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Fluorescent in situ hybridisation for juvenile Fasciola hepatica

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We use this protocol and it's working

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Abstract

This FISH protocol has been adapted for use in *Fasciola hepatica*, specifically 4-week-old juveniles, grown *in vitro* in 50% Chicken Serum, 50% RPMI culture medium. The majority of this protocol comes from those of the Newmark Lab (King and Newmark, 2013) and Collins Lab (Collins and Collins, 2017). We are grateful to members of these labs for several helpful discussions on this technique.

Guidelines

Following a long period of relative success with this technique, we had noticed difficulties emerged. After trying various tweaks, we decided to purchase the vast majority of reagents again. This seemed to have helped significantly.

For all steps prior to and including the riboprobe incubation, all tips and tubes should be RNase free. Reagents should be made up with DEPC-treated double distilled H_2O - for 1 litre of DEPC-treated water, add 1ul Diethyl pyrocarbonate (D5758, Sigma) per 1ml of H_2O. Shake vigorously for 15 seconds. Leave at room temperature overnight, or 2 hours at 37^{o}C, then autoclave.

We have found that longer riboprobes (800-1000 bases) have generally worked better than shorter (150-300 bases) ones.

Alongside every positive test, we run the sense probe as a negative control. This is especially helpful when we see probes with otherwise ambiguous/questionable staining.

Highlighted solutions have recipes at the bottom of the protocol.

Safety warnings

Various reagents through this protocol have hazards associated with them. Consider these before use.

Before start

Ensure in situ oven is at the desired temperature. We usually switch the oven on at the start of the day 1 and allow the oven to reach a stable temperature.

Riboprobe Preparation

1 Amplify target of interest with an end-point PCR using FastStart Taq polymerase, dNTPack (4738381001, Sigma). Primers should have a 5′-TAATACGACTCACTATAGGGT-3′ promoter sequence. For the antisense probe (positive) use the regular forward primer and the T7-tagged reverse primer. For the sense probe (negative control) use the T7-tagged forward and the regular reverse. 2h 30m

2 Purify resulting PCR products with the ChargeSwitch PCR clean-up kit (CS12000, Thermo) according to the manufacturers guidelines. Only 15µl of elution buffer is used to maximise amplified target concentration. 20m

3 Perform a transcription reaction containing 2µl transcription buffer, 1µl T7 polymerase (both from T7 polymerase kit, EP01111), 6µl purified PCR product, 1µl DIG-RNA labelling mix (11277073910, Sigma). Incubate Overnight at 27 °C. 20m

4 DNase treat the probes by adding 0.25µl DNase I (18047019, Thermo), 0.25µl transcription buffer (from previous step) and 2µl RNase-free H_2O. Incubate at 37 °C for 00:15:00. 15m

5 Add 1.25µl **4M LiCl** and 37.6µl molecular-grade ethanol (51976, Sigma). Precipitate probes Overnight at -80 °C. 5m

6 16000 x g, 4°C, 00:20:00 20m

7 Remove supernatant, wash pellet with 70% ethanol, 16000 x g, 4°C, 00:05:00, discard supernatant and allow to air-dry for 00:15:00. 20m

Expected result

Pellet is barely visible to the eye. We would recommend removing supernatants while viewing the pellet under a microscope.

8 Resuspend pellet in 20µl RNase-free H_2O. Check purity and concentration on a spectrophotometer/fluorometer (we use a DeNovix DS-11 FX). 20m

9 Adjust concentration to 50ng/µl with **Hybridisation Buffer**, store probes at -20 °C until use. 10m

Preparation of yeast RNA (mainly from Jiang [2012])

10 Add 1g of torula RNA (RNA from torula yeast, type VI [R6625, Sigma]) to a 50ml conical tube. 5m

11 Add 10ml phenol (P4557, Sigma) and 15ml Tris-EDTA buffer (12090015, Thermo). Vortex tubes until the yeast RNA has dissolved. 16000 x g, 4°C, 00:10:00 10m

12 Collect the aqueous phase into a new tube (50ml), add 1:1 phenol/chloroform (P2069, Sigma), mix well, 16000 x g, 4°C, 00:10:00 . 10m

13 Collect aqueous phase into a new tube, add 1:1 chloroform, mix well, 16000 x g, 4°C, 00:10:00 10m

14 Collect aqueous phase into a new 50ml tube, add 1ml 3M sodium acetate (71196, Sigma) and 30ml molecular-grade ethanol. Leave at -20 °C Overnight to precipitate.

15 16000 x g, 4°C, 00:20:00 , discard supernatant, wash pellet in 70% ethanol, 16000 x g, 4°C, 00:10:00 30m

16 Remove supernatant, allow pellet to airdry for 00:15:00 , add 5ml formamide pre-heated to 50 °C 15m

17 Vortex until the pellet is resuspended

18 Measure concentration and purity on a spectrophotometer/fluorometer. Adjust concentration to 50mg/ml and store in 1ml aliquots at -20 °C .

