Day 1. Polymerase chain reaction (PCR)

The purpose of today's exercise is to amplify (produce more of) DNA fragments encoding for specific planarian genes. The products obtained from today's PCR reaction will be used as templates for riboprobe synthesis in *in situ* hybridization experiments, as well as for double-stranded RNA (dsRNA) synthesis used in RNA-interference (RNAi) experiments.

Procedure

You should have 14 DNA gene fragments tube (labeled somewhere 1-100, plus *ccdB* (non planarian DNA negative control) and PC2 (planarian prohormone convertase 2; positive control).

PCR REACTION

5 ul Buffer *shake before use*
1.5 ul forward primer
1.5 ul reverse primer
1 ul dNTPs [10mM mix]
0.5 ul *Taq* polymerase
2 ul DNA gene fragment
13.5 ul MilliQ water

1) On ice, mix enough material for all +1 reactions in the following in order (this is a **master mix**):
   Buffer -> water -> primers -> dNTPs -> *Taq* polymerase (exclude DNA gene fragment)
2) Mix well and distribute 23 ul into individual PCR tubes
3) Add 2 ul of DNA gene fragment
4) Cap tube and spin down briefly
5) Place tubes in the Thermocycler and run the following protocol:

   95C for 10 minutes
   95C for 30 seconds
   56C for 30 seconds
   72C for 1 minute
      repeat last 3 steps for 40 cycles
   72C for 5 minutes
   4C forever

6) Analyze your PCR products by running 2 ul with 1 ul loading dye in a 0.8% agarose gel.
   Include 3 ul of ladder in your sample
   *store the rest of the PCR product at 4C overnight or -20C for longer periods.

Day 2.

In vitro transcription of double stranded RNA (dsRNA)

The purpose of today's exercise is to produce dsRNA by *in vitro* transcription of PCR product templates. Each dsRNA transcribed corresponds to a specific planarian gene (or a non-planarian gene for the negative control). The products obtained from today's dsRNA transcription reaction will be used to inhibit gene expression in RNA-interference (RNAi) experiments.

Before you begin

You should have 14 PCR products of 200-600 base pairs in size at a concentration of 50-200 ng/ul . These PCR products should have been extracted from all other ingredients in the PCR reaction mix and resuspended in 20 ul of **RNase-Free** water using RNase-free **filter** tips. We will use the *Zymo DNA clean-up kit for this procedure.

VERY IMPORTANT NOTE: Working with RNA can be compromised by RNA-degrading enzymes all around us (your saliva, skin, breath, laboratory space and materials). It is therefore imperative that we work carefully using RNase-free plastics, wear clean gloves, and do not touch anything that would compromise the stability of our RNA during this procedure.

Procedure

<u>dsRNA *in vitro* Transcription Reaction</u>

2 ul 10X Txn Buffer
3 ul RNAse free water
5 ul rNTPs [25mM each, made from 1:1:1:1 mixture of rATP, rUTP, rCTP and rGTP]
1 ul TIPP inorganic pyrophosphatase
0.5 ul RNAse inhibitor
1 ul **T7** RNA polymerase
7.5 ul PCR product (**specific template for each transcription reaction**)

1) On ice, mix enough material for all +1 reactions in the following in order (this is a **master mix**):
   Buffer -> water -> rNTPs -> RNAse inhibitor -> TIPP -> T7 RNA pol
2) Mix well and distribute 12.5 ul into individual 1.5 ml RNase-free eppi tubes
3) Add 7.5 ul PCR product and mix by flicking
4) Cap tube and spin down briefly
5) Place tubes in the 37C incubator for 3-4 hours
6) Analyze your dsRNA products by running 1 ul with 1 ul loading dye in a 0.8% agarose gel.
   Include 5 ul of ladder in your sample
   *store the rest of the PCR at -20C

Day 2.2.

In vitro transcription of digoxigenin (DIG) containing riboprobe

The purpose of this exercise is to produce DIG-containing riboprobes by *in vitro* transcription of PCR product templates. Each riboprobe transcribed will correspond to a specific planarian gene (or a non-planarian gene for the negative control). The riboprobes obtained this transcription reaction will be used to assess the expression distribution of corresponding genes in planarians.

Before you begin

You should have 14 PCR products of 200-600 base pairs in size at a concentration of 50-200 ng/ul (same as ones used for dsRNA synthesis). These PCR products should have already been resuspended in 20 ul of **RNase-Free water**.

VERY IMPORTANT NOTE: Working with RNA can be compromised by RNA-degrading enzymes all around us (your saliva, skin, breath, laboratory space and materials). It is therefore imperative that we work carefully using RNase-free plastics, wear clean gloves, and do not touch anything that would compromise the stability of our RNA during this procedure.

