

Implementation of Fusion Primer-Driven Racket PCR Protocol for Genome Walking

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Abstract

Genome-walking protocols have been extensively used to clone unknown genomic sequences next to known DNAs. Existing genome-walking protocols need further improvement in methodological specificity or operation. Here, we describe a novel genome-walking protocol based on fusion primer-driven racket PCR (FPR-PCR). FPR-PCR involves four sequence-specific oligos (SSO), SSO1, SSO2, SSO3, and SSO4, which are sequentially chosen from known DNA in the direction 5'→3'. The fusion primer, mediating primary FPR-PCR, is generated by attaching SSO3 to the 5' end of SSO1. The SSO3 encourages the target DNA of primary PCR to form a racket-like structure by mediating intra-strand annealing. SSO2 and SSO4 are directly used as sequence-specific primers (SSP) in secondary FPR-PCR, which selectively amplifies this racket-like DNA. This protocol was verified by cloning several unknown genomic sequences. Compared to traditional PCRs, FPR-PCR offers the advantages of higher specificity and fewer rounds, primarily attributed to the omission of arbitrary walking primers typically required in traditional methods.

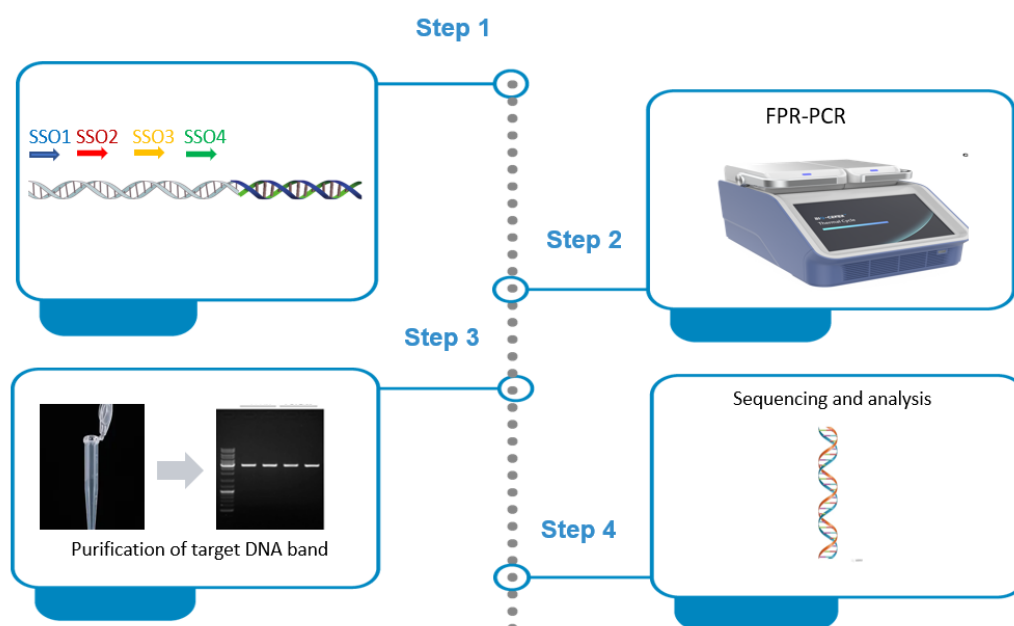
Key features

- This FPR-PCR protocol builds upon the method constructed by Pei et al. [1].
- The FPR-PCR protocol relies on a multi-functional fusion primer (FP) that mediates the primary amplification and the formation of racket-like DNA.
- The FPR-PCR comprises only two rounds of amplification reactions.

Keywords: Genome-walking PCR, Sequence-specific primer, Fusion primer, Partial annealing, Intra-strand annealing of DNA, Racket-like DNA

This protocol is used in: *Frontiers in Genetics* (2022), DOI: 10.3389/fgene.2022.969840

Graphical overview



Background

Genome walking (GW) is a molecular technique for identifying unknown genomic sequences next to known DNAs [2–4]. GW has been extensively used to obtain regulatory sites of genes, amplify non-conserved regions based on conserved DNAs, identify T-DNA, discover new functional genes, or screen microbes [5–7]. Therefore, GW has made significant contributions to the development of life sciences [8–10].

To date, there are many protocols for genome walking, mainly including genomic library methods, ligation-based PCRs, and arbitrary PCRs [11–14]. The genomic library method has been abandoned due to its time-consuming nature [15]. The ligation-based PCRs are also showing a tendency to be phased out, as they require pretreatment of the genomic plate prior to PCR [16–19]. In contrast, arbitrary PCRs directly mediate walking by randomly annealing a walking primer to the unknown flank; in general, the target DNA is obtained after two to three nested amplifications [20–23]. Therefore, arbitrary PCRs are a more rapid and straightforward approach. However, existing arbitrary PCRs typically realize GW by the differential amplification between target DNA and non-target DNA. Obviously, non-target background arising from walking primer challenges these PCRs [24–26].

