, efficiency of polymeric GQ synthesis (ePG) were calculated for each reaction as with Eq. 2.

\[
\text{PA (TMB)}_{\text{sample}} = \frac{\text{PA}_{\text{sample}} - \text{average PA}_{\text{hemin only samples}}}{\text{average PA}_{\text{hemin only samples}}}
\]  

(1)

ePG (TMB)_{\text{sample}} = \frac{\text{PA (TMB)}_{\text{sample}} / \text{PA (TMB) sample}}{0.1 \mu \text{M monomeric GQ}}

In the cases of fluorescent signals of bound ThT to the reaction products the, fluorescent intensities were taken for calculation of RFU (ThT)_{sample} as in Eq. 3. In this case, the background signal (RFU (ThT) only samples) was calculated as the average of three samples that contained buffer, ThT and no GQ (7.5 mM KCl, 50 mM tris-HCl and 25 \mu M ThT).

\[
\text{RFU (ThT)}_{\text{sample}} = \frac{\text{RFU}_{\text{sample}} - \text{average RFU}_{\text{ThT only samples}}}{\text{average RFU}_{\text{ThT only samples}}}
\]  

2.7. Reverse transcription and UniAmPER for clinical samples

Positions 21377–21412 from the reference genome Mn908947 were selected as the target site for the cDNA synthesis. Genomic RNA was extracted by commercial kits from nasopharyngeal clinical samples. Half of the RNA samples were used for reverse transcription real time PCR validation (commercial kit). The other half was used for reverse transcription protocol that was appropriate for UniAmPER. The reverse transcription was performed only in presence of dDTP (dATP, dTTP, dGTP) and in the absence of dCTP. The reverse transcripted cDNA was utilized for UniAmPER. The UniAmPER was performed with 15 \mu M cDNA, in presence of TPol buffer, 0.1 mM dDTP and 1 \mu M H1-H2-SARS-CoV-2. The reaction mixture was incubated for 2 h at 37 \textdegree C, subsequently, 10 \mu M ThT (100 \mu M) was added to the samples and fluorescent measurements were taken by Synergy HTX multimode reader.

3. Results and discussion

3.1. Investigating for an appropriate molecular set up for nucleic acid detection

Concatamer DNA sequences that were synthesized by PER have been shown to be applicable in a variety of fields [14,15,17–20]. Example include detection of a single stranded nucleic acids [16]. The highest yield in PER would guarantee the lowest limit of detection for such analyses. Thus, in order to explore for a highly efficient amplifier hairpin in PER, several sets of hairpins have been designed and studied here (Table 1). The initial molecular setup in the report by Kishi et al. applied a coupled-hairpin protocol for miRNA detection [13]. The detector hairpin started the reaction and added a constant sequence (human telomerase monomer sequence, TTAGGG) to the 3’ end of the input primer. Then, a universal hairpin (amplifier) started concatemerization. The amplifier had an acceptor and flexible arm that were complementary and identical to the human telomerase monomer sequence, respectively. Here, H1 and H2 were designed to make the same concatamer GQ sequence that resembles the human telomerase. A minor change was applied in the design, since the original report by Kishi et al. had shown an increase in the synthesis efficiency when the flexible arms started with an adenosine, thus, the flexible arms of H1 and H2 were started with adenosine (AGGGTT, Fig. 2 A). The sequence of the products of H1 + H2 were identical to the sequence of the PER products in the original report [13]. Several sets of hairpins then were designed and compared with H1 + H2 for their synthesis efficiency. The sequences products of the PER reaction of all hairpins up to 4 rounds are depicted in Table 1. The lengths of the products up to 30 rounds are shown in supplementary Table SII. As the product of the synthesis was a polymeric GQ, both colorimetric and fluorimetric readout systems could be utilized finally for analysis.