Procedure

DIG Riboprobe *in vitro* Transcription Reaction using **Promega** T3 RNA Polymerase

4 ul 5X Txn Buffer
2 ul DTT, 100 mM
5.6 ul RNase-free water
3 ul 10/10/10/7 nM A/C/G/U (respectively)+ U-DIG rNTP mix
*made with 10 ul of 100 mM ATP, CTP and GTP; 7 ul UTP; 10 ul DIG-UTP; 57 ul DEPC water
0.4 ul RNase inhibitor
1 ul **T3** RNA polymerase
4 ul PCR product (as specific template for transcription reaction) [30-200 ng/ul]

1) On ice, mix enough material for all +1 reactions in the following in order (this is a **master mix**):
Buffer -> water -> rNTP+DIG mix -> RNAse inhibitor -> T3 RNA pol
2) Mix well and distribute 16 ul into individual 1.5 ml RNase-free eppi tubes
3) Add 4 ul PCR product, cap tube, mix by flicking, and spin down briefly
4) Place tubes in the 37C incubator for 2-3 hours
5) Add 1ul of DNAse to each tube
incubate at room temperature for 10 minutes

Clean up riboprobe by ethanol precipitation (next page)

Day 2.2. (continued)

Clean up riboprobe by ethanol precipitation

1) Add 10 ul of 6.6 M LiCl
2) Add 75 ul of 100% Ethanol
3) mix by vortexing and store at -20C or below overnight
4) Centrifuge at maximum speed for 20 minutes at 4C
5) Remove supernatant by decanting of careful pipetting
6) Wash pellet carefully with 500 ul of cold 70% ethanol/RNAse free water
7) Centrifuge at maximum speed for 10 minutes at 4C
8) Remove supernatant by careful pipetting (I recommend using p200, brief spin and p10)
9) Allow to air-dry for 3 minutes
10) Resuspend pellet in 50 ul of Hybridization buffer
11) Heat to 70C for 10 minutes, vortex and spin briefly, decap for 10 seconds and store at -20C

12) Analyze your riboprobe by running 5 ul with 1 ul loading dye in a 0.8% agarose gel.
    Include 3 ul of ladder in your sample
    *store the rest of the riboprobe at -20C

Day 2.2.

In vitro transcription of digoxigenin (DIG) containing riboprobe

The purpose of this exercise is to produce DIG-containing riboprobes by *in vitro* transcription of PCR product templates. Each riboprobe transcribed will correspond to a specific planarian gene (or a non-planarian gene for the negative control). The riboprobes obtained this transcription reaction will be used to assess the expression distribution of corresponding genes in planarians.

Before you begin

You should have 14 PCR products of 200-600 base pairs in size at a concentration of 50-200 ng/ul (same as ones used for dsRNA synthesis). These PCR products should have already been resuspended in 20 ul of **RNase-Free water**.

VERY IMPORTANT NOTE: Working with RNA can be compromised by RNA-degrading enzymes all around us (your saliva, skin, breath, laboratory space and materials). It is therefore imperative that we work carefully using RNase-free plastics, wear clean gloves, and do not touch anything that would compromise the stability of our RNA during this procedure.

Procedure

DIG Riboprobe *in vitro* Transcription Reaction using **Thermo** T3 RNA Polymerase

4 ul 5X Txn Buffer
7.6 ul RNase-free water
3 ul 10/10/10/7 nM A/C/G/U (respectively)+ U-DIG rNTP mix
    *made with 10 ul of 100 mM ATP, CTP and GTP; 7 ul UTP; 10 ul DIG-UTP; 57 ul DEPC water
0.4 ul RNase inhibitor
1 ul **T3** RNA polymerase
4 ul PCR product (as template for transcription reaction) [30-200 ng/ul]

1) On ice, mix enough material for all +1 reactions in the following in order (this is a **master mix**):
    Buffer -> water -> rNTP+DIG mix -> RNAse inhibitor -> T3 RNA pol
2) Mix well and distribute 16 ul into individual 1.5 ml RNase-free eppi tubes
3) Add 4 ul PCR product, cap tube, mix by flicking, and spin down briefly
4) Place tubes in the 37C incubator for 2-3 hours
5) Add 1ul of DNAse to each tube
    incubate at room temperature for 10 minutes

Clean up riboprobe by ethanol precipitation (next page)

Day 2.2. (continued)

Clean up riboprobe by ethanol precipitation

1) Add 10 ul of 6.6 M LiCl
2) Add 75 ul of 100% Ethanol
3) mix by vortexing and store at -20C or below overnight
4) Centrifuge at maximum speed for 20 minutes at 4C
5) Remove supernatant by decanting of careful pipetting
6) Wash pellet carefully with 500 ul of cold 70% ethanol/RNAse free water
7) Centrifuge at maximum speed for 10 minutes at 4C
8) Remove supernatant by careful pipetting (I recommend using p200, brief spin and p10)
9) Allow to air-dry for 3 minutes
10) Resuspend pellet in 50 ul of Hybridization buffer
11) Heat to 70C for 10 minutes, vortex and spin briefly, decap for 10 seconds and store at -20C

12) Analyze your riboprobe by running 5 ul with 1 ul loading dye in a 0.8% agarose gel.
    Include 3 ul of ladder in your sample
    *store the rest of the riboprobe at -20C

Day 2.3. (and repeated in later days)

RNA-interference (RNAi) of gene expression by dsRNA feeding

The purpose of this exercise is to establish the requirement of specific genes for spermatogenesis. This will be done by disrupting expression of such genes by RNAi. The dsRNA synthesized in Day 2.1 will be mixed with planarian food (liver) and this will induce cleavage of corresponding gene mRNAs over the next few days. We will repeat this treatment for two weeks and ultimately observe the effect that each gene RNAi has on testes development.

