

Inverse PCR and Sequencing of *P* element, *piggyBac* and *Minos* Insertion Sites in the *Drosophila* Gene Disruption Project

Protocol for determining sequences flanking insertions in the *Drosophila* Gene Disruption Project, including insertions in PZ, PlacW, EP, BG, KG, KV, EY, DG, LA, PA, PC, PL, Carnegie and Yale protein and enhancer trap, and MB lines. For details of the insertion collection, see Bellen, H.J., *et al.* (2004) "The BDGP gene disruption project: Single transposon insertions associated with 40% of *Drosophila* genes." *Genetics* 167(2):761-81.

This protocol is an updated version of the original BDGP protocol of Jay Rehm. The original protocol is available at <http://www.fruitfly.org/about/methods/inverse.pcr.html>.

If you have questions or comments about the protocol, please contact RHoskins@lbl.gov

Roger Hoskins, September 18, 2006

I. Fly Genomic DNA Prep

Reagents

<u>Buffer A</u>	<u>per prep</u>	<u>50 preps</u>
1 M Tris·HCl, pH7.5	40 μ l	2.0 ml
500 mM EDTA, pH8.0	80 μ l	4.0 ml
4 M NaCl	10 μ l	0.5 ml
10% SDS	20 μ l	1.0 ml
H ₂ O	250 μ l	12.5 ml

<u>LiCl/KAc solution</u>	<u>per prep</u>	<u>50 preps</u>
5 M KAc	230 μ l	11.5 ml
6 M LiCl	570 μ l	28.5 ml

Mix fresh from separate stock solutions. Mixture will precipitate salts with time.

Genomic Prep

Collect samples of ~15 adult flies per Eppendorf tube. Use fresh or store frozen at -80° . On ice, add 3 autoclaved ball bearings and 400 μ l Buffer A to each sample. Place the samples in a 96-tube Eppendorf rack. Wrap the tubes and rack with a sheet of Saran Wrap to hold tubes in place. Vortex on highest setting to homogenize flies (VWR VX2500 Multitube Vortexer). Invert rack and tubes, and continue vortexing. Repeat until flies are visibly disrupted. Vigorously shaking the rack of tubes by hand is also effective. Incubate 30 min @ 65°C . We use a dry heat block designed for Eppendorf tubes. Add 800 μ l LiCl/KAc solution, invert several times to mix, and incubate 10 min on ice. Spin 15 min @ 14,000 rpm, room temperature, in a microfuge. Transfer 1 ml supernatant to 2 ml ClickSeal Eppendorf tube, excluding floating solids. Add 800 μ l isopropanol and invert several times to mix. Spin 10 min @ 14,000 rpm in a microfuge at room temperature. Aspirate and discard supernatant. Quick spin. Aspirate again. Wash pellet with 500 μ l cold 70% ETOH. Spin 5 min @ 14,000 rpm in a microfuge at room temperature. Aspirate and discard supernatant. Quick spin. Aspirate again. Air dry pellets about 2 hours. Resuspend in 75 μ l TE (or ~5 μ l per fly) overnight at room temperature. Store at -20° . For processing multiple samples, transfer genomic DNA preps to a 96-well Nunc plate.

Notes

We prepare two batches of 48 samples to fill one 96-well plate for subsequent steps. Ball bearings and vortexing are used to process large numbers of samples. Alternatively, samples can be homogenized individually using disposable tissue grinders (Kontes). High quality genomic DNA is critical to the success of the protocol. Samples must be readily digested with restriction enzymes.

Stock Solutions for Genomic DNA Prep

Buffer A

100 mM Tris, pH7.5
100 mM EDTA
100 mM NaCl
0.5% SDS

5M KAc

KAc (98.14 g/mol)	245 g
H ₂ O	up to 500 ml

6 M LiCl

LiCl (42.39 g/mol)	254 g
H ₂ O	up to 1000 ml

TE

10 mM Tris, pH8.0
1 mM EDTA, pH8.0

Ball Bearings

Wheels Manufacturing, tel 303-410-7336, www.wheelsmfg.com

1/8" bearings, 500/bottle, catalog # BALL-1B, \$5.50/bottle

Each sample requires three bearings, or 300 per batch of 96 fly strains.

