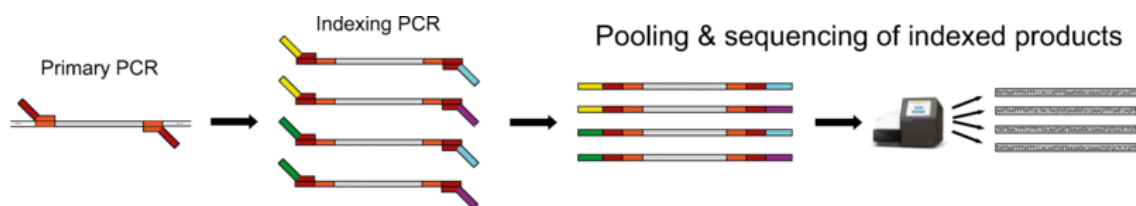


TITLE: METH030_METABARCODING_LIBRARY_PREP**1 INTRODUCTION****1.1 SCOPE**

Amplicon library preparation for Illumina sequencing.

1.2 OBJECTIVE

The metabarcoding library prep consists of two main steps as shown below: a PCR-1 where the target of interest is amplified and a PCR-2 where each library is labeled with a unique index combination.

**1.3 DEFINITIONS AND ABBREVIATIONS**

-

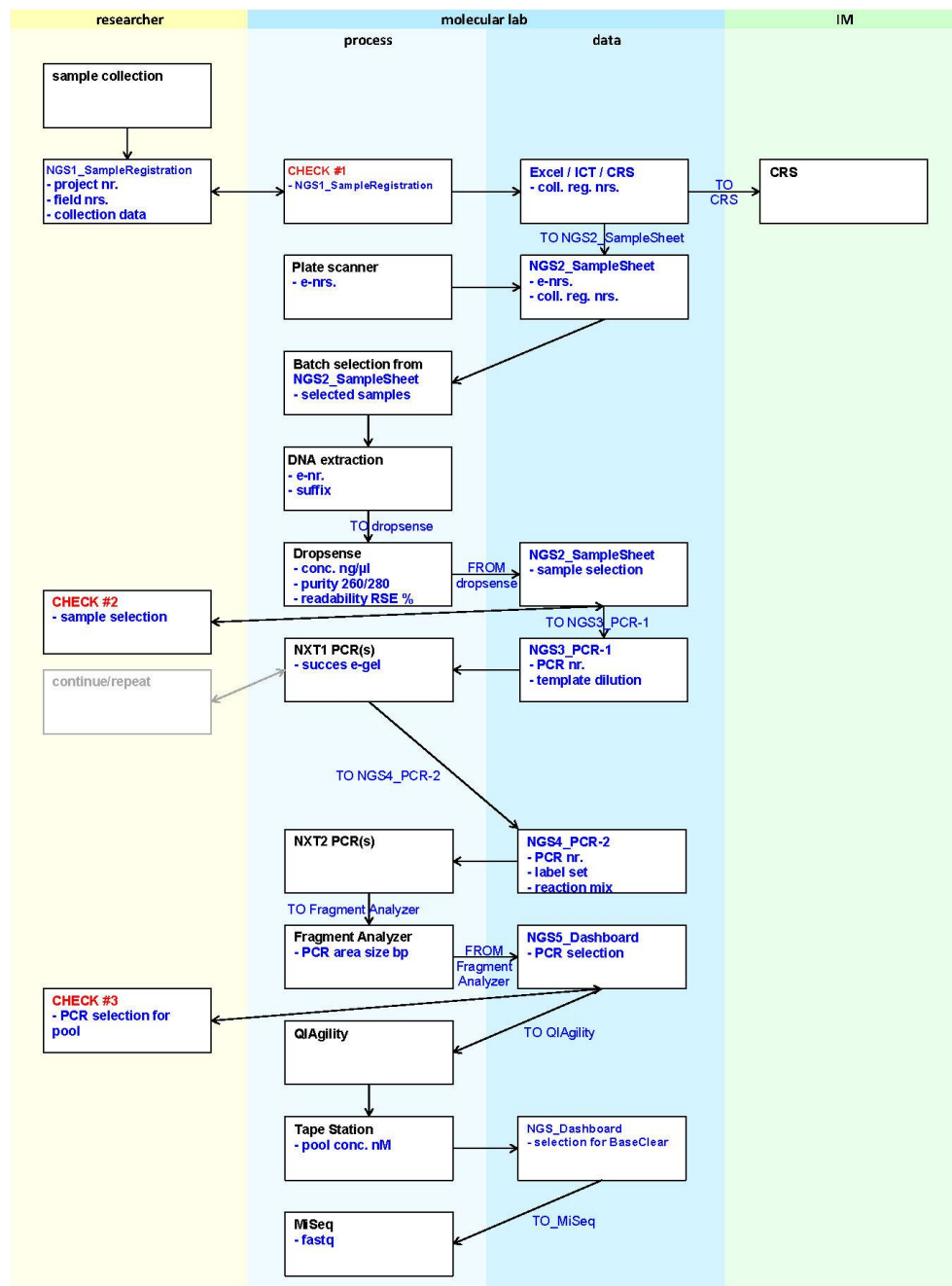
1.4 STATUS

The status and management of this document are regulated in the AODocs platform, the foundation wide accessibility is provided on [N=Info](#).

2 RESPONSIBILITIES & PEOPLE**2.1 RESPONSIBILITIES**

FUNCTION	ROLES and RESPONSIBILITIES
Lab workers (Researchers, Students, Guests)	Is able to use this document if contributing to the metabarcoding work process
(Senior) Research Technicians	Knows these document instructions and is able to instruct other lab workers.
Head Laboratories	Has final responsibility for all laboratory workflow and instructions

3 FLOW CHART



Metabarcoding workflow flow chart. **Administrative** processes are depicted in blue, **lab** processes in black and processes which require **feedback** in red

4 INSTRUCTIONS

This is the lab part of the metabarcoding workflow, the administrative part is described in [LAB PROC024 METABARCODING WORKFLOW](#). Use this workflow (up to paragraph 4.4 NGS3_PCR-1) to register the samples, before proceeding with the following PCR steps.