Worm Preparation 1d 0h 40m

19 Carry out excystment (see McVeigh *et al*., 2014), transfer newly excysted juveniles to 96-well round-bottomed plates, in groups of <25 NEJs per well. Add 200µl of pre-warmed, pre-mixed 50:50 chicken serum (New Zealand Origin, 16110082, Thermo) and RPMI (11835030, Thermo). Replace this every other day. Worms are maintained in a 37 °C incubator with 5% CO_2. 1d

20 At 4 weeks old, wash juveniles out of 50:50 and add RPMI. Transfer worms into a small petri dish (Starstedt – 83.3925) containing **0.25% Tricaine** diluted in RPMI. Leave worms in this for 00:03:00 . 3m

Expected result

Worms should initially curl ventrally but this should be greatly reduced following this incubation.

21 Transfer worms in a minimal amount of tricaine/RPMI into a 20µl droplet of **4% Formaldehyde** and set a glass coverslip on top. Leave for 00:10:00 . Remove the coverslip and collect worms into 1.5ml hydrophobic tubes (1210-10, SSIBio). Add 1ml 4% formaldehyde. Rotate tubes for 00:10:00 . 20m

22 Remove 4% formaldehyde and wash once with **PBST**, then replace this with a pre-mixed 1:1 ratio of PBST and methanol (32213, Sigma) rotating for 00:10:00 . Worms can then be stored in 100% methanol at -20 °C until use. We are yet to test how long worms can be stored for and still be suitable. We would be reluctant to use worms stored for longer than 6 months. 10m

Day 1 FISH (each step begins with removing the solution from the last) (volumes are 1ml unless otherwise stated)

23 Remove 100% methanol from worms and replace with 1:1 PBST:Methanol for 00:10:00 rotating. 10m

24 Add PBST, incubate for 00:10:00 rotating 10m

25 Add **1xSSC** and incubate for 00:10:00 with no rotation 10m

26 Incubate worms in 200µl **Bleaching solution** under a bright light for 01:00:00 1h

27 Add 1xSSC and incubate for 00:10:00 with no rotation 10m

28 Add PBST, incubate for 00:10:00 rotating. 10m

29 Add **Proteinase K solution**, incubate for 00:15:00 with no rotation. 15m

30 Add 4% formaldehyde, incubate for 00:10:00 rotating. 10m

31 Add PBST

32 Transfer worms in 500µl PBST into in situ baskets (12.444, Intavis) in a 24-well plate. Add 500µl **Prehybridisation buffer**.

33 Add plate to a shaker for 00:10:00 set at 100rpm (any future shaking steps are at 100rpm) 10m

34 Add Prehybridisation buffer (pre-warmed to 52 °C) to the next well and transfer the baskets to these. Place the plate in an in situ oven pre-warmed to 52 °C. Have the plate rocking. Our in situ oven (Boekel Scientific Shake 'N Bake™ Rocking Hybridization Oven/Incubator, 136400) only allows for gentle rocking.

34.1 Approximately 10 minutes before the end of the above 2 hours, prepare the probe as follows - heat 20µl of 50ng/µl probe to 80 °C for 00:05:00 then place on ice. Add 980µl Hybridisation buffer to the probe. 5m

35 Add the probe mix to the next well, transfer baskets to this well and incubate Overnight rocking at 52 °C.

Day 2 FISH (volumes are 1ml unless otherwise stated) (each basket wash is carried out in a new well unless otherwise stated)

36 Remove 500µl of probe mix from the well with the worms and add 500µl pre-heated **2xSSC**, incubate for 00:20:00 rocking at 52 °C . 20m

37 Wash in 2xSSC, incubate for 00:20:00 rocking at 52 °C . 20m

38 Repeat step 36) 2 more times, moving baskets into new wells each time.

39 Wash with **0.2xSSC**, incubate for 00:20:00 rocking at 52 °C 20m

40 Repeat step 38) 3 more times, moving baskets into new wells each time.

41 Wash with **TNT**, incubate for 00:10:00 shaking at Room temperature 10m

42 Repeat step 41) once

43 Incubate in **Blocking solution** for 01:00:00 shaking at Room temperature 1h

44 Incubate in **Antibody solution** Overnight shaking at 4 °C 1h

Day 3 FISH (volumes are 1ml unless otherwise stated)

45 Wash with TNT for 00:05:00 , then 00:10:00 , and then 6x 00:20:00 shaking at Room temperature . 35m

46 Protect plates from light from this point on. 4h 10m

FOR FLUORESCENT ISH

Incubate in freshly-made **Tyramide solution** for 00:10:00 , shaking at Room temperature . Go to step 46).

FOR CHROMOGENIC ISH

Transfer worms to small petri dishes and incubate in **Exposure buffer** for up to 04:00:00 with no rotation, checking every 20 minutes or so. If no signal has developed after 4 hours, incubate the dishes at 4 °C . This slows any subsequent development but this allows you to leave it overnight. When a sufficient signal has developed, halt the reaction by washing worms with TNT. Wash the worms in 100% ethanol to reduce any background/non-specific staining. Wash worms with TNT. Go to step 47).

47 Incubate worms in **DAPI solution** for 00:20:00 , followed by two 10 minute washes in TNT. 20m

48 Mount worms in 10µl Vectashield (H-1000, Vector Labs) on standard microscope slides and seal coverslips with clear nail polish.

