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Purification of DNA oligonucleotides to improve hybridization chain reaction performance

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ABSTRACT

Hybridization Chain Reaction (HCR) is a technique to generate a linear polymerization of oligonucleotide hairpins, used in multiple molecular biology methods. The HCR reaction is dependent on every hairpin being metastable in the absence of a triggering oligonucleotide and that every hairpin can continue the polymerization, which puts a strong demand on oligonucleotide quality. We show how further purification can greatly increase polymerization potential. It was found that a single extra PAGE-purification could greatly enhance hairpin polymerization both *in solution* and *in situ*. Purification using a ligation-based method further improved polymerization, yielding *in situ* immunoHCR stains at least 3.4-times stronger than a non-purified control. This demonstrates the importance of not only good sequence design of the oligonucleotide hairpins, but also the demand for high quality oligonucleotides to accomplish a potent and specific HCR.

Introduction

Cellular states are coordinated by complex signaling transduction circuits that are relayed by protein-protein interactions and post-translational modifications. In order to investigate both physiological and pathological mechanisms molecular biology methods are required to monitor these cellular states. Among the many developed molecular tools, Hybridization Chain Reaction (HCR) has emerged as a system that could trigger self-assembly of DNA nanostructures [1]. With creative use of the triggering sequence, HCR has been used in multiple applications including *in situ* hybridization (ISH) [2,3], Immunosignal hybridization chain reaction (isHCR) [4], and ProxHCR [5,6].

A typical HCR reaction utilizes a pair of hairpin oligonucleotides and a triggering oligonucleotide (Fig. 1a). The driving force behind the chain reaction lies in the potential energy stored in the loop region of the hairpins [1]. This region is protected by an adjacent stem that prevents the hairpins from rapidly equilibrating, as such a solution containing only the hairpin pair should remain in an unpolymerized state. Polymerization is initiated by the addition of the triggering oligonucleotide that binds the foothold of one of the hairpins and proceeds to release the stem through toe-hold mediated strand displacement [7]. The displaced oligonucleotide region is now free to act as a new trigger oligonucleotide for the other hairpin, furthering the polymerization. This process will continue until a long nicked double stranded DNA molecule is formed.

While some branched HCR systems have been developed [8,9] the typical and most easily applied HCR system utilizes a linear polymerization. However, even a linear polymerization is not without its limitations. Presence of faulty hairpins can disrupt both the metastability and the polymerization process (Fig. 1b). Errors in the 'triggering part' of the hairpin will be kinetically hindered or unable to continue the polymerization. Similarly, sequence errors in the 'starting region' can result in inert or unstable hairpins. As such, both design of the hairpins and their quality is paramount for an HCR system.

Hairpins for HCR are typically small and can be produced through synthetic oligonucleotide synthesis, which makes them both inexpensive and readily available. Oligonucleotides are often synthesized through a step-wise coupling of one nucleotide at a time through a synthesis cycle [10]. In each coupling there is a small error rate that will generate a fraction of hairpins with substitutions, deletions or insertions [11]. Larger errors can be removed by post process purifications such as

Abbreviations: HCR, Hybridization Chain Reaction; PAGE, Polyacrylamide gel electrophoresis; RP-HPLC, Reverse phase – high performance liquid chromatography; IE-HPLC, Ion exchange - high performance liquid chromatography; FISH, Fluorescent *in situ* hybridization; isHCR, Immunosignal hybridization chain reaction; ProxHCR, Proximity-dependent initiation of hybridization chain reaction; SEC, Size exclusion chromatography; Np, Non-purified; Pp, PAGE-purified; L, Ligated; LPp, Ligated PAGE-purified; LBP, Ligated bead-purified; LBPp, Ligated bead and PAGE-purified; LBSEC, Ligated bead and size exclusion chromatography purified.

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polyacrylamide gel electrophoresis (PAGE), reverse phase high performance liquid chromatography (RP-HPLC) and ion-exchange (IE)-HPLC, but smaller errors can be hard to resolve. The problem increases as the secondary structures in the hairpins makes them more difficult to purify.

In this paper different purification strategies are investigated in order to improve reproducibility and quality of HCR hairpins.

Materials and methods

Hairpin design

The oligonucleotides were designed *in silico* with the help of the NUPACK design tool (http://www.nupack.org) [12]. The hairpins were designed taking previous optimization guidelines into consideration [3, 13]. All oligonucleotides (supplementary Table 1) were synthesized and HPLC purified (Integrated DNA Technologies, IDT, Coralville, USA).

