User Guide

5R-PLEX Kit

Ultra-sensitive 16S amplicon based NGS library prep
for degraded & low biomass DNA input

For the Singular Genomics G4 Sequencing Platform

MBD6000-1KT

Product Overview

The 5R-PLEX is an NGS amplicon-based library preparation kit that targets five short variable regions along the 16S rRNA gene (V2, V3, V5, V6, V8) that are co-amplified in a multiplexed PCR within a single tube. After sequencing, the data can be uploaded and analyzed using the 5R-PLEX module of the M-CAMP™ bioinformatic platform (Microbiome Computational Analysis for Multi-omics Profiling). The 5R-PLEX module is a unique algorithm which computationally combines the data from all amplified regions allowing a high-resolution of microbial profiling even in harsh conditions of low bacterial biomass and fragmented DNA (for example, in the case of formalin-fixed and paraffin-embedded samples, fossil-derived DNA, or DNA exposed to other degrading conditions).

23177722 Rev 02/25

Kit Contents, Storage and Shelf life

The 5R-PLEX 16S Amplicon-Seq Kit contains enough material to prepare 96 samples for sequencing on the Singular Genomics G4 Sequencing Platform. The shelf life of all reagents is 12 months when stored properly. Store all components at –20 °C.

Component	Amount
5R-PLEX PCR1 Primer mix	25 µL
5R-PLEX PCR2 Forward Primer mix	25 µL
5R-PLEX index plate	96-plate
Water, microbial DNA-free	10X1.5 mL
HF DNA Polymerase	0.1 mL
5X HF Buffer	2 mL
dNTP's	0.2 mL
Elution Buffer (EB), microbial DNA-free	8 mL
5R-PLEX Positive Control (10 ng/µL)	30 µL

Required Materials (Not Provided)

Make sure that all the necessary user-supplied equipment, reagents and consumables are available before proceeding to sample preparation. Catalogue numbers in parenthesis can be ordered at SigmaAldrich.com unless otherwise indicated.

- GenElute™ PCR Clean-Up Kit (NA1020-1KT)
- Ethanol (1.08543)
- Water for molecular biology (W4502-1L)
- 5R-PLEX Magnetic beads for PCR clean-up (MBD6009) or AMPure™ XP Reagent for PCR Purification 5 mL (Beckman Coulter, A63880) or equivalent.
- KAPA Library Quantification Kit (Roche, KK4873)
- 96-well PCR Plate Non-skirted
- Adhesive PCR Plate Seal
- 2, 10, 20, 200 and 1000 µL pipettes/multichannel pipettes
- Nuclease-free barrier pipette tips
- RNase/DNase-free multichannel reagent reservoirs, disposable
- Fluorometric quantification method that uses dsDNA binding dyes, such as Qubit™ or equivalent.
- Multiplexed Capillary Electrophoresis (CE) such as Bioanalyzer®, TapeStation® or equivalent.
- Thermocycler
- Magnetic Stand-96 (Thermo Fisher Scientific®, AM10027) or equivalent
- Vortex

Precautions and Disclaimers

The 5R-PLEX kit is for research use only. Not for use in diagnostic procedures, not for drug, household, or other uses.

Please consult the Safety Data Sheet (SDS) for information regarding hazards and safe handling practices.

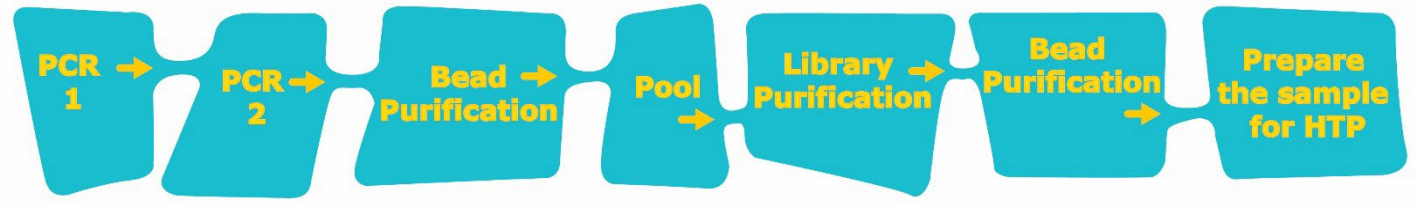
Preparation Protocol

Tips and Techniques

- Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
- Physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed.
- When working with very low-biomass samples, it is highly recommended to work in a sterile space such as a biological hood or a UV cabinet.
- Clean lab areas using Lookout™ DNA erase (L8917) followed by 70% Ethanol.
- Use barrier pipette tips to avoid exposure to potential contaminants.
- Always change tips between each sample.
- Run appropriate controls with each preparation to monitor background contaminations.

Note: This assay was developed to detect extremely low biomass in an ultra-sensitivity manner. Therefore, it is highly recommended to include negative control samples, which should be sequenced to allow bioinformatic subtraction of background.

