Nested Patch PCR for Highly Multiplexed Amplification of Genomic Loci

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INTRODUCTION

Nested Patch polymerase chain reaction (PCR) amplifies a large number (greater than 90) of targeted loci from genomic DNA simultaneously in the same reaction. These amplified loci can then be sequenced on a second-generation sequencing machine to detect single nucleotide polymorphisms (SNPs) and mutations. The reaction is highly specific: 90% of sequencing reads match targeted loci. Nested Patch PCR can be performed on many samples in parallel, and by using sample-specific DNA barcodes, these can be pooled and sequenced in a single reaction. Thus, the Nested Patch PCR protocol that is described here provides an easy workflow to identify SNPs and mutations across many targeted loci for many samples in parallel.

RELATED INFORMATION

The Nested Patch PCR protocol (see Fig. 1 for an overview) was developed to amplify 94 loci simultaneously from human genomic DNA that was extracted from cancer patient samples. Nested Patch PCR products can be sequenced to identify SNPs and mutations using the Roche/454 Life Sciences FLX machine (Varley and Mitra 2008).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!,> and recipes for reagents marked with <R>.

Reagents

- Agarose gel (3%) prepared with MetaPhor agarose (Lonza)
- Ampligase Reaction Buffer (10X; Epicentre Biotechnologies)
- Ampligase Thermostable DNA Ligase (Epicentre Biotechnologies)
- AmpliTaq DNA polymerase (Stoffel fragment; Applied Biosystems)
- Apyrase (New England BioLabs)
- Betaine (5 M in H2O; Sigma [Fluka] BioChemika, anhydrous, ≥98.0%)
- DNA ladder for agarose gel electrophoresis
- dNTP mix (Bioline BIO-39029)
- Endonuclease VIII (New England BioLabs)
- Exonuclease I (United States Biochemical)
- Exonuclease III (Escherichia coli; Epicentre Biotechnologies)
- Forward and reverse uracil PCR primers
  
  See “Design of PCR Primers and Oligonucleotides” at beginning of Method.

<,R>Gel electrophoresis buffer
Genomic DNA
MgCl₂ (50 mM in H₂O)
Nested Patch oligonucleotides (oligos), left and right

See “Design of PCR Primers and Oligonucleotides” at beginning of Method.

<Nested Patch PCR buffer (10X)
Platinum Taq DNA polymerase (Invitrogen)
Qiaquick PCR Purification Kit (QIAGEN)

The kit provides spin columns, buffers (including buffer EB) and collection tubes.

Reagents for agarose gel electrophoresis
Tailed barcoded universal PCR primer 454A [GCCTCCCTCGGACATCAG(barcode)
CTACACGAGCTTCCGATC]
Tailed barcoded universal PCR primer 454B [GCCTTGGCAGCGCGCTCAG(barcode)
CAACGAGACGCGGATAG]
Universal primer 1 (ACACTCTTTCCCTACACGACGCTCTTCCGATC)
Universal primer 2 (5'-phosphate TCGTATGCCGTCTTCTGCTTG-3'-C3 spacer; Integrated DNA
Technologies)
Uracil-DNA glycosylase (heat-labile; USB/Affymetrix)

Equipment

Agarose gel electrophoresis equipment (Owl Separation Systems Model B1; Thermo Scientific)
Centrifuge
PCR machine/thermocycler (DNA Engine Peltier Thermal Cycler; Bio-Rad)
PCR plates (96-well, 200-µL capacity; VWR) or PCR tubes (eight-tube strips, thin-walled, 200-µL
capacity; Eppendorf)
Sequencing machine (Roche/454 Life Sciences)

Use the GS emPCR Kit II (Amplicon A, Paired End) for reads starting from 454A primer and the GS emPCR Kit
III (Amplicon B) for reads starting from 454B primer.

Spectrophotometer for DNA quantification (Nanodrop; Thermo Scientific or Qubit fluorometer;
Invitrogen)