The flexible arms of H1 and H2 had one G-run and therefore, four rounds of PER reactions were required for synthesis of a single GQ. Thus, H3 and H4 were designed with flexible arms that contained two G-runs, started with adenosine and accommodated synthesis of a single adenosine or thymidine as the linker (AGGGTTGG, Fig. 2 B, Table 1). Molecular setups like H1 + H2 and H3 + H4 suffer from intrinsic complexities including the competition between the two hairpins for binding to the extended form of the primer. Affirmatively, successful applications of PER, e.g. SABER and related technologies, applied a single hairpin for concatemerization [3,4]. Thus, here, to elevate the efficiency of concatemerization, the detector and amplifier were merged...
to obtain a unified-amplifier. As the starting point, two archetypes of unified-amplifiers were designed, H5 and H6.

H5 was designed with two acceptor sequences. Acceptor 1 was complementary to the target and acceptor 2, which was flanked by acceptor 1 and flexible arm was complementary to the flexible arm. The reaction started upon binding of the primer (target) to the acceptor 1. The polymerase extended the primer over the acceptor 2 and reached the flexible arm. The polymerase continued synthesis by displacing the flexible arm up to the point of the stopper which were three cytosines in the flexible arm. The polymerase continued synthesis by displacing the binding of the target to the acceptor arm of sequence to the target. It could be predicted that synthesis starts with 1% H presence of PER products, hemin and appropriate buffer were measured. peroxidase activity of the product of each set. Oxidation of TMB in desired reaction and the observed extensions were primer-dependent.

Hairpin only samples, confirming that hairpins do not extend in an un-directed reaction and the observed extensions were primer-dependent.

The major products of PER reactions with hairpins H3 + H4 were visual on the denaturing PAGE by making 11 bonds starting nearly at 50 nucleotides. Considering the size of the product in each round (supplementary Table SII) and also taking the sizes of hairpins as the size marker on the gel, it could be deduced that H3 + H4 were capable to synthesize 8–28 G-runs that equals to 4–14 rounds of PER reactions. Although H3 + H4 were more efficient in comparison to H1 + H2, but their products were limited to lengths below 150 nucleotides. The best performance in the four initial setups of this study was achieved by H5. H5 was a unified-amplifier and the major product of it was extended above ca. 150 nucleotides. It was deduced that H5 could make at least 34 G-runs, equal to 17 PER cycles, based on the supplementary Table SII. The unified-amplifier H6, however, did not achieve long products and could not perform PER cycles more than 2 rounds (Fig. 2B).

Peroxidase activity of the same samples were measured with TMB substrate. The results showed the average PA (TMB) value for H1 + H2 as 0.13 The PA (TMB) values for H3 + H4, H5 and H6 were 0.29, 0.33 and 0.99 respectively. Taking the average PA (TMB) of a monomeric GQ (supplementary Fig SIV) with a concentration of 0.1 µM (equal concentration to the primer), reflects that H1 + H2 had equal activity to 1.4 monomeric GQ, and H3 + H4, H5 and H6 were 3.1, 3.6 and 1.0 times more active in comparison to monomeric GQ with the same concentration as the primer. In the other words, peroxidase activities (PA) of individual samples may be noticed from a different point of view. It would be beneficial to calculate the ratio of the PA of a special sample to the PA of a single monomer GQ. This would result in a new vision about the efficiency of polymeric GQ (ePG) synthesis. Therefore, ePG values were also calculated from PAs, wherever applicable. The ePG values for the same data at Fig. 2 C are presented in Supplementary Fig SVA. Notably, the higher lengths of PER products resulted in higher PA (TMB) values, however, the increments were not linear in length above 150 nucleotides. The reason may be attributed to the fact that GQ peroxidase activity reaches its maximum plateau level. To address this, increasing concentrations of monomeric GQs were investigated for their peroxidase activities (Supplementary Fig. SIV). The peroxidase activity of GQ monomers reached to the saturation point at concentrations above 5 µM. This could be taken as an assumption that PER products that have equivalent GQs (albeit in tandem) would reach to saturation at some level of synthesis. In summary, our initial analyses showed significantly elevated GQ synthesis and also higher PA (TMB) values with the unified-amplifier H5. Thus, optimization of the archetype unified-amplifier H5 was addressed in the next steps to reach the most efficient unified-amplifier based PER (UniAmPER) products.

