Protocoll for 4sU-tagging, thiol-specific biotinylation and separation of total RNA into nascent and pre-existing RNA

This protocol was completed on the 29.11.2010 by Lars Dölken but may be subject to change and improvements. Feel free to contact me at Doelken@mvp.uni-muenchen.de

Material to order and prepare:

- **15 ml Polypropylene Tubes SuperClear™ Gatefree™** (tolerate up to 15,000 g)
  (VWR International, Order no. 525-0153)

- **4-thiouridine** (Sigma, T4509 4-thiouridine, 100mg**)**
  Dissolve in sterile PBS (or water)
  Stock concentration: 50 mM
  Store in small aliquots at -20°C, thaw only once

- **Trizol (Invitrogen)**

- **RNA precipitation buffer**
  0.8 M NaCl
  1.2 M NaCitrate

- **Biotin-HPDP (**Pierce, 50mg EZ-Link Biotin-HPDP, Cat. Nr. 21341)
  Stock concentration: 1 mg/ml dissolved in Dimethylformamide (DMF)
  Gentle warming will ensure complete solubilisation
  Store at 4°C

- **RNAse free 10x Biotinylation Buffer (BB)**
  100 mM Tris pH 7.4
  10 mM EDTA
  => store in aliquots of 1 – 1.5 ml at 4°C

- **Phase Lock Gel Heavy Tubes (2.0 ml)**, Eppendorf (Order No. 0032 005.152)

- **µMacs Streptavidin Kit (Miltenyi, Order No. 130-074-101)**
  ⇨ use only the beads, but not the supplied buffers

- **1x Washing Buffer (WB)**
  100 mM Tris pH 7.5
  10 mM EDTA
  1 M NaCl
  0.1% Tween20

- **Dithiothreitol (DTT)**
  Always prepare fresh: 100 mM DTT in H_2O

- **Magnetic Stand (2 - 3 required, one stand holds 4 columns)**
  => has to fit the small columns of the µMacs Streptavidin Kit
- Chloroform/Isoamylalcohol (24:1)
- Isopropanol
- Ethanol

RNA labeling:

Cell culture medium volumes required for efficient RNA labelling:

- 10 cm cell culture dish = 5 ml
- 14 cm cell culture dish = 10 ml
- ⇨ **using too little volume may reduce labelling efficiency as 4sU is very efficiently taken up by the cells.**

Begin of labelling:

Thaw 4-thiouridine just before use.

Add 4-thiouridine to cell culture medium in the required concentration (500 µM will probably be fine for most applications). Do this by pipetting the required amount of 4sU into a fresh falcon tube. Take the cell culture medium off the plates to which 4sU is to be added and add it to the 4sU containing falcon tube. Mix well and immediately put the medium back onto the plates. Do not do more than one condition (3-5 plates) at a time. Try to handle cells at room temperature as quickly as possible.

End of labelling:
Quickly remove cell culture medium from cells (one condition at a time, max. 3-5 plates) and immediately add 5ml of Trizol or other cell lysis reagent (RNeasy) used for RNA preparation (it is best, if this step is done by two people, one removing the medium, the other adding Trizol and ensuring cell lysis).
It is important to obtain very clean RNA. Therefore, do not use too little lysis reagent.

Trizol RNA preparation:

Add **5 ml** of Trizol per 15 cm dish**.** Incubate at room temperature for 5 min to allow nucleoprotein complexes to dissolve. Transfer lysate to 15 ml Falcon tubes (special tubes).

Add 1ml Chlorform (0.2 ml per ml Trizol) and shake vigorously for 15 sec
Incubate at room temperature for 2-3 min
Centrifuge at 13,000 g for 15 min at 4°C

Transfer aqueous upper phase (containing the RNA) to new 15 ml Falcon tube.
Add ½ the reaction volume of both RNA precipitation buffer and isopropanol (e.g. to 3 ml supernatant add 1.5 ml RNA precip. sol. and 1.5 ml isopropanol).
Mix well. Incubate at room temperature for 10 min.
Centrifuge at 13,000 g for 10 min at 4°C.

Immediately remove supernatant
Add an equal volume of 75% Ethanol and vortex until the pellet dissolves into small pieces.
Centrifuge at 13,000 rpm for 10 min at 4°C.

Immediately remove supernatant.
Centrifuge again briefly to spin down remaining ethanol.
Remove remaining ethanol by pipetting using 200 µl pipett.
Repeat step using 20 µl pipet. After these two steps no further drying of the pellet is required.

Add 100 µl of 1x TE (10 mM Tris, 1 mM EDTA) per 100 µg expected RNA yield.
Dissolve RNA by heating to 65°C for 10 min (shaker).

Comments:
Using this modified Trizol protocol by Chomczynski et al. improves the removal of DNA and glycoproteins. 5 ml Trizol per 15 cm dish produces nice clean RNA. As higher centrifugal force is used the RNA pellets are more solid and easier to handle. This requires the use of special Falcon tubes as the regular tubes do not survive more than 6,000 g.
By removing the remaining ethanol twice by pipetting, the RNA does not have to be dried. This avoids the risk over-dried RNA, which may be difficult to dissolve again.

Biotinylation assay:

RNAse free 10x Biotinylation Buffer (BB)
100 mM Tris pH 7.4
10 mM EDTA
=> store in aliquots of 1 – 1.5 ml at 4°C

Biotin-HPDP (Pierce, 50mg EZ-Link Biotin-HPDP, Cat. Nr. 21341)
Stock concentration: 1 mg/ml dissolved in Dimethylformamide (DMF)

Labeling Reaction (use 30 – 100 µg total RNA):

- 2 µl Biotin-HPDP (1mg / ml DMF) per 1 µg RNA
- 1 µl 10x Biotinylation Buffer per 1 µg RNA
- 7 µl RNAse free H_2O per 1 µg RNA

Incubate at room temperature for 1.5 h with rotation.

