Technical Advance

Simultaneous Sequencing of Multiple Polymerase Chain Reaction Products and Combined Polymerase Chain Reaction with Cycle Sequencing in Single Reactions

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DNA sequencing is considered the gold standard for nucleic acid identification and mutation detection. However, sequencing is labor intensive because it requires previous amplification and only a single sequence is analyzed at a time. We developed two novel strategies that substantially improve DNA sequencing. The first allows multiple polymerase chain reaction (PCR) products to be sequenced in a single sequencing reaction and analyzed simultaneously in a single lane or capillary. Simultaneous sequencing by this method, designated "SimulSeq," can provide either simultaneous single-direction sequencing of multiple genes or simultaneous forward and reverse sequencing from a single gene. In the second approach, designated "AmpliSeq," we demonstrate a technique combining PCR amplification and sequencing in a single reaction that is analyzed in a single lane or capillary. We demonstrate combined PCR with short bidirectional sequencing, and combined PCR with unidirectional sequencing. We anticipate that these methods will have utility in research and clinical settings where panels of mutations or large numbers of samples are analyzed and/or when turnaround time is critical. (*Am J Pathol* 2002, 161:27-33)

DNA sequencing¹ has been the standard against which other types of DNA testing is compared. Major advances in DNA sequencing include the development of automated sequencers,² discovery of fluorescent terminator chemistry,³ and cycle sequencing.⁴ These developments have made sequencing easier to perform and therefore more widely used. Currently, sequencing is used to identify microbial drug resistance mutations,⁵ cancer predisposition, somatic mutations,⁶ and genetic diseases.⁷

With the cloning and sequencing of the human genome^{8,9} and the new era of molecular medicine, one can only expect the use of DNA sequencing to increase.

Despite advances in sequencing technology, significant limitations remain. First, most applications require polymerase chain reaction (PCR) amplification of the target sequence, and purification of the product before sequencing. Second, standard Sanger-sequencing reactions are performed with a single primer and therefore yield only a single sequence. These limitations are costly and have somewhat hindered the widespread application of DNA sequencing in clinical and research settings.

In this article we report two novel sequencing strategies that directly address these limitations. In the first, we engineered sequencing reactions to permit simultaneous sequencing of multiple PCR products in a single lane. Under normal conditions, multiple sequencing reactions run simultaneously are superimposed on each other because the sequencing products overlap in size. SimulSeq prevents this because of two principles: sequencing products stop when the end of a PCR product is reached, and long oligonucleotide primers can be used to prevent otherwise overlapping short sequencing products. In the second sequencing strategy, we designed conditions and primer modifications to permit combined PCR and sequencing in a single reaction. AmpliSeq uses a single primer pair, in which one primer contains both an abasic region and a long region of nontemplated nucleotides tailed on the 5' end. Because both of these novel strategies can easily be implemented in any lab currently performing sequencing, we believe that these new approaches have broad potential applicability.

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Materials and Methods

PCR

PCR was performed in a 50- μ l reactions containing a final concentration of 1 $\times$ PCR Buffer (Applied Biosystems, Foster City, CA), 50 μ mol/L each of dNTP, 1.25 U *Taq* Gold (Applied Biosystems), 0.01% gelatin, and 0.2 μ mol/L each forward and reverse primer. The reaction mixture was subjected to 95°C for 5 minutes followed by 35 cycles of 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute, followed by 72°C for 10 minutes. The PCR products were identified on 10% polyacrylamide gel electrophoresis and then purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. All oligonucleotides were synthesized and purified by Oligo's Etc. (Wilsonville, OR). After PCR for *prothrombin*, to some samples 3 μ l (1 U/ μ l) of uracil-*N*-glycosylase (Life Technologies, Carlsbad, CA) was added to the 50 μ l PCR product, and incubated at 37°C for 30 minutes. The enzyme was then heat inactivated by incubation at 94°C for 10 minutes.

Cycle Sequencing

Cycle sequencing was performed using the BigDye version 2.0 terminator cycle-sequencing kit according to manufacturer's instructions (Applied Biosystems). Primers were added to a final concentration of 300 nmol/L in a 20- μ l reaction volume. Cycle-sequencing conditions were 95°C for 30 seconds, followed by 35 cycles of 95°C $\times$ 15 seconds, 50°C $\times$ 15 seconds, and 60°C $\times$ 4 minutes. Products were analyzed using an ABI Prism 3700 (Applied Biosystems).

