

TITLE: PROC024_METABARCODING_WORKFLOW**1 INTRODUCTION****1.1 SCOPE**

For all researchers using the NBC facilities for metabarcoding sample preparations and for technicians assisting and performing such projects this procedure is obligatory.

1.2 OBJECTIVE

Administering all steps as described is compulsory. The whole process is monitored and controlled following all the steps in this procedure. The NGS workflow includes: Collection and registration, transfer of (sub)samples, DNA extraction, scanning of e-numbers, selection, PCR, Fragment Analyser measurements, QIAgility equimolar pooling and Bioanalyzer or TapeStation measurements.

1.3 DEFINITIONS AND ABBREVIATIONS

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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

2.2 LIST OF PEOPLE ADDRESSED

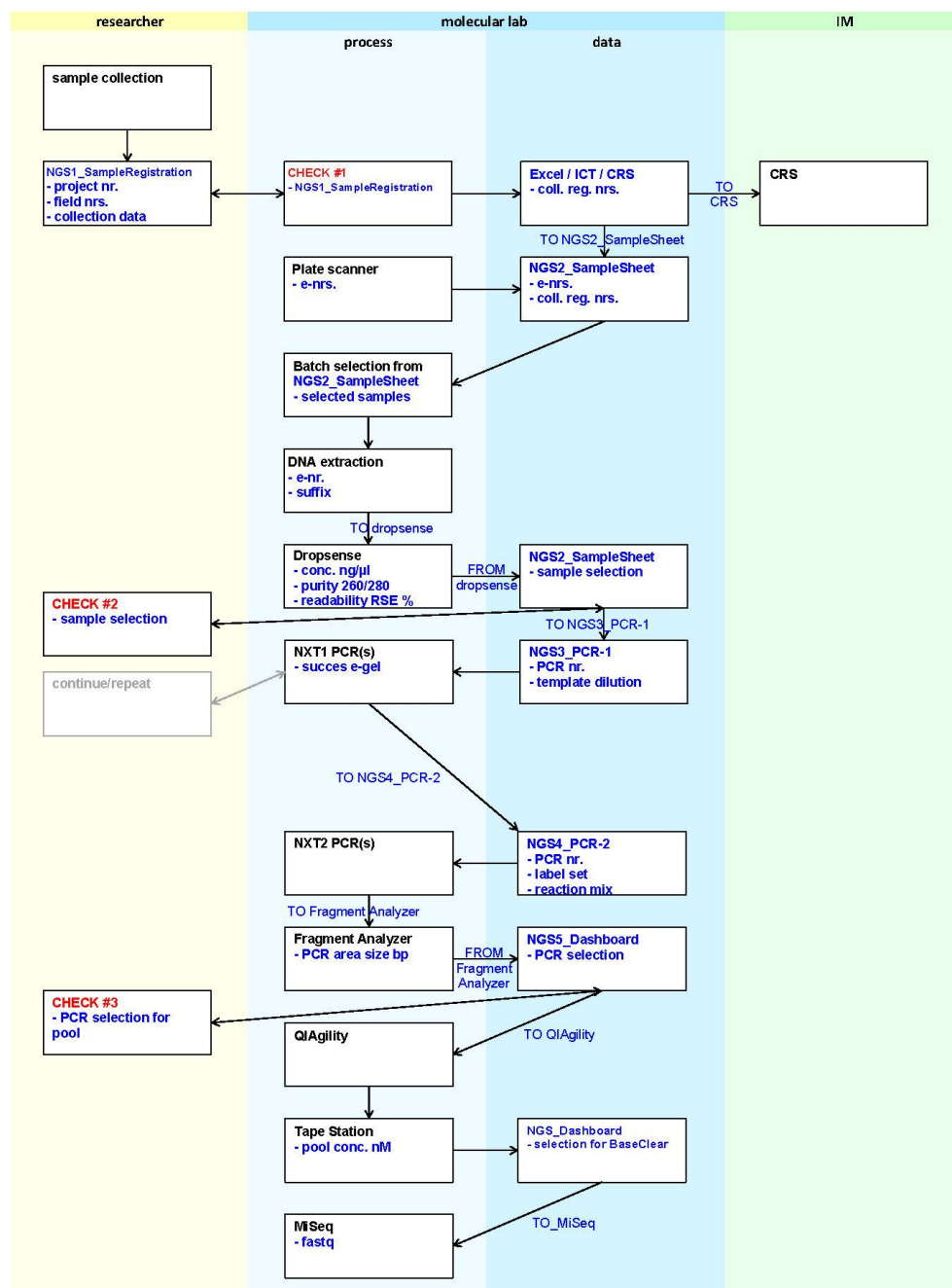
DNA Technicians performing or assisting in NGS library preparations.

Marina Ventayol: 1st responsible for NGS post lab including all equipment.

Marcel Eurlings: 2nd responsible for NGS post lab including all equipment.

[SHEET010_OVERVIEW_ROOM MANAGERS & TELEPHONE NUMBERS](#)

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 PROCEDURE

This is the administrative part of the metabarcoding workflow, the lab part is described in [METH030_Metabarcoding_LIBRARY_PREP](#)

This workflow is guided by, and comes with 5 consecutive Excel files and also holds 3 checkpoints. After finishing the whole administration and lab work of a single Excel file the relevant values are copied to the next Excel file. This transfer of data is crucial to start the work and administration in the next Excel file. Three checks need to be confirmed in writing by the researcher/submitter. Workflow

cannot continue if these checks are not confirmed. The consecutive files can be downloaded from the following links:

[NGS1_SampleRegistration_v0.8_TEMPLATE.xlsx](#)

[NGS2_SampleSheet_v0.21_TEMPLATE.xlsx](#)

[NGS3_PCR-1_v0.25_TEMPLATE.xlsx](#)

[NGS4_PCR-2_v2.3_TEMPLATE.xlsx](#)

[NGS5_Dashboard_v3.999_TEMPLATE.xlsx](#) Usage described in [section 4.6](#)

Always make a copy for your own use before working with these files!

Follow the order of the Excel files as described in this procedure.

4.1 Instruction

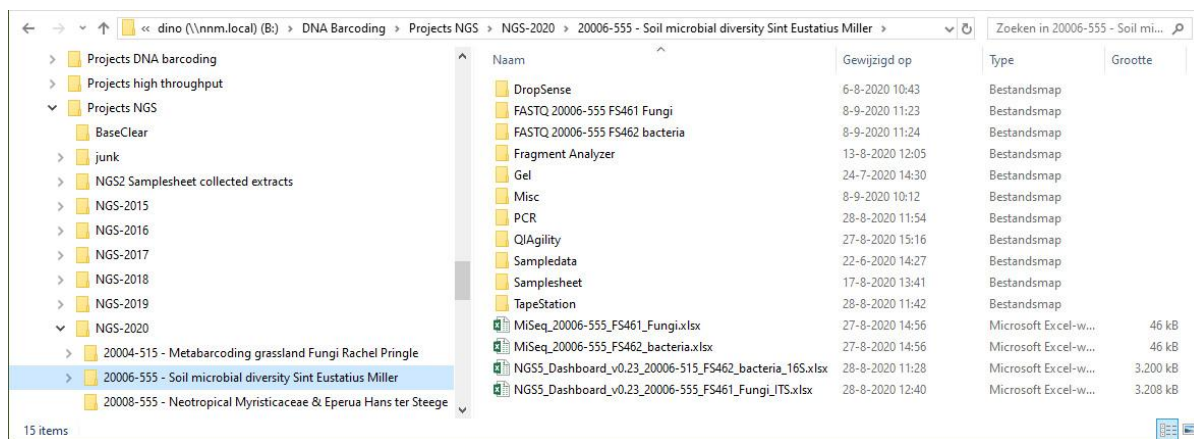
- Create a folder for your project:

[project number] - [Subject] [Locality] [Name] e.g.

