SNP genotyping

Detection of single-nucleotide polymorphisms using a ligation–rolling circle amplification approach

Key findings

- Thermo Scientific™ *Thermus thermophilus* DNA Ligase is a high-fidelity enzyme capable of discriminating single-nucleotide mismatches.
- The RCA reaction using Thermo Scientific™ EquiPhi29™ DNA Polymerase enables efficient signal amplification.
- The utility of the ligation-RCA approach was demonstrated for SNP detection.

Introduction

Single-nucleotide polymorphisms (SNPs) are the most prevalent form of genetic variation in the human genome and the primary source of individual differences. SNPs are linked to a variety of serious conditions, including cardiovascular, metabolic, and autoimmune diseases as well as cancer. Consequently, SNPs are becoming crucial markers in clinical diagnosis. However, distinguishing SNPs can be challenging because they involve variations in just a single base of the DNA sequence, representing different alleles.

Efficient and high-throughput detection of SNPs can be achieved by utilizing DNA ligase–mediated single-nucleotide discrimination and signal amplification via rolling circle amplification (RCA). In this method, the 5′ and 3′ ends of a specially designed oligonucleotide, known as a padlock probe (PLP), anneal to the target sequence containing the SNP and form a circular structure. When the 3′ end of the PLP is completely complementary to the SNP, DNA ligase can join it to the 5′ end of the PLP. Conversely, a single-nucleotide mismatch between the PLP and SNP prevents circularization (Figure 1). This helps ensure single-base selectivity through two means: specific hybridization of the PLP ends to sequences adjacent to the SNP, and the precise nick closure activity of the DNA ligase. Once the PLP is circularized, an isothermal RCA reaction driven by a DNA polymerase with strand displacement activity rapidly generates multiple copies of the PLP, amplifying the signal. The amplified signal can then be detected using various methods, such as molecular beacons, CRISPR-Cas12a systems, nucleic acid stains, and more.

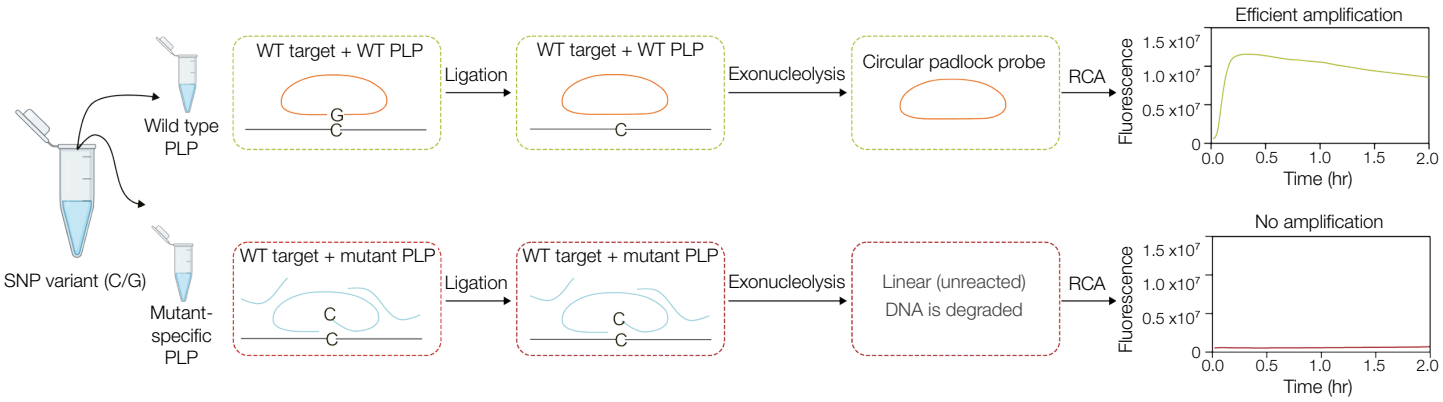

Figure 1. SNP detection using high-fidelity DNA ligation and RCA. Each SNP sample is hybridized with a wild type or mutant-specific PLP to enable ligation of fully complementary hybrids using high-fidelity *Thermus thermophilus* DNA Ligase. After linear (unreacted) probes are removed, circular DNA is amplified by RCA. RCA products can be detected with various techniques; in this case, we used fluorescence detection methods.

We present proof of principle for a SNP detection workflow that employs *Thermus thermophilus* DNA Ligase and EquiPhi29 DNA Polymerase. *Thermus thermophilus* DNA Ligase offers precise discrimination between matched and mismatched ligation sites on PLPs at elevated temperatures ranging from 45°C to 65°C, and EquiPhi29 DNA Polymerase enhances assay sensitivity by amplifying the circular probes. EquiPhi29 DNA Polymerase is an engineered version of Thermo Scientific™ Phi29 DNA Polymerase that features greater thermostability, faster reaction speed, and higher product yields while maintaining the advantages of the wild type enzyme. Its high processivity and strand displacement activity result in significant DNA amplification from minimal template amounts, making it an excellent choice for applications requiring high sensitivity. The protocol described here is versatile and can be paired with various detection systems, depending on your specific needs. We employed a fluorescence-based detection method and used the double-stranded DNA dye Invitrogen™ Quant-iT™ PicoGreen™ dsDNA Reagent.

