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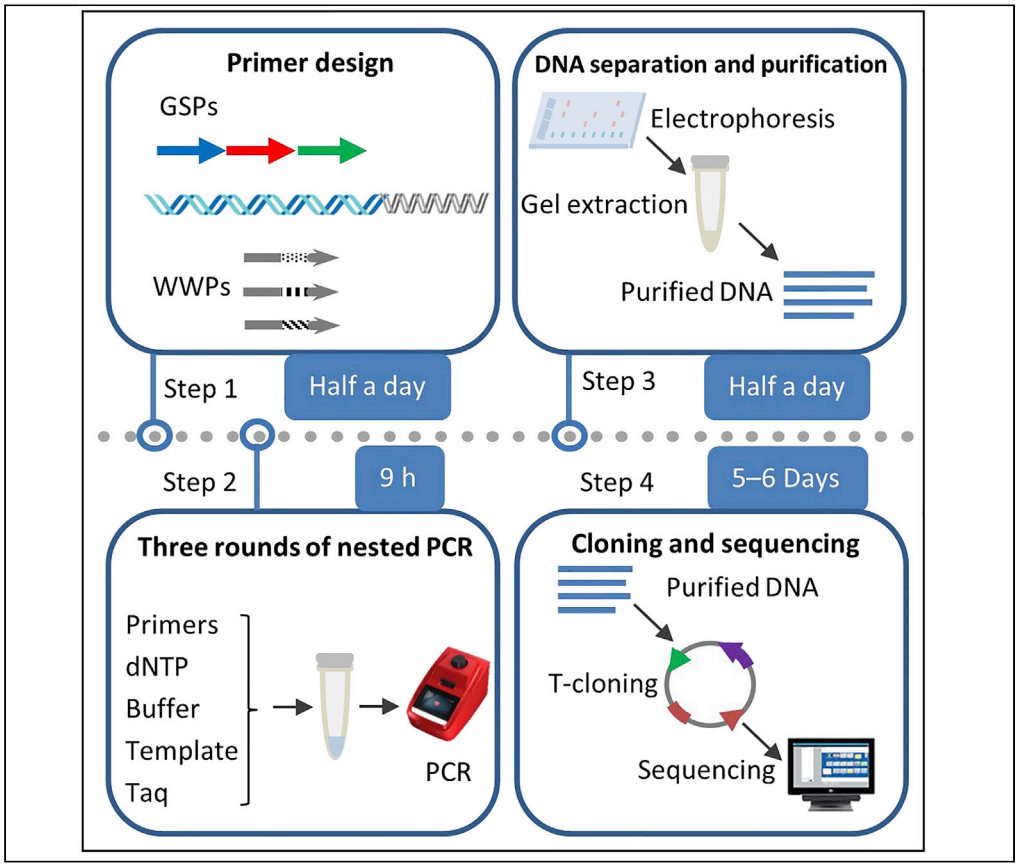
Protocol to access unknown flanking DNA sequences using Wristwatch-PCR for genome-walking



Here we describe a protocol for wristwatch PCR, an approach based on wristwatch-like structure formed between walking primers to obtain unknown flanks. We specify the criteria for designing wristwatch primers and gene-specific primers. We detail how to set wristwatch primer permutations to obtain personalized walking outcomes and improve walking efficiency. We describe experimental procedures for isolating a DNA of interest using three rounds of nested wristwatch PCR as well as the subsequent steps for DNA purification, cloning, and sequencing.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Design of a wristwatch primer set to selectively enrich target DNA

Parallel use of wristwatch primer permutations to ensure walking efficiency

Conduct three successive nested PCRs implemented for genome-walking

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Protocol to access unknown flanking DNA sequences using Wristwatch-PCR for genome-walking

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SUMMARY

Here we describe a protocol for wristwatch PCR, an approach based on wristwatch-like structure formed between walking primers to obtain unknown flanks. We specify the criteria for designing wristwatch primers and gene-specific primers. We detail how to set wristwatch primer permutations to obtain personalized walking outcomes and improve walking efficiency. We describe experimental procedures for isolating a DNA of interest using three rounds of nested wristwatch PCR as well as the subsequent steps for DNA purification, cloning, and sequencing.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2022).[1]

BEFORE YOU BEGIN

Genome-walking is a group of techniques for capturing unknown genomic regions contiguous to a targeted DNA, which is integral to molecular study of chromosomes and significant genetic elements.[2,3] As the advancement of molecular biology, genome-walking relying on PCR has been promising given its simplicity and clarity.[4,5] According to the involved rationales, the available genome-walking strategies can generally be classified into three types: 1) inverse PCR[6]; 2) cleavage-ligation-mediated PCR[7,8]; and 3) randomly primed PCR.[9–11]