20006-555 - Soil microbial diversity Sint Eustatius Miller

in B:\DNA Barcoding\Projects NGS\NGS-[year]

Access to B:\DNA barcoding can be requested via [TopDesk](#) by [Aanvraag extra rechten](#)



- Save all your data in the created project folder

4.2 NGS1_SampleRegistration

The researcher/submitter is responsible for

- collecting,
- registration of collection data (e.g. date, coordinates, depth) and
- providing collection registration numbers (RMNH- or L-numbers) for the samples.

Usage of file [NGS1_SampleRegistration_v0.8_TEMPLATE.xlsx](#) is mandatory for the NGS workflow. Only samples registered in the Naturalis collection registrations systems CRS or BRAHMS and provided with collection registration numbers can be processed by the lab.

Only if samples will **not** be included into the **Naturalis collection**, and therefore no RMNH- or L-numbers are applicable, the lab will provide the samples with collection registration numbers (NBC.LAB-numbers) and register them in CRS. In all other cases the researcher/submitter is responsible for registration in the Naturalis registration system.

If extra controls (besides the obligatory negative NXT PCR-1 control) are required, make sure to adjust the number of samples to the 96-well format. E.g. in a plate with a positive control and an extraction blank, there is only space left for 93 samples.

4.2.1 Entering data into NGS1

Download a copy of [NGS1_SampleRegistration_v0.8_TEMPLATE.xlsx](#)

- Provide the following data in the tab **Start**: name of submitter, date and project number.
Proceed with either 4.2.1.1 (if samples/specimens [vouchers] will be stored in the Naturalis collection) or 4.2.1.2 (in other cases).

4.2.1.1 If samples/specimens will be stored in the Naturalis collection

Researchers should contact the relevant collection manager (Collection Department) about the samples/specimens they want to deposit. The collection manager will provide registration numbers (either RMNH- or L-numbers) and assist in registration in the relevant collection registration system (CRS or BRAHMS). The researcher is responsible for the registration.

Nr	field nr.	collection registr. nr.	collector	collecting date (dd-mm-yyyy)	country	state_province	island	locality	locality detail	geo_lat (in decimal, WGS84)	geo_lon (in decimal, WGS84)	geo_radius in meters	altitude (m asl)	depth (m)
1		RMNH 1234567												
2		RMNH INS 123456												
3		RMNH 5000000a												
4		RMNH 5000000b												
5		L 123456												
6		L 123457												
7		L 123458												

- In this case it will suffice to fill out the collection registration numbers (RMNH- or L-numbers) in the NGS1-file (tab **DATA**).

4.2.1.2 If samples/specimens are NOT stored in the Naturalis collection

If samples/specimens are not stored, e.g. in case of environmental samples that are used completely, or stored elsewhere, then the lab will provide NBC.LAB-nrs and take care of registration in CRS.

Nr	field nr.	collection registr. nr.	collector	collecting date (dd-mm-yyyy)	country	state_province	island	locality	locality detail	geo_lat (in decimal, WGS84)	geo_lon (in decimal, WGS84)	geo_radius in meters	altitude (m asl)	depth (m)
1	voorbeeld1		P. Pluk	13-12-2011	Netherlands	Drenthe		Luljebroek		12.13474	-68.98485			
2	voorbeeld2		Vorst, O.	14-12-2011	Baltic Sea			E-bay		55.71319				
3	voorbeeld3		A & B & C	15-12-2011	Netherlands	Antilles	Curacao	Curacao		-68.98253	12.13865		10	
4	sloot1		Vorst, O.	16-12-2011	Netherlands	Drenthe		Luljebroek		12.13474	-68.98485			
5	sloot2		Vorst, O.	17-12-2011	Netherlands	Drenthe		Luljebroek		12.13474	-68.98485			
6	sloot3		Vorst, O.	18-12-2011	Netherlands	Drenthe		Luljebroek		12.13474	-68.98485			
7	sloot		Vorst, O.	19-12-2011	Netherlands	Drenthe		Luljebroek		12.13474	-68.98485			

- In this case the researcher/submitter should fill out the sample/specimen registration in the **DATA**-tab of NGS1. At least the fields with a **green header**: field nr., collector, coll. date, country, locality, geo_lat., geo_lon., geo_radius.

Once the Data has been filled in, inform a technician about the file. The technician will assign NCB.LAB numbers to each sample by using the file NBC.LAB.1-9999_REGISTRATION.xlsx in B:\DNA Barcoding\RMNH-NBC.LAB-numbers. The technician will then inform back that the numbers have been assigned. S/he will also register the samples in the CRS; large numbers of samples (95+) in batch via [TopDesk](#), smaller numbers are entered into CRS individually ([DNA lab training CRS](#)).

NBC.LAB-numbers are for specimens not deposited in the Naturalis collection, mixed samples etc.

[METH027_SAMPLESHEET](#) and [WI_SP006_FOLDER_STRUCTURE_DNA_BARCODING_DRIVE](#)

4.2.2 CHECK #1

After delivery of samples, the responsible molecular lab technician checks if the samples are properly labeled and if the renamed `NGS1_SampleRegistration_TEMPLATE.xlsx` is correctly filled out. If this is the case, this is noted and shared in the respective NGS project form. After the registration of the (sub)samples is complete and the check is done DNA extractions can be carried out. The DNA extraction method is in agreement with the submitter/researcher.

4.3 NGS2_SampleSheet

4.3.1 96-well NCBN plates

The DNA extracts, generated in NGS projects, will be permanently stored in a -80°C freezer, in dedicated, pre-numbered NCBN Micronic® 96-well plates. These plates have matrix codes at the bottom of the tubes, coding for unique extraction IDs (e-numbers). It is critical that each extract is provided with an e-number for downstream data linkage.

- Each NCBN plate has to be registered in both [SHEET027 ProtoLIMS V08](#) and the log booklet (LOG_REGISTRATION_NCBN_PLATES) kept with the extract plate stock in L.03.10.

- All NCBN-plates will be stored permanently in a dedicated -80°C ULT freezers (FRE-10 and FRE-33) [SHEET015 OVERVIEW FREEZER CONTENT](#)

- The matrix codes at the bottom of the tubes can be scanned, according to [METH027 SAMPLESHEET section 5.2](#)

4.3.2 The NGS2 file

Each Excel file `NGS2_SampleSheet.xlsx` represents a **single NCBN plate** (permanent storage). An NCBN plate holds extracts from a single NGS project or from several, tiny projects. So, this file allows to store samples from multiple projects in a single NCBN plate, as long as the total number of samples fits in one plate (95 samples).

Purpose of this file is to link samples/specimens (registration numbers) with well positions (A01-H12) and extract IDs (e-number). Also, DNA concentration measurements are entered here. The tab `TO_NGS3_PCR-1` facilitates the transfer of data to the next file in the workflow.

If you have a full NCBN plate which you want to analyze completely, the downstream processing (PCRs etc) is straightforward. Alternatively you can select a **subset** of samples from an NCBN-plate to partly fill a working plate, thus allowing to combine extracts from several NCBN-plates (different NGS2 files!) in a single working plate for analysis (see [4.3.7 Combining extracts](#)).

When pipetting a subset of DNA extracts into an already existing NCBN plate, keep the contents of the plate on ice so they do not thaw.

4.3.3 Entering data into NGS2_SampleSheet

- Download a copy of the template [NGS2_SampleSheet_v0.21_TEMPLATE.xlsx](#) or, in case of a non-full plate, open an existing `NGS2_SampleSheet.xlsx` from `B:\DNA Barcoding\Projects NGS\NGS2 Samplesheet collected extracts`. In the latter case the last part of the filename

shows the projects involved and the number of available wells e.g.