3.2. Escalating UniAmPER activity

Since the unified-amplifier H5 had the maximal activity in initial investigations, few more amplifiers were designed with the same architecture but with various length and nucleotide sequence. Adenines were known to increase the peroxidase activity of HRP mimicking deoxyribozymes, when positioned at the end of a monomer GQ [43]. In addition, a report by Chen et al. have shown that dimer GQs have maximal activity when linkers were adenines. They have shown that the best activity was obtained with A4 internal linkers and A7 connecting linkers [44]. Thus, here three unified-amplifiers were designed with adenine linkers. Unified-amplifier H7 consisted of a flexible arm that provided synthesis of a single G-run (AGGGAAA). The product of H7 was a tandem GQ with A4 linkers. H8 accommodated 2 G-runs per PER cycle and alternating linkers with A1 or A4 sequence (AGGGAGGGAAA). H9 was designed to synthesize a single G-run per cycle and had A7 as linkers (AGGGAAAAA). All three unified-amplifiers were designed in a way that the flexible arms started with an adenine as suggested by Kishi et al. [13]. For visual depiction of the hairpins and their products see Fig. 3 A. The hairpin H7 did not provide PER reactions more than 2 cycles and the peroxidase activity of it, in presence and absence of primer, were below PA_hemin and thus, the PA (TMB) had negative values (Fig. 3B and the
Fig. 3. The effect of the lengths of the flexible arm and acceptor 2 on the peroxidase activity of UniAmPER products. A) The sequence composition of unified-amplifiers with flexible arms that start with adenine and produces GQs with adenine linkers. B) Denaturing PAGE analysis of the results of polymerization by unified amplifiers explained in A. C) The PA (TMB) calculated based on the peroxidase activities of the samples in B. D) The sequence composition of unified-amplifiers that have 1, 2 or 3 G-runs in flexible arms and 2 or 3 G-runs in acceptor 2. E) Denaturing PAGE analysis of the results of polymerization by unified-amplifiers in D. F) The PA (TMB) calculated based on the peroxidase activities of the samples in E. A and D) The size of each hairpin is mentioned in the respected parentheses. The size of the PER products in consecutive PER cycles are also depicted beside each setup B and E) The reactions were performed in presence of 1 µM amplifier hairpins, Bst and in presence of 1x TPol - buffer in the total volume of 20 µl. The samples incubated at 37 °C for 2 h. Hairpins were taken as size markers to estimate the size of the products. Upon the size of the products and the sequence of the flexible arms, the estimated cycles of PER reactions and G runs were calculated. The major products of each sets are marked with a bracket on the gel. C and F) The peroxidase activities were performed with 15 µl of PER products in presence of 50 mM tris-HCl, 150 mM ammonium chloride, 0.5 µM hemin, 40 µl TMB solution and 1% H2O2. Error bars are standard deviations. G) The UniAmPER reactions were performed with 0.1, 1 and 10 µM concentrations of H13 in presence of 0.1 µM primer and standard reaction buffer. For each hairpin concentration a “no primer” control was included. H) The UniAmPER reaction in presence of increasing concentrations of primer. The reactions were performed in presence of 1 µM hairpin and standard conditions. Error bars are standard deviation. I) The kinetics of UniAmPER reaction monitored by PA (TMB). The reactions were performed with 0.1 µM primer and 1 µM H13 in presence of standard reaction condition, for all time points except for time point zero. Time point zero lacks Bst to make sure that no extension is performed in the very short time frame prior to quenching.
cases the PA values in the absence of primer were negligible. The full gel image in Fig. 3 E is also shown in supplementary Fig. SIII with no marked brackets. Thus, H13 was taken as the best unified-amplifier in this report for synthesis of tandem GQs with single thymidine linkers.

