Subject Categories: RNA *in situ* hybridization, High throughput, 96-well format

Determination of gene expression patterns using high-throughput RNA *in situ* hybridization to whole-mount Drosophila embryos

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Abstract

We describe a high-throughput protocol for RNA *in situ* hybridization (ISH) to Drosophila embryos in 96-well format. cDNA or genomic DNA templates are amplified by PCR and then digoxigenin-labeled ribonucleotides are incorporated into anti-sense RNA probes by in vitro transcription. The quality of each probe is evaluated prior to *in situ* hybridization using a RNA Probe Quantification (dot blot) assay. RNA probes are hybridized to fixed, mixed-staged Drosophila embryos in 96-well plates. The resulting stained embryos can be examined and photographed immediately or stored at 4°C for later analysis. Starting with fixed, staged embryos, the protocol takes 6 days from probe template production through hybridization. Preparation of fixed embryos requires a minimum of two weeks to collect embryos representing all stages. The method has been used to determine the expression patterns of over 6000 genes throughout embryogenesis.

Introduction

In situ hybridization is a general method for localizing DNA [1, 2] and RNA[3] in chromosomes, cells, tissues and whole animals. It has been used in Drosophila since the early 1980's to determine accurate spatial and temporal gene expression patterns in tissue sections of embryos, larvae and adults [4-7]. A major technical advance in the late 1980's by Tautz and Pfeifle [8] was the development of *in situ* hybridization to whole-mounted embryos using non-radioactive probes. This advance allowed three-dimensional image analysis using differential interference contrast (Nomarski) or confocal fluorescence microscopy and eliminated tedious reconstructions from tissue sections. It also eliminated long exposure times required when using radioactively labeled probes. The original protocol was based on DNA probes, but subsequent studies showed that RNA, PCR and oligonucleotide-derived probes can improve the sensitivity in detecting rare mRNAs (reviewed in [9]). Another advantage of this method has been the relative ease of adapting it to high-throughput methods [10-13].

Overview of the Procedure

Here, we describe a protocol for RNA probe production from cDNAs and genomic DNA followed by RNA hybridization to embryos or imaginal discs in 96-well format. We use this procedure routinely as part of an ongoing project to document embryonic expression patterns for all Drosophila genes as described [12, 13]. Images from this project are posted on a public website (http://www.fruitfly.org/cgi-bin/ex/insitu.pl) and are periodically updated as new data are generated. For templates, we use a large collection of cDNAs,

the Drosophila Gene Collection (DGC) [14-16] representing over 70% of the genes in the Drosophila genome, as well as genomic DNA for the genes not represented in the DGC. The protocol can be used to determine expression patterns in fixed, mixed staged embryos (as described in the main Procedure) or fixed, mass isolated imaginal discs (collected as described [17] and processed as described in Box 1). A flow diagram outlining all the major steps in the protocol is shown in Figure 1.

DNA templates are generated from cDNA clones by PCR using vector-specific primers, or from genomic DNA using gene-specific primers (described in BOX 2), and the PCR product is purified by iso-propanol precipitation. The purified PCR product is transcribed using digoxigenin-labeled UTP and an RNA polymerase, Sp6, T7 or T3, depending on which vector was used to clone the cDNA target (see Table 1); for genomic DNA targets, Sp6 RNA polymerase promoter is added to the 3' gene specific PCR primer. The resulting RNA probe is treated with DNase I to remove any remaining DNA template then the sample is ethanol precipitated and the pellet is re-suspended in a formamide solution. To assay the labeled RNA probes, diluted probes are spotted on and cross-linked to a nylon membrane. The membranes are treated with anti-digoxigenin Fab Fragments coupled to alkaline phosphatase (AP) and the color substrates 4-nitrobluetetrazolium chloride (NBT) and 5-bromo 4-chloro 3-indoyl phosphate (BCIP). The quality of the probe is determined by comparing the intensity of individual probe spots to control spots.

Embryos stored in methanol are gently placed in the wells of a 96-well filter plate with a multi-channel pipette and wide-orifice disposable tips, then are re-hydrated, washed, and pre-hybridized. A vacuum is applied to remove methanol, subsequent re-hydration washes and hybridization solution. Imaginal discs are stored in fixation solution instead of methanol and do not need to be re-hydrated; the imaginal disc hybridization protocol starts at the washing step prior to pre-hybridization and requires filter plates with a smaller pore (.45µm) than those used for the embryo hybridizations. Following pre-hybridization, DIG-labeled RNA probes are added, and the samples are incubated overnight at 55ºC. Three genes (engrailed [18], hunchback [19] and brinker [20]) with well-described expression patterns are included in each plate and used to monitor hybridization efficiency.

The embryos and imaginal discs are stained using the same reagents used in the dot blot assay of probe quality. First, they are reacted with antidigoxigenin Fab fragments conjugated to alkaline phosphatase. Following washes in PBT and AP buffer, NBT/BCIP color substrates are used to detect the hybridization patterns. Embryos are washed with ethanol to enhance contrast and stored in 70% (v/v) glycerol in PBS. Imaginal discs are washed in methanol instead of ethanol. After immunohistochemical staining, each plate is examined under low magnification (Zeiss Stemi 2000-C microscope) to determine the quality of embryo or imaginal disc morphology and staining as well as the proportion of wells with samples that show an expression pattern.

Applications of high-throughput RNA ISH

High throughput ISH is a useful tool for determination of temporal and spatial patterns of expression where large numbers of samples are required, such as for genomic scale studies. We have used this procedure both for determination of gene expression patterns in wild type Drosophila [12, 13] and for studies of regulation of gene expression by cis-regulatory modules (CRM) using transgenic Drosophila embryos carrying CRM-driven reporter gene constructs to screen for functional CRMs [21]. With minor modifications, we adapted the embryo protocol for use with mass isolated imaginal discs (See Box 1) and the procedure could be similarly adapted for other tissues.

Advantages and Limitations of high throughput RNA ISH

Information about the spatial localization of gene expression in developmental context provided by ISH is complementary to other more quantitative methods of cataloging gene expression such as microarrays or RNA sequencing by massively parallel sequencing technologies. Alkaline phosphatase-based probe visualization, as used in the protocol presented here, can detect dynamic expression patterns at the tissue level for ~18 hours of Drosophila embryonic development, after which the newly formed cuticle begins to interfere with penetration of the probe. Fluorescent in situ hybridization (FISH) produces high contrast data [22] and with advanced imaging techniques can be used to extract quantitative gene expression data [23], but the technical complexity of these subcellular detection methods limits the range of development that can be analyzed in one experiment. The chief advantage of the procedure described here over other ISH protocols is that we have optimized it for robust performance with multiple samples using standard laboratory equipment. To minimize variability and simplify the procedure, we

have eliminated a number of steps from the original hybridization protocol. In the embryo fixation step, proteinase K traditionally is used to permeabilize the embryos, improving signal and reducing background. We removed the proteinase K step, and reduced the amount of probe from nanograms to picograms to compensate for background problems. This also allowed us to eliminate the following background reducing agents from the hybridization solution; Denhardts; tRNA; ssDNA; and heparin. Using this streamlined protocol, we can process up to four 96-well plates simultaneously with reliable results. The main disadvantage of a high throughput procedure is the corollary of its advantage: optimal conditions for multiple samples are not always the best conditions for every sample, so a few samples (~10%) on each plate may require additional experiments for best results.

