STORE

SOP Title:

RNA extraction and testing

The STORE processing methods were shown to be fit-for purpose for DNA, RNA and protein extraction from FFPE material. The STORE characterisation methods were shown to be fit for-purpose for Quality Control of the extracted DNA, RNA and proteins.

Standard Operation Procedures (SOPs) for

RNA extraction and testing

Task 3.1 (HMGU)

Herein we describe the material we used for establishing our protocol for RNA extraction
as well as
how we characterized the quality of RNA using
RIN (RNA Integrity Number).

Three different extraction methods are tested (to whom it may concern), although our method of choice is Qiagen kit.

RNA- Extraction from FFPE-tissue blocks

To avoid contamination of samples with ribonucleases, use solely disposable plastic-ware from new packages and pipet tips with aerosol filters. Everything else (lab benches, microtomes, etc.) should daily be decontaminated with RNaseExitus decontamination reagent. Solutions should be passed trough sterilfilters (0.22 µm diameter) if they could not be autoclaved. RNA solutions should be supplied with ribonuclease inhibitor for storage at -70°C.

- **samples: 10 FFPE blocks:**

Block No.	Label	Amount
TP-4295-71	**71**	**3x8µm sections**
K-4313-74	**74**	**3x8µm sections**
4206-85	**85**	**3x8µm sections**
5930-86	**86**	**3x8µm sections**
14647-00	**00**	**3x8µm sections**
18163-01K	**01**	**3x8µm sections**
T47D-pH4-1	**pH4/1**	**6x8µm sections**
T47D-pH4-3	**pH4/2**	**6x8µm sections**
T47D-pH7-3	**pH7**	**6x8µm sections**
10269-02	**Colon**	**3x8µm sections**

- Put FFPE-blocks at 4°C 1 h before starting
- Prepare reaction tubes: 2 per FFPE block, label and put oN at -20°C to minimize effects of electrostatics, transport in a thermoblock

I. Paraffin removal

This procedure has been standardized and differs from the Qiagen and Norgen protocol!

- From each block: for **3 parallel batches** cut **3x or 6x 8µm each** and put into labeled, cold reaction tubes
- clean microtome, tweezers etc. with isopropanol after each block

+ 500 µl Xylene, vortex and centrifuge 2 min at 14.000 rpm, remove supernatant **very carefully! tissue might not pelletize properly!**

+ 500 µl EtOH abs, vortex and centrifuge 2 min at 14.000rpm, remove supernatant

+ 500 µl EtOH 90%, vortex and centrifuge 2 min at 14.000rpm, remove supernatant

- Check for Xylene smell! Xylene inhibits lysis! If necessary, repeat EtOH 90% step!

- Air dry pellet for 10-15 min at RT (**DO NOT dessicate pellet completely!**)

--> from here proceed different for each extraction methode:
(Please note: Modifications of the manufacturers protocol are marked in red within the protocol)

II. RNA extraction

II. 1 QIAGEN RNeasy FFPE kit

- Resuspend deparaffinized pellet from step I. in **150 µl Buffer PKD**, add **20µl proteinase K** from QG, vortex

  --> **55°C**, 450 rpm, 15 min
  --> **80°C**, 450 rpm, 15 min

- Lysis of connective tissue rich samples might not be complete; in case visible debris remains, centrifuge 3 min at 8000 rpm and transfer supernatant into a new tube

- **+ 320 µl Buffer RBC**, mix immediately by pipetting,

- **transfer to gDNA Eliminator column, centrifuge 30s at 12.000rpm**
  --> discard column, save flow-through

- **+ 720 µl 100% EtOH to flow through, mix immediately by pipetting**

- **load in 700µl steps (including any precipitates that may have formed) onto RNeasy MinElute columns, centrifuge each time 15 sec at 12.000 rpm, discard flow-trough**

- **+ 500µl buffer RPE, 15 sec at 12.000 rpm, dicard flow-through**

- **+ 500µl buffer RPE, 2 min at 12.000 rpm, dicard flow-through**

- place column in a new collection tube, centrifuge 5 min at 14.000 rpm with open lids of the spin columns to dry the membranes

- place column in a new 1.5ml Eppendorf tube

- apply 25 µl Ampuwa to the membrane without touching it

- elute for 1 min at 14.000 rpm

➔ **perform NanoDrop measurements immediately**
➔ **after NanoDrop measurements supply RNA with 0,4µl RiboLock Rnase inhibitor**
➔ **store at -70°C**

II.2. NORGEN (NG)

- Resuspend deparaffinized pellet from step I. in **300µl Digestion Buffer**, add **10 µl proteinase K** from NG, vortex

--> **50°C, 450 rpm, 3h**

- Lysis of connective tissue rich samples will not be complete; in case visible debris remains, centrifuge 3 min at 8000 rpm and transfer supernatant into a new tube

- Add 10 µl mercaptoethanol to each 1 ml Binding Solution, vortex
  (20 probes: 7ml + 70µl)

