Protocol for the study of the gene MUC1 (DupC in VNTR Ex2)

**Day 1**

1 – Digestion of 100 ng genomic DNA: DNA-Lab

Dilute the DNA to a concentration of 20-25 ng/µl, wait a few hours and quantify with nanodrop. Change the filter tip between each step. Transfer 100 ng of DNA in a PCR tube and fill up with water to 10µl. In the DNA-Lab prepare 5 µl of digestion master-mix with 1µl of enzyme MWO1 (5 U/µl), buffer3 1.5 µl (10X) and 2.5 µl of water. Mix 10 µl of DNA and the 5 µl of the digestion master-mix, mix, centrifuge, and incubate at 60 °C for 2-3 hours. Change the filter tip between each tube.

Enzyme MWO1 (Site - GCNNNNNNNGC) does not recognize the mutated sequence of the VNTR and only digests in the presence of 7 Cs.

Seq WT 7 Cs: ccGCCCCCC-CAGCcc – cut

Seq Mut 8 Cs: ccGCCCCCCCCAGCcc – uncut

After the first digestion, prepare in the DNA-Lab 5 µl of digestion master-mix with MWO1 (5 U/µl) 1 µl, buffer3 0.5 µl (10X) and 3.5 µl of water. Remove the tubes with the digested DNA from the thermocycler and add 5 µl of digestion master-mix, mix and incubate 2-3 h at 60 °C, then at 37 °C o/n if necessary. Change the filter tip between each tube.

After the second digestion (or the next day morning), prepare 5 µl of digestion master-mix in the DNA-Lab with MWO1 (5 U/µl) 1 µl, buffer3 0.5 µl (10X) and 3.5 µl of water. Remove the digested DNA tubes, add 5 µl of this digestion master-mix, mix and incubate 1-2 h at 60 °C. Change the filter tip between each tube.

**Day 2**

2 – PCR amplification of the VNTR: prePCR-Lab and DNA-Lab

Remove the primers MUC1-Repeat F and R, as well as the Sigma taq from freezer. Transfer 11.5 µl of digested DNA in new tubes. Prepare a PCR master-mix with Sigma taq (2x) 12.5 µl, 0.5 µl primer MUC1-Repeat F (10 µM), 0.5 µl primer MUC1-Repeat R (10 µM). Add these 13.5 µl of PCR master-mix to the 11.5 µl of digested DNA. Change the filter tip between each tube.

(Perform in parallel fluorescent PCR under the same conditions to control the sizes of VNTR, digestion quality with a FAM-tagged R-Primer)

Mix, centrifuge and run the PCR:

* 5 min. 95 °C
* (94 °C, 30 sec. - 67 °C, 30 sec. - 72 °C, 30 sec.) **45X**
* 10 min. 72 °C

The PCR amplifies the non-digested 60 bp repeat, because of the presence of the 8C mutation (or other affecting the site of digestion), or due to incomplete digestion.

The primers MUC1-Repeat F and R are located in 2 contiguous repeats flanking the 7C/8C and are tagged with a 21 bp sequence.

GCCCACGGTGTCACCTCGGCCCCGGACACCA**GGCCGGCCCCGGGCTCCACC**G**CCCCCCC**A GCCCACGG**TGTCACCTCGGCCCCGGA**CACCAGGCCGGCCCCGGGCTCCACCGCCCCCCCA

3 – Digestion of the PCR product: PostPCR-Lab

\*\* AMPure-Purification of the PCR (1.8 Vol. AMPure, 2x Wash with 500 µl 70 % EtOH, elution with 15 µl Water.

Transfer 12 µl of the (purified) PCR product in new tubes and prepare 3 µl of digestion master-mix in the DNA-Lab with MWO1 (5 U/µl) 1 µl, buffer3 1 µl (10X) and 1 µl of water. Mix the 12 µl PCR product and these 3 µl in the PostPCR-Lab and digest 2-3 h at 60 °C. Change the filter tip between each tube.

Addition of a 0.5 µl access of buffer3 will optimize the concentration of salt for digestion.

After the first digestion, prepare 5 µl of digestion master-mix in the DNA-Lab with MWO1 (5 U/µl) 1 µl, buffer3 0.5 µl (10X) and 3.5 µl of water. Remove the digested DNA tubes, add these 5 µl of digestion master-mix, mix and incubate for 2-3 hours at 60 °C, then at 37 °C o/n if necessary. Change the filter tip between each tube.