Herein, we propose an efficient but specific genome-walking protocol, fusion primer-driven racket PCR (FPR-PCR). This method utilizes a fusion primer (FP) to mediate the target amplicon of primary FPR-PCR to form a racket-like structure; then, a secondary PCR, driven by a sequence-specific primer (SSP) pair, selectively enriches this racket-like DNA. As a result, non-target amplification is basically overcome in FPR-PCR. The FPR-PCR was validated by successfully acquiring several unknown flanking genomic DNAs [27–29].

Materials and reagents

Biological materials

1. Genome of *Levilactobacillus brevis* [30–35], isolated with the TIANamp Bacteria DNA kit

Reagents

1. TIANamp Bacteria DNA kit (TIANGEN, catalog number: 4992448)

2. 1× TE buffer (Sangon, catalog number: B548106)
 3. LA Taq polymerase (Takara, catalog number: RR02MA)
 4. 6× Loading buffer (Takara, catalog number: 9156)
 5. DiaSpin column DNA Gel Extraction kit (Sangon, catalog number: B110092)
 6. DL 5,000 DNA marker (Takara, catalog number: 3428Q)
 7. Agarose (Sangon, catalog number: A620014)
 8. 0.5 M EDTA (Solarbio, catalog number: B540625)
 9. GoldView I nucleic acid staining agent (10,000 ×) (Solarbio, catalog number: G8140)
 10. Tris (Solarbio, catalog number: T8060)
 11. Boric acid (Solarbio, catalog number: B8110)
 12. Oligos (Sangon)
- gadC-FPα: TGTTTTCTTCTTGCTCT|ATGGTTATTCTCTGGGG
gadC-FPβ: TGTTTTCTTCTTGCTCT|TCTCTGGGGATTGATTG
gadC-SSP2: TTGGGCGTTATAATTCTGTTTTCTTCTTG
gadC-SSP4: GGAGCGGTAGTGTGTTAGTTGGGTT

Note: The two parts (SSO1 and SSO3) in an FP are separated by a vertical line. The left part is SSO3, and the right part is SSO1.

Solutions

1. 100 μM primer (see Recipes)
2. 10 μM primer (see Recipes)
3. 2.5× TBE buffer (see Recipes)
4. 0.5× TBE buffer (see Recipes)
5. 1.5% agarose gel (see Recipes)

Recipes

1. 100 μM primer

Reagent	Final concentration	Quantity or volume
Primer powder	100 μM	n/a
1× TE buffer	1×	Volume (μL) specified by the supplier
Total	n/a	Volume (μL) specified by the supplier

2. 10 μM primer

Reagent	Final concentration	Quantity or volume
100 μM primer	10 μM	10 μL
Ultrapure water	n/a	90 μL
Total	n/a	100 μL

3. 2.5× TBE buffer (pH 8.3)

Reagent	Final concentration	Quantity or volume
Tris	225 mM	27 g
Boric acid	225 mM	13.75 g
0.5 M EDTA	5 mM	10 mL
Ultrapure water	n/a	950 mL
Total	n/a	1,000 mL

4. 0.5× TBE buffer (pH 8.3)

Reagent	Final concentration	Quantity or volume
2.5 × TBE buffer	0.5×	200 mL
Ultrapure water	n/a	800 mL
Total	n/a	1,000 mL

5. 1.5% agarose gel

Reagent	Final concentration	Quantity or volume
Agarose powder	1.5%	1.5 g
0.5× TBE buffer	0.5×	100 mL
GoldView I nucleic acid staining agent (10,000×)	1×	10 μL
Total	n/a	100 mL

Laboratory supplies

1. 0.2 mL PCR tubes (Kirgen, catalog number: KG2311)
2. 10 μL pipette tips (Sangon, catalog number: F600215)
3. 0.2 mL pipette tips (Sangon, catalog number: F600227)
4. 1 mL pipette tips (Sangon, catalog number: F630101)
5. 1.5 mL tubes (Labselect, catalog number: MCT-001-150)

Equipment

1. PCR cycler (Analytikjena, model: Biometra TOne 96G PCR)
2. Electrophoresis apparatus (Beijing Liuyi, model: DYY-6C)
3. Gel imaging system (Bio-Rad, model: ChemiDoc XRS+)
4. Microcentrifuge (Tiangen, model: TGear)