Procedure

You should have 14-16 (plus control) dsRNA samples. These will be mixed with planarian liver containing food dye and fed to groups of 5 planarians each. After eating, planarians will be washed and placed under clean husbandry conditions until feedings is repeated or phenotype is assessed.

NOTE: dsRNA is very stable, so we do not have to worry about using RNase-free conditions. Use normal plastics, wear clean gloves, and do not touch anything that would compromise the stability of our RNA during this procedure.

<u>Food + dye preparation (calculated for 20-30 groups)</u>

1) Mix food, dye and water with a pestle in a 1.5 ml Eppi tube.
    -600 ul Minced Beef Liver
    -300 ul MilliQ water
    -10 ul McCormick food dye (blue, green, and red all work well
2) Centrifuge for 10 seconds
3) Distribute Liver paste into 40 ul aliquots in 1.5 mL tubes (do as many tubes as # genes tested)

<u>Clean up riboprobe by ethanol precipitation</u> (next page)

1) Add 4 ul of dsRNA (1-2 ug/ul) in each liver paste tube and mix by swirling in a circular motion on the bottom of the tube, then suction the liver (avoid bubbles!) and dispense in the bottom of the respective petri dish.
2) Allow planarian to eat in the dark for 15-30 minutes (they change color due to food dye and swim away from liver when done.
3) After the planarians are done feeding, rinse them in 0.75X salts and place them in a new (and labeled) petri dish with fresh salts until next feeding**.

** Feedings are repeated every 3-4 days until assessment of phenotype

Day 3 to 6. *in situ hybridization*

>>This protocol is based on published steps from Pearson et al. (Dev Dyn), 2009; and King and Newmark (BMC Dev Dyn), 2012.

*Animals should not be fed for at least 7 days prior to use for *in situ* hybridization.

First Day (kill, remove mucus, fix, reduce/permeabilize, dehydrate, bleach).

1) Transfer up to 20 sexual (of at least 1 cm in size) planarians to a 50-ml Falcon tube (repeat w/# tubes as needed).

2) Replace planarian water with a solution of 7.5% NAC in PBS. Rock semi-gently for 10 minutes at room temperature (RT). NAC kills the worms and removes their mucus.
***Note: this is the most sensitive step. Practice with fewer planarians on first try and do not exceed the recommended times**

3) Replace NAC solution with 4% Fixative soution (1 ml of 36.5% formaldehyde solution per 8 mls of PBSTx). Rock for 2 hrs at 4°C.

4) Remove 4% Fixative and rinse worms 2× with PBSTx for 5 minutes each.

5) Replace PBSTx with 50% Methanol in PBSTX and rock for 5 minutes.

6) Replace 50%Methanol with 100% Methanol rock for 5 minutes.

7) Replace 100% Methanol with new 100% methanol and store at -20C for 1 hr or longer.

Second Day (rehydrate, bleach, proteinase K, post-fix, hybridization).

1) Replace 100% Methanol with 50% Methanol in PBSTx, rock for 5 min at room temp.

2) Replace 50% Methanol with PBSTX. Rock for 5 minutes at room temp.

3) Replace PBSTX with 1XSSC. Rock for 5 minutes at room temp.

4) Replace 1xSSC with Formamide bleaching solution and place under bright light for 2 hours at room temperature.

> Formamide bleaching soltution contains 44 ml MilliQ water, 2.5 ml of Formamide, 1.25 ml of 20x SSC, and 2 ml of 30% H2O2.
>
> ****Caution**** at high concentrations formamide and H2O2 undergo a violent reaction. Always dilute these reagents into the water before mixing.

5) Wash for 5 minutes in 1X SSC

6) Wash twice with PBSTX for 5 minutes

7) Incubate for 12 minutes in 5 ml of Protainase K solution, at room temp. **No rocking** – just swirl gently every 5 minutes.

8) Rock in 4% fixative, 10 min, RT.

9) Remove fixative and rinse samples twice with PBSTx (shake by hand for 10 seconds).

10) Transfer planarians to **pre-labeled** 24-well plate (3 to 4 planarians per gene; 1 gene per well)

11) Wash in 1:1 (PBSTx:PreHyb), 10 min at room temperature.

12) Replace 1:1 mix with 10 ml of Prehyb and incubate for 1 hour at, 56°C.

13) Replace Prehyb with Riboprobe mix*** and incubate overnight at 56°C.