4.1 PCR-1. TARGET PCR

DNA template has to be amplified with IDT10 tailed primers.

Sequences IDT10 tails:

Forward: 5' -ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

Reverse: 5' -GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'

These IDT10 tails should be placed at the 5' position of the target specific part of the primer. Let a technician check a newly designed primer. To order primers use [SHEET009 OLIGO ORDERS](#).

For tips on the PCR see [METH008 PCR and GEL ELECTROPHORESIS](#).

Do not use more than 35 cycles for this first round PCR! More will introduce artifacts.

4.2 GEL ELECTROPHORESIS

Check the success of the PCR on a gel.

Work instructions for the e-gel can be found on [WI SO004 WI E-GEL](#). For the agarose gel use [METH008 PCR and GEL ELECTROPHORESIS](#).

Make sure you use filter tips for pipetting from your PCR sample.

4.3 MN BEADS CLEANUP OF PCR-1

PCR-1 products have to be cleaned before they can be labeled with sample-unique labels in PCR2. Do so with a 0.9 times ratio of magnetic beads. Use any of the available methods (magnetic stamp, traditional pipetting or C.Wash).

4.3.1 MN beads cleanup of 96-well plate, using magnetic extractor stamp

- Let the MN beads get to room temperature (remove from fridge 15 min before use)
- Fill a 96- well round bottom plate with appropriate amount of magnetic beads (in case of a standard clean-up 0.9 times the PCR volume)
- Add the PCR product to the magnetic beads, mix and incubate 5 minutes
- Fill 2 fresh round bottom plates with 50µl 80% EtOH (wash plates)
- Fill 1 fresh round bottom plate with appropriate (usually the volume of PCR product) amount of MQ (elution plate)
- Load the magnetic stamp with a cover plate and make sure the plate is locked into place
- Place the magnetic stamp in the sample / beads plate and wait for 1 minute
- Bring the stamp to the first wash plate and leave for 30 seconds
- Bring the stamp to the second wash plate and leave for 30 seconds
- Take out the stamp from the wash plate and let the beads dry for 30 seconds by holding it in the air
- Place the stamp in the elution plate and release the cover plate in a very quick snapping motion to prevent beads from following the magnetic pins up the sides of the cover plate.
- Swirl the cover plate in the MilliQ to release all beads. It takes some effort to swirl carefully and thoroughly. Keep checking if all the beads are released in the MQ.
- **Optional** --- *If the beads do not dissolve properly in the MQ then put the cover plate back on the stamp (in the same position!) and mix the beads by pipetting the solution up and down (multichannel) to maximize the DNA release from the beads --- optional --- Moving the cover plate between the MQ plate and the stamp is prone to cross contamination between samples.*
- Place the stamp back into the cover plate and wait for 5 minutes
- Take out the stamp with the cover plate and discard the cover plate (with attached beads)
- Transfer the purified PCR product to a 0.2 ml PCR plate for storage

4.3.2 MN beads cleanup of 96-well plate, using tips (optional)

- Let the MN beads get to room temperature (remove from fridge 15 min before use)
- Prepare fresh 80% EtOH (200µL per sample, e.g. 41,7 ml EtOH 96% + 8,3 ml MQ)
- Mix the MN beads well by vortexing
- Add 0.9x (PCR product volume) MN beads to the wells of a new 96-well plate
- Add the PCR product to the MN beads and mix well by pipetting
- Incubate at room temperature for 5 minutes
- Place the plate on the magnetic rack
- When the solution is clear (5 min), carefully remove the supernatant (LEAVE THE PLATE ON THE MAGNET)
- Wash the beads 2x with 100µl 80% ethanol (LEAVE THE PLATE ON THE MAGNET)
- Let the beads air dry for 1 minute (LEAVE THE PLATE ON THE MAGNET)
- Take the plate off the magnet and resuspend the beads with e.g. 25µl MQ, by pipetting up and down
- Place the plate back on the magnet
- When the solution is clear (5 min), move 22µl of the the supernatant to a clean plate

4.3.3 MN beads cleanup of 96-well plate, using C.Wash

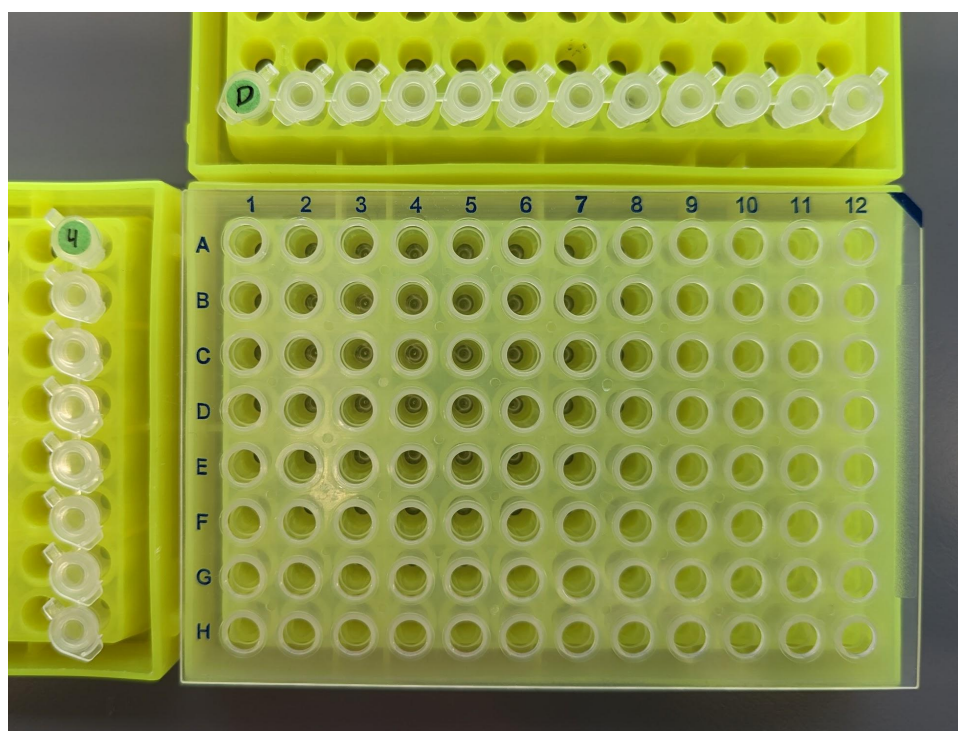
Follow the instructions on [WI AP046 Bead clean-up using the Cytena C.Wash](#)

4.4 PCR-2. Labels

After PCR-1 has been cleaned up, the individual libraries can be labeled with unique index combinations by performing a second PCR with IDT10 indexes.