NGS2_SampleSheet_v0.20_NCBN001190_20009_20006_FRE-10-01_19_left.xlsx

- Rename the file as follows: NGS2_SampleSheet_[version number]_[NCBN plate number]_[project number(s)]_[freezer number]_[number of wells left].xlsx
e.g. NGS2_SampleSheet_v0.20_NCBN001190_20009_20006_FRE-10-01_19_left.xlsx

- In the tab \Start enter: person entering data, date and NCBN plate number.

The purpose of this form is to:

- register storage position of the DNA extracts
- link the DNA extract IDs (e-nmbr) to the physical sample IDs (both the collection registr. nmbr in CRS and the field nmbr).
- create exports for import into Geneious, PCR sheets and DropSense

Use a single Samplesheet for each NCBN 96-well extraction plate.
IMPORTANT When extracts of different projects are stored in the same NCBN plate, please combine in ONE Samplesheet. The green cells always should be filled out.

data entered by: Oscar Vorst
on (dd-mm-yyyy): 17-Feb-2019

NCBN plate ID: NCBN001127

number of plate positions used: 96
number of plate positions selected: 90

projects included: 19001-555, 1234
technician (each project): ☒ Oscar Vorst

Start FROM_plate_scan FROM_NGS1_SampleRegistration PLATE_Setup TO_DropSense FROM_DropSense TO_NGS3_PCR-1 TO_Geneious

- In the same tab enter the project numbers in the green fields (projects included) as well as for each project the technician involved.

The project numbers entered here should be **identical** to the numbers in the data imported from NGS1_SampleRegistration.

To retrieve the sample details from one or more NGS1 files:

collection registr. nmbr.	field nmbr.	project nmbr.	taxon	lat	long	country	locality
NBC.LAB.1	voorbeeld1	19001-555	Carabus Linnaeus, 1758	12,13474	-68,98485	Netherlands	Lutjebroek
NBC.LAB.2	voorbeeld2	19001-555		12,13474	-68,98485	Baltic Sea	E-bay
NBC.LAB.4	slot	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
RMNH.123456	slot2	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
RMNH.123457	slot3	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
RMNH.123458	slot4	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
RMNH.123459	slot5	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
NBC.LAB.5	slot6	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek

Start DATA TO_SampleSheet TO_CRS

- Copy the data from the tab \TO_NGS2_SampleSheet in NGS1_SampleRegistration.xlsx (ctrl+c)

collection registr. nmbr.	field nmbr.	project nmbr.	taxon	lat	long	country	locality
1 NBC.LAB.1	voorbeeld1	19001-555	Carabus Linnaeus, 1758	12,13474	-68,98485	Netherlands	Lutjebroek
2 NBC.LAB.2	voorbeeld2	19001-555		12,13474	-68,98485	Baltic Sea	E-bay
4 NBC.LAB.4	sloot	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
6 RMNH.123456	sloot2	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
7 RMNH.123457	sloot3	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
8 RMNH.123458	sloot4	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
9 RMNH.123459	sloot5	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek
9 NBC.LAB.5	sloot6	19001-555		12,13474	-68,98485	Netherlands	Lutjebroek

- Paste the data in the tab \FROM_NGS1_SampleRegistration in NGS2_SampleSheet.xlsx (ctrl+v). **Always paste values only!**

If needed, data from several NGS1 files can be copied to this tab below each other.

- Scan the tube matrix codes of each new NCBN plate (see section 5.2 Scanning Micronic plate of method [METH027 SAMPLESHEET](#)) and copy the data in the tab \FROM_plate_scan

4.3.4 Populating the NCBN plate

- Open tab \PLATE_Setup

pos	select	registr.nr.CRS	suffix	remarks
A-01	☐	NBC.LAB.1		
B-01	☐	NBC.LAB.1		opmerking2
C-01	☐	NBC.LAB.3		
D-01	☐	NBC.LAB.4		
E-01	☐	RMNH.123456		qw
F-01	☐	RMNH.123456		
G-01	☐	RMNH.123458		
H-01	☐	RMNH.123459		

project	registr.nr.CRS	fieldnr-suffix	extract ID
19001-555	NBC.LAB.1	fieldnr+suffix NOT unique	e4012502781
19001-555	NBC.LAB.1	fieldnr+suffix NOT unique	e4012502773
19001-555	NBC.LAB.3	voorbeeldig_3	e4012502785
19001-555	RMNH.123456	sloot	e4012502797
19001-555	RMNH.123456	fieldnr+suffix NOT unique	e4012502809
19001-555	RMNH.123458	fieldnr+suffix NOT unique	e4012502821
19001-555	RMNH.123458	sloot4	e4012502833
19001-555	RMNH.123459	sloot5	e4012502845

- Enter the collection registration numbers of the samples/specimens used for the DNA extraction in column registr.nr.CRS Only numbers available from the imported NGS1 data are allowed.

At position H-12 no registration can be entered as this holds the obligatory *negative control*.

pos	select	registr.nr.CRS	suffix	remarks
A-01	☐	NBC.LAB.1	filter	
B-01	☐	NBC.LAB.1	EtOH	opmerking2
C-01	☐	NBC.LAB.3	PhCl	
D-01	☐	NBC.LAB.4	soil	
E-01	☐	RMNH.123456	specimen	qw
F-01	☐	RMNH.123456	EtOH	
G-01	☐	RMNH.123458	specimen	
H-01	☐	RMNH.123459	specimen	

project	registr.nr.CRS	fieldnr-suffix	extract ID
19001-555	NBC.LAB.1	voorbeeldig-filter	e4012502781
19001-555	NBC.LAB.1	voorbeeldig-EtOH	e4012502773
19001-555	NBC.LAB.3	voorbeeldig_3-PhCl	e4012502785
19001-555	NBC.LAB.4	sloot-soil	e4012502797
19001-555	RMNH.123456	sloot2-specimen	e4012502809
19001-555	RMNH.123456	sloot2-EtOH	e4012502821
19001-555	RMNH.123458	sloot4-specimen	e4012502833
19001-555	RMNH.123459	sloot5-specimen	e4012502845

- Optionally fill in suffixes for the samples (e.g. filter or EtOH). The columns under NCBN Storage plate should now become yellow.

Suffixes can be used to distinguish the same sample if it is used multiple times e.g. in different extraction methods. The suffixes will remain linked with the sample name throughout the

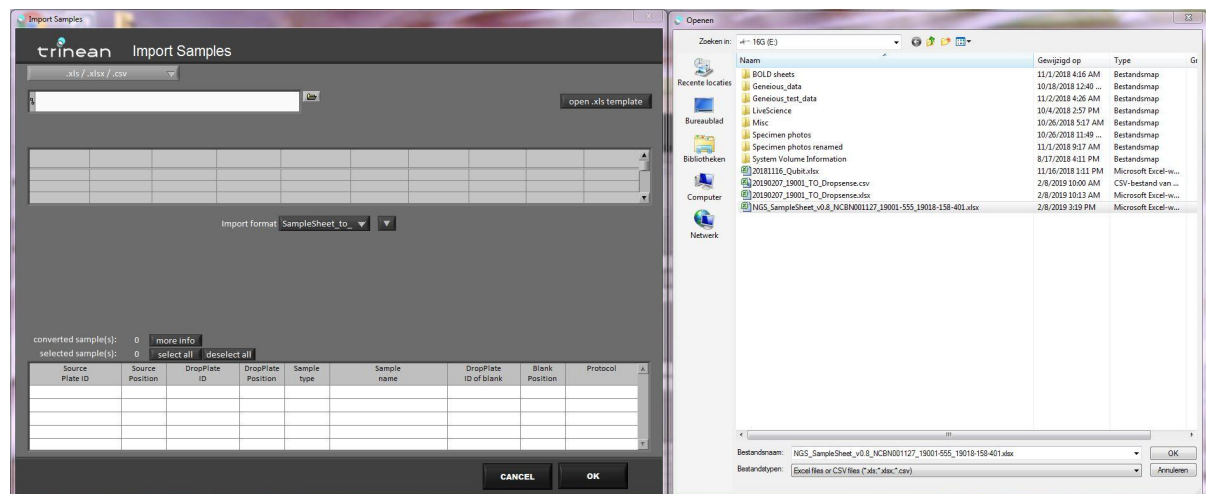
metabarcoding workflow. Do not make the suffixes too long as they will be included in the MiSeq sample names e.g. e4012502761_NBCLAB1_voorbeeld1-**filter**_RP998_A_D1

