Inverse PCR and Sequencing Protocol on 5 Fly Preps

For recovery of sequences flanking XP elements
This protocol is an adaptation of

"Inverse PCR and Cycle Sequencing Protocols" by E. Jay Rehm
Berkeley Drosophila Genome Project
And
"Single-Fly DNA Preps for PCR" by Greg Gloor and William Engels
Dept. of Genetics, University of Wisconsin
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South San Francisco, CA 94083

To prep the DNA for use with this protocol use the “5 Fly Drosophila Genomic Prep for iPCR in 96-well Format” protocol.

I. Things to keep in mind before you start this protocol

- Read the whole protocol before you start to make sure each step is clear.
- Ensure that you have all reagents and primers before you start.
- Keep all reactions on ice until they go into incubators or tetrads. Use metal 96-well plate holders that have been cooled to 4°C for best results. Place the plate in the metal holder, which is sitting on the ice for the duration of the setup. Use these to keep the DNA/donor reaction cool during reaction setup.
- Always add enzyme last to reagent mixtures. Do this just before you are ready to add the DNA or aliquot from the previous step which then starts the reaction.
- After reagent mixture has been added to plate wells, be sure to quick spin the covered plate to pull all liquid to bottom of plate.
- The polymerase chain reaction (PCR) is covered by patents owned by Hoffman-La Roche, Inc. and F. Hoffman-La Roche Ltd. Users should obtain a license to perform the reaction.
- AT ALL STEPS USE AEROSOL TIPS
## II. Reagents

This protocol optimized with the following reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Manufacturer</th>
<th>Catalog</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate</td>
<td>Axygen</td>
<td>PCR-96-HS-C</td>
</tr>
<tr>
<td>Tape Pad</td>
<td>Qiagen</td>
<td>19570</td>
</tr>
<tr>
<td>Adhesive PCR film</td>
<td>Abgene</td>
<td>AB-0558</td>
</tr>
<tr>
<td>Aluminum Sealing Film</td>
<td>Axygen</td>
<td>47734-816</td>
</tr>
<tr>
<td>Sau3A I enzyme</td>
<td>New England Biolabs</td>
<td>R0169</td>
</tr>
<tr>
<td>HinP1 I enzyme</td>
<td>New England Biolabs</td>
<td>R0124</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>New England Biolabs</td>
<td>M0202</td>
</tr>
<tr>
<td>Tetrad thermal cycler</td>
<td>MJ Research</td>
<td>PTC-225</td>
</tr>
<tr>
<td>AmpliTaq DNA polymerase</td>
<td>Perkin Elmer</td>
<td>E09425</td>
</tr>
<tr>
<td>ExoSAP-IT kit</td>
<td>USB Corp.</td>
<td>78200</td>
</tr>
<tr>
<td>96-well working rack</td>
<td>Stratagene</td>
<td>410094</td>
</tr>
<tr>
<td>BigDye</td>
<td>Applied Biosystems</td>
<td>4331186</td>
</tr>
<tr>
<td>ABI Prism 3700</td>
<td>Applied Bioystems</td>
<td>4308058</td>
</tr>
<tr>
<td>70% ethanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>multichannel pipet, aerosol tips, centrifuge, vortex</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
III. Digestions (Sau3A I and HinP1 I done separately)

**Sau3A I digests are for 5’ and 3’ iPCR**
**HinP1 I digests are for 3’ iPCR only**

Protocol per reaction is as follows:
20 µl reactions done in 96-well plate

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA (~0.5 fly)</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>10X buffer (NEB Sau3AI or NEB 2)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>10X BSA (Sau3AI only)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Sau3AI or HinP1</td>
<td>4 units Sau3AI or 5 units HinP1</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>add to 20 µl total</td>
</tr>
</tbody>
</table>

1) Cover plate with Adhesive PCR film.
2) Incubate @ 37°C for 1 hr in MJ Tetradyne.
3) Incubate @ 65°C for 20 min. to heat inactivate.
4) Briefly centrifuge plate to spin down condensation.
5) Remove film to aliquot for ligations.
6) For storage @ -80°C, use Aluminum Sealing Film. Apply sheet to plate and incubate again @ 65°C for 15 min to seal plate. Briefly centrifuge plate to spin down condensation. Store @ -80°C.