Bidirectional SimulSeq

Factor V forward primer, 5'-TGCCCAGTGCTTAACAA-GACCA-3', and reverse primer, 5'-AAGGTTACTTCAAG-GACAAAATAC-3', were designed to amplify a 145-bp product encompassing the mutation site. Forward sequencing primer was 5'-AGGACTACTTCTAATCTGTA-AG-3'. The reverse sequencing primer was identical to the reverse PCR primer with the 5' addition of four abasic sites followed by 90 thymidines and was gel purified. Equal amounts of two sequencing primers were used.

AmpliSeq Reactions

Primers for bidirectional AmpliSeq were identical to the sequencing primers described for bidirectional SimulSeq.

For unidirectional AmpliSeq the forward primer was identical to the *Factor V* SimulSeq forward primer (Table 1), and the reverse primer that was used in bidirectional AmpliSeq with the tail extended to a total of 126 thymidines (total length, 150 bases). Reactions were performed with 50 to 500 ng of genomic DNA, 0, 12.5, or 125 μ mol/L supplemental dNTPs in 20- μ l reactions of BigDye version 2.0 terminator cycle-sequencing kit, and cycling conditions according to the manufacturer's instructions. Primers and cycling conditions were performed as described above. After PCR/sequencing, the products were purified with spin columns (Biomax, Odenton, MD) and analyzed on an ABI 3700.

Results

The experimental strategy is depicted in Figure 1A. PCR of each gene was designed with the known mutation site near the distal end of the PCR strand to be sequenced. This feature is required in the SimulSeq strategy so that sequencing products terminate shortly after the site of interest, allowing sequencing products generated from other targets to be detected downstream. To demonstrate simultaneous sequencing of multiple DNA targets, we simultaneously sequenced three genes. We present this data as proof of principle of these new technologies, not to suggest that these methods are limited to these specific mutations. *Factor V Leiden* (Arg506Gln),¹⁰ *prothrombin* (G20210A),¹¹ and the *methylenetetrahydrofolate reductase* (*MTHFR*, Ala223Val)¹² mutations each result in an increased risk of thrombosis, and mutations in combination seem to have a synergistic effect on thrombosis risk.¹³

PCR amplification of each gene was performed in separate PCR reactions using the primers listed in Table 1. The three PCR products were mixed at equal concentrations and simultaneously sequenced using a mixture of three forward sequencing primers (Table 1), one for each gene, in a single tube. The results of simultaneous sequencing of the three genes are shown in Figure 1, B to E. During simultaneous sequencing, the 22-base *MTHFR*-sequencing primer extends up to 42 bases to the end of the PCR product such that the largest *MTHFR* sequence product was 64 bases in length. A 69-base *prothrombin*-sequencing primer was designed with 24 complementary bases tailed with an additional 45 thymidines on the 5' end of the primer. This design creates a 6-base gap in sequencing products between the final *MTHFR*-sequencing product (64 bases) and the beginning of *prothrombin*-sequencing products (70 bases) making it easy to distinguish the two. *Prothrombin* se-

Figure 1. SimulSeq of three genes. **A:** Experimental design. PCR products (green, red, and blue rectangles) for three different genes were designed such that the mutation site (asterisk, highlighted in yellow) was near the distal end of the PCR strand to be sequenced. Sequencing primers (arrows) increasing in size with complimentary (solid arrows) and noncomplimentary (striped region of arrows) bases were designed for each gene. The large sequencing primers were designed to be several bases longer than the largest sequencing product of the previous reaction with the shorter sequencing primer. This creates a dead space between the sequencing products of different reactions. The left ends of the PCR products are not shown (wavy lines). Simultaneous sequencing of PCR products from the *MTHFR*, *prothrombin* (PROT), and *factor V* (FV) genes demonstrating *factor V Leiden* (B), *prothrombin* (C), and *MTHFR* (D) heterozygotes. The first bases detected are the result of *MTHFR* sequencing, which is followed by a dead space, the *prothrombin* sequencing products, a second dead space, and the *factor V* sequence products. Only the first ~35 bases of *factor V* sequencing (which contains the *Leiden* mutation site) are shown. Yellow highlights indicate the known mutation/polymorphic site for each gene; red arrows demonstrate heterozygous sequence. **E:** Use of uracil-*N*-glycosylase to eliminate sequence products resulting from the reverse PCR primer. Base sizes indicated are not accurate because of cropping for the figure.