In the next step, hairpin concentration was changed by factors of 0.1 and 10. The respected values for PA (TMB) were 0.29 and 0.27 (Fig. 3 G). The PA (TMB) values for all “no primer” samples were negligible that were an indication that all the amplifications were primer-dependent and even high concentrations of hairpins did not disturb the results. In addition, keeping the concentration of the hairpin as 1 µM, titration of the target primer in the range of 0.01–10 µM showed that the PA (TMB) values reach to saturation point with ca. 1 µM primer. Primer concentrations equal or below 0.05 µM were in the other hand, so less active that their peroxidase activities were negative (Fig. 3H). In summary, TMB oxidation with GQs had a limited dynamic range for analyzing the UniAmPER products and thus binding to ThT was followed in consequent reactions.

3.3. UniAmPER kinetics

In all previous experiments, the amplifiers had the chance to amplify for 2 h. To address the kinetics of the reaction, seven reactions were setup. The first sample was a size control as well as peroxidase control and thereby, no Bst enzyme was added to the sample. This sample was shown as time zero in the Fig. 3I, pointing that zero second reactions would have resulted in such banding pattern and PA (TMB) values. The rest of the samples included Bst and were stopped at 30 s up to 240 min. At each time point, part of the samples was quenched and kept at –20 °C prior to be loaded on gel and part of the samples was directly gone through peroxidase assay. It was observed at the very short time point, 30 s, the amplifier and Bst enzyme had amplified the primer up to ca. 50 nucleotides and only 15 min was enough for synthesis of products that were significantly long. However, longer time points, up to 2 h, resulted in an increase in the peroxidase activity of the system, which was not clearly observed by PAGE analysis, perhaps due to presence of little amounts of products with various lengths [13]. These set of experiments confirmed that 2 h reaction was appropriate when the extension of the UniAmPER products were monitored by peroxidase activity (Fig. 3I and supplementary Fig. SVI).

3.4. UniAmPER fluorescence analysis

Inspection methods for detection of presence of a GQ in a system varies but may be divided in two main strategies. Either the peroxidase activity of the GQs are monitored, as it was utilized in previous sections, or binding of a specific dye to the GQs is investigated. In the latter, the quantum yield of a fluorescent dye is increased upon binding to GQs. Dyes such as ThT and malachite green (MG) have been used in this respect [32,33]. Here intercalation of ThT to the GQs were investigated. Initially, monomer GQs were titrated to validate the signal generation upon ThT to the specific GQ sequence that is generated by H13 (Fig. 4A). Next, the products of the unified-amplifiers H10–H13 were investigated

![Fig. 4. Fluorescent signal generation by UniAmPER products](image-url) A) Fluorescent signal generation by monomeric GQ. Increasing concentrations of monomeric GQ (TGGGTGCGTGCGTGCG) were incubated with Tpol buffer, MgSO₄, 7.5 mM KCl and 50 mM Tris-HCl in presence of 25 µM ThT. The excitation filter was 420/20 nm and the emission filter was 485/20 nm. B) The RFU (ThT) values for UniAmPER products. Primer concentration for UniAmPER reactions were 0.1 µM except for no-primer control.
for their fluorescence signal generation in comparison to H1 + H2 (Fig. 4B). A no-primer reaction in presence of H13 was also performed as a control reaction. The RFU (ThT) values were in agreement with peroxidase activities and confirmed that H13 was the most active amplifier for synthesis of GQs. The RFU (ThT) value for H13 was significantly above other hairpins. In summary, H13 unified-amplifier with 2 acceptor sequences and a flexible arm that synthesized GQs with T linkers was the best performance amplifier in this study.

