Chapter 13

Hyperbranching Rolling Circle Amplification, An Improved Protocol for Discriminating Between Closely Related Fungal Species

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Abstract

Hyperbranching Rolling Circle Amplification (HRCA) is a technique derived from Rolling Circle Amplification (RCA) in which DNA polymerase replicates circularized oligonucleotide probes under isothermal conditions with either linear or geometric kinetics. Since its first introduction HRCA has been proven to be a robust DNA amplification technique for detecting pathogenic fungi and other applications, allowing rapid detection of nucleic-acid sequences with high specificity. Here we describe an improved protocol of HRCA, which is both specific and sensitive for detecting low copy numbers of template DNA. The test can be performed within one working day in routine molecular labs.

Key words: Rolling circle amplification, Isothermal polymerase, Padlock probe, Ligation, Single nucleotide polymorphism, Identification

1. Introduction

Nucleic-acid amplification technology has greatly increased our ability to clarify detailed questions about genotype or transcriptional phenotype in small biological samples, and has provided the impetus for many significant advances in biology. The methods have revolutionized fungal identification, which has moved from the specialist to the routine laboratory. To the wealth of available methodologies, the use of circularizable oligonucleotides, termed "padlock probes" initially used in the detection of repeated alphoid sequences in metaphase chromosomes of humans (1), has been added. The high sequence specificity of padlock probes allowing discrimination

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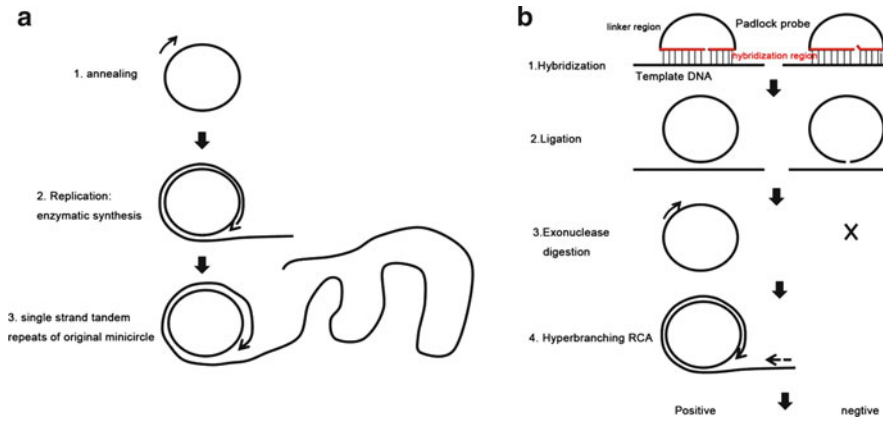

Fig. 1. Diagramatic representation of RCA (*Panel A*) and HRCA (*Panel B*) method. *Panel A*: The original RCA reaction can be divided in three major steps: (1) *mini-circle* denaturation and annealing of RCA primer, (2) Replication, (3) Single-strand tandem repeats of original *mini-circle*. During the first step the mini-circle is denatured followed by annealing of the RCA primer. Then, catalyzed by specific enzymes with strand replication activity (Bst DNA polymerase or Phi 29 DNA polymerase) the single-strand tandem replication reaction occurs. *Panel B*: The improved HRCA comprises four major steps: (1) DNA denaturation and hybridisation of Padlock probe, (2) Ligation reaction, (3) Exonuclease digestion, (4) Hyperbranching PCR reaction. In the first step the DNA is denatured and incubated overnight with Padlock probes which consist of two regions for RCA PCR primer annealing. The two ends of Padlock probe hybridize adjacent to each other on the target sequences. Only when the two ends of the probes are a perfect complement to the template DNA can hybridization occur followed by ligation during the ligation reaction (step 2). Unligated probes will be digested by Exonuclease (step 3) giving negative results. Ligated probes will be exponentially amplified during the subsequent PCR reaction (step 4) and resulting in long single-strand DNA being produced. Following this the RCA primer will be start another strand replication using the new single-strand DNA, resulting in different length double-strand DNA giving positive signals.

of point mutations in situ has prompted the generation of series of techniques such as HRCA suitable for the differentiation of molecular siblings that are increasingly being described (2).

