Isothermal reactions for the amplification of oligonucleotides

Faculty Opinions – Post-Publication Peer Review of the Biomedical Literature · 2003 · 10.3410/f.1014367.193401

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Communicated by Eric H. Davidson, California Institute of Technology, Pasadena, CA, February 11, 2003 (received for review November 30, 2002)

Abstract

We have devised a class of isothermal reactions for amplifying DNA. These homogeneous reactions rapidly synthesize short oligonucleotides (8–16 bases) specified by the sequence of an amplification template. Versions of the reactions can proceed in either a linear or an exponential amplification mode. Both of these reactions require simple, constant conditions, and the rate of amplification depends entirely on the molecular parameters governing the interactions of the molecules in the reaction. The exponential version of the reaction is a molecular chain reaction that uses the oligonucleotide products of each linear reaction to create producers of more of the same oligonucleotide. It is a highly sensitive chain reaction that can be specifically triggered by given DNA sequences and can achieve amplifications of >10^6-fold. Several similar reactions in this class are described here. The robustness, speed, and sensitivity of the exponential reaction suggest it will be useful in rapidly detecting the presence of small amounts of a specific DNA sequence in a sample, and a range of other applications, including many currently making use of the PCR.

Introduction

The invention of the PCR changed the practice of molecular biology. It has become a mainstay for biological research and diagnostics in providing a method for the rapid detection, isolation, and measurement of DNA sequences through their specific amplification. There are currently two widely used methods for amplifying specific DNA sequences: PCR (1, 2) and the rolling-circle amplification method (3–5). The PCR method is the simpler and more flexible of these and has the added advantage of being geometric rather than linear in character, so that amplification levels of 10^5 or more can be achieved. It is by far the most widely used amplification method in biology. It has the disadvantage relative to the isothermal rolling-circle amplification method, however, of needing a temperature cycling protocol to achieve amplification. This imposes instrumentation constraints on the PCR method that make it more complex and limit the rate of the amplification to the temperature cycling schedule. Another limitation of the rate of PCR derives from the nature of the reaction itself in that a maximum 2-fold amplification can be achieved in each cycle. Advances in speed, accuracy, and sensitivity, in addition to simplicity, would be most welcome for applications in biology and medicine.

We report here a class of isothermal reactions for amplifying DNA that overcomes all the above disadvantages of PCR. This class includes a linear amplification method, which is fundamental to the others, and several versions of an exponential amplification scheme. These reactions are simple, flexible, and require no special cycling of conditions. They depend entirely for their rate of amplification on the molecular parameters governing the interactions of the molecules in the reaction. Because of the balance between the thermal properties of the DNA oligonucleotides and the enzymes used, the optimum temperature of the reaction with these enzymes is 60° C. The exponential version of the method, designated the exponential amplification reaction (EXPAR), is an isothermal molecular chain reaction in that the products of one reaction catalyze further reactions that create the same products.

Materials and Methods

Oligonucleotides and Enzymes. Sequences used in experiments described in the text: oligonucleotides used in the linear reaction (Figs. 1 and 2): ITAtop, 5′-CCGATCTAGTGAGTCGCTC-3′; NBbt12, 5′-ACGACTGGAACTGAGCGACTCACTAGATCGG-3′; NBbt16, 5′-ACCTACGACTGGAACTGAGCGACTCACTAGATCGG-3′; NBbt20,

5'-TGAAACCTACGACTGGAACTGAGCGACTCACTAGATCGG-3'. Oligonucleotides used in the exponential reaction (Figs. 3 and 4): template oligo, ceap, 5'-CCTACGACTGGaacAGACTCACCTACGACTGGAP-3'; trigger, seqS, 5'-ACCAGTCGTAGG-3' (spacer bases in lowercase; P indicates phosphate group; the nicking enzyme site or its complement is underlined). The trigger oligo (seqS) is one base longer than that produced by the primer template, enabling us to distinguish the initial trigger from the amplified sequence. Oligonucleotides were synthesized by Midland Certified Reagent Company (Midland, TX), MWG Biotech (High Point, NC), or Sigma-Genosys (The Woodlands, TX). All enzymes were purchased from New England Biolabs. The DNA polymerase used was Vent exo- (6, 7). The nicking enzyme (N.BstNBI) has a specific activity of ≈10^6 units/mg.

