Protocol 3: Whole mount *in situ* hybridization (WISH)

The WISH protocol is probably the most complex experiment suggested because of the many steps and reagents required. The aim of this experiment is to show where a gene of interest is expressed by visualizing its mRNA with a riboprobe able to specifically recognize it. The bravest and more curious student can explore expression pattern of genes different from those proposed if they would like to look for mRNA sequence, clone it and produce the specific riboprobes.

On the other hand, students at any level can look at the figures published in the paper "Hands-on, classroom studies of regeneration, and stem cell biology using freshwater planarians" and try to dissect with the teacher the steps of the protocol.

3.1. Identification of mRNA species-specific sequences and primer design

This WISH protocol is pretty specific and significantly reduces the risk of non-specific binding of the riboprobes or background signal. Accordingly, we prepared species-specific riboprobes for each planarian species considered. To accomplish this, the first step is finding the mRNA sequence of the gene of interest, download it and design the specific primers.

Among the tools available in this website, the search for mRNA sequences of four planarian species is provided with the relative tutorials.

If you know the name or the function of the gene of interest, please, look at the tutorial "Search for planarian genes based on gene name or function" and use the tool "Search genes". The tool "Sequence similarity search" allow you for looking for the planarian sequences that are highly similar to one sequence already identified and available in

another planarian species (such as *Schmidtea meditteranea*) or in a different animal (such as the human *Homo sapiens* or the mouse *Mus musculus*). In order to learn how to use this tool, please, look at the tutorials “Search for planarian genes based on protein homology” and “Search for planarian genes based on sequence similarity”.

Once one mRNA sequence for each gene of interest for each planarian species was obtained, the specific primers were designed. The tutorial “Design primer specific for a sequence of interest” explains how to correctly exploit the “Primer design” tool that is available each time that a sequence of interest is found.

One primer forward (F) and one primer reverse (R) were designed for each sequence of interest for the *in situ* hybridization protocol, identified in *Girardia sp.* (Table 2)*, D. dorotocephala* (Table 3)*, P. morgani* (Table 4) and *P. gracilis* (Table 5). All the primers are 20 bp long, their melting Temperature (Tm) is 54-58°C and the amplified region will be 700-1200 bp long.

Before each primer the two following sequences were added in order to allow the cloning step specifically into the PR-T4P plasmid:

- 5’-CATTACCATCCCG + Primer F sequence-3’
- 5’-CCAATTCTACCCG + Primer R sequence-3’

The final primer sequences were ordered from the Integrated DNA Technologies, Inc. using their “Custom DNA Oligos” service.

REQUIRED MATERIALS 3.1:

Laptop

Internet connection

3.2. cDNA production and gene cloning

Total RNA for each species was purified from 3-5 adult worms 5-7 mm in length using TRIzol® (Ambion). Tissues were dissociated using a pestle and then the manufacturer's instructions were followed. The RNA was dissolved in Milli-Q water with a concentration of about 1 µg/ml and the quantity and quality of the RNA were evaluated with the NanoDrop 1000 Spectrophotometer.

Purified total RNA (1-2 µg) was used as template during the retrotranscription reaction with the SuperScript III reverse transcriptase enzyme (Invitrogen) that synthesizes cDNA (DNA complementary to the RNA template). Following the manufacturer's instructions, 20 µl of cDNA were obtained and then stored at -20°C.

One PCR reaction per each primer pair was performed with 1 µl of cDNA as template and the enzyme Phusion DNA polymerase (New England Biolabs) following the manufacturer's instructions. The following touchdown PCR program was used for all of the primer pairs reported (Table 2-5):

- 1 cycle: 2 min at 98°C
- 8 cycles: 30 sec at 98°C, 30 sec from 65°C to 58°C (the Tm is decreased of 1°C each cycle), 90 min at 72°C
- 32 cycles: 30 sec at 98°C, 30 sec at 58°C, 90 sec at 72°C
- 1 cycle: 5 min at 72°C.

A small fraction of the amplified DNA (1-2 µl) was run on a 1% agarose gel in 1x TAE buffer. If a single band of the proper size was obtained, the residual fraction (17-19 µl) was purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. If multiple bands were present, the residual fraction (17-19 µl) was run on a 1% agarose gel in 1x TAE buffer and the band with the expected size

was specifically cut out from the gel using a blade and purified with the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. In both cases, the quantity and quality of the amplified and purified DNA were analyzed with the NanoDrop 1000 Spectrophotometer.

