Colorimetric *in-situ* for *Drosophila* pupal genitalia

Nuclease Free H20
GE SH30538.02

Formamide
Molecular Biology grade
Fisher BP227-100, kept at 4C

Heparin sodium salt
Sigma-Aldrich H4784-1G

Tween-20
Fisher BP337-500

EtOH 200 proof
Decon Laboratories #2716

MeOH
Fisher A412P-4

16% Paraformaldehyde
EMS #15710

NBT
Promega S380C

BCIP
Promega S381C

BSA
Sigma A2153-100G

Glycerol
EMD Millipore 56-81-5

PBT (1L)

10X PBS pH 7.2	100mL
TritonX-100	1mL
ddH2O	to 1L

1% w/v BSA in PBT in 4°C
= 1g BSA for 100mL sol'n,

BSA : Sigma A7888

Hybe (200 mL) - kept in -20

Formamide	100 mL
20X SSC, pH 4.2	50 mL
ssDNA (10mg/ml)	2 mL
Heparin	.02g
TritonX-20	0.2 mL
Nuclease free H20	to 200mL

Anti-DIG AP - 4°C
Roche 11093274910

Here we describe an in situ protocol that uses the InSitu Pro robot from Intavis. If performing in situ hybridization by hand, see the accompanying protocol that elucidates the steps performed by the robot.

Start up *in situ* robot

Turn on robot and heating block.

Turn on computer.

Open InSituPro program and select the correct method. *Contact the Rebeiz lab if you need the file.*

Aliquot pupal genitalia

Take microcentrifuge tube with pupal genitalia out from the -20C.

Transfer pupal genitalia with 200µL tips into a clean glass viewing dish in 100% EtOH. Separate samples if they are stuck together using forces or tungsten wires. *We use P200 pipettes to transfer samples.*

Set up Intavis baskets in a clean tissue plate.

Aliquot samples into baskets with 600ul of 100% EtOH and leave at room temperature. *We usually aliquot 10 samples for each time point to compare replicates. Make sure to keep the tube containing pupal genitalia on ice so that quality doesn't decrease with repeated aliquoting.*

Run *in situ* robot

Add all regents to the *in situ* robot.

Remove the baskets from the tissue culture plate containing the baskets and immediately add to the robot.

Start the robot. *The protocol generally takes 2-3 days to complete depending on sample number.*

Take samples out of the robot

Remove baskets from the robot.

Move the baskets to a clean tissue plate and add PBT until the bottom half of the basket is submerged.

Transfer the pupal genitalia from the baskets to a clean glass viewing dish. *We use P200 pipettes to transfer samples.*

Transfer the pupal genital discs from the clean glass viewing well to a micro-centrifuge tube or PCR tube.

Store at 4C until ready for colorimetric reaction. *We try to perform the colorimetric reaction within 24 hours after the robot completes the method.*

Reagents cont'd

Staining Buffer [make fresh]

5mM NaCl	1mL
50 mM $MgCl_2$	2.5mL
100mM Tris HCl pH 9.5	5mL
Tween-20	0.050mL
H_2O	to 50mL

Staining solution [make fresh]

Staining Buffer	4.5mL
NBT	0.0612mL
BCIP	0.01575mL

Glycerol Mountant

0.1 M Tris Hcl pH 8.0	10mL
Glycerol	40mL

Coverslips
Fisher 12-542A 18x18mm #1

Slides
Fisher 12-550-343

Poly-L-Lysine
Thermo scientific 86010

*Quick wash = add wash solution and wait until pupal genitalia settle to do the next addition of wash solution.

Prepare for the colorimetric reaction

In the morning, make fresh aliquots of staining buffer and staining solution.

Run colorimetric reaction

Transfer the pupal genitalia to a clean glass viewing dish with 200µL pipet tip.

3 quick washes of 200µL Staining Buffer.

Remove all liquid and add 0.4mL staining solution.

Cover the samples and check every 20 mins for purple staining (total staining time can be 20mins - 4hours+ depending on probe).

To stop the reaction, wash 2X quick with 400µL Staining Buffer.

Wash 2X quick with 200µL PBT.

Add 500µL of Glycerol Mountant and mix.

Transfer pupal genitalia to micro-centrifuge tubes or PCR tubes.

Store at room temperature. *While samples can be mounted immediately after the colorimetric reaction is complete, we usually wait until the following day.*

Prepare slides for mounting samples

Place 2 layers of double sided tape on a slide and use a razor to cut a square well in the middle.

Add Poly-L-Lysine into the well and then remove some of the liquid, leaving behind a thin layer.

Let the Poly-L-Lysine dry (~5mins)

Transfer samples from the micro-centrifuge or PCR tube to the well on the slide.

Gently push the each sample with the posterior side facing up so it just touches the bottom of the well. The Poly-L-lysine should adhere the sample to the slide.

Pipet up mounting solution until it the surface lies slightly above the sticky tape.

Place a coverslip over your samples making sure to press the sides down to seal. *Slides can be stored at room temperature indefinitely.*