PAGE purification

All oligonucleotides that were PAGE-purified were initially mixed 1:1 with TBE-Urea Sample Buffer (Thermo Fisher Scientific, Waltham, MA, USA) and heated to 95 °C for 10 min. The oligonuclotides were then loaded to 10% TBE-Urea Gels (Thermo Fisher Scientific) preheated to 55 °C. The gels were then run at 100 V for about 75 min. To visualize the oligonucleotide bands the gel was briefly stained with SYBR Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO) (Thermo Fisher Scientific). The gels were then placed on a UV-table and the hairpin bands were cut out. The excised gel fragments were then shredded by passing through an 18 G syringe. Oligonucleotides were extracted in extraction buffer (8 mM Tris, 0.8 mM EDTA, 1 M NaCl, pH 8) equal to about 3x of the gel volume, at 4 °C for at least 48 h. The solution was then centrifuged and the supernatant collected, gel fragments were washed again with a small amount of extraction buffer and pooled with the supernatant. The solution was then passed through a spin-filter to remove remaining gel fragments. To precipitate the DNA, ice cold 98% ethanol equal to 4x the volume of supernatant was added and the solution was incubated at − 20 °C for 2 h. The solutions were then centrifuged at − 10 °C for 10 min at 21,130 xg. The pellet was then washed 5 times in ice cold 70% ethanol and air dried. Oligonucleotides were resuspended in PBS and concentration was measured by nanodrop spectrophotometer.

Ligation

All hairpins that were ligated were ordered as two parts, a short 6-mer and a longer 34-mer. Before the ligation, the larger 34-mer was snap cooled by heating to 95 °C followed by cooling for 30 min at room temperature (RT). Both oligonucleotides were then mixed to a final concentration of 20 μM in 1x Cutsmart buffer (NEB, Ipswich, MA, USA), with added ATP (final concentration 1 mM, Thermo Fisher Scientific), DTT (final concentration 10 mM, Sigma-Aldrich, Saint Louis, MO, USA). To the ligation mix, both polynuclotide kinase (PNK) (final concentration 0,25 U/μl, NEB) and T4-ligase (final concentration 0025 U/ul, Thermo Fisher Scientific) was added, and the mix was incubated overnight in a thermocycler cycling between 30 min at 4 °C and 30 min at 37 °C.

Bead purification

To remove unligated 34-mers from the ligated oligonucleotides, first 750 μl of streptavidin bead solution (Cytiva, Marlborough, MA, USA) was washed once in water and three times in extraction buffer (8 mM Tris, 0.8 mM EDTA, 1 M NaCl, pH 8). To this mix 300 μl of the ligated oligonucleotides were added (6000pmol) and incubated overnight at

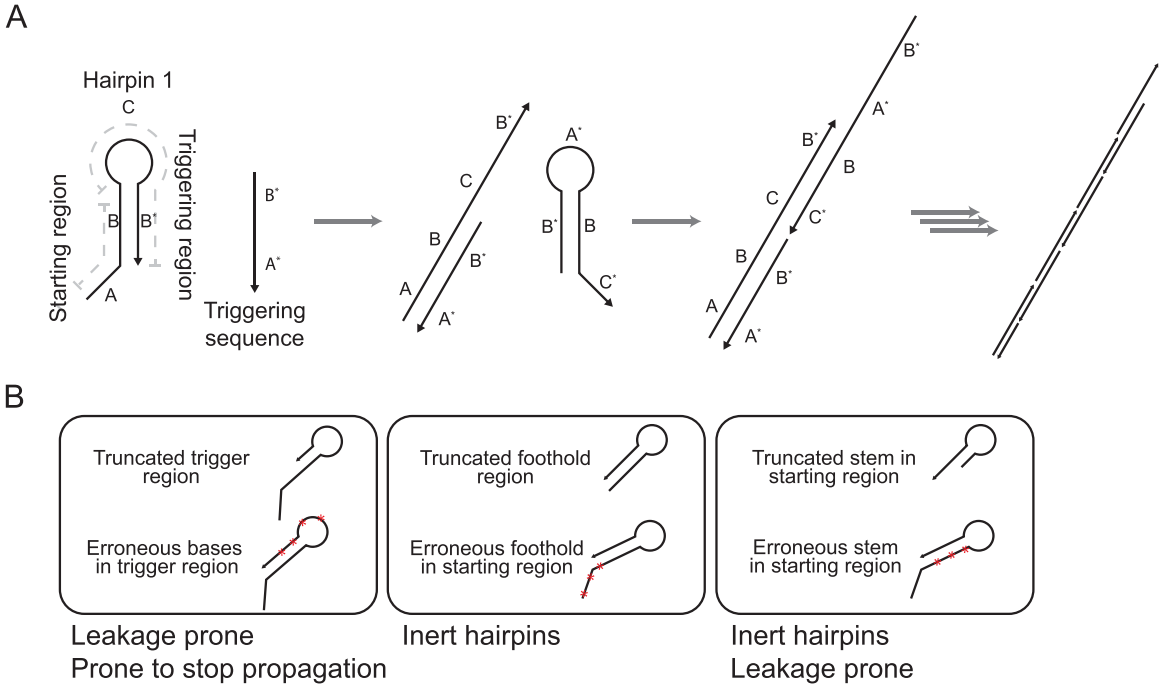

Fig. 1. Mechanism of HCR polymerization. (a) HCR-hairpins consists of two separate hairpins, both containing a 'starting region' consisting of a foothold and a stem region, and a 'triggering region' consisting of a loop and the complementary stem-sequence. HCR is initiated with the addition of a triggering sequence complementary to the starting region of either hairpin. Consequently, the triggering sequence binds to and opens up the hairpin, exposing the triggering region. In turn, this region binds and opens the paired hairpin exposing the next triggering region. The reaction continues until either the hairpins are consumed, or equilibrium is reached. (b) Potential errors that could be introduced in oligo synthesis. Hairpins containing faults in the 'triggering region' will be obstructed from binding to the paired hairpins starting region, resulting in a slowed or stopped polymerization or a destabilized stem with reduced stability. An incorrect foothold would remain inert, unable to be initiated upon by a triggering sequence. Erroneous stem sequence in the 'starting region' could become unstable resulting in an exposed, intact, 'triggering region' causing unintended polymerization.