Prepare Riboprobe mix by heating your probe at 80°C for 5 minutes and mixing 2 ul with 1 ml 500 ul of 56°C Hyb. USE FILTER TIPS FOR THIS STEP.

Day 3 (washing and antibody incubation).

Note: You do not need to take precautions against RNAse contamination after hybridization, because RNAses from your hands and bacteria etc. are generally single-stranded RNAses and will not cleave dsRNA.

NOTE: Preheat 50 ml of 2×SSC + 0.1% Triton-X and 0.2×SSC + 0.1% Triton-X to 56°C.

1) Remove Riboprobe mix and wash three times with 0.5 ml of 2×SSC + 0.1% Triton-X for 15 minutes each at 56°C.

2) Wash twice with 0.5 ml of 0.2×SSC + 0.1% Triton-X for 15 minutes each at 56°C.

3) Return specimens to RT and wash with TNTx twice for 10 min each at room temp.

4) Incubate samples with 400 ul of Blocking Solution for 1 to 2 hours

5) Incubate samples with DIG-Alkaline Phosphatase antibody solution rocking overnight at 4°C.

Day 4 (antibody washes and development).

1) Remove antibody solution and rinse samples at room temperature with TNTx

2) Exchange TNTx with new TNTx and wash samples six times at room temperature for 15 minutes each.

3) Replace TNTx with Alkaline Phosphatase (AP) buffer and incubate for 10 minutes at room temp.

4) Replace AP buffer with Development buffer (AP buffer containing 4.5 µl/ml NBT and 3.5 µl/ml BCIP) and placed in the dark **without** agitation.

**Development of signal begins now. Check every 15-20 minutes for signal under a dissection microscope and proceed when ready (check w/instructors)

5) Stop development by exchanging Development buffer with PBSTX

6) Fix signal into specimens by incubating in 4% Fixative for 10 minutes at room temp.

7) Dispose of Fixative properly and rinse samples in PBSTX for 5 minutes

8) Replace PBSTX with 100% Ethanol and incubate rocking at room temperature for 10 minutes.

9) Replace 100% Ethanol with a solution of 50% Ethanol:PBSTX and incubate for 10 min.

10) Replace 50% Ethanol solution was replaced with PBSTx.

11) Replace PBSTX with a solution of 80% Glycerol/20% PBS and store at 4°C until mounting on slides.

Solution formulas for animal preparation.

5% NAC solution: 5% N-acetyl cysteine (NAC; Sigma) dissolved in 1× PBS. *Note:* 5% NAC solution is best when made fresh, but may be stored at 4°C and appears to be stable > 6 months, although the activity declines somewhat during this period.

PBSTx: 1× PBS + 0.1–0.5% Triton X-100.

4% Fixative: prepared fresh for each experiment by dilution of a 36.5% formaldehyde stock solution (Formalin; Sigma) into PBSTx.

Reduction solution: 50 mM DTT, 1% NP-40, 0.5% SDS, in 1× PBS.

50% Methanol solution: equal volumes of 1× PBSTx and 100% Methanol.

50% Ethanol solution: equal volumes of 1× PBSTx and 100% Ethanol.

6% Bleach solution: 6% H_2O_2 (30% stock; Sigma) in Methanol.

Proteinase K solution: 2µg/ml Proteinase-K (Invitrogen), 0.1% SDS, in 1× PBSTx.

Solution formulas for hybridization.

Hyb:50–55% De-ionized Formamide (Roche): 5–10% Dextran Sulfate (Sigma, from 50% stock); 5× SSC; 1 mg/ml yeast torula RNA (Sigma); 1% Tween-20 (Sigma, from 10% stock). *Notes:* SDS may be used instead of Tween-20, but tends to precipitate during storage at −20°C; Fresh de-ionization of formamide appears to affect staining differently in different organisms, but is critical for planarians. We de-ionize 1 L of formamide (Roche) with 50 g of Bio-Rad AG 501-X8 (D) Resin for 1 hr at RT, then filter, aliquot, and store at −80°C.

Prehyb / Wash Hyb: Hyb without the dextran sulfate.

Riboprobe mix: 400 µl of Hyb plus ~400 ng (~4 µl) of riboprobe. *Note:* Riboprobe mix was denatured at 72–90°C for 5 min, then placed at 56°C before use.

TNTx: 0.1 M Tris pH 7.5, 0.15 M NaCl, and 0.3% Triton X-100 (for 1 L: 12.11g Tris Base, 8.77 g NaCl, 3 ml 100% Triton X-100. pH to 7.5 and filter sterilize).

SSC: 20× SSC stock (Sigma).

Solution formulas for antibody incubation and development.

Blocking solution: 5–10% horse serum in TNTx. *Note:* Head-to-head comparisons indicate that bovine serum albumin (BSA) in the blocking solution is detrimental to signal detection.

Antibody solution: Antibody diluted into Blocking solution. *Note:* anti-DIG-AP (Roche) was used at 1:4,000 for all NBT/BCIP experiments.