Materials and equipment

- Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL
- Applied Biosystems™ ProFlex™ PCR System
- Applied Biosystems™ QuantStudio™ 7 Pro Real-Time PCR System, 96-well, 0.1 mL
- *E. coli* genomic DNA
- Invitrogen™ E-Gel™ 1 Kb Plus Express DNA Ladder
- Invitrogen™ E-Gel™ EX 2% Agarose Gels
- Invitrogen™ Quant-iT™ PicoGreen™ dsDNA Reagent
- Thermo Scientific™ dNTP Mix (10 mM each)
- Thermo Scientific™ *E. coli* DNA Ligase
- Thermo Scientific™ EquiPhi29 DNA Polymerase
- Thermo Scientific™ Exonuclease I
- Thermo Scientific™ Exonuclease III
- Thermo Scientific™ FastDigest™ Hpy8I
- Thermo Scientific™ FastDigest™ RsaI
- Thermo Scientific™ Pyrophosphatase, inorganic (0.1 U/µL)
- Thermo Scientific™ T4 DNA Ligase
- Thermo Scientific™ *Thermus thermophilus* DNA Ligase
- Ampligase™ Thermostable DNA Ligase (LGC Biosearch Technologies)
- HiFi *Taq* DNA Ligase (New England Biolabs)

Oligonucleotide sequences

Table 1 shows the oligonucleotide sequences used for ligase fidelity tests and rolling circle amplification. Red segments in the sequences denote the ligation junction sites in splint oligonucleotides; the 5′ and 3′ ligation nucleotides in donor and acceptor oligonucleotides, respectively; the nucleotide of interest in SNP variant oligonucleotides; and the nucleotides in padlock probes that hybridize to the nucleotide of interest in SNP variants. Green, orange, and blue sequences in the padlock probes indicate the binding sites for Primer 1, Primer 2, and Primer 3, respectively. Sequences highlighted in yellow and gray represent the restriction recognition sites for RsaI and Hpy8I, respectively.

Methods

Ligase fidelity analysis

Five DNA ligases—T4 DNA Ligase, *E. coli* DNA Ligase, *Thermus thermophilus* DNA Ligase, HiFi *Taq* DNA Ligase, and Ampligase Thermostable DNA Ligase—were tested for specificity. Specificity analysis was based on the detection of 3,6-fluorescein (FAM dye)–labeled ligation products by capillary gel electrophoresis (CE). Briefly, substrate pools were prepared in 1X annealing buffer (10 mM Tris-HCl, 50 mM KCl, 0.1 mM EDTA, pH 7.5) containing 2.2 µM of one target strand (splint), 2.4 µM of one upstream probe, and 0.5 µM of each downstream probe, each differing only in the base at the ligation junction and the length (Figure 2A). Thus, all four bases at either side of the ligation junction were represented by analyzing four reactions per splint. Overall, 64 separate pools were prepared to cover all 16 possible NN pairs in the splint. Oligonucleotides were hybridized by heating to 95°C for 2 minutes and slowly cooling to 20°C at a rate of 0.1°C/sec. The hybridized oligonucleotides were then diluted 1:10, from which 2.5 µL was used in a 10 µL ligation reaction with 50 relaxed plasmid ligation units (RPLUs*) of each ligase separately in 1X reaction buffer of the corresponding mesophilic or thermophilic DNA ligase for 30 minutes at 37°C or 55°C, respectively. Reactions were inactivated by the addition of EDTA to a final concentration of 5 mM and allowed to cool. The ligation products were analyzed by CE on an Applied Biosystems™ 3500xL Genetic Analyzer.

*1 RPLU of ligase is defined as the amount of enzyme required to catalyze the ligation of 50% of 0.5 pmol nicked (relaxed) plasmid DNA in 15 minutes by a mesophilic ligase at 37°C or a thermophilic ligase at 55°C.

Table 1. Oligonucleotide sequences.