To prepare for use, add 1000 bearings to a beaker, rinse well in H₂O, air dry to completion on paper towels, and autoclave in a beaker on dry cycle. We no longer re-use bearings.

II. Restriction Digestion

Select the appropriate restriction enzyme(s) from the table.

strain name	vector	enzyme
Lethal lines	P{PZ}, P{lacW}	HinP1 I
EP lines	P{EP}	HinP1 I
BG lines	P{GT1}	HinP1 I
KG, KV lines	P{SUPor-P}	Hpa II
EY lines	P{EPgy2}	Hpa II
DG lines	P{wHy}	HinP1 I
LA lines (5' end only)	P{Mae-UAS.6.11}	Rsa I
PA, PC lines (5' end)	PBac{5HPw +}, PBac{3HPy +}	Sau3A I (5' end)
PA, PC lines (3' end)	PBac{5HPw +}, PBac{3HPy +}	HinP1 I (3' end)
PL lines	PBac{GAL4D, EYFP}	Hae III
CA, CB, CC lines	P{PTT-GA}, P{PTT-GB}, P{PTT-GC}	Hpa II or HinP1 I
YB, YC, YG, YP, YZ	P{PTT-GA}, P{PTT-GB}, P{PTT-GC}	HinP1 I
BA, YD lines	PBac{HpaI;y+}	Hae III
MB lines	Mi{ET1}	Hpa II

Restriction Digestion Reactions

	per reaction	per 96 reactions
genomic DNA (~2 flies)	10.0 μ l	N/A
10X restriction buffer	2.5 μ l	250 μ l
RNase A (100 μ g/ml)	2.0 μ l	200 μ l
restriction enzyme	0.5 μ l (5 to 10 units)	50 μ l
w/o BSA:		
ddH ₂ O	10.0 μ l	1.0 ml
w/ BSA (for Sau 3A I only)		
100X BSA (10 mg/ml)	0.25 μ l	25 μ l
H ₂ O	9.75 μ l	975 μ l
Total	25 μl	1.5 ml mix (15 μl/rxn)

Aliquot 10 μ l of each genomic DNA sample. For 96 samples, make 1.5 ml reaction mix and aliquot 15 μ l per sample. Incubate @ 37°C for 2.5 hrs.

Heat inactivate enzyme @ 65°C for 20 mins.

Run 5 μ l of the digestion reaction on a 0.8% agarose gel. If DNA samples do not digest relatively well to produce a bright smear, then subsequent steps will likely fail.

RNase A (1 mg/ml)

Dissolve 10 mg RNase A in 1.0 ml 15mM NaCl, 10mM Tris, pH7.5 in an Eppendorf tube. Clamp cap shut and float sample in a beaker of boiling water for 15 minutes. Remove beaker from heat and allow sample to cool slowly to room temperature. Dispense into ten 100 μ l aliquots and store at -20° . Dilute to 100 μ g/ml with dH₂O (10 μ l RNase stock solution in 990 μ l H₂O) before use.

Alternatively, a commercially prepared solution of RNase A may be used.

III. Ligation

10X Ligation Buffer with ATP

1 M TrisHCl, pH7.5	2.63 ml
1 M MgCl ₂	200 µl
1 M DTT	40 µl
100 mM ATP	400 µl
ddH ₂ O	1.13 ml
Total	4.4 ml

Ligation Reactions

For 96 samples, use a Beckman deep well block. Need >1.5 ml capacity for precipitation.

	per reaction	per 100 reactions
Digestion reaction (~1 fly)	10 µl	N/A
10x Ligation Buffer with ATP	40 µl	4 ml
ddH ₂ O	348 µl	34.8 ml
T4 DNA Ligase (0.2 U)	2 µl	200 µl
Total	400 µl	39 ml mix (390 µl/rxn)

Aliquot 10 µl each restriction digest sample into plate. Make 39.2 ml reaction mix and aliquot 392 µl into each well. Incubate @ 4°C overnight to favor circularization.

