SSH PCR

Material and reagents

Endonuclease digestion
• x µl 10X *Rsa* I Restriction Buffer
100 mM Bis Tris Propane-HCl (pH 7.0)
100 mM MgCl2
1 mM DTT
• x µl *Rsa* I (10 units/µl)

Adaptor ligation
• x µl T4 DNA Ligase (400 units/µl; contains 3 mM ATP)
• x µl 5X DNA Ligation Buffer
250 mM Tris-HCl (pH 7.8)
50 mM MgCl2
10 mM DTT
0.25 mg/ml BSA
• x µl Adaptor 1 (10 µM)
• x µl Adaptor 2 (10µM)

Hybridization
• 200 µl 4X Hybridization Buffer
• 1.4 ml Dilution buffer (pH 8.3)
20 mM HEPES (pH 6.6)
20 mM NaCl
0.2 mM EDTA (pH 8.0)

PCR amplification
• x µl PCR Primer 1 (10 µM)
• x µl Nested PCR primer 1 (10 µM)
• x µl Nested PCR primer 2 (10 µM)
• x µl *gpd-1* 5' Primer (10 µM)
• x µl *gpd*-1 3' Primer (10 µM)

General reagents
• x µl dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP)
• x µl 20X EDTA/Glycogen Mix (0.2 M EDTA; 1 mg/ml glycogen)
• x µl NH4OAc (4 M)
• x ml Sterile H2O

• ***Hae* III digest of bacteriophage *F X174*** (#6310-1,-2)
DNA size markers for agarose gels
• **0.5-ml PCR reaction tubes**.
• **80% Ethanol & 96% Ethanol**

• 50X PCR enzyme mix
Advantage® cDNA Polymerase Mix (#8417-1; also
Also provided in the Advantage cDNA PCR Kit [#K1905-1,-y]).

• 10X PCR buffer
Use the 10X reaction buffer supplied with DNA polymerase or mix (included with Advantage cDNA Polymerase Mix and Advantage cDNA PCR Kit).
• dNTP Mix for PCR (10 mM each dATP, dCTP, dGTP, dTTP)
• 50X TBE electrophoresis buffer
or TAE electrophoresis buffer
242 g Tris base
57.1 ml Glacial acetic acid
37.2 g Na2EDTA•2H2O
Add H2O to 1 L. For 1X TAE buffer, dilute 50X stock solution 1:49 with H2O.

E. *Rsa* I Digestion

1. Add the following reagents into the tube:
ds cDNA 43.5 µl
10X *Rsa* I Restriction Buffer 5.0 µl
Rsa I (10 units/µl) 1.5 µl
2. Mix by vortexing and centrifuging briefly.
3. Incubate at 37°C for 1.5 hr.
4. Set aside 5 µl of the digest mixture to analyze the efficiency of *Rsa* I digestion. Check *Rsa* I-digested cDNA from Step E.4 using agarose/EtBr gel electrophoresis
5.QIAgen PCR purification kit, *Rsa* I digested cDNAs dissolve in 5.5 -10 µl ddH2O. These samples of *Rsa* I digested cDNA will serve as experimental driver cDNA.

F. Adaptor Ligation

To perform subtractions in both directions, to prepare tester cDNA corresponding to each of poly A+ RNA samples. To perform a positive control mock subtraction. In step 2 (below), prepare tester cDNA for this control subtraction by mixing the worm cDNA with ΦX174/*Hae* III DNA and diver cDNA using the worm cDNA only. Three adaptor ligations must be performed for each experimental tester cDNA, as well as the control mock tester cDNA. Each cDNA is aliquotted into two separate tubes: one aliquot is ligated with Adaptor 1 (Tester 1-1, 2-1, and 3-1), and the second is ligated with Adaptor 2 (Tester 1-2, 2-2, and 3-2). After the ligation reactions are set up, portions of each tester tube are combined so that the cDNA is ligated with both adaptors (Unsubtracted tester control 1-c, 2-c, and 3-c).

Adaptors will not be ligated to the driver cDNA.

