SOP Title:

DNA 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

DNA extraction and testing

Task 3.1 (HMGU)

Herein we describe the material we used for establishing our protocols for DNA extraction as well as how we characterized the quality of DNA using electrophoresis, OD-measurements, and PCR.

From the three different extraction methods we used only our method of choice is described (commercional Qiagen kit).

- **samples we used: 10 FFPE blocks**

Block No.	**Label**	**Amount of material**
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 min. 1 h before starting
- Prepare reaction tubes: 3 per FFPE block, label and put oN at -20°C to minimize effects of electrostatics, transport in a thermoblock

I. Paraffin removal

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

Work quickly during these steps!

- From each block: for **3 parallel running tests** cut **3x or 6x 8µm each** as indicated in the table above and put into labeled, cold reaction tubes
- put a 4µm section/block on a microscope slide for subsequent HE staining,
- 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 at RT for 10-15 min (**DO NOT dessicate pellet completely!!!**)

--> from here we proceeded different for the different extraction methods:

(Please note: Modifications of the manufacturers protocol are marked in red within the DNA extraction protocol)

II. DNA extraction
using QIAGEN (QG) kit

(QIAmpDNA FFPE tissue kit (Cat #56404, Qiagen, Hilden, Germany)

- Resuspend deparaffinized pellet from step I. in **180 µl Buffer ATL**, add **20µl proteinase K** from QG, vortex

--> 56°C, 450 rpm, 24h (vortex and add additional proteinase K to samples, that shown no signs of clarification after 4 h)

--> 90°C 1h (**max!**)

- Lysis of connective tissue-rich samples may be incomplete. In this case (visible debris remains) centrifuge 3 min at 8000 rpm and transfer supernatant into a new tube
- **+ 2 µl 100mg/ml RNaseA**

--> RT, 2min

- + **200 µl Buffer AL** (vortex each sample immeadiately!), **+ 200 µl EtOH abs** (voretx each sample immediately!) and spin down

(all centrifugation steps are carried out at RT!)

- Transfer lysate to QG-columns, centrifuge at 8000 rpm for 1 min
- Put column into new collection tube
- + **500 µl Buffer AW1** centrifuge at 8000 rpm for 1 min
- Put column into new collection tube
- **+500µl Buffer AW2**, centrifuge at 8000 rpm for 1 min
- Put column into new collection tube

(Do not mix up AW1 and AW2, otherwise discard experiment and start from the beginning!)

- Centrifuge at 13.000 rpm for 3 min to dry the membrane
- Place column into a new labeled 1.5ml reaction tube

(Label of tube: "**method_label-batch_date", e.g. QG_84-1_15.6.10)**

- and apply **30 µl TE Buffer to the center of the membrane without touching it**

(TE-buffer: 10 mM Tris, 1 mM EDTA, pH 8.0 with 5 M HCl (all reagents from Sigma-Aldrich, Steinheim, Germany) in molecular grade water (Ampuwa from Fresenius Kabi, Bad Homburg, Germany) and autoclaved)

--> RT, 5 min

- Eluate at 13.000 rpm for 1 min

III. Spectrophotometrical measurements

(NanoDrop ND-100 from PEQLAB Biotechnologie, Erlangen, Germany)

- **Vortex each probe and determine**
  - DNA concentration
  - OD260/230
  - OD260/280

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

  Save data

- **adjust one aliquot for each probe to 100 ng/µl**
  (-->10µl on for gele electrophoresis + 11µl for PCR => min 21µl!)

- **remeasure concentration for each probe (only once)**

IV. PCR assays

IV.1. Preparation of primer supermixes

→ **for each batch for the entire study (500µl):**

- multiplex primers at **6,25 pmol/µl each F/R** (for final conc. 12,5 pmol each)
  4x 31,25µl F + 3x 31,25µl R + 250 µl Ampuwa
- single primer pairs at **12,5 pmol/µl each F/R** (for final conc. 25 pmol each)
  62,5 F + 62,5 R + 375µl Ampuwa

primer pair	fragment length	(Ref. No.)	**Ref. No./Label**
TBP-100F/R	100bp	**1**	
ACTB-142F/R	142bp	**2**	**m**
GAPDH-200mod F/R	200bp	**3**	
ACTB-307F/968R	307bp	**4**	

primer pair	fragment lenght	**Ref. No.**
ACTB-411F/R	411bp	**5**
GAPDH-500F/R	500bp	**6**
GAPDH-687F/R	687bp	**7**
GAPDH-781F/R	781bp	**8**
ACTB-968F/R	968bp	**9**
ACTB-1338F/R	1338bp	**10**

--> split into 2 aliquots á 125µl (QG+1)

IV.2. Preparation of 96-well plates:

Vortex each supermix rigorously and put 2 µl respective supermix on the left side of each well, according to the following scheme:

- check again, if there is a drop in each well
- tapp MTP (multi titer plate) carefully on bench, so drops slip to the bottom of each tube
- check again, if all drops are on the bottom
- close with adhesive film and move with caution, so drops don't fling to sealing

- put at 4°C until use (or -20°C for more than 2 weeks of storage)

IV.3. Mastermix (MMix)

Allways vortex Pink MMix before use! Sucrose concentrates at the bottom!

