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

Protocol for recovery of sequences flanking insertions in the *Drosophila* Gene Disruption Project, including insertions in lethal P{lacW} and P{PZ}, EP, G(enexel), BG, KG, KV, EY, DG, LA, PA, PC, PL, SH, S, Carnegie and Yale enhancer and protein trap, MB and MI lines. For details of the insertion collection, see

1. Spradling, A.C., *et al*. (1999) *Genetics* 153(1):135-77.
2. Bellen, H.J., *et al*. (2004) *Genetics* 167(2):761-81.
3. Bellen, H.J., *et al.*, (2011) *Genetics*, Epub ahead of print.
4. http://flypush.imgen.bcm.tmc.edu/pscreen/

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

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Roger Hoskins and Martha Evans-Holm, June 11, 2004, updated May 16, 2011.

I. Fly Genomic DNA Prep

Reagents

Buffer A	per prep	50 preps
1 M Tris·HCl, pH7.5	40 µl	2.0 ml
500 mM EDTA, pH8.0	80 µl	4.0 ml
4 M NaCl	10 µl	0.5 ml
10% SDS	20 µl	1.0 ml
H_2O	250 µl	12.5 ml

LiCl/KAc solution	per prep	50 preps
5 M KAc	230 µl	11.5 ml
6 M LiCl	570 µl	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°C.
On ice, add 3 autoclaved ball bearings and 400 µl 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 at 65°C. We use a dry heat block designed for Eppendorf tubes.
Add 800 µl LiCl/KAc solution, invert several times to mix, and incubate 10 min on ice.
Spin 15 min at 12,000 rpm, room temperature, in a microfuge.
Transfer 1 ml supernatant to 2 ml ClickSeal Eppendorf tube, excluding floating crud.
Add 600 µl isopropanol and invert several times to mix.
Spin 20 min at 12,000 rpm in a microfuge at room temperature.
Aspirate and discard supernatant. Quick spin. Aspirate again.
Wash pellet with 500 µl cold 70% EtOH
Spin 10 min at 12,000 rpm in a microfuge at room temperature.
Aspirate and discard supernatant. Quick spin. Aspirate again.
Air dry pellets about 1 hour.
Resuspend in 75 µl TE (or ~5 µl per fly) overnight at room temperature. Store at −20°C.
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_2O	up to 500 ml

6 M LiCl

LiCl (42.39 g/mol)	254 g
H_2O	up to 1000 ml

TE
10 mM Tris, pH7.5
1 mM EDTA

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 H_2O, air dry to completion on paper towels, and autoclave in a beaker on dry cycle. Bearings can be re-used twice. To re-use, rinse well in several changes of H_2O. Dry well, and autoclave.

II. Restriction Digestion

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

strain name	vector	enzyme
BA, YD lines	PBac{Hpa1-GFP, y^+}	Hae III
BG lines	P{GT1}	HinP1 I
CA, CB, CC, YB, YC, YG, YP, YZ lines	P{CASPR4[GFP]}	HinP1 I or Sau3A I
DG lines	P{wHy}	HinP1 I
EP, G lines	P{EP}	HinP1 I
EY lines	P{EPgy2}	Hpa II
KG, KV lines	P{SUPorP}	Hpa II
LA lines (5' end only)	P{Mae-UAS.6.11}	Rsa I
lethal P, S, SH lines	P{PZ}, P{lacW}	HinP1 I or Hpa II
MB lines	Minos{MiET1}	Hpa II
MI lines	Minos{MIMIC}	Mbo I or Sau3A I
PA, PC lines (3' end)	PBac{5HPw$^+$}, PBac{3HPy$^+$}	HinP1 I (3' end)
PA, PC lines (5' end)	PBac{5HPw$^+$}, PBac{3HPy$^+$}	Sau3A I (5' end)
PL lines	PBac{GAL4D, EYFP}	Hae III
VK docking sites	PBac{y^+, attP}	Sau3A I or Hpa II

Restriction Digestion Reactions

	per reaction	per 96 reactions
genomic DNA (~2 flies)	10.0 µl	N/A
10X restriction buffer	2.5 µl	250 µl
RNase A (100 µg/ml)	2.0 µl	200 µl
restriction enzyme	0.5 µl (5 to 10 units)	50 µl
w/o BSA:		
ddH_2O	10.0 µl	1.0 ml
w/ BSA (for Sau 3A I only)		
100X BSA (10 mg/ml)	0.25 µl	25µl
H_2O	9.75 µl	975 µl
Total	**25 µl**	**1.5 ml mix (15 µl/rxn)**

Aliquot 10 µl of each genomic DNA sample. For 96 samples, make 1.5 ml reaction mix and aliquot 15 µl per sample. Incubate at 37°C for 2.5 hrs.
Heat inactivate enzyme at 65°C for 20 mins.
Run 5 µl of the reaction on a 0.8% agarose gel. If DNA samples do not digest relatively well to produce a bright smear on agarose, then subsequent steps will likely fail.