The PR-T4P plasmid (Adler et al., 2014; Rink et al., 2009) was digested overnight (ON) with the restriction enzyme SmaI, run on a 1% agarose gel in 1x TAE buffer and the open plasmid was purified from the gel with the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions.

Both plasmid and fragment were treated separately with T4 DNA polymerase and GTP nucleotides or CTP nucleotides, respectively (as described by the manufacturer) to create specific and complementary sticky ends. The reactions were incubated for 30 min at 22°C and for 20 min at 75°C.

This treatment of plasmid and fragment allows a directional ligation, which means that the fragment binds the plasmid only in the predicted direction and not upside-down.

The ligation happened mixing 2 µl of the fragment with 1 µl of the PR-T4P plasmid and incubating for 5 min at room temperature (RT).

The obtained constructs (the fragment bound to the plasmid) were stored at -20°C.

REQUIRED MATERIALS 3.2:

TRIzol®
Plastic tubes (1.5 ml)
Pestels
Chloroform, Isopropanol and Ethanol
Milli-Q water
NanoDrop 1000 Spectrophotometer
SuperScript III kit (Oligo(dT)$_{20}$ primer, dNTPs Mix, 5x First strand buffer, DTT, RNase Inhibitor and SuperScript III retro transcriptase)
Plastic tubes (0.2 ml)
Thermocycler

Phusion DNA polymerase, together with 5x High Fidelity buffer, dNTPs mix and $MgCl_2$

Primers F and R (Section 3.1)

Agarose and 1x TAE buffer

Electrophoresis chamber

Loading dye and 1 kb DNA ladder

UV transilluminator

QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (blade and thermoblock)

Plasmid PR-T4P

SmaI restriction enzyme and 10x NEB buffer #2

T4 polymerase, its buffer and BSA

GTP and CTP nucleotides

3.3. Transformation and insert sequencing

Keeping the construct (3 µl) on ice, 48 µl of competent *Escherichia coli* DH5α strain was added to it. The mix was incubated for 10 min on ice, and then a heat shock was performed for 35 sec at 42°C after which the tube was moved back into the ice. After 5 min, the transformed *E. coli* were placed at RT and 1 ml of Luria Broth (LB) without antibiotics were added. The cells were placed on a shaker at 250 rpm for 1 h at 37°C to recover and, finally, 200 µl of them were spread on a LB-Kanamycin (Kan, 50 µg/ml) plate that was subsequently incubated ON at 37°C.

Only bacteria transformed with a plasmid containing the insert are Kan-resistant and able to form a colony on the plate.

Optional step: To double-check the presence of the construct inside the bacteria, a colony screening was performed by PCR. From each plate, 3-5 colonies were selected, picked up, used for both colony screening PCR and 5 ml LB-Kan (50 µg/ml) broth culture. The PCR was constituted of the plasmid transformed inside the bacteria as template, the Taq DNA polymerase and two primers (AA18: 5'-

CCACCGGTTCCATGGCTAGC-3' and PR244: 5'-CACATAACCCCTTGGGGCCTC-3') that bind the plasmid sequence at the two sides of the insert, in the proportion suggested by the manufacturer. The PCR program used was:

- 1 cycle: 5 min at 95°C
- 35 cycles: 30 sec at 95°C, 30 sec at 58°C, 120 sec at 72°C
- 1 cycle: 5 min at 72°C.

Half of the amplification reaction (10 µl) was run on a 1% agarose gel in 1x TAE buffer to distinguish the positive colonies (one signal with the insert size) from the negative one (no band).

If the colony screening is not performed, 2-3 colonies were selected from the plate, picked up and used only for 5 ml LB-Kan (50 µg/ml) broth culture, that were incubated ON in a shaker at 250 rpm and at 37°C. The plasmid-insert construct contained in the bacteria grown ON was purified using the QIAprep® Spin Miniprep Kit (Qiagen) by following the manufacturer's instructions. The quantity and quality of the eluted plasmid were evaluated with the NanoDrop 1000 Spectrophotometer.

An aliquot of the construct, together with the primer AA18, was submitted to sequencing. The instruction for preparing the sample and for shipping it are always reported in detail on the website of the Company that offers the sequencing service.

The sequencing output is used to verify that the correct gene was cloned.