1. Dilute 1 µl of each *Rsa* I-digested experimental cDNA (Step E.5)
with 5 µl of sterile H2O.

2.Prepare a mock positive control tester cDNA

a. Briefly centrifuge the tube containing Control DNA (*Hae III*-digest of f X174 / *Hae* III DNA [3 ng/ µl]).

b. Dilute 2 µ l of the Control DNA with 38 µl l of sterile H_2O (to 150 ng/ml).

c. Mix 1 µ l of control worm cDNA (or gDNA) (Step E.5) with 5 µl l of the diluted f X174/ *Hae* III Control DNA (150 ng/ml).

Prepare your adaptor-ligated tester cDNA:

3. Prepare a ligation Master Mix by combining the following reagents in a 0.5-ml microcentrifuge tube. To ensure that you have sufficient Master Mix, prepare enough for all ligations plus one additional reaction.

per rxn

Sterile H2O 3µl

5X Ligation Buffer 2 µl (NEB)

T4 DNA Ligase (400 units/µl) 1 µl (NEB)

Note: The ATP required for ligation is in the T4 DNA Ligase (3 mM initial, 300 µM final).

4. For each experimental tester cDNA, combine the reagents in Table I in the order shown in 0.5-ml microcentrifuge tubes. Pipet mixture up and down to mix thoroughly.

Table I Setting up the ligation reactions

Component	Tube #: 1 Tester 1-1* (µl)	2 Tester 1-2* (µl)
Diluted tester cDNA	2	2
Adaptor 1 (10 µM)	2	–
Adaptor 2R (10 µM)	–	2
Master Mix	6	6
Final volume	10	10

* Use the same setup for Tester 2-1 and 2-2, 3-1 and 3-2.

5. In a fresh microcentrifuge tube, mix 2 µl of Tester 1-1 and 2 µl of Tester 1-2. After ligation is complete, this will be Unsubtracted tester mock control 1-c. Do the same for each additional experimental tester cDNA. After ligation, approximately 1/3 of the cDNA molecules in each Unsubtracted tester control tube will bear two different adaptors.

6. Centrifuge tubes briefly, and incubate at 16°C overnight.

7. Stop ligation reaction by adding 1 µl of EDTA/Glycogen Mix.

8. Heat samples at 72°C for 5 min to inactivate the ligase.

9. Briefly centrifuge the tubes. Preparation of experimental Adaptor-Ligated Tester cDNAs and Unsubtracted tester controls is now complete.

10. Remove 1 µl from each Unsubtracted tester control (1-c, 2-c, 3-c) and dilute into 1 ml of H2O. These samples will be used for PCR (Section.I).

11. Store samples at –20°C.

Perform the ligation efficiency analysis (see last SSH chapter, Figure 9)

G. First Hybridization

Note: Before begin the hybridization, warm the 4X Hybridization buffer to room temperature for at least 15–20 min.

1. For each of the experimental subtractions, combine reagents in Table II (below) in 0.5-ml tubes in the order shown.
2. Overlay samples with one drop of mineral oil and centrifuge briefly.
3. Incubate samples in a thermal cycler at 98°C for 1.5 min.
4. Incubate samples at 68°C for 8 hr, then proceed **immediately** to Section H.

Table II Setting up of the first hybridization

Component	Hybridization sample 1(μl)	Hybridiazion sample 2(μl)
***Rsa I*-digested driver cDNA(E.5)**	**1.5**	**1.5**
Adaptor 1-ligated tester 1-1*	**1.5**	**-**
Adaptor 2R-ligated tester 1-2*	**-**	**1.5**
4X Hybridization Buffer	**1.0**	**1.0**
Final volume	**4.0**	**4.0**

***use the same setup for tester 2-1 and 2-2,3-1 and 3-2**

H. Second Hybridization

1. Add the following reagents into a sterile tube:
Driver cDNA (Step E.5) 1 ul
4X Hybridization Buffer 1 ul
Sterile H2O 2ul
2. Place 1 ul of this mixture in a 0.5-ml microcentrifuge tube and overlay it with 1 drop of mineral oil.
3. Incubate in a thermal cycler at 98°C for 1.5 min.
4. Remove the tube of freshly denatured driver from the thermal cycler.
a. Set a micropipettor at 15 ul.
b. Touch the pipette tip to sample interface of the tube containing hybridization sample 2.
c. Carefully draw the entire sample partway into the pipette tip.
d. Remove the pipette tip from the tube, and draw a small amount of air into the tip, creating a slight air space below the droplet of sample.
e. Repeat steps b–d with the tube containing the freshly denatured driver. The pipette tip should now contain both samples separated by a small pocket of air.
f. Transfer the entire mixture to the tube containing hybridization sample 1.
g. Mix by pipetting up and down.
5. Briefly centrifuge the tube.
6. Incubate reaction at 68°C overnight.
7. Add 200 μl of dilution buffer to the tube and mix by pipetting.
8. Heat in a thermal cycler at 68°C for 7 min.
9. Store at –20°C.