3.5. UniAmPER assisted detection of SARS-CoV-2 viral RNA

The unified-amplifiers accomplished synthesis of tandem GQs as a tail for a primer sequence. The results were promising in the sense of detection of nucleic acids. A single stranded DNA with a defined 3′ end sequence and 3′ hydroxyl group could be a target of such a system. A reversely transcribed cDNA from RNA samples is expected to accommodate the desired 3′ hydroxyl group, but usually, the reverse transcription takes long for several hundreds of nucleotides and ends with undefined 3′ sequence. As a proof-of-concept, here, in an orthogonal reaction with the “three letter code” PER reactions, the reverse transcription was performed only in presence of dDTP, i.e. dATP, dTTP and dGTP (Fig. 5A). The primer binding site for reverse transcription was chosen to bind to a conserved sequence in SARS-CoV-2 genome that was upstream of a short guanine-free sequence that ended with a string of consecutive guanines (Fig. 5B). The position 21377–21412 of the reference SARS-CoV-2 sequence accommodated the required criteria and ended in four guanines in a row. It was expected that in the absence of dCTP in the reaction, this G run would have stalling effects on the reverse transcriptase, at least to some extents. Consequently, the generated primers with the new defined 3′ ends could get released spontaneously. The extended primer now may act as an input for the UniAmPER and promotes synthesis of tandem GQs at its new 3′ end. ThT intercalation to the products were measured in SARS-CoV-2 positive and control samples (Fig. 5C and supplementary Fig SVII). The results validated that high concentrations of RNA samples (groups of samples with reverse transcription real time PCR Ct values below 15) could generate meaningful results in comparison to control experiments. Control samples included RNA samples that were proven to be negative in SARS-CoV-2 genome (C1) as well as buffer-only samples (C2 to C4). The notable fluorescence of buffer-only samples indicated that buffer components of reverse transcription and UniAmPER reactions have elevated the ThT fluorescence and increased backgrounds. Further analyses are required to address such effect which were beyond the scope of this paper.

4. Conclusion

Primer exchange reaction (PER) was increasingly intriguing in recent years as a method of ssDNA synthesis. In this report, synthesis of concatenemer GQs was investigated with the aim of detection of a single stranded DNA molecule. GQ concatemers have been utilized as the signal generating part of many biosensors [45,46]. These GQs may generate colorimetric signals that are detectable by bare eyes or promote formation of fluorescent signals with specific intercalating dyes [10,47].

PER reactions were shown to be beneficial for detection of single stranded nucleic acids [13]. In this special application, PER was performed with two coupled hairpins, a detector and an amplifier. The detector hairpin needed to be designed and synthesized for each new target. The design of such systems was complicated in thermodynamic aspects due to competitions between the four players i.e. primers,

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Fig. 5. UniAmPER enabled detection of reversely transcribed cDNA from SARS-CoV-2. A) The workflow of the procedure. Extracted RNA samples from nasopharyngeal clinical samples were reverse transcribed in presence of dDTP. dCTP was absent from the mixture to stop the reaction and generate the desired 3′-OH. The nascent cDNA bonded H13-SARS-CoV-2 hairpin and the G rich sequence was synthesized at its 3′ end repeatedly in consecutive cycles. The results were monitored after ThT binding by fluorescence analysis. B) sequence information on the primer binding site for reverse transcription and H13-SARS-CoV-2 hairpin. B) The RFU (ThT) values for UniAmPER products of SARS-CoV-2 H13 hairpin in the groups of clinical samples with respected Ct values. To see the individual samples of the graph, please see supplementary Fig SVII. Note that C1 control samples contain RNA samples that obtained from nasopharyngeal swabs and had confirmed to be SARS-CoV-2 negatives. Other controls (C2, C3 and C4) contain no RNA sample and are buffer controls.
extended primers, detector and amplifier. In addition, the molar ratio of hairpins, detectors and amplifiers need to be optimized. Here we introduced unified-amplifier based PER (UniAmPER) that simplified the sequence design. UniAmPER applies unified-amplifiers that perform both reactions of detector and amplifier with only one hairpin. Unified-amplifiers have two acceptor regions, the first binds the target nucleic acid and the second is complementary to the flexible arm of the amplifier and binds its own product for further extension.