1536 index combinations are available at Naturalis but only 384 (plate combinations B2, D2, B4 and D4) are suitable for a shared run at BaseClear. Choose the indexes according to the type of sequencing run needed for the project in question.

The indexes are stored in tube strips. They need to be combined one column and one row. In the pictures below an example is illustrated.



D	13	14	15	16	17	18	19	20	21	22	23	24
4												
09	D.13 - 4.09	D.14 - 4.09	D.15 - 4.09	D.16 - 4.09	D.17 - 4.09	D.18 - 4.09	D.19 - 4.09	D.20 - 4.09	D.21 - 4.09	D.22 - 4.09	D.23 - 4.09	D.24 - 4.09
10	D.13 - 4.10	D.14 - 4.10	D.15 - 4.10	D.16 - 4.10	D.17 - 4.10	D.18 - 4.10	D.19 - 4.10	D.20 - 4.10	D.21 - 4.10	D.22 - 4.10	D.23 - 4.10	D.24 - 4.10
11	D.13 - 4.11	D.14 - 4.11	D.15 - 4.11	D.16 - 4.11	D.17 - 4.11	D.18 - 4.11	D.19 - 4.11	D.20 - 4.11	D.21 - 4.11	D.22 - 4.11	D.23 - 4.11	D.24 - 4.11
12	D.13 - 4.12	D.14 - 4.12	D.15 - 4.12	D.16 - 4.12	D.17 - 4.12	D.18 - 4.12	D.19 - 4.12	D.20 - 4.12	D.21 - 4.12	D.22 - 4.12	D.23 - 4.12	D.24 - 4.12
13	D.13 - 4.13	D.14 - 4.13	D.15 - 4.13	D.16 - 4.13	D.17 - 4.13	D.18 - 4.13	D.19 - 4.13	D.20 - 4.13	D.21 - 4.13	D.22 - 4.13	D.23 - 4.13	D.24 - 4.13
14	D.13 - 4.14	D.14 - 4.14	D.15 - 4.14	D.16 - 4.14	D.17 - 4.14	D.18 - 4.14	D.19 - 4.14	D.20 - 4.14	D.21 - 4.14	D.22 - 4.14	D.23 - 4.14	D.24 - 4.14
15	D.13 - 4.15	D.14 - 4.15	D.15 - 4.15	D.16 - 4.15	D.17 - 4.15	D.18 - 4.15	D.19 - 4.15	D.20 - 4.15	D.21 - 4.15	D.22 - 4.15	D.23 - 4.15	D.24 - 4.15
16	D.13 - 4.16	D.14 - 4.16	D.15 - 4.16	D.16 - 4.16	D.17 - 4.16	D.18 - 4.16	D.19 - 4.16	D.20 - 4.16	D.21 - 4.16	D.22 - 4.16	D.23 - 4.16	D.24 - 4.16

To prepare for the PCR-2, fill in the `NGS4_PCR-2.xlsx` template according to [section 4.5 of PROC024](#). It is best to stay with the same enzyme for the 2nd PCR as used in the first PCR. Anyway do not combine KAPA with Environmental mix. These hotstart mixes are not compatible.

Do not switch well positions within your plate (e.g. when there are empty positions) and always use a multichannel pipette to avoid confusion.

When preparing PCR-2, take into account the following:

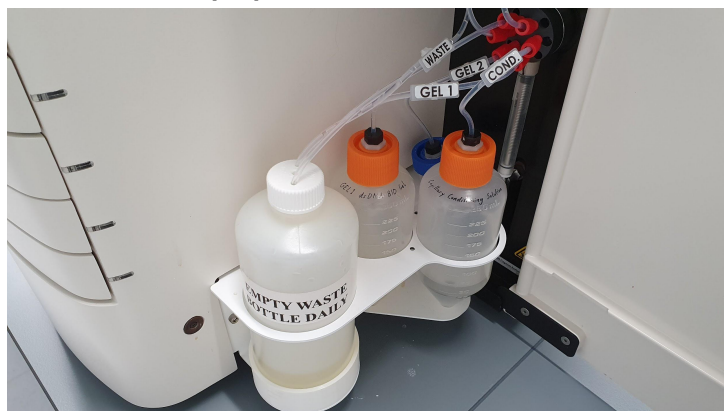
- Prepare the master mix in a clean (pre-PCR) lab
- Use fresh pipette tips when adding primers from 96-well plate
- Add the PCR-1 template in a post-PCR lab
- 5 to 10 cycles are enough to add the indexes to the libraries. Do not exceed a total of 40 cycles on both PCR-1+PCR-2 as it will introduce artifacts.

4.5 MEASURING PCR-2 PRODUCTS ON FRAGMENT ANALYZER

Detailed instructions in [WI_AP041_Fragment_Analyzer](#)

- Turn on Fragment Analyzer with switch on the back
- Start Fragment Analyzer software
- Username: administrator
- Remove Agilent chemicals from freezer, fridge and cabinet

4.5.1 Chemicals preparation



- Empty waste bottle (reagents compartment) and waste tray (drawer W) in red waste container below fume hood (organic chemicals)

- Add the necessary amount of Gel (fridge) to the bottle in the reagents compartment e.g. for 40ml for 1 plate: 40ml **Gel dsDNA 810** + 4µl **Dye** (freezer, brown bag, purple cap ●), carefully swirl to mix, do not shake Gel to avoid creating bubbles!

