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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To prep the DNA for use with this protocol use the "5 Fly *Drosphila* 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.

Reagent	Manufacturer	Catalog
96-well plate	Axygen	PCR-96-HS-C
Tape Pad	Qiagen	19570
Adhesive PCR film	Abgene	AB-0558
Aluminum Sealing Film	Axygen	47734-816
*Sau*3A I enzyme	New England Biolabs	R0169
*Hin*P1 I enzyme	New England Biolabs	R0124
T4 DNA Ligase	New England Biolabs	M0202
Tetrad thermal cycler	MJ Research	PTC-225
AmpliTaq DNA polymerase	Perkin Elmer	E09425
ExoSAP-IT kit	USB Corp.	78200
96-well working rack	Stratagene	410094
BigDye	Applied Biosystems	4331186
ABI Prism 3700	Applied Bioystems	4308058
70% ethanol		
multichannel pipet, aerosol tips, centrifuge, vortex		

III. Digestions (*Sau*3A I and *Hin*P1 I done separately)

_Sau_3A I digests are for 5' and 3' iPCR
_Hin_P1 I digests are for 3' iPCR only

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

Genomic DNA (~0.5 fly)	10.0 µl
10X buffer (NEB Sau3AI or NEB 2)	2.0 µl
10X BSA (Sau3AI only)	2.0 µl
Sau3AI or HinPI	4 units Sau3A1 or 5 units HinP1
ddH_2O	add to 20 µl total

1) Cover plate with Adhesive PCR film.
2) Incubate @ 37°C for 1 hr in MJ Tetrad.
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

Digested genomic DNA (~0.075 fly)	3.0 µl
NEB 10X T4 DNA Ligase Buffer (w/ 10mM ATP)	1.0 µl
ddH_2O	6.0 µl
NEB T4 DNA Ligase (200 Weiss units)	0.5 µl

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.

1st round iPCR: 20.0 µl reaction

Ligated genomic DNA (~0.035 fly)	5.0 µl
10X dNTP (2mM each)	2.0 µl
forward primer (10 µM)	0.4 µl
reverse primer (10 µM)	0.4 µl
10X PE AmpliTaq buffer w/ 15mM $MgCl_2$	2.0 µl
ddH_2O	9.9 µl
PE AmpliTaq (2 units)	0.3 µl

XP iPCR

1) 95°C 5 min
2) 95°C 30 sec
3) 60°C 1min
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 1st Round PCR by adding 180 µl H_2O.

2nd round iPCR: 20.0 µl reaction.

1:10 diluted 1st round DNA	5.0 µl
10X dNTP (2mM each)	2.0 µl
forward primer (10 µM)	0.4 µl
reverse primer (10 µM)	0.4 µl
10X PE AmpliTaq buffer w/ 15mM $MgCl_2$	2.0 µl
ddH_2O	9.9 µl
PE AmpliTaq (2 units)	0.3 µl

4) Cycle on MJ Tetrad using **"XP iPCR"** program (~3hr run) with heated lid.
5) Optional: Examine 5 µl of the 3' 2nd round and 5' 2nd round iPCRs on 1.0% agarose gel.

Primers for 1st and 2nd round iPCR:

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

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:

Exonuclease I (10U/µl)	1 µl
Shrimp Alkaline Phosphatase (2U/µl)	1 µl
ddH_2O	3 µl

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° 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

DNA (1 µl from 10 µl SAP prep)	1.0 µl
Primer (0.8 µM)	4.0 µl
5X BigDye buffer	1.5 µl
ABI BigDye (v3.0) Mix	1.0 µl
ddH_2O	2.5 µl