Software and datasets

1. Oligo 7 software (Molecular Biology Insights, Inc., USA)
2. DNASTAR Lasergene software (DNASTAR, Inc., USA)
3. All data are available at <https://www.frontiersin.org/articles/10.3389/fgene.2022.969840/full#supplementary-material> (access date, 10/18/2022)

Procedure

A. Primer design

1. Sequentially pick a set of four SSOs—SSO1, SSO2, SSO3, and SSO4—from known DNA in the direction 5'→3' (Figure 1).

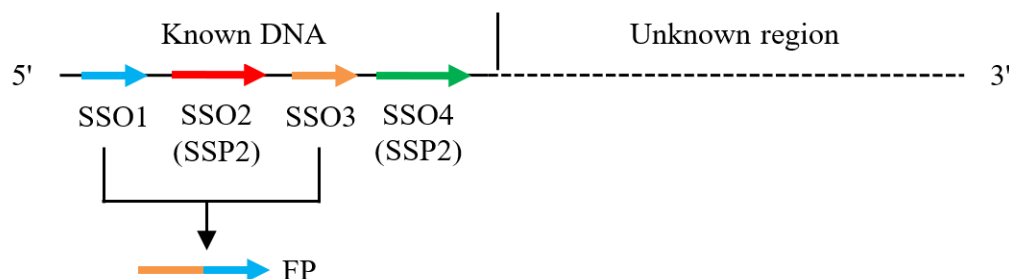


Figure 1. Picking sequence-specific oligos (SSOs). The four SSOs are from known DNA. The fusion primer (FP) is made by attaching SSO3 to the 5' end of SSO1. FP performs primary FPR-PCR, while SSO2 and SSO4 are directly used as sequence-specific primers (SSPs) to perform secondary FPR-PCR.

Note: We suggest picking more than one SSO1 (SSO1 α , SSO1 β , ...), one SSO2, one SSO3, and one SSO4. Then, attach SSO3 to SSO1s to obtain FPs, so as to perform parallel FPR-PCRs for a walking experiment. In this study, two SSO1s (SSO1 α and SSO1 β) were picked from each gene to make FP α and FP β , respectively.

Critical: SSO1s can partially overlap or be completely different. For the former, the difference at the 3' ends must not be less than 3 nt. SSO1s are 17–21 nt with a melting temperature (T_m) of 50–55 °C. SSO3 is 17–18 nt with a T_m of 45–55 °C. SSO2 or SSO4 is 25–30 nt with a T_m of 60–66 °C.