RNase A (1 mg/ml)
Dissolve 10 mg RNase A in 1.0 ml 10 mM NaOAc, pH5.2 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. Add 100 µl of 1 M Tris, pH8.0 to neutralize the pH. Transfer to a 15 ml Falcon tube, add 8.9 ml of dH_2O, and mix gently. Dispense into ten 1 ml aliquots, label "1 mg/ml RNase A", and store at −20°C. Dilute to 100 µg/ml with dH_2O 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_2O	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_2O	348 µl	34.8 ml
T4 DNA Ligase (0.25 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 at 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 at –80°C.
Spin at 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 at 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 dH_2O. Adjust to pH7.5 with 10 M NaOH (~1 ml). Adjust the volume to 50 ml with dH_2O. The ATP solution should be stored at –20°C 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 µl	N/A
2.5mM dNTP mix	1.0 µl	100 µl
10µM primer 1	0.5 µl	50 µl
10µM primer 2	0.5 µl	50 µl
10x Taq buffer	2.5 µl	250 µl
Taq polymerase (1 U)	0.2 µl	20 µl
ddH_2O	15.3 µl	1.53 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.

Aliquot 5 µl each ligation reaction sample into PCR plate. Make 2 ml reaction mix and aliquot 20 µl 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, 5 min
35 X (95°C, 30 sec; appropriate annealing temperature, 1 min; 68°C, 2 min)
72°C, 10 min
4°, 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. For insertion flanks that fail to amplify, increasing the extension time may help.

Run 5 µl 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.

PCR Primers and Annealing Temperatures

strain name	primer 1	primer 2	annealing temp
BA, PL, YD 3' end	PRF	PRR	65°
BA, PL, YD 5' end	PLF	PLR	65°
BG 3' end[#]	Pry1	Pry4	55°
BG 5' end	pGT1.5a	pGT1.5d	55°
DG 3' end[#]	Pry1	Pry4	55°
DG 5' end	Plac1	Pwht1	60°
EP, G 3' end[#]	EY.3.F	EY.3.R	55°
EP, G 5' end	Plac1	Pwht1	60°
EY 3' end[#]	EY.3.F	EY.3.R	55°
EY 5' end	Plac1	Pwht1	60°
KG, KV 3' end	3.rev.hpa2	Pry4	55°
KG, KV 5' end	Plac1	Pwht1	60°
LA 5' end	LA(f).1	LA(r).1	55°
lethal P{PZ}, P{lacW} 3' end[#]	EY.3.F	EY.3.R	55°
lethal P{PZ}, P{lacW} 5' end	Plac1	Plac4	60°
MB, MI 3' end	MI.3.F	MI.3.R	50°
MB, MI 5' end	MI.5.F	MI.5.R	50°
P{CASPR4[GFP]} 3' end	EY.3.F	EY.3.R	55°
P{CASPR4[GFP]} 5' end	Plac1	Pwht1	60°
PA, PC 3' end	3F1	3R1	55°
PA, PC 5' end	5F1	5R1	55°
S, SH P{lacW} 3' end*	EY.3.F	PlacW-3R	55°
S, SH P{lacW} 5' end*	Plac1	PlacW-5F	60°
VK 3' end	HY.3.F	HY.3.R	55°
VK 5' end	HY.5.F	HY.5.R	55°

[#] The Pry1/Pry4 primer pair always performed relatively poorly. We have replaced it with an improved EY.3.F/EY.3.R primer pair, which performs very well in inverse PCR amplification of the 3' ends of insertions in P{PZ}, P{lacW}, EP, EY and P{CASPR4[GFP]} lines. The pair should also work well for 3' ends in BG and DG lines.

* S and SH lines carry lethal P{lacW} inserts and also FRT-bearing P inserts. These alternative iPCR primer sets amplify P{lacW} flanking sequences specifically.

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 µl 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 µl of cocktail to each sample well and mix by gentle pipetting.

# of Plates	**1**	**2**	**4**	**6**	**8**	**10**	**12**
dH_2O	250 µl	500 µl	1.0 ml	1.5 ml	2.0 ml	2.5 ml	3.0 ml
10X SAP buffer	50 µl	100 µl	200 µl	300 µl	400 µl	500 µl	600 µl
Exo I	50 µl	100 µl	200 µl	300 µl	400 µl	500 µl	600 µl
SAP	200 µl	400 µl	800 µl	1.2 ml	1.6 ml	2.0 ml	2.4 ml

Incubate reactions at 37°C for 30 min.
Inactivate enzymes by incubation at 70° for 15 min.
Store samples at 4°C 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 an 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_2O	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°, 1 min; 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
BA, PL, YD 3’ end	PRF
BA, PL, YD 5’ end	PLR
BG, DG 3’ end	Spep1
EP, EY, P{CASPR4[GFP]} 3’ end	EY.3.F
EY, KG, KV, P{CASPR4[GFP]} 5’ end	5.SUP.seq1
KG, KV 3’ end	3.SUP.seq1
LA 5’ end	LA(f)seq1
MB, MI 5’ & 3’ ends	MI.seq
P{PZ}, P{lacW}, EP 3’ end	EY.3.F
P{PZ}, P{lacW}, EP, BG, DG 5’ end	Sp1
PA, PC 3’ end	pB-3SEQ
PA, PC 5’ end	pB-5SEQ
VK 3’ end	HY.3.F
VK 5’ end	HY.5.R

Primer Sequences for Inverse PCR and Sequencing

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