Polymerase-endonuclease amplification reaction for

large-scale enzymatic production of antisense oligonucleotides

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ABSTRACT

Synthetic oligonucleotides are contaminated with highly homologous failure sequences. Oligonucleotide synthesis is difficult to scale up because it requires expensive equipments, hazardous chemicals and tedious purification process. Here we report a novel thermocyclic reaction, polymerase-endonuclease amplification reaction (PEAR), for the amplification of oligonucleotides. A target oligonucleotide and a tandem repeated antisense probe are subjected to repeated cycles of denaturing, annealing, elongation and cleaving, in which thermostable DNA polymerase elongation and strand slipping generate duplex tandem repeats, and thermostable endonuclease (PspGI) cleavage releases monomeric duplex oligonucleotides. Each round of PEAR achieves >100-fold amplification. The product can be used in one more round of PEAR directly, and the process can be further repeated. In addition to avoiding dangerous materials and improved product purity, this reaction is easy to scale up and amenable to full automation, so it has the potential to be a useful tool for large-scale production of antisense oligonucleotide drugs.

Keywords: *antisense oligonucleotide; microRNA; thermostable DNA polymerase; thermostable endonuclease; DNA amplification*

Introduction

MicroRNAs (miRNAs) are a family of short noncoding regulatory RNA molecules. The miRNA pathway serves as an important post-transcriptional regulation mechanism (*1*). Synthetic antisense oligonucleotides to target miRNAs or their mRNA targets are proving to be powerful tools for molecular biology research (*2*) and may eventually find application as new therapeutic agents (*3, 4*).

Large quantities (possibly from kilograms to tons) of a specific oligonucleotide have to be produced for commercial production of antisense oligonucleotide drugs. Therefore, the development of an economical and safe method for industrial production of short oligonucleotides has become necessary. Traditional method for *de novo* oligonucleotide synthesizing is the phosphodiester method (*5*). Using automatic synthesizers, micrograms to kilograms of a specific oligonucleotide can be produced in a few hours. Unfortunately, however, synthetic oligonucleotides are contaminated with a significant fraction of truncated failure sequences, make the product purification process difficult. Moreover, the oligonucleotide synthesis process requires not only expensive equipments, but also costly and hazardous chemicals. For example, an organic solvent (dichloromethane or toluene) must be used to dissolve the deblocking reagent. Therefore, large-scale production of oligonucleotides is not only costly, but also involves disposing dangerous waste that pose threats to human health and environmental safety.

In modern molecular biology laboratories, nucleic acids are routinely amplified by polymerase chain reaction (PCR). PCR has the limitation, however, of needing a pair of synthetic primers. This imposes not only a length constraint on the target DNA, but also a yield limit on the PCR product defined by primer concentrations. Therefore, PCR-based methods are generally not applicable for the amplification of short oligonucleotides that are only about 20 nucleotides (nt) in length. Enzymatic amplification of oligonucleotides has been reported by successive rounds of ligation, rolling circle replication (RCR) and cleaving (*6*) or nicking (*7*), but a laborious recircularization process is required for each round of RCR. Here we report a novel thermocyclic reaction, polymerase-endonuclease amplification reaction (PEAR), for fast amplification of oligonucleotides. In addition to avoid using dangerous materials and improved product purity, this reaction is easy to

scale up and amenable to full automation, thus enabling large-scale and pollution-free antisense oligonucleotide production.

The principle of PEAR

A PEAR reaction contains a target oligonucleotide (*X*), an antisense probe, a thermostable DNA polymerase (Taq polymerase), a thermostable endonuclease (PspGI), four dNTPs and an appropriate buffer solution. The antisense probe, denoted by *X'R'X'*, is designed to be a single-stranded oligonucleotide contains at least two tandem repeated complements of the target sequence (*X'*) that are separated from one another by an intervening complementary recognition site (*R'*) for PspGI.

As shown in Fig 1, PEAR consists of repetitive cycles of: (1) heat denaturation, (2) annealing, (3) elongation and (4) cleaving. In the first cycle, a target oligonucleotide and an antisense probe were heat denatured and annealed to form a partial duplex (*X/X'R'X'*). When a target oligonucleotide binds to a probe in the upstream (Fig 1, top right), there is no elongation, because it provides no primer/template structure for the Taq polymerase. However, as both target and probe are present in a large number of copies, according to the law of probability, nearly half of the target oligonucleotides bind to the probe in the downstream. In the presence of dNTPs, they are elongated by Taq DNA polymerase to form fully matched duplex tandem repeats (*XRX/X'R'X'*). Subsequently, PspGI cleavage of the recognition site releases monomeric oligonucleotides (*X/X'*). Thereafter, a next cycle of denaturation, annealing, elongation and cleaving is started again, resulting in exponential amplification of the duplex oligonucleotide.

In addition, the tandem repeated duplexes are not fully digested by PspGI, because the duration of cleaving is rather short. When the remaining tandem repeated duplexes are subjected to more cycles of denaturing, reannealing and elongation, the number of repeat units increases continually through slipped strand pairing and polymerase elongation (Fig 1, right). When PspGI cleavage monomerizes the elongated tandem repeats in a following cycle, many more duplex oligonucleotides are released. It is this *slipping-and-cleaving mechanism* that promotes not only the rate of amplification, but also the yield of product. Moreover, the PEAR products can be used in a next round of

PEAR amplification directly without any treatment, and the process can be further repeated. The tandem repeated oligonucleotides are thus called *seeds,* because they can reproduce themselves.