IV. Ligations

Protocol per reaction is as follows:
10.5 µl reactions done in 96-well plate

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested genomic DNA (~0.075 fly)</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>NEB 10X T4 DNA Ligase Buffer (w/ 10mM ATP)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>6.0 µl</td>
</tr>
<tr>
<td>NEB T4 DNA Ligase (200 Weiss units)</td>
<td>0.5 µl</td>
</tr>
</tbody>
</table>

1) If doing PCR immediately following ligation;
   Incubate @ Room Temp for 30 min (cover plate with Tape Pad)
   Remove Tape Pad and aliquot to 1st round PCR.
   For storage @ -80°C, use Aluminum Sealing Film. Apply film to plate and incubate @ 65°C for 15 min to seal plate. Briefly centrifuge plate to spin down condensation, store @ -80°C.
2) If not doing PCR immediately following ligation;
   Apply Aluminum Sealing Film to plate and incubate @ Room Temp for 30 min, then incubate @ 65°C for 15 min to seal plate. Briefly centrifuge plate to spin down condensation, store @ -80°C.
V. iPCR

PCR to be done in 96-well plates covered with Adhesive PCR film.

1\textsuperscript{st} round iPCR: 20.0 µl reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligated genomic DNA (~0.035 fly)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>10X dNTP (2mM each)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>forward primer (10 µM)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>reverse primer (10 µM)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>10X PE AmpliTaq buffer w/ 15mM MgCl\textsubscript{2}</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>ddH\textsubscript{2}O</td>
<td>9.9 µl</td>
</tr>
<tr>
<td>PE AmpliTaq (2 units)</td>
<td>0.3 µl</td>
</tr>
</tbody>
</table>

**XP iPCR**

1) 95°C 5 min  
2) 95°C 30 sec  
3) 60°C 1 min  
4) 72°C 2 min  
5) GOTO 2 x40  
6) 72°C 10 min  
7) 12°C hold

1) Cycle on MJ Tetrad using “XP iPCR” program with heated lid.  
2) Centrifuge briefly to spin down condensation.  
3) Do 1:10 dilution of 1\textsuperscript{st} Round PCR by adding 180 µl H\textsubscript{2}O.

2\textsuperscript{nd} round iPCR: 20.0 µl reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 diluted 1\textsuperscript{st} round DNA</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>10X dNTP (2mM each)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>forward primer (10 µM)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>reverse primer (10 µM)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>10X PE AmpliTaq buffer w/ 15mM MgCl\textsubscript{2}</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>ddH\textsubscript{2}O</td>
<td>9.9 µl</td>
</tr>
<tr>
<td>PE AmpliTaq (2 units)</td>
<td>0.3 µl</td>
</tr>
</tbody>
</table>

4) Cycle on MJ Tetrad using “XP iPCR” program (~3hr run) with heated lid.  
5) Optional: Examine 5 µl of the 3\textsuperscript{rd} 2\textsuperscript{nd} round and 5\textsuperscript{th} 2\textsuperscript{nd} round iPCRs on 1.0% agarose gel.
### Primers for 1st and 2nd round iPCR:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>PCR Round</th>
<th>XP-element end</th>
<th>Primer Sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>51A</td>
<td>1st</td>
<td>5' end</td>
<td>5'-AAT GAT TCG CAG TGG AAG GCT-3'</td>
</tr>
<tr>
<td>51B</td>
<td>1st</td>
<td>5' end</td>
<td>5'-CAC CCA AGG CTC TGC TCC CAC AAT-3'</td>
</tr>
<tr>
<td>52A</td>
<td>2nd</td>
<td>5' end</td>
<td>5'-TAC CAG TGG GAG TAC ACA AAC-3'</td>
</tr>
<tr>
<td>52B</td>
<td>2nd</td>
<td>5' end</td>
<td>5'-TTT ACT CCA GTC ACA GCT TTG-3'</td>
</tr>
<tr>
<td>31A</td>
<td>1st &amp; 2nd</td>
<td>3' end</td>
<td>5'-CGA CAC TCA GAA TAC TAT TCC-3'</td>
</tr>
<tr>
<td>31B</td>
<td>1st &amp; 2nd</td>
<td>3' end</td>
<td>5'-AAT TTG CGA GTA CGA AAA GC-3'</td>
</tr>
</tbody>
</table>

### VI. Pre-Sequencing Preparation

Strong and unique bands as well as smears from the iPCRs can be directly sequenced without extensive purification. Prior to sequencing, use the USB ExoSAP-IT kit to clean up an aliquot of the 2nd round reactions. This kit uses Exonuclease I (degrades primers) and Shrimp Alkaline Phosphatase (degrades unincorporated nucleotides) to prepare the template for sequencing.