2. Open the Oligo 7 software and click *File* and *Open* to enter a known DNA sequence (Figure 2A).

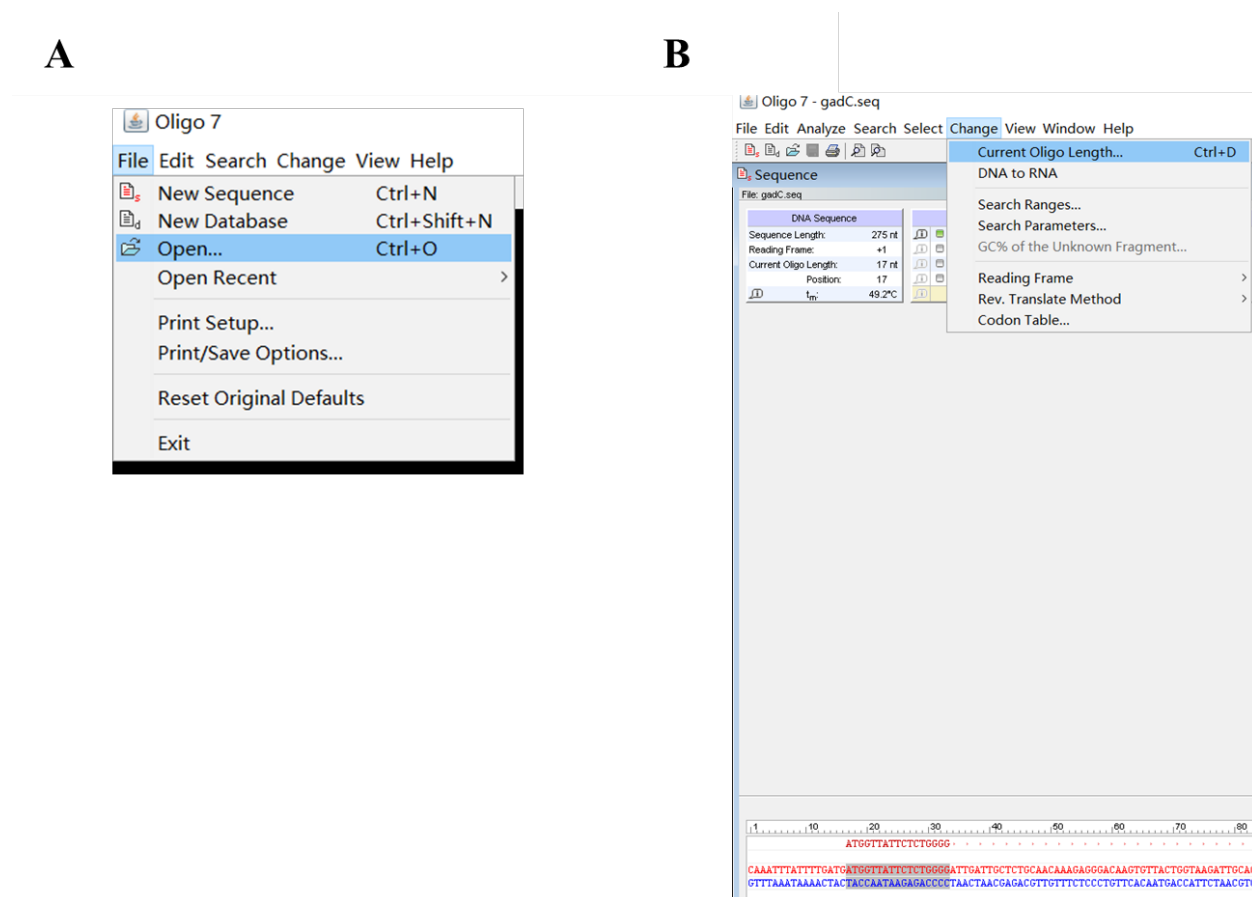


Figure 2. Screenshots exhibiting how to design a sequence-specific oligo (SSO). (A) How to enter known sequence. (B) How to define the length of SSO.

3. Click *Change* and *Current Oligo Length* to define SSO length (Figure 2B).

4. Click *Analyze*, *Duplex Formation*, and *Current Oligo* (Figure 3A) to assess SSO dimer (Figure 3B).

5. Click *Analyze*, *Hairpin Formation*, and *Current Oligo* (Figure 4A) to assess the SSO hairpin (Figure 4B).

Note: If the current SSO forms an obvious dimer(s) or hairpin(s) with a $T_m \geq 40$ °C (Figure 4C), pick a new SSO and then assess it by repeating steps A3–A5 until a satisfactory one is obtained.

6. Attach SSO3 to the 5' end of SSO1 to form FP and assess the FP by repeating steps A3–A5.

Note: If the current FP is unsatisfactory, redesign SSO3 or SSO1 to make a new one until a satisfactory FP is obtained.