AP buffer: 100 mM Tris, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$; 0.1% Tween-20 brought up to volume with 10% polyvinylalcohol solution (PVA; Sigma P8136). This was prepared fresh before every experiment from stocks of 1 M Tris, pH 9.5; 5M NaCl; 1 M $MgCl_2$; 10% Tween-20. The PVA solution is a 10% w/v stock in H_2O stored at RT.

Development buffer: Freshly made AP buffer with 4.5 µl/ml NBT (Roche) and 3.5 µl/ml BCIP (Roche). *Note:* PVA greatly increases AP activity and helps the most for weak probes.

80% Glycerol solution: 80% Glycerol; 10 mM Tris, pH 7.5; 1 mM EDTA.

Day 6. Total RNA purification from planarian flatworms

The purpose of today's exercise is to extract total RNA from whole planarians using the acid guanidinium thiocyanate-phenol-chloroform extraction method (commercially known as TRIzol). The RNA obtained from today exercise will be used as template for complementary DNA (cDNA) synthesis. cDNA produced will be used as template to amplify genes for next year's class by PCR.

Procedure

You should have large (> 1 cm) sexually mature planarians (2-3/tube; 2 tubes per person). The cells that make up the planarian will be opened up using a RNase-free pestle and TRIzol reagent. Then RNA will be separated from protein, lipids, and DNA using an acid/phenol extraction.

***RNase free materials are crucial for this procedure. Phenol, Chloroform, Propanol, and TRIzol are toxic irritants. Dispose waste in special containers and work under hood or in well-ventilated areas.*

1) Place 2-3 planarians in a 1.5 ml eppendorf tubes, remove media and add 1 ml of TRIzol.
2) Use a new RNase-free pestle to crush and grind the planarians in TRIzol. This works best if one captures planarians against the wall in the bottom of the 1.5 ml tubes.
3) Incubate lysate at room temperature for 5 minutes.
4) Add 0.2 ml of chloroform, cap tube securely and shake by hand for 15 seconds.
5) Incubate samples at room temperature for 3 minutes.
6) Place tubes in 4C centrifuge (with the tube hinge facing outside rotor) and spin for at 12,000×*g* for 15 minutes.
7) Taking care not to disturb the layering inside the 1.5 ml tubes** and transfer top (aqueous) layer to a new RNase-free tube.
   ***Note: it is more important to avoid contamination from lower layers, than to obtain ALL the aqueous one.*
8) Add 0.5 ml of 2-propanol to each of the RNAse of the isolated aqueous fractions, cap and mix by hand for 15 seconds.
9) Incubate samples at room temperature for 10 minutes.
10) Place tubes in 4C centrifuge (with the tube hinge facing outside rotor) and spin for at 12,000×*g* for 10 minutes
11) Dispose supernatant and wash pellet (RNA) w/ 1 ml of cold 70% ethanol in DEPC water. Vortex briefly.
12) Centrifuge at 10,000×*g* for 5 minutes
13) Remove <u>all</u> ethanol and air-dry RNA pellet for 1 minute (perform and extra spin if needed).
14) Add 100 ul of RNase-free water to dissolve the RNA pellet at for 5 minutes at 60C.
15) Immediately place tubes on ice and analyze by running 2 ul with 1 ul loading dye in a gel.
16) Store RNA at below -70C for long term or -20C overnight.

Day 7. cDNA synthesis from Total RNA using Promega's GoScript system

The purpose of today's exercise is to synthesize complementary DNA (cDNA) from mRNAs present in total RNA purified from planarians. The cDNA will be used as template to amplify genes of interest by PCR using gene-specific primers.

Procedure

Single stranded DNA can be synthesized from RNA templates using a viral Reverse Transcriptase. This is done using oligo(dT) primers that anneal to the polyadenosine tail in the 3'end of the mRNA. Random primers will be added to this reaction to assist the Reverse Transcriptase synthesize cDNAs more inclusive of 5' mRNA ends.

***RNase free materials are crucial for this procedure.*

1) Combine 5 ug of total RNA with 1 ul of oligo(dT) primer and 1 ul of random primer in an RNase free 1.5 ml eppi tube.
2) Add RNase free water to bring mix up to 10 ul. Close tightly.
3) Denature the RNA and reverse transcription primer at 70°C for 5 minutes. Chill on ice until use in step .
4) Prepare the GoScript™ Mastermix on ice by adding the following in the order listed below.

Per Reaction
1.5 ul RNase-free water
4 ul of 5X reaction buffer (thaw completely and vortex before use)
2ul of 25 mM magnesium chloride
1 ul of 10 mM dNTPs
0.5 ul of RNase Inhibitor
1 ul Reverse Transcriptase
(this adds up to a final volume of 10 ul)

5) Add 10 ul of GoScript™ Mastermix to the10ul mix of total planarian RNA primers for a final volume of 20 ul.

6) Incubate total reaction mixture at room temperature for 5 minutes.
7) Incubate total reaction mixture at 42°C for an hour.
8) Inactivate reaction by incubating at 70°C for 15 minutes.
9) Store cDNA at 4°C or on ice for immediate use. Alternatively, store the cDNA at –20°C.

