# Detection of polyadenylated RNA

## Stock solutions to be prepared:

***5 × AMV reverse transcriptase buffer***

250 mM Tris–HCl, pH = pH 8.3
250 mM KCl
50 mM $MgCl_2$
2.5 mM spermidine
50 mM DTT

## 1. Preparation of the samples

### *A. Cells grown on coverslips*

a) Fix the cells with 2% formaldehyde in 1× PBS (5 min, room temperature).
b) Wash in 1× PBS (3×).
c) Permeabilise the samples with 0.2% Triton X-100 in 1× PBS (10 min, room temperature).
d) Wash the samples in 1× PBS (3×).

### *B. Cells conventionally embedded in resins*

a) Fix the cells with 8% formaldehyde in 0.2 m PIPES, pH = 6.95 (2 hrs, room temperature).
b) Then, exchange formaldehyde for the new one and fix the samples for an additional 12 hrs at 4°C.
c) Wash in 1× PBS (3×).
d) Add 5 ml 1× PBS and scrape off the cells from the culture flask.
e) Transfer 2 ml of suspension into the Eppendorf tube and centrifuge 5 min at 500× g and 37°C.
f) Prepare 10% gelatine and leave it at 37°C.
g) Remove supernatant and add ca 200 µl of 10% gelatine and 1 µl of Cibacron Blue (not necessary, only help to visualise clump of cells).
h) Resuspend and centrifuge 5 min at 500× g and 37°C.
i) Remove the excess of gelatine and incubate the cells on ice for 10 min.
j) Post-fix cells with 8% formaldehyde in 0.2 m PIPES, pH = 6.95 (20 min on ice).
k) Wash in 1× PBS (3×).
l) Cut the tip of the Eppendorf tube and remove the gelatine sample into the Petri dish with 1× PBS.
m) Cut the gelatine sample into small pieces (ca < 1 $mm^3$).
n) Dehydrate small pieces in the methanol or ethanol and embed the samples in Lowicryl K4M or LR White or Epon resin (follow the common protocols for embedding samples into resins for electron microscopy).
o) After polymerisation, cut ultrathin sections (70 nm or 200 nm thick, depending on if you will observe them using light or electron microscope).
p) Put 70 nm thick sections on nickel grids coated with formvar and carbon (electron microscopy), and 200 nm thick sections on the coverslips coated with poly-D-lysine (light microscopy).

### *C. Cells embedded using high pressure freezing and freeze substitution*

a) Remove the culture medium from the cell cultivated in the petri dish and replace it with the $CO_2$-independent culture medium supplemented with 20% bovine serum albumin.

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**Perform this step under sterile conditions, all the following steps are performed under non-sterile conditions. Work as quickly as possible while freezing the cells.*

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b) Remove the $CO_2$-independent culture medium, leave only 200 – 300 µl in the Petri dish. Scrape off the cells.
c) Add 1 µl of Cibacron blue to the cell suspension.
d) Transfer 1 µl of the cell suspension to the membrane carriers (Leica, 1.5 mm cavity diameter) and freeze the cells, e.g. in the Leica EM PACT high-pressure freezer.
e) Transfer the frozen samples in the carriers in liquid nitrogen to the freeze substitution machine (e.g. Leica AFS machine) and place them in the substitution solution pre-cooled to -90°C.

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**You can use various freeze substitution solutions depending on your samples. In the case of HeLa cells, we used the following solutions: acetone, acetone with 0.25% glutaraldehyde and 0.1% uranyl acetate, acetone with 0.5% uranyl acetate.*

**Dissolve uranyl acetate in acetone from a 20% methanol stock solution*

**Dehydrate acetone using a dried molecular sieve.*

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f) Freeze substitute the cells 48 hrs at -90°C.
g) Raise the temperature at a rate of 10°C per hour to −40°C and incubate for 12 hrs.
h) Then, transfer samples into special plastic capsules (Leica Reagent bath and Leica Flow-through rings) filled with cold acetone to wash out the residues of the substitution solutions.
i) Infiltrate samples with the cold mixtures of acetone and Lowicryl HM20 at -40°C in this order:
   Acetone : Lowicryl HM20
   (i) 2:1 for 30 min
   (ii) 1:1 for 1 h
   (iii) 1:2 for 2 h.
j) Incubate the samples in the Lowicryl HM20 for 2 h at -40°C.