Implementation of PEAR

A synthetic oligonucleotide and an antisense probe derived from human microRNA miR-375 were used to validate the proposed reaction mechanism. PEAR reactions with complete and incomplete (lacking Taq DNA polymerase, PspGI or target) components were conducted under previously optimized reaction conditions with target concentration at 1 nM and probe concentration at 100 nM. As shown in Fig 2, a lower band represents the duplex product *X/X'* and several upper bands represent tandem repeats are observed in the complete PEAR reactions, but such bands are not observed if any of the four essential components, the two enzymes, the target and the probe, is omitted. We tested another three pairs of targets and antisense probes, amplification are all dependent on the presence of both of the two enzymes, the target, and the probe (Data not show).

To determine the sensitivity of PEAR, a series of reactions were conducted with target concentrations ranging from 0.1 pM to 1 nM and probe concentration at 100 nM. As shown in Fig 3, the yield is very high when target concentration was at 0.1 to 1 nM, whereas relatively low when target concentration was < 10 pM.

Hpy99I digestion of the PEAR product

Although duplex oligonucleotides showed increased cellular uptake when compared to single-stranded antisense oligonucleotides (*8*), and demonstrated improved *in vitro* potency and stability compared to small interfering RNA (*9*), single-stranded antisense oligonucleotides have been more frequently used in practice (*10-13*). Therefore, it is necessary to separate the antisense oligonucleotides from its complementary strands. To facilitate the separation of the two complementary strands, we introduced a recognition site for restriction enzyme Hpy99I into the probe positioned between the tandem repeats and downstream to the recognition site for PspGI. At the end of PEAR, PspGI cleavage of the product have resulted in a 5-nt overhang in the 3'-end of the sense strand. After

PEAR, the product were pooled and further digested with Hpy99I, resulted in another 5-nt overhang in the 5'-end of the the sense strand (Fig 4). Note that the antisense strand is cleaved into 20-nt monomers, and the sense strand is cleaved into monomers that are 25 or 30 nt in length, allowes convenient separation of them by chromatographic methods.

Separation and analysis of the antisense oligonucleotides

For separation of the antisense oligonucleotides, we used an anion exchange chromatography using a SOURCE 15Q column (*14*). The high pH stability of SOURCE 15Q allows facile separation of the antisense oligonucleotides from its complementary strands under denaturing alkaline conditions at pH 12. The chromatogram shown in Fig 5 represents the purification of 1 nmol of antisense oligonucleotides. The antisense fraction was collected within the window indicated in Fig 5. The 20-nt antisense oligonucleotides were separated from the sense strands, short by-products and remaining tandem repeats. Collected fraction was further analyzed by anion exchange chromatography and capillary electrophoresis. As shown in Fig 6, the purity of the collected fraction analysis by chromatography was > 99.0%. Fig 7 shows further analysis of the purified antisense oligonucleotide by capillary electrophoresis. The purity was confirmed to be better than 99.0%. The purity level of this oligonucleotide product is significantly improved when compared to that of HPLC-purified synthetic oligonucleotides (*14, 20*). This is primarily due to avoiding of highly homologous impurities, especially (n-1) deletions.

Scale-up and productivity evaluation

PEAR reactions were run in 96-well plates, with 95x100 μL PEAR reactions and a no target control (NTC) in each run. To demonstrate the ability to scale up, three successive rounds of PEAR were conducted, respectively at scales of 1, 100 and 500 runs. The product of round 1 was used diluted 1:100 as seeds for round 2 directly without any treatment, and that of round 2 for round 3. After cleavage and purification, the quantity of purified antisense oligonucleotide was given by measuring its optical density at a wavelength of 260 nm. The average concentrations, purities, yields and total yields of recoverable (purified) products are estimated and summarized in Table 1. On average, the

concentration of purified product is about 10 µM or 70 ng/µl, which is ~100-fold higher than the input concentrations. The average yield of purified product of a single run is about 0.67 *mg*, which is greater than the typical yield of HPLC-purified product of a 1-µmole scale commercial synthesis. The total yield of round 2 (a 100-fold scale-up of round 1) was increased to ~67 mg; and that of round 3 (a 5-fold scale-up of round 2), was further increased to ~336 mg, demonstrated that this method is well suited to scale up.

Discussion

MiRNAs plays an important role in post-transcriptional gene silencing that is highly conserved among species (*1*). Synthetic antisense oligonucleotides to target miRNAs or their target mRNAs have been proving to be powerful tools to specifically and selectively regulate the expression of genes, and to investigate the roles of dysregulated genes in human diseases (*2*). MiR-375, for example, is highly expressed in pancreatic islets and is required for glucose homeostasis that is conserved in zebrafish, mice and human (*15*, *16*). Obese mice exhibit increased miR-375 expression, and genetic deletion of miR-375 resulted in a severe diabetes (*17*). Targeted inhibition of miRNA with a modified antisense oligonucleotide reveals the role for miR-375 in pancreatic islet (*18*).