Name	5′ → 3′ sequence	Description
splint_GG	GACGCTTCTGGTACATTGTAGGTCCCATCGGCAATAGAAGTAAAGTTATAGTAG	Splint oligonucleotides used for ligase fidelity assessment
splint_TT	GACGCTTCTGGTACATTGTAGGTCCCATCTTCAATAGAAGTAAAGTTATAGTAG	
splint_GT	GACGCTTCTGGTACATTGTAGGTCCCATCGTCAATAGAAGTAAAGTTATAGTAG	
splint_CT	GACGCTTCTGGTACATTGTAGGTCCCATCCTCAATAGAAGTAAAGTTATAGTAG	
splint_AT	GACGCTTCTGGTACATTGTAGGTCCCATCATCAATAGAAGTAAAGTTATAGTAG	
splint_TG	GACGCTTCTGGTACATTGTAGGTCCCATCTGCAATAGAAGTAAAGTTATAGTAG	
splint_CG	GACGCTTCTGGTACATTGTAGGTCCCATCCGCAATAGAAGTAAAGTTATAGTAG	
splint_AG	GACGCTTCTGGTACATTGTAGGTCCCATCAGCAATAGAAGTAAAGTTATAGTAG	
splint_TC	GACGCTTCTGGTACATTGTAGGTCCCATCTCCAATAGAAGTAAAGTTATAGTAG	
splint_GC	GACGCTTCTGGTACATTGTAGGTCCCATCGCCAATAGAAGTAAAGTTATAGTAG	
splint_CC	GACGCTTCTGGTACATTGTAGGTCCCATCCCCAATAGAAGTAAAGTTATAGTAG	
splint_AC	GACGCTTCTGGTACATTGTAGGTCCCATCACCAATAGAAGTAAAGTTATAGTAG	
splint_TA	GACGCTTCTGGTACATTGTAGGTCCCATCTACAATAGAAGTAAAGTTATAGTAG	
splint_GA	GACGCTTCTGGTACATTGTAGGTCCCATCGACAATAGAAGTAAAGTTATAGTAG	
splint_CA	GACGCTTCTGGTACATTGTAGGTCCCATCCACAATAGAAGTAAAGTTATAGTAG	
splint_AA	GACGCTTCTGGTACATTGTAGGTCCCATCAACAATAGAAGTAAAGTTATAGTAG	
Donor-pA	P-AGATGGGACCTACAATGTACCAGAAGCGTC-6-FAM	Downstream oligonucleotides used for ligase fidelity assessment
Donor-pC	P-CGATGGGACCTACAATGTACCAGAAGCGTCTC-6-FAM	
Donor-pG	P-GGATGGGACCTACAATGTACCAGAAGCGTCTCTC-6-FAM	
Donor-pT	P-TGATGGGACCTACAATGTACCAGAAGCGTCTCTCTC-6-FAM	
A Acceptor_24	CTACTATAACTTTACTTCTATTGA	Upstream oligonucleotides used for ligase fidelity assessment
C Acceptor_24	CCAGGAGACTACTATAACTTTACTTCTATTGC	
G Acceptor_24	TCGTTTAGCCAGGAGACTACTATAACTTTACTTCTATTGG	
T Acceptor_24	CCTCTACTTCGTTTAGCCAGGAGACTACTATAACTTTACTTCTATTGT	
rs1800795_C	CACTTTTCCCCCTAGTTGTGTCTTGCCATGCTAAAGGACGTCACATT	Model SNP variants used for SNP detection
rs1800795_G	CACTTTTCCCCCTAGTTGTGTCTTGCGATGCTAAAGGACGTCACATT	
rs11119982_C	TTTGAAAATGTAAGTGCTCTTTAGACACTCGATGGCCAAGGATTGGCCAT	
rs11119982_T	TTTGAAAATGTAAGTGCTCTTTAGACACTTGATGGCCAAGGATTGGCCAT	
rs1800797_A	CTTGAAGTAACTGCACGAAATTTGAGGATGGCCAGGCAGTTCTACAAC	
rs1800797_G	CTTGAAGTAACTGCACGAAATTTGAGGGTGGCCAGGCAGTTCTACAAC	
PLP1_C	P-GCAAGACACAACTAGGGGGAGAGTATGCTGCTGCTGTACTACGAGCAAGG ACTCGTCATGTCTCAGCTTTAGTAATTCCTTTAGCATG	Padlock probes for SNP detection
PLP1_G	P-GCAAGACACAACTAGGGGGATCGTGTGCTGGGTCGTTCACTTGTGGAAGG ACTCGTCATGTCTCAGCTTTAGTAATTCCTTTAGCATC	
PLP2_C	P-AGTGTCTAAAGAGCACTTACATTTTCAAACTGAGTATGCTGCTGCTGTACTA CGAGCAAGGACTCGTCATGTCTCAGCTTTAGTAATTTGGCCATCG	
PLP2_T	P-AGTGTCTAAAGAGCACTTACATTTTCAAACTTCGTGTGCTGGGTCGTTCACT TGTGGAAGGACTCGTCATGTCTCAGCTTTAGTAATTTGGCCATCA	
PLP3_A	P-CCTCAAATTTCGTGCAGTTACTTCAAGGAGTATGCTGCTGCTGTACTACGAG CAAGGACTCGTCATGTCTCAGCTTTAGTAATCCTGGCCAT	
PLP3_G	P-CCTCAAATTTCGTGCAGTTACTTCAAGTCGTGTGCTGGGTCGTTCACTTGTG GAAGGACTCGTCATGTCTCAGCTTTAGTAATCCTGGCCAC	
Primer 1	GACTCGTCATGTCTCAGCTT*T*A*G	Primers used for RCA reaction
Primer 2	CTCGTAGTACAGCAGC*A*G*C	
Primer 3	CACAAGTGAACGACCC*A*G*C	
P = phosphate; FAM = FAM dye (3,6-fluorescein); * = phosphorothioate bond		

