In situ hybridization probe design and synthesis

Reagents

Nuclease Free H20
HyClone HyPure Molecular Biology Grade Water. GE SH30538.02

DNeasy Blood and Tissue Kit
Qiagen catalog #69504

PCR Master Mix
Phusion Flash High-Fidelity PCR Master Mix. ThermoFisher F548S

T7 RNA polymerase and buffer
ThermoFisher EP0112

DIG-labelled NTP mix
Roche / Sigma 11277073910

RNAse inhibitor
SUPERase-in, ThermoFisher AM 2694

Linear polyacrylamide
VWR K548-1mL

Formamide
Fisher, BP 227-500

Design probes

We design 200-300bp probes, although we note that other labs design longer probes for fluorescent in situ hybridization experiments in Drosophila (http://fly-fish.ccbr.utoronto.ca/).

For a given gene of interest, Identify a large exon using the Get Decorated FASTA tool from flybase.org. Ensure that exon is present in all annotated transcripts using the UCSC Genome Browser.

Use Primer3Plus to select primers that will amplify a 200-300 bp region of the selected exon. Use the In Silico PCR tool from the UCSC Genome Browser to ensure that the selected pair will amplify a single region from the genome. If desired, use the In Silico PCR tool to screen suggested primer pairs from Primer3Plus for their ability to amplify the same region of DNA in multiple Drosophila species.

Order your selected primers using IDTDNA or another service, ensuring that the reverse primer (the primer that forms the 5' end of the antisense strand) has a T7 RNA polymerase binding sequence (5' TAATACGACTCACTATAG 3') on its 5' end.

Amplify probe template

Isolate genomic DNA from your experimental strain using the (Qiagen) DNeasy kit.

PCR the probe template from genomic DNA. We use Phusion Flash Master Mix (Thermo Fisher) and associated protocol.

Run a small amount of the reaction (2uL + 8uL water) on an agarose gel to ensure that no off-target amplification has occurred. You should see a single band of the correct size.

Synthesize probes

Use *in vitro* transcription to synthesize antisense RNA probes labelled with digoxigenin (DIG). *We find that raw PCR products work well as template DNA.*

	20 uL Reaction
5X buffer	4 uL
10x NTP mix	2 uL
Template DNA (PCR reaction)	2 uL
RNAse Inhibitor	1 uL
T7 RNA polymerase	1.5 uL
Sigma water to final volume	9.5 uL

Incubate at 37˚C overnight. *We find that overnight incubation can improve yields over standard incubation times of 2-4 hours.*

If desired, run an agarose gel after incubation but prior to precipitation that compares equal volumes of transcription reaction and a 1:10 dilution of the template DNA. *This step checks the efficacy of the in vitro transcription reaction.*

Precipitate probes

Add 1:10 volume (2uL) 3M NaOAc pH 5.2.

Add 2 volumes (44uL) 100% ethanol.

Add 2 uL linear polyacrylamide. *This decreases the amount of incubation time needed for ethanol precipitation.*

Incubate at -20˚C for at least 30 minutes.

Spin at 4˚C at max speed for 10 minutes. *A white pellet should be visible after this step*

Remove supernatant, and replace with 100uL 70% ethanol.

Spin at 4˚C at max speed for 10 minutes.

If desired, repeat wash with 100% ethanol.

Remove supernatant, and air dry for 10 minutes.

Resuspend in 27 uL RNAse-free water. Use 2 uL to quantify concentration and purity on a Nanodrop. Add 1 volume (25uL) formamide and store at -20˚C.