Table 1. Oligonucleotide Primers Used for PCR and Sequencing

Gene		PCR primers	Sequencing primer
<i>Factor V</i>	(F)*	5'-TGCCCAGTGCTTAACAAGACCA-3'	5'-T ₉₀ -GGCTAATAGGACTACTTCTAATC-3'
	(R)	5'-TGTTATCACACTGGTGCTAA-3'	(gel purified)
<i>Prothrombin</i>	(F)	5'-GGATGGGAAATATGGCTTCTACAC-3'	5'-T ₄₅ -TAAACTATGGTTCCCAATAAAG-3'
	(R)	5'-ATGAATAGCACTGGGAGCATTG-3' [†]	(gel purified)
<i>MTHFR</i>	(F)	5'-CTCCTGACTGTCATCCCTATTG-3'	5'-CCTGAAGCACTTGAAGGAGAAG-3'
	(R)	5'-AAAGAAAAGCTGCGTGATGATG-3'	

*F, forward; R, reverse.

[†]Underlined thymidines (reverse *prothrombin* primer) were replaced with uracils for the uracil *N*-glycosylase experiment. Thymidine tails are shown in bold.

quence extends up to 39 bases to the end of the PCR product such that the final *prothrombin* sequence product is 108 bases. A 113-base *factor V*-sequencing primer was designed with 23 complimentary bases tailed with an additional 90 thymidines on the 5' end. This creates a gap between the final *prothrombin*-sequencing product and *factor V*-sequencing products, which begin at 114 bases and continue up to 183 bases to the end of the PCR product. Figure 1, B to D, demonstrates simultaneous sequencing of the three prothrombotic genes on each of three patients heterozygous for *factor V Leiden* (Figure 1B), *prothrombin* (Figure 1C), or *MTHFR* (Figure 1D) mutations.

We investigated an additional modification to this method to obtain even shorter stretches of sequence to permit larger numbers of sequencing reactions to be run simultaneously. Our strategy was to eliminate the majority of sequencing downstream of the mutation site because it reflects the known sequence of the reverse primer, providing no additional information. We designed a *prothrombin* reverse PCR primer identical to that used in Figure 1, B to D, except that two thymidines near the 3' end of the primer were replaced with uracils (which should not limit its ability to function as a PCR primer). After PCR, the *prothrombin* PCR products were treated with uracil-*N*-glycosylase and then mixed with *MTHFR* and *factor V* PCR products, and simultaneously sequenced with the three sequencing primers as above. Uracil-*N*-glycosylase treatment creates abasic sites in the *prothrombin* PCR products that selectively terminate the *prothrombin* sequence at the beginning of the reverse primer (Figure 1E). This technique could be used to simultaneously acquire very short (eg, 10 to 20 bases) segments of sequence from many different gene sequences, making SimulSeq a viable method to detect a large panel of mutations or single nucleotide polymorphisms.

In general, it is desirable to confirm the sequence of a mutation in both the forward and reverse directions. We therefore wished to demonstrate that SimulSeq could perform bidirectional sequencing. To obtain both forward and reverse sequence from a single gene product using SimulSeq, we redesigned the *factor V* PCR reaction such that the mutation site was located near one end of the 145-bp PCR product. A forward sequencing primer, 22 bases in length, was designed to yield up to 54 bases of sequencing (to the end of the PCR product). We imagined that this would be a straightforward modification of

the original strategy and therefore designed a large reverse primer with 24 complimentary bases, and 56 non-coding thymidines. When used in a SimulSeq reaction, we surprisingly found overlapping sequencing with adenines at the end of the sequence (data not shown).

We realized that sequencing products from the reverse primer can serve as templates for the forward primer such that some forward primer-sequencing products terminate within the noncoding thymidine region of the reverse primer producing runs of adenine and are superimposed on those generated from the reverse primer. To solve this problem, we designed a new reverse primer with abasic sites between the coding and noncoding tail. This experimental design is depicted in Figure 2A. Bidirectional sequencing for both a *factor V* wild-type homozygote and *Leiden* heterozygote is demonstrated in Figure 2B. As shown, when the forward and reverse primers are used to simultaneously cycle-sequence, there is a short (~5 base) gap between the end of the forward sequencing products and the beginning of the reverse sequence, making it easy to distinguish the two.

Because simultaneous forward- and reverse-sequencing reactions include two primers pointed toward each other, a thermostable polymerase, and are thermocycled, we realized that such conditions could support PCR if the reaction were supplemented with additional dNTPs. Using this approach, early cycles should be dominated by PCR amplification (because the free deoxynucleotide concentration is relatively high), and later cycles by cycle sequencing (because depletion of free deoxynucleotides during PCR increases the relative dideoxynucleotide concentration, Figure 3A). To test whether PCR and cycle sequencing can be combined in a single reaction, we set up standard cycle-sequencing reactions containing genomic DNA and the *factor V* forward and reverse primers described above. The anticipated PCR product is diagrammed in Figure 3B. To support PCR, the reactions were supplemented with additional dNTPs at varying concentrations. Without deoxynucleotide supplementation, no discernable sequencing products were identified. With the addition of 12.5 μ mol/L or 125 μ mol/L of deoxynucleotides, both forward- and reverse-sequencing products were generated (Figure 3C). Our results demonstrate that this strategy, designated "AmpliSeq," supports combined PCR and sequencing in single reactions.