The inverse PCR and cleavage-ligation-mediated PCR have been extensively utilized in acquiring flanking sequences.[12] Nevertheless, extra experimental steps, including restriction digestion and ligation, are enforced prior to PCR in these methods. The randomly primed PCRs, like thermal asymmetric interlaced PCR, partially overlapping primer-based PCR, and self-formed adapter PCR, are relatively straightforward strategies.[13–15] However, the thermal asymmetric interlaced PCR cannot overcome the background arising from random walking primers[9]; the self-formed adapter PCR is susceptible to the non-universality of walking primers[15]; and the partially overlapping primer-based PCR requires many walking primers.[10,13]

In this protocol, we describe wristwatch PCR (WW-PCR), an approach based on wristwatch-like structure formed between walking primers, to obtain unknown flanks. We devise a walking primer set of three primers having a 5′- and 3′-overlap and a middle mismatch. Any two walking primers can form a wristwatch-like structure under sufficiently low temperatures. The walking primer is thus called

Table 1. Primers used in this protocol

Primer	Oligo sequence (5′-3′)	Melting temperature (°C)	GC content (%)
WWP1	CGTCTCCAGTCTCCATGTGTTCGTC	63.5	56
WWP2	CGTCTCCAGTCTTAGGCACAGTGTC	63.4	56
WWP3	CGTCTCCAGTCTAGTCAGTCAGGTC	62.3	56
*gadA*GSP1	TCCATACCCTCATCTCCATTTCCAT	60.4	44
*gadA*GSP2	AACTATCACCCCACAACGTCATCTC	61.4	48
*gadA*GSP3	ACCGTTCATAGGCGAAATTGTTTGT	60.7	40
*hyg*GSP1	CGGCAATTTCGATGATGCAGCTTGG	64.1	52
*hyg*GSP2	CGGGACTGTCGGGCGTACACAAATC	66.2	60
*hyg*GSP3	GACCGATGGCTGTGTAGAAGTACTC	61.3	52

wristwatch primer (WWP). A WWP set selectively enriches target DNA and simultaneously excludes undesired DNAs, due to the fact that in each round of PCR, the single relaxed-stringency cycle restricts partial annealing of any primer to once only. The feasibility of WW-PCR has been verified by isolating flanks of the glutamate decarboxylase (*gadA*) locus[16–18] and hygromycin gene (*hyg*).[13]

Design of primers

Timing: half a day

This step describes how to design WWPs and gene-specific primers (GSPs).

1. Design of WWPs.
   a. All the WWPs are 25 nt in length and completely random.
   b. All the WWPs possesses identical 5′-parts (12 nt) and 3′-parts (3 nt) and mutually heterologous spacers (10 nt) (Table 1).
   c. A WWP has a high melting temperature (60°C–65°C).
   d. The four bases evenly distribute in a primer, with a 56% GC content.
   e. The annealing temperatures between the WWPs are approximate 40°C.
2. Design of GSPs.
   a. Select GSPs according to a known DNA.
   b. A GSP has a high melting temperature (60°C–65°C).
   c. The four bases evenly distribute in a primer, with a 40%–60% GC content.

Pause point: Email the designed sequences to the Sangon Biotech Co., Ltd. (Shanghai, China) for synthesis.

⚠ CRITICAL: A primer or primer pair avoids forming an obvious dimer or hairpin. A GSP has an equal melting temperature to its paired WWP.

Note: Use the software package Oligo 7 (Molecular Biology Insights, Inc., USA) to design and evaluate a primer.

Note: Heterologous spacers in WWPs are underlined.

Preparation of ready-to-use primer solution

Timing: 30 min

This step details how to prepare a ready-to-use primer solution.

3. Centrifuge a powdery primer at 4,000 × *g* for 30 s.

4. Carefully open the tube cover.
5. Add 1 × TE buffer then fully mix to prepare a 100 μM stock solution of primer.
6. Produce ready-to-use primer solution by diluting a portion of the stock ten folds with ddH_2O.
7. Divide the ready-to-use primer solution into aliquots.
8. Store the aliquots and rest stock solution at −20°C.