Please, look at the provided tutorial "Search for planarian genes based on sequence similarity" and use the available tool "Sequence similarity search" to compare the mRNA sequence found in Section 3.1 with the sequencing output (cloned fragment). If the correct sequence was cloned in the plasmid, the construct can be used for the riboprobe synthesis.

REQUIRED MATERIALS 3.3:

Construct (Section 3.2), ice and plastic tubes (1.5 ml)

Competent *Escherichia coli* DH5α

Water bath or thermoblock

Luria Broth (LB)

Incubator at 37°C and shaker

LB-Kan (50 µg/ml) plate and LB-Kan (50 µg/ml) broth

Optional step: Thermocycler, plastic tube (0.2 ml), Taq DNA polymerase, Taq buffer, dNTPs mix and $MgCl_2$, agarose and 1x TAE buffer, electrophoresis chamber, loading dye and 1 kb DNA ladder, UV transilluminator

Primers AA18 and PR244

Plastic tube (5 or 15 ml)

QIAprep® Spin Miniprep Kit

NanoDrop 1000 Spectrophotometer

Laptop and Internet connection

3.4. Riboprobe synthesis

Once the gene of interest is successfully cloned (Section 3.3), 0.8-1 µg of amplified insert was obtained by PCR, using 10 ng construct as template, the primer AA18 and PR244 and the enzyme Phusion DNA polymerase (New England Biolabs) following the manufacturer's instructions. To obtain the required amount of amplified insert sequence, 160 ul (4 reaction/40 µl total each) of PCR were prepared with 100 ng of template per each reaction. The touchdown PCR program reported in the Section 3.2 was used.

A small aliquot of the resultant amplified DNA (1-2 µl) was run in 1% agarose gel in 1x TAE to check if the reaction worked properly. The residual aliquot (157-159 µl) was purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's

instructions and the quantity and quality of the amplified and purified DNA were analyzed with the NanoDrop 1000 Spectrophotometer.

This amplified and purified DNA represents the template for the riboprobe synthesis reaction. The riboprobe synthesis reaction consists of the ON incubation (about 16 hours) at 37°C of 0.8-1 ug of purified PCR product, T7 RNA polymerase (Promega), the transcription optimized buffer (Promega), Digoxigenin (DIG)-nucleotides (Roche) and RNase Inhibitor (Promega) in Milli-Q water following the dilution suggested by the manufacturer. The nucleotide that constitute the riboprobes are labeled with DIG, in order to be able to detect the riboprobe localization with Ab anti-DIG.

The following day the synthesized riboprobe was purified:

1. Add to the mix 2 µl RNase-free DNase RQ1 and incubate for 1 h at 37°C.
2. Add 0.5 volumes of 7.5 M ammonium acetate and mix gently.
3. Add 2 volumes of ice-cold 100% Ethanol.
4. Add 2 µl of glycogen and incubate for 45 min at -20°C.
5. Centrifuge for 45 min at 4°C at max speed.
6. Remove the supernatant.
7. Wash twice with 500 µl ice-cold 70% Ethanol.
8. Centrifuge for 5 min at 4°C at max speed.
9. Remove the supernatant.
10. Resuspend the pellet in 100 µl deionized formamide.
11. Store the riboprobes at -20°C or -80°C for long-term storage.

A small aliquot of the synthesized and purified riboprobe (4 µl) was run in 1% agarose gel in 1x TAE to check if both synthesis and purification steps worked properly. It could

happen that the band is not well defined or exactly at the expected size because of the different migration property of the RNA compared to the DNA.

REQUIRED MATERIALS 3.4:

- Plasmid purified with Miniprep Kit (Section 3.3)
- Thermocycler and plastic tube (0.2 ml)
- Phusion DNA polymerase, together with 5x High Fidelity buffer, dNTPs mix and $MgCl_2$)
- Primers AA18 and PR244
- Agarose and 1x TAE buffer
- Electrophoresis chamber
- Loading dye and 1 kb DNA ladder
- UV transilluminator
- QIAquick PCR Purification Kit
- NanoDrop 1000 Spectrophotometer
- Thermoblock
- T7 RNA polymerase and the transcription optimized buffer
- DIG-nucleotides
- RNase Inhibitor
- Milli-Q water
- DNase RQ1 RNase-free
- 7.5 M ammonium acetate
- 100% and 70% Ethanol
- Glycogen
- Deionized formamide