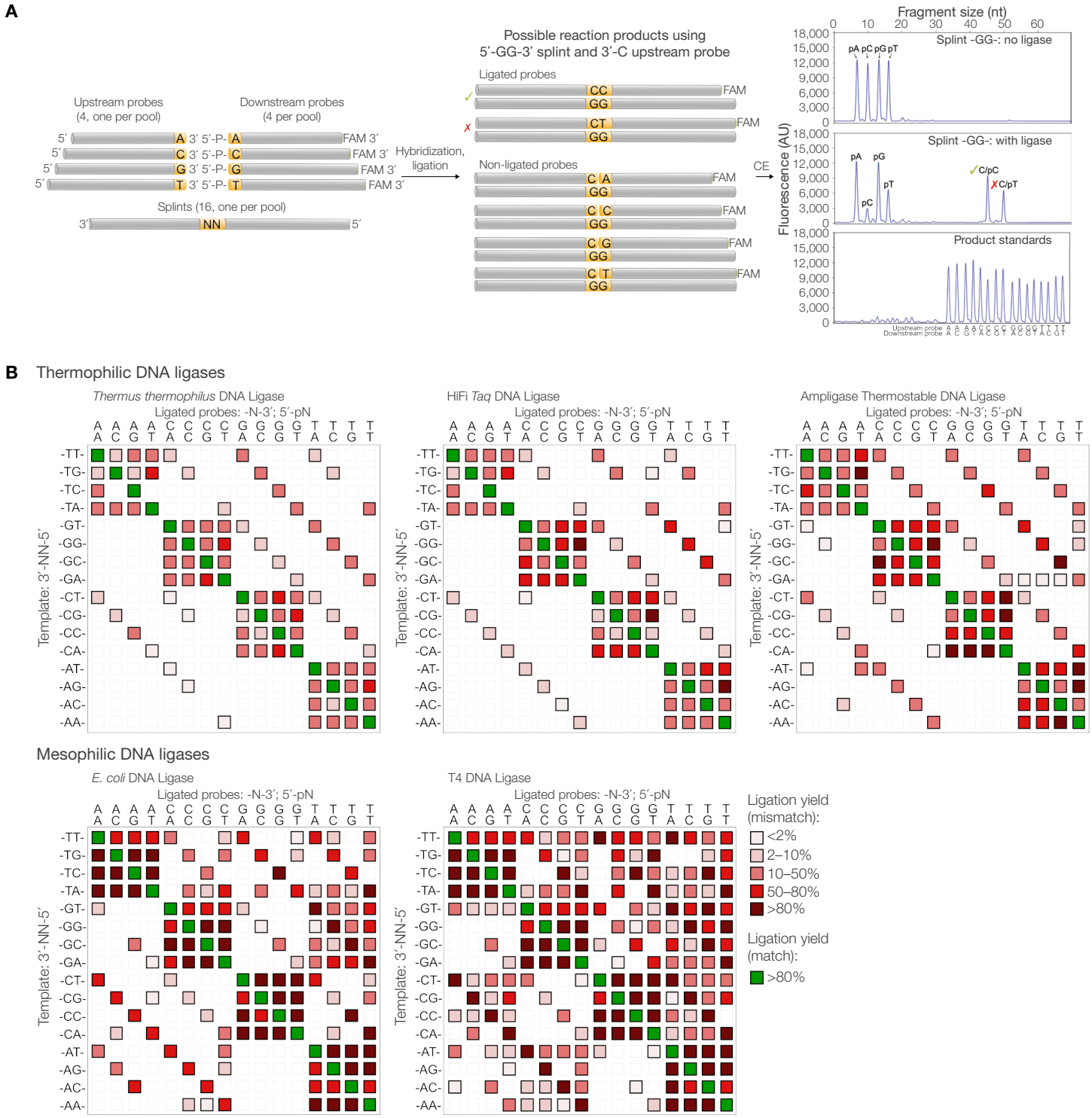

Figure 2. Ligase fidelity analysis. (A) Substrate pools for ligation fidelity assessment. Each substrate pool contained a single splint characterized by a specific NN combination at the ligation junction (e.g., AA, AC, AG, AT—16 in total), as well as all four FAM-labeled downstream probes and a single upstream probe with an A, C, G, or T at the 3′ end. Pools were hybridized and analyzed via CE. Theoretically, only fully complementary hybrids should be ligated by a high-fidelity DNA ligase (see representative substrate pool in the middle of the schematic). The products of each ligation reaction were identified using product standards. **(B)** Comparison of the ligation fidelity of thermophilic DNA ligases (*Thermus thermophilus* DNA Ligase, Ampligase Thermostable DNA Ligase, and HiFi *Taq* DNA Ligase) and mesophilic DNA ligases (*E. coli* DNA Ligase and T4 DNA Ligase). Fidelity measurements utilized 50 RPLUs of each ligase in a 20 µL reaction volume with the supplied buffers at 1X concentration. The reactions were incubated for 30 minutes at 55°C or 37°C for thermophilic and mesophilic DNA ligases, respectively, using multiplexed substrate pools prepared as described in panel A. Rows in the data represent individual splint sequences, while columns denote specific ligation products from probe pairs joining at the indicated bases of the ligation junction. A colored square signifies detection of a product based on ligation product yield (see legend). The diagonal from the top left to the bottom right represents ligation products with Watson-Crick base pairing, while other spaces represent mismatch ligation products.

SNP detection via L-RCA

Circular padlock probe preparation

Three SNPs present in the human genome (rs1800795 (C/G), rs11119982 (C/T), and rs1800797 (A/G)) were selected as model systems for SNP detection using the ligation–rolling circle amplification (L-RCA) approach. For each SNP variant, 0.2 µM 5´-phosphorylated PLP was hybridized with 0.2 µM–0.2 nM specific or nonspecific model SNP target in 1X *Thermus thermophilus* ligation buffer with 1 µL *Thermus thermophilus* DNA Ligase (50 U) by heating at 95°C for 3 minutes and cooling to 65°C at a rate of 0.1°C/sec in a total volume of 10 µL. The ligation reaction proceeded at 65°C for 30 minutes. Unreacted linear DNA was removed by exonuclease degradation. Briefly, 2 µL of ligation products were incubated with 1.2 U/µL Exonuclease I and 24 U/µL Exonuclease III in 1X EquiPhi29 DNA Polymerase reaction buffer supplemented with 1 mM DTT in a total reaction volume of 10 µL. The reactions were performed in a MicroAmp Fast Optical 96-Well Reaction Plate at 37°C for 30 minutes, followed by heat inactivation at 80°C for 15 minutes.