After the second digestion (or the next day morning), prepare 5 µl of digestion master-mix in the DNA-Lab with MWO1 (5 U/µl) 1 µl, buffer3 0.5 µl (10X) and 3.5 µl of water. Remove the digested DNA tubes, add these 5 µl of digestion master-mix, mix and incubate for 1-2 hours at 60 °C. Change the filter tip between each tube

**Day 3**

4 – Purification of the digested PCR: PostPCR-Lab

\*\* Take AMPure out of the refrig. 30 min. before use and resuspend. Remove the tubes of digested DNA from the cycler, transfer the total volume of the solution in Eppendorf LowBind tubes and add 50 µl of freshly resuspended beads. Change the filter tip between each tube. Mix and incubate for 5 min. Prepare 1 ml of 70 % ethanol for each tube to purify. Place the tubes on the magnetic rack and wait 3 min. for formation of the bead pellet. Remove the supernatant and wash 2 times with 500 µl of freshly prepared 70 % ethanol. Aspirate remaining ethanol completely. Change the filter tip between each tube. Let dry 5 minutes with open lids. Once dry, remove the tubes from the magnetic rack and resuspend the beads adding 6 µl of water. Wait 5 min. and replace the tubes on the magnetic rack again. Change the filter tip between each tube. Wait 3 min. for formation of the bead pellet and transfer the supernatant into a new PCR tube.

5 – ExoSAP digestion: PostPCR-Lab

Transfer 5 µl of the digested and purified PCR product and add 1µl of ExoSAP (or 3 µl of a mixture of 1 µl ExoSAP and 2 µl water), incubate 20 min. at 37 °C and 15 min at 80 °C. Change the filter tip between each tube. This dual enzymatic digestion eliminates the dNTP (SAP) and all remaining PCR primers (Exo) that have not been eliminated during purification with magnetic beady.

6 – SnapSHOT: PostPCR-Lab

Remove buffer tubes from the freezer. Prepare a solution containing 2 µl of SNAPShot-Mix, 0.8 µl of primer 7C (10µM, 19 bp) and 0.2 µl of primer repeat R (10µM, 18 bp plus 21 bp tag). Mix this mix with ExoSAP reaction. Change the filter tip between each tube.

Mix, centrifuge and run the the reaction:

* 2 min. 94 °C
* (94 °C, 10 sec. - 52 °C, 5 sec. - 60 °C, 5 sec.) **55X**
* 60 °C 1 min.

SNAPShot reaction allows the interrogation of one fluorescent nucleotide directly adjacent to the probe by extending it.

GCCCACGGTGTCACCTCGGCCCCGGACACCAGGCCGGCCCCGGGCTCCACCGCCCCCCCA GCCCACGGTGTCACCTCGGCCCCGGACACCAGGCCGGCCCCGGGCTCCACCGCCCCCCCA

7 – SAP digestion: PostPCR-Lab

Digest the SNAPShot reaction with 1µl of SAP (or 3 µl of a mixture of 1 µl SAP and 2 µl water), incubate 20 min. at 37 °C and 15 min. at 80 °C, in order to eliminate the non-fluorescent dNTPs used. Change the filter tip between each tube.

8 – Sequencer

Prepare 10 µl of formamide with 0.20 µl size marker (LIZ120). Add 1 µl of the final product, mix, and denature 5 min. at 96 °C. Run in the 3500DX sequencer in the 3rd floor with the SnapSHOT program (Matrix E5).

9 – Analysis of the results

With GenMapper, analyze the SnapShot files and the corresponding LIZ scale.

Primer repeat R produces a black 45 bp peak (39pb + 1pbFLUO, elongation of nucleotide C), and corresponds to the presence of the VNTR amplified during repeat PCR (not digested PCR if 8C or PCR fragment digested during 2nd Mwo1 digestion from first incomplete digestion)

Primer 7C produces a 28 bp peak (19pb + 1pbFluo):

* Green (nucleotide A), if the Mwo1 digestion was not 100 % effective and reflects an incomplete digestion or the presence of a variation in the Mwo1 site
* Black (nucleotide C) reflecting the presence of the 8C mutation. The absence of results for this probe indicates a complete Mwo1 digestion and therefore the absence of 8C