I. PCR Amplification

A minimum of 6 PCR reactions are recommended: (1) forward-subtracted experimental cDNA, (2) the unsubtracted tester control (1-c), (3) reverse-subtracted experimental cDNA, (4) the unsubtracted tester control for the reverse subtraction (2-c), (5) mock subtracted experimental cDNA, (6) the unsubtracted mock tester control (3-c), using digested ΦX174 DNA/*Hae*III as a positive control when run–gel. To perform a standard PCR control to ensure that your enzyme is performing efficiently (see last SSH chapter, Figure 10 a,b).

1. Prepare the PCR templates:

a. Aliquot 1 µl of each diluted cDNA (i.e., each subtracted sample from Step H.9 and the corresponding diluted Unsubtracted tester control from Step F.10) into an appropriately labeled tube.

b. Aliquot 1 µl of the PCR control subtracted cDNA into an appropriately labeled tube.

2. Prepare a Master Mix for all of the primary PCR tubes plus one additional tube. For each reaction planned, combine the reagents in

Table III Preparation of Primary PCR master mix in the order shown:

Reagent	Amount per reaction (µl)	For a 7-rxn Experiment (µl)*
Sterile H_2O	19.5	156.0
10X PCR reaction buffer	2.5	20.0
dNTP Mix (10 mM)	0.5	4.0
PCR Primer 1 (10 µM)	1.0	8.0
50X Advantage cDNA Polymerase Mix	0.5	4.0
Total volume	24.0	192.0

* For each additional experimental cDNA, prepare Master Mix for one additional reaction.

3. Mix well by vortexing, and briefly centrifuge the tube.

4. Aliquot 24 µl of Master Mix into each of the reaction tubes prepared in step 1.

5. Overlay with 50 µl of mineral oil.

6. Incubate the reaction mix in a thermal cycler at 75°C for 5 min to extend the adaptors. (Do not remove the samples from the thermal cycler.)

7. Commence thermal cycling using the following:

• 95°C 1 min • 95°C 1 min

• 27 cycles:

95°C 20 sec 66°C 30 sec 72°C 2 min

8. Analyze 8 µl from each tube on a 2.0% agarose/EtBr gel run in 1X BE/TAE buffer. Alternatively, set these 8-µl aliquots aside and run them on the same gel used to analyze the secondary PCR products (step 16).

9. Dilute 3 µl of each primary PCR mixture in 27 µl of H2O.

10. Aliquot 1 µl of each diluted primary PCR product mixture from Ste 9 into an appropriately labeled tube.

11. Prepare Master Mix for the secondary PCRs plus one additional reaction by combining the reagents in Table IV in the order shown:

Table IV Preparation of secondary PCR master mix

Reagent	Amount per reaction (µl)	For a 7-Rxn Experiment (µl)*
Sterile H_2O	18.5	148.0
10X PCR reaction buffer	2.5	20.0
Nested PCR primer 1 (10 µM)	1.0	8.0
Nested PCR primer 2R (10 µM)	1.0	8.0
dNTP Mix (10 mM)	0.5	4.0
50X Advantage cDNA Polymerase Mix	0.5	4.0
Total volume	24.0	192.0

* For each additional experimental cDNA, prepare Master Mix for one additional reaction.

12. Mix well by vortexing, and briefly centrifuge the tube.
13. Aliquot 24 µl of Master Mix into each reaction tube from step 10.
14. Overlay with 1 drop of mineral oil.
7. Commence thermal cycling using the following:
• 95°C 1 min • 95°C 1 min
• 12 cycles:
95°C 20 sec 68°C 30 sec 72°C 2 min
16. Analyze 8 µl from each reaction on a 2.0% agarose/EtBr gel run in 1X TBE/TAE buffer.
17. Store reaction products at –20°C.