Note: All the primers, purified with polyacrylamide gel electrophoresis, are provided in the form of freeze-dried powder by the Sangon Biotech Co., Ltd. The concentration of a ready-to-use primer solution is 10 μM.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Levilactobacillus brevis	Gao et al.[16]	CD0817
Escherichia coli	TAKARA	DH5α
Biological samples		
Rice genomic DNA	Nanchang University	Wang et al.[1]
T-Vector pMD™19 (Simple)	TAKARA	Cat# 3271
Chemicals, peptides, and recombinant proteins		
Tris	Solarbio	Cat# T8060
Boric acid	Solarbio	Cat# B8110
Sodium hydroxide	Sinopharm	Cat# 10019762
Hydrochloric acid	Sinopharm	Cat# 10011018
EDTA	Solarbio	Cat# E8040
Agarose	Sangon	Cat# A620014
Tryptone	Angelyeast	Cat# FP318
Yeast extract	Angelyeast	Cat# FM408
Agar powder	Aobox	Cat# 01023
Sodium chloride	Sinopharm	Cat# L8290
Luria-Bertani (LB) nutrient agar	Solarbio	Cat# L8290
LB broth	Solarbio	Cat# L8291
Ampicillin	Solarbio	Cat# A8180
1 × TE buffer	Sangon	Cat# B548106
5 × TBE buffer	Sangon	Cat# B548102
0.5 M EDTA (pH 8.0)	Sangon	Cat# B540625
1 M NaOH	Yuanye	Cat# B28412
100 mg/mL ampicillin	Solarbio	Cat# A1170
dNTP (dATP, dTTP, dCTP, and dGTP)	TAKARA	Cat# RR02MQ
LA Taq polymerase (Non-hot-start version)	TAKARA	Cat# RR02MQ
10 × LA PCR buffer (Mg^{2+} plus)	TAKARA	Cat# RR02MQ
6 × Loading buffer	TAKARA	Cat# 9156
DL 5000 DNA marker	TAKARA	Cat# 3428Q
GoldView II Nuclear Staining Dyes (5000 ×)	Solarbio	Cat# G8142
Critical commercial assays		
Bacterial Genomic DNA Isolation Kit	TIANGEN	Cat# DP302-02
MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0	TAKARA	Cat# 9762
DNA Ligation Kit (Mighty Mix)	TAKARA	Cat# 6023Q
Sanprep Column Plasmid Mini-Preps Kit	Sangon	Cat #B518191
Oligonucleotides		
WWP1: CGTCTCCAGTCTCCATGTGTTCGTC	Sangon	Wang et al.[1]
WWP2: CGTCTCCAGTCTTAGGCACAGTGTC	Sangon	Wang et al.[1]
WWP3: CGTCTCCAGTCTAGTCAGTCAGGTC	Sangon	Wang et al.[1]
*gadA*GSP1: TCCATACCCTCATCTCCATTTCCAT	Sangon	Wang et al.[1]

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
*gad*AGSP2: AACTATCACCCCACAACGTCATCTC	Sangon	Wang et al.[1]
*gad*AGSP3: ACCGTTCATAGGCGAAATTGTTTGT	Sangon	Wang et al.[1]
*hyg*GSP1: CGGCAATTTCGATGATGCAGCTTGG	Sangon	Wang et al.[1]
*hyg*GSP2: CGGGACTGTCGGGCGTACACAAATC	Sangon	Wang et al.[1]
*hyg*GSP3: GACCGATGGCTGTGTAGAAGTACTC	Sangon	Wang et al.[1]
M13F: CGCCAGGGTTTTCCCAGTCACGAC	Sangon	Wang et al.[1]
M13R: CACACAGGAAACAGCTATGAC	Sangon	Wang et al.[1]
Software and algorithms		
Oligo 7	Molecular Biology Insights, Inc.	www.oligo.net
DNASTAR Lasergene	DNASTAR, Inc.	www.dnastar.com
Other		
Biometra TOne 96G Thermal Cycler	Analytikjena	4109222
ChemiDoc XRS+ Gel Imaging System	Bio-Rad	721BR12310
DYY-6C Electrophoresis Apparatus	Beijing Liuyi	112-0630

MATERIALS AND EQUIPMENT

Thermal cycler: The Biometra TOne 96G Thermal Cycler is used in this protocol.

Alternatives: Any PCR instruments can be used to complete this protocol.

1 M NaOH		
Reagent	Final concentration	Amount
NaOH	1 M	40 g
ddH_2O	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

The 1 M NaOH can be stored at 25°C–30°C for up to 3 months.

Alternatives: Purchase pre-prepared 1 M NaOH (Yuanye, Cat # B28412).

0.5 M EDTA solution (pH 8.0)		
Reagent	Final concentration	Amount
EDTA	0.5 M	18.61 g
ddH_2O	N/A	100 mL
Total	**N/A**	**100 mL**

Adjust pH to 8.0 using NaOH.
The 0.5 M EDTA solution can be stored at 25°C–30°C for up to 6 months.