Antisense oligonucleotides to target miRNAs or mRNA are expected to be capable of curing a wide variety of human diseases, including cancer, obesity, cardiovascular and metabolic diseases (*3, 4*). By far, antisense oligonucleotides are all manufactured by solid-support phosphoramidite-coupling chemistry (*8-14*). Dichloromethane (CH_2Cl_2), a halogenated solvent, was used to dissolve the deblocking reagent (trichloroacetic acid, TCA, or dichloroacetic acid, DCA). As oligonucleotide synthesizers are now available for syntheses in large scale, the quantity of chlorinated waste generated becomes quite large (*19*). The high toxicity, high volatility and carcinogenicity of dichloromethane are leading to stringent regulation by regulatory agencies. Replacement of dichloromethane with toluene, a recyclable non-halogenated solvent, eliminated the production of large quantities of chlorinated waste (*19*). Toluene, however, is a highly volatile, flammable liquid with strong odor, and is still harmful to human health and environmental safety.

Typically, crude products from solid-support organic synthesis is contaminated with

a significant fraction of highly homologous failure sequences that arise from incomplete detritylation, coupling, sulfurization, capping or deprotection (20). The most significant impurities found in synthetic oligonucleotides are the (n-1) deletions that differ from the full-length product by lacking only one of the necessary nucleotides. For oligonucleotides 18–21 nucleotides in length, it is difficult to remove the (n-1) deletions completely from full-length oligonucleotides by HPLC (20).

As a novel, simple method for the amplification of short oligonucleotides, PEAR is capable of solving all above problems. The basic raw materials, nucleotides and enzymes, are all safe, inexpensive and pollution-free. Hence, although a small-scale synthesis of the seeds is still a prerequisite, the use of hazardous chemicals is reduced to a minimum. Moreover, due to avoiding of (n-1) deletions, purification of the antisense oligonucleotide is made easy, and the purity level of the product is also improved.

For the majority of existing DNA amplification technologies, including polymerase chain reaction (PCR) (23), ligase chain reaction (LCR) (24), rolling circle amplification (RCA) (25), loop-mediated isothermal amplification (LAMP) (26), strand displacement amplification (SDA) (27) and helicase-dependent amplification (HDA) (28), all needs a pair of short synthetic primers to achieve exponential amplification. The yields of the final products are inherently dependent on and limited by input primer concentrations.

Exponential amplification reaction (EXPAR) is a fast isothermal reaction that can achieve 10^6-fold amplification of a target oligonucleotide in 10 min (29). However the utility of EXPAR is currently limited, as serious nonspecific background amplification was reported (30). Moreover, obviously in EXPAR DNA synthesis proceeds in only one of the two strands (the target strand), but not in the other (the antisense strand), so the product yield is still limited by the input concentration of the antisense template.

Noteworthy, rolling-circle replication (RCR) proceeds in a linear fashion using the highly processive phi29 DNA polymerase can copy a circular probe into a DNA strand containing >1,000 tandem repeated complements of a circularized DNA molecule (25). Circle-to-circle amplification (C2CA), a multistep RCR-based process for strand-specific amplification of circularized DNA (6), has been used for the amplification of DNA circles, in which tandem repeated complements of DNA circles are generated by RCR,

and converted to monomeric circles of opposite polarity to that of the starting material. Billion-fold amplifications was achieved through successive rounds of ligation, RCR and cleaving, however a laborious cleavage, ligation and recircularization process is required for each round of RCR amplification. Moreover, a synthetic *ligation template* is required to recircularize and monomerize the tandem repeated complements, so this technique requires the additional production of large amounts of ligation templates. This problem was eliminated by using a hairpin-containing self-templating oligonucleotide that contains a *suicide cassette,* a recognition site for a nicking enzyme, and recircularizing and monomerizing the tandem repeated complements by nicking the suicide cassette (*7*), however, a laborious nicking, ligation and recircularization process is still needed for each generation of RCR, makes it not readily amenable to automation.

In contrast, PEAR is a simple but effective method for the amplification of short oligonucleotides through the unique slipping-and-cleaving mechanism. As is evident from the data presented here, one round of PEAR amplification can result in >100-fold increment in product yield. We have observed >1000-fold in PEAR reactions (data not show), but we adopted a one hundred-fold amplification for the scale-up reactions in this study, because it provides more reliable and uniform product yield. Three rounds of PEAR starting from micrograms of seeds produced more than three hundred milligrams of products. Thus more rounds of PEAR starting from these hundred-milligram seeds can yield dozen-grams, then kilograms, and then tons, of products. Thus, one hundred-fold scale-up is sufficient for large-scale oligonucleotide production.

In this preliminary study, we demonstrated only the production of an unmodified antisense oligonucleotide. Modified antisense oligonucleotides, such as phosphorothioate oligodeoxynucleotides (*21*), 2'-O-methoxyethyl (*10-12*), 2'-O-methyl (*11-12*) or 2'-fluoro substituted oligonucleotides (*11-12*), and locked nucleic acid (*11-13*), are reported to demonstrate increased stability and potency when compared to its native counterparts. Inspiringly, modified DNA bearing 5(methoxycarbonylmethyl)-2'-deoxyuridin has been prepared in large scale by PCR and postsynthetic derivatization (*22*), implies that it might be feasible to prepare modified oligonucleotides in large scale by PEAR amplification and/or postsynthetic derivatization.

