Optimised method for RNA isolation from mammalian cells following glyoxal fixation, antigen staining and flow sorting

This protocol was optimised to recover good quality RNA from human cancer cells following glyoxal fixation, antigen staining and flow sorting

Starting cell density 1-2 x106 cells

**Required**

Bovine serum albumin (BSA) – Sigma-Aldrich, A7906

Ice-cold methanol 100%

RNasin Plus RNase Inhibitor-Promega #N2615

40% glyoxal Sigma‐Aldrich, #50649

1.5 mL RNase free eppendorf tubes

Polypropylene round bottom tubes (Scientific laboratory supplies, 352063)

CellTrics 30µm sterile filter green - Sysmex, 04-004-2326

4,6-diamidino-2-phenylindole (DAPI) Sigma, D9542

TRI reagent – Sigma T9424

**To prepare ~4mL 3% glyoxal**

Water 2.835 mL

Ethanol 100% (v/v) 0.789 mL

40% Glyoxal – 0.313 mL

Acetic acid – 0.03 mL

Vortex the solution and adjust the pH to 4-5 value with a few drops of 1 M NaOH. Keep the solution cool and use within few days, otherwise glyoxal may precipitate. Upon reuse of solution, ensure the pH is within the range.

Glyoxal stock solution may precipitate upon storage. Precipitates can be dissolved by heating the solution to 50-60˚C

**RNasin Plus Rnase inhibitor**

We find that the effect of RNase inhibitor (RI) is dependent on the cell type. For the above cell type, we use RNase inhibitor at 1:25 dilution for all incubation steps in the protocol and 1:100 dilution for all washes

**Protocol**

Trypsinise cells and collect cell pellet by centrifugation at 300 x *g* for 5 minutes. Wash cells once with 1 ml PBS and remove the supernatant

Gently resuspend the cell pellet in 100µl 3% Glyoxal solution supplemented with RNase inhibitor (RI) and incubate for 15 minutes on ice

From this point, ensure cells are kept cold and use pre-chilled reagents to minimise the activity of RNases

Add 1ml 1xPBS (or 1% BSA in PBS) supplemented with RI and centrifuge at ~2000g for 3 minutes at 4˚C. Discard the supernatant

Add 100µl ice-cold methanol (100%) supplemented with RI (add drop by drop while gently vortexing cells) to the cell pellet and incubate on ice for 30 mins

Add 1ml 1%BSA in PBS supplemented with RI and centrifuge at ~2000g for 3 minutes at 4˚C. Discard the supernatant

Gently re-suspend cells in 100 μl of diluted antibody (in this case anti-CCNB1, CST 12231S, diluted 1:200) in 1% BSA in PBS with RI and incubate for 1 hour on ice.

Add 1ml 1%BSA in PBS supplemented with RI and centrifuge at ~2000g for 3 minutes at 4˚C. Discard the supernatant

Resuspend cells in 100µl Alexa Fluor 488 donkey anti-rabbit secondary antibody diluted 1:1000 in 1% BSA in PBS supplemented with RI. Incubate on ice for 30 minutes in dark

Add 1ml 1%BSA in PBS supplemented with RI and centrifuge at ~2000g for 3 minutes at 4˚C. Discard the supernatant

Resuspend the cell pellet in 200µl 1% BSA in PBS supplemented with RI (1:100)

Filter cells by passing the cell suspension through CellTrics 30µm sterile filter into 5 mL polypropylene round bottom tubes

Add DAPI to cells at a final concentration of 1µg/ mL and sort cells (ensure that the sample and collection chamber is chilled) into 1.5 ml RNase-free 1.5 mL eppendorf tubes (pre-coat the tubes with 1% BSA in PBS supplemented with RNase inhibitor)

Centrifuge sorted cells at 2000 x *g* for 3 minutes at 4˚C. Discard supernatant. Add 1 mL TRI reagent to lyse cells and proceed to RNA isolation procedure with standard TRI reagent protocol