Alternatives: Purchase pre-prepared 0.5 M EDTA (pH 8.0) (Sangon, Cat# B540625).

5 × TBE buffer		
Reagent	Final concentration	Amount
0.5 M EDTA solution	10 mM	20 mL
Tris	0.45 M	54 g
Boric acid	0.45 M	27.5 g
ddH_2O	N/A	980 mL
Total	**N/A**	**1,000 mL**

Adjust pH to 8.3.
The 5 × TBE buffer can be stored at 25°C–30°C for up to 3 months.

Alternatives: Purchase pre-prepared 5 × TBE buffer (Sangon, Cat# B548102).

1% agarose gel		
Reagent	Final concentration	Amount
Agarose	1%	1 g
0.5 × TBE buffer	0.5 ×	100 mL
GoldView II Nuclear Staining Dyes (5000 ×)	0.5 ×	10 μL
Total	**N/A**	**100 mL**

The 1% agarose gel can be stored at 25°C–30°C for up to 7 days.

LB medium		
Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
Yeast extract	5 g/L	5 g
NaCl	10 g/L	10 g
ddH_2O	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

Adjust pH to 7.0 using 1 M NaOH.
Autoclave the LB broth at 121°C for 20 min. LB agar plate is made by adding 10 g agar to 1 L LB broth, autoclaving at 121°C for 20 min, cooling to 50°C–60°C, pouring into 15 cm plates (15–20 mL/plate), and solidifying at 25°C–30°C. The preparation of LB/Amp agar plate is the same as that of the LB agar, except that ampicillin is added at 100 μg/mL before pouring plates.

Alternatives: Purchase pre-prepared LB broth (Solarbio, Cat# L8291) and LB nutrient agar (Solarbio, Cat# L8290).

100 mg/mL ampicillin		
Reagent	Final concentration	Amount
Ampicillin	100 mg/mL	1 g
ddH_2O	N/A	10 mL
Total	**N/A**	**10 mL**

The 100 mg/mL ampicillin can be stored at −20°C for up to 6 months.

Alternatives: Purchase pre-prepared 100 mg/mL ampicillin (Solarbio, Cat# A1170).

STEP-BY-STEP METHOD DETAILS

PCR procedures

Timing: 9 h

The WW-PCR implements retrieving an unknown DNA using the partial overlap among three WWPs. A WW-PCR comprises three consecutive nested amplification reactions. And parallel WW-PCRs involve permutations of the WWPs. This section details the principle and operations of WW-PCR.

Figure 1 presents the rationale and process of WW-PCR. For convenience and clarity, only employ the permutation WWP1-WWP2-WWP3 to illustrate WW-PCR.

1. Permutate the three WWPs into three combinations (Table 2).

Note: A WWP permutation is shown in the same column.