3.5. WISH protocol

The WISH protocol is applied to visualize the mRNA target, meaning where the cells that express the gene of interest are localized. WISH data are mainly qualitative, but an indication about the abundance of mRNA in the different planarian tissues is possible to obtain. Because the specificity of the riboprobe binding is fundamental, both the sequence of the riboprobe, the hybridization conditions and stringency washes are equally and fundamentally important. The following protocol requires 4 days:

-Day 1: Animal fixation (planarians are killed and the layer of mucous is removed with N-Acetyl Cystein, they are fixed with paraformaldehyde treatment, then the tissues

are permeabilized with Triton X-100 and dehydrated for the long conservation in methanol)

-Day 2: Incubation with the riboprobes (planarians are rehydrateted, bleached for removing the pigment to help the visualization of the signal, treated with proteinase K that increase the accessibility to the tissue and incubated with the riboprobes labeled with DIG and diluted in the hybridization buffer)

-Day 3: Removal of excess riboprobe and incubation with the antibody (planarians are washed thoroughly to remove the unbound or non-specifically bound riboprobes and incubated with the antibody anti-DIG diluted in blocking solution, which deters binding of the antibodies in nonspecific locations)

-Day 4: NBT/BCIP colorimetric signal development (planarians are washed thoroughly to remove the unbound or non-specifically bound antibodies, and incubated in the solution that develop the colorimetric signal; then the non-specific signal and the background are washed out with Ethanol and the worms are fixed to preserved them for long-term storage in 80% Glycerol or 80% ScaleA2)

Day 1: Animal fixation

1. Collect 3-5 planarians (2-5 mm long and starved for 1 week) for each probe of interest (the triplicate is important to reconcile possible the differences between animals and ensure reproducibility, robustness, and consistency of the results).
2. Transfer the planarians into 1.5 ml plastic tubes (for processing up to 20 worms).
3. Rinse the worms two times before fixation to remove debris.
4. Incubate the worms with 5% N-Acetyl Cystein (NAC; mucolytic reagent that both

removes the mucus and kills the worms) in 1x PBS for 5 min at RT.

5. Incubate the samples in the fixative solution 4% paraformaldehyde in 1x PBSTx for 40 min at RT.
6. Rinse the worms twice with 1x PBSTx for 10 min at RT.
7. Incubate the worms in 50% Methanol for 10 min at RT.
8. Rinse the worms with 100% Methanol.
9. Replace the 100% Methanol and incubate the worms for 10 min at RT.
10. The worms are now completely dehydrated and can be stored in 100% Methanol at -20°C until use.

Day 2: Incubation with the riboprobes

BLEACHING OPTION A) The majority of the worms and probes work fine with the formamide bleach solution.

1. Move the worms into baskets placed into a 24 multi-well dish (1.5 ml plastic tube can be used as well through the entire protocol)
2. The 100% Methanol used for long-term storage is replaced by 50% Methanol for 10 min at RT.
3. Incubate the worms with 1x PBSTx for 10 min at RT.
4. Rinse the worms with PBSTx.
5. Incubate the worms in formamide bleach solution for 1 h at RT under direct light.
6. Rinse the worms twice with 1x PBSTw.

BLEACHING OPTION B) Some reactions required the methanol bleach solution instead of the formamide bleach solution because the latter is less gentle and cause the loosening of the epithelium and superficial structure.

1. The 100% Methanol used for long-term storage is replaced by the methanol bleach solution ON at RT under direct light.
2. Rinse the worms twice with 100% Methanol.
3. Incubate the worms in 50% Methanol for 10 min at RT.
4. Rinse the worms with 1x PBSTw.
5. Incubate the worms with 1x PBSTw for 10 min at RT.

Note: The bleaching step A was used on *Girardia sp.* for *frz-4, PC2, laminin, innexin-10*, *collagen* genes, on *D. dorotocephala* for *slit-1, sfrp-1, frz-4* genes, on *P. morgani* for all the riboprobes and *P. gracilis* for all the riboprobes. The bleaching step B was used on *Girardia sp.* for *piwi-1, ifb, slit-1, sfrp-1, opsin, porcupine-1* genes and on *D. dorotocephala* for *piwi-1, ifb, PC2, opsin, porcupine-1, laminin, innexin-10*, *collagen* genes.