Finally, PEAR amplification of antisense oligonucleotides uses much less expensive thermocyclers instead of highly expensive DNA synthesizers. The cost of equipments in large-scale production of oligonucleotide could be reduced greatly. The cost of raw materials could also be cut down sharply if they were used in bulk quantities. Because the product can be used as seeds in a next round of amplification directly without any treatment, so the process is readily amenable to full automation. In conclusion, the ability of PEAR to avoid handling of dangerous chemicals, simplified product purification, improved product purity, easy to scale up and amenable to automation would all be beneficial when compared to traditional organic synthesis. So it might be worthwhile to replace organic synthesis with this simple, safe and economic method in large-scale production of oligonucleotide drugs.

Materials and methods

Enzymes and oligonucleotides

Taq DNA Polymerase and restriction enzymes, PspGI and Hpy99I, were purchased from New England Biolabs. A target oligonucleotide and two antisense probes to target human microRNA miR-375 were custom synthesized and HPLC-purified by Invitrogen Life technologies. The sequence of the target oligonucleotide is 5'-TGT TCG TTC GGC TCG CGT GA-3'. Both of the two probes contain three tandem repeated complements of the target sequence (X'). The first probe (X'R'X'R'X') was used for validation of the reaction mechanism, in which the three complements are separated from each other by a recognition site (R') for PspGI. The second probe (X'R'H'X'R'H'X') was used in scale-up reactions, in which two recognition sites (R' and H'), respectively for PspGI and Hpy99I, are embedded between the repeats. The sequence of the first probe is 5'-TCA CGC GAG CCG AAC GAA CAC CAG GTC ACG CGA GCC GAA CGA ACA CCA GGT CAC GCG AGC CGA ACG AAC A-3', and that of the second probe is 5'-TCA CGC GAG CCG AAC GAA CAC CAG GTT TTC GAC GTC ACG CGA GCC GAA CGA ACA CCA GGT TTT CGA CGT CAC GCG AGC CGA ACG AAC A-3'. The purity of the target oligonucleotide and probes received was ~95.0%. They were repurified at home by

anion exchange chromatography to achieve >99.0% purity.

PEAR reactions

PEAR were carried out in 96-well plates on a Applied Biosystems 9700 Thermal Cycler, each in a 100 µL volume reaction mixture containing 200 µM each dNTP, 15 mM Tris-HCl, 30 mM KCl, 5 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.02 % BSA, 0.08 u/µl Taq DNA polymerase, 0.4 u/µl PspGI restriction enzyme, desired amount of target oligonucleotide and antisense probe. The reactions were initiated at 95°C for 1 min, followed by 30 cycles of denaturing at 94°C for 15 sec, annealing at 55 °C for 35 sec, elongation and cleaving at 75°C for 5 min. If desired, PspGI digestion of the product is conducted by a final incubation at 75°C for 30 min. PEAR products were separated by 15% non-denaturing polyacrylamide gel electrophoresis (PAGE), and visualized under an ultraviolet illuminator after SYBR Gold staining (Molecular Probes).

Cleavage, separation, purification and quantification

The PEAR products were pooled and fully digested by the addition of 1 volume of cleavage mixture containing 1× NEBuffer 4, and 1.0 u/µl of Hpy99I. Cleavage reactions were incubated for 3 hours at 37°C and stopped by heat inactivation at 65°C for 20 minutes. Separation and purification of the antisense oligonucleotide were performed by anion exchange chromatography using ÄKTA explorer™ 10 system (GE Healthcare) as described (*14*). SOURCE Q PE 4.6/100 prepacked analytical columns filled with polymer-based, 15 μm monosized beads were purchased from GE Healthcare. The column effluent was fractionated and collected using a Frac-950 Fraction Collector (GE Healthcare). The volume of collected fraction was 0.5 column volume (CV). The chromatographic conditions are described in the legend to Fig 5. A HiPrep™ 26/10 column (GE Healthcare) was employed for desalting the purified oligonucleotide using MilliQ™ ultrapure water (Millipore Corporation, Billerica, USA) as eluent. The mass estimates of the purified antisense oligonucleotide were given by 260 nm absorbance measurements of diluted samples. Data were analyzed in Microsoft Excel.

Purity analysis

The purity of purified antisense oligonucleotide was analyzed by anion exchange chromatography as described (*14*). The anion exchange HPLC analysis was conducted on Hewlett-Packard Model 1100 liquid chromatography (Palo Alto, CA, USA) fitted with a non-porous medium DNAPac PA-100 4/250 mm analytical column (Dionex Corporation, Sunnyvale, CA, USA), thermostatted to 25C and operated at a flow rate of 480 cm/h. The chromatographic conditions are described in the legend to Fig 6.