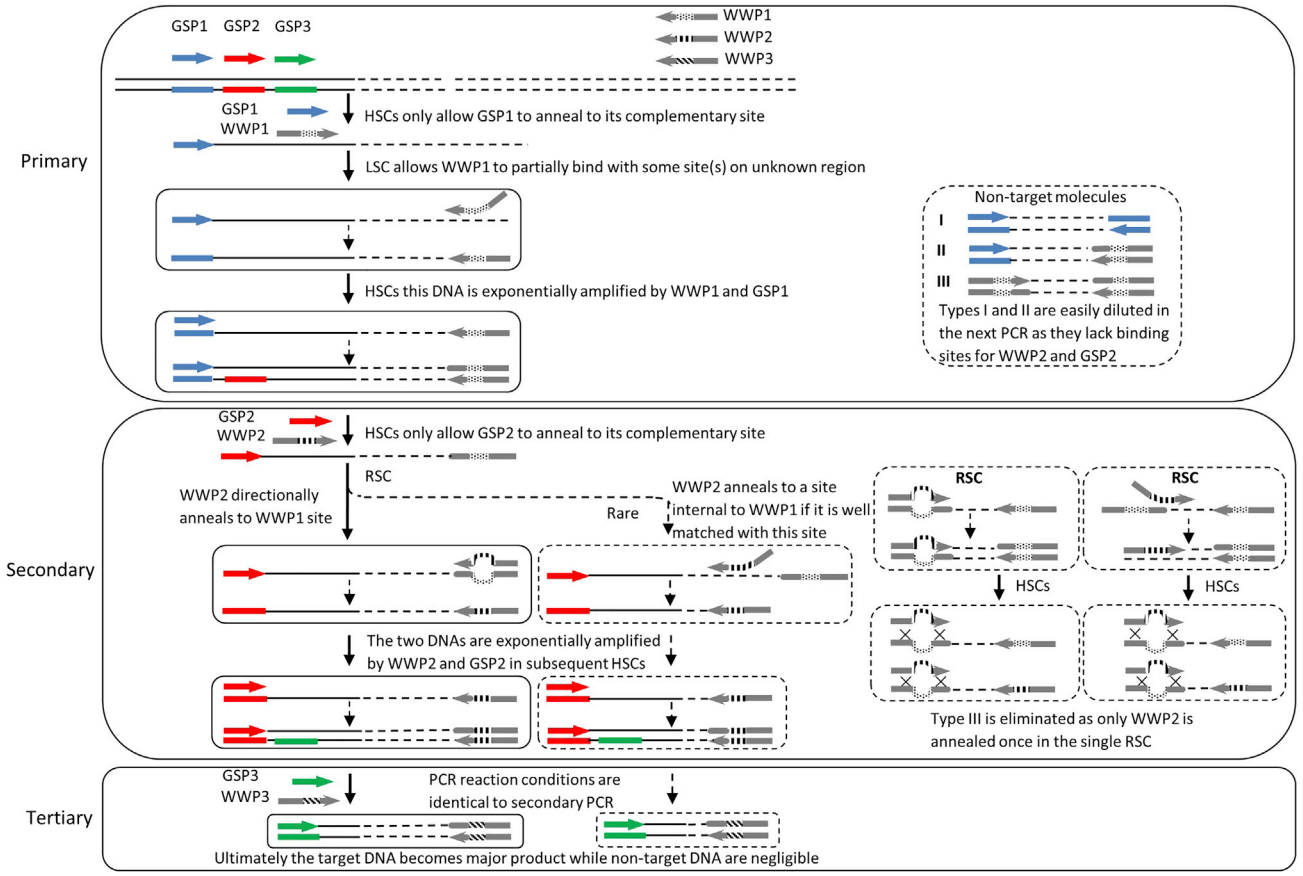

Figure 1. Overview of wristwatch PCR

GSP1, GSP2, and GSP3: gene-specific primers for primary, secondary, and tertiary PCRs, respectively; WWP1, WWP2, and WWP3: wristwatch primers for primary, secondary, and tertiary PCRs, respectively; thin solid line: known sequence; thin dotted line: unknown sequence; colorful thick line: primer complement; HSC: high-stringency cycle; LSC: low-stringency cycle; and RSC: reduced-stringency cycle. Only WWP permutation WWP1-WWP2-WWP3 is presented here to illustrate wristwatch PCR. This figure is reprinted from our previous publication.[1] Copyright of © 2022 Wang, Jia, Li, Liu, Sun, Pei, Wei, Lin and Li.

⚠ CRITICAL: Each walking experiment includes three parallel sets of WW-PCRs, performed by the three WWP permutations individually pairing with the GSP set. Each WW-PCR set consists of three successive rounds of nested PCRs, executed by the WWPs and GSP in the same row.

2. Primary WW-PCR.
   a. Assemble PCR reaction components into a 0.2 mL thin-walled PCR tube on ice.

Reagent	Final concentration	Amount
DNA template	0.2–2 ng/μL, Prokaryotic gDNA; and 2–20 ng/μL, Eukaryotic gDNA	1 μL
LA Taq polymerase	0.05 U/μL	0.5 μL
WWP1	0.2 μM	1 μL
GSP1	0.2 μM	1 μL
10 × LA PCR buffer II (Mg^{2+} plus)	1 ×	5 μL
dNTP mixture	0.4 mM each	8 μL
ddH_2O	N/A	33.5 μL
Total	**N/A**	**50 μL**

Note: Keep all the involved reagents on ice. Use a genomic DNA as template.

b. Slightly pipet 2–3 times to mix the contents.
c. Centrifuge at 4,000 × *g* for 10 s to gather the mixture at the bottom.
d. Transfer the tube to the programmed thermal cycler and run PCR.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 s	5
Annealing	65°C	30 s	
Extension	72°C	2 min	
Denaturation	94°C	30 s	1
Annealing	25°C	30 s	
Extension	72°C	2 min	
Denaturation	94°C	30 s	25
Annealing	65°C	30 s	
Extension	72°C	2 min	
Final extension	72°C	3 min	1
Hold	4°C	forever	

e. Place the reacted solution on ice.
f. Pipet 1 μL the solution as template of the secondary PCR.
g. Keep the rest solution at −20°C until agarose gel electrophoresis.