4 °C. After binding the hairpins to the beads, the bead solution was washed 8 times with phosphate buffered saline (PBS) at 60 °C with a 5 min incubation per wash. After the final wash the oligonucleotides were eluted using a molar excess of biotin and incubated overnight at 4 °C. A part of the bead purified oligonucleotides were further purified by size exclusion chromatography using an ÄKTA Pure HPLC (GE Healthcare, Chicago, IL, USA) with a superdex 200 10/300 GL. After purification all oligonucleotides had their concentrations determined by nanodrop spectrophotometer.

In solution amplification

For the analysis of oligo amplification in gel, all hairpins were diluted separately to a concentration of 1,25 μM in 5x saline sodium citrate (SSC) and were snap cooled by heating to 95 °C and then allowing it to cool for at least 30 min at RT. To start the amplification reaction, 4 μl of hairpin 1 and 4 μl of hairpin 2 were mixed in a PCR-strip, to this mix 1 μl of triggering sequence (0.1 μM) diluted in 5xSSC and 1ul of 5xSSC was added (in non-initiated controls 2 μl of 5xSSC was added). Final concentration of the amplification mix results in 0.5 μM of each hairpin and 0,01 μM of triggering sequence in 5xSSC. The solution was then mixed by pipetting up and down and incubated at RT for 2 h. After the incubation, 2 μl of 6xloading dye (Thermo Fisher Scientific) was added to each tube and the solution was loaded onto a 4–20% gradient TBE gel and 100 V was applied. Bands were then visualized by staining the gels for 15 min with SYBR Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO). Stained gels were scanned using an Odyssey Fc imaging system (LI-COR, Lincoln, NE, USA).

Conjugation

Antibodies were conjugated as described previously [6]. Initially anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PE, USA) were concentrated using Amicon Ultra 10 K centrifugal filter units (Merck Millipore, Burlington, VT, USA) to a concentration of at least 3 mg/ml. For activation, the antibodies were incubated with a 25-molar excess of succinimidyl 6-hydrazinonicotinate acetone hydrazine (SANH) (Trilink, San Diego, CA, USA) for 2 h at RT. After activation, the buffer was exchanged to 150 mM NaCl and 100 mM NaH_2PO_4 pH 6.0 using a Zeba Spin Desalting Columns 7 K MWCO (Thermo Fisher Scientific). To the activated antibody, aldehyde-modified triggering sequence was added at 3-times molar excess. To catalyse the reaction, 10 mM of aniline (Sigma-Aldrich) was added and the mixture incubated for 2.5 h at RT. Directly following the incubation, the buffer was exchanged to TBS (50 mM tris(hydroxymethyl)aminomethane, 150 mM NaCl, pH 7.5) and stored at 4 °C. The conjugated antibodies were then purified using an ÄKTA Pure HPLC (GE Healthcare) with a superdex 200 10/300 GL.

Cell culture

Cells were maintained in a humidified incubator at 37 °C, 5% CO_2 atmosphere. The cells were grown using high glucose DMEM supplemented with Glutamax, sodium pyruvate (Cat#31966047, Thermo Fisher Scientific) and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) with routine passaging when confluent using 0.25% Trypsine-EDTA (Thermo Fisher Scientific). HaCaT cells were trypsinized and seeded to 8-well Lab-Tek II Chamber Slides (Sigma-Aldrich) and grown to 90–100% confluence. Following overnight growth, all cells were fixed with 3.7% formaldehyde (Sigma-Aldrich) on ice for 15 min, washed in PBS, dried and stored at − 20 °C until further use.

ImmunoHCR

Slides were first permeabilized using 0.2% Triton-X100 (Sigma-Aldrich) in TBS for 10 min followed by a short rinse in TBS. Slides were then blocked with Intercept blocking buffer (LI-COR) mixed with 2.5 mg/ml salmon sperm DNA (Thermo Fisher Scientific) for 1 h at RT. After blocking, primary antibodies (mouse anti-E-cadherin, BD Transduction laboratories, San Jose, CA, USA, diluted 1:100 or rabbit anti-Histone H3, Abcam, Cambridge, UK, diluted 1:500) diluted in intercept blocking buffer mixed with 2.5 mg/ml salmon sperm DNA were added and incubated overnight at 4 °C. The slides were then washed three times in TBS. Secondary antibodies conjugated with a triggering sequence were then diluted to a concentration of 5 μg/ml in Intercept blocking buffer mixed with 2.5 mg/ml salmon sperm DNA, added to the slide and incubated at RT for 1 h, followed by three washes with TBS-T (added 0.05% Tween-20 (Sigma-Aldrich)). Following the wash, 100 nM of each detection hairpin mixed in 5xSSC was added and incubated for 60 min at RT. Slides were then washed twice in TBS, followed by a 10 min incubation with Hoechst-33342 (Thermo Fisher Scientific) before a final wash in TBS followed by sealing the slide with Slowfade Gold antifade reagent (Thermo Fisher Scientific). All tests were repeated at least three times. Control stains were produced in the same way with the exception of removal of the primary antibody.