To test L-RCA reaction specificity, 0.2 µM 5´-phosphorylated PLP was hybridized with 0.2 nM specific model SNP target mixed with 0.2 µM nonspecific target or 5 ng/µL *E. coli* genomic DNA in 1X *Thermus thermophilus* ligation buffer with 50 U *Thermus thermophilus* DNA Ligase by heating at 95°C for 3 minutes and cooling to 65°C at a rate of 0.1°C/sec in a total volume of 10 µL. Ligation and exonucleolysis were performed as described previously.

Rolling circle amplification

The prepared circular DNA was directly amplified via RCA using a two-primer combination to enhance SNP detection. The RCA reaction mixture was prepared as shown in Table 2. The reactions were incubated for 2 hours at 42°C followed by 10 minutes at 65°C, then held at 4°C in the QuantStudio 7 Pro Real-Time PCR System. The RCA reaction was monitored by measuring the fluorescence of Quant-iT PicoGreen dsDNA Reagent every minute over the two-hour reaction period. The time-to-signal was calculated as the time required to reach 2.5 x 10^6 fluorescence units. Specificity was assessed by analyzing intact and digested RCA products on an E-Gel EX 2% Agarose Gel. Briefly, 1 µL of RCA product was digested with FastDigest RsaI (PLP1_C, PLP2_C, and PLP3_A) or FastDigest Hpy8I (PLP1_G, PLP2_T, and PLP3_G) in a 20 µL reaction at 37°C for 5 minutes, followed by heat inactivation at 80°C for 5 minutes. Then 2 µL of digested RCA product, as well as 2 µL of intact RCA product (diluted 1:20),were analyzed on the gel.

Table 2. RCA reaction setup using EquiPhi29 DNA Polymerase.

Component	Volume for 1 reaction (µL)	Final composition
10X EquiPhi29 DNA Polymerase Reaction Buffer	1	1X
100 mM DTT	0.2	1 mM
10 µM Primer 1*	0.2	0.1 µM
10 µM Primer 2 or Primer 3*, **	0.2	0.1 µM
10 mM dNTP Mix	2	1 mM each
Quant-iT PicoGreen dsDNA Reagent (20X)	1	1X
Prepared sample after exonucleolysis	10	10 µL/reaction
EquiPhi29 DNA Polymerase	1	0.5 U/µL
Pyrophosphatase	1	0.005 U/µL
Nuclease-free water		Add to 20 µL volume

* For best results, test different primer concentrations (0.1–1.0 µM) to determine the optimal concentration for your experiment.

** Primer 2 was used when RCA was performed using the PLP1_C, PLP2_C, or PLP3_A padlock probe as the template, while Primer 3 was used with PLP1_G, PLP2_T, and PLP3_G.

Results

Analysis of the ability of ligase to discriminate single-nucleotide variations

A SNP is a genomic variant occurring at a single base in a DNA sequence. To achieve high sensitivity and low background in SNP detection via the L-RCA approach (Figure 1), the chosen ligase must efficiently join fully complementary substrates while discriminating against substrates with even a single mismatched base pair at the ligation site. To identify the most suitable ligase for the SNP detection assay, we tested five different DNA ligases: mesophilic T4 DNA Ligase and *E. coli* DNA Ligase, and thermophilic *Thermus thermophilus* DNA Ligase, HiFi *Taq* DNA Ligase, and Ampligase Thermostable DNA Ligase. Their ability to discriminate mismatches at the ligation site was evaluated using a high-throughput assay as described in the Methods section. Briefly, ligation substrates were designed to form a double-stranded DNA structure with a nick in the middle, similar to the ligation substrate in the SNP detection method (Figure 1). The substrates were prepared by combining three types of oligonucleotides: upstream oligonucleotides that provided a 3′ OH group at the ligation junction; downstream oligonucleotides that provided a 5′ phosphate at the ligation junction and a 3′ FAM label; and splint oligonucleotides that covered all possible combinations of bases (NN) at the ligation junction (Figure 2A). To cover all possible variants at the ligation junction (total 256), 64 reaction mixtures were prepared for each ligase. Each reaction contained one splint oligonucleotide, one upstream probe, and four downstream probes that provided different 5′ bases at the ligation junction site. In each reaction, four hybrid types could potentially form. The products of each reaction were analyzed via CE by detecting FAM fluorescence from products of different lengths.

Following the analysis of ligation products by CE, we identified clear differences in the ability of the ligases to discriminate mismatches. Notably, *Thermus thermophilus* DNA Ligase demonstrated the highest fidelity (Figure 2B). This ligase did not ligate any substrates with two mismatches and incorrectly ligated only 7 single-mismatched substrates at >50% efficiency. In contrast, HiFi *Taq*, Ampligase, *E. coli*, and T4 DNA ligases incorrectly ligated 21, 37, 73, and 78 single-mismatched substrates at >50% efficiency, respectively. The mesophilic *E. coli* and T4 DNA ligases ligated 4 and 17 substrates, respectively, with two mismatches at the ligation junction at >50% yield. Therefore, mesophilic DNA ligases should be avoided in applications requiring high selectivity. To minimize nonspecific ligation and help ensure high fidelity in applications like SNP detection that require stringent accuracy, we recommend using *Thermus thermophilus* DNA Ligase.

