Chapter 2

Materials and Methods

2.1 *Drosophila* stocks and maintenance

*Drosophila melanogaster* imagos (all the strains tabulated below) were maintained at 24 °C in standard cornmeal agar medium with 14-hour light and 10-hour dark cycle. Standard procedures were used for handling the cultures (Roberts, 1986). Flies were allowed to lay eggs and transferred every 24 hours for expansion. For larval work, flies were allowed to lay eggs in media bottles for 12 hours.

Table 2.1 *Drosophila* fly strains maintained

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Fly strain</th>
<th>Source of the strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Canton Special Benzer (CSBz)</td>
<td>Wild type stock, TIFR Stock Centre, Mumbai</td>
</tr>
<tr>
<td>2</td>
<td>003</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>3</td>
<td>080</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>4</td>
<td>191</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>5</td>
<td>1110</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>6</td>
<td>OK66</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>7</td>
<td>OK140</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>8</td>
<td>OK284</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>9</td>
<td>OK294</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>10</td>
<td>OK301</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>11</td>
<td>OK309</td>
<td>Cahir O’Kane, University of Cambridge, Cambridge, UK</td>
</tr>
<tr>
<td>12</td>
<td>288Y</td>
<td>Josh Dubnau, CSHL</td>
</tr>
<tr>
<td>13</td>
<td>P{XP}d04570</td>
<td>Exelixis Collection at Harvard (#d04570)</td>
</tr>
</tbody>
</table>
14  P{EPgy2}EY01663  Bloomington Drosophila Stock Center (# 19988)
15  P{GT1}BG01047  Bloomington Drosophila Stock Center (# 12534)
16  PBac(RB)ffz61685  Exelixis Collection at Harvard (# e01695)
17  yw ; + ; Ki p{A2-3} (99B)  NCBS fly stock
18  w / w ; +/+ ; TM3Sb / TM6Tb  NCBS fly stock
19  UAS-2xEGFP on IIIrd Chr.  NCBS fly stock
20  UAS-mCD8::GFP on IIIrd Chr  NCBS fly stock
21  UAS-n-syb.eGFP on IIIrd Chr  NCBS fly stock

2.2  Materials

2.2.1  Drosophila growth medium:

Flies grown in bottles or vials at room temperature in corn meal agar with the following composition –

Maize flour 90.0 g
Dextrose 50.0 g
Sucrose 30.0 g
Agar 10.0 g
Yeast tablet powder 10.0 g
Propionic acid 5 ml
Ortho phosphoric acid 0.6 ml
Benzoic acid (10 %) 0.7 ml
Distilled water up to 1 litre.

2.2.2  Adult behavior solutions:

Conditioning medium (Thorpe’s medium):

Potassium sodium tartrate tetrahydrate (C4H4O6KNa4H2O) 8.0 g/L
Ammonium sulphate (NH4)2SO4 2.0 g/L
L(+)-Tartaric acid cryst (C4H6O6) 5.0 g/L
Magnesium sulphate 0.5 g/L
(MgSO$_4$·7H$_2$O)  
Potassium dihydrogen ortho phosphate \( \text{(KH}_2\text{PO}_4\text{)} \) 0.65 g/L  
Calcium chloride (Dihydrate) \( \text{(CaCl}_2\cdot2\text{H}_2\text{O)} \) 0.25 g/L  

1.5 % Sucrose and 4 % Agar is added fresh before use.

2.2.3 Larval behavior solution:

Ringer’s solution:

**Solution A:**
- Sodium chloride \( \text{(NaCl)} \) 7.48 g/L
- Potassium chloride \( \text{(KCl)} \) 0.35 g/L
- Calcium chloride (Dihydrate) \( \text{(CaCl}_2\cdot2\text{H}_2\text{O)} \) 0.27 g/L

**Solution B:**
- Di-Sodium hydrogen ortho phosphate anhydrous \( \text{(Na}_2\text{HPO}_4\text{)} \) 0.13 g/L
- Potassium dihydrogen ortho phosphate \( \text{(KH}_2\text{PO}_4\text{)} \) 0.05 g/L

Solution A and B are prepared separately, autoclaved, cooled down completely and then mixed together to form Ringer’s solution.

20 mM Lithium chloride \( \text{(LiCl)} \):
- Lithium Chloride 0.85 g/L

2.2.4 Molecular Biology Solutions:

Plasmid Rescue:

**Homogenization Buffer:**
- Tris HCl, pH 7.5 50 mM
- NaCl 60 mM
- EDTA, pH 8.0 10 mM
Sucrose 5 %

**Lysis Buffer :**
- Tris HCl, pH 9.0 300 mM
- EDTA, pH 8.0 100 mM
- SDS 0.625 %
- Sucrose 5 %

**RNase A :**
- Stock solution 10 mg/ml
- For stock solution, dissolve DNase free RNase A in,
  - Tris HCl, pH 7.5 10 mM
  - NaCl 15 mM
  - Double distilled water as per required
- Heat the solution at 100 °C for 15 minutes on heating block. Allow to cool slowly to room temperature and dispense into aliquots and keep at -20 °C.

**Proteinase K :**
- Stock solution 20 mg/ml
- Dissolved in,
  - Tris HCl, pH 8.0 50 mM
  - Calcium acetate 1.5 mM
  - Double distilled water as per required
- Dispensed in small aliquots and kept at -20 °C.

**Phenol : Chloroform : Isoamyl alcohol :**
25 : 24 : 1

**TBE Buffer, pH 8.3 :**
- Stock solution 5 X
- Working solution 0.5 X
- In 5 X stock solution,
  - Tris-Base 0.445 M
  - Boric acid 0.445 M
  - EDTA, pH 8.0 0.01 M
- To make 1 litre of 5 X stock solution, add
  - Tris-Base 54 g
  - Boric acid 27.5 g
EDTA (0.5 M), pH 8.0
Distilled water

20 ml
as per required

Autoclave and dilute to make working solution and prefer to use freshly diluted.

Ethidium bromide (Etbr):
Stock solution

10 mg/ml

Add 1 g of Etbr to 100 ml of double distilled water. Wrap and the container in aluminium foil or transfer the solution to a dark bottle and store at room temperature.

LB Medium (Luria–Bertani Medium/Broth):
To make 100 ml, add

Tryptone
Yeast Extract
NaCl

1 g
0.5 g
1 g

Shake until the solutes are dissolved, adjust the pH to 7.0 with 5 N NaOH, then make up the volume to 100 ml using distilled water. Sterilize by autoclaving and keep at room temperature.

For LB-agar plates, just add 1.5 g of Agar powder to 100 ml of LB broth and then autoclave it.

SOB-agar plate with Antibiotic:
To make 100 ml of SOB medium/broth, add

Tryptone
Yeast Extract
NaCl
KCl (250 mM)
MgSO₄ (0.5 M)

2 g
0.5 g
0.06 g
1 ml (final concentration 2.5 mM)
2 ml (final concentration 10 mM)

Shake until the solutes are dissolved, adjust the pH to 7.0 with 5 N NaOH, then make up the volume to 100 ml using distilled water. To make SOB-agar, add 1.5 g Agar and boil to dissolve it. Then get it autoclaved.

When using, melt SOB-Agar and add

MgCl₂ (2 M)

0.5 ml

Swirl mix, and when temperature drops to ~50 °C – 60 °C, add

Ampicillin (50 mg/ml)

100 µl for 100 ml SOB medium
(final concentration 50 µg/ml)
SOC Medium:
After SOB broth is autoclaved and its temperature is dropped down to 60 °C or less, add 200 µl of 1 M Glucose (filter sterilized using 0.22 µm filter) into 10 ml of SOB medium.

And before use, add 50 µl of 2 M MgCl₂ in 10 ml of SOC medium.

Antibiotics:

Tetracycline —
Stock solution 5 mg/ml in Ethanol (light sensitive)
Working concentration 50 µg/ml (1:100)

Store at -20 °C.