The protocol per reaction is as follows:

**ExoSAP protocol**

Done in 96-well plates covered with Adhesive PCR film

Make a master mix per reaction of:

<table>
<thead>
<tr>
<th>Exonuclease I (10U/µl)</th>
<th>1 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shrimp Alkaline Phosphatase (2U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>3 µl</td>
</tr>
</tbody>
</table>

1) Remove 5 µl 2nd Rd iPCR and add to 5 µl SAP mix (to make 10 µl total).
2) Run on “SAP” program on tetrad using heated lid.

**SAP**

1) 37°C 30min.
2) 85°C 15min.
3) 4°C hold

3) Do not hold @ 4°C overnight. The SAP prep should be done on the day that the sequencing reactions are to be done.
VII. Cycle Sequencing Protocol for 3700 ABI Machine

The protocol per reaction is as follows
10 µl reaction done in 96-well plate

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (1 µl from 10 µl SAP prep)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Primer (0.8 µM)</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>5X BigDye buffer</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>ABI BigDye (v3.0) Mix</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>2.5 µl</td>
</tr>
</tbody>
</table>

1) Cycle Sequence (~2.5 hours)

**BigDye**
1) 96°C 4min
2) 96°C 30sec
3) 50°C 15sec
4) 60°C 4min
5) GOTO 2 X24
6) 12°C hold

2) To purify reactions add 75µl 70% ethanol, cover, let stand 30 minutes at room temp in the dark. Centrifuge for 30 minutes @ 2,470 RCF. Remove cover, invert on paper towel and spin @ 700 RCF for 1 min.

3) Register plate in the LIMS for runs on ABI 3700 machines.

Sequencing Primers:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>XP-element End</th>
<th>Sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP-5SEQ</td>
<td>5' end</td>
<td>5'-ACA CAA CCT TTC CTC TCA ACA A-3'</td>
</tr>
<tr>
<td>XP-3SEQ</td>
<td>3'end</td>
<td>5'-TAC TAT TCC TTT CAC TCG CAC TTA TTG-3'</td>
</tr>
</tbody>
</table>
Figures:

**XP 5' end**

```
XP 5' end
10 20 30 40 50
CATGATGAAATAACATAGGTTGGTCGCACTATACGCAAGCTTACCAGA
GTACTACCTTATGTATTTCCACCAGGGGAAGTCATCGGCTTCGAATGGCTT
60 70 80 90 100
GTATACATCTCTAAATTCATAGCGACCTTTGGTTGGAGAAAGTGGTTG
CATATGTGAATTAAAGTCACGTGCAAACGAACAACTCTCCTTTCCAACT

XP-5SEQ
110 120 130 140 150
TGCGGACGAATTTTTTTTTTTTTAATACATTACCCTTACGTGGAATAAAAA
ACGCTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
160 170 180 190 200
AAATGAATAATGGCAAATTTGGCTGAAAGCTGAGTGAATTTGTTGGGAGCAGATC
TAAGTGACACCTTACACGATAATTCTCTT
```

**Sau3AI**

```
Sau3AI
510 520
GTCTCTCCGGGCTCGAAGATC
CAGGAGGGCCGGACGTCTCTAG
```

**5'B**

```
5'B
310 320 330 340 350
ATTCCGAGTGGAAGGCT
TCACCTGCAAAAGGTCAGACATTTAAAAGGAGG
TAAGCGTCACCTTCCGACGTGGACGTTTTCCAGTCTGTAAATTTTCCTCC
360 370 380 390 400
CGACTCAACGGCAGATGCTGACCTAGTTAGTAAGTGAAGTACTGAACCCAGA
GCTGAGTTGCGGCTTCTACGGCATTCTGACATTTTAATCAGCTCTGGAATCG
410 420 430 440 450
AAAGATAAAAGAAGGCTAT
ACCAGTGGGAGTACACAAACAGAGTAAGTTT
TTTCTATTTTTCTCCGATAATGTCCACCTCATTGTGCTATTCAA
460 470 480 490 500
GAATAGTAAAAAAAAATCAATTTATGTAACAAATTACGTCATGCTGCTTAG
CTTATCATTTTTTTTAGTAAAATAACATTTTTTATTGGCAGCTACGGCAATC
```