Capillary electrophoresis was performed using a 50-µm I.D.×20-cm (effective length 18 cm) capillary. The capillary was treated with PlusOne™ Bind-Silane (GE Healthcare) and filled with polyacrylamide cyclodextrin gel. Buffer was consisted of 0.1 M Tris-0.25 M boric acid, pH 8.5, containing 7 M urea. Injection was made at 1 kV for 5 sec. Analysis was performed at 1.5 kV for 30 min.
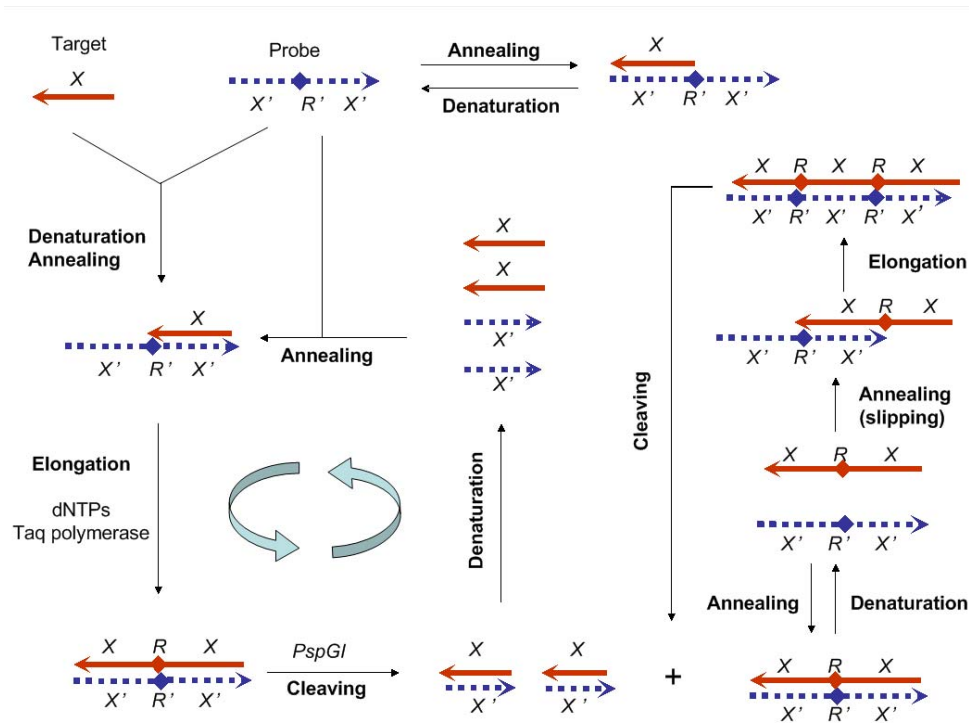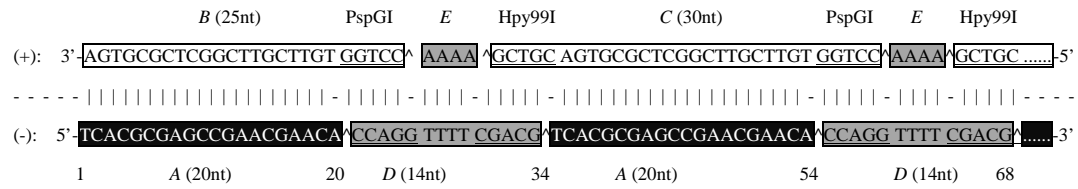
Fig 1. Schematic description of PEAR. Sense and antisense strands are represented respectively by solid and dashed lines, the 3' ends are indicated by arrows and the restriction sites for PspGI are indicated by solid diamonds. When a target oligonucleotide (*X*) binds to a probe in the upstream, it is elongated by a Taq DNA polymerase, a full-duplex contains tandem repeats is produced. If the repeats are cleaved by PspGI, short duplex oligonucleotides (*X/X'*) are released; and if they are not cleaved, the number of tandem repeats increases by slipping and elongation.
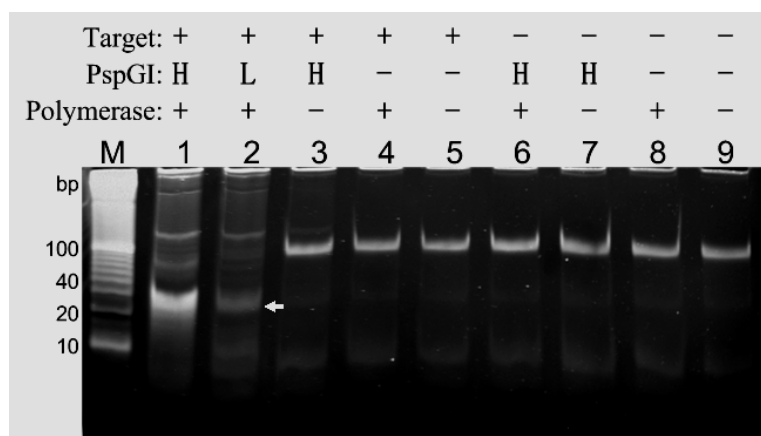
Fig 2. PEAR reactions with complete and incomplete components. Target (X) and probe (X'R'X'R'X') concentrations were respectively at 1nM and 100 nM. For PspGI, H and L stand respectively for high (0.4 unit/μL) and low (0.1 unit/μL) concentrations. Lane M: Invitrogen Trackit™ 10bp DNA ladder; Lane 1-2: complete PEAR reactions containing Taq DNA polymerase, PspGI, the target and the probe. The lower band (show by an arrow) represents the 20-bp duplex monomers, and the upper bands represent tandem repeats; Lane 3-9: incomplete PEAR reactions lacking one or both of the two enzymes or the target. No product band is observed. The bands represent probe self dimers formed by intermolecular interactions.
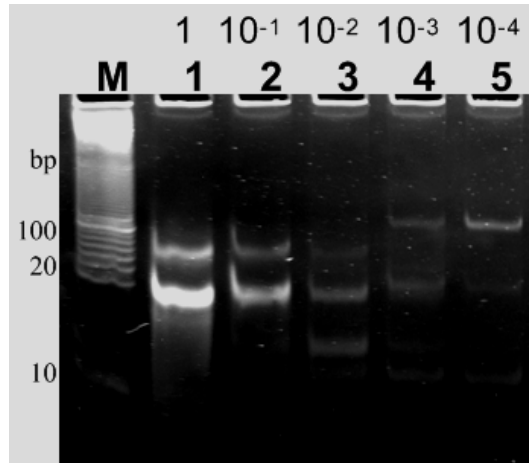
Fig 3. PEAR reactions with different target concentrations. Lane M: Invitrogen Trackit™ 10bp DNA ladder; lane 2-8: PEAR reaction with 1 to 10^{-4} nM of input oligonucleotides. Probe (X'R'X'R'X') concentration is at 100 nM. A final incubation at 75C for 30 min was conducted to cleave the product.