Chloramphenicol —
Stock solution 34 mg/ml in Ethanol (light sensitive)
Working concentration 170 µg/ml (1:200)
25 µg/ml (for XL·10 Gold E. coli)

Store at -20 °C.

Ampicillin —
Stock solution 50 mg/ml in sterile water
Working solution 100 µg/ml (1:500)

Sterilize through 0.22 µm syringe filter unit.
Store at -20 °C.

Plasmid Isolation:

Solution I:
Glucose 50 mM
Tris HCl, pH 8.0 25 mM
EDTA, pH 8.0 10 mM

The solution should be autoclaved and kept at 4°C.

Solution II:
NaOH 0.2 N
SDS 1 %

The solution should be prepared fresh and used at room temperature.
Solution III:
For 100 ml,
Potassium acetate (5 M) 60.0 ml
Glacial acetic acid 11.5 ml
Double distilled water 28.5 ml

The resulting solution is 3 M with respect to Potassium and 5 M with respect to Acetate.
The solution should be stored at 4 °C and transferred to ice bucket just before use.

Inverse PCR:

Buffer A:
Tris HCl, pH 7.5 100 mM
EDTA, pH 8.0 100 mM
NaCl 100 mM
SDS 0.5 %

The first three components should be autoclaved and SDS should be added later and kept at room temperature.

LiCl/KAc Solution:
KAc 5 M
LiCl 6 M

The above two components should be autoclaved separately.
Mix 1 part 5 M KAc stock : 2.5 parts 6 M LiCl stock just before the experiment, the mixture should be freshly prepared.

TE Buffer (pH 8.0):
Tris HCl, pH 8.0 10 mM
EDTA, pH 8.0 1 mM

The buffer should be autoclaved and kept at room temperature.

RNase A:
Stock solution 10 mg/ml
Working solution 100 μg/ml with autoclaved double distilled water.

Prepared as described for Plasmid Rescue above.
RNA Extraction and Electrophoresis:

DEPC-treated (RNase-free water):

- Add 0.1 % DEPC in Milli-Q water.
- Stir properly and incubate over night at room temperature in dark.
- Autoclave the solution to decompose the active DEPC, and use further.

RNase-free glasswares/plasticwares/pipettors:

- Soak in 3 % H\textsubscript{2}O\textsubscript{2} (Hydrogen peroxide) for 10 minutes keeping it light protected.
- Rinse thoroughly in DEPC-treated water.
- Glasswares can also be baked at 180 °C for at least 8 hours, or at 300 °C for 4 hours.
- To remove RNases from polypropylene plasticwares, rinse with Chloroform.
- Use baked metal spatulas.
- Fully autoclavable pipettors should be used and wiped with paper towel soaked in RNase Zap at regular intervals before and while using.

MOPS Buffer (5 X):

For 1 litre, add —

\[
\begin{align*}
\text{MOPS, pH 7.0} & \quad 0.1 \text{ M} & \quad (\text{Final concentration}) \\
\text{Sodium acetate} & \quad 10 \text{ mM} & \quad (\text{Final concentration}) \\
\text{EDTA, pH 8.0} & \quad 5 \text{ mM} & \quad (\text{Final concentration})
\end{align*}
\]

- Dissolve 20.93 g of MOPS (free acid, Molecular weight = 209.3) and 0.8203 g of Sodium acetate anhydrous (Molecular weight = 82.03) in 700 ml of sterile DEPC-treated water.
- Stir until completely dissolved.
- Add 10 ml of DEPC-treated 0.5 M EDTA (pH 8.0) and adjust the pH to 7.0 with 10 N NaOH/Acetic acid.
- Adjust the volume with DEPC-treated water to make one litre.
- Dispense into 200 ml aliquots and autoclave.
- The solution will turn yellow, but this will not affect the quality of the buffer.
- Store the MOPS buffer at 4 °C, protected from light.
- Also, buffer yellows with age if it is exposed to light or is autoclaved. Straw-colored buffer works well, but darker buffer doesn't.
RNA Gel/Denaturing Gel (50 ml):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X MOPS</td>
<td>10.0 ml</td>
<td>(1 X final concentration)</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>30.9 ml</td>
<td></td>
</tr>
<tr>
<td>Agarose</td>
<td>0.45 g</td>
<td>(0.9 %)</td>
</tr>
</tbody>
</table>

- Boil Agarose in DEPC-treated water, cool till ~60 °C, then add 10 ml of 5 X MOPS and 9.1 ml of Formaldehyde (37 %) (12.3 M) in a chemical fume hood, mix well. Then cast the gel.
- Before running an RNA gel, soak the gel tank, gel tray and comb in 3 % H₂O₂ for 10 minutes, then remove peroxide by extensively rinsing with DEPC-treated water prior to use.
- Add enough 1 X MOPS running buffer in the gel tank.

Ethidium bromide (Ethbr):

Stock concentration 0.2 mg/ml in DEPC-treated water
Working concentration 10 µg/ml

RNA sample loading (15 µl):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA sample</td>
<td>2.5 µl (~1 µg)</td>
</tr>
<tr>
<td>Formamide</td>
<td>6.0 µl</td>
</tr>
<tr>
<td>Formaldehyde (37 %)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>5 X MOPS</td>
<td>3.0 µl</td>
</tr>
</tbody>
</table>

- Heat denature the above mixture at 65 °C for 10 minutes.
- Keep on ice and add 1 µl of 6 X Gel loading dye and 0.5 µl of Ethbr (10 µg/ml final concentration).
- At least 200 ng of RNA must be loaded in order to visualize with Ethbr.
- Run in 1 X MOPS buffer at 60 V for 45 minutes.

2.2.5 Molecular Biology Products:

**Fine chemicals** –
Sigma-Aldrich Company
USB Corporation
Invitrogen Corporation

**Analytical grade chemicals** –
Qualigens Fine Chemicals (Fisher Scientific)
Merck Chemicals Limited
Enzymes and other reagents –

<table>
<thead>
<tr>
<th>Enzymes/Reagents</th>
<th>Company/Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt; DNA Ligase, Restriction enzymes, Taq DNA Polymersase, Deep Vent™ DNA Polymerase, RNase H</td>
<td>New England Biolabs (NEB)</td>
</tr>
<tr>
<td>TRIzol Reagent, Random Primers, 5 X First-Strand Buffer, dNTP Mix, DTT, Ribonuclease Inhibitor, M-MLV Reverse Transcriptase, RNA Ladder</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>RnaseZap, RNase-free water</td>
<td>Ambion Inc.</td>
</tr>
<tr>
<td>RQI RNase-Free DNAse</td>
<td>Promega Corporation</td>
</tr>
<tr>
<td>DNA Ladders</td>
<td>Bangalore Genei</td>
</tr>
<tr>
<td>SYBR® Green JumpStart™ Taq ReadyMix™ for quantitative PCR (Product Code : S4438)</td>
<td>Sigma-Aldrich Company</td>
</tr>
<tr>
<td>MESA GREEN qPCR MasterMix Plus for SYBR® Assay No ROX (Product Code : RT·SY2X·03+NRWOU)</td>
<td>Genex India (Eurogentec)</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit, MinElute PCR Purification Kit</td>
<td>Qiagen</td>
</tr>
</tbody>
</table>

Special equipments –

<table>
<thead>
<tr>
<th>Equipments</th>
<th>Company/Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel Documentation and Imaging System</td>
<td>Alpha Innotech ALPHAIMAGER HP and FluorChem FC2</td>
</tr>
<tr>
<td>Thermal Cycler</td>
<td>DNA Engine (PTC-200) from MJ Research (now Bio-Rad)</td>
</tr>
<tr>
<td>Real Time Thermal Cycler</td>
<td>Corbett Research Rotor-Gene 3000 (RG-3000)</td>
</tr>
<tr>
<td>DNA Sequencer</td>
<td>3130x Genetic Analyser (Applied Biosystems,</td>
</tr>
<tr>
<td>ABI-Hitachi</td>
<td>Bio Rad Gene Pulser Electroporation System</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Electroporator</td>
<td>Thermo Scientific NanoDrop™ 1000 (ND-1000) UV-Visible Spectrophotometer</td>
</tr>
</tbody>
</table>

Custom DNA Sequencing —
NCBS Sequencing Facility
MWG BIOTECH
Macrogen Inc.

