# Purification of Crosslinked RNA-Protein Complexes by Phenol-Toluol Extraction, PTex

# Protocol

## Cells and reagents

- Selected cell line.
- Culture medium according to experiment.
- DPBS (Gibco).
- Solution D (modified from Chomcznski y Sacchi 2006):

    - 5.85 M guanidine isothiocyanate.
    - 31.1 mM sodium citrate.
    - 25.6 mM N-lauryosyl-sarcosine
    - 1% 2-mercaptoethanol.

- Phenol (Roti-Phenol, Roth 0038).
- Toluol (Th.Geyer, 752.1000).
- 1,3-Bromochloropropane (Merck, 8.01627.0250).
- Ethanol absolute.
- Distilled, sterile water.

## Equipment

- Incubator (shaker)
- Cross-linking device: 254 nm bulbs (CL-1000, Ultra-Violet Products Ltd.).
- Refrigerated bench-top centrifuge for 2 ml microtubes.
- Refrigerated bench-top centrifuge with rotor for 5 ml tubes.
- ThermoMixer (Eppendorf).

# Cell culture and crosslinking

Culture cells at 80% confluence in DMEM with D-glucose (10% FCS, 1% P/S). Wash cells in monolayer with 10 ml cold DPBS per dish. Remove the PBS and place the culture dishes without their lids on a cold try inside the cross-linker device. Irradiate with 0.15-0.25 J/cm$^2$ at 254-nm UV light. Add 500 μl of DPBS to each dish and scrape the cells with a rubber; place the –CL and +CL pools separately in a 15 mL falcon tube. Take a sample of each, experimental (+CL) and control (-CL) for cell counting. Make aliquots of 1x10$^7$ cell and centrifuge 2mL sterile micro-tubes (500 xg, 3 min, 4 °C). Remove and discard the supernatant, store at -20°C for its use within a week, or -80°C for longer.

# PTex: Phenol-Toluol extraction in three steps

**TIMING: ~40 min for extraction / 1h ethanol for precipitation and centrifuging**
**PERFORM ALL STEPS UNDER THE HOOD. USE SAFETY GEAR!**

# STEP ONE

This step must be carried out under physiological conditions. Use of detergents, high concentrations of salts or other denaturing conditions affect the correct behaviour of the complexes during the extraction.

Pre-cool the centrifuge at 4°C. Prepare three tubes 2 ml, safety cap and one 5 ml tube per sample, label accordingly with an ethanol-resistant marker.

- To one set of 2 ml tubes add 300 uL phenol, 300 μl toluol and 200 μl bromo-chloro-propane (BCP). The second set of tubes will contain 300 μl of solution D, reserve. Add 4.5 ml of Ethanol absolute to 5 ml tubes, reserve. Use the third set of tubes for input control.

- Resuspend –CL and +CL cell pellets in 1 ml DPBS to obtain a cell suspension equivalent to 1x10$^7$ cells, transfer 60 μl of the cell suspension as input control into the third set of 2 ml tubes, snap freeze and store until use. Take 600 μl and mix with the organic solution during 1 min at RT on ThermoMixer at 2.000 r.p.m.

- Centrifuge at 20.000xg, 4° C, 3 min. You will obtain three visible phases: the upper aqueous-phase (aq1), a middle membraneous-like inter-phase (int1), and the organic phase (org1).

- Carefully remove as much aq1 as possible avoiding contact with the int1. Place the aq1 in the corresponding tube with the solution D, mix pipetting up and down. CRITICAL: do not to contaminate the aq1 with the int1. Help yourself with a syringe, blunt needle (21G).

## STEP TWO

- Add 600 µl phenol and 200 µl BCP to the aq1-solutionD tube; mix during 1 min at RT, 2.000 r.p.m. (ThermoMixer), and centrifuge as before.

- Carefully with the help of a fresh syringe remove the upper three quarters of the resulting aqueous-phase (aq2) and the lower three quarters of the organic-phase (org2), approximately 600 µl each. CRITICAL: a whitish-homogeneous layer adhered to the wall of the tube in the aqueous/interphase area might appear, don't discard it!.

- Keep the resulting interphase in the same tube and proceed with the final step.

## STEP THREE

- Mix the int2 with 400 µl of water and 200 µl of ethanol absolute, shake slightly. Then, add 400 µl phenol and 200 µl BCP, mix and centrifuge as before.

- Remove 3/4th of the aqueous- and organic- phases using fresh syringes. Transfer the remaining inter-phase 3 (~200 µl) into the 5 ml tubes previously prepared for ethanol precipitation. Cool-down at -20°C during at least 30 min. PAUSE POINT.

- Precipitate the complexes by centrifuging at 20.000xg, 30min, 4°C. Carefully decant the supernatant and let pellets dry under the hood for no more than 10 min. Resuspend in 30-50 µl of distilled water, Laemmli buffer, or the respective solution according to downstream applications.