Precipitate Ligation Reaction

Add 40 µl 3M NaOAc and 1 ml EtOH. Seal tightly, invert to mix, and chill 1 hr @ -80°. Spin @ maximum rpm for 30 mins at 4°C.

For 96 samples, we use an Eppendorf 5810R at 4000 rpm.

Decant supernatant and blot inverted block on paper towels.

Wash pellets with 500 µl cold 70% EtOH.

Spin @ maximum rpm for 15 mins at 4°C.

Decant supernatant and blot inverted block on paper towels.

Air dry at room temperature.

Resuspend in 150 µl TE (~1/150 fly per µl) for at least 1 hr at room temperature.

Transfer resuspended samples to 96-well flat bottom NUNC plate.

100mM ATP, pH 7.5

Dissolve 2.75 g ATP (disodium salt; e.g. Sigma A3377) in 40 ml dH₂O. Adjust to pH7.5 with 10 M NaOH (~1 ml). Adjust the volume to 50 ml with dH₂O. The ATP solution should be stored at -20° in 1 ml aliquots and is stable for up to 1 year.

IV. Inverse PCR

Inverse PCR Reactions for 5' and 3' Flanks

	per reaction	per 100 reactions
Ligation reaction (~1/30th fly)	5.0 μ l	N/A
2.5mM dNTP mix	2.0 μ l	200 μ l
10 μ M primer 1	0.5 μ l	50 μ l
10 μ M primer 2	0.5 μ l	50 μ l
10x reaction buffer B	2.5 μ l	250 μ l
HOT FIREPol (1 U)	0.2 μ l	20 μ l
25 mM MgCl ₂	2.5 μ l	250 μ l
ddH ₂ O	11.8 μ l	1.2 ml
Total	25 μl	2.0 ml (20 μl/rxn)

We have obtained comparable results using various thermostable DNA polymerases, including Taq polymerase from Amersham and Pharmacia, and Applied Biosystems AmpliTaq and AmpliTaq Gold. We currently use Solis Biodyne HOT FIREPol polymerase, and the reactions conditions above and temperature cycling conditions below are for this enzyme.

Aliquot 5 μ l each ligation reaction sample into PCR plate. Make 2 ml reaction mix and aliquot 20 μ l into each well. Use the appropriate PCR primers listed in the table below. Conduct temperature cycling as described below, using the annealing temperature designated in the table for the 5' or 3' end of the appropriate vector.

Temperature cycling conditions*

95°C, 15 min

35 X (95°C, 30 sec; appropriate annealing temperature, 1 min; 72°C, 2 min)

72°C, 10 min

4°C, hold

These conditions work well for the MJ Research, and the Perkin-Elmer Applied Biosystems 9600 and 9700 thermal cyclers. Other machines may have different cycling profiles and so adjustments may be necessary to achieve optimal results.

Run 5 μ l samples of PCR products on a 1.2% agarose gel with a 100-bp marker ladder. In most cases, each sample should yield a single PCR product.

*Use the supplier's recommended temperatures and times for the optional hot start and the extension steps. Optimal conditions vary by polymerase.