In another words, the new approach of PER reaction is an adaptation of a previously reported coupled-hairpin strategy for detection of single stranded nucleic acids. In the coupled-hairpin strategy, detection and amplifications were distinct reactions and the molar ratio of Primer: detector: amplifier was a question [13,16]. Unified-amplifiers, however, makes the system simpler and the users only needs to adjust the concentration of the unified-amplifier which is intrinsically both detector and amplifier. This study, designed and investigated various unified-amplifiers that each were intrinsically different in their sequence content, flexible arms and acceptor lengths. The hairpins and their products were monitored for their synthesis efficiencies, peroxidase activities as well as binding to ThT. It was found that some handle sequences (flexible arms) do not accommodate repetitive cycles and only produce short products, while others could extend longer. However, the aim of the project was not only about the produced length, but also about the amount of tandem GQ synthesis as well as the ability of the tandemly synthesized GQs for binding to hemin and ThT. It was found that some handle sequences (flexible arms) do not accommodate repetitive cycles and only produce short products, while others could extend longer. However, the aim of the project was not only about the produced length, but also about the amount of tandem GQ synthesis as well as the ability of the tandemly synthesized GQs for binding to hemin and ThT.

Here we showed that the synthesis efficiency of various unified amplifiers were different. The difference could be attributed to the strand displacement phenomenon that provides separation of the nascent oligo and re-binding of it. Notably, a PER system bears two phenomenon of strand displacement. Phenomenon 1 is performed by Bst polymerase (Supplementary Fig SVIIA). This phenomenon is distinct to the toehold-exchange reactions [13]. The most important difference is that the toehold based DNA circuits are dependent on pre-synthesized DNA strands that associate and dissociate via thermodynamic rules, while PER reactions synthesize the strands via a strand-displacing DNA polymerase.

The phenomenon 2, is the phenomenon that provides branch migration. Branch migration follows thermodynamic rules for strand displacement and is assisted by toeholds. It is known that the ability of toeholds for promotion of strand displacement is in accordance to their length and GC content [48] Two competing toeholds react to result in the open or closed form of the hairpins (supplementary Fig SVIIB and C). The favorite structure however, is the closed state at which the product has higher chance to be released spontaneously and start the new cycle. Little is known about the complex competition between the two present toeholds in UniAmPER since it is a competition among the two phenomena that one is intra- and the other is inter-molecular [49] and a wealth of experimental data in accordance to in silico studies are required to address such a special case.

Among all the investigated hairpins, the synthesis efficiency and the peroxidase activity and ThT binding of the product of the unified-amplifier H13 were significant. The flexible arm of the amplifier resulted in tandem GQs that contained a thymidine as the loops and linkers. The efficient synthesis of GQ concatamers at the tail of a target nucleic acid molecule was pointing toward the potentials of the UniAmPER system for detection of nucleic acids in biological systems. As a proof-of-principle, the structural elements of the H13 hairpin was adapted to design a new hairpin that has a binding site for a SARS-CoV-2 cDNA. The preliminary data showed significant increase in the ThT signal at clinical samples with Ct values below 15. Further studies are required to optimize the reaction components to make the system more sensible to clinical samples.

CRediT authorship contribution statement

R.T was involved in conceptualization, methodology, investigation, data curation and reviewing & Editing the draft, F. J.-Z. was involved in conceptualization, methodology, data curation, writing the original draft, reviewing and editing the draft, funding acquisition and supervision of the project, H. M. involved in providing clinical samples, validation of the clinical results, reviewing & editing the draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflict of Interest

The authors declare no competing interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.131409.

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