Solution Levels

Check the fluid volumes before proceeding. Ensure that the waste is empty and that the gel and conditioning solutions are full.

Record the solution volumes here:

	Volume (mL)	Solutions
Gel 1	50.0	DNF-810
Gel 2	50.0	NaOH
Conditioning Solution	50.0	
Waste	0.0	

OK Cancel

- Update the amount of Gel in the software \Utilities\Solution Levels
- Add the necessary amount of Capillary Conditioning Solution to the bottle in the reagents compartment e.g. 40ml for 1 plate: 8ml **5x Capillary Conditioning Solution** (cabinet) + 32ml MilliQ
- Update the amount of Gel in the software \Utilities\Solution Levels
- Change the Inlet Buffer (drawer B) every day / 5 plates: 20ml **5x 930 dsDNA Inlet Buffer** (fridge) + 80ml MilliQ (1ml per well)
- Change the Markers plate (drawer M) every 5 plates: 30µl **Markers 35bp & 1500bp** (freezer, 3.2ml tube with green cap ●) per well + drop of **Mineral Oil** (cabinet)

4.5.2 Sample preparation

- Fill wells A01-H11 of sample plate with 22µl **Dilution Buffer 1x TE** (fridge) + 2µl sample, use 24µl Dilution Buffer 1x TE for wells without sample
- Fill well H12 with 24µl **100bp Ladder** (freezer, 1,5ml tube with green cap ●)
- Seal plate, vortex on plate vortex, spin

4.5.3 Running experiment

- Open your NGS4_PCR-2.xlsx ([PROC024_METABARCODING_WORKFLOW](#))
 - Copy the data from tab TO_Fragment_Analyzer
 - Paste the data into a new .txt and save
 - In the Fragment Analyzer software, select \Load from file\All file types (open .txt)
- Do this before adding the plate to the queue or your sample names will not be in the export!**

- \Run Entire Tray\Add to queue
- \Method e.g. DNF-910
- \Tray Name (enter filename)
- Optionally Add up to 2 more plates to the queue
- Final check:
 - remove seal from sample plate
 - Enough Gel present
 - Enough Capillary Conditioning Solution present
 - Inlet Buffer replaced/present
 - Waste tray and bottle empty
 - Markers plate present

- Sample plate + ladder present
- Start the run(s) by clicking the  button
- After run, seal the Markers plate (drawer M) and store at 4°C
- When 3 or more plates are measured, put back the storage buffer plate in the instrument/
- Switch off Fragment Analyzer with switch on the back

4.5.4 Data analysis in PROSize 3.0

- Start PROSize 3.0 software
 - \File\Open File
 - Search for folder with corresponding date
 - Open the run's .raw file
 - Check all samples for correct UM en LM depiction, using the arrow keys on the keyboard.
- When given an error regarding a mismatch of the ladder, increase the minimum RFU for peak detection

- \Smear Analysis\Set Individual Parameters  \Smear Analysis (e.g. 250-550bp)\Apply to all

- Click the  button
- Deselect ☐ Export All
- Select ☒ Smear Analysis Only
- \Export File Path (create a project folder in \Documents)
- \Export .csv

- Click the  button \Export PDF to project folder in \Documents
- Open the .csv file in Notepad (Kladblok) and copy the contents (ctrl+a, ctrl+c)
- Paste the contents into NGS4_PCR-2.xlsx tab FROM_Fragment_Analyzer and proceed with [PROC024 METABARCODING WORKFLOW](#)

4.6 QIAGILITY EQUIMOLAR POOLING

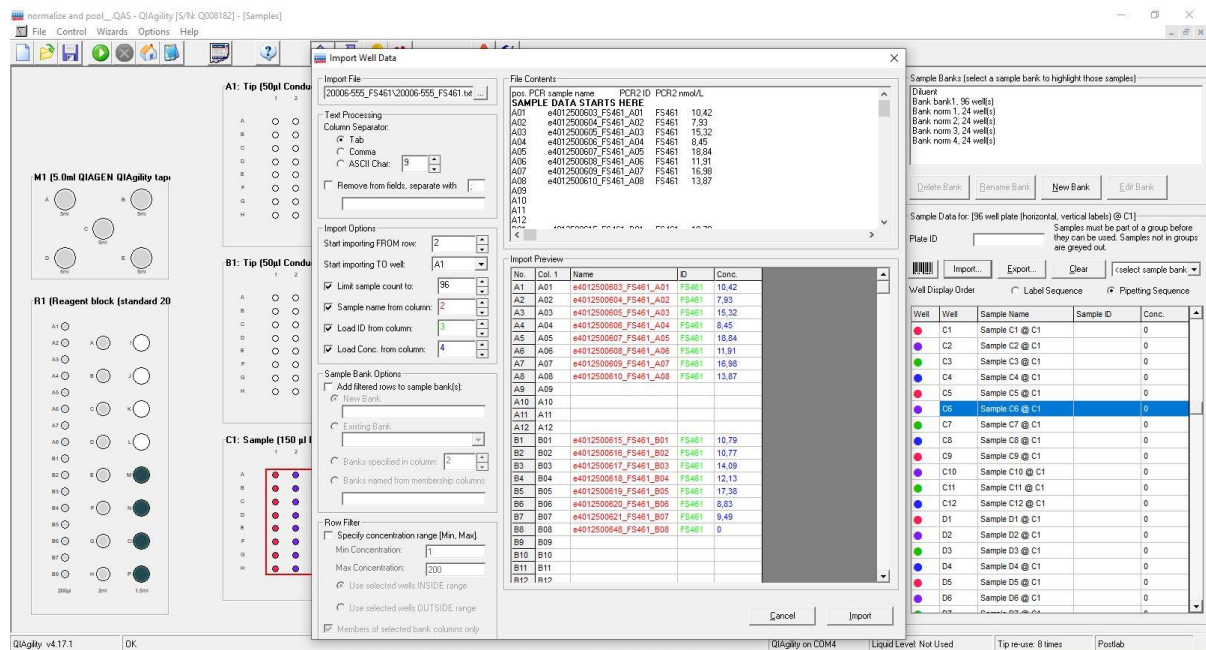
Follow [PROC024 METABARCODING WORKFLOW](#) till paragraph 4.6.2

4.6.2 QIAgility import

- Switch on the QIAgility liquid handling workstation before opening the QIAgility software
- Open QIAgility software

When the QIAgility robot cannot communicate with the PC, switch off the QIAgility, unplug and replug the QIAgility USB from the PC and restart QIAgility.