4.3.5 Determining the DNA concentration with the Lunatic

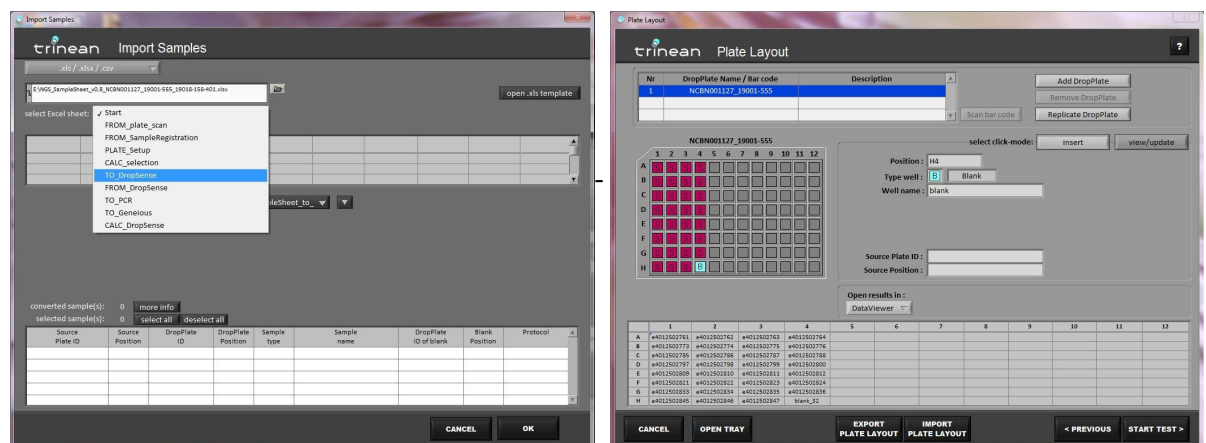
Once the extractions are done, the DNA/RNA concentration (ng/ul), purity (260/280) and unidentified fraction of the measured spectrum (Root Relative Squared Error RRSE %) of extracted DNA should be measured on the DropSense, according to [WI AP031 SPEC-07](#).

The DropSense software can read the plate setup directly from the NGS2 Excel file. It uses the data in the tab \TO_DropSense This layout corresponds with the working plate, not the NCBN plate! So, take care to select all extracts when measuring a complete NCBN plate.

- In the DropSense DropQuant software, press /IMPORT PLATE LAYOUT button



- Press  button and select NGS2_SampleSheet.xlsx



In dropdown menu select /TO_DropSense/OK

- Indicate the position of the negative control
- After measurement select /EXPORT PLATE LAYOUT and save as
- Copy the data from the DropSense export to tab /FROM_DropSense in NGS2_SampleSheet.xlsx

4.3.6 Creating a working plate

If needed, a work plate with a **subset** of the samples in an NCBN plate can be composed. This is particularly useful if an NCBN plate holds samples from several projects.

In case the complete plate will be analyzed the layout of the working plate should be identical to that of the NCBN plate. So, make sure all extracts are selected!

pos	select	registr.nr.CRS	suffix	remarks	project	registr.nr.CRS	fieldnr-suffix	extract ID	position
A-01	☒	NBC LAB 1	EtOH	opmerking2	19001-555	NBC LAB 1	voorbeeld1-EtOH	e4012502781	A01
B-01	☒	NBC LAB 3	PhCI		19001-555	NBC LAB 3	voorbeeldig_3-PhCI	e4012502773	B01
C-01	☒	NBC LAB 4	soil		19001-555	NBC LAB 4	stoot-soil	e4012502785	C01
D-01	☒	RMNH 123456	specimen	qvw	19001-555	RMNH 123456	stoot-specimen	e4012502797	D01
E-01	☒	RMNH 123456	EtOH		19001-555	RMNH 123456	stoot-EtOH	e4012502809	E01
F-01	☒	RMNH 123456	specimen		19001-555	RMNH 123456	stoot4-specimen	e4012502821	F01
G-01	☒	RMNH 123456	specimen		19001-555	RMNH 123456	stoot5-specimen	e4012502833	G01
H-01	☒	RMNH 123456	specimen		19001-555	RMNH 123456	stoot5-specimen	e4012502845	H01

- To include extracts in the work plate check the boxes in the column **select**. The corresponding cells in the column **Working plate** now show the work plate position.

- Fill out the work plate **START** position. The default value is A01, but this can be changed, e.g. if extracts from several projects are combined in a single NCBN plate.

H12 cannot be unchecked because this is the mandatory negative control.

The tab **PLATE_Setup** can be used as a pipetting scheme if the extract positions in the NCBN storage plate are different from the working plate

4.3.7 Combining extracts from multiple NCBN plates in a single plate

If extracts from multiple NCBN plates are *to be combined in a single plate for PCR-ing*, the easiest way is to have the complete NCBN plate data copied to NGS3_PCR-1 and select the desired extracts from the multiple NCBN plates in this file.

Only in some cases it might be necessary to combine extracts from several NCBN plates directly in a single working plate. To do so, select the relevant extracts from each individual NCBN plate in the cognate NGS2 files. Take care to enter the correct work plate **START** position in each NGS2 file, avoiding overlapping plate position ranges in the final work plate. This strategy enables the combination of several **TO_DropSense** outputs to be used for a **DropSense** measurement of combined working plate.

4.3.8 CHECK #2

The **NGS2_SampleSheet.xlsx**, including the details of the filled in tab **FROM_DropSense** needs to be e-mailed to the researcher/submitter, who has to confirm the samples will be PCR-ed. If the researcher/submitter agrees, he/she has to note this in the **CHECK** tab of the project proposal form in Google drive.

4.4 NGS3_PCR-1

The **NGS3_PCR-1** file can be used to make selections for the 1st Nextera PCR, from the samples included in the working plate created in **NGS2_SampleSheet**. It also provides the option for duplicate and triplicate PCR repeats. A Fragment Analyzer input and export function is included, as well as tabs to store PCR mixes, primers and programs.