Image quantification

Histone stained HaCat cells were imaged as described above. All image data was analysed using CellProfiler 4.2.4 [14]. For each image, cells were identified using the Hoechst-33342 nuclear stain. The histone stain that overlapped with the nuclear stain was then quantified with median intensity measurement for the nucleus area with the median background signal removed. The average was then taken for measurements of the three repeats.

Statistical analysis

Histone stained with ImmunoHCR was analysed using a one-way ANOVA test to determine statistical significance. Normality was tested using a Shapiro-Wilk test and multiple comparison test against non-purified hairpins was performed using a Dunnett test.

Results

Purification and initial analysis of hairpins

HCR requires three separate oligonucleotides, a triggering oligonucleotide together with a pair of oligonucleotide hairpins. After initiating the reaction with the triggering oligonucleotide, the pair of oligonucleotide hairpins will stepwise unfold and bind to the pairing hairpin, building a long nicked double stranded DNA molecule. The reaction requires that each and every hairpin can trigger the next hairpin to continue the reaction, as a single faulty hairpin could terminate the elongating polymer resulting in an incomplete polymerization.

To investigate the link between amplification and hairpin purity, the same hairpin pairs were purified using various strategies (Fig. 2). Initially the complete hairpins were purified using PAGE-purification. They were also purified using a ligation-based protocol, where the hairpin was split into two parts; a 6-mer and a 34-mer. Both hairpins were split at the 'triggering part' of the hairpin. The rationale for this intentional truncation was that erroneous 6-mers would either be less likely or unable to ligate to the longer 34-mer. Similarly, if the 34-mer was truncated around the ligation site no ligation would occur. In addition, presence of internal sequence errors would disallow the hairpin structure of the 34-mer, which also would prevent ligation. As such, the ligation is a pseudo quality control of the produced oligonucleotides, where only correct or close to correct sequences would be ligated. These ligation products were then further purified by PAGE, where the ligated 40-mer could be separated from the unligated 34-mer. As the 6-linker contained a desthiobiotin-modification, the ligation product could also be pulled down using streptavidin coated magnetic

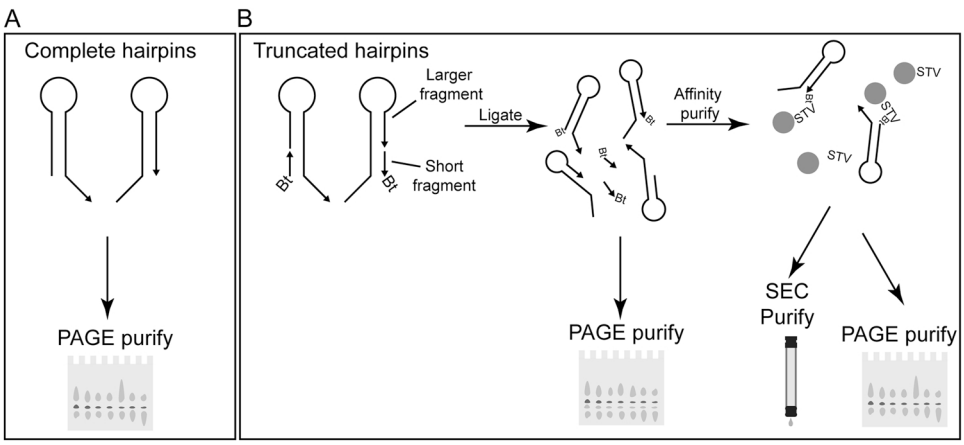

Fig. 2. Purification methods explored. (a) Oligonucleotides were purchased as complete HPLC purified hairpins and either used as is (Non-Purified, NP), or further purified with denaturing PAGE-purification (Page purified, Pp). (b) Hairpins were purchased in truncated parts consisting of a short oligonucleotide with a biotin modification and a longer oligonucleotide. These were initially ligated and used as is (Ligated, L) or further purified with either PAGE (ligated PAGE-purified, LPp) or affinity purified with streptavidin beads (ligated bead-purified, LBp). Finally, the bead-purified hairpins were further purified with either denaturing PAGE (ligated bead- and PAGE-purified, LBPp) or Size exclusion chromatography (SEC) (ligated bead- and SEC-Purified, LbSEC).

beads, removing unligated 34-mers. The bead purified hairpins were then further purified with either PAGE or SEC. The purified hairpins were then investigated using PAGE with a non-denaturing TBE-gel (Fig. 3a). The gel shows that almost all hairpin bands appear identical, despite the different purification methods applied. Further investigation of the hairpins using denaturing PAGE revealed a smearing pattern of erroneous oligonucleotides below some of the non-purified hairpins (Fig. 3b), which are dramatically reduced upon purification. The band below the ligated hairpins shows that there is a fraction of non-ligated oligonucleotides, which are removed by the subsequent purifications.