Input genomic DNA concentrations ranging from 50 to 500 ng yielded approximately equivalent amounts of se-

Figure 2. Bidirectional SimulSeq. **A:** Experimental design of simultaneous forward and reverse sequencing. The light blue rectangle represents the double-stranded PCR product. The mutation site (asterisk) is highlighted in yellow. The forward- and reverse-sequencing primers are represented by blue arrows with the complementary bases in solid blue. In the reverse-sequencing primer, the dots represent the abasic sites. The line, 5' to the abasic sites, represents nontemplated thymidines. **B:** Results of simultaneous forward and reverse sequencing of homozygous wild-type (WT/WT) and heterozygous *Leiden* mutant (WT/L) individuals. Yellow highlighting indicates the mutation site in both the forward and reverse sequence products. Red arrows demonstrate heterozygous sequence.

quencing products. We have also used AmpliSeq technology to generate forward and reverse sequence data of the APC I1307K mutation (data not shown). To generate longer unidirectional sequencing, we designed a Factor V AmpliSeq reaction that results in a larger PCR product, which therefore requires lengthening the reverse primer tail (Figure 3D). To accomplish this, we moved the forward primer further upstream of the *Leiden* mutation and lengthened the reverse primer tail to a total of 150 bases, including 126 thymidines. Experiments are in progress to develop alternate reverse primer modifications that can be used in lieu of the long thymidine tail. Therefore, AmpliSeq reactions yield either bidirectional or long unidirectional sequence in combination with PCR amplification.

Discussion

In this report, we have demonstrated two novel methods that significantly improve the ease and efficiency of DNA sequencing. The first method, SimulSeq, allows either

simultaneous single-direction sequencing of multiple genes or simultaneous bidirectional sequencing from a single gene after PCR. Previously described methods of simultaneous sequencing require significant deviations from standard sequencing protocols. For example, one method uses two fluorescently labeled primers, and specialized detection equipment and software to sort the sequence data,^{14–16} whereas another method requires strand separation after the sequencing reaction and separate sequence analysis.¹⁷ This strategy is similar to multiplex SNUPE (single nucleotide primer extension) reactions. However, SNUPE reactions contain only ddNTP terminators, extend only a single base, and therefore do not provide sequence context for the fluorescent molecule. Although SimulSeq is probably best applied to known mutation sites, reactions can be designed to yield many short sequences, fewer long sequences, or a combination of short and long sequences. Thus SimulSeq can be adapted for many different types of simultaneous sequencing applications.

Figure 3. AmpliSeq. **A:** Theoretical AmpliSeq reaction. Because of supplemental dNTPs, PCR amplification is supported during initial cycles of the reaction. As PCR occurs, dNTPs are consumed resulting in an increase in the ddNTP/dNTP ratio. This causes the reaction to convert itself to cycle sequencing in latter cycles. **B:** Anticipated PCR product generated during AmpliSeq. Forward and reverse primer sequences are shown in dark blue and the rest of the PCR product in light blue. In the reverse primer, **dots** denote the abasic region and **stripes**, the nontemplated thymidines. Yellow highlight designates the mutation site (**asterisk**). **C:** Bidirectional AmpliSeq results of a *factor V* wild-type homozygote. **D:** Unidirectional AmpliSeq of a *factor V* wild-type homozygote. Yellow highlighting indicates the potential mutation site in sequencing products.

The second method, AmpliSeq, combines PCR and cycle sequencing in a single reaction that yields both forward and reverse sequence data. We know of no other published method effectively combining PCR and sequencing in a single reaction. Previously attempts require that the samples be partitioned after several cycles of amplification so that radioactively labeled primers and dideoxynucleotides can be added to eight individual reactions.^{18,19} We anticipate that SimulSeq and AmpliSeq can be combined to simultaneously amplify and sequence multiple genes at the same time. Although AmpliSeq and SimulSeq require attention to primer design, they require no additional steps, sample manipulations, or reagents such that any lab currently performing DNA sequencing reactions can perform either of these techniques. These techniques significantly reduce the cost, time, and labor of nucleic acid sequencing, making direct sequencing a competitive alternative to other mutation detection methods. In fact, AmpliSeq and SimulSeq are ideally suited for a variety of clinical and research applications, such as single nucleotide polymorphism panels, large-scale genetic testing, analysis of bioterrorism organisms, and drug resistance testing.

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