- Tab \Recent\normalize and pool__\Open
- Highlight area C1 by left clicking on it
- \Sample Data (right window)\Clear
- \Sample Data (right window)\Import...
- Select .txt import \Import

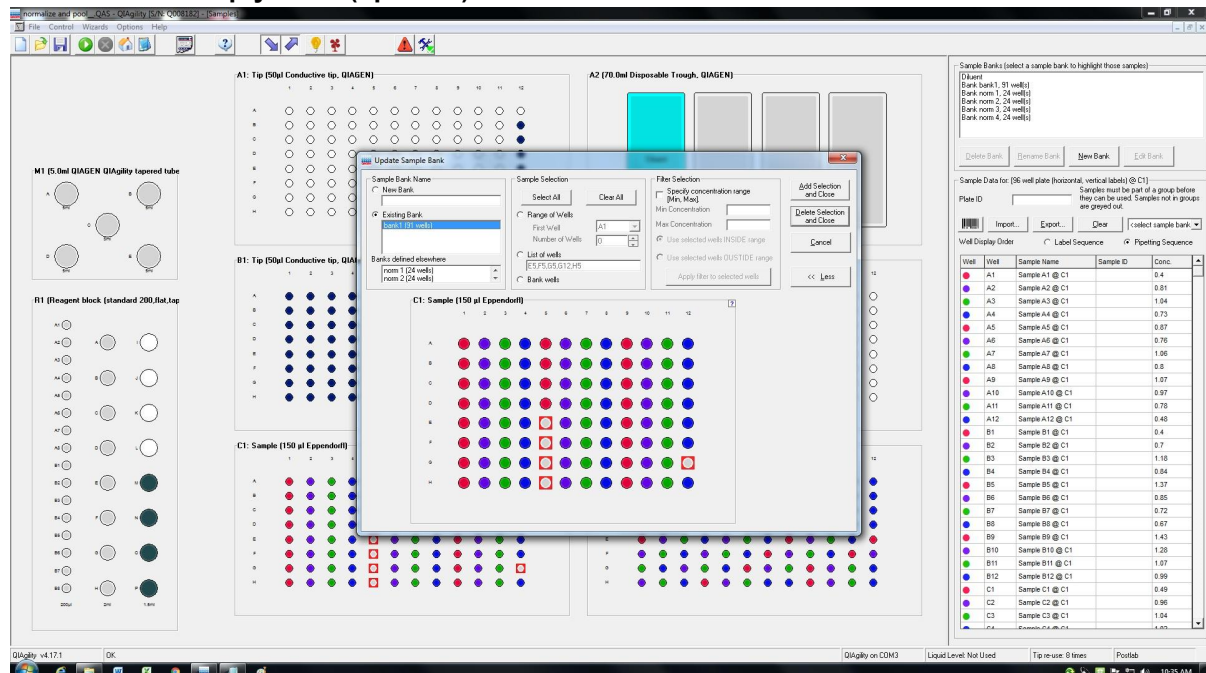


- Check if the QIAgility software columns correspond to the .txt import:

- ☒ Sample name from column: 2
- ☒ Load ID from column: 3
- ☒ Load Conc. from column: 4

- Import/Finish

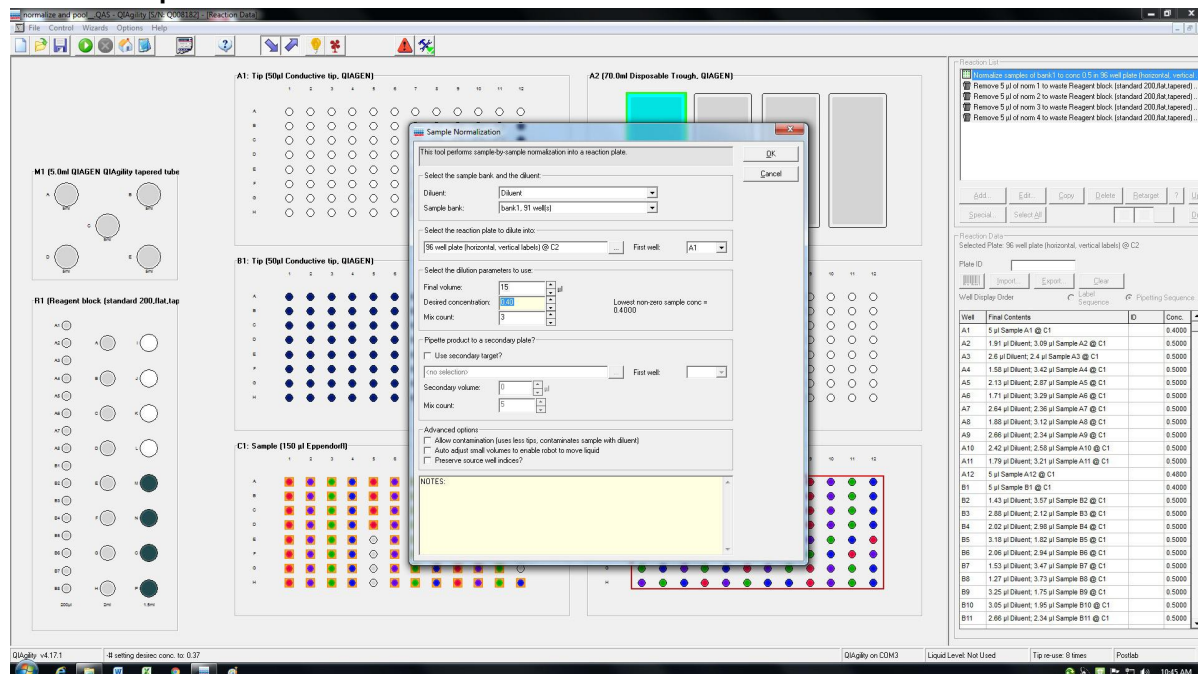
4.6.2.1 Delete empty wells (optional)



- Empty wells can be removed by right-clicking on area C1
- \Remove selected wells from sample bank...
- In the window Update Sample Bank, select wells to be removed by left-clicking on them while holding down Ctrl
- In the field Existing Bank, select bank1 (... wells)
- Click button Delete Selection and Close

- If you have deleted empty wells from the sample plate C1 then also delete the same positions from area C2. Otherwise the QIagility will report error messages while performing the last pipetting steps from plate C2 to the 1,5 ml (collection) tubes.