Amplification of purified hairpins

As the non-denaturing PAGE analysis of the purified hairpins showed virtually no difference in the hairpin bands and the denaturing PAGE only revealed a small amount of smearing, the investigation was continued by performing an HCR-reaction with the non-purified and purified hairpin pairs (Fig. 4a). Unlike the previous analysis, the various purifications provided different amplification results. Despite not showing any large amount of impurities, the non-purified hairpin pair barely polymerized at all. By performing a single PAGE purification on the complete hairpin, the HCR was far more complete. Unsurprisingly, the non-purified ligated hairpins also showed a very poor polymerization, likely due to remaining 34-mers being unable to propagate the reaction. Affinity-purified hairpins produced far larger HCR-product compared to non-purified control, but the non-initiating control also showed a pronounced leakage. The three remaining purifications with ligated hairpins performed roughly equally, with a strong amplification and faint leakage. Further comparison between three separately purchased non-purified hairpins reveal a large difference between the three batches (Fig. 4b). It should however be noticed that the difference between separate purification batches (based on separate purchase batches) was far smaller, indicating that batch difference can be overcome by further purification (supplementary Figure 1).

ImmunoHCR

HCR is used for signal amplification in several *in situ* staining methods, such as fluorescent *in situ* hybridization (FISH) [2,3] and proxHCR [5,6]. To analyse the effect of the different purification strategies in *in situ* methods. isHCR was utilized. Here, the stain is visualized by polymerizing HCR hairpins to triggering sequences conjugated to secondary antibodies. The conjugated secondary antibodies were used to detect E-cadherin in HaCat cells (Fig. 5a). Similar to the PAGE analysis of amplification, non-purified hairpin pairs produced a weak HCR stain. The other stains using purified hairpins were notably better, with all the ligated variants yielding stronger stainings. It should be noted that the non-purified hairpins gave a correct staining pattern, but the required exposure time was far longer compared to other hairpin pairs (supplementary Figure 2).

To quantify better the difference, HaCat cells were also stained using a histone H3 antibody (Fig. 5b). Similar to the E-cadherin results, the non-purified antibodies resulted in far weaker staining compared to the other purification variants. In order to quantify better the data, the signals in stained and unstained cells (with the primary antibody removed) were measured. PAGE purifying the hairpins once resulted in

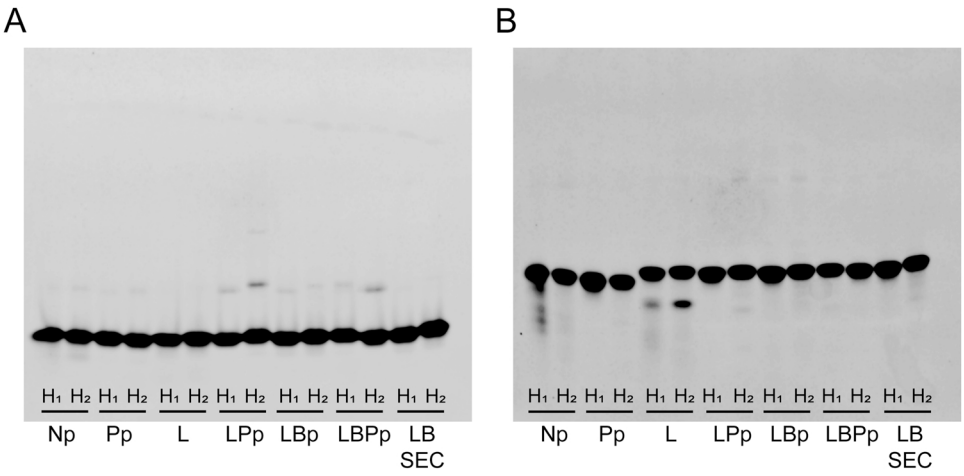

Fig. 3. PAGE analysis of purified and non-purified hairpins. (a) Non-denaturing 4–20% TBE gel loaded with hairpin pairs of different purification methods. (b) Denaturing 10% TBE-urea gel heated to 55 °C loaded with hairpin pairs of different purification methods. In both gels (a-b) hairpin pairs are loaded as non-purified (lane 1–2), PAGE purified (lane 3–4), Ligated (5–6), Ligated PAGE-purified (7–8), Ligated Bead purified (9–10), Ligated Bead- and PAGE-purified (11–12) and Ligated Bead- and SEC-purified (13–14). Odd lanes are loaded with hairpin 1 and even lanes are loaded with hairpin 2.

