Superscript cDNA Post Labelling Kit

Protocol

February 21, 2008 V9.1

Changes from previous version are in blue

Remarks

- use PCR machine for incubation steps
- place all the reagents (except for the enzyme) on ice to thaw
- **Before pipetting the enzyme: spin down all at the bottom of the tube; be sure that all enzyme is finished, before starting a new kit!**

!! Immediately notify Anne or Siger when you take a tube from the stock !!

1. Annealing

Perform Annealing in 300 µl PCR tube.

X	µl	totalRNA (10-20 ug)
Y	µl	nuclease free water
0,25	µl	lucidea spike RNA (Optional)
2	µl	Random nonamers (1.6 µg/µl)
18	**µl**	**annealing mix** Total end volume

- mix gently by pipetting up and down
- 5' 70°C
- cool on R.T. for 10' (annealing)
- spin down the reactions in the bottom of the tube
- place on ice

2. Reverse Transcription

18	µl	annealing mix		
12	µl	Master mix :	**6** µl	5x Superscript III buffer
			3 µl	0.1 M DTT
			1,2 µl	25x AA-dUTP / nucleotide mix (blue cap)
			1,8 µl	Superscript III (keep short as possible outside –20°C)
30	µl	**Reverse transcription mix** Total end volume		

- mix 5-10x by very gently pipetting up and down (vigorous pipetting will denature the enzyme !!!)
- 16 hrs 42°C
- cool on ice for immediate purification
- or place at – 20 °C for storage (max overnight)

3. Degredation of mRNA

30	µl	Reverse transcription mix
3	µl	2,5 M NaOH (Yellow cap)
		• *mix (vortex) and spin down*
		• *15' 37°C*
15	µl	2 M HEPES free acid (Purple cap)
		• *mix (vortex) and spin down*
48	**µl**	**Total volume**
		ready for purification or store at – 20 °C

4. Purification of amino allyl-modified cDNA

(usage of NucleoSpin Extract II columns)

- Take a fresh 0.1 M Sodium Bicarbonate pH 9.0 from the freezer (do not freeze it again !). (0.84 g NaHCO3 in 90 ml mQ; adjust pH to 9.0 (excact) with 1 M NaOH; add mQ to 100 ml. Divide in aliquots of 1 ml and freeze at -20°C)

- Add 200 µl of NTC buffer to each column, placed in a clean tube.
- Add the unpurified cDNA to each column; mix the cDNA by gently pipetting up and down 5 times.
- Centrifuge for **1 min** at **11,000 x g**. Discard flow-through and place the NucleoSpin® Extract II column back into thecollecting tube.
- Wash **600 µl buffer NT3**. Centrifuge for **1 min** at **11,000 x g**.
- Wash **500 µl 80% Ethanol**. Centrifuge for **1 min** at **11,000 x g**.
- Centrifuge for **2 min** at **11,000 x g** to remove **Ethanol** quantitatively. Make sure the spin column doesn't come in contact with the flow-through while removing it from the centrifuge and the collecting tube.

Residual ethanol from buffer NT3 might inhibit subsequent reactions and has to be removed in this step. In addition to centrifugation, total removal can be achieved by incubation of NucleoSpin® Extract II columns for 2-5 min at 70°C prior to elution.

- Transfer column to a fresh 1.5 ml micro centrifuge tube and add 60 µl **0.1 M sodium bicarbonate pH9.0** directly to the top of matrix in each column. It is crucial that the elution buffer completely covers the membrane.
- Incubate the column at room temperature for 1 minute. Centrifuge at 11.000 x g for 1 minute to collect the purified labelled cDNA.
- Proceed immediately to the coupling reaction

5. Measurement of cDNA concentration

- Use Nanodrop to measure cDNA concentration. This should be arround 200 ng/ul in total volume of 60 ul.
- 60 ng/ul is considered as lower limit to continue with labeling

6. Labeling of amino allyl-modified cDNA with CyDye

- Add the amino allyl modified cDNA (in 0.1 M sodium bicarbonate) directly into one aliquot (5 µl) of CyDye NHS ester.

  CyDye-NHS-ester:

  Use *Cy3/Cy5* 'remaining' tubes from –80^{0}C.

  If there are no remaining tubes:

  - Dissolve one tube *Cy3- or Cy5 mono reactive Dye* (PA23001/PA25001) in 40 µl DMSO (Sigma D-8418).
  - Divide into 8 portions of 5 µl.
  - Store remaining tubes, up to one month, at –80^{0}C.

- Incubate at room temperature, in the dark for 60 to 90 minutes.
- Add 15 µl 4 M Hydroxylamine to each coupling reaction.
- Mix by vortex and incubate at 30°C , in the dark, for 15 minutes.
- Proceed directly to purification of CyDye-labelled cDNA.

7. Purification of CyDye labelled cDNA (NucleoSpin ExtraxtII columns)

- Add 200 µl of NTC buffer to each column, placed in a clean tube.
- Add the unpurified cDNA to each column; mix the cDNA by gently pipetting up and down 5 times.
- Centrifuge for **1 min** at **11,000 x g**. Discard flow-through and place the NucleoSpin® Extract II column back into thecollecting tube.
- Wash **600 µl buffer NT3**. Centrifuge for **1 min** at **11,000 x g**.
- Wash **500 µl 80% Ethanol**. Centrifuge for **1 min** at **11,000 x g**.
- Centrifuge for **2 min** at **11,000 x g** to remove **Ethanol** quantitatively. Make sure the spin column doesn't come in contact with the flow-through while removing it from the centrifuge and the collecting tube.

Residual ethanol from buffer NT3 might inhibit subsequent reactions and has to be removed in this step. In addition to centrifugation, total removal can be achieved by incubation of NucleoSpin® Extract II columns for 2-5 min at 70°C prior to elution.

- Transfer column to a fresh 1.5 ml micro centrifuge tube and add **50 µl elution buffer NE** directly to the top of matrix in each column. It is crucial that the elution buffer completely covers the membrane.
- Incubate the column at room temperature for 1 minute. Centrifuge at 11.000 x g for 1 minute to collect the purified labelled cDNA.
- ready for hybridisation or store **at – 80 °C**

8. Measurement of labelled cDNA.

- Use Nanodrop to measure incorporation of Cy dyes in the cDNA.
- cDNA should be arround 200ng/ul
- Concentration of Cy3 and Cy5 should be at least **0.5 pmol/ul** in a total volume of 50 ul.

9. Mixing labelled cDNA's

- Use all the labelled cDNA for hybridisation but mix **equal quantities** cDNA of Cy3/Cy5 for hybridization. Unequal quantities will lead to biased ratio's