3. Secondary PCR.
a. Assemble PCR reaction components into a 0.2 mL PCR tube on ice.

Secondary PCR system

Reagent	Final concentration	Amount
Primary PCR product	N/A	1 μL
LA Taq	0.05 U/μL	0.5 μL
WWP2	0.2 μM	1 μL
GSP2	0.2 μM	1 μL
10 × LA PCR buffer II (Mg^{2+} plus)	1 ×	5 μL
dNTP mixture	0.4 mM each	8 μL
ddH_2O	N/A	33.5 μL
Total	**N/A**	**50 μL**

Note: Keep all the involved reagents on ice. Use the primary product as template.

b. Slightly pipet 2–3 times to mix the contents.
c. Centrifuge at 4,000 × *g* for 10 s to gather the mixture at the bottom.
d. Transfer the tube to the programmed thermal cycler and run PCR.

Secondary PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 s	5
Annealing	65°C	30 s	
Extension	72°C	2 min	
Denaturation	94°C	30 s	1
Annealing	40°C	30 s	
Extension	72°C	2 min	

(*Continued on next page*)

Continued			
Steps	Temperature	Time	Cycles
Denaturation	94°C	30 s	25
Annealing	65°C	30 s	
Extension	72°C	2 min	
Final extension	72°C	3 min	1
Hold	4°C	forever	

e. Place the reacted solution on ice.
f. Pipet 1 μL the solution as template of the tertiary PCR.
g. Keep the rest solution at −20°C until agarose gel electrophoresis.

4. Tertiary PCR.
   a. Assemble PCR reaction components into a 0.2 mL PCR tube on ice.

Tertiary PCR system		
Reagent	Final concentration	Amount
Secondary PCR product	N/A	1 μL
LA Taq	0.05 U/μL	0.5 μL
WWP3	0.2 μM	1 μL
GSP3	0.2 μM	1 μL
10 × LA PCR buffer II (Mg^{2+} plus)	1 ×	5 μL
dNTP mixture	0.4 mM each	8 μL
ddH_2O	N/A	33.5 μL
Total	**N/A**	**50 μL**

Note: Keep all the involved reagents on ice. Use the secondary product as template.

b. Slightly pipet 2–3 times to mix the contents.
c. Centrifuge at 4,000 × *g* for 10 s to gather the mixture at the bottom.
d. Transfer the solution to the programmed thermal cycler and run PCR.

Tertiary PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 s	5
Annealing	65°C	30 s	
Extension	72°C	2 min	
Denaturation	94°C	30 s	1
Annealing	40°C	30 s	
Extension	72°C	2 min	
Denaturation	94°C	30 s	25
Annealing	65°C	30 s	
Extension	72°C	2 min	
Final extension	72°C	3 min	1
Hold	4°C	forever	

e. Keep the solution at −20°C until agarose gel electrophoresis.

Pause point: Store the PCR samples at −20°C if the next step is not immediately followed.

Table 2. Pairing of WWP permutations with a GSP set

Round of PCR	WWP permutation			GSP primer set
Primary	WWP1	WWP2	WWP3	GSP1
Secondary	WWP2	WWP3	WWP1	GSP2
Tertiary	WWP3	WWP1	WWP2	GSP3

Agarose gel electrophoresis and DNA extraction

Timing: Half a day

This step describes how to perform agarose gel electrophoresis and the followed recovery of amplicons.

5. Agarose gel electrophoresis.
   a. Mix 5 μL PCR reaction solution with 1 μL 6 × loading buffer.
   b. Load the mixture into agarose gel well.
   c. Set the DYY-6C electrophoresis apparatus with voltage of 5 V/cm (30 cm long).
   d. Carry out electrophoresis for 30 min.
   e. Visualize DNA products by the ChemiDoc XRS+ imaging system.
6. PCR product extraction.
   a. Extract PCR product using the TAKARA agarose gel DNA extraction kit Ver. 4.0.
   b. Measure DNA concentration.

Pause point: Store the PCR samples at −20°C if the next step is not immediately followed.

T-cloning and sequencing

Timing: 5–6 days

This step describes cloning and sequencing of a recovered amplicon.