- Download a copy of the Excel file [NGS3_PCR-1_v0.25_TEMPLATE.xlsx](#)

project	pos	work	extract pos	extract ID	registr. code	field nmbr	remark	ss	taxon	lat	long	country
19001-555	A01	NCBN001127-A01	4012502761	NBC.LAB.1	voorbeeld1-filter				0	Carabus Linnaeus, 1758	12,13474	-68,98485 Netherlands
19001-555	B01	NCBN001127-B01	4012502773	NBC.LAB.2	voorbeeld2-ETOH	opmerking2			0		12,13474	-68,98485 Baltic Sea
19001-555	C01	NCBN001127-C01	4012502785	NBC.LAB.4	sloot-soil				0		12,13474	-68,98485 Netherlands
19001-555	D01	NCBN001127-D01	4012502797	RMNH.123456	sloot2-specimen				0		12,13474	-68,98485 Netherlands
19001-555	E01	NCBN001127-E01	4012502809	RMNH.123457	sloot3-specimen	qw			0		12,13474	-68,98485 Netherlands
19001-555	F01	NCBN001127-F01	4012502821	RMNH.123458	sloot4-specimen				0		12,13474	-68,98485 Netherlands
19001-555	G01	NCBN001127-G01	4012502833	RMNH.123459	sloot5-specimen				0		12,13474	-68,98485 Netherlands
19001-555	H01	NCBN001127-H01	4012502845	NBC.LAB.5	sloot6-filter				0		12,13474	-68,98485 Netherlands

- Copy the data in NGS2_SampleSheet.xlsx tab TO_NGS3_PCR-1

project	pos	work	extract pos	extract ID	registr. code	field nmbr	remark	ss	taxon	lat	long	country	locality
19001-555	A01	NCBN001127-A01	4012502761	NBC.LAB.1	voorbeeld1-filter				0	Carabus Linnaeus, 1758	12,13474	-68,98485 Netherlands	Lutjebroek
19001-555	B01	NCBN001127-B01	4012502773	NBC.LAB.2	voorbeeld2-ETOH	opmerking2			0		12,13474	-68,98485 Baltic Sea	E-bay
19001-555	C01	NCBN001127-C01	4012502785	NBC.LAB.4	sloot-soil				0		12,13474	-68,98485 Netherlands	Lutjebroek
19001-555	D01	NCBN001127-D01	4012502797	RMNH.123456	sloot2-specimen				0		12,13474	-68,98485 Netherlands	Lutjebroek
19001-555	E01	NCBN001127-E01	4012502809	RMNH.123457	sloot3-specimen	qw			0		12,13474	-68,98485 Netherlands	Lutjebroek
19001-555	F01	NCBN001127-F01	4012502821	RMNH.123458	sloot4-specimen				0		12,13474	-68,98485 Netherlands	Lutjebroek
19001-555	G01	NCBN001127-G01	4012502833	RMNH.123459	sloot5-specimen				0		12,13474	-68,98485 Netherlands	Lutjebroek
19001-555	H01	NCBN001127-H01	4012502845	NBC.LAB.5	sloot6-filter				0		12,13474	-68,98485 Netherlands	Lutjebroek

- Paste the data (values only!) in NGS3_PCR-1.xlsx tab FROM_NGS2_SampleSheet

project	extract pos.	work plate pos.	extract ID	registr.nr.CRS	fieldnr-suffix
19001-555	NCBN001127-A01	A01	e12001	NBC.LAB.1	voorbeeld1-filter
19001-555	NCBN001127-B01	B01	e4012502773	NBC.LAB.2	voorbeeld2-ETOH
19001-555	NCBN001127-C01	C01	e4012502785	NBC.LAB.3	voorbeeld3-PhCI
19001-555	NCBN001127-D01	D01	e4012502797	NBC.LAB.4	sloot-soil
19001-555	NCBN001127-E01	E01	e4012502809	RMNH.123456	sloot2-specimen
19001-555	NCBN001127-F01	F01	e4012502821	RMNH.123457	sloot3-specimen
19001-555	NCBN001127-G01	G01	e4012502833	RMNH.123458	sloot4-specimen
19001-555	NCBN001127-H01	H01	e4012502845	RMNH.123459	sloot5-specimen
19001-555	NCBN001127-A02	A02	e4012502762	NBC.LAB.5	sloot6-filter
19001-555	NCBN001127-B02	B02	e4012502774	NBC.LAB.6	sloot7-filter
19001-555	NCBN001127-C02	C02	e4012502786	NBC.LAB.7	sloot8-filter
19001-555	NCBN001127-D02	D02	e4012502798	NBC.LAB.8	sloot9-filter
19001-555	NCBN001127-E02	E02	e4012502810	NBC.LAB.9	sloot10-filter
19001-555	NCBN001127-F02	F02	e4012502822	NBC.LAB.10	sloot11-filter
19001-555	NCBN001127-G02	G02	e4012502834	NBC.LAB.11	sloot12-filter
19001-555	NCBN001127-H02	H02	e4012502846	NBC.LAB.12	sloot13-filter
19001-555	NCBN001127-A03	A03	e4012502783	NBC.LAB.13	sloot14-filter
19001-555	NCBN001127-B03	B03	e4012502775	NBC.LAB.14	sloot15-filter
19001-555	NCBN001127-C03	C03	e4012502787	NBC.LAB.15	sloot16-filter
19001-555	NCBN001127-E03	E03	e4012502811	NBC.LAB.17	sloot18-filter

- In the tab SELECT, select the samples for NXT PCR-1 by filling in "1"

From NCBN plate(s) To PCR plate FS366 There are 17 samples selected for 51 PCR reactions. START pos.: A01 N empty duplicate: 0 N empty triplicate: 0

Extracts:

project	extract pos.	work plate pos.	extract ID	registr nr/CRS	field nr/suffix	select	PCR plate pos.	select	PCR plate pos.	select	PCR plate pos.
19001-555	NCBN001127-A01	A01	e12001	NBC.LAB.1	voorbeeld1-filter	1	A01	1	B03	1	C05
19001-555	NCBN001127-B01	B01	e4012502773	NBC.LAB.2	voorbeeld2-ETOH	1	B01	1	C03	1	D05
19001-555	NCBN001127-C01	C01	e4012502785	NBC.LAB.3	voorbeeld3-3-PhCl	0		0		0	
19001-555	NCBN001127-D01	D01	e4012502797	NBC.LAB.4	sloot-soil	1	D01	1	D03	1	E05
19001-555	NCBN001127-E01	E01	e4012502809	RMNH.123456	sloot2-specimen	1	D01	1	E03	1	F05
19001-555	NCBN001127-F01	F01	e4012502821	RMNH.123457	sloot3-specimen	1	E01	1	F03	1	G05
19001-555	NCBN001127-G01	G01	e4012502833	RMNH.123458	sloot4-specimen	1	F01	1	G03	1	H05
19001-555	NCBN001127-H01	H01	e4012502845	RMNH.123459	sloot5-specimen	1	G01	1	H03	1	A06
19001-555	NCBN001127-A02	A02	e4012502782	NBC.LAB.5	sloot6-filter	1	H01	1	A04	1	B06
19001-555	NCBN001127-B02	B02	e4012502774	NBC.LAB.6	sloot7-filter	1	A02	1	B04	1	C06
19001-555	NCBN001127-C02	C02	e4012502786	NBC.LAB.7	sloot8-filter	1	B02	1	C04	1	D06
19001-555	NCBN001127-D02	D02	e4012502798	NBC.LAB.8	sloot9-filter	1	C02	1	D04	1	E06
19001-555	NCBN001127-E02	E02	e4012502810	NBC.LAB.9	sloot10-filter	0		0		0	
19001-555	NCBN001127-F02	F02	e4012502822	NBC.LAB.10	sloot11-filter	0		0		0	
19001-555	NCBN001127-G02	G02	e4012502834	NBC.LAB.11	sloot12-filter	1	D02	1	F04	1	F06
19001-555	NCBN001127-H02	H02	e4012502846	NBC.LAB.12	sloot13-filter	1	E02	1	F04	1	G06
19001-555	NCBN001127-A03	A03	e4012502783	NBC.LAB.13	sloot14-filter	1	F02	1	G04	1	H06
19001-555	NCBN001127-B03	B03	e4012502775	NBC.LAB.14	sloot15-filter	1	G02	1	H04	1	A07
19001-555	NCBN001127-C03	C03	e4012502787	NBC.LAB.15	sloot16-filter	1	H02	1	A05	1	B07
19001-555	NCBN001127-E03	E03	e4012502811	NBC.LAB.17	sloot18-filter	1	A03	1	B05	1	C07

- Optionally, select duplo's or triplo's, if they fit in one 96-well plate, by checking duplicate or triplicate. If the duplicates or triplicates do not fit inside one 96-well plate, create multiple NGS3_PCR-1 sheets.