Experimental Design

Embryo Collections High quality embryos are required for successful in situ hybridization. Embryo morphology should be monitored during collections and fixation steps should be adjusted as described in Troubleshooting if necessary. To assure that all developmental stages are represented in the embryo mix for the hybridization, embryos are collected in 3 hr intervals and aged until the desired developmental stage before fixing. Collections are kept separate through the fixation process, and then a master mix with equal proportions from each age range is assembled for the hybridizations as described in Box 3. For high throughput ISH, embryo collection becomes labor intensive. Properly prepared fixed embryos stored in methanol at –20°C and are stable for at least one year. Therefore it is most efficient to collect enough embryos for all

experiments that are anticipated within a one–year period and scale embryos collections accordingly. Following the collection schedule shown in Table 2, collection from two cages for two weeks will yield enough embryos for a little more than single 96 well hybridization plate. For large scale projects with wild type embryos additional cages are recommended. We collect embryos from eight cages over the course of six weeks to accumulate enough embryos for approximately 50 hybridization plates. For testing multiple mutant or transformed lines with a single probe, smaller scale collection methods can be devised. We have used minicages that hold a standard petri plate as a collection tray.

Probe templates and primer design. For genes available as cloned cDNA in one of the standard vectors (e.g. pOT2a, pOTB7, pBS SK-, or pFlc-1), primers from the vector are used to generate PCR products (see Table 1). The cDNAs are directionally cloned and the vectors include sequences for a different RNA polymerase promoter on opposite ends so that choice of RNA polymerase determines which strand is transcribed. For genes not represented as cDNA clones, successful probe templates can be generated from genomic DNA by designing primers to the largest exon of target genes as described in Box 2. These primers should include an RNA polymerase promoter sequence at the 5' end of the reverse primer to allow transcription of the RNA probe. Other RNA polymerase promoter sequences could be used, but we have had best results with the SP6 tail (5'ATTTAGGTGACACTATAGAAGTG 3'). The gene specific primers should be designed with TM of 60°C, GC content 50 ± 20%, and contain 18-25 bp of the gene specific sequence in addition to the SP6 polymerase sequence.

RNA Probe Controls. After the preparation of the initial set of RNA probes, sufficient RNA probe reference controls for all anticipated hybridization experiments should be prepared according to a procedure described in Box 4. These controls are used during probe quantification as reference controls for evaluation and tracking of probe quality.

Materials

Reagents

- Distilled de-ionized H_2O
- Collection tray medium (see Reagent Setup
- Agar, Gelidium, Fine Ground (Moorhead 41004)
- Sucrose (Sigma, S0389)
- Hydroxybenzoic Acid Methyl Ester (Sigma, H5501)
- Grape Juice (Welches)
- Foam Trays, (Sealed Air Corporation, Cryovac Division, NatureWorks Tray 10S)
- Yeast (Fleishman)
- 6% sodium hypochlorite (Chlorox, regular bleach)

Heptane (J.T. Baker. 9338-03)

Caution Heptane is a poison and is extremely flammable. Handle with care.

Formaldehyde (Sigma, F-8775-25ml).

Caution Formaldehyde is toxic and is a classified as a human carcinogen and should be handled with caution and discarded into a properly labeled Formaldehyde waste container.

- Diethyl pyrocarbonate (DEPC) (Sigma, D-5758, store at 4 °C)

  Caution Harmful. Combustible. May develop pressure. Moisture sensitive. Wear suitable protective clothing

- DEPC treated H_2O
- NaCl (Sigma, S-7653)
- KCl (Sigma, P-9541)
- KH_2PO_4 (Sigma, P-0662)
- $Na_2HPO_4 \bullet 7H_20$ (Sigma, S-9390)
- HCl (Sigma, H-1758)

  Caution Toxic by inhalation. Causes burns. Irritating to the respiratory system.

- PBS (see Reagent Setup)
- Agarose (GibcoBRL, 15510-027)
- 1x TAE (see Reagent Setup)
- Tween 20 (polyoxyethykenesorbitan monolaurate) (Sigma, P-9416)
- PBT (0.1% Tween 20 in PBS)
- Methanol (J.T. Baker, 9077)

  Caution Methanol is a poison. May be fatal or cause blindness if swallowed. Harmful if inhaled or absorbed through skin. Can not be made nonpoisonous. Flammable liquid and vapor.

- Bacterial stock carrying cloned cDNA of interest (user supplied)
- Lennox L Broth Base (LB Broth Base) (Invitrogen, 12780-029)
- LB Broth (dissolve 20 g of LB Broth Base in 1 L of ddH_2O)
- Antibiotic (Ampicillin or Chloramphenicol)
- 10mM Deoxynucleotide Mix (Invitrogen, 18427-088, store at -20°C)
- Vector specific primers (Invitrogen, store at –20°C) See Table 1
- Gene specific primers (user supplied) for genomic DNA templates (see Box 2)
- DNA Polymerase (DyNAzyme EXT,Finnzymes, F-505L, store at –20°C)
- DNA Polymerase (PfuUltra High Fidelity Polymerase, Stratagene 600384, store at –20°C)
- 2-propanol, for molecular biology, minimum 99% (Sigma, I9516)

  Caution Highly flammable. Irritant. May cause drowsiness and dizziness.
- RNA Polymerase SP6 (Roche, 11487671001, store at -20°C)
- RNA Polymerase T3 (Roche,11031171001, store at -20°C)
- RNA Polymerase T7 (Roche, 10881775001, store at –20°C)
- RNase inhibitor (Amersham Pharmacia Biotech, 27-0815-01, store at –20°C)
- DNase I, RNase-free (Roche, 10776785001, store at –20°C)
- 100 mM Ribonucleotide Set (Finnzymes, F-622, store at –20°C)
- Digoxigenin-11-UTP (Roche, 11209256910, store at –20°C)
- Sodium Acetate (Fluka, S9513)

  Caution Avoid contact and inhalation.
- Dithiothreitol (DTT)(Sigma, D8255, store at 4°C)

- 1 M DTT (see Reagent Setup)
- Tris Base (Roche, 03118142001)
- 1 M Tris pH 8 (see Reagent Setup))
- 10x DNase I Buffer (see Reagent Setup)
- DNase I mix (see Reagent Setup)
- Ammonium Acetate (Sigma, A7330, store at 4°C)

  Caution Irritating to the skin, skin and mucous membrane
- 7.5 M CH_3COONH_4 (see Reagent Setup)
- Absolute ethanol (non-denatured) (Pharmco-Aaper, 111000200 111ACS200)

  Caution Flammable liquid and vapor.
- Formamide (Sigma, F-5786-250 ml)).

  Caution Toxic. May cause harm to a developing fetus. Irritating to the respiratory system and skin.
- Resuspension buffer (see Reagent Setup).
- (ethylenedinitrilo)tetraacetic acid, disodium salt, dihydrate (EDTA) (J.T. Baker, 5632)

  Caution Harmful if swallowed or inhaled. Causes irritation to skin, eyes and respiratory tract. Use only with adequate ventilation.
- TE pH 7.5 (10 mM Tris-HCl pH 7.5, 1 mM EDTA).
- NaOH anhydrous pellets, (Sigma, S-8045, store at 4°C)

  Caution Corrosive. Causes burns.
- Maleic Acid (Sigma, M-0375)

Caution Corrosive. Causes burns. Harmful by inhalation, in contact with skin and if swallowed.

