RNA extraction

Reagents and materials

- Trizol
- Chloroform
- Eppendorf tubes
- Centrifuge
- Isopropanol
- 75% ethanol (etOH)
- Heating block
- Nanodrop

Trizol is highly toxic. Therefore, always handle it in the hood and with gloves and a lab coat, and use special waste containers.

Step 0

Pre-cool down the centrifuge (4°C) and turn on the heating block (60°C).

Step 1

Aspirate the media of the cells and add 400µL Trizol per well (from the 6cm plate).

Step 2

Pipette up and down and transfer all to an Eppendorf tube. Wait 5 minutes at RT.

Step 3

Add 80µL of chloroform. Invert the tubes five times and incubate 2-3 minutes at RT.

Step 4

Centrifuge 15 minutes at 12.000g (4°C). Transfer the aqueous phase (upper part) into a fresh Eppendorf tube. BE CAREFUL TO NOT TAKE ANYTHING FROM OTHER PHASES!

Step 5

Add 200µL of isopropanol and incubate 10 minutes at 4°C.

Step 6

Centrifuge 10 minutes at 12.000g (4°C). Discard the supernatant with a pipette and resuspend the pellet with 400µL of 75% etOH.

Step 7

Mix well and centrifuge 5 minutes at 7.500g (4°C). Discard the supernatant with a pipette and air dry the pellet for 10 minutes at 60°C in the heating block.

Step 8

Resuspend the pellet in 20-50µL of RNAse free water, depending on the size of the pellet.

Step 9

Quantify the RNA using the Nanodrop and store at -80°C until cDNA synthesis.

cDNA synthesis

Reagents and materials

- SCRIPT cDNA synthesis kit (#PCR-511L) from Jena Bioscience.
- Eppendorf tubes
- Heating block

Step 1

Add the following components to a nuclease-free microtube. Pipette the mix on ice and mix gently by pipetting up and down. Use the document “cDNA synthesis calculations template”.

Component	Stock concentration	Final concentration	10µL assay
RNAse-free water	-	-	Fill up to 10µL
RNA template	-	1µg	1µg (calculate depending on RNA concentration)
Primer (random primer)	100µM	50pmol (100ng)	0.25µL
SCRIPT RT Buffer complete	5x	1x	2µL
dNTP Mix	10mM each	500µM each	0.5µL
DTT stock solution	100mM	5mM	0.5µL
SCRIPT Reverse Transcriptase	200 units/µL	100 units	0.25µL

Step 2

First strand cDNA synthesis:

- Incubate at 42°C for 10 minutes.
- Incubate at 50°C for 45 minutes.

Step 3

Inactivation of the Reverse Transcriptase:

- Heat the mixture to 70°C for 10 minutes.

Step 4

Store at -20°C in a 1:20 dilution (10µL cDNA + 190µL H_2O).

qPCR

<u>Reagents and materials</u>

- SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, #1725275)
- MicroAmp Fast Optical 48-Well Reaction Plate
- MicroAmp™ 48-Well Optical Adhesive Film (25 films)
- Primers
- cDNA
- H_2O
- Centrifuge
- StepOne qPCR machine

<u>Step 1</u>

Mix to a final volume of 5µL/well:

- 2,5µL Power SYBR™ Green PCR Master Mix (2x)
- 0,15µL forward primer (10µM)
- 0,15µL reverse primer (10µM)
- 2µL cDNA
- 0,2 µL H2O

Use the document "qPCR calculations and plate design template".

<u>Step 2</u>

Seal the plate with the adhesive film.

<u>Step 3</u>

Spin down the plate in the centrifuge and start the following PCR program.

Holding stage:
- 10 min at 95°C

Cycling Stage (40 cycles):
- 15 sec 95°C
- 1 min 60°C

Melt curve stage:
- 15 sec 95°C
- 1 min 60°C
- 60°C → 95°C