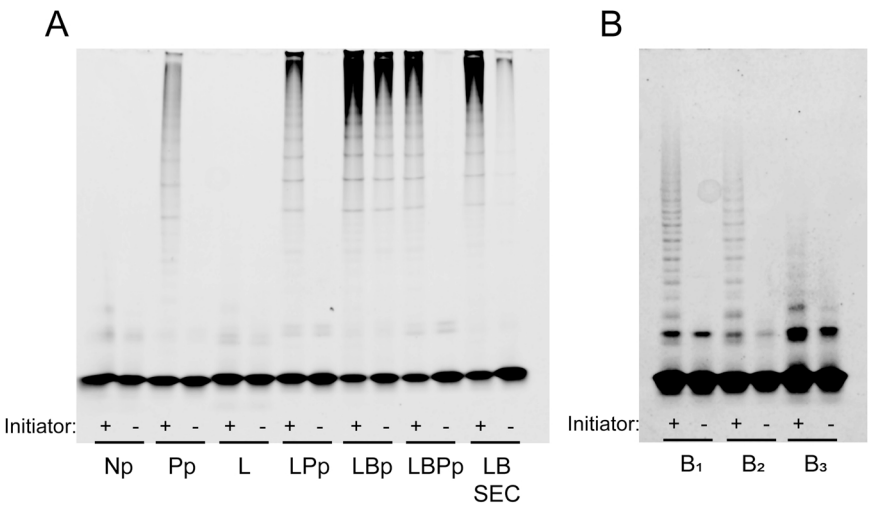

Fig. 4. In solution polymerization of purified and non-purified hairpins. For all reactions, hairpins were diluted and snap-cooled separately before being mixed together to a final concentration of 0.5 μM. Polymerization was either triggered with the addition of 1:50 triggering sequence (odd lanes, marked with +) or left untriggered (even lanes, marked with -). (a) Triggered and untriggered HCR reactions for all purification strategies. (b) Triggered and untriggered HCR reaction for three separate batches of purchased complete hairpins.

a 2.5-fold increase in intensity, compared to the non-purified hairpins (Fig. 5c). The non-metastable bead-purified hairpins, while deviating between batches, increased signal over 9-fold compared to non-purified hairpins. While the increase in signal strength was fairly large, there were only minor differences in background signal (supplementary Figure 3).

Applicability to other hairpin sequences

In order to verify if the problem with truncations along with the improvement *via* purification applied to other oligonucleotide sequences as well, two more hairpin pairs were tested *in solution.* The first hairpin pair contained a 6nt foothold with a 12nt stem described in [15]. The second pair contained a 9nt foothold and a 12nt stem and was initially described in [3]. Both hairpin pairs were purchased either as complete hairpins with or without fluorophores conjugated, or split into two truncation variants featuring one larger and one smaller fragment. The first variant splits the stem at the 'triggering region' resulting in a larger fragment and a short 6-mer similar to previous experiments. The second variant splits the hairpins at the 'starting region' (the end where the foothold is situated), resulting in a larger fragment and a small fragment containing 6 nucleotides along with the foothold. These two variants were initially ligated overnight. Next, both the complete hairpins and the ligated variants were purified using denaturing PAGE to further improve purity. As the affinity based bead purification coupled with SEC or PAGE only gave a small increase in amplification over just ligation coupled with PAGE, this purification variant was excluded.

To test if the purification affected the HCR amplification for these hairpin sequences both hairpin sets and all purification variants were mixed separately with or without initiator sequence and allowed to polymerize for 2 h. Starting with the 9nt foothold and 12nt stem hairpin pair, it is possible to see a clear improvement in amplified product and consumption of the monomers for the purified variants (Fig. 6a). Although the difference between PAGE purified complete hairpins and ligated hairpins is fairly small, slightly less leakage was observed for ligated and purified hairpins. Furthermore, using fluorophore-conjugated hairpins, the non-purified hairpins gave a considerably worse amplification which could be improved by further purification. For the 6nt foothold and 12nt stem hairpin pair the PAGE-purified and non-purified resulted in fairly similar amplifications for the non-fluorescent hairpins. There was a slight improvement for the hairpins that were both ligated and PAGE purified. For the fluorophore coupled hairpins, there was a clear improvement in amplification for both purified variants with a clear decrease in non-specific amplification for the ligated variant.

Discussion

We have herein shown that increased purity of HCR hairpins has a profound impact on its amplification properties. The erroneous hairpins can be divided into separate categories: inert hairpins that neither hinder nor start the HCR reaction by itself; and reaction starting hairpins, where the triggering sequence of the hairpin remains intact but the complementary stem is destabilized enough to allow for triggering sequence independent polymerization. There are also reaction stopping hairpins, most likely where the triggering sequence itself is damaged and are unable to propagate the reaction. Our proposed ligation-based purification, assuming that non-ligated hairpins can be removed, should reduce reaction stopping and starting. The method splits the hairpin in the triggering sequence. If too many mismatches occur in the hairpin structure it becomes unlikely to fold in on itself; likewise the linker sequence would be less likely to bind. On the other hand, a truncation in the hairpin part would result in a gap at the ligation site rendering it unable to ligate.

Synthesis of oligonucleotides inherently carries a risk of miss-incorporations and potential truncations over the synthesis cycles [11]. As such, any produced oligonucleotide requires additional post-synthesis purification. Typically, oligonucleotides are purified with either HPLC methods or PAGE, however mismatches will most likely not be resolved in these methods and small truncations can be hard to completely remove in a reasonable scale.