Add an equal volume of Chloroform/Isoamylacohol (24:1).
Mix vigorously. Incubate for 2 – 3 minutes until phases begin to separate and bubbles start to disappear.
Centrifuge at full speed (20,000 g) for 5 min.
Carefully transfer upper phase into new tubes.
(This step can also be done using **phase lock gel heavy** tubes to avoid loss of material).

RNA precipitation:
Add 1 /10 the reaction volume of 5 M NaCl
Add an equal volume of isopropanol and centrifuge at 20,000 g for 20 min.
Remove supernatant.
Add an equal volume of 75% ethanol
Centrifuge at 20,000 g for 10 min.
Resuspend RNA in 100 µl 1x TE

Comments:
The chloroform extraction is required to remove unincorporated biotin-HPDP. To reduce the loss due to the extraction procedure the initial volume should be at least 500 µl. Smaller volumes should be increased by the addition of 1x TE.
To further reduce RNA loss we perform the second chloroform extraction using Phase Lock Gel Heavy tubes (2.0 ml, Eppendorf) following the manufacturer's instructions.
In principle, a single chloroform extraction step is enough to remove virtually all unincorporated Biotin-HPDP. Still, we initially perform two rounds to ensure complete removal. Usually we only use

the phase-lock tubes for the second round as 1 ml biotinylation volume is too much for these tubes. After the initial chloroform extraction only about 80% of the volume remains as the DMF is also removed.

Separation of labeled and unlabeled RNA using Streptavidin-coated magnetic beads

Washing Buffer (50 ml):
100 mM Tris pH 7.5
10 mM EDTA
1 M NaCl
0.1% Tween20

⇨ Heat Washing Buffer to **65°C**.

Elution Buffer (always prepare fresh):
100 mM Dithiothreitol (DTT) in RNAse free H_2O

Protocoll:
Heat biotinylated RNA samples to 65°C for 10 min and immediately place on ice for 5 min.
Add up to 100 µg (max. 100 µl) of biotinylated RNA to 100µl of streptavidin beads.
Incubate with rotation for 15 min.

Place µMacs columns into magnetic stand. Do not process more than 12 samples at a time (6-8 samples are optimal).

Add 0.9 ml of washing buffer to columns (pre-run and equilibrate).
To initiate the flow through the column you can gently press on the top of the column with your finger. Once the flow through the column has started it drains rapidly.

Apply beads (RNA) to the columns. Discard the flow-through.
Wash 3x with 0.9 ml 65°C washing buffer (*pipet tips shrink when pipetting buffers at 65°C*)
Wash 3x with 0.9 ml room temperature washing buffer

Pipet 700 µl Buffer RLT (RNeasy MinElute Cleanup Kit) into new 2 ml Eppis.
Elute RNA directly into Buffer RLT by placing the Eppis underneath the columns and adding 100 µl Elution Buffer (100 mM DTT) to the columns.
Perform a second elution round into the same tubes 3 min later.

Recovery of newly transcribed RNA
Continue with the RNease MinElute Cleanup Protocol following the manufacturer's instructions (*shown below*).

Add 500 µl 96-100% ethanol to the diluted RNA and mix thoroughly by pipetting.
Do not centrifuge.

Apply 700 µl of the sample to an RNAeasy MinElute Spin Column in a 2 ml collection tube. Close the tube gently and centrifuge for 15 s at >8000 g. Discard the flow-through.
Apply the remaining 700 µl and repeat the centrifugation. Discard the flow-through.

Transfer the spin column into a new 2 ml collection tube.

Pipet 500 µl Buffer RPE onto the spin column. Close the tube gently and centrifuge for 15 s at >8000 g to wash the column. Discard the flow-through.

Add 500 µl of 80% ethanol to the spin column. Close the tube gently and centrifuge for 2 min at >8000 g to dry the silica-gel membrane. Discard the flow-through and collection tube.

Transfer the spin column into a new 2 ml collection tube. Open the cap of the spin column and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.

To elute, etransfer the spin column to a new 1.5 ml collection tube. Pipet 20 µl RNase free-water directly onto the center of the silica-gel membrane. Close the tube gently and centrifuge for 1 min at maximum speed to elute.

The RNA is now ready for microarray analysis.

Recovery of unlabeled, unbound RNA
In case the unbound RNA needs to be recovered, collect the flow-through and the first wash for subsequent precipitation (together these contain >90% of the unbound RNA). Combine the two fractions and recover the unbound RNA by isopropanol/ethanol precipitation as performed after the biotinylation reaction (no salt need to be added as the washing buffer already contains 1 M NaCl).

Comments:
Small differences in sample volume do not matter when adding the biotinylated RNA to 100 µl beads. In case RNA input concentrations vary by >2-fold simply add the required volume of 1x TE to the beads to equalize conditions.
Like many column based assays collection of newly transcribed RNA using the RNAeasy minelute kit may leave something in the final sample which absorbs at 230-260 nm and may interfere with OD260 measurements. You therefore can not trust OD measurements at all if OD260/280 ratios are <1.7!!!
Recovery of newly transcribedRNA is highly quantitative. If you started with the same RNA concentration you can expect the same amounts of newly transcribed RNA. For down-stream analysis (microarrays) it is usually best to decide on one RNA concentration to be used for all samples.
*In case you want to quantify 4sU incorporation you have to precipitate the eluted RNA with isopropanol/ethanol adding 20 - 40 µg glycogen. In this case you have to add 10% 5M NaCl as the eluate does not contain high salt concentrations. Quantifying 4sU concentration for RNA prepared with the RNeasy kit does **not** work!!!*

Good Luck!!!

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