PCR primers and annealing temperatures

strain name	primer 1	primer 2	annealing temp
P{PZ}, P{lacW} 5' end	Plac1	Plac4	60°
P{PZ} 3' end	Pry1	Pry4	55°
P{lacW} 3' end	Plw3-1	Pry4	55°
P{PZ}, P{lacW} 3' end*	Pry1	Pry2	60°
EP 5' end	Plac1	Pwht1	60°
EP 3' end	Pry1	Pry4	55°
EP 3' end*	Pry1	Pry2	60°
BG 5' end	pGT1.5a	pGT1.5d	55°
BG 3' end	Pry1	Pry4	55°
KG, KV 5' end	Plac1	Pwht1	60°
KG, KV 3' end	3.rev.hpa2	Pry4	55°
EY, CA, CB, CC 5' end	Plac1	Pwht1	60°
EY, CA, CB, CC 3' end	Pry1	Pry4	55°
DG 5' end	Plac1	Pwht1	60°
DG 3' end	Pry1	Pry4	55°
LA 5' end	LA(f).1	LA(r).1	55°
PA, PC 5' end	5F1	5R1	55°
PA, PC 3' end	3F1	3R1	55°
PL, BA, YD 5' end	PLF	PLR	65°
PL, BA, YD 3' end	PRF	PRR	55°
EY, CA, CB, CC, (PZ, EP, BG, DG, YB, YC, YG, YP, YZ) 3' end [#]	EY.3.R	EY.3.F	55°
MB 5' end	MI.5.F	MI.5.R	50°
MB 3' end	MI.3.F	MI.3.R	50°

* The Pry2/Pry1 primer combination can be used in cases in which strong, unique bands do not result from the Pry4/Pry1 or Pry4/Plw3-1 primer combinations. Pry2 should then be used as the sequencing primer, but it maps very near the vector-genome sequence junction, and so the precise insertion site may not be recovered in the sequence data.

[#] The Pry1/Pry4 primer pair has always performed relatively poorly. We have recently replaced it with an improved EY.3.F/EY.3.R primer pair, which has performed very well in inverse PCR amplification of the 3' ends of insertions in EY, CB and CC lines. The pair should also work well for 3' ends in PZ, EP, BG and DG lines, and perhaps other lines, but we have not yet tested these.

V. Exo/SAP Reaction

Exonuclease I (Exo I) degrades single stranded primers. Shrimp alkaline phosphatase (SAP) dephosphorylates dNTPs. Excess primers and dNTPs from the PCR reaction will interfere with the sequencing reaction and must be removed. No further clean-up of the PCR reactions is required.

Aliquot 10 μ l of each inverse PCR reaction product into a fresh PCR plate on ice. Prepare the volume of enzyme cocktail appropriate to the number of 96-well plates to be sequenced. Mix water and buffer first, and chill on ice for 5 minutes. Add the enzymes to the chilled buffer solution, and mix gently but thoroughly by inversion. Add 5 μ l of cocktail to each sample well and mix by gentle pipetting.

# of Plates	1	2	4	6	8	10	12
dH ₂ O	250 μ l	500 μ l	1.0 ml	1.5 ml	2.0 ml	2.5 ml	3.0 ml
10X SAP buffer	50 μ l	100 μ l	200 μ l	300 μ l	400 μ l	500 μ l	600 μ l
Exo I	50 μ l	100 μ l	200 μ l	300 μ l	400 μ l	500 μ l	600 μ l
SAP	200 μ l	400 μ l	800 μ l	1.2 ml	1.6 ml	2.0 ml	2.4 ml

Incubate reactions at 37° for 30 min.

Inactivate enzymes by incubation at 70° for 15 min.

Store samples at 4° until sequencing reactions are performed.

VI. Sequencing Reaction (10 µl; 1/8th scale)

We sequence using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit and a ABI3700 or ABI3730 model DNA sequencer. For other sequencing chemistries and machines, follow the manufacturers recommended protocols.

Prepare the volume of sequencing cocktail appropriate to the number of 96-well plates.

# of Plates	1	2	3	4	5	6
BigDye3	100 µl	200 µl	300 µl	400 µl	500 µl	600 µl
5X buffer	150 µl	300 µl	450 µl	600 µl	750 µl	900 µl
Primer (1 µM)	200 µl	400 µl	600 µl	800 µl	1000 µl	1200 µl
ddH ₂ O	250 µl	500 µl	750 µl	1000 µl	1250 µl	1500 µl

Aliquot 7 µl of sequencing cocktail into each well of a PCR plate.