We also observed different discriminatory properties toward different types of mismatches. All of the tested DNA ligases were more tolerant of mismatches on the downstream side of the ligation junction (providing the phosphate in a ligation reaction) than they were of mismatches on the upstream side. For example, 7, 18, 32, 48, and 48 downstream mismatches were ligated using *Thermus thermophilus*, HiFi *Taq*, Ampligase, *E. coli*, and T4 DNA ligases, respectively, while 0, 3, 5, 25, and 30 upstream mismatches were tolerated. Note that *Thermus thermophilus* DNA Ligase did not ligate any upstream mismatches at >50% yield. Thus, when designing a SNP detection assay, we recommend positioning the base to be analyzed so that it will be complementary to the 3′ end of the padlock probe to minimize nonspecific ligation as much as possible.

Design of padlock probes for SNP detection

In this study, we used a model system to evaluate the feasibility of SNP detection using the L-RCA approach. Three SNPs found in the human genome were selected for analysis: rs1800795 (C/G), rs11119982 (C/T), and rs1800795 (A/G). Two 5′-phosphorylated PLPs were designed to discriminate between the two alleles for each SNP: one specific to the wild type variant (PLP1_C, PLP2_C, PLP3_A) and the other to the mutant variant (PLP1_G, PLP2_T, PLP3_G). Only a specific match between the PLP and SNP variant would be expected to lead to circularization by high-fidelity DNA ligase (Figure 1). Each PLP comprised several specific parts to help ensure precise SNP detection. The 3′ end nucleotide of the PLP was complementary to the base being analyzed, allowing for accurate discrimination between wild type and mutant SNP variants since the DNA ligases better discriminated mismatches at the 3′ OH ligation site. The 5′ and 3′ ends of the PLP probes were complementary to sequences flanking the SNP region. The 5′ end complementary region was designed with a melting temperature (T_m) of approximately 65°C to help ensure stable hybridization, while the 3′ end complementary region had a T_m of approximately 11°C to facilitate selective binding and enhance ligation specificity. Upon ligation, the DNA circle created a template for the RCA reaction. The region of the PLP between the target-specific ends provided primer binding sites for RCA reaction initiation. The PLPs also contained restriction sites: for FastDigest RsaI in PLP1_C, PLP2_C, and PLP3_A, and for FastDigest Hpy8I in PLP1_G, PLP2_T, and PLP3_G. These sites enabled digestion of RCA products into fragments of a length corresponding to one repeat of the PLP.

L-RCA–based SNP detection

The feasibility of the L-RCA protocol for SNP detection was thoroughly evaluated using a model system comprising three SNPs: rs1800795 (C/G), rs11119982 (C/T), and rs1800795 (A/G). Each SNP was targeted by two 5´-phosphorylated padlock probes specific to the wild type and mutant alleles in different reactions using high-fidelity *Thermus thermophilus* DNA Ligase. Ligation products were subjected to exonucleolysis using Exonucleases I and III to remove unreacted padlock probes and reduce subsequent ligation-independent amplification events in the following RCA reaction. The remaining circular PLPs were amplified via RCA using EquiPhi29 DNA Polymerase and two primers: Primer 1, which bound universally within the PLP sequence, and a reverse primer (Primer 2 or Primer 3). Primer 2 and Primer 3 were specific to wild type and mutant-specific PLPs, respectively. This dual-primer system helped ensure efficient amplification of circular DNA. The RCA reaction kinetics were monitored using the fluorescent Quant-iT PicoGreen dsDNA Reagent, measuring the fluorescence intensity every minute, thereby providing real-time amplification data. We conducted experiments with varying concentrations of target DNA—0.4 fmol, 4 fmol, 40 fmol, and 400 fmol. Real-time profiles of the RCA reactions indicated that an amplification signal was obtained only from reactions in which the PLP matched the SNP nucleotide. For each concentration tested, the amplification graphs clearly demonstrated signal detection for matched target–PLP combinations. This indicated successful amplification for all target levels, while nonspecific target–PLP combinations resulted in no amplification signal (Figure 3A).

The time-to-signal, defined as the time taken to reach a detectable fluorescence threshold, was calculated for each target concentration for each SNP model system. The results indicated a clear correlation between the target quantity and the time-to-signal. Higher concentrations of target DNA resulted in shorter time-to-signal, consistent with the expected kinetics of the RCA reaction (Figure 3B). Time-to-signal is slightly different between samples, and it can be affected by PLP sequence (PLP1_C, PLP2_C, PLP3_A vs. PLP1_G, PLP2_T, PLP3_G). In our case, time-to-signal was consistently slightly longer for RCA reactions with the PLP1_G, PLP2_T, and PLP3_G padlock probes. Thus, we highly recommend testing your padlock probes in a model system before proceeding to genotyping samples.