7. DNA ligation.
   a. Assemble ligation components into a 0.2 mL PCR tube on ice.

Reagent	Final concentration	Amount
T-vector pMD-19 (simple)	5 ng/μL	1 μL
Ligation mighty mix	1 ×	5 μL
Purified amplicon	1–10 ng/μL	4 μL
Total	**N/A**	**10 μL**

Note: Keep all the involved reagents on ice.

   b. Incubate at 16°C for 30 min.
8. DNA transformation.
   a. Add the ligation solution into 100 μL competent cells of *E. coli* DH5α and gently mix.
   b. Place on ice for 30 min.
   c. Heat shock for 60 s, and immediately place on ice for 90 s.
   d. Add 900 μL antibiotic-free LB broth.
   e. Incubate at 37°C and 50 rpm for 40 min.
   f. Plate 100 μL the broth onto LB agar plate containing 100 μg/mL ampicillin.
   g. Culture at 37°C for 12–16 h.

9. Validation with colony PCR.
   a. Assemble master mixture for PCRs on ice.

⚠ CRITICAL: Prepare 220 μL of the master mixture to ensure sufficient volume for 10 PCRs (20 μL/tube).

PCR reaction mixture		
Reagent	Final concentration	Amount
Transformant	N/A	N/A
LA Taq	0.05 U/μL	0.2 μL
M13 forward primer	0.2 μM	0.4 μL
M13 reverse primer	0.2 μM	0.4 μL
10 × LA PCR buffer II (Mg^{2+} plus)	1 ×	2 μL
dNTP mixture	0.4 mM each	3.2 μL
ddH_2O	N/A	13.8 μL
Total	**N/A**	**20 μL**

   b. Slightly pipet 5–6 times to fully mix the contents.
   c. Centrifuge at 4,000 × *g* for 10 s to gather the mixture at the bottom.
   d. Add 20 μL aliquots of the master mixture into 10 PCR tubes.
   e. Transfer some cells with a sterile toothpick from a bacterial colony to a PCR tube.
   f. Place the tubes in the programmed thermocycler and run PCR.

Colony PCR cycling conditions			
Steps	Temperature	Time	Cycles
Lysis of cells	95°C	5 min	1
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	30 s	30
Annealing	55°C	30 s	
Extension	72°C	2 min	
Final extension	72°C	3 min	1
Hold	4°C	forever	

   g. Detect the PCR products with agarose gel electrophoresis.
   h. Select positive transformants.

Pause point: Store the PCR samples at −20°C if the sequencing step is not immediately followed.

10. Sequencing.
   a. Inoculate a positive colony to LB broth containing 100 μg/mL ampicillin.
   b. Culture at 37°C and 200 rpm for around 18 h.
   c. Extract plasmids from the culture using the Sangon Sanprep column plasmid mini-preps kit.
   d. Sequence the inserted fragment with the M13 primers.

Pause point: Send the plasmids to the Sangon Biotech Co., Ltd. for sequencing.

11. Align and analyze the sequences.

Note: Conduct sequence alignment and analysis using the "By Cluster W Method" function of MegAlign tool in the Lasergene software.