2.2.6 Primers used for molecular biology:

RT-PCR:

**Rp49 (RpL32):**
Forward Primer : 5’ - GCTAAGCTGTCGCACAAATG - 3’
Reverse Primer : 5’ - GACAATCTCCTTGCGCTTC - 3’
cDNA PCR product = 252 bp, gDNA PCR product = 314 bp

**rpII140:**
Forward Primer : 5’ - CCTTCAGGAGTACGGCTATCATCT - 3’
Reverse Primer : 5’ - CCAGGAAGACCTGAGCATTAATCT - 3’
cDNA PCR product = 89 bp, gDNA PCR product = 148 bp

**or83b:**
Forward Primer : 5’ - GCCCAGGTGTTCCACCTTTTGC - 3’
Reverse Primer : 5’ - TCGCTCCCGATATGCTGATC - 3’
cDNA PCR product = 170 bp, gDNA PCR product = 530 bp

**or59b:**
Forward Primer : 5’ - CGCAGTGAGGCAGACAACATC - 3’
Reverse Primer : 5’ - CAGGATGGTGATGACGAAACAGGAG - 3’
cDNA PCR product = 219 bp, gDNA PCR product = 277 bp

or92a:
Forward Primer : 5' ~ ATTGCCGCATCGGTCGTGATTTG - 3'
Reverse Primer : 5' ~ GGCCAATCCGAGAGCTTGGAAG - 3'
cDNA PCR product = 163 bp

or42b:
Forward Primer : 5' – GCCTCCGTTCCGATGAGAACCC – 3'
Reverse Primer : 5' – GCAGTATCCTTAGAATGAGCTTGTGGTC – 3'
cDNA PCR product = 92 bp

or10a:
Forward Primer : 5' – TGGGCGCAATGCTCTATGTGG – 3'
Reverse Primer : 5' – AGTGCTACTTTTCGGCCACCA – 3'
cDNA PCR product = 95 bp

fz:
Forward Primer : 5' – TGCTTGTGGTACGGTGCTCACCTTC – 3'
Reverse Primer : 5' – CGCCACCTTAGCCATCCCAAC – 3'
cDNA PCR product = 114 bp

(A) Amplification of PGawB in 238Y GAL4 mutant by inverse PCR:

5' end amplification:

PGaw2: 5' – CAGATAGATTGGCTTCAGTGGAGAC – 3'
PGaw3: 5' – CGCATGGTCTTAGATGAGAC – 3'

3' end amplification:

Plw3.2: 5' – TAACCCTTAGCATGTCCGTGGGTGTTG – 3'
PRY4: 5' – CAATCATACTGGCTGTCACCTCA – 3'
(B) End sequencing in 238Y GAL4 mutant:

Inverse PCR product:

SP1 (for 5' end sequencing):
5' – ACACAACCTTTCTCTCAACAA – 3'

SPEP1 (for 3' end sequencing):
5' – GACACTCAGAATACATATCC – 3'

Plasmids with insert obtained from Plasmid rescue:

T3 (for 3' end sequencing):
5' – AATTAACCCTCACTAAAGGG – 3'

T7 (for 5' end sequencing):
5' – TAATACGACTCACTATAGGG – 3'

For amplifying and sequencing DNA from excision lines:

FP1: 5' – GCAATGTCTTCTAACGCGAC – 3'

FP2: 5' – CTATTAGCCCTGTCGATTGC – 3'

RP3: 5' – GGCTCTTTAGGACTTAGTAGC – 3'

RP4: 5' – ACTCCCACGAAACACTTAACAC – 3'

2.2.7 Immunohistochemistry:

1X Phosphate-buffered saline (PBS) (500ml):

\[
\begin{align*}
\text{NaCl} & : & 4.0 \text{ g} \\
\text{KCl} & : & 0.1 \text{ g} \\
\text{Na}_2\text{HPO}_4 & : & 0.72 \text{ g} \\
\text{KH}_2\text{PO}_4 & : & 0.12 \text{ g}
\end{align*}
\]

- Dissolve the above salts in 400 ml of distilled water.
- Adjust pH to 7.4 with HCl. Make up volume to 500 ml.
- Filter using Whatman filter paper no. 1
• 1X PBS should be stored at 4 °C and 10X PBS should be stored at room temperature.

4 % Paraformaldehyde (PFA):

Should be prepared fresh before use.

• Take 10 ml of 1X PBS in a Schott 100 ml bottle and heat in microwave for 20 seconds.
• Add 0.4 g of PFA (Sigma-Aldrich), put magnetic bead and stir with heating at 60 °C. PFA is carcinogenic.
• After ~20 minutes, when the PFA is dissolved completely, cool it to room temperature and add 20 μl of Triton® X-100 (Sigma-Aldrich). Stir very slowly for ~15 seconds, avoiding froth formation. Keep on ice to chill.

0.1 % PTX:
(PBS+Triton X-100)
• 0.1 ml Triton X-100 in 100 ml of 1X PBS. Store at 4 °C.

0.1 % PBTX:
(PBS+Triton X-100+BSA)
• 0.1 g Albumin from bovine serum (Bovine Serum Albumin; BSA) (Sigma-Aldrich Cat # A7906) in 100 ml of 0.1 % PTX. Store at 4 °C.

Primary antibody mix (1° Ab) (100 μl):

<table>
<thead>
<tr>
<th>Antibodies/Solvent</th>
<th>Company</th>
<th>Concentration of the stock</th>
<th>Volume taken from the stock</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAbnc82</td>
<td>Developmental Studies Hybridoma Bank (DSHB)</td>
<td>1</td>
<td>10 μl</td>
<td>1:10</td>
</tr>
<tr>
<td>rabbit anti-GFP</td>
<td>Molecular Probes, Invitrogen</td>
<td>1:100</td>
<td>1 μl</td>
<td>1:10,000</td>
</tr>
<tr>
<td>0.1 % PBTX</td>
<td></td>
<td></td>
<td>90 μl</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>101 μl</strong></td>
<td></td>
</tr>
</tbody>
</table>
Secondary antibody mix (2° Ab) (100 μl):

<table>
<thead>
<tr>
<th>Antibodies/ Solvent</th>
<th>Company</th>
<th>Concentration of the stock</th>
<th>Volume taken from the stock</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa 568 goat anti-mouse</td>
<td>Molecular Probes, Invitrogen</td>
<td>1</td>
<td>0.5 μl</td>
<td>1:200</td>
</tr>
<tr>
<td>Alexa 488 goat anti-rabbit</td>
<td>Molecular Probes, Invitrogen</td>
<td>1</td>
<td>0.5 μl</td>
<td>1:200</td>
</tr>
<tr>
<td>0.1 % PTX</td>
<td></td>
<td></td>
<td>100 μl</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>101 μl</td>
<td></td>
</tr>
</tbody>
</table>
2.3 Methods:

2.3.1 Adult behavioral assay:

T-trap assay was used to test the adults for their olfactory behavior (Krishnan, 1996).