Importantly, no fluorescence signal was detected in the negative control reactions, which included nonspecific ligation events (Figure 3B). This lack of signal further corroborated the high specificity of the padlock probes and the ligation step, which helped ensure that only fully matched SNP variants would enable successful circularization and subsequent RCA amplification. The absence of nonspecific product detection highlights the fidelity of the *Thermus thermophilus* DNA Ligase, which discriminates efficiently between matched and mismatched 3´ OH sites. The results demonstrated that the L-RCA approach was effective in detecting SNPs at all tested concentrations.

To further assess the success and specificity of the reaction, the RCA products were analyzed on E-Gel EX 2% Agarose Gels (Figure 3C). The analysis included intact samples and those digested with FastDigest RsaI (PLP1_C, PLP2_C, and PLP3_A) or with FastDigest Hpy8I (PLP1_G, PLP2_T, and PLP3_G), which cut at a single recognition site within the PLPs. For undigested samples, significant amounts of high molecular weight products were observed as smears or remained trapped in the gel wells, confirming the generation of RCA products when a specific combination of PLP and target was used. In contrast, nonspecific combinations of target and PLP did not give any visible signal, confirming the absence of RCA products in real-time data. Upon digestion with restriction enzymes, the RCA products of specific target–PLP combinations yielded bands corresponding to single repeat units of the padlock probes, further validating the successful and specific amplification.