Indeed similar problems have been observed for other DNA-based circuits. In a paper investigating non-covalent DNA catalytic reactions, catalytic DNA activity was investigated for a DNA circuit containing a catalyst-, substrate- and fuel-strand with either purified and non-purified DNA-strands [16]. The paper revealed a clear improvement in catalytic activity when the DNA-strands were purified. Similar results could be seen for the catalysed hairpin assembly (CHA) DNA circuit. Here, leakage could be reduced by further purification, and perhaps most interestingly, the best reactions were achieved by enzymatically produced DNA-strands [17]. Considering these papers, perhaps an enzymatic production could provide better and more robust HCR hairpins.

As shown in Fig. 3, judging the quality of the oligonucleotides using a typical in-house analysis such as non-denaturing PAGE does not reveal the degree of impurity. Evaluation of different hairpin designs for HCR, will hence be severely affected by the purity of the hairpins. Amplification efficiency and kinetics of HCR hairpins becomes a function of both purity and sequence.

Based on the amplifications in Figs. 4 and 5, the overall quality of the purified hairpins is increased. Unsurprisingly the non-purified ligated

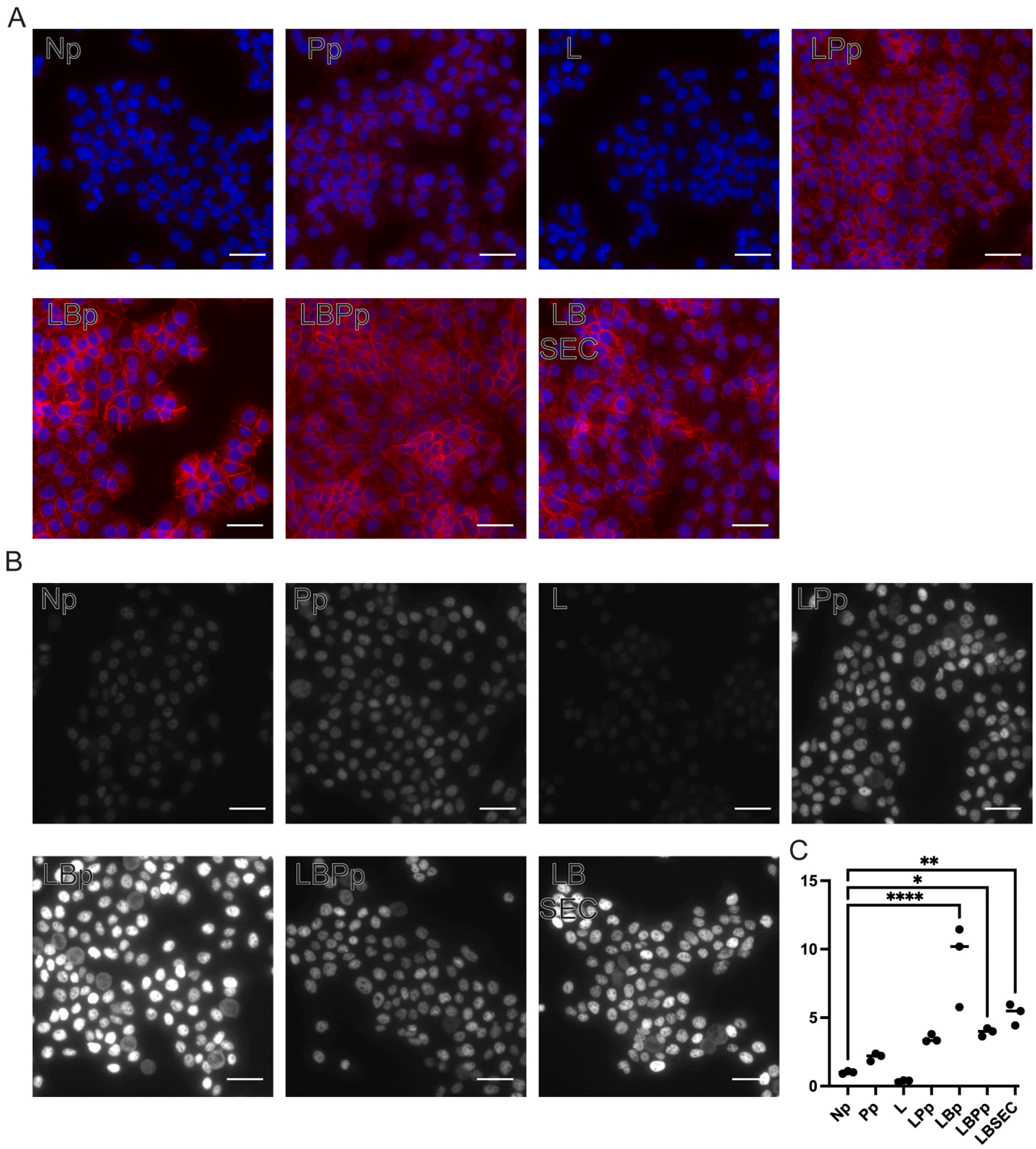

Fig. 5. ImmunoHCR stain with purified and non-purified hairpins. (a) HaCat-cell were stained with primary antibodies targeting E-cadherin and secondary antibodies conjugated with a triggering sequence. The slides were then visualized by adding purified hairpin pairs. (b) HaCat-cell were stained with primary antibodies targeting histone-H3 and secondary antibodies conjugated with a triggering sequence. (c) Intensities measured from the histone-H3 stain, Significant differences (*P < 0.05, **P < 0.01, ****P < 0.0001).