Materials for making a T-trap:

Nalgene T-Connectors (7.7 cm long with a stem of 4.2 cm)
Eppendorf Microfuge tubes (1.5 ml)
Tarsons pipette tips (200 μl) cut at tapering end with internal diameter 1.5 mm and at broader end internal diameter 3.27 mm)
Caps (Nunc),
Aluminum Rings (internal diameter of 6.4 mm, and external diameter of 9.87 mm)

Construction of Trap:

The 200 μl pipette tip was cut at 3.1-mm internal diameter and at 1.58-mm internal diameter at the broader end. The tip of the (1.5 ml) microfuge eppendorf tube also was cut according to the size of the pipette tip. The microtip was then fitted into the eppendorf. This is used as the trap.
The traps are connected to the T-connector. The diameter of one arm of the T-connector is slightly bigger (10.7 mm) than the other arm (10.19 mm). To the narrower arm, the trap fits correctly. This arm is called the odor arm. Since the diameter of the other arm is slightly larger, a strip of parafilm was wrapped around the trap at the point of contact, and then it is fit to the other arm. This is used as the escape arm. First the trap was fit to the odor arm, and then to the escape arm.

**Conditioning:**

- 0-12 hours old flies (~400) were collected from the corn meal media bottles into an empty glass bottle.
- To 50 ml of Thorpe's medium, 2 g (4 %) Agar-Agar was added and the mixture was boiled till the agar melted completely giving rise to clear bubbles.
- The solution was cooled a little and then 0.75 g (1.5 %) sucrose was dissolved in it.
- The solution was further cooled, when it was lukewarm, the odor Ethyl acetate at required dilution was added to it. The medium was shaken well to ensure uniform mixing of odor, then was poured into a clean glass bottle and plugged with non-absorbent cotton.
- The bottle with the medium was kept in the freezer compartment of the refrigerator to solidify for ~10 minutes. Then, it was removed and allowed to come to the room temperature by keeping it in open for 3-4 minutes.
- The moisture accumulated on the inner walls of the bottle and over the surface of the solidified medium was wiped off carefully using a piece of tissue paper, to avoid the flies from getting stuck.
- The flies collected from the corn meal media bottles were then transferred into this bottle and maintained at 20 °C in a BOD incubator set at 12 hrs day/night period.
- The same conditioning procedure was done for four days by transferring the same flies into another fresh conditioning bottle every 24 hours.
In the same way, another set of flies should be treated, but without any odor, that will serve as unconditioned flies. After this step, the conditioned and unconditioned flies were either taken to the microscope for cutting and collecting antenna to extract RNA for further experiments, or taken to the further steps for adult behavioral testing.

Separation and Starvation:

- The four days conditioned (or unconditioned separately) flies were transferred into a glass vial and females were separated by anaesthetizing them using cold shock, and six glass vials with 30 females in each were prepared. These six glass vials contained a moist bed of tissue paper moistened with 1 ml of distilled water.
- Then the six glass vials with 30 female flies in each were transferred to the BOD incubator maintained at 20 °C.
- The flies were starved for four hours.

Testing:

- The flies were tested for their olfactory response to Ethyl acetate in the T-trap in white light at 20 °C in a closed room.
- The odor was prepared in 1 % low melting point (LMP) Agarose, in double distilled autoclaved water.
- 315 µl of this solution was then added to the caps of the traps marked ‘O’ with the help of 1 ml pipette. In the same way, LMP Agarose solution without odor was added to the opposite trap caps marked as ‘E’.
- After the LMP Agarose solidified the caps of the traps were closed properly in the same order as it was added to them.
- Overall, six tubes were used in each experiment and the odor arms and the escape arms of the T-mazes were kept in an alternate arrangement to avoid the light induced biasness in the experiment.
- The four hours starved flies were then introduced into the T-maze through their stems using a funnel.
- The number of flies was counted in the odor trap and the escape trap at 2.5 min, 5 min, 7.5 min, 10 min, 15min, 20 min, 25 min, 30 min and 40 min.

Calculation of response:

The olfactory response of the flies to Ethyl acetate was calculated in terms of the following response indices:
Response index 1 (RI1):

It is the difference in the total probabilities of flies entering the odor arm and those entering the escape arm. It is given by,

$$RI_1 = p_o - p_e$$

$$= (N_o - N_e)/30$$

* Where, $p_o$ and $p_e$ are the probabilities of entry into odor trap and the escape trap respectively at the specified time interval (20 or 30 min).
* $N_o$ and $N_e$ are the number of flies in the odor trap and escape trap respectively at the specified time interval (20 or 30 min).

$RI_1$ tells us what fraction of the flies out of the total has been attracted to the odor. Hence, it is a good measure of olfactory response/conditioning of the flies.

Response index 2 (RI2):

It is the difference in the relative probabilities of the flies entering odor arm and those entering the escape arm. It is given by,

$$RI_2 = pr(o) - pr(e)$$

$$= (N_o/N_o + N_e) - (N_e/N_o + N_e)$$

$$= (N_o - N_e)/(N_o + N_e)$$

* Where, $pr(o)$ and $pr(e)$ are the relative probabilities of entry into the odor trap and the escape trap respectively at the specified time interval (20 or 30 min).
* $N_o$ and $N_e$ are the number of flies in the odor trap and escape trap respectively at the specified time interval (20 or 30 min).

$RI_2$ tells us what fraction of the flies out of the total that have participated in the test responded to the odor by attraction. However, this index does not tell anything about the number of the flies that have actually participated in the test i.e. which have been conditioned.
2.3.2 Larval plate test:

A modified version of larval plate test (Khurana, 2003) was used for testing the olfactory behavior of the larvae. (Fig. 2.3)

![Fig. 2.3 Schematic of the Larval plate test paradigm](image)

- The flies were transferred to a fresh corn meal agar media bottle and allowed to lay eggs for 12 hours at 25 °C. The early third instar larvae were used for experiments.
- 10 ml of Ringer's-Agar (1 %) was poured in each 9 cm glass Petri-dish (testing plate) and after solidification kept in the larval behavior testing room maintained at 24 °C.
- Early third instar larvae were separated from corn meal agar media using water (at 25 °C) and 30 % Polyethylene Glycol (PEG) in a sieve.
- The separated larvae were kept in a glass Petri-dish with 1 ml of Ringer's solution and brought to the larval behavior testing room.
- The testing plate is kept on the template, and two round filter discs are placed in the two zones O1 and O2.
- 50 larvae were taken using a soft brush (No. 000), dried on a filter paper, and transferred to the centre of the circular zone in the testing plate kept over the template.
- 20 μl of odor is dispensed on each of the two filter discs and the lid of the testing plate is kept back quickly.
- After two minutes of testing, the numbers of larvae were counted in different zones of the testing plate (odor zone O1, odor zone O2 and control zone C).
- The response index (R.I.) is calculated using the following formula:

\[
R.I. = \frac{O1 + O2}{O1 + O2 + C} \times 100
\]

The response index was calculated from the total of six plates for each experiment, and for any point, at least four such experiments were done and the error was calculated as standard error of mean.

2.3.3 Electroshock conditioning of larvae:

For electroshock conditioning of larvae, a modified method described by Khurana (2003) was used. (Fig. 2.4)

![Fig. 2.4 Schematic of the electroshock conditioning apparatus for larvae](image)

- 10 ml of 20 mM LiCl·Agarose (1.5 %) was poured in a 9 cm Petri dish (shocking plate). Before solidification, two silver electrodes (6 cm x 2 cm x 0.08 cm) were placed at the opposite ends of the plate, using alligator clips. There were two other alligator clips holding these two electrodes and connecting to the AC power source of 110 volts, 50 Hz.
After partial solidification of the LiCl-Agarose, one hollow plastic cylinder (1.5 cm height x 4.5 cm diameter) was placed in the centre for the bigger Petri dish. This cylinder was made by cutting the bottom of a 4.5 cm plastic Petri dish. 2 ml of 20 mM LiCl-Agarose was poured inside the cylinder to seal the gaps at the bottom.

~200 larvae were placed inside the hollow cylinder and 500 µl of 20 mM LiCl was dispensed into that.