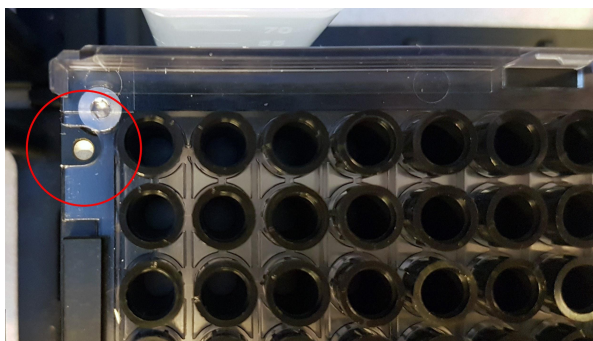
4.6.3 Sample normalization



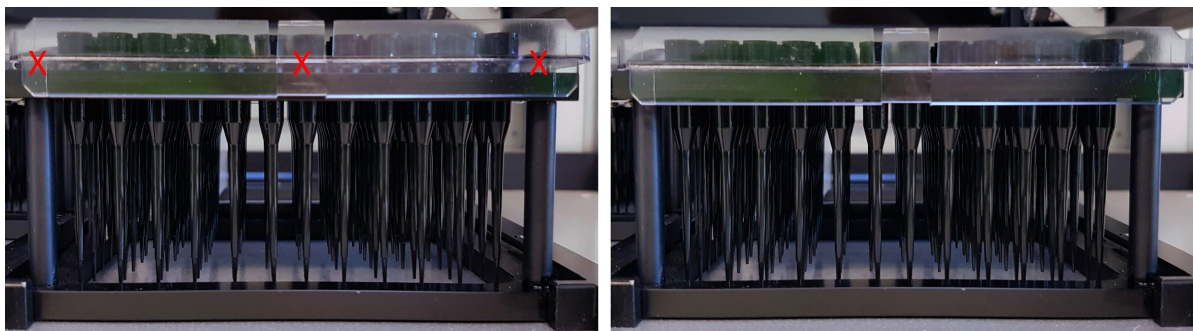
- Highlight area C2 by left-clicking on it
- \Reaction List (top right)
- Double-click on Normalize samples of bank1 to conc...
- In the Sample Normalization window, adjust Final volume to the amount of available PCR product. If you have > 15µl left after the FA measurement(s), add 3µl to this number.
- Adjust Desired concentration to the Lowest non-zero sample conc (in this case 0.40)
- Press Tab to apply
- The NOTES: field should be empty

4.6.4 Refill tips and MilliQ

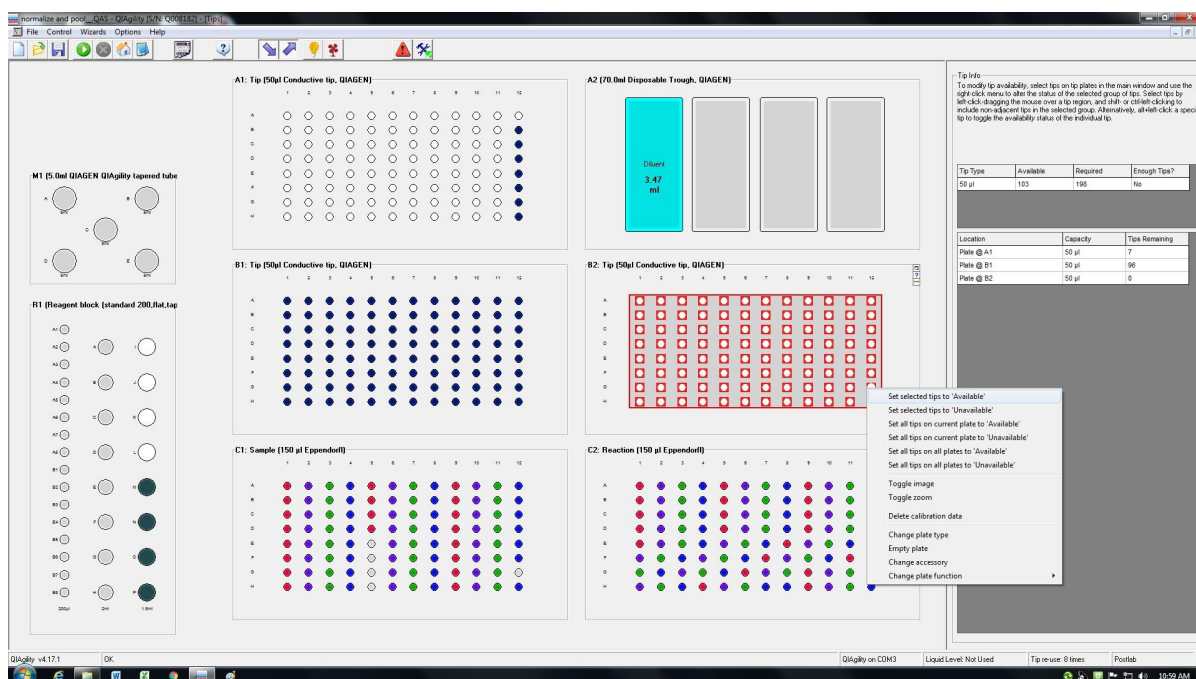
- Wearing gloves, remove empty tip racks from the QIagility and replace them with full racks.