hairpins performance was poor, most likely due to remaining unligated 34-mers. Due to the truncated triggering sequence, an unligated hairpin will be unable to propagate the HCR reaction. Perhaps more surprising is the triggering sequence-independent propagation of the bead-purified hairpins. These hairpins properly propagate the reaction, hinting to a lack of stopping truncations. However, they will propagate regardless of triggering sequence. This could be caused by remaining biotinylated link-oligonucleotides after the pull-down. Further purification, either *via* PAGE or SEC seems to resolve the problem. This non-specific amplification seems to have aided in increasing signal strength for the histone stains in Fig. 5b. One possibility would be the pre-amplification of this purification batch in the stain solution, allowing for "pre-polymerized"

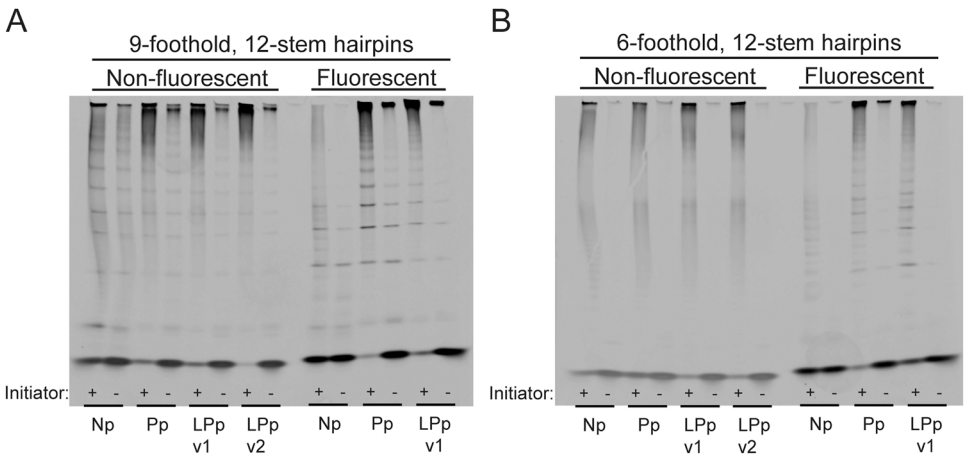

Fig. 6. Purification and amplification of other hairpin sequences. For all reactions, hairpins were diluted and snap-cooled separately before being mixed together to a final concentration of 0.5 μM. Polymerization was triggered by adding a triggering sequence at a concentration of 0.01 μM. All hairpin sets were allowed to react for 2 h before being loaded to a polyacrylamide gel. Lanes marked with a + have added triggering sequence, lanes marked with - are without. Np= non-purified, Pp=PAGE purified, LPp v1 =ligated PAGE purified variant 1 (split at 'triggering region'), LPp v2 = ligated PAGE purified variant 2 (split at the 'starting region', the foothold end). (a) Amplification of 9nt foothold, 12nt stem. **(b)** Amplification of 6nt foothold, 12nt stem.

fragments to bind to the triggering sequence-conjugated antibodies.

As seen in Fig. 6, the problem with truncations was also apparent for other hairpin sequences. Perhaps most significant for other groups was the finding that non-purified fluorophore-conjugated hairpins performed considerably worse compared to both their purified counterparts and their non-fluorescent counterparts. This is likely to be due to more difficulties in post-synthesis purification of the modified hairpins. However, even for the non-modified hairpins there was an improvement with the purifications, but as the starting material was better, subsequent purifications made less of a difference. Based on these data, it could potentially be better to split fluorescent hairpins into a larger fragment containing the loop and a shorter linear fragment containing the fluorophore to be ligated together.

The choice of purification strategy will depend on time and cost invested, along with required amplification strength. PAGE-purifying either complete hairpins or ligated hairpins give a strong increase in amplification at a fairly low time investment and decent yields. Bead purifying gives a large increase in amplification, but a leakage prone result. Further treatment of bead-purified hairpins will alleviate the leakage propensity, but comes at the cost of lower yields and greater time commitment. As a reference point, our bead purifications typically resulted in a yield of around 70%, SEC around 60% and PAGE yields varying between 20% and 60%. Generally, each purification batch started with about 1500pmol.

The current ligation-based strategy proposed here most likely does not remove all sequence errors from hairpins, and small mismatches and minor truncations will probably remain. Further optimization of linker length, ligation conditions and post-ligation purification strategies could potentially further improve the oligo quality.

Conclusion

In this paper it is shown that by further purifying the HCR oligonucleotides, one can greatly improve amplification both *in solution* and *in situ*. The amplifications become more robust and less variable over batches, while only requiring a simple additional purification available to most laboratories.

Declaration of Competing Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.04.004.

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