The odor was placed as a drop, in the center of the lid of the cut 4.5 cm Petri dish. The odor was exposed for one minute, but after 30 seconds of presenting the odor, the electric current was switched on. And after 30 seconds, current was switched off and the lid with odor was removed simultaneously. So, the larvae feel both odor and shock in the last 30 seconds (odor associated with shock).

The hollow cylinder was covered with a fresh lid (without any odor) and the larvae were given rest for 5 minutes.

The same cycle of training and rest was repeated for required number of training cycles.

After the training, larvae were transferred using a thick brush to a fresh 9 cm glass Petri dish containing little Ringer’s solution. The trained larvae were tested in the previously described larval plate test paradigm.

**Schematic of the shock protocol:**

Response Learning

```
1 minute       1 minute
Odor A       Odor A
```

```
30 sec
Shock
```

```
Rest interval (5')
```

The learning index (L.I.) is defined as the fractional decrease in response index (R.I.) after conditioning, and could be calculated by using the following formula –
Response Learning Index (L.I.) = \frac{R.I. \text{ (unconditioned)} - R.I. \text{ (conditioned)}}{R.I. \text{ (unconditioned)}}

2.3.4 Molecular Biology:


Plasmid Rescue:

Extraction of genomic DNA from flies:

Homogenization and lysis:

- Take 30 anaesthetized flies in a 1.5 ml eppendorf tube.
- Freeze the tube by dipping in liquid N\textsubscript{2}.
- Homogenize in 300 \mu l of chilled homogenization buffer using a polypropylene pestle homogenizer. Crush gently till cuticle of the fly is visibly broken into small pieces. Leave on ice.
- Add 300 \mu l of lysis buffer. Mix gently by inversion.

RNase treatment:

- Add 10 \mu l of RNase A (from 10 mg/ml stock). Mix gently. Incubate at 37 °C for 30 minutes.

Proteinase treatment:

- Add 5 \mu l of Proteinase K (from 20 mg/ml stock). Mix gently. Incubate at 37 °C for 30 minutes.
PhenoL'Chloroform/Isoamyl alcohol extraction:

- Extract at least two times (optimum three times) with equal volume of Phenol : Chloroform : Isoamyl alcohol (25:24:1).
  Add 600 µl of Phenol : Chloroform : Isoamyl alcohol.
  Mix by inverting 10-15 times until the aqueous layer turns milky.
  Spin at 13000 rpm for 10 minutes at room temperature.
  Separate aqueous phase (top layer) and collect ~550 µl of aqueous phase.
  Add 550 µl of Phenol : Chloroform : Isoamyl alcohol.
  Repeat as above.
  Collect ~500 µl of aqueous phase.

Chloroform extraction:

- Add 500 µl of Chloroform.
  Mix by inverting 10-15 times vigorously for two minutes.
  Spin at 13000 rpm for 5 minutes at room temperature.
  Collect top 450 µl of aqueous layer.
  Repeat as above.

Alcohol precipitation:

- Add 900 µl (two volumes) of absolute alcohol.
  Mix gently, DNA precipitates.
  Let stand on ice for 15 minutes.
  Spin at 13000 rpm for 10 minutes.
  Discard ethanol.
  Add 600 µl of 70 % alcohol for washing.
  Rinse briefly.
  Spin at 13000 rpm for 15 minutes at room temperature.
  Discard ethanol.
  Remove excess with pipette.
  Leave at room temperature for ~20 minutes to evaporate.
  Resuspend the pellet in 30 µl of sterile water by leaving stand for 5 minutes and tapping. About 0.5 µg/µl of DNA is obtained. Store at 4 °C.

Digestion of genomic DNA (50 µl):

Set up the following digestion reaction (with a PL3/cloning enzyme):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA (~2 flies)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>10 X buffer</td>
<td>5.0 µl</td>
</tr>
</tbody>
</table>
Sterile water 37.5 µl
BSA 0.5 µl
Restriction enzyme (Xho I) 2.0 µl

- Incubate in water bath at 37 °C for 3 hours.
- Heat the tube at 65 °C for 20 minutes on heating block to stop the reaction.
- Check 10 µl of the reaction on 0.8 % TBE-agarose gel.

Precipitation of digested genomic DNA:

- Add 60 µl of sterile water to the leftover 40 µl of digested genomic DNA reaction.
- Put on the heating block at 42 °C.
- Extract once with Phenol: Chloroform : Isoamyl alcohol (25:24:1)
  Add 100 µl of Phenol : Chloroform : Isoamyl alcohol
  Vortex for 20 seconds.
  Spin at 13000 rpm for 10 minutes at room temperature.
  Collect 90 µl of supernatant in a fresh tube.
- Extract once with Chloroform
  Add 180 µl of Chloroform.
  Vortex for 20 seconds.
  Spin at 13000 rpm for 5 minutes at room temperature.
  Collect 80 µl of aqueous top layer in a fresh tube.
- Precipitation of the digested DNA
  Add 9 µl of 3 M Sodium acetate (pH 5.0)
  Add 180 µl of chilled absolute alcohol.
  Mix gently.
  Keep on ice bath for 30 minutes.
  Spin at 13000 rpm for 30 minutes at 4 °C.
  Discard the supernatant.
  Rinse with 100 µl of 70 % alcohol.
  Spin at 13000 rpm for 15 minutes at 4 °C.
  Remove ethanol.
  Spin briefly again.
  Remove traces of ethanol.
  Let air dry for ~20 minutes at room temperature.
  Re-dissolve the pellet in 20 µl of sterile water.

Ligation of the digested genomic DNA (200 µl):

Set up the following ligation reaction:
- Digested genomic DNA 10.0 µl
- 10 X ligation buffer 20.0 µl
Sterile water 169.0 μl
T4 DNA Ligase 1.0 μl

- Incubate at 16 °C for 16 hours.

Precipitation of the ligated DNA:
- Add 20 μl of 3 M Sodium acetate (pH 5.0)
- Add 400 μl of chilled absolute alcohol.
- Invert mix gently.
- Leave over night at -20 °C.

Pelleting the ligating DNA:
- Spin at 13000 rpm for 30 minutes at 4 °C.
- Discard the supernatant.
- Rinse with 100 μl of 70 % alcohol.
- Spin at 13000 rpm for 30 minutes at 4 °C.
- Remove ethanol.
- Spin briefly again.
- Remove traces of ethanol.
- Let air dry at room temperature for ~20 minutes.
- Re-dissolve in 5 μl of sterile water.

Transformation and analysis:
- Thaw out XL 10 Gold E. coli electrocompetent cells on ice for 15 minutes.
- Add ligated DNA at volume no greater than 4 μl to 40 μl of electrocompetent cells. Mix gently with pipette keeping it cooled.
- Transfer the content to the bottom of sterile Bio-Rad 0.2 cm electroporation cuvette kept on ice, remove any air bubble.
- Wipe out the moisture from the outer surfaces of the cuvette using a tissue paper, and pulse (electroporate) at the following settings -
  Voltage 2.5 KV
  Resistance 200 Ω
  Capacitance 25 μF
Ideal time constant obtained: 4.5 seconds
- Immediately add 1 ml SOC medium containing 20 mM Glucose kept at room temperature. Mix gently using pipette. Addition of medium at room temperature provides a heat shock that increases the efficiency of transformation.
- Transfer to 1.5 ml sterile eppendorf tube and incubate at 37 °C with mild shaking for one hour.
Plate out required dilution (up to 200 µl per 90 mm plate) on SOB-Agar medium containing 20 mM MgSO₄ and the appropriate antibiotics.

Pouring LB-Agar plates:

For XL 10 Gold E. coli cells –
Melt the LB-Agar medium and cool it to ~50 °C – 60 °C, and then add
- Tetracycline 2 ml in 200 ml of LB-Agar
- Chloramphenicol 148 µl in 200 ml of LB-Agar
- Ampicillin 400 µl in 200 ml of LB-Agar

Pick up at least 6 independent transformants per each line and re-streak on fresh LB-Agar plates with appropriate antibiotics added like above.