Linear Amplification Reaction. Conditions for the linear reaction: 85 mM KCl/25 mM Tris-HCl (pH 8.8, 25° C)/2.0 mM MgSO4/5 mM MgCl2/10 mM (NH4)2SO4/0.1% (vol/vol) Triton X-100/0.5 mM DTT/0.4 units/l N.BstNBI nicking enzyme/0.05 units/l Vent exo- polymerase/400 M dNTPs/10 g/ml BSA/0.05 M template and primer oligonucleotides. Reactions were assembled at 4° C, initiated by transferring to a preheated thermocycler at 60° C, and stopped by incubation at 4° C.

Exponential Amplification Reaction (EXPAR). The exponential reactions were carried out at 60° C, temperature controlled to within 0.1° C, using the same conditions as the linear reaction except with 0.1 M template oligonucleotide only. Triggering oligonucleotides were added as described for each experiment. For fluorescence monitoring, SYBR green (Molecular Probes) was added to 5× concentration.

Chromatography and MS. The chromatography system was an Agilent 1100 Series HPLC with a Waters Xterra MS C18 column (2.1 mm × 20 mm) run at 30° C with a gradient of acetonitrile in 5 mM dimethyl-butylamine acetate. The MS was a Micromass LCT time-of-flight instrument run in electrospray negative mode, 800–2,000 amu. Oligonucleotides exhibit different ionization efficiencies; a survey of >80 different 12 mers indicated the variation between sequences attributable to this difference is <30%. Almost all relevant quantitative comparisons are with the same oligo sequence.

Real-Time Fluorescence Measurement. Fluorescence measurements were made on an MJ Opticon instrument using an isothermal protocol with a 30-sec interval read beginning 10 sec after the lid and chamber reached 60° C.

Results

Linear Amplification. To produce an amplification reaction, we devised a cyclic chain of reactions that restores the reactants to their initial state after each synthesis of the molecule to be amplified. The reaction synthesizes short oligonucleotides and depends on the idea that, at the reaction temperature, oligonucleotides above a certain length form stable duplexes, whereas those below this critical length form unstable duplexes that dissociate readily. By arranging a specific single-strand nicking site and nicking enzyme and a compatible DNA polymerase (Fig. 1), a cycle of polymerization and subsequent oligonucleotide release is created. This cycle depends on the nicking reaction cleaving a phosphodiester bond to create an oligonucleotide that is below the threshold of stability in a duplex and is thereby released from the duplex, regenerating the initial primer template. The synthesized oligonucleotide is fully stable at 60° C when covalently joined to the rest of the upper strand, but is only transiently stable as a 12 mer after the nicking reaction.

In Fig. 1b, the experiment produced a 12 mer as its amplified product; products were analyzed on the LC-MS system after incubation at 60° C. The initial rate is about one molecule (12 mer) per primer template every 2.5 sec, or ≈0.4 molecules per primer template per sec. The reaction slows noticeably after 10 min, consistent with the reaction rate declining exponentially, as if an essential component is being inactivated. We expect the nicking enzyme is responsible, as its optimum temperature (≈55°) is lower than the 60° C of the reaction. The absolute initial rate of the reaction is proportional to the primer-template concentration over a wide range of concentrations. The reaction is completely dependent on the presence of both enzymes, the template, and the primer oligonucleotide, but the yield is a complex function of the amounts of both enzymes. Tuning the reaction conditions via enzyme concentrations is important for maximizing the yield of any particular product.

Exponential Amplification. We devised a simple way to use the linear amplification to create an

exponential amplification reaction. The key idea is to arrange it so that the oligonucleotide product of the linear reaction serves to create a new primer that anneals to a target template and creates a new primer template, which in turn produces more of the same oligonucleotide product, creating a chain reaction. The product oligonucleotide, although unstable as a duplex, will form a transient duplex with its complement, and this transient duplex can act as a primer for extension by the DNA polymerase. Once extension has occurred, the duplex is stabilized by the additional complementary section. The amplification templates are single-stranded oligonucleotides containing two copies in tandem of the complement of the oligonucleotide product, separated by the complement of the nicking enzyme recognition site (3'-CTCAG-5') and a four-base spacer. We take the precaution of blocking the 3' ends of the template oligonucleotides (with 3' PO4 groups or tethered amines) to prevent spurious self-priming by pairs of template molecules.