As the original formulation of Hyperbranching Circle Amplification (HRCA) (3, 4), the Rolling Circle Amplification (RCA) reaction involves multiple rounds of DNA polymerase extending a circle-hybridized primer by continuously progressing around the circularized DNA probe isothermally (Fig. 1a). Several thousands of sequence-complementary tandem repeats of the original DNA mini-circle are yielded. RCA was normally used to generate line probes or repeat sequences of circled target sequence, while HRCA, a more complicated version of RCA, could be used to discriminate point mutation. During the HRCA reaction (Fig. 1b), specially designed padlock probes hybridize to target DNA or RNA, whereby the two ends of the probe become juxtaposed and are joined by a DNA ligase only when both ends of the probe show perfect complementarity. This permits the detection of single-nucleotide mismatches and prevents nonspecific amplification. In the presence of two primers, each hybridizing to one of the complementary strands, a complex pattern of DNA strand displacement generates over 10^9 copies of each circle in 90 min when catalyzed by a powerful

isothermal DNA polymerase (2). To date, HRCA has mainly been used for the detection of bacteria (5) and viruses (6). In fungi, the technique has been applied to a number of genera, among which are *Cryptococcus*, *Trichophyton*, *Candida*, *Aspergillus*, *Scedosporium*, *Penicillium*, and *Fonsecaea* (7–12). The technique can be used for the detection of any species of fungi if the informative site (sequence heterogeneity) can be found. Here we introduce the workflow of HRCA with a practical protocol to be performed in the routine molecular laboratory.

2. Materials

Prepare all solutions using ultrapure water (MQ H_2O, 18 MΩ cm at 25°C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Follow all waste disposal regulations carefully.

2.1. DNA Extraction

TEx-buffer: 400 mM Tris, 10 mM Na–EDTA, pH 8.5–9.0.

Proteinase K: Dissolve 10 mg Proteinase K in 1 mL ultrapure water. Prepare a 1/200 dilution, aliquot and store at –20°C.

SEVAG: Chloroform:isoamyl-alcohol (24:1).

RNAse: Dissolve 10 mg pancreatic RNAse in 1 mL 0.01 M Na-acetate, pH 5.2. Heat the solution to 100°C for 15 min. Cool slowly to room temperature. Adjust pH by adding 1 M Tris, pH 7.4. Make a 1/100 dilution, aliquot and store at –20°C.

Tris-buffer: 10 mM Tris, pH 8.

TE-buffer: 10 mM Tris, 1 mM Na–EDTA, pH 8.

10% CTAB: 10 g cetyltrimethylammoniumbromide solved in 100 ml MQ H_2O.

Glass beads (Sigma)..

Bench top centrifuge.

NanoDrop DNA concentration detector (Thermo Scientific).

3. Pre-amplification

10× PCR buffer: 80 mM Tris-SO_4, 2 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 5% Glycerol, 5% DMSO, 0.06%. 0.05% Tween-20, pH 9.2 25°C.

dNTPs: 1.0 mM dNTPs each in 20 μL reaction system.

DNA polymerase: 5 U DNA polymerase (Takara, Japan) in a 50 μL reaction.

3.1. Ligation Reaction

10× Ligation buffer: 20 mM Tris–HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% Igepal, 0.01 mM rATP, 1 mM DTT.

Isothermal DNA ligase: 2 U *pfu* DNA ligase (Stratagene, USA) in each 10 μL reaction.

3.2. Exonucleolysis

10× Exonucleolysis buffer: 670 mM glycine–KOH, 67 mM $MgCl_2$, 100 mM 2-mercaptoethanol, pH 9.5, 25°C.

Exonuclease I and III: 10 U each of exonucleases I and III (New England Biolabs) in each 20 μL reaction volume.

3.3. RCA Reaction

10× RCA amplification reaction buffer: 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0.1% Triton X-100.

Bst DNA polymerase: 4 U *Bst* DNA large fragment polymerase (New England Biolabs) in each 20 μL reaction.

DNTPs: 1.0 mM dNTPs in each 20 μL reaction.

3.4. Amplicon Detection

50× TAE buffer: 2 M Tris–acetic acid, 100 mM EDTA, pH 8.5.

6× Loading buffer: 15% Ficoll-400, 66 mM EDTA, 17.8 mM Tris–HCl (pH 8.0, 25°C), 0.102% SDS, 0.09% bromophenol blue.

1% Agarose gel: 1 g agarose in 100 mL 1×TAE buffer.

Ethidium bromide stain: 10 mg/mL ethidium bromide, use 2 μL for 100 mL 1% agarose gel.

4. Methods

Carry out all procedures at room temperature unless specified otherwise.

4.1. DNA Extraction (see Note 1)

1. Transfer approximately 0.5 g mycelium of 14-day-old cultures are transferred to a 2 mL Eppendorf tube containing 400 μL TEx and glass beads.
2. Homogenize the fungal material using a vortex for 1 min.
3. Add 120 μL SDS 10% and 10 μL Proteinase K and incubate for 30 min at 55°C.
4. Vortex the mixture for 3 min.
5. Add 120 μL 5 M NaCl and 1/10 (V/V) CTAB 10% solution and incubate for 60 min at 55°C.
6. Transfer the mixture is then vortexed for 3 min.
7. Add 700 μL SEVAG and mix carefully by hand.

