RNA Extraction (originally written by T. Fujita and edited by M. Hasebe)

last up dated 2004.06.01

A. Precaution

(1) Use gloves, disposable tubes (usually not necessary to autoclave)
(2) Make a distinction between RNA utensils including regents and others (Do not use RNA utensils for other purpose. It is bette to make special shelf for RNA regents)
(3) Do not open the caps of the bottles of RNase and Pronase in the laboratory.
(4) Do not weight RNase, DNase, and Pronase. Believe the label and dissolve the contents at a time with appropriate buffer.
(5) Do not discard high concentration of the solution or used tubes in the lab.
(6) Be careful for conatamination. Use species specific motor and pistle if necessary.
(7) Plant tissue is the biggest source of RNase, and mix tissue with buffer as quick as possible.
(8) You yourself are another source of RNase. Keep your mouth shut without having spit.

B. Pretreatment

(1) Sterilize all of utensils by autoclave (120C 20 min) or oven (200C 3 hours). Plastic tubes are not necessary to pretreat.
(2) Inactivate RNase with **DEP** (Diethylpyrocarbonate):
All of reagent except Tris
Add 2% (v/v) DEP in Draft
Keep at least 2 hours at room temp in Draft
Autoclave (121C 40min)
In case of **Tris**
Use RNase free reagents (usually no problem to use regular grade Tris, but do not share the bottle with other purposes)
Dissolve in DEP treated H2O with sterilized utensils
Adjust PH with PH paper or clean PH electrode

1) RNeasy (QIAGEN) --- for protonemata: for RACE, RTPCR
2) GuSCN and CTAB method --- for protonemata: for northern, RTPCR
3) DYNABEADS mRNA DIRECT kit (DYNAL) --- for both protonemata and gametophores: for any purposes

You can extract relatively pure total RNA from protonemal tissue, while it may be rather difficult to get pure RNA from gametophores. During RNA isolation steps from gametophore, polysaccharide-like moldy contaminant co-precipitates with extracted RNA after centrifugation, and this pellet never dissolve. Centrifugation is not involved in the method 4), and is useful to extract RNA from gametophores.

We use different RNA extraction method dependent on purposes.

For RACE

Total RNA and mRNA extracted by 1) and 3) work for 3'RACE System kit (Invitrogen) and Marathon cDNA amplification system kit (CLONTECH). Total RNA by 3) may sometimes have troubles because of purity.

For Northern hybridization

It should be better to use about 1ug poly(A)+RNA par lane for northern blotting, although 10ug of total RNA par lane may work.

For RT-PCR and semi-quantitative RT-PCR

DNase treatment is necessary for every method.

1) RNeasy (QIAGEN)

Follow manufacturer's manual.
Note:
- This method is as easy as the method 1).
- Avoid overloading sample onto column.
- genomic DNA usually contaminate with RNA.
- 100ug total RNA from 0.2 g fresh weight of 2-cell stages of protonema regenerated from protoplasts.
- RLT buffer in the kit works well for protonemata.

2) GuSCN and CTAB method

<Extraction of total RNA> (large scale) ((~8 h))

1. Buffer GnSCN	final conc.	15ml	30ml	50ml
GuSCN (Guanidine thiocyanate , mw=118.2)	4 M	7.1 g	14.2 g	24 g
NH4SCN (ammonium thiocyanate, mw=76.12)	1 M	1.14 g	2.28 g	3.8 g
1 M Tris-HCl (pH 7.5)	100 mM	1.5 ml	3 ml	5 ml
N-lauryl sarcosine	1%	0.15 g	0.3 g	0.5 g
Antifoam A Emulsion (sigma A5758)	trace	1 drop	1 drop	1drop
PVP 360,000	< 0.5%	0.075 g	0.15 g	0.25 g
beta-mercaptoethanol	1%	150 ul	300 ul	500 ul

2. CTAB buffer	final conc.	50 ml	100 ml
CTAB	2%	1 g	2 g
1M Tris.HCl , pH7.5	50 mM	2.5 ml	5 ml
0.5M EDTA.Na2	5 mM	0.5 ml	1 ml
5M NaCl	0.84 M	8.4 ml	16.8 ml
up to		50 ml	100 ml
Add beta-mercaptoethanol just before use		500 ul	1 ml

3. 10% CTAB buffer	final conc.	50 ml
CTAB	10%	5 g
5M NaCl	0.7 M	7 ml
DEPC water up to 50ml		

4.CTAB precipitation buffer	final conc.	50 ml
CTAB	1%	0.5 g
1M Tris.HCl, pH 7-8	50 mM	2.5 ml
0.5M EDTA	5 mM	500 ul
DEPC water up to		50 ml