PCR plate ID: FS366 marker: ITS2 24/08/2020 09:22

N reactions: 96 fudge: 3 template dilution: 10 Technician: Frank Stokvis Source plate: NCBN001127_19001-555

PCR reaction mix: Env Master Mix

Brand	Chemical	Conc.	reaction (μl)	mastermix (μl)	Chemical	per 2ml tube (μl)
ultrapure	mQ	2 X	5,5	544,5	mQ	272,3
Life	Env Master Mix	2 X	10,0	990,0	Env Master Mix	495,0
Primer F	mICOintFNKT	10 μM/ul	1,00	99,0	mICOintFNKT	49,5
Primer R	mICOintRNKT	10 μM/ul	1,00	99,0	mICOintRNKT	49,5
Template (10 times diluted)			2,5	= 0,25 μL undil.		
Total			20,0	1732,5		866,25

PCR program: Env 50-35

Number of cycles: 35

Temp. Time

Init. Denature 95 °C 0:10:00

Denature 95 °C 0:00:15

Annealing 50 °C 0:00:30

Extension 72 °C 0:00:40

Final Extension 72 °C 0:05:00

Pause 12 °C 0:00:00

PCR machine: Env 50-35

N of bands: 0,9

Cleanup bead ratio: 0,9

Insert gel photo here

- In the tabs "PCR mixes", "PCR primers" and "PCR programs" add your PCR conditions if needed.
- In tab PCR_Setup, fill in all green fields: a unique PCR number under PCR plate ID, marker, number of reactions, fudge (extra mix for pipetting errors), template dilution (1 = undiluted, 10 = 10x diluted etc.), Technician name, Source plate number (NCBN and project number), PCR reaction mix (dropdown content in tab PCR mixes), Primer F and R (dropdown content in tab PCR primers), μl Template and PCR program (dropdown content in tab PCR programs)
- Perform PCR-1 ([METH030 Metabarcoding LIBRARY PREP](#))
- Check your PCR results using an agarose (e-)gel ([WI SO004 E-GEL](#))
- After editing, insert the gel photo in tab PCR_Setup

If PCR-1 was successful, continue with NGS4 PCR-2

4.5 NGS4_PCR-2

- Download a copy of [NGS4_PCR-2_v2.3_TEMPLATE.xlsx](#)

	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
	lat	long	country	locality	plate	DS dsDNA	DS 260/280	DS RRSE	PCR1 ID	marker	primer F	primer R	bead ratio	template c
1	12,13474	-68,98485	Netherlands	Lutjebroek	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10
2	12,13474	-68,98485	Baltic Sea	E-bay	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10
3	12,13474	-68,98485	Netherlands	Lutjebroek	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10
4	12,13474	-68,98485	Netherlands	Lutjebroek	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10
5	12,13474	-68,98485	Netherlands	Lutjebroek	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10
6	12,13474	-68,98485	Netherlands	Lutjebroek	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10
7	12,13474	-68,98485	Netherlands	Lutjebroek	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10
8	12,13474	-68,98485	Netherlands	Lutjebroek	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10
9	12,13474	-68,98485	Netherlands	Lutjebroek	NCBN001127	not available	not available	not available	FS366	ITS2	mIColintFNXT	igHCO2198NXT	0,9	10

- Copy the data from of NGS3_PCR-1.xlsx tab TO_NGS4_PCR-2.

	A	B	C	D	E	F	G	H	I	J	K	L
	project	pos_work	extract pos	extract ID	registr.code	field nmbr	extractiemethode	taxon	lat	long	country	locality
1	19001-555	A01	NCBN001127-A01	4012502761	NBC.LAB.1	voorbeeld1-filter		0 Carabus Linnaeus, 1758	12,13474	-68,98485	Netherlands	Lutjebroek
2	19001-555	B01	NCBN001127-B01	4012502773	NBC.LAB.2	voorbeeld2-ETOH	opmerking2		12,13474	-68,98485	Baltic Sea	E-bay
3	19001-555	C01	NCBN001127-C01	4012502785	NBC.LAB.4	sloot-soil		0	12,13474	-68,98485	Netherlands	Lutjebroek
4	19001-555	D01	NCBN001127-D01	4012502797	RMNH.123456	sloot2-specimen		0	12,13474	-68,98485	Netherlands	Lutjebroek
5	19001-555	E01	NCBN001127-E01	4012502809	RMNH.123457	sloot3-specimen	qw		12,13474	-68,98485	Netherlands	Lutjebroek
6	19001-555	F01	NCBN001127-F01	4012502821	RMNH.123458	sloot4-specimen		0	12,13474	-68,98485	Netherlands	Lutjebroek
7	19001-555	G01	NCBN001127-G01	4012502833	RMNH.123459	sloot5-specimen		0	12,13474	-68,98485	Netherlands	Lutjebroek
8	19001-555	H01	NCBN001127-H01	4012502845	NBC.LAB.5	sloot6-filter		0	12,13474	-68,98485	Netherlands	Lutjebroek

- Paste the data (values only!) in NGS4_PCR-2.xlsx tab FROM_NGS3_PCR-1. Multiple NGS3_PCR-1.xlsx outputs can be pasted here.

project	extract pos.	work plate pos.	extract ID	registr.nr.CRS	fieldnr-suffix	PCR1 ID	PCR1 pos.	marker	select	PCR2 plate pos.
20035-298-545	NCBN001075-A01	A01	e4012511305	NBC.LAB.2830	UPG77-rf4-Cal1	AL005	A01	18S SSU	1	A01
20035-298-545	NCBN001075-B01	B01	e4012511317	NBC.LAB.2831	UPG77-rf4-Cal2	AL005	B01	18S SSU	1	B01
20035-298-545	NCBN001075-C01	C01	e4012511329	NBC.LAB.2832	UPG77-rf4-Cal3	AL001	A01	18S SSU	1	C01
20035-298-545	NCBN001075-C01	C01	e4012511329	NBC.LAB.2832	UPG77-rf4-Cal3	AL001	A02	18S SSU		
20035-298-545	NCBN001075-C01	C01	e4012511329	NBC.LAB.2832	UPG77-rf4-Cal3	AL002	A01	18S SSU	1	D01

- In NGS4_PCR-2.xlsx tab SELECT, select all samples to be included in PCR-2.