- DIG Wash and Block Buffer Set (Roche; 11585762001)
- Blocking Reagent (Roche; 1096176)
- 1X blocking solution (See Reagent Setup)
- Anti-Digoxigenin-AP Fab Fragments (Roche, 11093274910, store at 4°C)
- Magnesium Chloride (Sigma, M-1028)
- AP Buffer (see Reagent Setup)
- Nitroblue tetrazolium chloride (NBT) (Roche, 11383213001, store at –20 °C)

  Caution Toxic
- 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche, 11383221001, store at –20 °C)

  Caution Toxic
- Developing solution (see Reagent Setup)
- 10X PCR Buffer (Sigma, store at –20°C)
- $Na_3C_6H_5O_7 \cdot 2H_2O$ (Sigma, S4641)
- 20X SSC (see Reagent Setup)
- Hybridization buffer (see Reagent Setup))
- Dextran Sulfate (Sigma, D6001, store at 4°C)
- 50% Dextran Sulfate (see Reagent Setup)
- Hybridization Buffer with dextran sulfate, (see Reagent Setup)
- Wash buffer (see Reagent Setup)
- Goat Serum (GibcoBRL, 16210-064, store at –20 °C)

- Anti-Digoxigenin-AP Fab Fragments (Roche, 11093274910, store at 4 °C)
- AP Buffer (0.1M NaCl, 0.05M $MgCl_2$, 0.1M Tris pH 9.5, 0.1% Tween 20; prepare fresh before use)
- Glycerol (Sigma, G5516)

  Caution Avoid Contact and Inhalation
- 70% (v/v) Glycerol (70:30 Glycerol: 1x PBS, filter sterilize)

EQUIPMENT

- Weigh boats, large (VWR, 25433–106)
- Weigh boats, small (VWR, 25433–102)
- Polarseal foil adhesive tape for multi-well plates (E&K Scientific, T592100)
- Paper Towels
- Parafilm “M” (VWR, 52858– 032)
- 96-well Filter Plates, for embryos (Millipore, Multi Screen HTS™, DV; Clear Plates, Non-Sterile, 0.65 µm Hydrophilic Low Protein Binding Durapore® Membrane, MSDVN6510)
- 96-well Filter Plates, for imaginal discs (Millipore, Multi Screen HTS™, DV; Clear Plates, Non-Sterile, 0.45 µm Hydrophilic Low Protein Binding Durapore® Memembrane, MSDVN4510)
- TC Microwell 96U plates (VWR Scientific, 163320)
- 96-well half-skirt PCR plates (E&K Scientific, 489096)
- Wide-orifice 250 µL pipette tips (Rainin, HR-250WS)
- 25 ml reagent reservoir (Matrix, 8093)

- AeraSeal™ (Sealing Tape; Gas Permeable for Sealing Microplates, E&K Scientific, T896100)
- DIG Quantification Teststrips (Roche, 85444122, store at 4°C)
- DIG Control Teststrips (Roche, 90519120, store at 4°C)
- Nylon Membranes, positively charged (Roche; 1417240)
- 96-well, 2 ml Square Well Round Bottom Polypropylene Plate (E&K Scientific, 662000).
- Beaker, polypropylene, 5 liter with handle (VWR)
- Fly cages (see Equipment Setup)
- Three-level sieve (nominal sieve openings (850 μm (20), 250 μm (60), 150 μm (100)) (Fisher)
- CELLECTOR® Tissue Sieve (ThermoEC)
- Ring Stand (Fisher)
- Funnel 150mm (Nalgene)
- Falcon Pipetaid
- GeneAmp PCR System 9700 (Applied Biosystems).
- Eppendorf Centrifuge 5810 (equipped with 96-well plate holders).
- Vortex Genie-2 (VWR Scientific)
- Gyrotory® shaker Model G2 (New Brunswick)
- Model A6 Millipede™ (for 192 samples) Wide Gel Electrophoresis System (Owl Separation Systems, 23cm W x 25cm L, A6 Millipede™)
- Model D3 Centipede (for 96 samples) Gel Electrophoresis System (Owl Separation Systems, 23cm W x 14cm L, D3-14 Centipede™)

- Transferpette®-12, 0.5-10uL (BrandTech Scientific Inc., 2703620)
- Transferpette®-12 , 2.5-25uL (BrandTech Scientific Inc., 2703624)
- Transferpette®-12, 10-100uL, (BrandTech Scientific Inc., 2703628)
- Transferpette®-12, 30-300uL (BrandTech Scientific Inc., 2703632)
- 8 Channel Impact2 Electronic Pipettor, 5-250µL (Matrix Technologies, Corporation, 2012)
- 8 Channel Impact2 Electronic Pipettor, 15-1250µL (Matrix Technologies, Corporation, 2024)
- Speedball roller (E&K Scientific)
- Incubator/Shaker (Innova 4300)
- UV Stratlinker (Stratagene)
- Pin-tool (V & P Scientific, Inc.)
- HybAid tubes (HybAid Limited)
- HybAid Hybridization oven (HybAid Limited)
- Genetix Q-Fill 2 plate filler
- Eppendorf 5’ Prime Vacuum Manifold
- Model 1545 Incubator (VWR)
- Clay Adams® Brand Nutator
- Low magnification microscope (Zeiss-Stemi 2000-C)

REAGENT SETUP

PBS: Dissolve 8 g of NaCl, 0.2 g KCl, 0.24 g KH_2PO_4 and 2.72 g $Na_2HPO_4 \bullet 7H_20$ in 800 ml of DEPC treated H_20, adjust pH to 7.4 with HCl, adjust volume to 1L, sterilize by autoclaving.

1X TAE : 40 mM Tris, 20 mM Glacial Acetic Acid, 2mM EDTA, pH 8.1

1 M DTT: Dissolve 3.09 g of DTT in 20 ml of 0.01 M sodium acetate pH 5.2, sterilize by filtration, aliquot and store at –20°C

1 M Tris pH 8: Dissolve 121.1 g Tris-base in H_2O, adjust pH with concentrated HCl; adjust volume to 1 L; pH of Tris is temperature sensitive; sterilize by autoclaving

10x DNase I Buffer: 0.2 M Tris pH 8, 0.1 M $MgCl_2$

7.5 M CH_3COONH_4: Dissolve 578.1 g of CH_3COONH_4 in H_2O, adjust volume to 1 L

Resuspension Buffer: 50% formamide, 5mM Tris-HCl pH 7.5, 0.5mM EDTA and 0.01% Tween 20

1X blocking solution: For 1L, dissolve 10 g of blocking reagent in 0.1M maleic acid, 0.15M NaCl adjusted pH to 7.5 with NaOH (solid), store at 4°C

AP Buffer: 0.1M NaCl, 0.05M $MgCl_2$, 0.1M Tris pH 9.5, 0.1% Tween 20; prepare fresh before use

Developing solution: 45 µl NBT and 35 µl BCIP per 10 ml of AP Buffer; add NBT and BCIP just before use

20X SSC: Dissolve 175.3 g NaCl and 88.2 g of Sodium Citrate in 800 ml of DEPC treated H_20, adjust pH to 7, adjust volume to 1L, sterilize by autoclaving

Hybridization Buffer: 50% formamide, 4X SSC, and 0.01% Tween 20, store in the dark at -20°C

50% Dextran Sulfate: Dissolve 25 g of dextran sulfate in 50 ml of ddH_2O

Hybridization Buffer with dextran sulfate: 50% formamide, 4x SSC, 5% dextran sulfate and 0.01% Tween 20 store in the dark at –20°C

Wash Buffer: 50% formamide, 2X SSC and 0.01% Tween 20; prepare fresh before use

10x dig-nucleotide mix: Prepare mix for one plate as tabulated below.

Reagent	1 rxn (µl)	1plate-110rxn (µl)	Final Conc.
10 mM Digoxigenin-11-UPT	0.32	35.36	6.43 mM
100 mM UTP	0.02	1.96	3.56 mM
100 mM ATP	0.05	5.5	10 mM
100 mM CTP	0.05	5.5	10 mM
100 mM GTP	0.05	5.5	10 mM
DEPC H2O	0.01	1.18	
Total	**0.50**	**55.00**	

Embryo collection trays: Heat 3500 ml of H_2O. Add 125 g of agar to the water and bring to boil while mixing continuously. Wait for agar to go into solution. Add 100 ml 1.25 N NaOH; then add 1360 ml Grape Juice**.**

CRITICAL Add NaOH before adding the grape juice.