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
7) 12°C hold

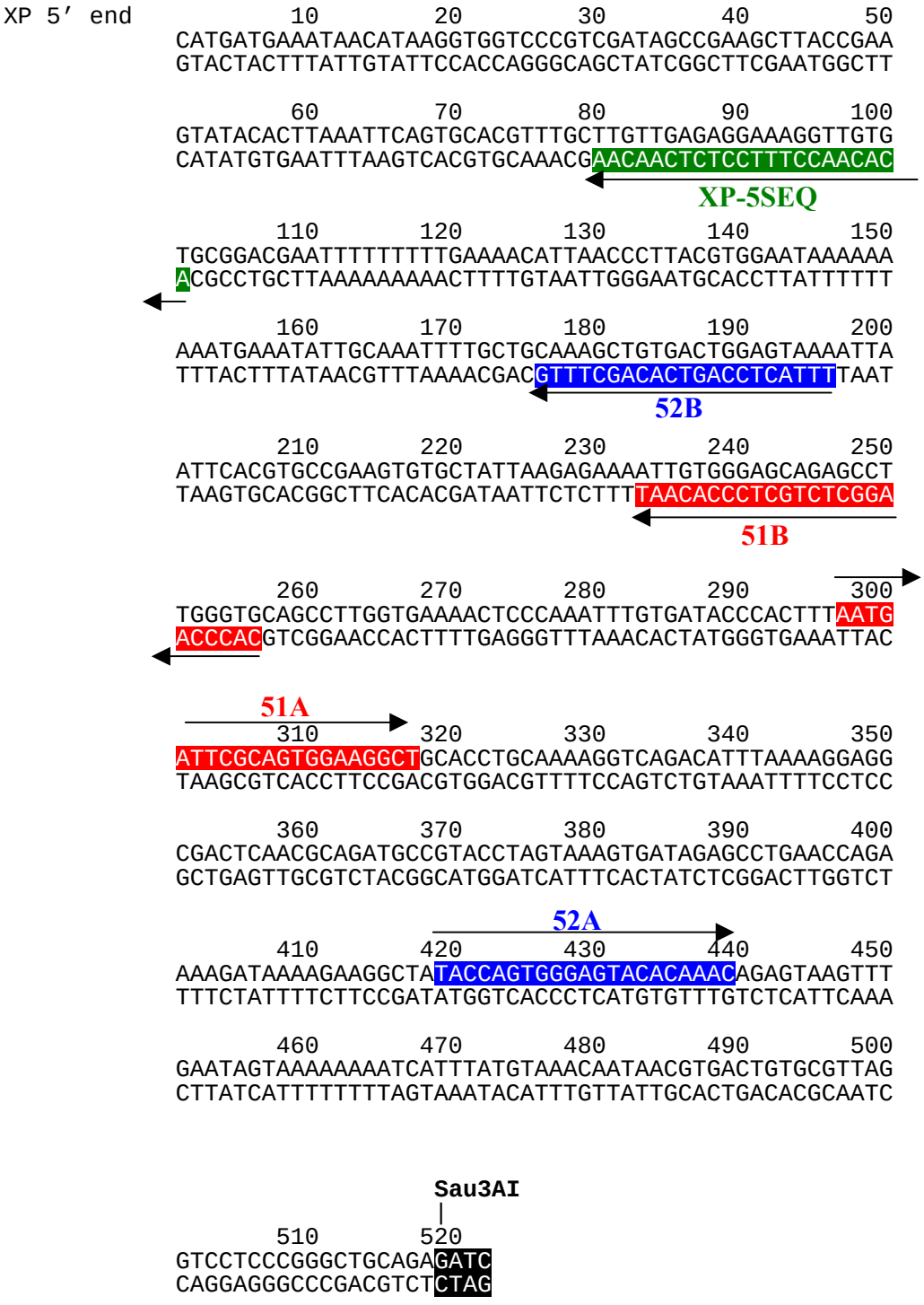
2) To purify reactions add 75µl 70% ethanol, cover, let stand 30 minutes at room temp in the dark. Centrifuge for 30 minutes @ 2,470 RCF. Remove cover, invert on paper towel and spin @ 700 RCF for 1 min.
3) Register plate in the LIMS for runs on ABI 3700 machines.