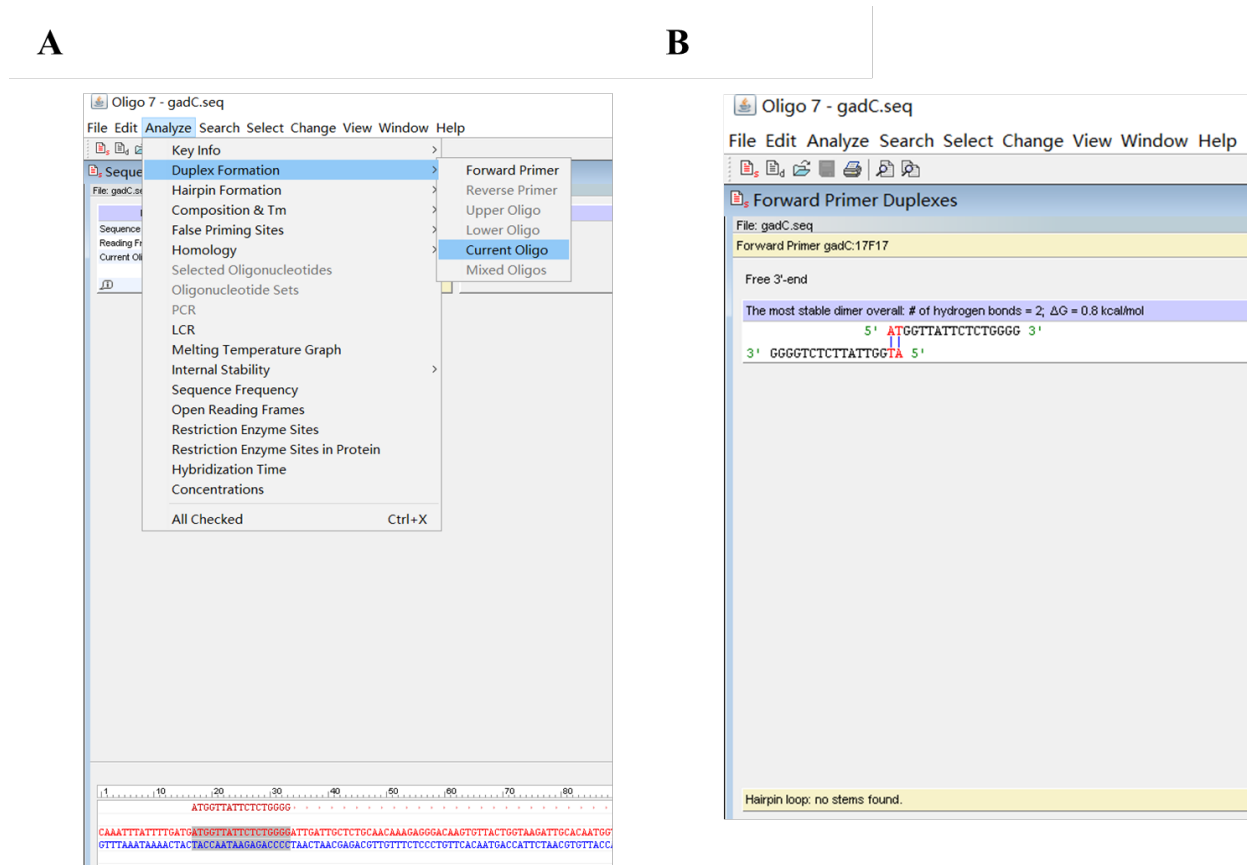


Figure 3. Screenshots exhibiting how to assess sequence-specific oligo (SSO) dimer. (A) How to find *Duplex Formation* and *Current Oligo*. (B) Output SSO dimer(s).

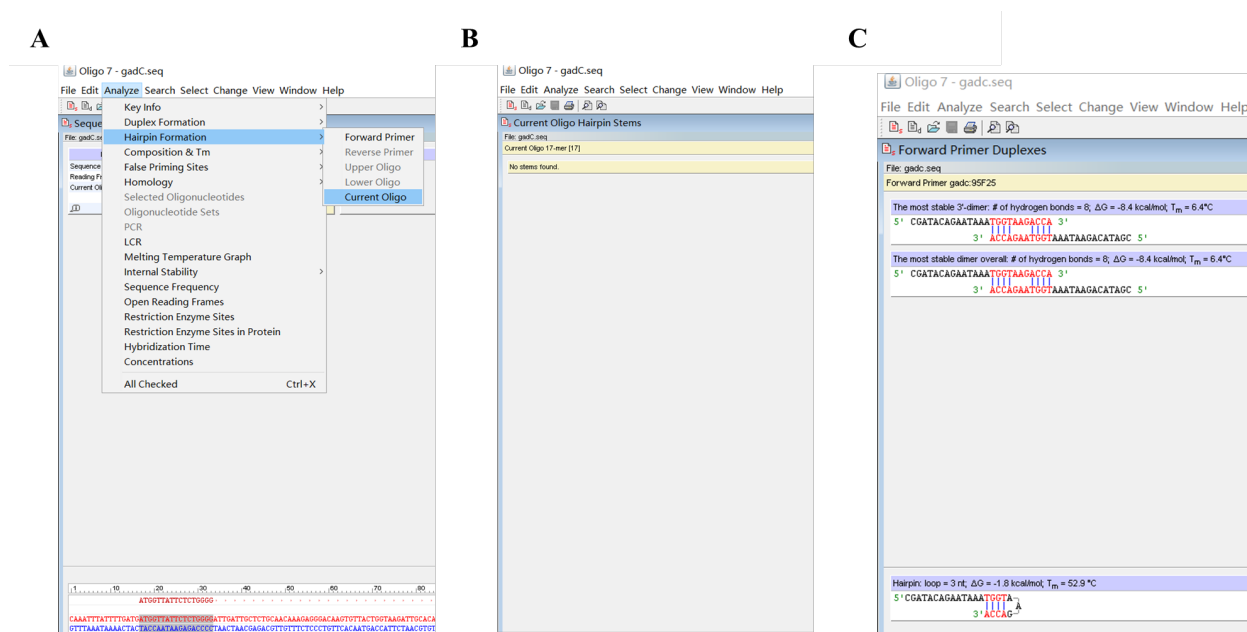


Figure 4. Screenshots exhibiting how to assess sequence-specific oligo (SSO) hairpin. (A) How to find *Hairpin Formation* and *Current Oligo*. (B) Output SSO hairpin(s). (C) An unsatisfactory SSO selection.

B. FPR-PCR amplification

The process of FPR-PCR is outlined in Figure 5.

