

Plant RNA Polymerase I Holoenzyme Purification

Saez-Vasquez and Pikaard (1997) PNAS 94: 11869-11874

Saez-Vasquez and Pikaard (2000) J. Biol. Chem. 275:37173-37180

You will need the following buffers :

Nuclei Resuspension Buffer (RB0)

	<u>1 liter</u>	<u>4 liter</u>
50 mM Hepes, pH 7.9	11.9 g	47.7 g
20% Glycerol	200 ml	800 ml
10 mM EGTA	3.8 g	15.2 g
10 mM MgSO ₄ x 7 H ₂ O	2.48 g	9.9 g

4 lt requires approx. 25 ml of 10 M KOH or 14 gr. of KOH

Nuclei Resuspension Buffer (RB100)

Resuspension buffer containing:

100 mM KCl	29.8 g
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Honda Buffer

0.44 M sucrose	150.6 g
1.25% ficoll	12.5 g
2.5% dextran T40	25 g
20 mM Hepes-KOH, pH 7.4	4.8 g
10 mM MgCl ₂	2 g
0.5% Triton X-100	5 ml

CB100 (one liter recipe)

20 mM Hepes-KOH, pH 7.9	5.95 g
20% Glycerol	200 ml
0.1 mM EDTA (0.5 M stock)	200 µL
0.1 M KCl	7.45 g

CB1000 (one liter recipe)

20 mM Hepes-KOH, pH 7.9	5.95 g
20% Glycerol	200 ml

0.1 mM EDTA (0.5 M stock)	200 μ L
1 M KCl	74.5 g

Proteases Inhibitors:

Amounts for **200** mL of Honda Buffer:

[final]	[stock]	amount of stock
0.5 mM DTT	1 M	0.1 ml
1 mM PMSF	100 mM	2 ml
2.5 μ g/ml antipain	1 mg/ml	0.5 ml
0.35 μ g/ml bestatin	1 mg/ml	0.07 ml
0.5 μ g/ml leupeptin	5 mg/ml	0.02 ml
4.0 μ g/ml pepstatin A	1 mg/ml	0.8 ml

Amounts for **12.5** ml of RB100:

[final]	[stock]	
0.5 mM DTT	1 M	0.00625 ml
1 mM PMSF	100 mM	0.125 ml
5.0 μ g/ml antipain	1 mg/ml	0.0625 ml
3.5 μ g/ml bestatin	1 mg/ml	0.045ml
5.0 μ g/ml leupeptin	5 mg/ml	0.0125 ml
4.0 μ g/ml pepstatin A	1 mg/ml	0.05 ml

Proteases inhibitors stock solutions (SIGMA)

1 M DTT (D-0632): 3.09 g in 0.01 M sodium acetate pH 5.2

PMSF (P-7626): 17.4 g/ml (100 mM) in Isopropanol

Antipain (A-6191): 1 mg/ml in water

Bestatin (B-8305): 1 mg/ml in water

Leupeptin (L-2884): 5 mg/ml in water in water; 6 moths at -20°C

Pepstatin A (P-4265): 1 mg/ml in methanol; 6 moths at -20°C

Bio-Rad Assay of columns steps elutions

2 ml Bio-Rad concentrate

7 ml H₂O

9 ml total

add 90 μ l/well of a microtiter dish , then add 10 μ l of CNE (crude nuclear extract) or fraction

PURIFICATION PROTOCOL

Crude nuclear extract

ALL THE STEPS MUST BE DONE IN COLD ROOM or at 4 degrees C

1. Start with 75 to 100 grams of Broccoli inflorescence. Take only the clusters of rapidly dividing cells; avoid the stems. Use a razor blade.
2. Grind (6 pulses of 5 seconds each) in stainless steel Waring blender containing 200 ml of Honda buffer
3. Filter through 2 layer of Miracloth
4. Spin at 7,500 rpm, 30 minutes in Beckman rotor JA10, 4 degrees C, to obtain a crude nuclear pellet
5. Resuspended pellet in 20 mL of RB0+DTT (0.5 mM)+PMSF (1 mM)
6. Add 1.16 grams of NaCl and mix by hand, incubate 10 minutes
7. Add 0.4 ml of PEG8000-50% (1/50, original protocol), mix gently w/magnetic bar for 20 minutes
8. Spin at 12,000 rpm, 30 minutes, JA20 rotor (or 11,000 rpm in JA14 if the volume is large), 4 degrees C
9. Recover the supernatant and spin it again at 12,000 rpm, 30 minutes, JA20 (or 11,000 rpm, JA14), 4 degrees C.
10. Recover the supernatant and dilute with RB0 to 100 ml (you will see viscous *chromatin* floating in the diluted supernatant)
11. Filter through 2 layers of Miracloth (do it twice if required).
12. Recover the supernatant and precipitate with 0.33 g/ml ammonium sulfate $\text{NH}_4(\text{SO}_4)$, (Add $\text{NH}_4(\text{SO}_4)$ very slowly over a period of 20-30 minutes then, stir for approximately 1 hr.)
13. Spin at 12,000 rpm, 30 minutes, JA20 (or 11,000 rpm, JA14), 4 degrees C.
14. After spinning you will see that the pellet forms along the walls of the centrifugation bottle. Very carefully discard supernatant-the pellet is loose.