Add 125 g Sucrose. Add 8 g p-Hydroxybenzoic Acid Methyl Ester and mix thoroughly. Use a beaker with handles to pour hot mixture onto the foam trays. Let the filled trays cool to room temperature (~25 °C) before storing at 4 °C. Embryo collection trays can be stored at 4 °C for up to a week.

Yeast Paste: Mix yeast with H_2O to form a paste.

CRITICAL The paste has to be easily spreadable, so that the collection medium will not separate from the tray, but not so thin that the flies stick to the paste.

EQUIPMENT SETUP

Fly cages: 30 cm diameter plexiglass cylinders, 43 cm long with attached stand and 5mm grooves cut around each end (custom made), covered on one end with a flat silk cloth and accessible on the other end through a 70 cm long silk cloth sleeve secured with elastic cord set into the end groove.

Procedure

Mass Embryo Collection TIMING variable, minimum 2 weeks

1. Prepare embryo collection trays and yeast paste as described in Reagent Setup.
2. Spread a thin layer of yeast paste on the fresh collection tray and place into a fly cage.

   Critical step For maximum egg deposition, approximately equal numbers of young male and female adult flies (~8000-12000, depending on fecundity of the stock used) should be introduced into the cage at least 2 days before first collection, with daily collection tray changes.

   Critical Step For maximum egg deposition allow the collection trays and yeast paste to reach room temperature before placing in the fly cage

3. Place a yeasted pre-lay collection tray into cage one hour before beginning first collection to encourage females to deposit retained eggs and synchronize subsequent egg collections. Discard this pre-lay collection tray.

4. Insert fresh collection tray to begin embryo collections. An example collection schedule is shown in Table 2.
5. After 3 hrs replace the collection tray with a fresh one taking care not to release any flies from the cage. Label trays to indicate collection time period.

Table 2: Example of a collection schedule

	Cage #1*		**Cage #2****	
	Week 1	Week 2	Week 1	Week 2
Collection Time	Age Interval	Age Interval	Age Interval	Age Interval
7:30 am-10:30 am	9—12 h	9—12 h	3—6 h	9—12 h
10:30 am-1:30 pm	6—9 h	6—9 h	0—3 h	6—9 h
1:30 pm-4:30 pm	3—6 h	3—6 h	15—18 h	15—18 h
4:30:00 pm-7:30 pm	0—3 h	0—3 h	12—15 h	12—15 h

* Embryos from Cage #1 are always fixed at 7:30pm the same day they are collected

** Embryos from Cage #2 are fixed at three different times depending on the desired interval (0-3, 3-6 at 1:30 pm same day as collected; 6-9,9-12 at 7:30 pm same day as collected; 12-15, 15-18 at 7:30 am the day after collection).

6. Store collection tray with deposited embryos at 25 °C until the embryos have developed to desired age interval before fixing.

   Critical Step Keep embryos of different age intervals separate during fixation.

   Critical Step, Timing Prepare 50-50 mix of heptane and 4% formaldehyde/PBS fixative just prior to starting the embryo fixation
7. Collect embryos from collection tray by rinsing the tray with de-ionized water and capture the embryos in the CELLECTOR® Tissue Sieve. The sieving removes yeast paste.

Mass Embryo Fixation TIMING 1.5 hrs per time period

8. Fill reservoir with 100% bleach and immerse tissue sieve containing embryos into the bleach, agitating gently for 3 min. to remove the chorion membrane.

Critical Step If the embryos are left in the bleach for too long they will be irreparably damaged.

9. Wash well with distilled water to completely remove any bleach residue.
10. Remove embryos from the sieve using a spatula and place in a 50ml falcon tube filled with the 50-50 mix of heptane and 4% formaldehyde/PBS.
11. Fix embryos in the 50-50 mix of heptane and 4% formaldehyde/PBS fixative by shaking on a gyrotory shaker for 25 min.
12. Remove *lower* aqueous phase and replace with equal volume of methanol.

    Critical Step To prevent embryos sticking to the outside of the pipette move the pipette through the liquid and the inter-phase very slowly while blowing out air bubbles.
13. Shake (manually) for 1 min then allow embryos to settle. DO NOT VORTEX.

    Critical Step Vortexing the embryos at this point will significantly damage the embryo morphology
14. Remove *upper* phase containing the vitelline membranes and embryos remaining at inter-phase. (Good quality embryos—unbroken and devoid of chorion and vitelline membranes—will sink to the bottom. Embryos remaining at inter-phase are damaged and should not be used in hybridization.)

    Critical Step When removing the *upper* (heptane) phase, start at the inter-phase and work up from there to prevent marginal embryos sinking into methanol.
15. Remove remaining methanol.
16. Wash 3x in methanol.
17. Top off 50 ml Falcon tube to 50 ml with methanol.

Pause Point Embryos can now be stored at -20°C for up to 1 year.

Critical Step Do not put more than 6 ml of embryos in a 50 ml falcon tube. Lay the falcon tube on its side to prevent crushing the embryos.

Cell Inoculation of cultures containing cloned DNA templates for RNA probes

TIMING 18 hours (overnight)

18. Add appropriate antibiotic (see Table 1) to a final concentration of 50 ug/ml in 110 ml of LB and mix thoroughly.

Table 1 Vector specific requirements

Vector	antibiotic	PCR Primers	PCR Primer sequence	RNA polymerase
pFlc-1	carb	M13 (-21) M13 (REV)	5' TGTAAAACGACGGCCAGT 3' 5' GGAAACAGCTATGACCATG 3'	T3
pOT2A pOTB7	chlor	PM001a PM002a	5' GTCGACGTTAGAACGCGGCTAC 3' 5' GGGTTAAATTCCCGGGTACTGC 3'	SP6 (for pOT2A) T7 (for pOTB7)
pBS SK-	carb	SK-30 SKMet	5' GGGTAACGCCAGGGTTTTCC 3' 5' ATGACCATGATTAGCCAAGC 3'	T7

carb = Carbenicillin; chlor = Chloramphenicol

19. Add 1.0 ml of antibiotic/LB mix into each well of a 96-well Ritter Riplate using a multi-channel pipette.
20. Thaw frozen bacterial stocks containing cloned genes of interest and add 10 µl of thawed cells per well using a multi-channel pipette.
21. Seal tightly with AeraSeal sealing tape.
22. Place Ritter Riplate in a 37 °C shaking incubator set at 300 RPM for 18 hrs.

Pause Point After incubation is complete, cells can be stored at 4ºC for up to seven days.

PCR TIMING 6.5 hrs

23. Dilute cells 1:50 in sterile de-ionized H_2O.

24. For one 96-well PCR plate prepare a total of 1.87 ml reaction mix, as tabulated below.

Reagent	1 rxn (µl)	1plate-110rxn (µl)	Final Conc.
10x PCR Buffer for DyNAzyme Polymerase	2	220	1x
10 mM dNTP mix	0.5	55	0.25 mM
100 µM PCR Primer 1	0.2	22	1 µM
100 µM PCR Primer 2	0.2	22	1 µM
DyNAzyme DNA Polymerase	0.2	22	1 U
dd H_2O	13.9	1529	
TOTAL	**17**	**1870**	

25. Dispense 17 µl of the mix to each well using a multi-channel pipette.

26. Add 3 µl 1: 50 cell dilution (from Step 21) into each well using a multi-channel pipette, mix thoroughly and centrifuge at 4000 rpm for one minute.