After the bleaching step A) or B):

6. Incubate the worms with 2 µg/ml Proteinase K solution for 10 min at RT.
7. Incubate the samples in the fixative solution 4% paraformaldehyde in 1x PBS for 10 min at RT.
8. Rinse the worms twice with 1x PBSTw.
9. Incubate the worms in 1:1 1x PBSTw : PreHybridization (PreHybe) solution for 10 min at RT.
10. Incubate the worms in PreHybe for 2 h at 56°C.

11. Incubate the worms in 1:1000 (1:500-1:2000) riboprobe in Hybe solution ON at 56-60°C. If the incubation are performed in the 24 multi-well dish, the evaporation of the riboprobe solution has to be avoid sealing the dish with parafilm.

Note: All the probes used in the paper “Hands-on, classroom studies of regeneration and stem cell biology using freshwater planarians” were diluted 1:1000, excepted for detecting *collagen* gene expression in *D. dorotocephala* and *P. morgani,* where the riboprobes were diluted 1:500.

Day 3: Removal of excess riboprobe and incubation with the antibody

1. Incubate the worms in WashHybe solution two times for 30 min at 56-60°C.
2. Incubate the worms in 1:1 WashHybe : 2x SSC two times for 30 min at 56-60°C.
3. Incubate the worms in 2x SSC three times for 30 min at 56-60°C.
4. Incubate the worms in 0.2x SSC three times for 30 min at 56-60°C.
5. Incubate the worms in MABT three times for 10 min at RT.
6. Incubate the worms in filtered blocking solution for 2 h at RT.
7. Incubate with 1:3000 anti-DIG-AP conjugated in filtered blocking solution ON at 4°C.

Day 4: NBT/BCIP colorimetic signal development

1. Incubate the worms in MABT six times for 20 min at RT.
2. Incubate the worms in AP buffer for 2 min at RT.
3. Incubate the worms in EQ buffer for 10 min at RT and in the meaning while pull worms apart with a tungsten wire or forceps to allow homogeneous signal development.
4. Incubate the worms in DEV buffer at RT in the dark until the signal is completely developed.

5. Stop the developing reaction rinsing the worms 3 times in 1x PBS.
12. Incubate the worms in the fixative solution 4% paraformaldehyde in 1x PBSTx for 20 min at RT.
6. Incubate the worms in 100% Ethanol for 45 min or until background is sufficiently cleared at RT.
7. Incubate the worms in 50% Ethanol in 1x PBS for 5 min at RT.
8. Incubate the worms in 1x PBS until the worms sink.
9. Rinse the worms in 1x PBS twice.
10. Incubate the worms in the mounting media 80% Glycerol or 80% ScaleA2 for long-term storage.
11. Mount the worms on the slides: place one spacer (Sigma-Aldrich) on the slide, fill the hole of the spacer with the mounting media and the stained worms, place the coverslip above it avoiding the formation of bubbles under the coverslip (the spacers are sticky on both sides and they strongly adhere to both slide and coverslip).

REQUIRED MATERIALS 3.5:

Plastic Pasteur pipettes
Plastic tubes (1.5 ml)
Instant Ocean® Sea Salt or 1x Montjuïc water
PBS (Solution Recipes)
N-Acetyl Cystein
Paraformaldehyde
Triton X-100 and Tween-20
50% and 100% Methanol
Optional: Baskets and 24 multi-well dish and parafilm
Filter paper or filter unit to filter the solutions
Formamide or methanol bleach solution (Solution Recipes)
Proteinase K (Solution Recipes)
PreHybe (Solution Recipes)
Incubator at 56°C

Riboprobes (Section 3.4)

Hybe (Solution Recipes)

WashHybe (Solution Recipes)

2x and 0.2x SSC (Solution Recipes)

MABT (Solution Recipes)

Blocking solution (Solution Recipes)

Syringe filter 0.45 µm pore size and syringes

Antibody anti-DIG-AP conjugated

AP, EQ and DEV buffer (Solution Recipes)

Tungsten wire or forceps

50% and 100% Ethanol

Glycerol or 80% ScaleA2 (Solution Recipes)

Slides, coverslips and spacers

SOLUTION RECIPES:

- 1x PBS: 137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4 (monobasic), 10 mM Na_2HPO_4 (dibasic) in Milli-Q H_2O. pH to 7.4. Filter.

- 1x PBSTx: 0.5% Triton X-100 in 1x PBS. Filter.

- 1x PBSTw: 0.1% Tween-20 in 1x PBS. Filter.