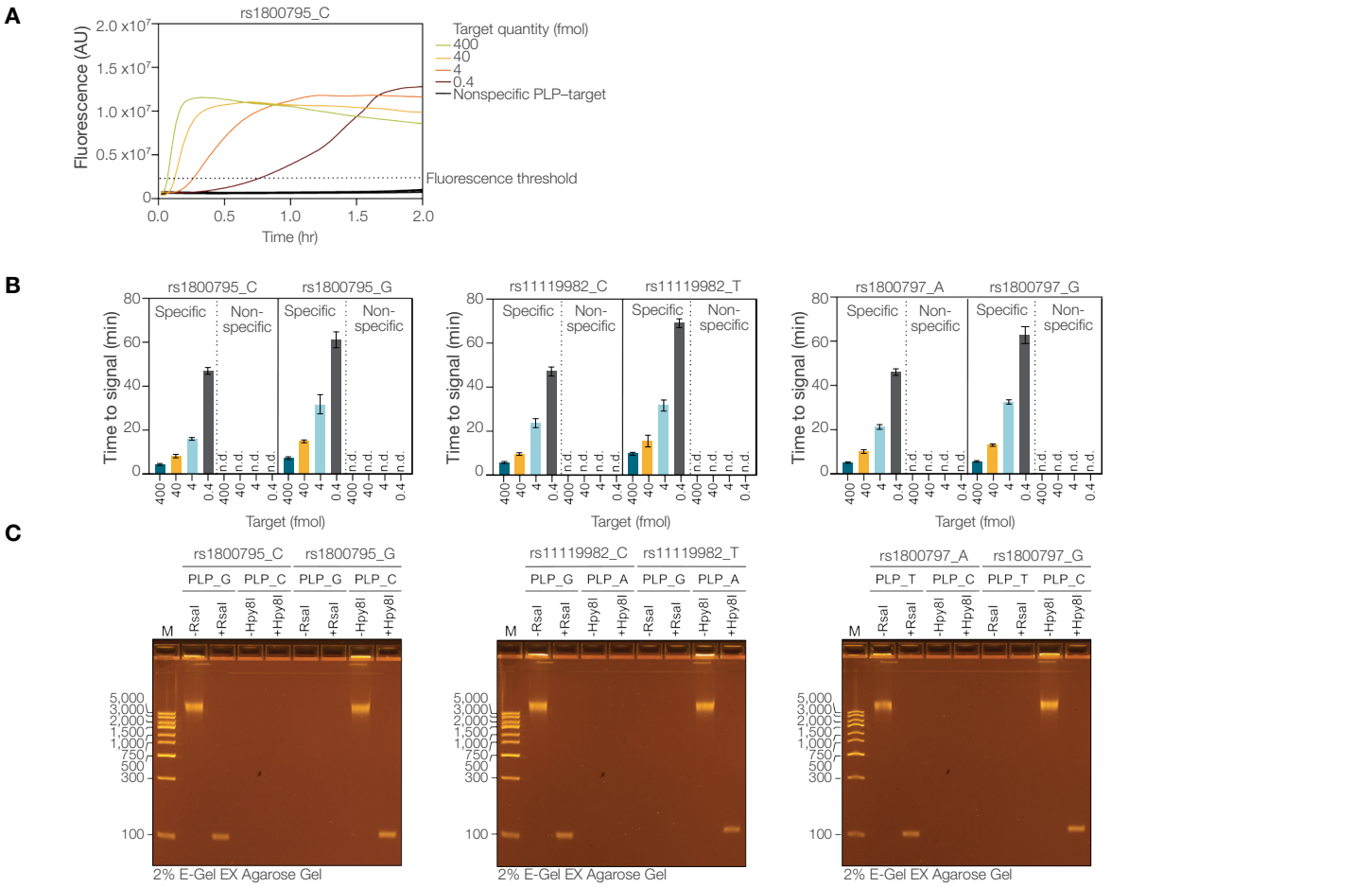

Figure 3. SNP detection using the L-RCA approach. (A) Representative real-time profile of the amplification signals obtained from 0.4–400 fmol of ligation products. **(B)** Detection of three different SNP variants (rs1800795, rs11119982, rs1800797) via the L-RCA approach using *Thermus thermophilus* DNA Ligase and EquiPhi29 DNA Polymerase. Amplification kinetics were demonstrated using different target inputs (0.4, 4, 40, and 400 fmol). SNP variants were detected using Quant-iT PicoGreen dsDNA Reagent. Error bars represent the standard deviation of reaction speed (time-to-signal) values calculated from technical repeats (n = 3). Signals for mismatched PLP–target combinations were not detected within 2 hours. **(C)** Gel electrophoresis analysis of undigested and digested RCA products. The endpoint products of SNP detection reactions were resolved on E-Gel EX 2% Agarose Gels. The characteristic high molecular weight DNA or DNA fragment corresponding to a single repeat unit length of the PLP was observed exclusively in reactions where matched PLPs and targets were used.

To further assess the specificity of our system, the matched combination of 0.4 fmol target and 400 fmol PLP was mixed with either 400 fmol nonspecific target or 10 ng *E. coli* genomic DNA, followed by ligation and RCA reaction. The control reaction contained only the matched combination of 0.4 fmol target and 400 fmol PLP. As shown in Figure 4, additional nonspecific DNA (nonspecific target or *E. coli* gDNA) did not change time-to-signal significantly. This indicates that the proposed system is able to specifically detect SNP variants, even in the presence of background DNA.

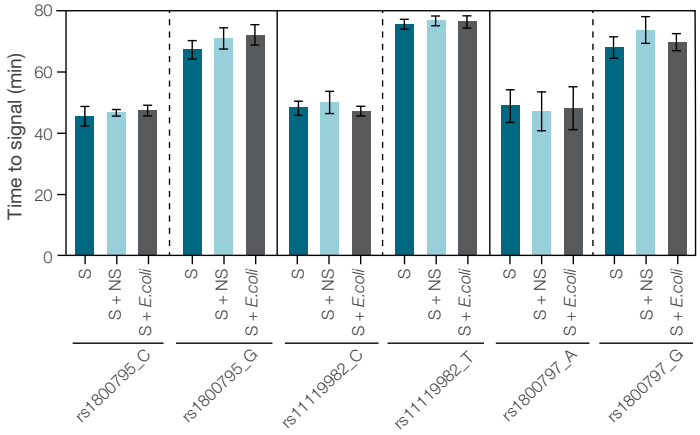

Figure 4. Specificity of L-RCA assay for SNP detection. Time-to- signal values of the L-RCA assay with 0.4 fmol specific target (S), a mixture of 0.4 fmol specific target and 400 fmol nonspecific target (S + NS), or a mixture of 0.4 fmol specific target and 10 ng *E. coli* genomic DNA (S + *E. coli*). In each reaction, the PLP concentration was 400 fmol. Error bars indicate the standard deviation calculated from technical repeats (n = 3).

These results collectively demonstrate that the L-RCA protocol, with its carefully designed padlock probes and precise ligation conditions, provides a reliable method for SNP detection. The ability to generate clear and distinguishable amplification signals emphasizes the potential of this method for applications in genetic analysis and diagnostic research. It is important to recognize that multiple detection methods are available for SNP analysis, each with varying degrees of sensitivity and specificity, and the overall sensitivity of the SNP detection workflow is heavily influenced by the chosen detection system. Therefore, we recommend considering different detection systems (e.g., based on colorimetry, fluorescence, or bioluminescence) and selecting a detection method that suits your experimental needs.

Conclusions

Thermus thermophilus DNA Ligase exhibited exceptional accuracy in discriminating single-nucleotide mismatches. The combination of *Thermus thermophilus* DNA Ligase and EquiPhi29 DNA Polymerase enabled the detection of SNPs; real-time RCA assays specifically identified all three tested SNPs. These findings indicate that *Thermus thermophilus* DNA Ligase and EquiPhi29 DNA Polymerase can be utilized to develop a highly precise and sensitive method for SNP detection.

Ordering information

Description	Quantity	Cat. No.
MicroAmp Fast Optical 96-Well Reaction Plate, 0.1 mL	10 plates	4346907
ProFlex PCR System, 3 x 32-well	1 unit	4484073
QuantStudio 7 Pro Real-Time PCR System, 96-well, 0.1 mL	1 system	A43163
E-Gel 1 Kb Plus Express DNA Ladder	2 x 1.25 mL	10488091
E-Gel EX 2% Agarose Gels	10 gels	G401002
Quant-iT PicoGreen dsDNA Reagent	10 x 100 µL tubes	P11495
dNTP Mix (10 mM each), 1 mL	1 mL	R0192
E. coli DNA Ligase	400 U	EL00361
EquiPhi29 DNA Polymerase	250 U	A39390
Exonuclease I (20 U/µL)	4,000 units	EN0581
Exonuclease III (200 U/µL)	4,000 units	EN0191
FastDigest Hpy8I	20 µL (20 reactions)	FD1574
FastDigest RsaI	100 µL (100 reactions)	FD1124
Pyrophosphatase, inorganic (0.1 U/µL)	10 U	EF0221
T4 DNA Ligase (5 U/µL), 200 units	200 U	EL0014
Thermus thermophilus DNA Ligase	1,000 U	EL07981

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