27. Amplify PCR reactions according to the appropriate cycling conditions (see Table 3, 4 or 5). Note that the extension time depends on the size of the clones: 1 minute per kb as recommended by the polymerase manufacturer

Table 3: Cycling conditions for pOT2A

Cycle number	Denature	Anneal	Extend	
1	94°C, 1 min	–	–	
2–6	94°C, 30 sec	66°C – 2°C/cycle, 45 sec	68°C, 1 min/kb	

7–35	94°C, 30 sec	56°C, 45 sec	68°C, 1 min/kb+6 sec/cycle	
36			68°C, 10 min	
37				4°C, ∞

Table 4: Cycling conditions for pFlc-1

Cycle number	Denature	Anneal	Extend	
1	94°C, 1 min	–	–	
2–6	94°C, 30 sec	56–1°C, 45 sec	72°C, 1 min/kb	
7–35	94°C, 30 sec	50°C, 45 sec	72°C, 1 min/kb	
36			72°C, 10 min	
37				4°C, ∞

Table 5: Cycling conditions for pBS SK-

Cycle number	Denature	Anneal	Extend	
1	94°C, 1 min	–	–	
2–6	94°C, 30 sec	60–2°C, 45 sec	72°C, 1 min/kb	
7–35	94°C, 30 sec	50°C, 45 sec	72°C, 1 min/kb	
36			72°C, 10 min	
37				4°C, ∞

28. Quantitate and size the PCR products by electrophoresis of 5 ul of each PCR reaction through 1% agarose in 1X TAE

    Pause Point PCR product can be stored at -20ºC indefinitely

    TROUBLESHOOTING

PCR Product Purification TIMING 1 hr

29. Add 80 µL of 75% (v/v) isopropanol to 20 µL of PCR Product, seal well and vortex
30. Incubate for 15 min at RT
31. Centrifuge for 30 min @ 3200 X G
32. Remove foil and dispose of supernatant appropriately.

33. Turn plates upside down and centrifuge for 1 min @ 700 G on a paper towel

34. Re-suspend DNA pellets in 35 µL of ddH_20.

35. Seal well, vortex at maximum speed for 25 sec and centrifuge at 3200 X G for one minute.

Pause Point Purified PCR product can be stored at -20ºC indefinitely

RNA Probe Preparation TIMING 4 hrs

36. For one 96-well plate prepare a total of 550µl of 2x RNA Polymerase reaction mix as tabulated below.

2x RNA Polymerase Mix			
Reagent	**1 rxn (µl)**	**1plate-110rxn (µl)**	**Final Conc.**
10x Transcription Buffer	1.00	110.00	2x
10x dig-nucleotide mix*	0.50	55.00	1x
RNAguard RNase Inhibitor 26200 U/ml	0.13	14.30	3.4 U
RNA Polymerase 20 U/µl**	0.50	55.00	10 U
DEPC H_2O	2.87	315.70	
Total	**5.00**	**550.00**	

*see Reagent Setup** **Critical Step** Determine the correct RNA Polymerase based on the vector used (see Table 1)

37. Dispense 5 µl of 2x polymerase mix into each well of 96-well plate using a multi-channel pipette.

38. Add 5 µl of PCR product using a multi-channel pipette.

39. Incubate in a PCR machine at 37 °C for 2 hrs.

40. Prepare DNase I mix as listed below and add 10 µl of the mix into each well using a multi-channel pipette.

Dnase I Mix			
Reagent	**1 rxn (µl)**	**1plate-110rxn (µl)**	**Final Conc.**

1M DTT	0.10	11.00	10 mM
10x Dnase I Buffer*	1.00	14.30	1x
Dnase I, Rnase-free 10 U/µl	1.00	110.00	1 U
DEPC H_2O	7.90	869.00	
Total	**10.00**	**1100.00**	

*see Reagent Setup

41. Incubate in a PCR machine at 37 °C for 15 min.
42. Add 20 µl of DEPC H_2O.
43. Place plate on ice and quickly add 20 µl of 7.5M NH_4OAc into each well using a multi-channel pipette.
44. Add 160 µl of ethanol into each well, seal well, vortex for 15 s to mix and centrifuge at 3200 X G for 1 min.
45. Incubate at room temperature for 10 min.
46. Centrifuge at 4000 rpm (3200 X G) in an Eppendorf 5819 for 30 min at room temperature.
47. Drain by inverting the plate 6X; centrifuge in an Eppendorf 5819 at 800 rpm (130 X G) with plate upside down to drain remaining liquid.
48. Quickly re-suspend in 50 µl of re-suspension buffer and reseal plate.

    Critical Step RNA probe must be re-suspended quickly, otherwise it is hard to get RNA into solution

    Caution Remainder of the re-suspension buffer must be properly discarded into Formamide waste container

49. Vortex at maximum speed for 25 sec and centrifuge at 3200 X G for one minute.

    Pause Point RNA Probe can be stored at -80ºC indefinitely

    RNA Probe Quantification (dot blot) TIMING 3.5 hrs

50. Dilute a 1µl aliquot of each of the 96 re-suspended RNA probes 1:300 in RNAse-free water.
51. Using a 96-spot pin-tool, spot 1 µl of diluted RNA probe onto a positively charged nylon membrane.
52. Using a multi-channel pipette spot 1µl of the 2 reference control dilution series onto the same nylon membrane above the 96 spots.
53. Crosslink RNA spots to the nylon membrane in a UV Stratalinker (Use the auto-crosslink function).

    CRITICAL STEP: For steps 54-59 use hybaid hybridization oven and tube and use 10 ml of appropriate solution per wash/incubation.

54. Incubate the membrane in blocking solution at room temperature for 30 min.
55. Incubate in a solution of 1:2000 dilution of Anti-Digoxigenin-AP Fab Fragment in Blocking Solution at room temperature for 30 min.
56. Wash 4X with Blocking Solution for 15 min each wash.
57. Wash 2X with AP Buffer for 5 min each wash.
58. Develop color at room temperature in the dark with Developing Solution (about 20-30 min).
59. Wash 3X in Blocking Solution for 3 min each wash to stop the color reaction.
60. Compare the 96 RNA probes with the two reference dilution series (3, 10, 30, 100 and 300 pg) to determine the success rate. If the probe spot is at least as intense as the 1:330 reference control spot (3pg) the RNA probe reaction was successful and the RNA probe is ready to be used in hybridization.

    Pause Point Quantified RNA Probe can be stored at -80ºC indefinitely

***In Situ* Hybridization** TIMING 3 days

61. Transfer1 ml of embryos from the master mix per 96-well plate into a 15 ml Falcon tube with 4 ml of methanol.
62. Carefully pour the embryos into a 25 ml reagent reservoir.
63. Add 20 µl of embryos into each well of a 96-well filter plate using a multi-channel pipette.

    Critical Step Use wide bore pipette tips to avoid damaging the embryo morphology

    Critical Step Even though there is no way to add exactly the same amount of embryos into each well, great care needs to be taken to get as close as possible. If the difference in the numbers of embryos between individual wells is too large, the wells with fewer embryos will drain consistently faster than the wells with greater numbers of embryos. Consequently, the embryos in these wells get flattened and stick together. As a result, the morphology of these embryos will be compromised.

    CRITICAL STEP: For steps 64 on, use 200µl per well unless otherwise specified and remove the liquid after each step using the vacuum manifold.

    CRITICAL STEP: For Steps 64-66, use a Matrix Impact2® pipettor to add solutions to the filter plate; for all other steps involving a filter plate use Q-Fill to fill the filter plates, except where indicated otherwise.

    Critical Step set the vacuum to the lowest setting to prevent embryos from getting flattened, crushed or stuck to the membrane.

Critical Step After all the liquid is gone, *quickly* turn off the vacuum to prevent embryos from getting flattened and crushed.