Upon completion of this protocol the cDNA is ready to use directly or diluted with 80 ul of water.

Day 7. cDNA synthesis from Total RNA using Thermo's SuperScript IV system

The purpose of today's exercise is to synthesize complementary DNA (cDNA) from mRNAs present in total RNA purified from planarians. The cDNA will later be used as template to amplify genes of interest by PCR using gene-specific primers.

Procedure

Single stranded DNA can be synthesized from RNA templates using a viral Reverse Transcriptase. This is done using oligo(dT) primers that anneal to the polyadenosine tail in the 3'end of the mRNA. Random primers will be added to this reaction to assist the Reverse Transcriptase synthesize cDNAs more inclusive of 5' mRNA ends.

***RNase free materials are crucial for this procedure.*

1) To anneal primers to RNA, mix the following reagents on ice:

    1 ul of 10 mM dNTPs (10 mM each)
    1 ul of 50 uM Oligo $d(T)_{20}$ primer
    2 ul of planarian total RNA (0.5 to 2.5 ng/ul concentration)
    9 ul of RNAse-free water

2) Mix and centrifuge briefly
3) Incubate at 65°C for 5 minutes
4) Place tube on ice for at least 1 minute Add 10 ul

5) Mix the following on a **separate RNAse-free tube on ice**

    4 ul of 5X SSIV buffer (thaw completely and vortex before use)
    1 ul of 100 mM DTT
    1 ul of RNase Inhibitor
    1 ul of SuperScript IV Teverse Transcriptase

6) Mix the contents of both tubes and incubate at 55°C for 10 minutes.
7) Inactivate reaction by incubating at 80°C for 15 minutes.
9) Store cDNA on ice for immediate use or at –20°C if not using immediately.

Upon completion of this protocol the cDNA is ready to use directly or diluted with 80 ul of water.

Day 7. Gene-specific cDNA amplification using Promega's PCR MasterMix

The purpose of today's exercise is to amplify cDNA from specific genes from a pool of cDNAs representative of total planarian mRNA. The gene-specific cDNA will be "cloned" into a bacterial vector for propagation and use in future RNAi as well as *in situ* hybridization experiments.

Procedure

Single stranded cDNA is used as template for PCR to amplify cDNA of a specific gene using primers containing the corresponding gene sequence. *Taq* Polymerase is the enzyme of choice for this PCR reaction, since it leaves "A-overhangs" on the PCR product and these will be used for annealing with "T-overhangs" in the vector used during the following cloning steps.

1) Thaw and mix the following items on ice in the following order in a 1.5 ml eppi tube

Per Reaction

10 ul of 2X PCR MasterMix

2 ul of cDNA template

7 ul of MilliQ water

2) Distribute 19 ul of the Mastermix/cDNA/water reaction mix into 0.2 ml PCR tubes

3) Add 1 ul of pre-mixed gene-specific sense and antisense primers to pre-labeled PCR tubes with reaction mix.

4) Place tubes in the Thermocycler and run the following protocol:

95C for 2 minutes

95C for 30 seconds

55C for 30 seconds

72C for 1 minute

repeat last 3 steps for 40 cycles

72C for 5 minutes

4C forever

6) Analyze your PCR products by running 2 ul with 1 ul loading dye in a 0.8% agarose gel.

Include 3 ul of ladder in your sample

*store the rest of the PCR product at -20C for long term or at 4C if continuing with ligation step within a day.

Day 8. cDNA PCR analysis and purification for cloning.

The purpose of today's exercise is to decide which primers yielded a PCR product, and to purify this product using Promega's Wizard PCR purification system. Once purified, the PCR product will be ligated to Promega's pGEM –T vector.

Procedure

Analysis

1) Analyze 5 ul of PCR reaction on a 0.8% agarose gel.
2) Transfer the remainder of the PCR reaction of genes that yielded a product to a new 1.5 ml eppi tube. Label it by its given code (for example, 1A).

Clean-up

3) Add an equal volume of Membrane Binding Solution to the PCR amplification mix.
4) Label one SV Minicolumn and corresponding collection tube with the code of each successful PCR product
5) Insert Minicolumn into Collection Tube.
6) Transfer prepared PCR product/Membrane Binding Solution mix into the Minicolumn assembly to allow it to bind to the membrane. **Incubate at room temperature for 1 minute.**
7) Centrifuge at 16,000 × g for 1 minute at room temperature.
8) Add 700µl Membrane Wash Solution to column.
9) Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
10) Repeat Step #8 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × g for **5 minutes**.
11) Empty the Collection Tube and re-centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

12) Carefully transfer each Minicolumn to a clean 1.5ml microcentrifuge tube labeled on its side.
13) Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute.
14) Centrifuge at 16,000 × g for 1 minute.
15) Discard Minicolumn and proceed to ligation procedure or store DNA at 4°C (briefly-overnight) or –20°C (long term).