For each extraction batch, a 12x mastermix/row on the MTP is required
--> label 30 tubes with label-batch
--> work on ice/thermoblocks

- 258µl mix + 3 µl BiothermAB Taq, vortex, put **18µl into negative control**
- 11µl of template solution, vortex, put **22µl each into each well of one row**

1 rxn:		**12 rxn:**
21,5 µl	Pink MMix	**258 µl**
0,25	Taq (GeneCraft,BiothermAB)	**3 µl**
1 µl	extracted DNA (100ng/µl in TE buffer) or lysate	**11 µl**

IV.4. PCR conditions

--> **cycling**:

4 min 94°C	initial denaturation	
1 min 94°C	}	**35x**
1 min 64°C	}	**35x**
1 min 72°C	}	**35x**
7 min 72°C	final elongation	

V. Electrophoresis

V.1. PCR- products (1.5% agarose)

- 300ml 1xTBE buffer + 4,5g agarose, boil up for 3 times
- cool down while stiring, add 7.5 µl EtBr solution
- cast into a 30cm gel tray
- insert 4 combs (20 pockets)
- allow polymerisation, wrap in cling film before storage at 4°C
- **loading:** - **5µl 100bp Plus ladder** (ready-to-use, by Fermentas), outer left and right in each row
  - **10µl of each PCR sample**

- add 7.5µl EtBr solution into electrophoresis chamber
  electrophoresis: 45 min at 180mV (electrode distance: 35cm)
- geldocumentation on UV table **--> make sure, cut corners of gels are visible on foto!**

V.2. genomic DNA (1% agarose)

- 300ml 1xTBE buffer + 3g Agarose, boil up for 3 times,
- cool down while stiring, add 7.5µl EtBr solution
- cast into a 30cm gel tray
- insert 5 combs (15 pockets)
- Load 1µg DNA from each extraction on a 1% agarose gel
  → 10µl of 100ng/µl aliquot
  + 2µl 6xLoadingDye (Invitrogen)
- electrophoresis: 120V for 1h, then 100V for 30min
  (electrode distance: 35cm)

VI. Appendix

VI.1. Oligonucleotides

name	sequence (5´->3´)	amplicon lenght	RefNo.
TBP-100F	AAC TTCGCTTCCGCTGGCCC	100bp	1
TBP-100R	AGTGCAGTGGTGGCCTTCGC		
ACTB142F	CCTGTGTTATCTTGGAGGTCCCCTGAAG	142 bp	2
ACTB-142R	ACCGTAGAGTGGTCACTCAATGAATGGG		
GAPDH-200Fmod	GGG AAG TCA GGT GGA GCG AGG C	200bp	3
GAPDH-200Rmod	TTGCGGTGGAAATGTCCTTTTCCAACTACC		
ACTB-968/307F	CTTGAGGAGGTGGGAAGGGACTATTTGG	307bp	4
ACTB-307R	CCCCTACCCCAACTTGACTTTGATTCCA		
ACTB-411F	AAGTTCCCAAGCACAGAAGAGAACCTGT	411bp	5
ACTB-411R	CTTCCTAGTGCTCTCCTATGCACCCTTC		
GAPDH-500F	GAAAAGGACATTTCCACCGCAAAATGGC	500bp	6
GAPDH-500R	GGTCAGAAATTAACTGGACAGGGCAAGC		
GAPDH-687F	ATTAGCCCAGTTTCATGCAGCAGAGAGA	687bp	7
GAPDH-687R	GGTGACTCAGCAGAGAAGACTTGAGGAG		
GAPDH-781F	AAAATCGGTAAAAATGCCCACCTCGCAT	781bp	8
GAPDH-781R	ATCAGCTAAAGATGTGCTTCCTCCGTGT		
ACTB-968/307F	CTTGAGGAGGTGGGAAGGGACTATTTGG	968bp	9
ACTB-968R	CTTCAGGGGACCTCCAAGATAACACAGG		
ACTB-1338F	CTGTGGCATCCACGAAACTACCTTCAAC	1338bp	10
ACTB-1338R	CCAAATAGTCCCTTCCCACCTCCTCAAG		

VI.2. TE buffer: (200 ml 1x TE)

- 10 mM Tris, pH 8.0 with HCl (0,24228g/200ml or 2ml 1M Tris-HCl, pH8.0/200ml)
- 1 mM EDTA (400µl 0.5M EDTA stock/200ml)

VI.3. PCR-ready lysis buffer (100ml)

40 mM Tris, pH 8.0 with HCl (0,485g/100ml)
1 mM EDTA (200µl 0,5M stock/100ml)
0.5% Tween-20 (500µl/100ml)

VI.4. 1.16x ready-to-use PCR mastermix

5 ml	10x reaction buffer	1.16x
100 µl	dATP 100mM	232.56 µM
100 µl	dGTP 100mM	232.56 µM
100 µl	dCTP 100mM	232.56 µM
100 µl	dTTP 100mM	232.56 µM
20 µl	cresol red sodim salt 100 mg/ml	46.51 µg/ml
3 g	sucrose	6.98% (w/v)

ad 43 ml with molecular grade water

→ 1 ml aliquots can bee stored at -20°C for several months

Please note:
Subsequent addition of 0.25 µl Polymerase, 2 µl primer solution and 1 µl template solution leads to the following final concentrations: 1x reaction buffer, 200 µM of each dNTP, 6 % (w/v) sucrose, 40 µg/ml cresol red, 0.4µM primer and 1.25U polymerase per PCR reaction in a total volume of 24.75 µl.