64. Re-hydrate embryos in 3:1 methanol: 2.5% formaldehyde in 1X PBS for 2 min.
65. Re-hydrate in 1:3 methanol: 2.5% formaldehyde in 1X PBS for 5 min.
66. Post-fix in 2.5% formaldehyde in 1X PBS for 10 min.
67. Rinse 6x in PBT.
68. Add hybridization buffer without dextran sulfate into each well, using Matrix Impact2®
69. Incubate with shaking at 125 rpm on the Gyrotory® shaker for at least 1 hr at room temperature to pre-hybridize the embryos.
70. During pre-hybridization put 200µl of hybridization buffer with dextran sulfate into each well of a TC Microwell 96U plate using multi-channel pipette.
71. Add 2 µl of probe into each well of the TC Microwell 96U plate the using multi-channel pipette.
72. Mix thoroughly on a vortex mixer at maximum speed for 25 s and centrifuge at 3200 X G for one minute.
73. Remove the hybridization buffer without dextran sulfate from the filter plate.
74. Transfer the probes from the 96-well plate into the 96-well filter plate using a multi-channel pipette.
75. Incubate at 55°C with shaking at 125 rpm on the Gyrotory® shaker overnight.
76. Add 100 µl of room temperature wash buffer.
77. Remove the hybridization buffer–wash buffer mix.
78. Rinse 2x with wash buffer.

79. Incubate 8x in wash buffer at 55°C with shaking for 45 min.

80. Incubate in wash buffer at 55°C with shaking overnight.

81. Rinse in PBT.

82. Incubate in PBT at RT with shaking for 30 min.

83. Using a Matrix Impact2® pipettor, add PBT, 5% goat serum, 1:2000 dilution Anti-Digoxigenin-AP Fab Fragments and incubate at RT with shaking for 2 hrs.

84. Rinse 2x with PBT.

85. Incubate 9x in PBT at RT with shaking for 10 min each.

86. Rinse 2x with AP buffer.

87. Wash in AP buffer at RT for 5 min.

88. Add Developing Solution using a Matrix Impact2® pipettor.

    Critical Step Prepare Developing Solution just before use, by adding 35 µl of BCIP and 45 µl NBT per 10 ml of AP Buffer

89. Incubate with shaking in the dark at RT until desired color development is achieved (about 75 min).

90. Rinse 3x in PBT to stop the color reaction.

91. Rinse 6x in ethanol.

92. Rinse 2x in PBT.

93. Add 70% glycerol, using a Matrix Impact2® pipettor .

94. Seal the top and the bottom of the filter plate with foil plate sealers.

    Critical Step Sealing the bottom of the plate prevents glycerol from leaking out over time

    Pause Point Stained Embryos can be stored at 4°C for at least 1 year.

95. Check individual wells on the plate under a low power magnification microscope.

TROUBLESHOOTING

96. Embryos are ready to be photographed

Timing

Steps 1–7 Mass Embryo Collection: variable, 2–6 weeks, changes at three hour intervals

Steps 8–17, Mass Embryo Fixation: 90 min for each time period

Steps 18–22, Cell Inoculation: 18 hrs (overnight)

Steps 23-28, PCR: 6.5 hrs

Steps 29–35, PCR Product Purification: 1 hr

Steps 36–49, RNA Probe Preparation: 4 hr

Steps 50–60, RNA Probe Quantification (dot blot): 3.5 hr

Steps 61–96, *In Situ* Hybridization: 3 days

Troubleshooting

Troubleshooting advice can be found in Table 6.

Anticipated results.

This protocol generated successful hybridizations for approximately 85% of the first 6000 genes tested during the first pass. Our protocol is optimized for high-throughput RNA ISH to Drosophila embryos. Consequently, a small percentage of genes will require re-work. The main failure modes of the protocol are over-staining and under-staining of embryos during color development step (~10%). Over-stained embryos can be collected for re-array and re-hybridization in hybridization plates that are stained for

shorter periods. Under-stained embryos can be collected for re-array and re-hybridization in hybridization plates that are stained for longer periods. Infrequent PCR errors due to mis-priming (multiple bands during agarose gel analysis), secondary structure interference, or poor cDNA template quality (no bands during agarose gel analysis) are further causes of unsuccessful RNA ISH. Where PCR errors occur, cDNA clones are re-arrayed and cDNA plasmid DNA is prepared and linearized prior to repeating the PCR reactions. If a single visible template band is produced at the PCR step (Figure 2), RNA probe production (Figure 3) is usually successful, however the RNA ISH outcome still may be negative. The possible causes are orientation of the insert or the size of the RNA probe. If cDNA is inserted in reversed orientation, the appropriate RNA polymerase can be used to transcribe the antisense strand. For long cDNAs, a restriction digest of the cDNA template prior to the RNA probe reaction can reduce the probe size, or the RNA probe can be digested by brief hydrolysis. Results for each cDNA throughout the RNA ISH process are tracked in detail. Analysis of these results then reveals where the failure occurred, the cDNAs are re-arrayed accordingly and re-work attempts are made. With rework we can achieve successful hybridization for ~97% of initially attempted experiments. Examples of acceptable hybridizations are shown in Figure 4.

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BOX 1 HYBRIDIZATION PROCEDURE FOR IMAGINAL DISCS

1. Fix freshly isolated imaginal discs (see reference 27) overnight at 4°C in 4% formaldehyde in PBS.

PAUSE POINT Imaginal discs can be stored in fixative at 4°C for up to 4 months.

2. For each 96 well plate, transfer ~200 µl settled, fixed imaginal discs into a 15 ml Falcon tube.

3. Rinse 3X with 10 mls PBT: let discs settle after each addition of PBT and then decant supernatant.

4. After third rinse suspend discs in 4 mls PBT and transfer to a 25 ml reagent reservoir.

5. Distribute 20 µl discs into each well of a 96-well filter plate as described for embryos in the main procedure Step 63.

CRITICAL STEP Use Filter plates with 0.45 µm pore size for discs (see equipment). Plates used for embryo hybridization have a larger pore size and will not retain imaginal discs.

6. Rinse with PBT 3X in the hybridization plate following procedure described for Step 67 of the main protocol.

7. Follow embryo protocol from Step 78 on except at Step 91 use methanol instead of ethanol for the rinses.

BOX 3 MASTER EMBRYO MIX PREPARATION

1. Add 1ml of embryos from each of the six collected time periods into a separate 1.5 ml eppendorf tube.
2. Turn the eppendorf tube with embryos from one time period upside down above a 50 ml Falcon tube and wash out the embryos with methanol using the pipetaid.
3. Repeat with all remaining time periods to pool in a single Falcon tube.
4. Once embryos from all time periods are transferred, fill the 50 ml Falcon tube with methanol and gently invert 15 times to mix.

   Critical Step Embryos may stick to the bottom of the Falcon tube. Care must be taken to mix all the embryos.

   Critical Step A maximum of 6 ml of master embryo mix should be aliquoted per 50 ml Falcon Tube. Seal tube with parafilm and lay it on its side to prevent crushing the embryos.

BOX 2 GENOMIC PCR

Critical Step Design gene specific primers (GSPs) with RNA Polymerase sequence on the 5' end of the reverse GSP to facilitate RNA probe preparation

1. Dilute gene specific primers (GSPs) to 1µM working concentration.
2. For one 96-well PCR plate prepare a total of 2.0 ml reaction mix, as tabulated below.

Reagent	1 rxn (µl)	1plate-100rxn (µl)	Final Conc.
10x PCR Buffer for Pfu Ultra Polymerase	2.5	250	1x
2.5 mM dNTP mix	2	200	0.2 mM
genomic DNA [225ng/µl]	0.5	50	112.5 ng
Pfu Ultra DNA Polymerase	0.5	50	1 U
dd H_2O	14.5	1450	
TOTAL	**20**	**2000**	
5' Gene specific Primer [1µM]	2.5	–	0.1 µM
3' Gene specific Primer* [1µM]	2.5	–	0.1 µM

* We add Sp6 tail (5'ATTTAGGTGACACTATAGAAGTG 3) to our 3 'GSP because in our experience the Sp6 RNA Polymerase is the most effective (see Experimental Design)

3. Dispense 20 µl of the mix to each well using a multi-channel pipette.
4. Add 2.5 µl of both 5' and 3' diluted GSPs (from Step 1) into appropriate well using a multi-channel pipette, mix thoroughly and centrifuge at 3220 X G for one minute.
5. Amplify PCR reactions according to the cycling conditions below. Note that the extension time depends on the size of the clones.