- When placing full racks in the QIagility frame, make sure to align them correctly, so the notch in the plate aligns with the white dot on the frame



- Ensure the new tip rack closely connects with the frame by pressing along the ridges with 2 hands
- Replace the diluent and make sure the reservoir is half-full with MilliQ



- Select the wells in the refilled areas by dragging a field around them and right clicking
- \Set selected tips to 'Available'
- Place 4 new Eppendorf low-bind 1.5ml tube in the marked positions in area R1
- Place 2nd PCR plate in area C1 (Standard is Eppendorf twin.tec PCR plate 96 skirted. If you have a different plate type, highlight area C1 by left-clicking on it\right-click\Change plate type\OK\)
- Place empty skirted PCR plate in area C2



- Click Start button
- Check all boxes and click Run

4.7 MN BEAD CLEANUP OF POOL

Sub pools have to be measured on the TapeStation so they can be pooled equimolarly [PROC024_METABARCODING_WORKFLOW](#) paragraph 4.6.3. The resulting end pool can be cleaned with MN beads.

Always save a portion of the non-cleaned pool (e.g. 100µl) in case something goes wrong with the clean-up.

- Let the MN beads get to room temperature (remove from fridge 15 min before use)
- Prepare fresh 80% EtOH (1ml per sample)

- Pool the contents of the 4 tubes into 1 tube and measure the total volume by pipetting
- Mix the MN beads well by vortexing
- Add 0.9x (pool volume) MN beads to the pool and vortex
- Incubate at room temperature for 5 minutes
- Place the tube on the magnetic rack
- When the solution is clear, carefully remove the supernatant. **Leave the tube on the magnet!**
- Wash the beads 2x with 500µl 80% ethanol. **Leave the tube on the magnet!**
- Let the beads air dry for 1 minute. **Leave the tube on the magnet!**

The elution volume depends on the Lowest non-zero sample conc to which the QIAgility has normalized the pool (4.6.3).

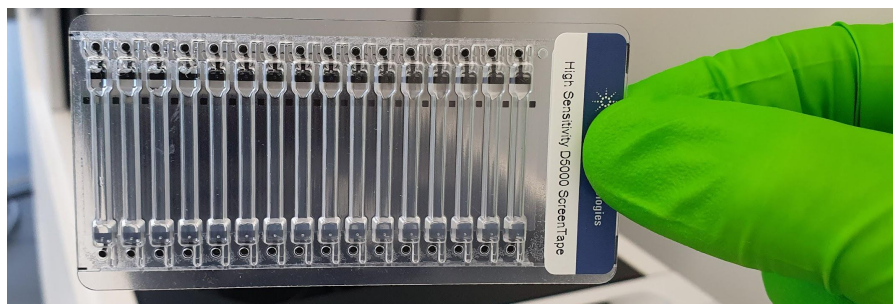
- Take the tube off the magnet and resuspend the beads with e.g. 50-200µl MilliQ by pipetting up and down
- Place the tube back on the magnet
- When the solution is clear, move the supernatant to a clean low-bind tube

4.8 MEASURE POOL ON TAPESTATION

Detailed instructions in [WI AP042 TapeStation](#)

4.8.1 High Sensitivity D5000

- Remove Screen High Sensitivity Tape D5000, Sample Buffer (brown tube, green cap ●) and High Sensitivity D5000 Ladder (yellow cap ●) from fridge 30 min. in advance
- Make a 1:20 dilution of the pool, e.g. 5µl pool in 95µl MilliQ
- Switch on the TapeStation
- Start the Agilent TapeStation Controller Software



- Remove Screen Tape from package, hold it upright with the 2D code facing away from you and flick to remove bubbles



- Open the lid of the TapeStation and place the Screen Tape loosely in the designated well



When the ScreenTape is placed correctly, it is detected by the TapeStation

Agilent TapeStation Controller Software 3.2

4150 TapeStation System

User: postlab

Notes:

Prefix:

Reagent Lot #: ScreenTape Lot #:

Required For Run

4 Tips

-

L: 2µl Ladder + 2µl Sample Buffer

1 2

A ☒ ☐

B ☒ ☐

C ☒ ☐

D ☒ ☐

E ☐ ☐

F ☐ ☐

G ☐ ☐

H ☐ ☐

...