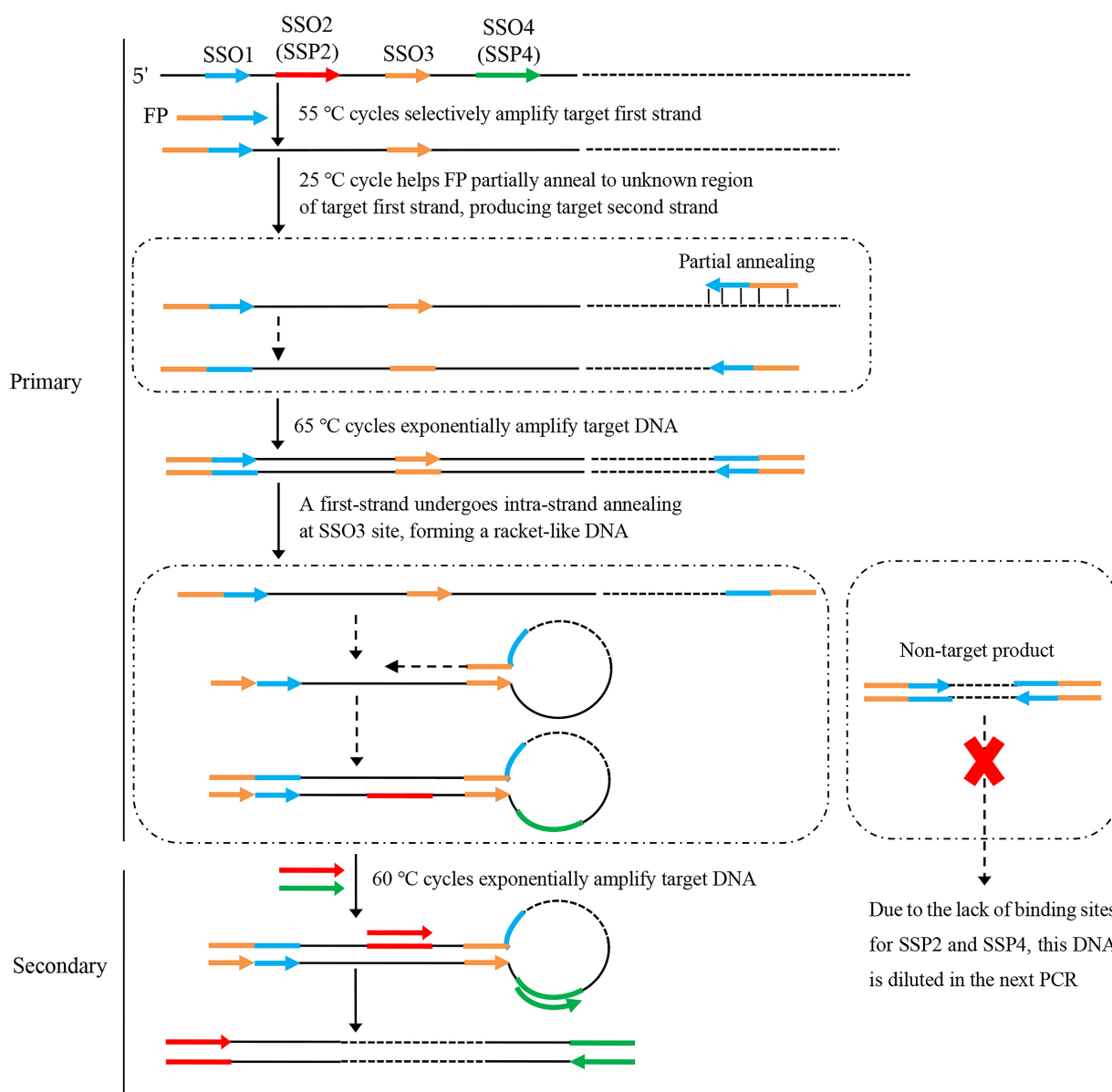


Figure 5. Outline of fusion primer (FP)-driven racket PCR. The FP's 3'-part is SSO1, while the 5'-part is SSO3. Primary PCR uses a single FP (FP = SSO3|SSO1), without involving a reverse primer, and the secondary PCR uses nested primers SSO2 and SSO4.

Notes:

- In primary PCR, the 25 °C cycle promotes FP to partially annealing to the unknown flank. The annealed FP synthesizes a target DNA by extending toward the known region, thereby mediating walking.
- One type of non-target DNA is also expected to be produced in primary FPR-PCR. In secondary FPR-PCR, however, only the target DNA can be amplified because only this DNA has the binding sites for both SSO2 and SSO4.

1. Primary FPR-PCR

- Mix primary FPR-PCR components into a PCR tube (Table 1).