To examine the kinetics, we carried out the full exponential reaction with differing initial amounts of amplifying oligonucleotide (σ) and measured amounts of σ at time points with the mass spectrometer. The oligonucleotide amplifies approximately exponentially for the first 2 min, until the concentration of σ approaches the concentration of the template pool, after which it proceeds approximately linearly. The total amplification of ≈10^6 to 10^7 is shown. In end-point measurements, amplification levels are all in the range of 10^6. Writing the mass action equations with the simplest assumptions shows that the kinetics of product generation are predicted to be exponential while the template lasts.

Because the EXPAR reaction is rapid and simple, it is potentially appealing as a "real-time" reaction. We carried out the reaction with SYBR-green added, and fluorescence was monitored on a temperature-controlled fluorescence reader. The fluorescence is generated from the double-stranded primer templates produced during the reaction (π), not by the amplified oligonucleotide itself. MS measurements show amplification of ≈10^6 to 10^7-fold.

Triggering Mechanisms. To initiate the exponential reaction, the first few oligonucleotide molecules must be produced from the sample to form the first primer templates. These initial products must be accurate representations of the sequence to be amplified; this is called the triggering mechanism for EXPAR. One scheme relies on the natural occurrence of nicking enzyme recognition sites (5'-GAGTC-3') in the DNA of interest. A linear amplifier of a genomic sequence is created by providing an oligonucleotide (trigger template) complementary to the genomic DNA flanking a specific nicking site. When annealed to the genomic DNA, the nicking enzyme converts this to a primer-template structure that produces the oligonucleotide corresponding to the sequence to the right of the nicking site, which is then used as the trigger for exponential amplification. Because the nicking enzyme recognition sequence occurs naturally in both bacterial and human DNA at the expected frequency for a five-base sequence (about 1 in 1,000), potential trigger sites provide a wide range of target sites. To demonstrate triggering from a naturally occurring nick site, we specifically amplified certain oligonucleotides contained in cDNA. The reaction is triggered only when the trigger oligonucleotide cognate to the specific cDNA is present; when no trigger is present, no reaction occurs.

Discussion

The amplification scheme described here appears to have several major advantages: isothermal conditions, relative speed, and flexibility with which it can be triggered and elaborated into multiple coupled reactions. The linear amplification reaction is quite distinct from strand displacement amplification (8); it depends fundamentally on the transformation of duplex thermal stability into instability by cleavage of a phosphodiester bond in the nicking reaction. It is difficult to be sure which step limits the speed of the overall cycle; these issues are being investigated to optimize speed, sensitivity, and accuracy. It seems likely that the diffusion and annealing of product oligonucleotides to the amplification template is a slow step. In addition to the triggering reaction demonstrated here, variant forms can be constructed using any technique that creates a discrete 3' end in the target DNA extendable by the polymerase, such as a restriction site.

Because the reaction is a true molecular chain reaction, once triggered it proceeds without change of

conditions or further stimulus. There is a concern that the reaction may spontaneously or spuriously trigger; blocking the 3' ends of all templates prevents spurious priming, and we do not see spurious priming at the concentrations used. The full range of possible amplification levels is not yet fully known; we routinely get 10^6- to 10^7-fold amplification within a few minutes but have observed as high as 10^8-fold.

There are several variant forms of the exponential amplification scheme. One ("copy EXPAR") provides an accurate copy of a polymorphic site that can subsequently be amplified. It uses a second template whose amplification is driven by the products of the first template, enabling detection and measurement of polymorphism in target DNA. The "copy EXPAR" reaction depends for its specificity on a phenomenon specific to the transient annealing and priming process: extension is sharply inhibited by mispairing near the 3' end, so much so that no detectable amplification occurs with a mispaired oligonucleotide.

Many potential variations of the coupled reactions are described here, including the use of one amplifying oligonucleotide to trigger another amplification reaction. We are currently investigating several variations and exploring the tethering of amplification templates to solid supports (slides and microbeads) for in situ amplification.

Acknowledgements

This work was supported in part by the W. M. Keck Foundation, the Norris Foundation, and Defense Advanced Projects Agency (contract no. MD4972-02-C-0047). We are grateful to Bill Jack of New England Biolabs and to Huimin Kong for unpublished information on the nicking enzyme N.BstNBI, and to Eric Davidson and an anonymous referee for comments. The technology described has been licensed to Ionian Technologies Incorporated, a company in which the authors have a financial interest.

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