**PROTOCOL:**

**Tumour cell invasion into Matrigel: optimized protocol for RNA extraction**

**Reagents and Materials**

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| **Product** | **Manufacter** | **code** |
| Matrigel matrix | Corning | 354234 |
| RNAse zap | ThermoFisher Scientific | AM9780 |
| TRIzol reagent | Life technologies | 15596018 |
| ReliaPrep™ miRNA Cell and Tissue Miniprep System | Promega | Z6211 |
| 100% isopropanol | Alfa Aesar | L10181 |
| 100% chloroform | Fisher Scientific | 10488400 |
| 95% ethanol, RNAse-free | Fisher Scientific | 10048291 |

**Invasion assay (24 well plate procedure)**

The procedure was as follows:

1. Thaw matrigel at +4°C on ice, over night (O.N.).
2. Calculate the number of samples and prepare a mixture of medium/matrigel (MM) 1:1. Consider a volume of 600 µl mixture needed for each sample.

***\* HINT*** do always an excess (e.g. calculate 700 µl for each sample)

***ATTENTION*** when working with matrigel, use cold tips (put at -20°, and change frequently cause they warm up quickly). Eppendorf microtubes and falcon tubes must be cold. Work on ice. Medium used for the mixture must be cold.

1. Put the 24 well plate on ice and let it get cold. Add 300 µl/well of the matrigel/medium mixture. Put the plate at 37°C for half an hour (at least).

***ATTENTION*** avoid the formation of bubbles. Eventually, remove them with a sterile needle.

1. Prepare the neurospheres (NS) cell suspension. NS should have been expanded in culture in order to obtain a diameter variable between 100-400 µm. Start from the NS in a T25 cm2 culture flask to have enough NS for 3 replicates (resuspension in 1 ml of MM – see step 6).
2. Pellet gently the neurospheres (900 rpm - 10 min), remove the supernatant and resuspend gently the pellet tapping the bottom of the tube.
3. Using an ice-cold p1000 tip, resuspend gently the neurospheres in the appropriate volume of the cold mixture.
4. Take the 24 well plate with the wells pre-coated with the bottom layer of matrix. Leave at room temperature few minutes.
5. Using an ice-cold p1000 tip, dispense 300 µl/well of the neurospheres suspension. Avoid the formation of bubbles.

***ATTENTION*** resuspend gently the neurospheres again immediatety before taking each aliquot, because neurospheres tend to precipitate and each time use a new cold tip.

1. Move the plate on each side in order to distribute the NS in the whole well.

***\* HINT*** This is important to avoid the neurospheres clump and to obtain an omogeneous distribution in the well. Check the neurospheres distribution at the microscope and eventually repeat the movement.

1. If not all the wells of the plate are used, dispense PBS in the empty wells in order to avoid evaporation. Incubate at 37°C.

***\* HINT*** It’s recommended to use the internal wells for the assay and put PBS in the external ones.

**RNA extraction procedure**

The procedure was performed as follow:

1. Put the plate at +4°C for 45 minutes (± 15 min) to let the matrigel melting.
2. Add cold TRIzol in a ratio matrigel/medium:TRIzol of 1:3 (1,8 ml TRIzol for 0,6 ml matrix in a well of a 24 well plate).

***\* HINT*** TRIzol is toxic, while using TRIzol do all the procedure under a chemical hood. In order to avoid any sample degradation due to RNAse activity, prepare your work location before starting the procedure cleaning the area with RNAse zap. Use always sterile gloves and RNAse free tips and tubes.

1. Leave 5 minutes on ice to let a partial dissociation of the sample.
2. Dissociate mechanically using a p1000, pipetting 15-20 times.

***ATTENTION*** After the mechanical dissociation, the solution should be viscous, opaque, and you should not observe any cell aggregate. If cell aggregates are still present, repeat the mechanical dissociation. It is important that while pipetting, the tip is kept on the wall of the well in order to better dissociate any complex.

1. Put the sample in eppendorf (1,2 ml x 2 eppendorf). Place on ice.

***REST*** Samples can be frozen at this step at -80°C for long storage and later on processing.

1. Incubate for 5 minutes at room temperature to allow the complete dissociation of the nucleoproteins complex.

***ATTENTION*** From this step on, all the volumes are referred to one eppendorf (1,2 ml sample volume).

1. Add 0,2 ml chloroform and shake the tube vigorously by hand for 15 seconds.
2. Incubate for 2-3 minutes at room temperature.
3. Centrifuge the sample at 12.000 x g for 15 minutes at +4°C.
4. Remove the aqueous phase of the sample (around 500-600 µl) by angling the tube at 45°C and place it in a new tube.

***ATTENTION*** Avoid disturbing the interface.

1. Add 400 µl of 100% isopropanol to the aqueous phase, shake the tube vigorously for 30 seconds.

Incubate at room temperature for 10 minutes. Shake again for few seconds.

***ATTENTION*** **From this point on, use the Promega ReliaPrep™ miRNA Cell and Tissue Miniprep System according to the manufacturer instructions.**You do not need to work anymore under the chemical hood.

1. Transfer homogenate to a ReliaPrep™ Minicolumn. Centrifuge at 12,000 × g for 30 seconds.
2. Discard the liquid in the collection tube.
3. Add 500μl of RNA Wash Solution (RWA) to each column. Centrifuge at 12,000 × g for 30 seconds. Discard liquid in collection tube.
4. Add 500μl of RWA to each column. Centrifuge at 12,000 × g for 2 minutes. Discard liquid in collection tube.
5. Transfer column to a 1.5ml Elution Tube.
6. Add 40μl of Nuclease-Free Water to each column. Centrifuge at 12,000 × g for 1 minute.
7. Transfer 5μl of DNase I and 5μl of DNase 10X Buffer to each eluate.

***ATTENTION*** Critical step. Mix gently each sample by tapping the bottom of the eppendorf.

1. Incubate 5 minutes at room temperature.
2. Add 150μl of Lysis Buffer (LBA) to the samples
3. Add 300μl of 95% ethanol to the mixture and vortex for 5 seconds. Do not vortex a lot.
4. Transfer mixture to a new ReliaPrep™ Minicolumn. Centrifuge at 12,000 × g for 30 seconds. Discard the liquid in the collection tube.
5. Add 500μl of RWA. Centrifuge at 12,000 × g for 30 seconds. Discard liquid in the collection tube
6. Add 500μl of RWA. Centrifuge at 12,000 × g for 2 minutes. Discard liquid in the collection tube.
7. Transfer column to a 1.5ml Elution Tube.
8. Add 30μl of Nuclease-Free Water. Centrifuge at 12,000 × g for 1 minute. If expected yields are greater than 15μg, add an additional 15μl of Nuclease-Free water and repeat the centrifugation step.

**Abbreviations:**

**MM**= mixture medium matrigel

**NS**= neurospheres