Incubate at 37 °C till well separated colonies are seen.

Plasmid miniprep from the transformants:

- Inoculate single colonies from each of the 6 independent transformants into 5 ml of LB-antibiotics medium in a culture tube.
- Grow over night at 37 °C in a mild incubator shaker.
- Chill culture on ice for 15 minutes.
- Transfer 1.5 ml of culture to sterile eppendorf tube.
- Harvest cells by spinning at 13000 rpm for 30 seconds at room temperature.
  Decant supernatant.
  Again transfer 1.5 ml of culture to the same tube.
  Spin at 13000 rpm for 30 seconds at room temperature.
  Decant supernatant.
  Re-spin cells for 30 seconds.
  Remove remaining LB medium.
  Leave tubes on ice.
- Add 150 µl of Solution I.
  Resuspend the bacterial pellet by vortexing.
  Vortex vigorously till cells are mixed properly.
  Leave on ice till chilled down (< 5 minutes).

Make Solution II in mean time as follows –
For 1 ml, add
- 10 N NaOH 20 µl
- 10% SDS 100 µl
- Sterile water 880 µl
• Add 300 µl of freshly prepared Solution II. Pour the Solution II very slowly with the wall of the tube and invert mix very gently for ~5 times.
  Leave on ice for 10 minutes till slight precipitation appear.
• Add 250 µl of Solution III, it leads to clump formation. Invert mix very gently. Leave on ice for 10 minutes.
• Pellet cell lysate by spinning at 13000 rpm for 5 minutes at room temperature. The white crap settles down.
  Carefully collect collect ~650 µl of supernatant avoiding the white crap completely. If white material is still visible in supernatant, re-spin the supernatant to remove the white material completely.

RNase treatment:

  Add 10 µl of RNase A (from 10 mg/ml stock). Vortex for a second to mix it properly.
  Incubate at 65 °C for 30 minutes on heating block. This step can be done at 37 °C as well.
• Add 650 µl of Phenol : Chloroform : Isoamyl alcohol (25:24:1).
  Mix by vortexing for 30 seconds. Also, mix thoroughly with jerks.
  Spin at 13000 rpm for 5 minutes at room temperature.
  Collect 600 µl of aqueous layer in a fresh tube.
  Repeat the above process.
  Collect 550 µl of aqueous layer in a fresh tube.
• Add 550 µl of Chloroform.
  Mix by vortexing for 30 seconds. Also, mix thoroughly with jerks.
  Spin at 13000 rpm for 5 minutes at room temperature.
  Collect 500 µl of aqueous layer in a fresh tube.
  Repeat the above process for two more times.
  After the third processing, collect 400 µl of aqueous layer in a fresh tube.
• Add 2 volumes (800 µl) of chilled absolute alcohol slowly.
  Mix gently by inversion.
  Leave at -20 °C for one hour or over night.
  Spin at 13000 rpm for 30 minutes at 4 °C. This spin can be done at room temperature as well.
  Decant ethanol.
• Add 400 µl of 70 % alcohol.
  Rinse pellet by tapping and inverting repeatedly, can be vortexed as well.
  Spin at 13000 rpm for 10 minutes at 4 °C. This spin can be done at room temperature as well.
  Remove ethanol.
  Re-spin briefly and remove excess ethanol with pipette.
Air dry the pellet at room temperature for ~20 minutes.
Resuspend in 20 µl of sterile water. Mix well by pipetting and tapping.
- Check the plasmid DNA obtained by digesting with cloning enzyme
  and running on a mini gel. A single band of size more than 3 Kb (size
  of pBlueScript plasmid) should be seen.

Plasmid DNA digestion (20 µl):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>10 X Buffer</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>14.3 µl</td>
</tr>
<tr>
<td>BSA</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Restriction enzyme (Xho I)</td>
<td>1.5 µl</td>
</tr>
</tbody>
</table>

- Double digest with the cloning enzyme and a PL4 enzyme to release
  the insert.

Preparation of electrocompetent cells:

- Inoculate a single colony of XL-10 Gold *E. coli* from a fresh Agar plate
  into a flask containing 50 ml LB media with antibiotics.
- Incubate the culture over night at 37 °C in a rotary shaker maintained
  at 250 rpm.
- Inoculate 25 ml each in two 500 ml pre-warmed LB media with antibiotics
  in conical flasks.
- Incubate at 37 °C in a shaker maintained at 250 rpm till the OD₆₀₀
  reaches to 0.4 (~2.5 hours).
- Rapidly transfer the flasks in ice-water bath. Keep for ~15-30 minutes.
  Swirl occasionally.
- Pellet down the bacterial cells by transferring the culture repeatedly
  into 250 ml pre-chilled centrifuge bottles, and by spinning at 2500 rpm
  for 15 minutes at 4 °C.
- Re-suspend the pellet in 250 ml of ice-cold sterile double distilled
  water. Swirl mix properly to wash the bacterial cells, maintaining the
  low temperature.
- Spin at 2500 rpm for 15 minutes at 4 °C. Repeat the above process.
- Re-suspend the pellet in 250 ml of ice-cold sterile 10 % Glycerol. Swirl
  mix properly maintaining the low temperature.
- Spin at 2500 rpm for 15 minutes at 4 °C. Repeat the above process.
- Re-suspend the pellet in 10 ml of ice-cold sterile 10 % Glycerol. Swirl
  mix properly maintaining the low temperature.
- Spin at 2500 rpm for 20 minutes at 4 °C.
• Decant the Glycerol from the bottles and re-suspend the pellet into the remaining Glycerol in the bottle by swirling, maintaining the low temperature.

• Make 40 µl aliquots of the cells in pre-chilled sterile eppendorf tubes and using cooled sterile pipette tips. Keep the tubes on ice.

• 40 µl checked in ice-cold electroporation cuvette for arcing.

• Immerse the tubes in liquid N₂ and transfer to a -80 °C freezer till further use for electroporation (transformation).

Storage of bacterial cultures growing in liquid media:

• To 1.5 ml of bacterial culture, add 0.5 ml of sterile 60% Glycerol.

• Vortex the culture to ensure that the glycerol is evenly dispersed.

• Transfer the culture to a labeled cryo-vial.

• Freeze the culture in liquid N₂ and transfer to a -80 °C freezer for long term storage.

• To recover the bacteria, scrap the frozen surface of the culture with a sterile inoculating loop, and then immediately streak the bacteria that adhere to the needle on to the surface of an LB-Agar plate containing the appropriate antibiotic(s).

• Return the frozen culture to storage at -80 °C.

• Incubate the plate over night at 37 °C.

Storage of bacterial cultures growing on Agar plates:

• Scrap the bacteria growing on the surface of an Agar plate into 2 ml of LB medium in a sterile tube. Add an equal volume of LB medium containing 30% sterile Glycerol.

• Vortex the mixture to ensure that the Glycerol is completely dispersed.

• Dispense aliquots of the culture into labeled cryo-vials.

• Freeze the culture in liquid N₂ and transfer to a -80 °C freezer for long term storage.

Inverse PCR:

Quick fly genomic DNA preparation from flies:

(modified from the methods described by E. Jay Rehm and Roger Hoskins of BDGP)

• Take 30 anaesthetized flies in a 1.5 ml eppendorf tube.