Sequencing Primers:

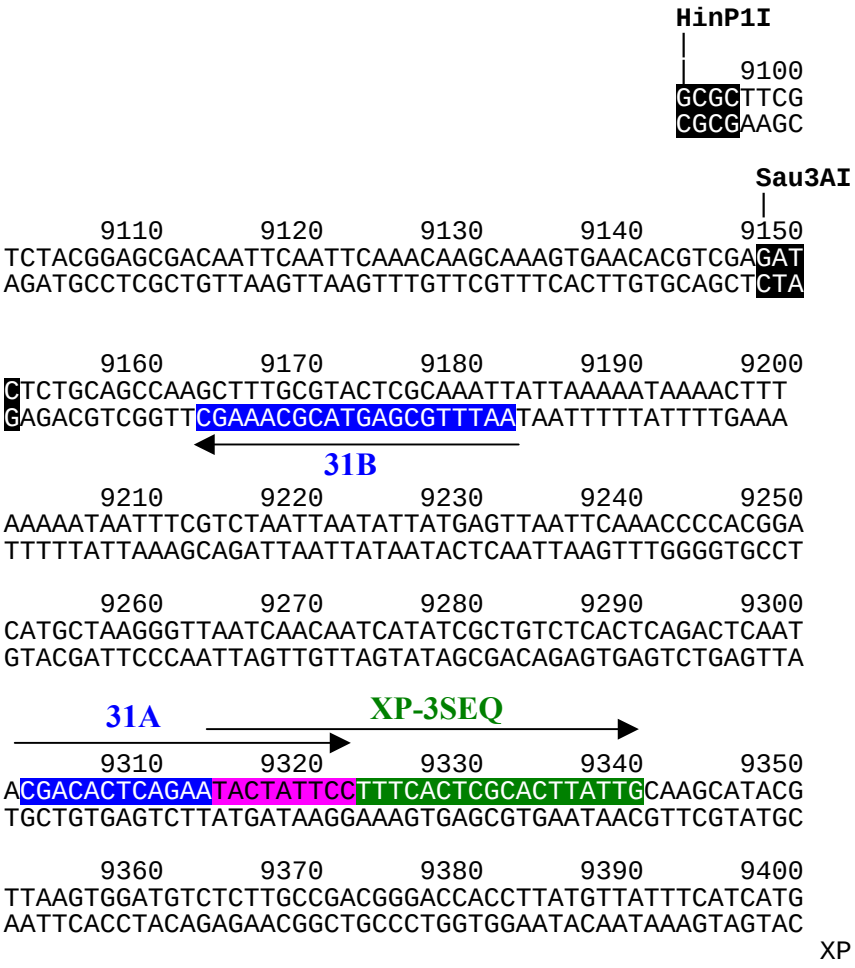
Primer Name	XP-element End	Sequence 5' to 3'
XP-5SEQ	5' end	5'-ACA CAA CCT TTC CTC TCA ACA A-3'
XP-3SEQ	3'end	5'-TAC TAT TCC TTT CAC TCG CAC TTA TTG-3'

Figures:

XP 5' end



XP 3' end


HinP1I
9100
GCGCTTCG
CGCGAAGC
Sau3AI
9110 9120 9130 9140 9150
TCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGAGAT
AGATGCCTCGCTGTTAAGTTAAGTTTGTTCGTTTCACTTGTGCAGCTCTA
9160 9170 9180 9190 9200
CTCTGCAGCCAAGCTTTGCGTACTCGCAAATTATTAAAAATAAAACTTT
GAGACGTCGGTTCGAAACGCATGAGCGTTTAATAATTTTTATTTTGAAA
31B
9210 9220 9230 9240 9250
AAAAATAATTTCGTCTAATTAATATTATGAGTTAATTCAAACCCCACGGA
TTTTTATTAAAGCAGATTAATTATAATACTCAATTAAGTTTGGGGTGCCT
9260 9270 9280 9290 9300
CATGCTAAGGGTTAATCAACAATCATATCGCTGTCTCACTCAGACTCAAT
GTACGATTCCCAATTAGTTGTTAGTATAGCGACAGAGTGAGTCTGAGTTA
31A
XP-3SEQ
9310 9320 9330 9340 9350
ACGACACTCAGAATACTATTCCTTTCACTCGCACTTATTGCAAGCATACG
TGCTGTGAGTCTTATGATAAGGAAAGTGAGCGTGAATAACGTTCGTATGC
9360 9370 9380 9390 9400
TTAAGTGGATGTCTCTTGCCGACGGGACCACCTTATGTTATTTCATCATG
AATTCACCTACAGAGAACGGCTGCCCTGGTGGAATACAATAAAGTAGTAC
XP 3' end