Add 3 µl of each Exo/SAP reaction product per well.

On a thermocycler, run the BigDye cycle sequencing protocol:

85°, 1 min

25 X (96°, 10 sec; 50°, 5 sec; 60° 4 min)

4°, hold

Prepare reaction products for the sequencer using any of the protocols described in the BigDye product literature.

Sequencing Primers

strain name	sequencing primer
P{PZ}, P{lacW}, EP 5' end	Sp1
P{PZ}, P{lacW}, EP 3' end	Spep1
P{PZ}, P{lacW}, EP 3' end*	Pry2
BG, DG 5' end	Sp1
BG, DG 3' end	Spep1
EY, KG, KV, CA, CB, CC 5' end	5.SUP.seq1
EY, KG, KV, CA, CB, CC 3' end	3.SUP.seq1
LA 5' end	LA(f)seq1
PA, PC 5' end	pB-5SEQ
PA, PC 3' end	pB-3SEQ
PL, BA, YD 5' end	PLR
PL, BA, YD 3' end	PRF
EY, CA, CB, CC, etc. 3' ends [#]	EY.3.F
MB 5' and 3' ends	MI.seq

* For Pry2/Pry1 inverse PCR products only.

[#] Only for inverse PCR products amplified with EY.3.F and EY.3.R.

Primer Sequences for Inverse PCR and Sequencing

primer name	primer sequence
Plac1	CAC CCA AGG CTC TGC TCC CAC AAT
Plac4	ACT GTG CGT TAG GTC CTG TTC ATT GTT
Pry1	CCT TAG CAT GTC CGT GGG GTT TGA AT
Pry4	CAA TCA TAT CGC TGT CTC ACT CA
Pry2	CTT GCC GAC GGG ACC ACC TTA TGT TAT T
Plw3-1	TGT CGG CGT CAT CAA CTC C
Pwht1	GTA ACG CTA ATC ACT CCG AAC AGG TCA CA
Sp1	ACA CAA CCT TTC CTC TCA ACA A
Spep1	GAC ACT CAG AAT ACT ATT C
pGT1.5a	CCG CAC GTA AGG GTT AAT G
pGT1.5d	GAA GTT AAG CGT CTC CAG G
5.SUP.seq1	TCC AGT CAC AGC TTT GCA GC
3.rev.hpa2	TTG CCA CTT GCT CAT ACG TC
3.SUP.seq1	TAT CGC TGT CTC ACT CAG
LA(f).1	GGG AAT TGG GAA TTC GTT AA
LA(r).1	TAG CGA CGT GTT CAC TTT GC
LA(f)seq1	CTC TCA ACA AGC AAA CGT GC
5F1	GAC GCA TGA TTA TCT TTT ACG TGA C
5R1	TGA CAC TTA CCG CAT TGA CA
pB-5SEQ	CGC GCT ATT TAG AAA GAG AGA G
3F1	CCT CGA TAT ACA GAC CGA TAA AAC
3R1	TGC ATT TGC CTT TCG CCT TAT
pB-3SEQ	CGA TAA AAC ACA TGC GTC AAT T
PLF	CTT GAC CTT GCC ACA GAG GAC TAT TAG AGG
PLR	CAG TGA CAC TTA CCG CAT TGA CAA GCA CGC
PRF	CCT CGA TAT ACA GAC CGA TAA AAC ACA TGC
PRR	AGT CAG TCA GAA ACA ACT TTG GCA CAT ATC
EY.3.F	CCT TTC ACT CGC ACT TAT TG
EY.3.R	GTG AGA CAG CGA TAT GAT TGT
MI.5.F	CAAAAGCAACTAATGTAACGG
MI.5.R	TTGCTCTTCTTGAGATTAAGGTA
MI.3.F	ATGATAGTAAATCACATTACG
MI.3.R	CAATAATTTAATTAATTTCCC
MiET.seq	TTTCGTCGTGAAGAGAAT