Agilent Technologies

High Sensitivity D5000 ScreenTape

Well	Description
L	Ladder
B1	FS462 1:10
C1	FS462 1:10
D1	FS462 1:10

4 Samples selected

Column selection

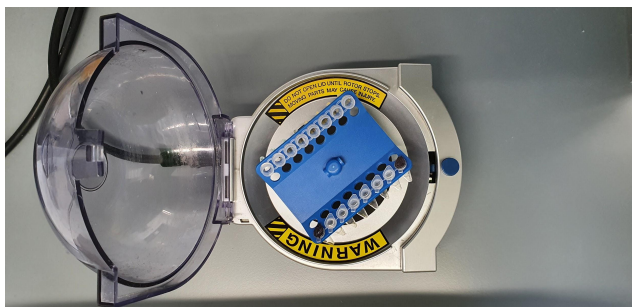
✓ DEDAB00503 : Idle

✓ High Sensitivity D5000 ScreenTape : Expires 10-Sep-2020

⚠ Instrument lid is open. Please close to continue

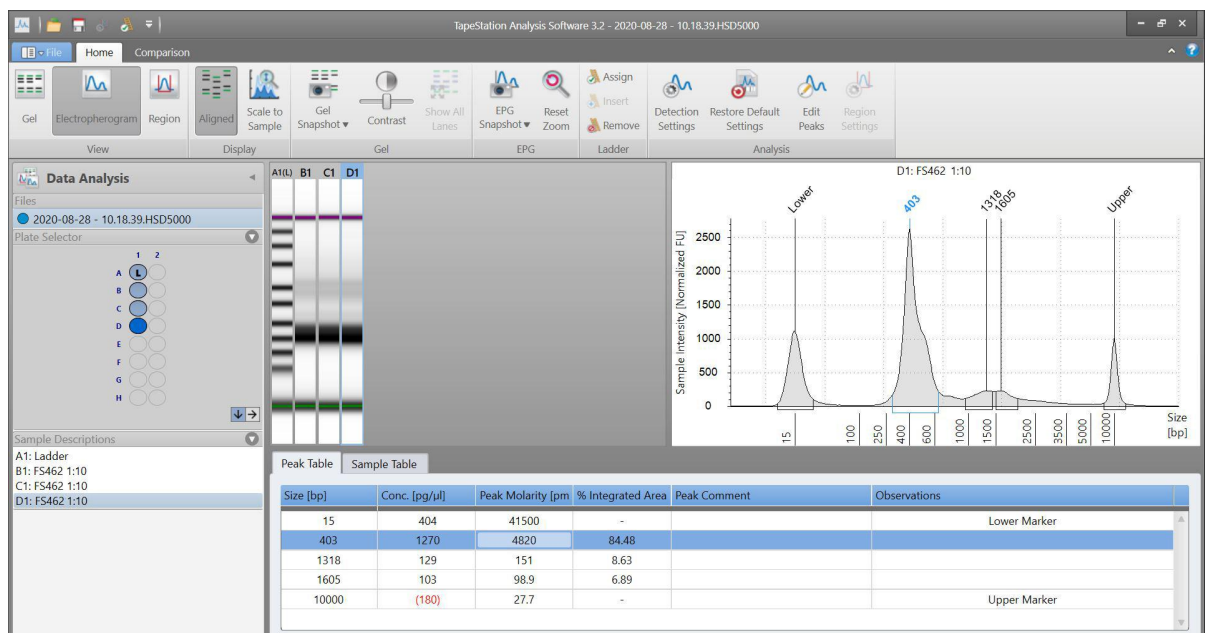
Start

- Fill in Sample descriptions in software
- Vortex and spin the Sample Buffer and Ladder
- Fill a tube strip (drawer below TapeStation) with 2µl Sample buffer and 2µl Ladder (A01) or sample
- Mark A01 on tube strip
- Seal tube strip with cap strip and vortex for 1 min. in plate vortex

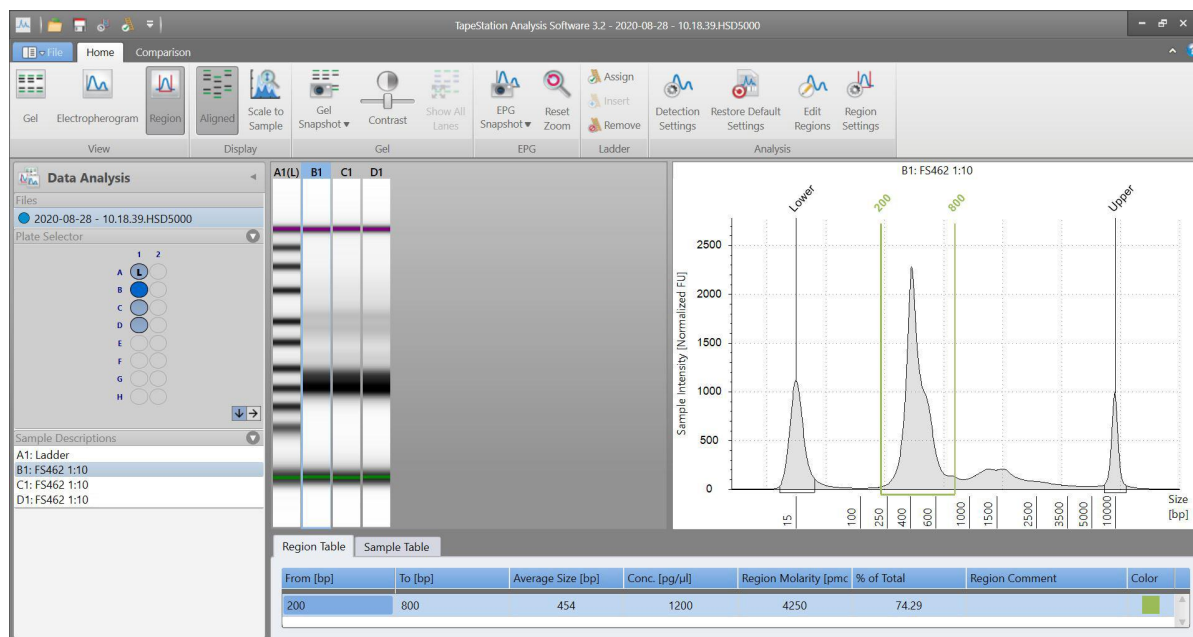


- Spin tube strip for 1 min.
- Place the tube strip in the TapeStation
- Remove cap strip
- Close the lid of the TapeStation
- Left click on Start in the software

When the TapeStation run is done, the TapeStation Analysis Software will automatically start:



- Left click on Electropherogram in the top menu and write down the Peak Molarity for each measurement of the desired fragment size e.g. 4820 pm/L for the 403bp peak



- Optionally, the region can be adjusted in the Region menu, by dragging the green boundaries
- Calculate the average of the 3 measurements
- Write the average of the 3 measurements of the end pool on the tube

The NGS5_Dashboard.xlsx can be used to calculate the equimolar pooling of multiple sub pools according to [PROC024_METABARCODING_WORKFLOW section 4.6.4](#).

Baseclear needs at least 20μl of a ≥ 5nM end pool. Always save a portion of the end pool, in case something goes wrong with sequencing.

Proceed with the preparation of the MiSeq run, according to [PROC024_METABARCODING_WORKFLOW](#)

5 ADDITIONAL PROCEDURES

5.1 For safety and lab regulations please reads the following documents:

[PROC002_GENERAL_LAB_REGULATIONS](#)

[PROC003_SAFETY_PROCEDURES](#)

[WI_WS001_PRE-LAB](#)

[WI_WS_NGS_LAB](#)

6 RELATED/ASSOCIATED DOCUMENTS

[METH030_Metabarcoding_LIBRARY_PREP](#)

[PROC024_METABARCODING_WORKFLOW](#)

[WI_AP041_Fragment_Analyzer](#)

[WI_AP042_TapeStation](#)