The screenshot shows the 'PCR Setup' tab of the NGS4_PCR-2_TEMPLATE.xlsx spreadsheet. The interface includes a ribbon with tabs like File, Home, Insert, Draw, Page Layout, Formulas, Data, Review, View, and Help. The main area contains several sections for PCR configuration:

- PCR plate ID:** RP998, **marker:** ITS2, **Date/Time:** 15/10/2020 16:40
- Reactions:** 96, **Fudge:** 3, **Owner:** Rob Pastoor, **Source plate:** NCBN001127_19001:555
- PCR reaction mix:** KAPA HiFi

Brand	Chemical	Conc.	reaction (µl)	mastermix (µl)	Location	Notes
Ultrapur	mQ		5,0	495,0	Clean room	
KAPA HiFi Roche	HS ReadyMix	2 X	10,0	990,0	Clean room	
Distribute			15,0		Clean room	
From plate	NXT_S	10 pMol/ul	1,00	99,0	Clean room	Refresh tips
From plate	NXT_N	10 pMol/ul	1,00	99,0	Clean room	Refresh tips
Template			3,00		NGS post lab	
Total			20,0	1683		
- PCR program:** KAPA HiFi 55-08

	Temp.	Time
Init. Denature	95 °C	0:03:00
Denature	98 °C	0:00:20
Annealing	55 °C	0:00:30
Extension	72 °C	0:00:30
Final Extension	72 °C	0:05:00
Pause	12 °C	∞
- Label set:** A

	1	2	3	4	5	6	7	8	9	10	11	12
A	●	●	●	●	●	●	●	●	●	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	●	●	●	●	●	●	●	●	●	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○

- In the tabs "PCR mixes", "PCR primers" and "PCR programs" add your PCR conditions if needed.
- In tab PCR_Setup, fill in all green fields: a unique PCR number under PCR plate ID, marker, number of reactions, fudge, template dilution, Owner name, Source plate number, PCR reaction mix, µl Template, PCR program and Label set
- Perform PCR-2 ([METH030 Metabarcoding LIBRARY PREP](#))

4.5.1 Fragment Analyzer

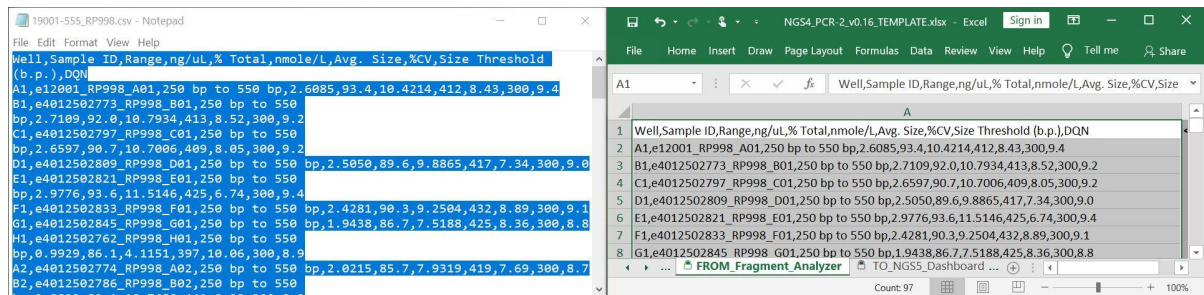
Check the PCR-2 results on the Fragment Analyzer (FA)

([METH030 Metabarcoding LIBRARY PREP, section 4.5](#), detailed instructions in [WI AP041 Fragment Analyzer](#))

The screenshot shows the 'TO_Fragment_Analyzer' tab of the NGS4_PCR-2_TEMPLATE.xlsx spreadsheet. The formula bar displays a complex Excel formula: `=IF("e" & IFERROR(INDIRECT("'"&FROM_NGS3_PCR-1"&"ID"&MATCH(CHAR(64+CEILING((ROW()-1)/12;1))&RIGHT("0"&MOD(ROW()-1;12)+1;2);"FROM_NGS3_PCR-1"!$Y:$Y;0));""))`. The spreadsheet grid shows columns A through Q. Column A contains sample IDs like e4012502761_RP998_A01, e4012502762_RP998_A02, e4012502763_RP998_A03, e4012502764_RP998_A04, followed by empty rows and e4012502773_RP998_B01. The status bar at the bottom indicates 'Count: 95' and '100%' zoom.

- Copy the data from of NGS4_PCR-2.xlsx tab TO_Fragment_Analyzer
- Paste the data into a new .txt document and save

The FA software uses the .txt as input, following [METH030 Metabarcoding LIBRARY PREP](#), in this SOP the measurement of the PCR-2 products and the subsequent export of those measurements as .csv are also described.



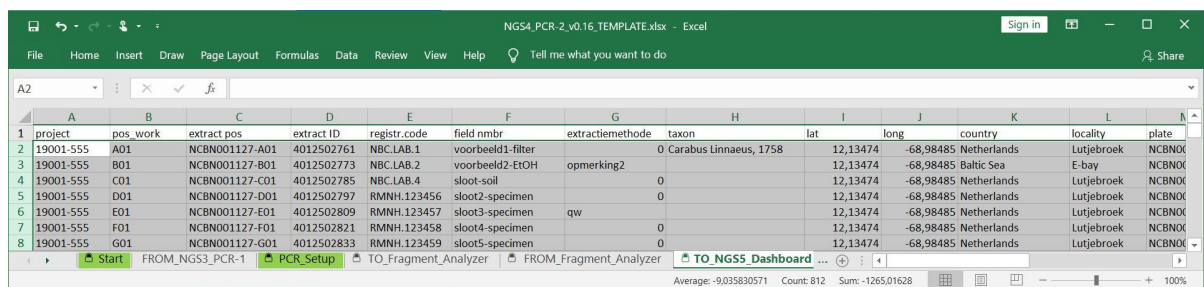
- After the FA run, open the .csv, exported by the FA software, with Notepad (NL Kladblok) and copy the data

- Paste the data (**values only!**) into NGS4_PCR-2.xlsx tab FROM_Fragment_Analyzer

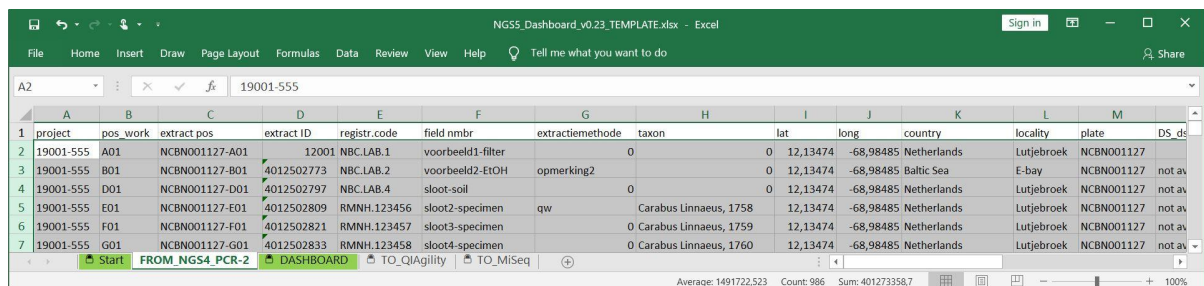
4.6 NGS5_Dashboard

The [NGS5_Dashboard_v3.999_TEMPLATE.xlsx](#) can be used to create QIAgility inputs for making equimolar pools (of 95 samples each) from up to 40 NGS4_PCR-2.xlsx exports.

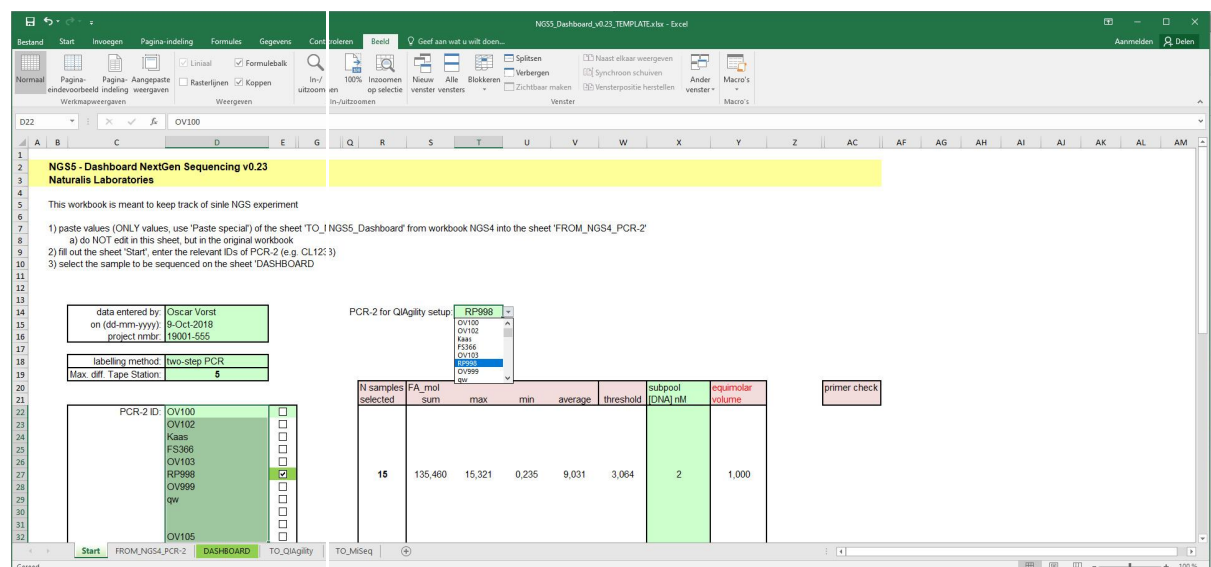
It can also be used to calculate the equimolar pooling of multiple sub pools.