Cycling Conditions for Genomic PCR

Cycle number	Denature	Anneal	Extend	
1	92°C, 2 min	–	–	
2–36	92°C, 30 sec	55°C, 30 sec	68°C, 1 min/kb	
37			68°C, 10 min	
38				4°C,∞

1. Quantitate and size the PCR products by electrophoresis of 5 ul of each PCR reaction through 1% agarose in 1X TAE
   Pause Point PCR product can be stored at -20ºC indefinitely

BOX 4 PREPARATION OF CONTROL PROBES

2. Choose 8 of the 96 probes to test against the Roche quantification control strips and use 5 µl of each to make 1:80 dilutions in RNAse-free water

   (The yield of DIG Reaction is about 40 µg/ml of DIG labeled product starting from 1 µg template after 1 hour incubation).

3. Prepare a dilution series (1:3.3, 1:10, 1:33, 1:100, and 1:330) of the 8 pre-diluted test probes from step 48.
4. Spot 1 µl of each of 5 dilutions (1:3.3, 1:10, 1:33, 1:100, and 1:330) of the 8 test probes onto 4 Roche DIG Quantification Teststrips.

   CRITICAL STEP: For Steps 4–9 use 2.5 ml reaction vials with 2 ml of appropriate solutions. Prepare a separate reaction vial for each dilution series.

5. Incubate the Quantification Teststrips and a Control Strip for 2 min in 1x Blocking Solution.
6. Incubate for 3 min in an antibody solution (1:2000 dilution of antibody in 1x Blocking Solution).
7. Incubate for 1 min in 1x Blocking Solution.
8. Incubate in Washing Solution for 1 min.
9. Equilibrate in Detection Buffer for 1 min.

   Caution NBT and BCIP are toxic. Handle with care. Developing solution must be discarded into a properly labeled NBT/BCIP waste container.

10. Incubate for 5-30 min in Developing Solution.
11. Determine the quantity of the probe by comparing the test strips to the control strip and select two reference probes that are the best match to the control strip concentrations.
12. Prepare an adequate amount of the selected probe dilution series for use as the reference dilution series during quantification of the 96 well probe plate.

    Pause Point Quantified RNA Probe Reference Controls can be stored at -80ºC indefinitely.

Table 6 **Troubleshooting**

Step	Problem	Possible Cause	Solution
Step 28	Agarose gel analysis shows no PCR band	Secondary structure may be causing PCR to fail	Linearize DNA template
	Agarose gel analysis shows multiple PCR bands	Mispriming Template quality problem	Adjust PCR conditions Prepare plasmid DNA for PCR template in place of cell dilution
Step 95	Massive embryo damage (torn or broken embryos)	Embryos were left in the bleach too long (Step 8) Embryos were shaken too hard (Step 13)	Observe bleach time limit Shake less vigorously
	Vitelline membranes attached	Embryos were shaken insufficiently (Step 13)	Shake for full minute
	Embryos flattened	Too many embryos in some wells can cause slow draining and excessive vacuum pressure	Add fewer embryos per well
		Vacuum may be set too high (Steps 64-92)	Reduce vacuum
		Too many embryos stored in the same 50 ml storage tube	Store storage tube horizontally and store fewer embryos in one tube
	No staining	Chorion membrane is attached	Leave embryos in bleach longer Use fresh bleach (Step 8)
		The probe is too long to penetrate embryo	Add base hydrolysis step to reduce size of probe: Substitute 20 ul 0.2 M Na_2CO_3 pH 10.2 for water in Step 42 and incubate at 60°C for 15 min before proceeding to probe precipitation (Step 43)
		Sense strand probe made instead of antisense (Step 36)	Use appropriate RNA polymerase

Figure Legends

Figure 1 Flowchart for high-throughput RNA *in situ* hybridization to whole-mount Drosophila embryos. The major steps required for in situ hybridization to whole mount embryos are shown in boxes linked by arrows. Boxes on the left describe preparation of embryos and boxes on the right describe probe preparation. Approximate duration of each step is indicated in parentheses inside the box.

Figure 2 Size verification of PCR products for probe templates.
A negative image of an agarose gel shows 5 ul from each of 96 PCR reactions. Size markers (Roche molecular weight marker X) are in the outside lanes. Reactions yielding a single visible band of the appropriate size were used to generate RNA probes.

Figure 3 Quantification of RNA probes.
Dot blot of 96 probes, with 1 ul of a 1:300 dilution of each probe, shows acceptable variability of probe concentrations. Two reference dilutions series of 1:3.3, 1:10, 1:33, 1:100, and 1:330 are spotted above row A. On this blot the 1:300 reference dilution is not visible for either reference control probe and the 1:100 dilution is barely visible for the weaker reference probe (set on the right). Of the 96 probes, all but 5 spots are darker than the 1;100 dilution of the weaker reference control. The weakest probes, D3, G11, G12, were noted as weak and marked for rework, pending hybridization results.

Figure 4 Anticipated results. Stained embryos were mounted in 70%glycerol in PBS on a microscope slide without a coverslip to record production images (**a, b**) and screen for genes with patterned expression in the embryo. Production images were captured using a dissection microscope (Leica Wild M10) and a ProGres 3012 digital camera. Shown are images for embryos hybridized with probes for *snail* (**a**) and *odd skipped* (*odd*) (**b**). Higher resolution images for both embryos and imaginal discs were taken with a Spot RT digital camera mounted on a Zeiss Axiophot microscope with DIC optics, using a 20X objective. Embryos in 70% glycerol were mounted on slides under a 22x40 mm cover slip with 18mm coverslip spacers. Imaginal discs were mounted in 70% glycerol under a 22 mm coverslip without spacers. Shown are stage 5 (**c**) and stage 9 (**d**) embryos hybridized with a probe for *odd* and a leg disc (**e**) and an eye-antennal disc (**f**) stained with a probe for the forkhead domain gene *fd96Cb*. Embryos in (**c**) and (**d**) are oriented anterior to the left and dorsal up. The leg disc (**e**) is oriented anterior left and dorsal up. The eye-antennal disc (**f**) is oriented eye portion at the top and antennal portion on the bottom, lateral to the left and posterior up. In both the leg disc and antennal portion of the eye-antennal disc, expression is detected primarily in the ventral regions with patches on either side of the anterior/posterior boundary. Scale bars are 50 μm for **c-f** and 200 μm for **a-b.**