• Freeze the tube by dipping in liquid N₂.
- Grind flies gently, without shearing genomic DNA, in 200 µl of Buffer A with polypropylene homogenizer on ice.
- Add an additional 200 µl of Buffer A and continue grinding on ice, until only cuticles remain.
- Incubate at 65 °C for 30 minutes on dry heating block.
- Add 800 µl of LiCl/KAc solution, invert mix several times slowly.
- Incubate on ice for at least 10 minutes.
- Spin at 12000 rpm for 15 minutes at room temperature.
- Transfer 1 ml of the supernatant into a fresh tube, avoiding floating crud.
  If crud transfers, re-spin to exclude the crud completely.
- Add 600 µl of Isopropanol and invert mix very slowly for several times.
- Spin at 12000 rpm for 20 minutes at room temperature.
- Aspirate away supernatant, leaving the pellet intact.
  Quick spin it again and aspirate using pipette.
- Wash pellet with 500 µl of 70 % alcohol.
- Spin at 12000 rpm for 10 minutes at room temperature.
- Aspirate and discard the supernatant.
  Quick spin again and aspirate using pipette.
- Air dry pellet for ~30 minutes at room temperature.
- Resuspend the pellet in 150 µl of TE buffer (pH 8.0).
  Leave it over night at room temperature to dissolve the pellet completely.

Digestion of genomic DNA (25 µl):

Set up the following digestion reaction (with DpnII restriction enzyme):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA (~2 flies)</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>10 X buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>9.5 µl</td>
</tr>
<tr>
<td>RNase A</td>
<td>2.0 µl (from 100 µg/ml stock)</td>
</tr>
<tr>
<td>Dpn II</td>
<td>1.0 µl (5-10 Units)</td>
</tr>
</tbody>
</table>

- Incubate at 37 °C in water bath for 3 hours.
- Heat the tube at 65 °C for 20 minutes on heating block to stop the reaction.
- Check 5 µl of the reaction on 0.8 % TBE-agarose gel.

Ligation of the digested genomic DNA (400 µl):

Set up the following ligation reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA digestion reaction (~1 fly)</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>10 X ligation buffer</td>
<td>40.0 µl</td>
</tr>
</tbody>
</table>
Sterile water 346.0 µl
T4 DNA Ligase (2 Weiss Units) 4.0 µl

- Incubate at 16 °C for 16 hours.
- Add 40 µl of 3 M Sodium acetate (pH 5.0) and 1 ml of absolute alcohol.
  Invert mix gently and chill for 1 hour at -80 °C.
- Spin at 16000 rpm for 30 minutes at 4 °C.
- Decant supernatant and aspirate the remaining alcohol using pipette.
- Air dry the pellet at room temperature for ~20 minutes.
- Resuspend in 150 µl of TE buffer (pH 8.0). Keep it for at least one hour
  at room temperature to dissolve the pellet completely.

Inverse PCR reaction (50 µl):

Set up the following PCR reaction (to amplify the 5' end):

Sterile water 33.0 µl
10 X PCR buffer 5.0 µl (1 X final concentration)
10 mM dNTP mix 2.0 µl (0.4 mM final concentration)
10 µM Primer PGaw2 2.0 µl (0.4 µM final concentration)
10 µM Primer PGaw3 2.0 µl (0.4 µM final concentration)
Taq polymerase 1.0 µl (5 Units)
Ligated genomic DNA 5.0 µl

PCR cycling parameters:

<table>
<thead>
<tr>
<th>Reaction Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
</tr>
<tr>
<td>15 mins</td>
</tr>
<tr>
<td>Denaturation</td>
</tr>
<tr>
<td>Extension</td>
</tr>
<tr>
<td>Final Extension</td>
</tr>
</tbody>
</table>
• Check 5 µl of PCR reaction on 1% TBE-agarose gel.
• Sequence with SP1 primer (for 5' end).
• To amplify the 3' end, use Plw3-2 and PRY4 primers set with Ta as 55 °C.
• Sequence with SPEP1 primer (for 3' end).

Gel extraction of DNA for sequencing:

The DNA (the PCR product) was extracted from the agarose gels using QIAquick Gel Extraction Kit (Qiagen) following company's protocol with some modifications.

• The DNA fragment was excised from the agarose gel using a clean, sharp scalpel. The gel slice was kept in an eppendorf tube and weighed.
• 3 volumes of the buffer QG was added to 1 volume of gel (100 mg ~ 100 µl). The tube was incubated at 50 °C until the gel slice had completely dissolved (~10 minutes), by vortexing every 2-3 minutes during the incubation.
• After the gel slice was dissolved completely, 10 µl of 3 M Sodium acetate (pH 5.0) was added and mixed.
• 1 gel volume of Isopropanol was added to the sample and invert mixed gently.
• QIAquick spin column was placed in the provided 2 ml collection tube, and the sample was applied to the column and centrifuged at 13000 rpm for 1 minute at room temperature.
• The collection tube was emptied and the column was kept back in the collection tube. 500 µl of the buffer QG was added to the column and centrifuged at 13000 rpm for 1 minute at room temperature.
• To wash the column, 750 µl of the buffer PE was added to the column and kept for 5 minutes, then was centrifuged at 13000 rpm for 1 minute at room temperature.
• The collection tube was emptied and the column was kept back in the collection tube, and centrifuged for one more minute at 13000 rpm at room temperature. The collection tube was discarded and the column was placed in a fresh 1.5 ml eppendorf tube.
• To elute DNA from the column, 30 µl of the buffer EB (10 mM Tris Cl, pH 8.5) was added directly to the center of the membrane in the column and kept for 5 minutes, then was centrifuged at 13000 rpm for 1 minute at room temperature.
• Then the column was discarded and the eluted DNA was stored at -20 °C or used for further experiments or sequencing.
Quantitative Reverse Transcription PCR (RT-PCR):

Total RNA Extraction from fly antennae:

- Detach and collect 200 antennae on ice bath, directly merge into 200 µl of Trizol in a 1.5 ml eppendorf tube kept on ice.
- Spin at 15000 rpm for 10 minutes at 4 ºC to pellet down the antennae.
- Homogenize with polypropylene pestle on ice till all the antennae are crushed, and the solution is almost clear and uniform.
- Wash the pestle with another 500 µl of Trizol into the same eppendorf tube.
  Invert mix properly.
  Leave on ice for 10 minutes.
- Add 200 µl of Chloroform (~0.2 volume).
  Shake and mix the solution vigorously by hand and then incubate on ice for 3 minutes.
- Spin at 15000 rpm for 15 minutes at 4 ºC.
- Transfer the aqueous phase to a fresh tube.
- Add 500 µl of Isopropanol.
  Invert mix gently.
  Incubate at room temperature for 15 minutes.
- Spin at 15000 rpm for 10 minutes at 4 ºC.
- Decant the supernatant.
  Wash the RNA pellet once with 1 ml of 75 % ethanol, break the pellet, mix the sample by vortexing for maximum salt dissolution.
- Spin at 15000 rpm for 5 minutes at 4 ºC.
- Decant the supernatant. Aspirate away the remaining ethanol with pipette.
- Air dry the pellet at room temperature for ~20 minutes. Do not over dry.
- Resuspend the pellet in 30 µl of DEPC-treated water. Pass water several times through pipette tip to aid in dissolving.
- Incubate at 60 ºC for 10 minutes on heating block.
- Quantify and store at -20 ºC (short-term) or -80 ºC (long-term).

DNase I treatment (30 µl):

Set up the following reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA extracted</td>
<td>x µl (up to 10 µg taken)</td>
</tr>
<tr>
<td>10 X DNase I reaction buffer</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>DNase I enzyme</td>
<td>y µl (1U/µg of total RNA)</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>to make up to 30 µl</td>
</tr>
</tbody>
</table>

- Incubate at 37 ºC for three hours in water bath.
• Inactivate the DNase I enzyme by addition of 3 µl of 20 mM EGTA (DNase stop solution) to a final concentration of 2 mM. Mix properly using pipette.
• Heat at 65 °C for 10 minutes on heating block. Cool at room temperature, quick spin to let the stuff down from the walls of the eppendorf tube.
• Add 3 µl of 3 M Sodium acetate (pH 5.2) (1/10th volume). Add 75 µl of absolute alcohol (2.5 volumes). Mix gently by pipette. Keep at -80 °C for 2 hours.
• Spin at 14000 rpm for 20 minutes at 4 °C. Decant the supernatant and wash the pellet with 500 µl of 75 % ethanol, break the pellet, mix the sample by little vortexing for maximum salt dissolution.
• Spin at 14000 rpm for 10 minutes at 4 °C.
• Remove the supernatant.
Air dry the pellet at room temperature for ~20 minutes. Resuspend the pellet in 16 µl of DEPC-treated water. Either use this DNase I treated RNA for further reverse transcription reaction or store at -80 °C.