**5'A**

```
5'A
310 320 330 340 350
ATTCCGAGTGGAAGGCT
GCACCTGCAAAAAGGTCAGACATTTAAAAGGAGG
TAAGCGTCACCTTCCGACGTGGACGTTTTCCAGTCTGTAAATTTTCCTCC
360 370 380 390 400
CGACTCAACGGCAGATGCTGACCTAGTTAGTAAGTGAAGTACTGAACCCAGA
GCTGAGTTGCGGCTTCTACGGCATTCTGACATTTTAATCAGCTCTGGAATCG
410 420 430 440 450
AAAGATAAAAGAAGGCTAT
ACCAGTGGGAGTACACAAACAGAGTAAGTTT
TTTCTATTTTTCTCCGATAATGTCCACCTCATTGTGCTATTCAA
460 470 480 490 500
GAATAGTAAAAAAAAATCAATTTATGTAACAAATTACGTCATGCTGCTTAG
CTTATCATTTTTTTTAGTAAAATAACATTTTTTATTGGCAGCTACGGCAATC
```

**52A**

```
52A
410 420 430 440 450
AAAGATAAAAGAAGGCTAT
ACCAGTGGGAGTACACAAACAGAGTAAGTTT
TTTCTATTTTTCTCCGATAATGTCCACCTCATTGTGCTATTCAA
460 470 480 490 500
GAATAGTAAAAAAAAATCAATTTATGTAACAAATTACGTCATGCTGCTTAG
CTTATCATTTTTTTTAGTAAAATAACATTTTTTATTGGCAGCTACGGCAATC
```

**51B**

```
51B
210 220 230 240 250
ATTACGTCGGCGAAGTGGTCTATTAAGAGAAATTTGTGGGAGCAGAGCCT
TAAGTGACACCTTACACGATAATTCTCTT
```

**52B**

```
52B
110 120 130 140 150
TGCAGACCAGGTTTTTTTTTTTTAATACATTACCCTTACGTGGAATAAAAA
ACGCTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
160 170 180 190 200
AAATGAATAATGGCAAATTTGGCTGAAAGCTGAGTGAATTTGTTGGGAGCAGATC
TAAGTGACACCTTACACGATAATTCTCTT
```

**51A**

```
51A
260 270 280 290 300
TGGGTGAGCGCTTTGTTGGAAGAATTTTTTGATACCCACTTTTAAATCC
AGCCAGTGGGAAACCACCTTTTTTGGGTTTAAACACTATGGGTGAAATTC
```

**XP 5' end**

```
XP 5' end

10 20 30 40 50
CATGATGAAATAACATAGGTTGGTCGCACTATACGCAAGCTTACCAGA
GTACTACCTTATGTATTTCCACCAGGGGAAGTCATCGGCTTCGAATGGCTT
60 70 80 90 100
GTATACATCTCTAAATTCATAGCGACCTTTGGTTGGAGAAAGTGGTTG
CATATGTGAATTAAAGTCACGTGCAAACGAACAACTCTCCTTTCCAACT

XP-5SEQ

110 120 130 140 150
TGCGGACGAATTTTTTTTTTTTTAATACATTACCCTTACGTGGAATAAAAA
ACGCTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
160 170 180 190 200
AAATGAATAATGGCAAATTTGGCTGAAAGCTGAGTGAATTTGTTGGGAGCAGATC
TAAGTGACACCTTACACGATAATTCTCTT
```

**Sau3AI**

```
Sau3AI

510 520
GTCTCTCCGGGCTCGAAGATC
CAGGAGGGCCGGACGTCTCTAG
```
XP 3' end

HinP1I

GCGTTCG
CGGGAAGC

Sau3AI

TCTACGGAGCGACATTCTGAAATTCAATCGACATGAGCAAGCGTAAT
AGATGCTTCGTGCTTAAAGTTAGTCTGTTTCTTCTTGACTGACTTGA

XP 3SEQ

A CGACACTCAGAA TACTATTCC TTTCACTCGCACTTATTG CAAGCATACG
TGCTGTGAGTCTTATGATAAGGAAAGTGAGCGTGAATAACGTTCGTATGC

XP 3' end