Day 8. Vector/Insert Ligation and Transformation.

The purpose of this procedure is create covalent bonds between our cDNA PCR product and a commercial vector. Vector/Insert ligated products are then transformed into chemically competent bacterial cells. The procedures below are based on the manufacturer's protocol.

Procedure

Taq Polymerase creates "A-overhangs" which can basepair with "T-overhangs" presents in commercial vectors (in this case Promega's pGEM-T) or verctors prepared within the lab. The base-pairing at both ends of the vector and insert creates a circular plasmid, which is covalently bonded after exposure to the enzymatic activity of T4 DNA Ligase. Circular plasmids are stable in bacteria. Appropriate origin of replication and antibiotic resistance genes allow propagation and selection of bacteria harboring the plasmid. Clone identity will be verified through sequencing.

Ligation

1) Use the Nanodrop at the GRC to quantify the concentration of your purified PCR products.
2) Calculate the volume of insert needed to have a 3:1 molar ratio of Insert:Vector.

    Given that most of the PCR products are of approximately 500 bps and pGEM-T is 3000 bps, and that the reaction includes 50 ng of vector, how much insert do you need?

3) Make a mastermix for all your ligation reactions plus a "background" and a "positive" control.

    Each reaction must contain the following:

    5 ul of 2X Rapid Ligation Buffer (Vortex well before use)

    1 ul of pGEM-T vector

    X ul of PCR product Insert (2ul for "positive" control, 0 ul for "background" control)

    1 ul of T4 DNA Ligase

    MilliQ water to bring the reaction to a final volume of 10 ul.

4) Mix reaction by pipetting and incubate with insert at room temperature for one hour.

Transformation

5) Centrifuge the ligation reactions briefly.
6) Add 2µl of each ligation reaction to a sterile 1.5ml tube on ice.
7) Place the JM109 High Efficiency Competent Cells in an ice bath until just thawed (~5 minutes).
8) Mix competent cells by gently flicking the tube.
9) Carefully transfer 50µl of cells to the ligation reaction tubes from Step #6.
10) Gently flick the tubes and incubate on ice for 20 minutes.
11) Heat-shock the cells for 45–50 seconds in a table top incubator at 42°C. DO NOT SHAKE.
12) Immediately return the tubes to ice for 2 minutes.
13) Add 950µl room temperature SOC medium to the ligation reaction transformations.
14) Incubate for 1.5 hours at 37°C with shaking (~150rpm).
15) Pipette 100µl of each transformation culture onto a separate LB/ampicillin/IPTG/ X-Gal plate.
16) Using sterile technique spread transformation on the plate with a spreader or beads.

Day 9. Cloning results analysis and start-up cultures for extraction of plasmid constructs.

The purpose of today's exercise is to decide whether there is evidence that yesterday's cloning experiments worked. If there is evidence for success, start bacterial cultures for extraction of new plasmid constructs by miniprep using commercial columns.

Procedure

Count number of colonies in each plate. If plate has too many colonies (>100) then count the colonies of a fraction (1/4, 1/8 or 1/16) of the plate and multiply to estimate the total number of colonies in each plate. Document your results.

Think about the following questions and write your answers:

What would you expect from a successful cloning experiment?
How do the controls used influence your conclusions?
Are your results promising enough to carry onto the next step?
What does each colony in your plate represent?
What do the different color colonies represent?

If your results show evidence of successful cloning, then proceed with the following:

1) Make a stock solution of Luria Broth (LB) supplemented with 100 ug of Ampicillin per mL of LB.
   Make enough for 3 tubes x 3 ml per tube x gene of interest cloned
2) Make aliquots of 3mls of LB+Amp in disposable tubes for bacterial culture
3) Label tubes with gene code and colony code (for example: 1.1, 1.2. and 1.3, for the first, second, and third colonies from gene #1 plate.
4) Pick a single <u>white</u> colony with a pipette tip and place it into the corresponding LB+Amp tube.
5) Place inoculated tubes into a shaking incubator for growth at 37C overnight.
6) Put Parafilm on the edge of the plates with colonies to avoid evaporation and save at 4C the correct clone for that plate is verified.

Day 10. Isolation of plasmid constructs by commercial column minipreps and verification of insert sequence by Sanger sequencing.

The purpose of today's exercise is to decide whether our cloning experiments worked. To do this, we will isolate plasmid DNA from single colony forming units (CFUs) and sequence it using primers that anneal to the SP6 promoter sequence in pGEM-T. The sequencing will be outsourced. We will mail the plasmids to Retrogen (San Diego, CA) and they will mail us the sequencing results. We will analyze the sequencing results next week.