| | *B* (25nt) | | PspGI | *E* | Hpy99I | | *C* (30nt) | | PspGI | *E* | Hpy99I |

(+): 3'-AGTGCGCTCGGCTTGCTTGT GGTCC^ AAAA ^GCTGC AGTGCGCTCGGCTTGCTTGT GGTCC^AAAA^GCTGC ......-5'

- - - - - | | | | | | | | | | | | | | | | | | | | | - | | | | | - | | | | - | | | | | - | | | | | | | | | | | | | | | | | | | | | - | | | | | - | | | | - | | | | | - - - -

(-): 5'-TCACGCGAGCCGAACGAACA^CCAGG TTTT CGACG^TCACGCGAGCCGAACGAACA^CCAGG TTTT CGACG^......-3'

| 1 | *A* (20nt) | 20 | *D* (14nt) | 34 | *A* (20nt) | 54 | *D* (14nt) | 68 |

Fig 4. Double digestion of the PEAR product by PspGI and Hpy99I. The sense and the antisense strand are indicated respectively by (+) and (-). Recognition sites for PspGI and Hpy99I are underlined and marked. Each position where cleavage was expected to occur is indicated by a caret ("^"). The antisense strands (*A*) are black boxed, the sense strands (*B* and *C*) are boxed, and the by-products (*D* and *E*) are grey boxed. The expected length for each strand is indicated in parenthesis.
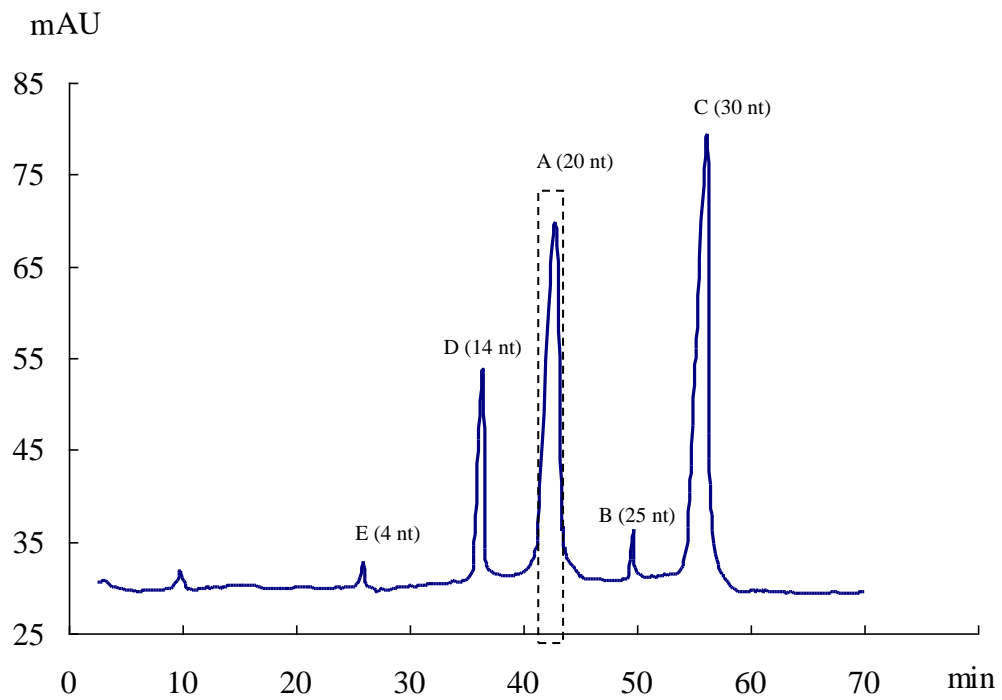
Fig 5. HPLC separation and purification of antisense oligonucleotide. Fractions are indicated by letter A to E as shown in Fig 4. Fraction A, which contains the antisense strands, was collected in the indicated interval. Sample: 10 μg PEAR product double digested by PspGI and Hpy99I; Column: SOURCE Q PE 4.6/100; Flow rate: 1 ml/min; Buffer A: 10 mM NaOH, pH 12; Buffer B: 10 mM NaOH + 2M NaCl, pH 12; Gradient: 20–35% B in 50 column volume.
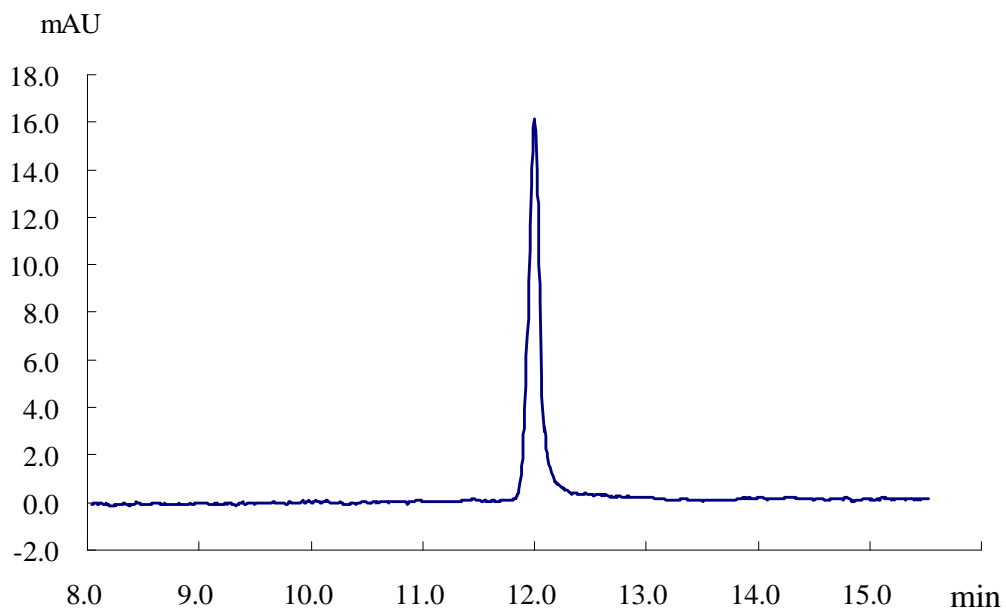
Fig 6. HPLC analysis of purified antisense oligonucleotide. Sample: 25 µL purified antisense oligonucleotide; Column: DNAPac PA-100 (4/250); Flow rate: 1 ml/min; Buffer A: 10 mM $NaClO_4$ + 1 mM Tris; Buffer B: 300 mM NaClO4 + 1 mM Tris; Gradient: 10–70% B, 7.6 CV.