METHOD

Design of PCR Primers and Oligonucleotides

Genomic DNA reference sequence for targeted regions, including ~150 bp of flanking sequence,
should be downloaded from the appropriate source; we often use the UCSC Genome Browser
(www.genome.ucsc.edu). Repeat sequences should be masked to Ns to avoid designing primers or
patch oligos that will anneal to repeats. We use Primer3 (http://frodo.wi.mit.edu/) to select PCR
primers pairs flanking the targeted regions. The design is constrained to PCR products between 50 and
500 bp, primer length 20-36 bp, primer melting temperature (T_m) = 61°C-67°C, the maximum differ-
ence in T_m between primer pairs of 5°C, and the GC content of the primer, which has to be between
10% and 80%. We allow Primer3 to generate up to 4000 possible primer pairs per region. Those PCR
primers pairs that end with a T as the 3' base are then selected. A Nested Patch oligo is then designed
to anneal at the end of the amplicon by extending into the sequence from the PCR primer until the
T_m of the Nested Patch oligo is 62°C-67°C. All of the oligo sequences are then aligned against them-
selves using WUBLAST BLASTN to approximate their cross-reactivity (http://blast.wustl.edu). For each
region, the oligo sets with the fewest BLASTN matches to the entire set are chosen. The forward PCR
primer sequences are substituted with a deoxyuridine in place of the 3' deoxythymidine to create the
forward uracil PCR primers. The reverse PCR primer sequences are substituted with a deoxyuridine in
place of every deoxythymidine to create the reverse uracil PCR primers. The reverse complement of
the appropriate universal primer sequences is then appended onto the end of the Nested Patch oligo
sequences. The reverse complement of universal primer 1 is appended onto the 3' of the left Nested
Patch oligo sequence, and the reverse complement of universal primer 2 is appended onto the 5'-end
of the right Nested Patch oligo sequence. The long and modified oligonucleotides can be ordered
from Sigma-Genosys. Standard desalting of synthesized oligonucleotides was performed for the
oligonucleotides used in this protocol.
Initial PCR

Requires a total of 2 h.

1. Prepare the initial PCR mix in a total of 10 µL with the following concentrations:
   
   Between $6.3 \times 10^{-15}$ M and $2.5 \times 10^{-14}$ M diploid genome equivalents (250 ng to 1 µg human genomic DNA)

   50 nM each forward PCR primer

   50 nM each reverse PCR primer

   200 µM each dNTP

   2 mM MgCl₂

   1X Nested Patch PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl)

   5 units of AmpliTaq DNA polymerase, Stoffel fragment
Prepare each reaction in thin-walled PCR tubes or plates. Suspend the genomic DNA in water, rather than TE, to avoid adding excess EDTA to this small reaction volume. Add the polymerase to the reaction mix last, just before the next step.

2. Perform PCR as follows:

<table>
<thead>
<tr>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>2 min</td>
</tr>
<tr>
<td>10</td>
<td>94°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>56°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>6 min</td>
</tr>
<tr>
<td>1</td>
<td>4°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Remove PCR Primers, Blunt PCR Products, Dephosphorylate Nucleotides

*Requires a total of 3 h.*

3. Add the following reagents directly to the PCR reactions:

- 1 unit of heat-labile uracil-DNA glycosylase
- 10 units of endonuclease VIII
- 10 units of exonuclease I

4. Incubate the reaction mix for 2 h at 37°C, followed by heat inactivation for 20 min at 95°C; hold at 4°C indefinitely.

5. Add 0.05 units of apyrase to the reaction. Incubate for 30 min at 30°C.

*This enzyme will remove the phosphates from unincorporated nucleotide in the mix to avoid further DNA polymerase extension.*

**Nested Patch Ligation of Universal Primers**

*Requires a total of 17 h; best performed overnight.*

6. Add the following reactants to the tube to yield the indicated concentrations in a total volume of 25 µL:

- 20 nM each Nested Patch oligonucleotide
- 40 nM universal primer 1
- 40 nM universal primer 2
- 1X Ampligase Reaction Buffer
- 5 units Ampligase (add last to reaction mix, just before next step)

7. Perform the following cycling program:

<table>
<thead>
<tr>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95°C</td>
<td>15 min</td>
</tr>
<tr>
<td>100</td>
<td>94°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>65°C</td>
<td>2 min</td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>5 min</td>
</tr>
<tr>
<td>1</td>
<td>4°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

**Exonuclease Degradation of Unselected DNA, Reaction Cleanup**

*Requires a total of 3 h.*

8. Add 10 units of exonuclease I and 200 units of exonuclease III to each reaction.

9. Incubate for 2 h at 37°C followed by heat inactivation for 20 min at 95°C; hold at 4°C indefinitely.

10. Purify selected DNA using the Qiaquick PCR Purification Kit with spin columns according to the manufacturer's instructions. In the final elution step, add 30 µL of EB to the column, let the column stand for 1 min, and then centrifuge to elute.
Universal PCR and Gel Electrophoresis of Products

Requires a total of 5 h.

11. Add the following PCR mix components to the 30 µL of eluted DNA from Step 10 to yield the indicated concentrations in a total volume of 50 µL:
   - 1X Nested Patch PCR Buffer
   - 0.5 µM 454A tailed barcoded universal PCR primer
   - 0.5 µM 454B tailed barcoded universal PCR primer
   - 0.5 mM each dNTP
   - 2 mM MgCl₂
   - 0.5 M betaine
   - 10 units Platinum Taq Polymerase (add last to reaction mix, just before next step)

12. Perform the following cycling program:

<table>
<thead>
<tr>
<th>No. of cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>93°C</td>
<td>2 min</td>
</tr>
<tr>
<td>27</td>
<td>93°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>6 min</td>
</tr>
<tr>
<td>1</td>
<td>4°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

13. Confirm the amplification of products in the expected size range by running 20 µL of PCR product from Step 12 in a 3% MetaPhor agarose gel with an appropriate DNA ladder. See Troubleshooting.