- 5% NAC: 5% NAC in 1x PBS. Make it fresh.

- 4% paraformaldehyde: 4% paraformaldehyde in 1x PBSTx. Make it fresh.

- Formamide bleach solution: 1.2% H_2O_2, 5% formamide in 0.5x SSC. Make it fresh.

- Methanol bleach solution: 6% H_2O_2 in 100% Methanol. Make it fresh.

- Proteinase K solution: 0.1% SDS, 2 µg/ml Proteinase K in 1x PBS. Make it fresh.

- PreHybe: 50% deionized formamide, 5x SSC, 1x Dehardts, 10 µg/ml Heparin, 1% Tween-20, 50 mM DTT, 1 mg/ml Torula Yeast RNA (Sigma) in Milli-Q H_2O. Shake until all the yeast RNA has gone into solution. Store at -20°C.

- Hybe: 50% deionized formamide, 5x SSC, 1x Dehardts, 100 µg/ml Heparin, 1% Tween-20, 50 mM DTT, 0.25 mg/ml Yeast RNA (Calbiochem), 5% dextran sulfate in Milli-Q H_2O. Shake and warm up the solution at 37°C until all the yeast RNA and the dextran sulfate have gone into solution and it is fully mixed. Store at -20°C.

- WashHybe: 50% formamide, 0.5% Tween-20, 5x SSC, 1x Dehardts in H_2O. Store at -20°C.

- 20x SSC (Saline Sodium Citrate): 3 M NaCl, 300 mM Sodium Citrate, 1% Tween-20 in Milli-Q H_2O.

- MABT: 100 mM Maleic Acid, 150 mM NaCl, 0.1% Tween-20 in Milli-Q H_2O. pH to 7.5. Filter.

- Blocking solution*:* 5% horse serum, 0.5% Roche Western Blocking Reagent in MABT. Filter with syringe filter 0.45 µm pore size. Make it fresh.

- 10% PVA: 10% PVA in Milli-Q H_2O. Stir with heat but be sure not to let it boil over. Filter.

NBT/BCIP Development Solutions:

- AP buffer: 0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$, 0.1% Tween-20 in Milli-Q H_2O. Make it fresh.

- EQ buffer: 0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$, 0.1% Tween-20, 5% PVA in Milli-Q H_2O. Make it fresh.

- DEV Buffer: 0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$, 0.1% Tween-20 in 10% PVA. Before usage add 80 µl BCIP and 40 µl NBT. Make it fresh.

Mounting media:

- 80% Glycerol: 80% Glycerol, 1 mM EDTA, 10 mM Tris in Milli-Q H_2O. pH to 7.4.

- 80% ScaleA2: 2 M Urea, 80% Glycerol in Milli-Q H_2O.

Table 2: Primer for WISH protocol on *Girardia sp.*

Gene Name	Primer Sequence F	Primer Sequence R	Sequence length
piwi-1	GCAAAGATGGTAGAGTCGTC	CGATTATGCTCTTGAACTCC	3975
pc2	CTGCAGTAAAACTGCTATCG	GCAAGATCGTATTCCACTTC	1680
opsin-like	GCCTATTTCAGTGCTCATTC	CTTTAGTTGGTTTGGGACAG	1329
porcupine-like	GGCCGGAATTGTAGAGTAAG	AGAAAGCTAGTGGTTCGATG	1480
laminin-like	GGTAAATATGGGTGTTCCG	TTTGGTTTCACAGGAGAGTC	3189
innexin	GAATGGAAGACTTTGCAGAC	GTTGTAATCAAATCACCCGC	1078
collagen	AGGAAAACAAGGACCTGAAG	CCTAAAGGACCATTTGAACC	1002
ifb	GTGTGAGTTGCCTTTTCTTC	ACTACAGGAAACCAGAATGC	1697
slit	CAATATATCTCCACATCCCG	TACCAGATGGACTGTTTTCC	3692
sfrp-1	ATAGGCAGCATATCGTTCTC	TATCAGACACCACCAAACAC	1005
frizzled-like	AGACTCCGAGATTACAATGC	ATACTCGACCAATTAACCCC	1343