Table 1. Composition of primary FPR-PCR

Reagent	Final concentration	Volume (μL)
Genomic template	Microbe, 0.2–2 ng/μL; <i>Oryza sativa</i> , 2–20 ng/μL	1
LA Taq polymerase (5 U/μL)	0.05 U/μL	0.5
FP (10 μM)	0.2 μM	1
10× LA PCR buffer II (containing Mg ²⁺)	1×	5
dNTP mixture (2.5 mM each)	0.4 mM each	8
Ultrapure water	n/a	34.5
Total	n/a	50

- b. Fully mix the components using a pipette.
- c. Centrifuge at 3,000× *g* for 10 s to gather the mixture.
- d. Run PCR amplification (Table 2).

Table 2. Primary FPR-PCR cycling program

Step	Temperature (°C)	Duration (min)	Cycle
Initial denaturation	94	2	1
Denaturation	94	0.5	5
Annealing	55	0.5	
Extension	72	3	
Denaturation	94	0.5	1
Annealing	25	0.5	
Extension	72	3	
Denaturation	94	0.5	30
Annealing	65	0.5	
Extension	72	3	
Final extension	72	10	1
Hold	4	forever	1

Note: The 25 °C annealing cycle enables PF to partially anneal to the unknown flank and then extend toward the known region, thus mediating genome walking.

- e. Take 1 μL of this PCR product as the template of the secondary FPR-PCR.
- f. Store the rest of the product at -20 °C.

2. Secondary FPR-PCR

- a. Mix secondary FPR-PCR components into a PCR tube (Table 3).

Table 3. Composition of secondary FPR-PCR

Reagent	Final concentration	Volume (μL)
Primary FPR-PCR product	n/a	1
LA Taq polymerase (5 U/μL)	0.05 U/μL	0.5
SSP2 (10 μM)	0.2 μM	1
SSP4 (10 μM)	0.2 μM	1
10× LA PCR buffer II (containing Mg ²⁺)	1×	5
dNTP mixture (2.5 mM each)	0.4 mM each	8
Ultrapure water	n/a	33.5
Total	n/a	50

- b. Fully mix the components.
- c. Centrifuge at 3,000× *g* for 10 s to gather the mixture.
- d. Run PCR amplification (Table 4).

Table 4. Secondary FPR-PCR cycling program

Step	Temperature (°C)	Duration (min)	Cycle
Initial denaturation	94	2	1
Denaturation	94	0.5	25
Annealing	60	0.5	
Extension	72	3	
Hold	4	forever	1

C. Gel electrophoresis

1. Mix 5 μ L of FPR-PCR product and 1 μ L of 6 $\times$ loading buffer.
2. Transfer the mixture into a 1.5% agarose gel with 1 $\times$ GoldView I nucleic acid staining agent.
3. Electrophorese at a voltage of 5 V/cm for 30 min.
4. Check the gel using the ChemiDoc XRS+ imaging system (Figure 6).

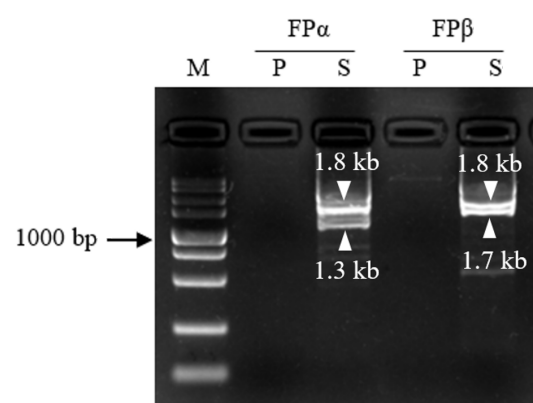


Figure 6. Mining the unknown flanking region of *gadC*. FP α and FP β denote the two parallel sets of FPR-PCRs. The bands indicated by white arrowheads are the secondary FPR-PCR products. Lane P, primary PCR; lane S, secondary PCR; lane M, TaKaRa DL5000 DNA marker. All four bands marked with arrows were sequenced and verified to be correct.

D. Purification of PCR product

1. Mix 40 μ L of secondary FPR-PCR product and 8 μ L of 6 $\times$ loading buffer.
2. Transfer the mixture into a 1.5% agarose gel with 1 $\times$ GoldView I nucleic acid staining agent.
3. Electrophorese at a voltage of 5 V/cm for 30 min.
4. Check the gel using the ChemiDoc XRS+ imaging system (Figure 6) and cut out target DNA band(s) with a knife.
5. Purify the DNA band(s) from the cut gel using the DiaSpin column DNA Gel Extraction kit.

E. DNA Sequencing

1. Mail the purified product(s) to Sangon Biotech Co., Ltd for sequencing.

Data analysis

1. Analyze sequencing data using the MegAlign software. Open the software and then click *File* and *Enter Sequences* (Figure 7A) to input DNA sequences to be analyzed (Figure 7B).