Procedure

To isolate plasmid DNA from bacterial cells (*E. coli*) we first grew a 3 ml culture from a single CFU overnight under ampicillin selection. The cells are collected in a 2 ml tube by centrifugation and lysed using an alkaline solution. Protein and bacterial chromosomal DNA, which is bound to the cell wall, are collected in an insoluble pellet by centrifugation. Then, the plasmid and other components of the cytoplasm are released to the soluble fraction. The soluble fraction is transferred to a column containing a silica membrane that retains the plasmid DNA under high-salt conditions and lets debris run through the column. The plasmid DNA bound to the column is washed from unwanted components of the lysate and salts using a solution containing large amounts of ethanol. Finally, the column is transferred to a new 1.5 ml tube and DNA is solubilized in water (or elution buffer) and collected in the 1.5 ml tube by centrifugation. **It is crucial to avoid contamination from one sample to the other, and contamination of stock solutions.**

Procedure

1) Pre-label columns and tubes (one 2 ml tube, a flow-through tube, & a 1.5 ml tube per miniprep) on the side of each tube.
2) Decant or pipette (using 1 ml pipette) 2 ml of bacterial culture in corresponding 2 ml tubes.
3) Close 2 ml tubes and centrifuge bacteria at 6800 x *g* for 2 minutes to pellet cells.
4) Decant supernatant in a waste beaker (treat later with chlorine and dispense in drain)
5) Add 250 ul of **Resuspension** buffer to pellet and vortex until no clumps are observed.
6) Add 250 ul of **Lysis** solution and invert the tube 4-6 times.
7) Add 350 ul of **Neutralization** solution and invert tube 4-6 times.
8) Centrifuge tubes for 5 minutes at max speed. The cytoplasm of cells including the plasmid will be on the supernatant..
9) Transfer supernatant to corresponding column, with flow-through tube and centrifuge for 1 minute at max speed.
10) Remove column from flow-through tube and dispose flow through liquid on waste basket. Place column back on flow-through tube.
11) Add 500 ul of **Wash** solution to column and centrifuge at max speed for 1 minute.

12) Dispose flow through and repeat.
13) Carefully, without contaminating with liquid from flow-through tube, transfer the column to its corresponding, pre-labeled 1.5 ml tube.
14) Add 50 ul of Elution buffer to column and incubate for two minutes at room temperature.
15) Centrifuge for 2 minutes at max speed. Your plasmid DNA will be in the 1.5 ml tube.

16) For sequencing: transfer 10 ul of the plasmid DNA to a new 1.5 ml tube and label it exactly the same as the tube with the plasmid DNA (e.g. 1A-1, 1A-2, 1A-3)…
17) Submit for sequencing.

Day 12-13. Testes analysis by whole-mount DAPI staining of planarian.

The purpose of this technique is to analyze the anatomy of planarian testes (and other structures) using DAPI staining of DNA. Due to the high cellular density in planarian testes, pharynx and brain, DAPI is a good option to see the anatomy of these structures. The pharynx anatomy can also be clearly observed by DAPI staining. Control and experimental RNAi animals will be "fixed" with a formaldehyde solution, and bleached with hydrogen peroxide. Then, the samples will be incubated with DAPI (a molecule that binds DNA and fluoresces under UV light) to visualize the nuclei of all cells. Excess DAPI is removed from the samples through a series of washes and samples will be mounted on slides. This analysis will reveal differences in spermatogenesis (and possibly other anatomical features) between control and RNAi planarians.

Procedure

As described above, this procedure involves fixing and exposing samples to a series of solutions, which include PBSTx (PBS containing 0.3% Triton X) supplemented with DAPI. All steps are done at room temperature except where indicated.

Protocol

1) Pre-label 5 ml tubes with the different gene names. Then, cover the label with a piece of tape going completely around each tube to protect the label will persist through the following steps.
2) Without damaging your samples, transfer the planarians into their respective 5 ml tubes. This can be achieved using a Pasteur pipette with the end cut off, using a squirt bottle or by decanting.
3) Remove salts with Pasteur pipette or by decanting and immediately replace with a **10% N-acetyl-cysteine (NAC) in 1X PBS**.
4) Immediately place tubes flat on your bench and immediately after on a rocking platform. Incubate with gentle rocking for **8 minutes** at room temperature (incubation length varies with planarian size).
5) Collect planarians at the bottom of the tubes and replace the NAC with **4% Formaldehyde in PBSTx (PBS w/0.3% Triton-X).** Dispose NAC solution in a special waste container and incubate samples in 4% Formaldehyde while **rocking gently** for 1 hr at 4C.
6) Dispose 4% Formaldehyde solution in its special waste container and replace with PBSTx. Rock for 5 minutes.
7) Replace PBSTx with new PBSTx solution. Incubate with agitation for 10 minutes.
8) Repeat step #7.
9) Incubate samples overnight at 4C in DAPI solution. The DAPI solution consists of PBSTx with 1:1000 dilution of 1mg/ml DAPI.
10) Replace DAPI solution with PBSTx and wash at least 4 times for 15 minutes each.
11) Mount samples on slides with dorsal side up (look for the eyes) on glass microscope slides.
12) Replace PBSTx with a solution of 80% Glycerol and 20% PBS once samples are on the slide.
13) Visualize samples under fluorescent microscope and document observations.