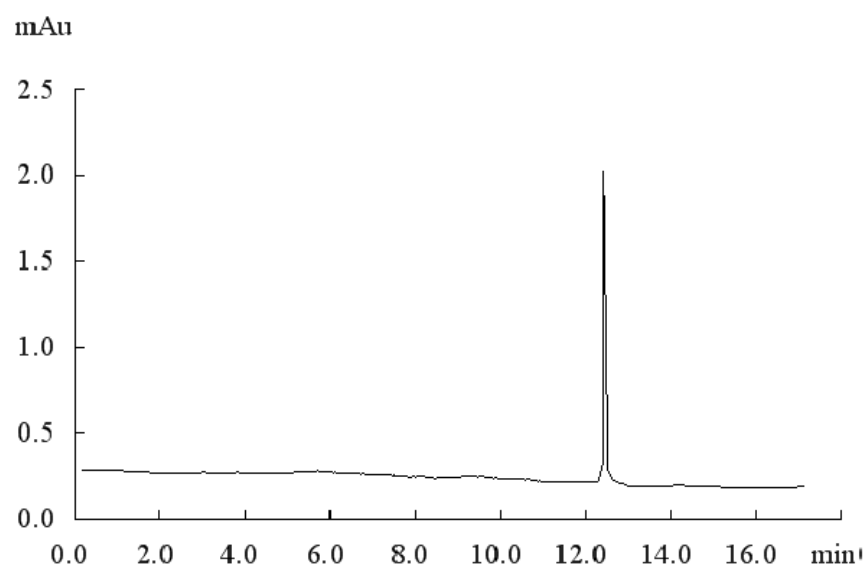
Fig 7. Capillary electrophoresis of HPLC purified antisense oligonucleotide. Capillary: 50 µm×18 cm, filled with polyacrylamide cyclodextrin gel; Buffer: Tris-borate/Urea; Running conditions: 1.5 kV/30 min; Sample application: 1 kV/5 s.

Table 1. Average recoverable concentration, purity and yield of purified antisense oligonucleotide

Round#[a]	Runs[b]	Average recoverable concentration		Purity[d]	Recoverable Yield (mg)	
		(µM)	(ng/µl)		Average	Total
1	1[c]	10.543	70.966	99.18%	0.674	0.674
2	100	10.579	71.209	99.17%	0.676	67.649
3	500	10.524	70.840	99.17%	0.673	336.490

a. The product of round 1 was used as seeds for round 2, and that of round 2 for round 3.

b. Each run consisted 95x100 µl reactions. Target and probe concentration are respectively at 1 nM and 100 nM.

c. Round 1 had two duplicate runs. As the first run had been used as seeds, the second run was used for purification and analysis.

d. Purity was calculated as peak area% at UV 260 nm.

References

[1]   Ambros V. 2004. The functions of animal microRNAs. *Nature* 431:350–355.

[2]   Esau CC. 2008. Inhibition of microRNA with antisense oligonucleotides. *Methods*. 44(1):55-60.

[3]   Weiler J, Hunziker J, Hall J. 2006. Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? *Gene Ther.* 13(6):496-502.

[4]   Rayburn ER., Zhang R. 2008. Antisense, RNAi, and Gene Silencing Strategies for Therapy: Mission Possible or Impossible? *Drug Discov Today*. 13(11-12): 513–521

[5]   Brown EL, Belagaje R, Ryan MJ, Khorana HG. 1979. Chemical synthesis and cloning of a tyrosine tRNA gene. *Methods Enzymol.* 68:109–151.