Figure 7. Screenshots exhibiting how to input DNA sequences. (A) How to find *Enter Sequences*. (B) Input sequences.

2. Click *Align* and *By Clustal W Method* (Figure 8A) to get the result (Figure 8B).

Note: The experiment is considered successful if the SSO4-sided segment of the FPR-PCR product overlaps known DNA (Figure 8B).

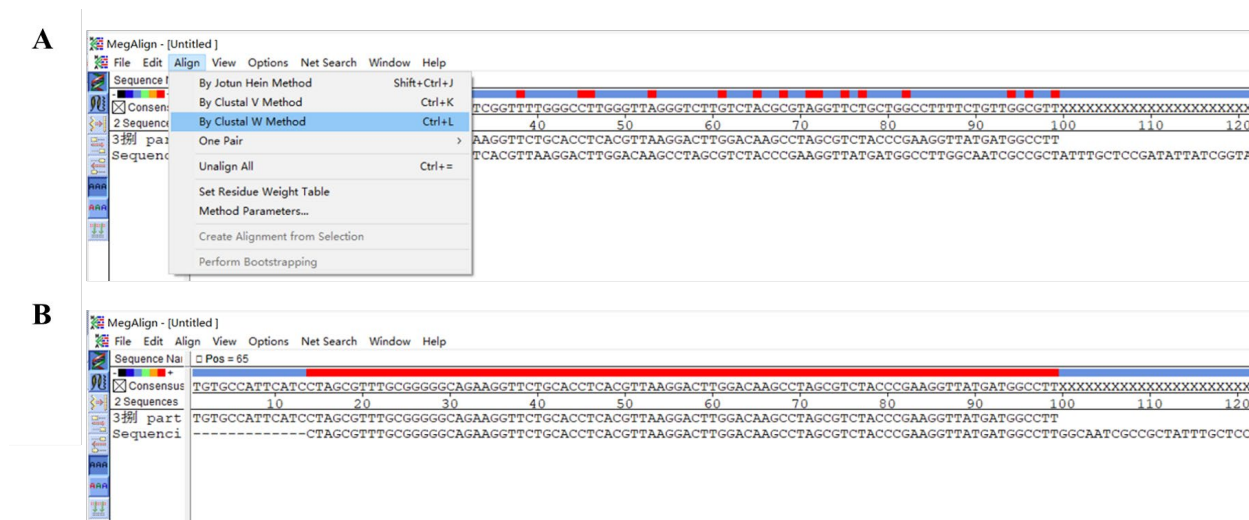


Figure 8. Screenshots exhibiting how to analyze the input sequences. (A) How to find *By Clustal W*. (B) Final outcome.

Validation of protocol

This protocol or parts of it has been used and validated in the following research article:

- Pei et al. [1]. Fusion primer driven racket PCR: A novel tool for genome walking. *Frontiers in Genetics* (Figure 6).

General notes and troubleshooting

General notes

1. FPR-PCR is a universal genome-walking protocol.
2. FPR-PCR relies on the formation of racket-like DNA mediated by SSO3.
3. Unlike other **arbitrary** PCRs that require three rounds of nested amplification, FPR-PCR only requires two amplifications because its secondary amplification is driven by an SSP pair.
4. Like other PCR genome-walking protocols, FPR-PCR also has the issue of multiple bands, but only the largest product

needs to be considered.

5. Although the walking length is unpredictable, experimental data indicate that the typical amplicon size range observed in the current FPR-PCR is 0.3–1.6 kb.

6. The band patterns of FPR-PCR are unpredictable, and there is no necessary connection between the band patterns between primary and secondary PCRs. However, secondary PCR generally produces 1–2 DNA bands.

7. Running parallel FPR-PCR sets increases the success rate and efficiency of a walking cycle.

Troubleshooting

Problem 1: No distinct amplicon(s) appear in secondary FPR-PCR.

Possible causes: (i) A weak target amplification or a strong non-target amplification occurs in primary FPR-PCR; or (ii) the SSO set may not be suitable.

Solutions: Appropriately dilute the primary FPR-PCR product, then use 1 µL of the dilution as the template of secondary FPR-PCR. If no distinct amplicon(s) appear yet, redesign an SSO set.

Problem 2: A clear DNA band(s) is the non-target product.

Possible cause: The genome has sites homologous to the SSP(s) in other region(s).

Solution: Redesign an SSP set.

Problem 3: Direct sequencing of the FPR-PCR product is difficult.

Possible cause: There exists interference from non-target background.

Solution: Clone the FPR-PCR product and then sequence.

Problem 4: Smear DNA is observed.

Solution: Reduce cycle numbers if smear DNA appears.

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Competing interests

The authors declare no competing interests.

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