8. Centrifuge for 5 min at 4°C at 20,400 × *g*.
9. Transfer the supernatant to an Eppendorf tube with 225 μL 5 M NH_4-acetate.
10. Incubated for 30 min on ice water and centrifuge for 5 min at 4°C at 20,400 × *g*.
11. Transfer the supernatant to another Eppendorf tube with 0.55 vol isopropanol and centrifuge for 5 min at 20,400 × *g* force.
12. Wash the pellet with 1,000 μL ice-cold 70% ethanol.
13. Dry the pellet at room temperature and resuspend in 48.5 μL TE-buffer.
14. Determine the DNA concentration using the nano-drop DNA concentration detector at 260 nm.

4.2. Pre-amplification

For pre-amplification, universal primers may be used. In fungi for example ITS4 and ITS5 (Table 1) are used to amplify the Internal Transcribed Spacer region of rDNA (see Note 2). PCR conditions differ depending on the target gene. The program using primers ITS4 and ITS5 primers is as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and72°C for 1 min, with final extension at 72°C for 10 min. Amplification products are detected by electrophoresis on 1% agarose gels.

4.3. Padlock Probe Design

The Padlock probe is used as template in the HRCA reaction after a successful ligation reaction. The Padlock probe comprises two parts. One is a universal linker region, the other is the hybridization region which needs to be designed by incorporating the specific DNA sequence of the target species of interest. The first step is to gather as much possible reference DNA sequences of the target gene from both the target and closely related species (in this case the ITS1 DNA sequence in target species of *Penicillium marneffei* and closely species in the genus *Penicillium*). Then, DNA sequences of candidate gene of the target species and closely species are aligned using software such as free version of Clustal X1.81 (13). The informative nucleotide polymorphisms are identified within the alignment file (Fig. 2). Padlock probes targeting the selected regions should be designed with minimum secondary structure (excluding dimers, hairpins, and mismatches) (see Note 3). The linker regions of the padlock probe should be designed to minimize cross-reaction with closely species. Specificity of the probes is confirmed by BLAST analysis in GenBank. The Padlock probe and RCA1, RCA2 primers used in this method (Table 1) are from a previously published paper targeting *Penicillium marneffei* (11).

Table 1
RCA padlock probes and padlock probe-specific primers used in this chapter

Oligonucleotides	Sequences
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG
RCA1	5′-CGCGCAGACACGATA-3′
RCA 2	5′-ATGGGCACCGAAGAAGCA-3′
PmPL1	5′-P-127AACGTCCCCCGGCGACCGG109 gatca**TGCTTC TTCGGTGCCCATT**ACGAGGTGCGGATAGCTA**CCGCGCAGACACGATA**gtcta 143CGCGGGCCCGGGGACA128-3′
PmPL2	5′-P-199GTACTCAGACAGTCCATCTT180 gatca**TGCTTCTTCGGTGCCCATT**ACGAGGTGCGGATAGCTA**CCGCGCAGACACGATA**gtcta 217TTTTGACAATTTTCATG200-3′
PmPL3	5′-P-421GGGACCACACCCAACACAC399 gatca**TGCTTCTTCGGTGCCCATT**ACGAGGTGCGGATAGCTA**CCGCGCAGACACGATA**gtcta 438TCGGGCAGGTCCCCGGA422-3′

The binding arms of the padlock probes which are joined by the backbone of the probe including the nonspecific linker region are underlined. The binding region of the RCA1 and RCA2 are in bold lettering. P: 5′ = phosphorylation

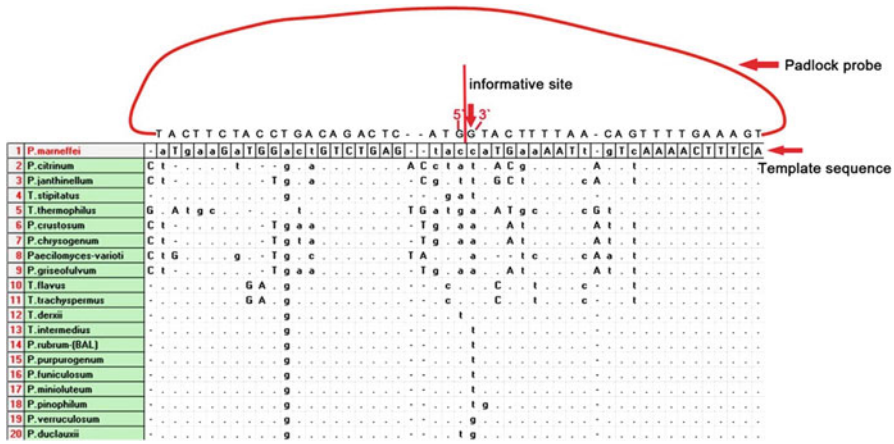

Fig. 2. Informative site detection in the ITS1 DNA sequence between *P.marneffei* and other closely fungi. The informative site, padlock probe and template DNA sequence are indicated with *arrow*. The linker region of Padlock probe is marked. The 5′ and 3′ end of Padlock probe are marked in figure.