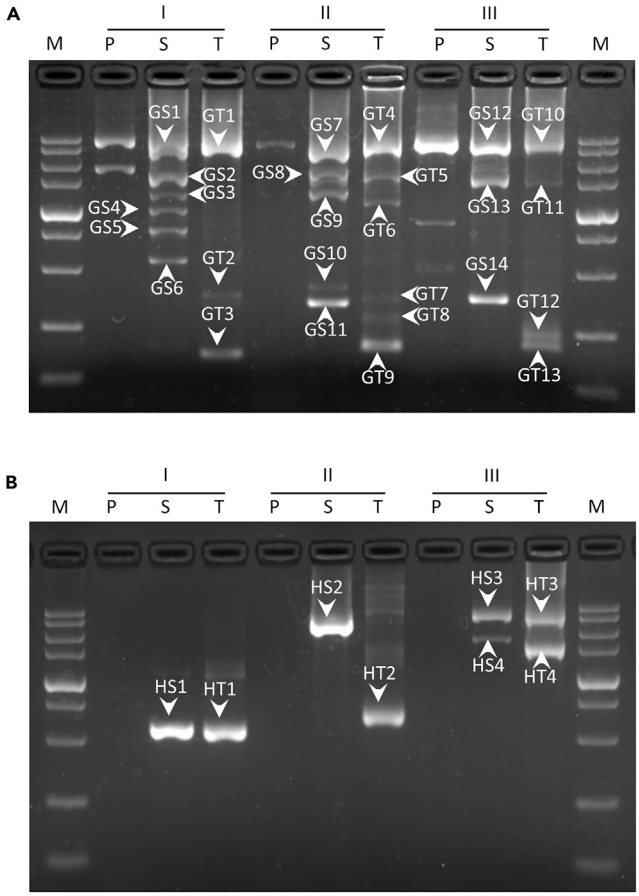

Figure 2. Validation of wristwatch PCR by walking the selected genes

Genome-walking for *gadA* of *L. brevis* CD0817 (A) and *hyg* of rice (B). I, II, and III represent three sets of WW-PCRs in a walking, individually participated by the three WWP permutations as indicated in Table 2. Lanes P, S, and T represent primary, secondary, and tertiary PCRs, respectively. Bands GS1-GS14 and GT1-GT13 indicate secondary and tertiary PCR products for *gadA*, respectively; and bands HS1-HS4 and HT1-HT4 indicate secondary and tertiary PCR products for *hyg*, respectively. M: DL 5000 DNA Marker (5000, 3000, 2000, 1500, 1000, 750, 500, 250, and 100 bp). This figure is reprinted from our previous publication.[1] Copyright of © 2022 Wang, Jia, Li, Liu, Sun, Pei, Wei, Lin and Li.

EXPECTED OUTCOMES

We herein detail the principle and experimental steps of WW-PCR, a genome-walking protocol completely based on PCR. The walking primers (WWPs) are universal to any genome. A positive outcome will generally release after two rounds of PCRs. To verify the feasibility of WW-PCR, we applied this method to isolate lateral segments of *L. brevis* CD0817 *gadA* and rice *hyg*. Each WW-PCR yielded a positive outcome, suggesting a high success rate of WW-PCR. All the major amplicons are desired products (Figure S1), meaning a high specificity of the proposed protocol. The largest product from each walking was up to 4 kb, demonstrating a high efficiency of WW-PCR. Some WW-PCRs produced multiple DNA bands, resulting from a primary WWP partially annealing to multiple sites on the unknown region in the low-stringency cycle. A DNA band in secondary PCR is slightly larger than the corresponding product in tertiary PCR (Figure 2), given the mutual position relationship between the nest GSPs.

LIMITATIONS

A possible multiple-band phenomenon challenges WW-PCR. This phenomenon is attributed to two aspects. First, the low-stringency cycle of primary PCR may permit a primary WWP to partially anneal to multiple sites on an unknown region. This partial annealing is the main origin of multiple bands. Second, in the reduced-stringency cycle of secondary/tertiary PCR, in the case that the WWP is well-matched with

some site(s) internal to the former WWP locus on the target region, the WWP may anneal to the site(s) and initiate DNA elongation (Figure 1). This internal annealing will generate a shorter target product(s). These two potential multiple-band mechanisms weaken the amplification of a major product(s).

TROUBLESHOOTING

Problem 1

Cloning of a PCR product is time-consuming and laborious (steps 7, 8, and 9).

Potential solution

The purified PCR product can also be directly sequenced.

Problem 2

In the reduced-stringency cycle of secondary/tertiary PCR, the WWP may anneal to some site(s) internal to the former WWP locus and initiate DNA elongation, producing some shorter DNA fragment(s) (Figure 1; steps 3 and 4).

Potential solution

In a genome-walking completed by WW-PCR, only the largest amplicon needs to be considered.

Problem 3

Three rounds of nested PCR are time-consuming (Figure 1; steps 2, 3, and 4).

Potential solution

In general, two rounds of nested PCRs suffice to give a positive outcome. Therefore, only two rounds of amplifications are required in an actual use of WW-PCR.

Problem 4

A WWP may partially anneal to more than one site on an unknown region in the low-stringency cycle (25°C) of primary PCR. Finally, multiple clear DNA bands appear (Figure 1; and step 2).

Potential solution

All the major bands are all correct. Only the longest band needs to be analyzed in practice.

Problem 5

What if any of three rounds of PCR steps do not work (steps 2, 3, and 4).

Potential solution

The three parallel WW-PCRs in each walking will ensure that at least one PCR gives a positive outcome. In fact, users can design n (> 3) WWPs so as to perform n parallel sets of WW-PCRs.

Problem 6

What if no *E. coli* transformants can be obtained (step 9).

Potential solution

In this case, directly sequence the purified PCR product.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Haixing Li (hxli@ncu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets presented in this study are available at GenBank: AYM03982.1 and KF206149.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.102037.

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AUTHOR CONTRIBUTIONS

Methodology, L.W.; Investigation, L.W.; Writing—Original Draft, L.W.; Investigation, M.J., T.S., C.W.; Data curation, Z.L., J.P.; Resources, X.L.; Software, Z.L.; Funding acquisition, H.L.; Conceptualization, H.L.; Writing—Review & Editing, H.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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