- Copy the data from NGS4_PCR-2.xlsx tab TO_NGS5_Dashboard.xlsx



- Paste the data (**values only!**) into NGS5_Dashboard.xlsx tab FROM_NGS4_PCR-2. Data from up to 40 NXT PCR-2's can be pasted in the tab FROM_NGS4_PCR-2



- In NGS5_Dashboard tab START, in the window on the left, under PCR-2 ID, fill in the relevant PCR-2 numbers.
- Check ☒ the PCR-2 number to be pooled by the QIAgility
- Under PCR-2 for QIAgility setup, select the PCR-2 number to be pooled by the QIAgility from the dropdown menu

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Sub pools have to be measured on the TapeStation so they can be pooled equimolarly. The resulting end pool can be cleaned with MN beads.

The end pool created by the QIAgility has to be cleaned with MN beads according to [METH030 Metabarcoding LIBRARY PREP section 4.7](#).

The molarity of the pool(s) has to be measured on the Tape station, according to [METH030 Metabarcoding LIBRARY PREP section 4.8.1](#) and [WI AP042 TapeStation](#).

- Write the average of the 3 measurements of the end pool on the tube

Baseclear needs at least 20µl of a ≥ 5 nM end pool.

4.6.4 Equimolar pooling of sub pools

When several sub pools have to be combined to create the end pool for sequencing on the MiSeq, the NGS5_Dashboard.xlsx can be used to calculate the volumes required for equimolar pooling.

N samples selected	FA_mol sum	max	min	average	threshold	subpool [DNA] nM	equimolar volume	primer check
15	135,400	15,321	0,235	9,031	3,064	9	0,196	WARNING: primers not unique
17	152,272	15,321	0,235	8,957	3,064	2	1,000	WARNING: primers not unique

- In NGS5_Dashboard.xlsx tab START under subpool [DNA] nM, fill in the TapeStation measurements converted to nM (e.g. 5123 pM = 5,123 nM)

The volumes per pool, required for an equimolar end pool, will appear in the column equimolar volume.

Explanation of possible warnings:

- WARNING: at least one reaction derived from more than one extract! Only combine markers from a single extract.: Several DNA extracts/e-numbers have the same NXT labels and marker and will thus not be able to be separated by demultiplexing after sequencing.
- WARNING: primers not unique: Several PCR-2's are performed on the same PCR-1 primer set

4.6.5 MiSeq

- Download a copy of [index sheet.xlsx](#)

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
e12001_NBCLAB1_voorbeeld1-filter_RP998_A_A1	N701	TAAGGCGA	S502	CTCTCTAT	43																		
e4012502773_NBCLAB2_voorbeeld2-EIOH_RP998_A_B1	N701	TAAGGCGA	S503	TATCTCTCT	46																		
e4012502797_NBCLAB4_sloot-soil_RP998_A_C1	N701	TAAGGCGA	S505	GTAAGGAG	41																		
e4012502809_RMNH123456_sloot2-specimen_RP998_A_D1	N701	TAAGGCGA	S506	ACTGCATA	49																		
e4012502821_RMNH123457_sloot3-specimen_RP998_A_E1	N701	TAAGGCGA	S507	AAGGAGTA	49																		
e4012502833_RMNH123458_sloot4-specimen_RP998_A_F1	N701	TAAGGCGA	S508	CTAAGGCT	49																		
e4012502845_RMNH123459_sloot5-specimen_RP998_A_G1	N701	TAAGGCGA	S510	CGTCTAAT	49																		
e4012502762_NBCLAB5_sloot6-filter_RP998_A_H1	N701	TAAGGCGA	S511	TCTCTCCG	44																		

- In NGS5_Dashboard.xlsx tab TO_MiSeq, copy the values from the relevant rows* of columns A-E:

* for a shared run or MiSeq, rows 1-384
for a full NovaSeq run, all rows

The last column (column F) depicts the number of characters used in the MiSeq sample names, this number should be ≤ 50 .

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
e12001_NBCLAB1_voorbeeld1-filter_RP998_A_A1	N701	TAAGGCGA	S502	CTCTCTAT	43																				
e4012502773_NBCLAB2_voorbeeld2-EIOH_RP998_A_B1	N701	TAAGGCGA	S503	TATCTCTCT	46																				
e4012502797_NBCLAB4_sloot-soil_RP998_A_C1	N701	TAAGGCGA	S505	GTAAGGAG	41																				
e4012502809_RMNH123456_sloot2-specimen_RP998_A_D1	N701	TAAGGCGA	S506	ACTGCATA	49																				
e4012502821_RMNH123457_sloot3-specimen_RP998_A_E1	N701	TAAGGCGA	S507	AAGGAGTA	49																				
e4012502833_RMNH123458_sloot4-specimen_RP998_A_F1	N701	TAAGGCGA	S508	CTAAGGCT	49																				
e4012502845_RMNH123459_sloot5-specimen_RP998_A_G1	N701	TAAGGCGA	S510	CGTCTAAT	49																				
e4012502762_NBCLAB5_sloot6-filter_RP998_A_H1	N701	TAAGGCGA	S511	TCTCTCCG	44																				

- Paste the values in index sheet.xlsx

- Fill in the [NGS runs form](#) to place the order at BaseClear.

Baseclear needs at least 20µl of a ≥ 5 nM end pool.

5 ADDITIONAL PROCEDURES

5.1 SAFETY & LAB REGULATIONS

[PROC002 GENERAL LAB REGULATIONS](#)

[PROC003 SAFETY PROCEDURES](#)

[WI WS001 PRE-LAB](#)

[WI WS NGS LAB](#)

[METH027 SAMPLESHEET](#)

[WI SP006 FOLDER STRUCTURE DNA BARCODING DRIVE](#)

6 RELATED/ASSOCIATED DOCUMENTS

[METH030 Metabarcoding LIBRARY PREP](#)

[PROC023 PROCEDURES CRS LIMS](#)

[SHEET027 ProtoLIMS V08](#)

[SHEET015 OVERVIEW FREEZER CONTENT](#)

[WI AP031 SPEC-07](#)

[WI SO004 E-GEL](#)

[NGS1_SampleRegistration_v0.8_TEMPLATE.xlsx](#)

[NGS2_SampleSheet_v0.21_TEMPLATE.xlsx](#)

[NGS3_PCR-1_v0.25_TEMPLATE.xlsx](#)

[NGS4_PCR-2_v2.3_TEMPLATE.xlsx](#)

[NGS5_Dashboard_v3.999_TEMPLATE.xlsx](#)

[index_sheet.xlsx](#)