4.4. Ligation of Padlock Probe

1. Combine 1 μL of amplified product (from Subheading 3.2) with 2 U *pfu* DNA ligase and 0.1 μM padlock probe in 10× ligation buffer final volume 10 μL.
2. Incubate 5 min at 94°C followed by 5 cycles at 94°C for 30 s and 4 min ligation at 63°C (see Note 4).

4.5. Exonucleolysis

1. Perform exonucleolysis (see Note 5) in a 20 μL reaction volume by adding 10 U of each exonuclease I and III to the ligation mixture (from Subheading 3.4).
2. Incubate at 37°C for 30 min, followed by 94°C for 3 min to inactivate the exonuclease.

4.6. HRCA Reaction

1. Perform RCA in 25 μL reaction volumes containing 0.25 μM each of RCA1 and RCA2, 4 U *Bst* DNA large fragment polymerase, 10× RCA amplification reaction buffer and 2 μL ligation product as template.
2. Incubate the reaction mixture at 65°C in a water bath for 60 min with final heating at 85°C for 2 min to terminate the reaction (see Note 6).

5. Data Analysis

1. Amplified products together with the Smart DNA ladder should be analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide (see Note 7) and photographed under UV Light (see Note 8). Positive signals are obtained

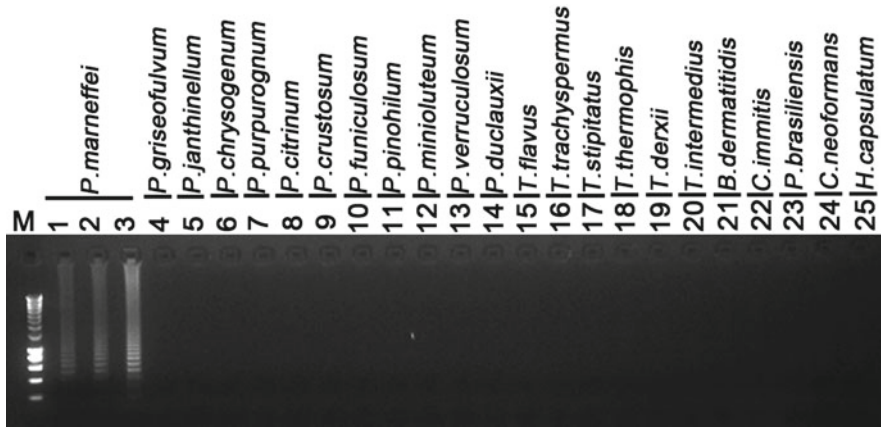

Fig. 3. RCA amplicons detected using electrophoresis on 1% agarose gel. *Lanes* 1–3 show positive samples giving a ladder pattern. *Lanes* 4–25 show no banding pattern indicating that the target sequence is not present.

when the designed padlock probes are a perfect complement to the targeted DNA sequence of *P.marneffei*, which are observed as a ladder-like pattern on a gel (Fig. 3 lane1-3). For closely related organisms there are no amplified products because the designed padlock probes are not a 100% match with the template DNA (Fig. 3 lanes 4–25). When the exonucleolysis step was omitted, unspecific bands appeared, but this did not interfere with the HRCA reaction (11).

6. Notes

1. DNA extraction protocols vary with the samples used. Here we showed the protocol frequently used for pure cultures of filamentous fungi.
2. The ITS region is widely used as target sequence for the identification of pathogenic fungi, applying universal primers ITS4 and ITS5. However, many fungi show insufficient diversity in ITS, and then hypervariable partial genes and introns are used, such as tubulin, actin, calmodulin, or translation elongation factor 1–α.
3. The T_m of the 5′ end probe binding arm is close to ligation temperature (63°C). To increase its discriminative specificity, the 3′ end binding arm was designed with a T_m 10–15°C below ligation temperature.
4. The ligation temperature may differ with each probe. A temperature gradient (58–65°C) amplification program was applied to optimize the annealing temperature using the optimized temperature, the padlock probes could hybridize with template DNA with the highest efficiency, resulting in the highest yield of HRCA products judged by the strongest signal on gel.

5. When the exonucleolysis step was omitted, unspecific background bands appeared, but this did not interfere with the RCA reaction.
6. This step is used to inactivate *Bst* DNA polymerase.
7. Can be replaced with other low-toxic compounds, such as SYBRgreen I dye.
8. DNA markers may be varied, depending on the size of the expected products.

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