5. High-salt TE
   0.5 ml Tris.HCl, pH 7-8
   0.1 ml 0.5 M EDTA
   10 ml 5 M NaCl (final 1M)
   DEPC water to 50 ml

6. Chloroform/Isoamylalcohol = 24 : 1

1. grind in liqN2 with moter and pestle
2. transfer powder to 50 ml tube with 15 ml GuSCN B (5 g moss/15 ml at most)
3. shake vigorously
4. add 15 ml of C/I (seal with parafilm)
5. shake vigorously for 5 min.
6. cfg @ 6K rpm (Hitachi himac CR20E, R12A2 rotor, #25) for 12 min. @RT
7. collect aqueous phase (should be a little brownish)
8. repeat C/I extraction two more (total 3 times)
9. collect aqueous phase (ca. 14 ml)
10. add 1/10 vol of 3 M NaOAc (pH 5.2)
    2-2.5 vol of 100% of EtOH (become cloudy)
11. on ice for 30 to 60 min.
12. cfg @9K rpm for 20 min. @4oC
13. rinse pellet with 80% EtOH, once
14. resuspend in 5 ml of CTAB B (combine tubes, 15-20 ml/tube, ideally 15 ml)*
    (warm @55oC for 20 min, pipetting to <1mm pieces, then cool to RT)
    (*) you may use falcon2059 and cfg 5k-6k (or 7k with adaptor) with angle roter #7
15. add 5 ml of C/I and vortex well
16. cfg @ 5K for 10 min. @RT (thin white interphase)
17. harvest the sup (~ 5 ml)
18. add 0.6 ml of 10% CTAB and mix well
19. add 5.6 ml (1 vol) of C/I, then mix

20. cfg @5K for 20 min. @RT
21. take sup (sup= about 5 ml, almost no interphase)
22. add 2-ME to 0.5%
    add 1.2 vol (6ml) of CTAB pptn B (become cloudy)
23. incubate @RT for 30 to 60 min.
24. cfg @9K for 15 min @RT
25. dissolve the pptn in 5 ml High-Salt TE B (add 2-ME @0.5% just before use),
    then heat to 50-60 oC for 2 – 3 min (by pipetting)
26. add 2.5 vol (12.5 ml) of 100% EtOH
27. on ice for 30 to 60 min.
28. cfg @9K for 20 min. @4oC
29. rinse with 80% EtOH once
30. dissolve in 300 ul TE (300 ul/ a 50 ml tube)

This resultant fraction contains total RNA and genomic DNA as a contaminant. To remove genomic DNA, we usually perform the following protocol by using ISOGEN-LS (NIPPON GENE).

(300 ul; convenient for ISOGEN-LS, see below)
(by pipetting and still cloudy)

<ISOGEN-LS (NIPPON GENE)> (2.5 h)
31. transfer the solution into 1.5 ml tube (<300 ul / a tube)
32. add 3 vol of ISOGEN-LS (900 ul /tube)
33. mix well and incubate for 5 min @RT
34. add 0.8 vol (300*0.8=240 ul) of CHCl3 (-IAA)
35. shake vigorously for 15 sec. and then incubate for 2 to 3 min. @ RT
36. cfg @15K rpm for 15 min. @4oC
37. collect aqueous phase (about a half of the above sum, ~750 ul)
38. add 1 vol of isopropanol
39. incubate for 10 min @RT
40. cfg @15K for 10 min. @4oC
41. rinse with 80% EtOH, once
42. dry
43. dissolve in H2O or TE (~150 ul / 1.5 ml tube = 50 ml tube, if 5 g, 200 ug RNA/150 ul)
44. AGE to check (loading 1ul is usually enough even in DNA gel)
45. Quantification by spectrophotometer

3) DYNABEADS mRNA DIRECT kit (DYNAL)

Meterials

- 3 g fresh or frozen samples

Reagents

DYNABEADS mRNA DIRECT kit (DYNAL) or prepare the following reagents

- Dynabeads Oligo (dT)25 (DYNAL) Magnetic beads
- Lysis/Bindingbuffer
  100 mM Tris-HCl pH 8.0
  500 mM LiCl
  10 mM EDTA pH 8.0
  1% SDS
  5 mM DTT

- SDS+Washingbuffer
  10 mM Tris-HCl pH 8.0
  0.15 M LiCl
  1 mM EDTA
  0.1% SDS

- Washingbuffer
  10 mM Tris-HCl pH 8.0
  0.15 M LiCl
  1 mM EDTA

- Elutionbuffer
  2 mM EDTA pH 8.0

Magnetic stand for recover magnetic beads

- Dynal MPC-1
- Dynal MPC-E-1
- heat block

Protocol

Follow the manufacturer's instruction, protocol B-for large scale mRNA isolation

Note

As the purity of poly(A)+ RNA obtained in this way is not good, we further purify the RNA by ISOGEN-LS (NIPPON GENE).

Yield and purity just after the extraction with

protonemata: 3 ug poly(A)+RNA/g fresh weight, OD260/OD280=1.6

gametophores: 1-2 ug poly(A)+RNA/g fresh weight, OD260/OD280=1.6

Traditionally we used the following method, and this also works well.

ISOGEN (NIPPON GENE)

equipments

Materials
1-3g fresh weight or frozen sample
This amount corresponds to protonemata from three plates (f 90mm)

Note:
- Follow manufacturer's manual.
- Remove gel-like precipitation (when for protonema) or soft precipitation (gametophore) by pipetting not to touch RNA pellet, when LiCl precipitation is carried out. When EtOH precipitation is performed, translucent pellet contains RNA.
- This method is easy going. However purity of total RNA from gametophore is poor and it is hard to concentrate total RNA more than 200ng/ul.
- Standard yield is
protonemata: 50ug/g fresh weight OD260/OD280=1.8
gametophore: 10ug/g fresh weight OD260/OD280=1.2 (yellowish color)