- **+ 300µl Binding Solution**, vortex

- **+ 600µl EtOH abs,** vortex

- Load onto columns in 600µl steps,
  centrifuge each time 1 min at 13.000 rpm, discard flowthrough

- **+ 400µl Wash Solution**
  centrifuge 1 min at 13.000 rpm, discard flowthrough

- **+ 400µl Wash Solution**
  centrifuge 1 min at 13.000 rpm, discard flowthrough

- centrifuge 1 min at 13.000 rpm to dry membranes, discard collection tube

- Place column into fresh elution tube

- Add 25µl Ampuwa to the center of the membrane without touching it

- centrifuge 2 min at 2000 rpm
- centrifuge 1 min at 14.000 rpm

➔ **perform NanoDrop measurements immediately**
➔ **after NanoDrop measurements supply RNA with 0,4µl RiboLock Rnase inhibitor**
➔ **store at -70°C**

II.3. PHENOL-CHLOROFORM-EXTRACTION (PC)

- Resuspend deparaffinized pellet from step I. in 2**00µl PCR-ready lysis buffer**, add **50µl proteinase K** from stock in the fridge, vortex

→ **55°C,** 450 rpm in a thermoshaker **for 2h**

- **+20µl 3M NaAc pH4,** vortex each probe immediately
- **+ 300 µl phenol-chloroform-Solution** (AppliChem, ready-to-use), vortex

→ **on ice for 15 min**

- centrifuge 2 min max speed, transfer supernatant into new reaction tube
- **+ 200µl chloroform**
- centrifuge 2 min at 13.000 rpm, transfer supernatant into new reaction tube
- **+ 1 µl (20µg/µl) glycogen**
  + 180 µl (0.6 Vol) isopropanol

→ **-20°C,** oN for precipitation

- centrifuge for 30 min with max speed at **4°C**
  (place tubes into centrifuge with hinge on the outside, so pellets have a defined location
  --> pellets from isopropanol precipitation are very loosely attached!)
- remove supernatant very carefully by pipetting
- save supernatant in a separate tube at -20°C until recovery of preticipated DNA has been veryfied
- **+500µl EtOH 70%** (RT!), wash 15 min with max speed at **4°C**
- remove supernatant very carefully, but as completely as possible by pipetting
- **+500µl EtOH 70%** (RT!), wash 15 min with max speed at **4°C**
- remove supernatant very carefully, but as completely as possible by pipetting
- air-dry pellets at RT until EtOH has evaporated
- resuspend in 25 µl Ampuwa
- dissolve for 15 min at 55°C

→ **perform NanoDrop measurements immediately**
→ **after NanoDrop measurements supply RNA with 0,4µl RiboLock Rnase inhibitor**
→ **store at -70°C**

III. Spectrophotometrical measurements

- **Vortex each probe and determine**
  - RNA concentration
  - OD ratio 260/230 *(should be above 1.8)*
  - OD ratio 260/280 *(should be above 1.8)*

--> 3x non sequentially for each sample, reblank several times

IV. Microcapillary electrophoresis (Determination of RIN values)

- Probes were used at a maximal concentration of 500ng/µl and diluted in Rnase-free molecular grade water, if neccessary

- Manufacturers instructions for setting up the Chip Priming Station and preparation of the RNA ladder after arrival (denature 2 min at 70 °C, cool down on ice, store aliquots at -70°C, thaw on ice) were followed, except we additionally denatured the ladder for 2 min at 70°C right before use

- Manufactureres instructions for preparation of gel aliquots (Pipette 550 µl of RNA 6000 Nano gel matrix into a spin filter, centrifuge at 1500 g for 10 min at RT, store filtered gel in 65 µl aliuots at 4°C) were followed.

- Analysis of RNA samples according to the manufacturers protocol includes the following steps:

  - Equilibrate RNA 6000 Nano dye concentrate to RT for 30 min, vortex for 10 sec, spin down
  - add 1 µl of dye into a 65 µl aliquot of filtered gel, vortex
  - centrifuge mixture at 13000 g for 10 min at RT
  - put RNA 6000 Nano chip on the chip priming station, load 9.0 µl of gel-dye mix trough the priming well, close the chip priming station and press plunger until it is held by the clip
  - after exactly 30 sec release clip, after 5 sec slowly pull back plunger
  - open chip priming station and load 9.0 µl of gel-dye mix in each filling well
  - load 5 µl of RNA 6000 Nano marker in all 12 sample wells and in the ladder well
  - load 1 µl of prepared ladder in the ladder well
  - load 1 µl of sample in each of the 12 sample wells.
  - put the chip horizontally in the adapter of the IKA vortexer and vortex for 1 min at 2400 rpm.
  - immediately run the chip in the Agilent 2100 bioanalyzer

Comment: all FFPE samples will show degradation of RNA; dependent on the analytical technique samples with a RIN value above 1.4 may be useful.