14. Purify the remaining 30 µL of PCR product using the Qiaquick PCR Purification Kit with spin columns according to the manufacturer’s instructions. Quantify the concentration of PCR products using a spectrophotometer.

   The PCR products are now ready for further enzymatic reactions.

15. Pool together equimolar quantities of the PCR products from different samples and prepare for sequencing on the Roche/454 Life Sciences sequencing machines following the manufacturer’s instructions. To obtain sequence reads starting from 454A primer, choose the GS emPCR Kit II (Amplicon A, Paired End), and for reads starting from 454B primer, choose the GS emPCR Kit III (Amplicon B).

   See Troubleshooting.

TROUBLESHOOTING

Problem: Primer-dimer (low-molecular-weight PCR product) is formed during universal PCR. [Step 13]

Solution: This product usually forms when low amounts of genomic DNA template are used or it is of poor quality. Consider the following:

1. Increase the amount of template genomic DNA in the initial PCR.

2. Perform a Qiaquick gel extraction (QIAGEN) to separate the smear of expected products from the primer-dimer before sequencing.

Problem: A targeted locus is missing from the sequencing. [Step 15]

Solution: Consider the following:

1. If the locus is present but amplified with poor efficiency, deeper sequencing (more coverage) may be required to observe it. Several changes to the protocol may improve the uniformity of amplification. Try the following:
DISCUSSION

Nested Patch PCR amplifies many genomic loci in a single reaction and provides an easy workflow for the analysis of many samples in 96- or 384-well format. The use of DNA barcodes allows the PCR products from many samples to be sequenced in the same reaction. This method enables the use of second-generation sequencing machines to detect SNPs and mutations in many candidate loci across many samples.

Increased numbers of primers in the exponential amplification step of a PCR lead to increased interprimer interactions that inhibit uniform amplification (Han et al. 2006) and increased formation of mispriming products (Fan et al. 2006; Chun et al. 2007). These issues have hampered efforts to perform high levels of multiplexing (greater than 30 loci) during PCR amplification. Nested Patch PCR was designed to reduce these two problems. The initial PCR that contains many different primer pairs (Fig. 1A) is performed for a low number of cycles (three to 10) to define the ends of the targeted loci. Performing a low number of cycles minimizes the amplification bias that is caused by primer inefficiency or interprimer interactions. This feature provides improved product uniformity for highly multiplexed PCR. Mispriming products that form during the initial PCR are excluded from the final pool of products. They are not targeted by patch oligos for the ligation of exonuclease-resistant universal primers. This allows mispriming products to be degraded by exonuclease before the final high-cycle number PCR. Only targeted loci are amplified during this final PCR because it is performed with the single pair of universal primers. This provides the high specificity obtained by Nested Patch PCR (90% of sequencing reads map to targeted loci) (Varley and Mitra 2008).

In addition to Nested Patch PCR, several other methods have recently been developed for the highly multiplexed selection, amplification, and sequencing of genomics subsets (Bashiardes et al. 2005; Dahl et al. 2005, 2007; Albert et al. 2007; Fredriksson et al. 2007; Hodges et al. 2007; Meuzelaar et al. 2007; Okou et al. 2007; Porreca et al. 2007). When compared to these other methods Nested Patch PCR is well-suited for the amplification of an intermediate number (100-1000) of targeted regions across a large number of samples. Several features distinguish it for this application, including high specificity (~90% of sequencing reads match targeted loci), precise definition of the target boundaries with PCR primers, reproducible amplification efficiently at each locus, and a simple workflow that is compatible with 96-well plates and sample-specific DNA barcodes.

The upper limit on the number of loci that can be amplified simultaneously in the same reaction has not been determined, but the amplification of 90 exons in a single reaction has been demonstrated (Varley and Mitra 2008). The cost of oligos (four oligos per target) becomes a consideration when attempting to amplify very large numbers of loci. A standard 25-nmol oligo synthesis of each primer is enough for 50,000 Nested Patch reactions. So, for the amplification of common sets of candidate regions, the upfront cost of synthesizing oligos by standard methods may be economical when amortized over the thousands of reactions that can be performed from one standard synthesis.

One limitation of the method is the non-uniform amplification of the targeted loci. The relative abundance of the different exons after amplification could likely be improved in several ways, including limiting the number of cycles in the initial PCR (Step 1), including betaine or other additives to improve the initial PCR, iteratively optimizing primer pairs that perform poorly, or partitioning similarly abundant targets into separate reactions.

We anticipate that Nested Patch PCR will be useful for a variety of applications that involve the detection of germline SNPs or somatic mutations at candidate genomic loci in model organisms or in human samples. For example, the Nested Patch PCR can be used to sequence germline SNPs in case
versus control studies of disease, to detect somatic mutations in malignant versus matched normal tissue (Varley and Mitra 2008), or to survey genetic diversity among populations.

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REFERENCES