Recipes (presented in order of first appearance) (DEPC-treated H_2O presented as DT-H_2O, double distilled water presented as ddH_2O) 2h

49 **4M LiCl** (100ml) 2h

Add 16.96g LiCl (L9650, Sigma) to 50ml DT-H_2O. Add this slowly as this is an exothermic reaction. Once dissolved, bring volume to 100ml with ddH_2O.

Hybridisation Buffer (50ml)
25ml deionised formamide (F9037, Sigma)
12.5ml 20xSSC (S6639, Sigma)
100µl 1mg/ml yeast RNA
5ml 10% (v/v) Tween-20 (5ml Tween-20 [P1379, Sigma] + 45ml DT-H_2O)
5ml 50% Dextran sulfate (S4030, Sigma)
2.4ml DT-H_2O

0.25% Tricaine
50mg Ethyl 3-aminobenzoate methanesulfonate (E10521, Sigma) in 2ml RPMI

4% Formaldehyde (4.5ml)

500µl 36.5% formaldehyde (F8776, Sigma)
4ml PBST (see below)

PBST (200ml)
1 tab PBS (P4417, Sigma)
200ml DT-H_2O
600µl Triton-X (T8787, Sigma)

1xSSC (20ml)
1ml 20xSSC
19ml DT-H_2O

Bleaching solution (2ml)
1.77ml DT-H_2O
100µl deionised formamide
50µl 20xSSC
80µl H_2O_2 (H1009, Sigma)

Proteinase K solution (~5ml)
4.95ml PBST
50µl 10% SDS (dissolve 5g SDS [L4509, Sigma] in 50ml DT-H_2O)
2.5µl Proteinase K (03115887001, Sigma) (check the bottle for exact stock concentration as this varies, adjust calculation accordingly. End concentration is to be 10ug/ml)

Prehybridisation buffer (50ml)
25ml deionised formamide
12.5ml 20xSSC
100µl 50mg/ml yeast
5ml 10% (v/v) Tween-20
7.4ml DT-H_2O

2xSSC (20ml)
17.98ml ddH_2O
2ml 20xSSC
20µl Triton-X

0.2xSSC (20ml)
19.78ml ddH_2O
200ul 20xSSC
20µl Triton-X

TNT (~1L)
1L ddH_2O
12.11g Tris Base (T1503, Sigma)
8.77g NaCl (27800.291, VWR)
3ml Triton-X
pH to 7.5 and filter sterilise

Blocking Solution (8ml)
400µl Horse serum (H0146)
40µl Western Blocking Solution (11921673001, Sigma)
7.56ml TNT

Antibody solution (4ml)
2µl Anti-DIG-AP (Chromogenic ISH) (11093274910, Sigma) / 3ul Anti-DIG-HRP (Fluorescent ISH) (11207733910, Sigma)
3998µl Blocking Solution

Tyramide Solution (~5ml)
5ml TSA Buffer (see below)
1µl 20mg/ml 4-iodophenylboronic acid (see below)
6µl 5% H_2O_2 (492µl TSA Buffer + 8µl H_2O_2)
2µl FAM-Tyramide (see below)

TSA Buffer (200ml)
58.44g NaCl
3.09g Boric acid (B6768, Sigma)
100ml ddH_2O, bring to 200ml when dissolved
pH to 8.5 and filter sterilise

20mg/ml 4-iodophenylboronic acid (2ml)
40mg 4-iodophenylboronic acid (IPBA, 471933, Sigma) in 2ml dimethylformamide (DMF, 227056, Sigma)

FAM-Tyramide
- Tyramine solution = Dissolve 10mg tyramine hydrochloride (T2879, Sigma) in 1ml DMF, add 10µl triethylamine (T0886)
- FAM-DMF = Dissolve 10mg 5(6)-FAM, SE (5-(and-6)-Carboxyfluorescein, Succinimidyl Ester) (C1311, Thermo) in 1ml DMF
Add 342.5µl tyramine solution to 1ml FAM-DMF
Incubate at for 02:00:00, rotating at room temperature
Add 8.6ml ethanol for a 1mg/ml stock

Split into 20µl aliquots and store at -20 °C

Exposure Buffer (~10ml)
Prepare 50ml tubes of each of;
- 1M Tris base = 6.06g
- 1M NaCl = 2.92g
- 1M $MgCl_2$ = 4.76g

Bring each to 50ml with ddH_2O

Prepare PVA - bring 80ml ddH_2O to 75 °C while stirring and slowly add 10g PVA (P8136, Sigma). If you add this too quickly it will form clumps and not dissolve.

1ml 1M Tris base
1ml 1M NaCl
500µl 1M $MgCl_2$
100µl 10% Tween-20
7.4ml PVA
45µl 4-Nitro blue tetrazolium (NBT, 11383213001, Sigma)
35µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, 11383221001, Sigma)

DAPI Solution (10ml)
10µl 1mg/ml DAPI (dissolved in ddH_2O) (D9542, Sigma)
9.99ml TNT