[6]   Dahl F, Banér J, Gullberg M, Mendel-Hartvig M, Landegren U, Nilsson M. 2004. Circle-to-circle amplification for precise and sensitive DNA analysis. *Proc Natl Acad Sci U S A*. 101 (13):4548-53.

[7]   Lohmann JS, Stougaard M, Koch J. 2007. A new enzymatic route for production of long 5'-phosphorylated oligonucleotides using suicide cassettes and rolling circle DNA synthesis. *BMC Biotechnol.* 7:49.

[8]   Astriab-Fisher A, Fisher MH, Juliano R, Herdewijn P. 2004. Increased uptake of antisense oligonucleotides by delivery as double stranded complexes, *Biochem. Pharmacol.* 68, 403–407

[9]   Allerson CR, Sioufi N, Jarres R, Prakash TP, Naik N, Berdeja A, Wanders L, Griffey RH, Swayze EE, Bhat B. 2005. Fully 2'-Modified Oligonucleotide Duplexes with Improved *in Vitro* Potency and Stability Compared to Unmodified Small Interfering RNA, *J. Med. Chem.* 48, 901-904

[10]   Butler M, Hayes CS, Chappell A, Murray SF, Yaksh TL, Hua XY. 2005. Spinal distribution and metabolism of 2'-O-(2-methoxyethyl)-modified oligonucleotides after intrathecal administration in rats, *Neuroscience*, 131 (3): 705-715

[11]   Davis S, Propp S, Freier SM, Jones LE, Serra MJ, Kinberger G, Bhat B, Swayze EE, Bennett CF, Esau C. 2009. Potent inhibition of microRNA in vivo without degradation. *Nucleic Acids Res*. 37(1):70-7

[12]   Davis S, Lollo B, Freier S, Esau CC. 2006. Improved targeting of miRNA with antisense oligonucleotides, *Nucleic Acids Res.*, 34 (8): 2294–2304

[13]   Lu Y, Xiao J, Lin H, Bai Y, Luo X, Wang Z, Yang B. 2009. A single anti-microRNA antisense oligodeoxyribonucleotide (AMO) targeting multiple microRNAs offers an improved approach for microRNA interference. *Nucleic Acids Res.* 37(3): e24.

[14]   Shanagar J. 2005. Purification of a synthetic oligonucleotide by anion exchange chromatography: method optimisation and scale-up. *Biochem Biophys Methods.* 64(3):216-25.

[15]   Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, et al. 2004. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432: 226–230.

[16]   Avnit-Sagi T, Kantorovich L, Kredo-Russo S, Hornstein E, Walker MD. 2009. The promoter of the pri-miR-375 gene directs expression selectively to the endocrine pancreas. *PLoS One.*

4(4):e5033

[17] Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M, Stoffel M. 2009. MiR-375 maintains normal pancreatic alpha- and beta-cell mass. *Proc Natl Acad Sci U S A.* 106(14):5813-8.

[18] Kloosterman WP, Lagendijk AK, Ketting RF, Moulton JD, Plasterk RH. 2007. Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. *PLoS Biol* 5:e203.

[19] Krotz AH., Carty RL., Scozzari AN., Cole DL., Ravikumar VT. 2000. Large-Scale Synthesis of Antisense Oligonucleotides without Chlorinated Solvents, *Organic Process Research & Development,* 4: 190-193

[20] Gilar M. 2001. Analysis and Purification of Synthetic Oligonucleotides by Reversed-Phase High-Performance Liquid Chromatography with Photodiode Array and Mass Spectrometry Detection, *Analytical Biochemistry,* 298, 196–206

[21] Balotta C, Lusso P, Crowley R, Gallo RC, Franchini G. 1993. Antisense phosphorothioate oligodeoxynucleotides targeted to the vpr gene inhibit human immunodeficiency virus type 1 replication in primary human macrophages. *J. Virol.* 67(7):4409-4414

[22] Mehedi Masud M, Ozaki-Nakamura A, Kuwahara M, Ozaki H, Sawai H. 2003. Modified DNA bearing 5(methoxycarbonylmethyl)-2'-deoxyuridine: preparation by PCR with thermophilic DNA polymerase and postsynthetic derivatization. *Chembiochem.* 4(7):584-8.

[23] Saiki, RK; Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230 (4732): 1350–1354

[24] Barany F. 1991. Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proc. Natl. Acad. Sci. USA.* 88(1):189-93

[25] Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC. 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat. Genet.* 19 (3): 225–232.

[26] Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 28: E63

[27] Walker GT, Little MC, Nadeau JG, Shank DD. 1992. Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proc Natl Acad Sci U S A.* 89(1):392–396.

[28] Vincent M, Xu Y, Kong H. 2004. Helicase-dependent isothermal DNA amplification, *EMBO reports* 5(8): 795–800

[29] Van Ness J, Van Ness LK, Galas DJ. 2003. Isothermal reactions for the amplification of oligonucleotides, *Proc Natl Acad Sci U S A.,* 100 (8): 4504–4509

[30] Tan E, Erwin B, Dames S, Ferguson T, Buechel M, Irvine B, Voelkerding K, Niemz A. 2008. Specific versus nonspecific isothermal DNA amplification through thermophilic polymerase and nicking enzyme activities. *Biochemistry.* 47(38):9987-9999

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