Table 3: Primer for WISH protocol on *Dugesia dorotocephala*

Gene Name	Primer Sequence F	Primer Sequence R	Sequence length
piwi-1	GCGAGGAGTACTACCAAATG	ACGGCTACAAGTAGAACAGG	2049
pc2	ACAACTTGATTGTCGAGGAC	TGTAAAACAAGGACCCACTC	2129
opsin-like	CACTTTCGTAAGCCTCATTC	CGTTCATCCTTACTGGAGAG	1139
porcupine-like	ATAATCGGCCTGAATCGTAG	AGGAGTAGAGTGGTTCGATG	1489
laminin-like	TTGTTCCCATAAGACCACTC	CGAATAGGTCGAATAGTTGG	3362
innexin	CTTCGTCTACGGTTTATTCG	GCGCATTGAGCTAAGTAATC	1007
collagen	GACCTCAAGGAATAGCTGGT	CCATGTCGGACCATTAAGTA	1001
ifb	GGATCACATCTGATAAACCG	ATAAAGTGCGACATCTGGAG	1526
slit	TTTACAGCTTCGAGGAAGAG	AAAACACCTGTCAACCAGAC	3812
sfrp-1	ATCGTTCCTGGAGACTTATG	TTGGTATTCGGGGAATG	903
frizzled-like	ACAACACGAAAGAGAGATGC	ATGTTCTCCGATTACCGAG	1949

Table 4: Primer for WISH protocol on *Phagocata morgani*

Gene Name	Primer Sequence F	Primer Sequence R	Sequence length
piwi-1	CCGGGATTAGGATTATTACC	ACCTTCACTTCAACCTGATG	1999
pc2	AGGAATTACAACGCAGAGAG	ATCGAACATGACGGTTAGTC	1203
opsin-like	AGCTCTGTCGATGAAGAAAG	ATCGAAGTAGGATTGGTGG	312
TNXB	TGTCTGTGCAAAAGATTCCT	CCGCCACAATTATTACACAT	803
laminin-like	ATACACGTGTGAGGGTATCG	TCATGACAACTATTGGTCCC	479
innexin	CATGTCTCAAATCACTGTGC	AAGTTGTCCAAGTATGTGGG	1414
collagen	CTCGAATAAGCAAACCACAG	GTTGGTGATCTCATTTGCAT	1300
ifb	ATGCTGCATAGAGTCCAGTC	GTCGGAGGTCAAAGTGTTAC	813
slit	GTCAAGTGCGTAGGTAGAGG	TATGCAGTCTTCGTCATGTG	636
sfrp-1	AAATCGCTTGCTTCCTG	CGTGAAGCAGGATAAAACTG	1005
frizzled-like	GCAGTTGGACTTTTTAGTGG	ATGCTTACTGGATACTTCGC	988

Table 5: Primer for WISH protocol on *Phagocata gracilis*

Gene Name	Primer Sequence F	Primer Sequence R	Sequence length
piwi-1	GTACCTTTAGTGGTTCGCTG	ATGGAGGAGAGAAAGGTAGC	2135
pc2	GTGAGTGCCGTACCTAGTTG	ACCATTATGTCATAGGGTCG	1758
opsin-like	AGTTGTCTCACGTTCCTTTG	AACTCCACCGTTAATGTGTC	1056
porcupine-like	TTACGGTGGTTCTACCATTC	GTTTTAACAGCAGACGTTCC	1436
laminin-like	ATGACCAACACTCTCTCCAG	CAGGTCAAATGAGGATAAGG	2950
innexin	ATGGTCTATCTCACCGTGTC	ACGTTACGACCTACAAATGG	1306
collagen	AGGAGATCCAGGAGAGACTG	GTCGGGTTTATTGGGATAAC	1300
ifb	GACGATTCTTCCGTTATCAG	ATGTAGTCATCAACCGAACC	1562
slit	CCTCCTCAAAGACAGACAAG	AACATCAGCCTCAAGTCATC	3731
sfrp-1	CATATCTCTTGCTTCCTTGG	CCAGTTTTCTGGCCTAATTC	578
frizzled-like	GGATGAAAACGTAGTCAAGG	GTCTGAGAAAGGATCGAATG	2023

References

Adler, C.E., Seidel, C.W., McKinney, S.A. & Sánchez Alvarado, A. (2014). Selective amputation of the pharynx identifies a FoxA-dependent regeneration program in planaria. *eLife*, 3, e02238.

Rink, J.C., Gurley, K.A., Elliott, S.A. & Sánchez Alvarado, A. (2009). Planarian Hh signaling regulates regeneration polarity and links Hh pathway evolution to cilia. *Science*, 326, 1406-1410.