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**Exchange Lowicryl HM20 three times to remove the residues of acetone.*

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k) Remove Lowicryl HM20 and add a fresh one.
l) Polymerase samples under UV light in this order:
   −40°C for 24 h
   Raise temperature at a rate of 10°C/h to 20°C and polymerise the samples by UV for 2 days.
m) After polymerisation, cut ultrathin sections (70 nm or 200 nm thick, depending on if you will observe them using light or electron microscope).
n) Put 70 nm thick sections on nickel grids coated with formvar and carbon (electron microscopy), and 200 nm thick sections on the coverslips coated with poly-D-lysine (light microscopy).¨

## 2. Reverse transcription

A. Wash samples on coverslips or on grids in deionised water (3×).

B. Prepare reaction mixture composed of 1 × AMV reverse transcriptase buffer, 0.2 U/µl AMV reverse transcriptase, 0.4 U/µl RNasin, 0.25 mM dATP, dGTP, dCTP and either 0.25 mM 5-bromo-2′-deoxyuridine triphosphate (BrdUTP) or 0.05 mM biotin-16-2′-deoxyuridine-5′-triphosphate (biotin-dUTP) or 0.05 mM digoxigenin-11-2′-deoxyuridine-5′-triphosphate (digoxigenin-dUTP), 0.05 mM Alexa Fluor® 555-aha-2′-deoxyuridine-5′-triphosphate (Alexa-dUTP), 0.05 mM ChromaTide® fluorescein-12-2′-deoxyuridine-5′-triphosphate (fluorescein-dUTP) and 0.01 µg/µl oligo dT15.

C. Incubate samples on 20 µl drops (coverslips) or 10 µl drops (grids) of the reaction mixture in the moisture chamber for 1 hour at 42°C.

D. Wash with 1× PBS (3×).

E. In the case of Alexa-dUTP and fluorescein-dUTP Wash with 1× PBS (3×). Remove the buffer by holding each coverslip on its edge with forceps and remove any excess of the liquid from the coverslip by touching its edge on filter paper. Apply drops of the mounting medium on the glass slide (3 – 5 µl) and carefully lay the coverslips onto the mounting medium. Avoid the creation of any air bubbles.

F. In the case of non-labelled nucleotides process the samples as follows:

a) Light microscopy: incubate the samples with the primary antibody diluted in 1× PBS for 1 hr at room temperature. Wash with 1× PBS (3×) and incubate in the solution of the secondary antibody conjugated with the fluorochrome (diluted in 1× PBS. Wash with 1× PBS (3×) and deionised water and stain with DAPI (10 µM) for 20 min at room temperature. Wash with 1× PBS (3×). Remove the buffer by holding each coverslip on its edge with forceps and remove any excess of the liquid from the coverslip by touching its edge on filter paper. Apply drops of the mounting medium on the glass slide (3 – 5 µl) and carefully lay the coverslips onto the mounting medium. Avoid the creation of any air bubbles.

b) Electron microscopy: incubate the samples with the primary antibody diluted in 1× PBS for 1 hr at room temperature. Wash with 1× PBS (3×) and incubate in the solution of the secondary antibody conjugated with 10 nm gold particles (diluted in 1× PBS). Wash with 1× PBS (3×) and deionised water. Stain with 3% uranyl acetate in water for 45 min at room temperature in the dark. Wash with 1× PBS (3×) and deionised water. Dry the grids.

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