In situ hybridization for *Drosophila* pupal genitalia

Reagents

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 at -20˚C

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

While we used an InSituPro robot to run this protocol, the same steps can be performed by hand. The protocol begins with dissected pupal genitalia stored at -20˚C in ethanol. Note that the exact volume can vary when performing many washes; we generally use 700 uL for each wash. If no time is indicated for a wash, assume that incubation lasts for as long as it takes samples to settle following addition of liquid. If no temperature is indicated, assume washes or incubations are performed at room temperature.

Rehydrate and post-fix

Wash 3X PBT

Incubate samples in PBT with 4% paraformaldehyde for 30 minutes RT

Wash 3X PBT

Wash 1X PBT for 30 minutes

Hybridization

Wash 1X in 1:1 hybridization buffer : PBT for 10 minutes

Wash 3X in hybridization buffer for 15 minutes at 65˚C

Wash X in hybridization buffer for 1 hour at 65˚C

Incubate samples with probe diluted in 600 uL hybridization buffer for 14 hours at 65˚C. *We generally ensured that at least 500 ng probe was included per reaction.*

Wash and block

Wash 3X in hybridization buffer at 65˚C

Wash 9X in hybridization buffer for 10 minutes at 65˚C

Wash 1X in 1:1 hybridization buffer : PBT for 5 minutes at room temperature

Wash 3X in PBT with 1% BSA

Wash 6X in PBT with 1% BSA for 20 minutes

Bind antibody

Incubate in anti-DIG diluted 1:25000 in PBT overnight at 4˚C. *This can also be performed for 2 hours at room temperature.*

Wash 6X in PBT for 20 minutes at room temperature.

At this point, samples are ready for the colorimetric reaction.