Reverse Transcription (RT):

• Add –
  2.0 µl of Random Primers (600 ng)
  16.0 µl of DNase I treated RNA
• Mix properly by pipette and heat at 65 °C for 5 minutes on heating block. Quick chill on ice (>5 minutes). Collect the contents of the tube by brief spin for 10 seconds.
• Add –
  5 X First-strand buffer 8.0 µl (1 X final concentration)
  10 mM dNTP mix 4.0 µl (1 mM final concentration)
  0.1 M DTT 4.0 µl (10 mM final concentration)
  Ribonuclease Inhibitor 4.0 µl (1 U/10 µl reaction or 4 U/reaction)
• Mix contents of the tube gently, quick spin, and incubate at 37 °C for 2 minutes in water bath.
• Divide the content of the tube into two with 19 µl in each tube.
• Add 1 µl of M-MLV Reverse Transcriptase (RT) in one of the tubes and 1 µl of DEPC-treated water in the other tube (no RT or negative control). Mix by pipetting gently up and down.
• Incubate the tubes at 25 °C for 10 minutes on heating block.
• Incubate the tubes at 37 °C for 2 hours in water bath.
- Quick spin to collect the contents of the tube.
- Inactivate the reaction by heating at 70 °C for 15 minutes on heating block.
- Cool it on ice. Quick spin to collect the contents of the tube.
- Add 2 µl of 10 X RNase H buffer and then 1 µl of RNase H enzyme (1.5 U/µl) to each tube.
- Incubate at 37 °C for 30 minutes in water bath.
- Use directly for PCR or freeze at -20 °C or -80 °C.

Set up the following PCR reaction (25 µl):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR grade water</td>
<td>16.5 µl</td>
</tr>
<tr>
<td>10 X PCR buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>10 µM forward primer</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>10 µM reverse primer</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>Deep VentR™ (2U/µl)</td>
<td>0.5 µl</td>
</tr>
</tbody>
</table>

**PCR cycling parameters:**

![PCR Cycling Parameters Diagram]
Real Time PCR:

- First make serial dilutions of cDNA as $1:10$, $1:100$, $1:1000$, $1:10000$.
  Mix properly and keep it aside for further use.

Set up the following Real Time PCR reaction (20 μl):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR grade water</td>
<td>3.8 μl</td>
</tr>
<tr>
<td>10 μM forward primer</td>
<td>0.6 μl</td>
</tr>
<tr>
<td>10 μM reverse primer</td>
<td>0.6 μl</td>
</tr>
<tr>
<td>Sybr Green Mix</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>cDNA diluted</td>
<td>5.0 μl</td>
</tr>
</tbody>
</table>

- Sybr Green should be mixed properly before using.
- cDNA should be added last into the cocktail of Sybr Green and primers, and mixed properly.
- The reactions should be set up in minimum light conditions as the Sybr Green is light sensitive.

Real Time PCR Cycling Profile:

```
<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>10 mins</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 secs</td>
</tr>
<tr>
<td>Annealing temp (Ta)</td>
<td>55 °C</td>
<td>30 secs</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>30 secs</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>5 mins</td>
</tr>
</tbody>
</table>
```
<table>
<thead>
<tr>
<th>Cycle</th>
<th>Cycle Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold @ 95°c, 10 min 0 secs</td>
<td></td>
</tr>
<tr>
<td>Cycling (15 repeats)</td>
<td>Step 1 @ 95°c, hold 30 secs</td>
</tr>
<tr>
<td></td>
<td>Step 2 @ 55°c, hold 30 secs</td>
</tr>
<tr>
<td></td>
<td>Step 3 @ 72°c, hold 30 secs,</td>
</tr>
<tr>
<td></td>
<td>Acquiring to</td>
</tr>
<tr>
<td></td>
<td>Cycling A([Green][l][l])</td>
</tr>
<tr>
<td>Hold 2 @ 72°c, 5 min 0 secs</td>
<td></td>
</tr>
<tr>
<td>Hold 3 @ 50°c, 1 min 0 secs</td>
<td></td>
</tr>
<tr>
<td>Melt (50-99°) , hold 30 secs on the 1st step, hold 5 secs on</td>
<td></td>
</tr>
<tr>
<td>next steps, Melt A([Green][l][l])</td>
<td></td>
</tr>
</tbody>
</table>

2.3.5 Immunostaining:

Immunostaining of adult fly brain:

- Take ~10 anaesthetized flies and submerge them completely in 70 % alcohol (dab) using soft brush. Keep for one minute.
- Transfer the flies to an eppendorf tube containing 1 ml of chilled 4 % PFA (freshly prepared), submerge the flies completely, and keep the tube on ice for ~ 3 hours (fixation).
- Take out the tube from ice, invert mix little, decant the PFA carefully and add 1 ml of 0.1 % PTX, invert mix slowly and decant the PTX (its first washing).
- Again add 1 ml of 0.1 % PTX, invert mix little and keep on gyratory shaker at low speed for 15 minutes (second washing).
- Repeat the above step for two more times (third and fourth washing). Decant the PTX and add little of 0.1 % PBTX, keep on ice.
- Dissect out brains and keep in a glass cavity block with 200 μl of PBTX (blocking), keep on rotary shaker (50 rpm) for ~15 minutes (first washing).
- Repeat the above washing step for three more times.
- After the fourth wash, take out PBTX using pipette, and add ~85 μl of primary antibody mix, all the brains should float in this. Put the cavity block in an empty slide box (to keep it in dark), keep on shaker (~50-55 rpm) at 4 °C for ~16 hours.
- Remove the primary antibody from the cavity block, put 400 μl of PBTX, wash and remove it. Then add 200 μl of PBTX and keep on shaker (55 rpm) at room temperature for 20 minutes (first washing).
- Repeat the above washing step for three more times.
• After the fourth wash, take out the PBTX using pipette, and add ~70 µl of secondary antibody mix, all the brains should float in this. Keep on shaker (55 rpm) at room temperature for 3 hours.
• Add 400 µl of PTX, wash, and remove the PTX. Then add 200 µl of PTX, wash and keep on shaker (55 rpm) at room temperature for 15 minutes (first washing).
• Repeat the above washing step for three more times.
• Mount the brain in 80 % Glycerol or Vectashield (Vector Labs, Peterborough, U.K.).
• Image on a Olympus (Tokyo, Japan) FV1000 confocal microscope, at 1 µm intervals, data processing using Confocal Assistant 5.0, Image J 1.37a (Wayne Rasband, NIH, USA) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

2.3.6 Computational tools:

Genome analysis:

FlyBase (http://flybase.bio.indiana.edu/)
Berkeley Drosophila Genome Project (BDGP) (http://www.fruitfly.org/)
Ensemble (http://www.ensembl.org/index.html)
UCSC Genome Bioinformatics (http://genome.ucsc.edu/)
European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/)
ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/)
Clustalx Version : 1.83 (http://bioinformatics.ubc.ca/resources/tools/clustalx)
TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html)
Primer designing:

Primer3 (http://fokker.wi.mit.edu/primer3/input.htm)

Oligo Explorer (http://www.genelink.com/tools/gl-oe.asp)

Sequence analysis by Basic Local Alignment Search Tool (BLAST):

FlyBase BLAST (http://flybase.net/blast/)

NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

WU-BLAST2 (http://www.ebi.ac.uk/blast2/)