15. Resuspend pellet in 12.5 ml of RB100+DTT and proteases inhibitors (PMSF, antipain, bestatin, leupeptin, antipain A) using a Dounce homogenizer

16. Spin at 12,000 rpm, 30 minutes, JA20 rotor, 4 degrees C

Dialyze against RB100+DTT (0.5 mM)+PMSF (1mM)
(at least 3 changes of 500 mL each)

Spin at 12,000 rpm, 30 minutes, JA20, 4 degrees C

Store at -80°C or begin chromatography procedures.

References for cell-free extract procedures

Chris Lamb Lab., Plant Journal 7(6), 1021-1030 (1995)

Ethan Signer Lab., Plant Mol. Biol. 18: 865-871 (1992)

4-column purification of the Pol I holoenzyme

DEAE-Sepharose Cl-6B

Approximately 12.5 ml of crude nuclear extract are loaded onto 10 ml DEAE-sepharose Cl-6B 9 (small column 2.5 cm diameter)

Wash the column with 5 volumes of RB100 (100 mM KCl)

Wash the column with 5 volumes of RB175 (175 mM KCl)

Elute RNA pol I holoenzyme fraction with RB400 (400 mM KCl)

Collect 4-5 ml fractions and test for proteins by mixing 10 ul of each fraction with protein reaction mix (70 ul H₂O + 20 ul Bradford reagent). The degree to which sample turns blue provides a visual way to follow the peaks eluting from the column and allows you to know when you have reached baseline during the washes.

Note: If making 10 extracts on one day, use a 100 ml DEAE sepharose Cl-6B column (big column 5.5 cm diameter)

Biorex70

Approximately 12 ml of fraction DEAE-F400 are loaded onto 2 ml Biorex 70 column (small column 1.5 cm diameter)

Wash with 10 ml RB100

Elute holoenzyme RNA pol I fraction with 10 ml of RB800 (800 mM KCl)

Collect 1 ml fractions and test for protein by mixing 10 ul of each fraction with protein reaction mix (70 ul H₂O + 20 ul Bradford reagent)

Note: If making 10 extracts at once, use a 10 ml Biorex (big column 2.5 cm diameter)

Sephacryl S300

Approximately 3 ml of Biorex F800 fraction is immediately loaded onto 190 ml Sephacryl S300 FPLC column equilibrated in CB100

FPLC program:

Flowrate: 1 ml/min

Speed of Chart: 0.25 cm/ml

Collector: 1 ml/min

Start collecting at 60 ml

Stop collecting at 120 ml

End of the program

Peak fraction should elute between fractions 10 to 20 (70 to 80 ml); due to desalting effect on the gel filtration column, these RNA Pol I fractions are now in CB100.

MonoQ

Approximately 10 ml of sephacryl fraction are immediately loaded onto a 2 ml MonoQ FPLC column equilibrated on CB100

FPLC program:

At 0 ml

Flow rate 1 ml/min (0.8 ml/min)

Chart speed 1 cm/min

Collector 1.25 ml/min

At 5 ml alarm goes ON

Start injection

At 15 ml alarm goes OFF

At 25 ml beginning of the gradient (100 mM KCl)

Start collecting

At 45 ml end of gradient (600 mM KCl)

Wash at 1000 mM KCl

At 5 ml stop Wash

Stop collecting

Salt goes down to 100 mM

Program stop at 55 ml

Collect fraction from 10 to 20 for dialysis against RB100 (0.5 mM DTT).

Holoenzyme RNA pol I should be between fraction 13 to 16. Verify by the assay of RNA pol I non-specific activity, promoter dependent activity and/or western blot using antibodies against LE-RNA pol I (largest RNA pol I subunit, last exon)