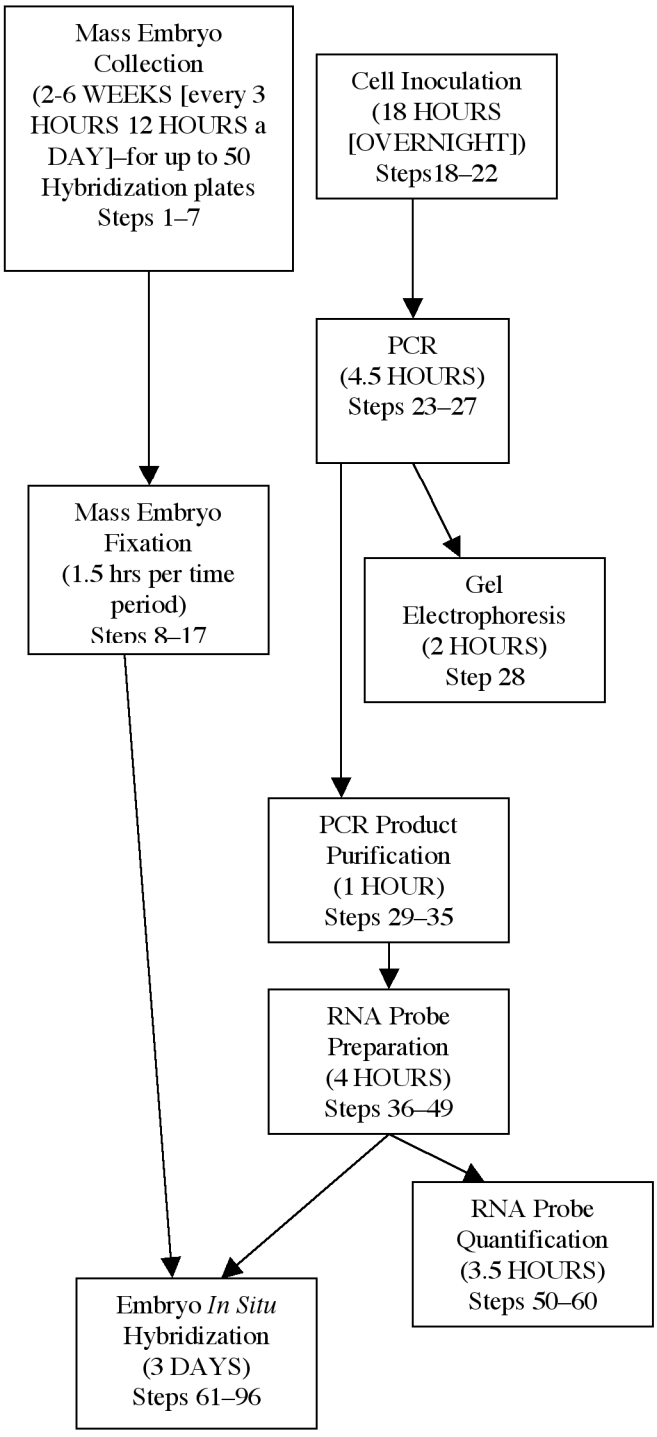
Mass Embryo Collection (2-6 WEEKS [every 3 HOURS 12 HOURS a DAY]–for up to 50 Hybridization plates Steps 1–7
Cell Inoculation (18 HOURS [OVERNIGHT]) Steps18–22
PCR (4.5 HOURS) Steps 23–27
Mass Embryo Fixation (1.5 hrs per time period) Steps 8–17
Gel Electrophoresis (2 HOURS) Step 28
PCR Product Purification (1 HOUR) Steps 29–35
RNA Probe Preparation (4 HOURS) Steps 36–49
RNA Probe Quantification (3.5 HOURS) Steps 50–60
Embryo *In Situ* Hybridization (3 DAYS) Steps 61–96

Figure 1

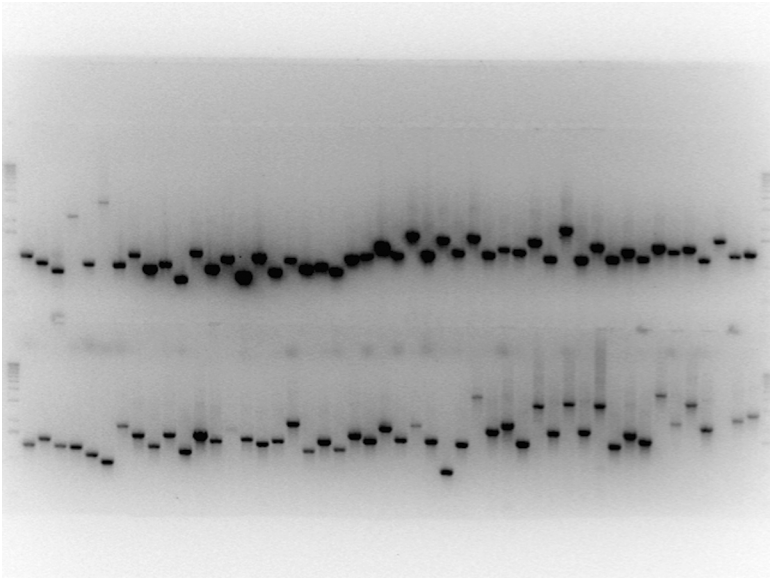

Figure 2

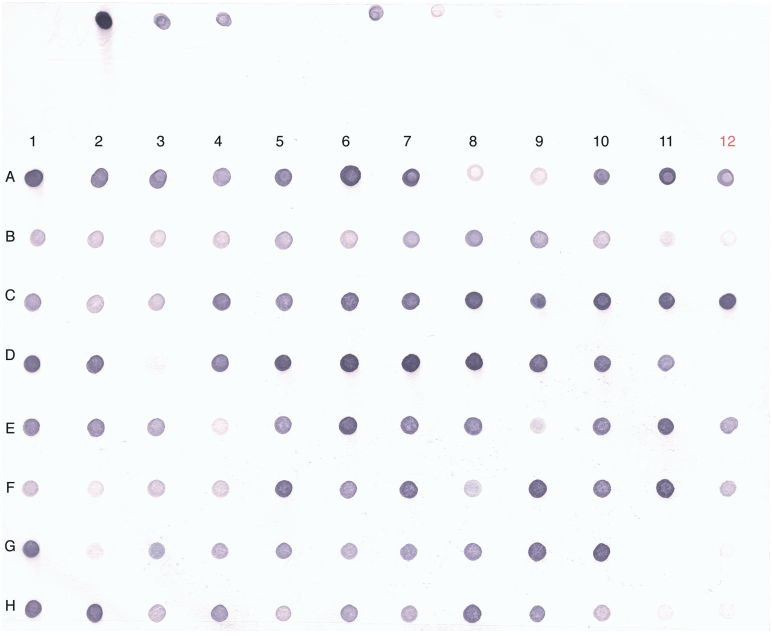

Figure 3

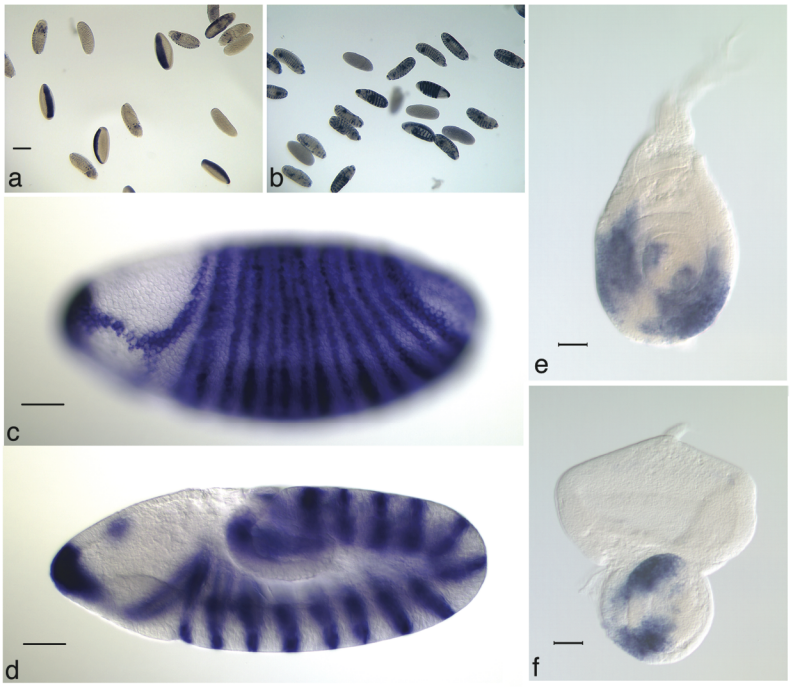

Figure 4