PAT

Homogenization

1. Prepare Homogenization Buffer with additives (HB+++)
2. Add 1ml/well on 6-well plate
3. Add PFS fresh before use
4. Place extracted spinal cord in well, dice with scalpel
5. Transfer mash to douncer. Dounce 15-20 times. Transfer to 2ml (safe-lock) tube
6. Aliquot 100ul per tube
7. Freeze at -80C or proceed directly to RNA isolation

RNA Isolation (100ul aliquots)

- Pre-heat DEPC water aliquots to 95C
- Let phenol chloroform settle

<u>Lysis (on ice)</u>

1. Add 1ml (10V) of cold Lysis/Binding Buffer to tissue aliquot
2. Homogenize by passing twice through 27G syringe needle
3. Add 110ul (0.1V) of Homogenate Additive and vortex
4. Incubate on ice for 10 minutes

<u>Phenol-chloroform extraction (RT)</u>

5. Add 800ul (1V) of Acid-Phenol:Chloroform and vortex 60 seconds (avoid adding aqueous phase)
6. Centrifuge 14k rpm RT for 5 minutes
7. Transfer the upper aqueous phase (~2.5ml) to 15ml tube

<u>Column purification (RT)</u>

8. Add 3.125ml (1.25V) 100% EtOH
9. Apply in aliquots to spin column (max 700ul each time)
10. Centrifuge 10k rpm RT for 15 seconds
11. Discard flow-through, repeat until all lysate went through column
12. Wash with 700ul Wash Solution 1, centrifuge 10k rpm RT for 15 seconds
13. Wash with 500ul Wash Solution 2/3, centrifuge 10k rpm RT for 15 seconds
14. Repeat with Wash Solution 2/3
15. Empty spin at 10k rpm RT for 1 minute
16. Transfer column to new collection tube, add 100ul 95C DEPC water to membrane
17. Centrifuge 10k rpm RT for 1 minute

<u>EtOH precipitation (on ice)</u>

18. Add 10ul (0.1V) 3M sodium acetate and vortex
19. Add 2ul Pellet Paint and vortex
20. Add 1ul glycogen and vortex
21. Add 250ul (2.5V starting) 100% EtOH and vortex

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22. Incubate O/N at -80C
23. Centrifuge 14k rpm at 4C for >30 minutes
24. Wash with cold 80% EtOH, centrifuge 14k rpm at 4C for 10 minutes
25. Wash with cold 100% EtOH, centrifuge 14k rpm at 4C for 10 minutes
26. Air dry pellet for ~5 minutes
27. Resuspend in 20ul DEPC H2O
28. Measure RNA quantity and quality with NanoDrop

DNAse Digestion

1. Reaction mix

RNA	5µg
RQ1 DNase Promega	5µL
DNAse Buffer	5µL
H2O	Make up to 50µL

2. Incubate at 37C for 30 minutes
3. Add 1µL RQ1 Stop Solution
4. Incubate at 65C for 10 minutes

<u>Phenol-chloroform purification</u>

1) Add 50µL Phenol-Chloroform-IAA (from the mirVana Kit)
2) Vortex for 1 minute
3) Centrifuge 14k rpm at RT for 5 minutes
4) Transfer the upper phase into a new tube
5) Add 2.5V (125ul) 100% EtOH, 0.1V (5ul) sodium acetate, 1µL glycogen and 1µL pellet paint
6) Incubate at -80°C for 2 hours or O/N
7) Centrifuge at 14k rpm at 4C for 30 minutes,
8) Wash with cold 80% EtOH (~100ul), centrifuge 14k rpm at 4C for 10 minutes
9) Wash with cold 100% EtOH (~100ul), centrifuge 14k rpm at 4C for 10 minutes
10) Air dry for ~5 minutes
11) Resuspend in 20µL DEPC H2O

Ligation

1. Reaction mix

RNA (DNAse treated)	1µg
P1 Primer (0,4µg)	0.67µL
DEPC H2O	Final volume 8µL

Sequence P1: 5´[Phos]ggtcaccttgatctgaagc[AmC3]3´

2. Incubate at 70C for 10 minutes

3. Add 1µL T4 RNA Ligase Buffer and 1µL T4 RNA Ligase (NEB)
4. Incubate at 4C O/N
5. Incubate at 95C for 5 minutes
6. Cool down to RT
7. Phenol-chloroform purification
8. Resuspend in 10ul

Reverse Transcription

1. Reaction mix #1

Ligation Mix	10µL
P1 **AS-4T**	0.55µL
H2O	17.5µL

Sequence **P1 AS-4T**: gcttcagatcaaggtgaccT

2. Incubate at 70C for 5 minutes
3. Reaction mix #2

10mM dNTP	5µL
MgCl2 25mM	5µL
10x Puffer Applied	5µL
RNAse Inhibitor (ABI)	0.5µL
H2O	1.25µL

4. Incubate at 37C for 5 minutes
5. Add 1µL MulV Reverse Transcriptase (ABI)
6. Incubate

42C	1 hour
70C	10 minutes
4C	forever

RNAse Digestion

1. Add 1ul RNAse H (NEB)
2. Incubate at 37C for 30 minutes
3. Phenol-chloroform purification
4. Resuspend in 50ul DEPC H2O

Gene specific PCRs

1. Reaction mix

cDNA	5µL

PAT

5X Buffer Mango Taq 5x	5µL
dNTP´s	1µL
MgCl2 (50mM)	1,6µL
Primer gene specific (10pmol / 1:10)	1µL
Primer reverse P1 AS (10pmol / 1:10)	1µL
Mango Taq (Bioline)	0,5µL
H2O	Make up to 25µL

2. Thermocycle

95C	5 minutes	
95C	30 seconds	35 cycles
Primer Tm	60 seconds	
72C	60 seconds	
72C	10 minutes	
4C	forever	

Polyacrylamide Gel

1. Cast gel (12%)

H2O	5.2
1X TBE	6
Acrylamide/Bis	4.8
10% APS	160ul
TEMED	16ul

2. Mix Loading Dye with samples, load everything
3. Run for 140V constant

Silver Staining

- Wear vinyl gloves over nitrile when handling gel
- Throw silver nitrate waste in waste container
- Mind which ladder to use (100bp)

1. Wash with 10% EtOH for 10 minutes
2. Wash with 1% HNO_3 for 3 minutes
3. Wash with 0.2% $AgNO_3$ (+ 100ul 37% Formaldehyde/100ml fresh before use) for 20 minutes (waste into waste bottle!!)
4. Wash with VE water for 1 minute (exact) for 3 times
5. Rinse with 3% Na_2CO_3 (+ 100ul 37% Formaldehyde/100ml fresh before use) for 3 times
6. Wash with 10% Acetic acid for 10 minutes
7. Wash with 70% MeOH for 30 minutesn

8. Sandwich the gel between shrink wraps, remove bubbles, assist with 15% Glycerol

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