Sample Preparation Flow Chart



Before first use of the kit

Starting Material

The 5R-PLEX 16S Amplicon-Seq Kit has been optimized and validated using degraded bacterial DNA input of 1-100 pg. Dilute DNA samples as required using microbial DNA-free Water, supplied in the kit. Do not use TE, as EDTA might inhibit PCR reaction.

Guidelines for DNA extraction

To reduce bias associated with DNA extraction (1), all samples should be extracted using the same, validated DNA extraction kit or protocol (5). Record extraction batches for downstream analysis. We strongly recommend using several technical replicates, as well as blanks. Blanks can be designated as negative controls in M-CAMP™ and contaminating sequences bioinformatically removed.

Reagent Preparation

PCR1

Reagents

- HF DNA Polymerase
- dNTPs
- 5X HF Buffer
- Water, microbial DNA-free
- 5R-PLEX PCR1 Primers mix

1. Thaw all reagents and prepare PCR1 mix according to the table below and keep on ice:

Components		Volume per reaction
Water, microbial DNA-free		___ µL
5X HF Buffer		10 µL
dNTPs 10 mM		1 µL
5R-PLEX PCR1 Primers mix		0.25 µL
HF DNA Polymerase*	Add last:	0.5 µL
PCR1 Product	Add separately:	2-5 µL
	Total Reaction Volume	50 µL

*__Note__: It is critical that the HF DNA Polymerase is the last component added to the PCR mixture.

2. Dispense the PCR1 mix in each well.
3. Add at least 6 Negative control samples to each batch (no DNA template).
4. Add the DNA separately into each well tube.
5. Dilute the 5R-PLEX positive control to 0.005 ng/µL using the supplied microbial DNA-free Water and add 2 µL to one of the wells.
6. Mix up and down with a pipette 10 times and spin.
7. Perform PCR1 on a thermal cycler using the following program:

Step	Temperature	Time	# Cycles
1	98 °C	2 minutes	1
2	98 °C	10 seconds	
3	62 °C	15 seconds	X30
4	72 °C	35 seconds	
5	72 °C	5 minutes	1

PCR2

Reagents

- HF DNA Polymerase
- dNTPs
- 5X HF Buffer
- Water, microbial DNA-free
- 5R-PLEX PCR2 Forward Primers Mix
- 5R-PLEX 96-well Index-plate

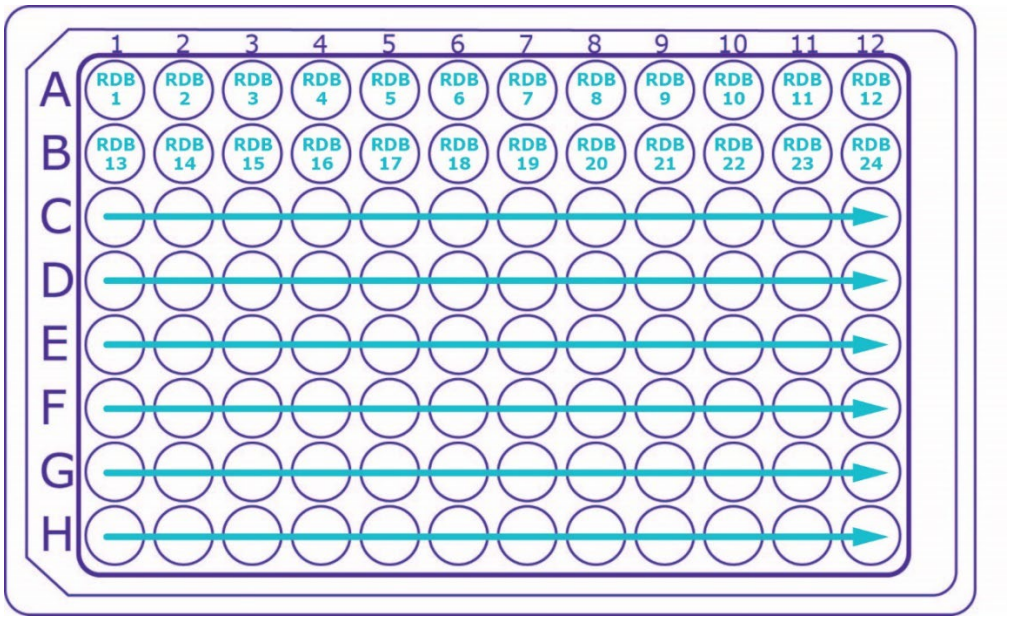
1. Thaw all reagents and prepare PCR2 mix according to the table below and keep on ice:

Components		Volume per reaction
Water, microbial DNA-free		___ µL
5X HF Buffer		10 µL
dNTPs 10 mM		1 µL
5R-PLEX PCR2 Forward Primer mix		0.25 µL
HF DNA Polymerase*	Add last:	0.5 µL
PCR1 Product	Add separately:	2-5 µL
	Total Reaction Volume	50 µL

*__Note__: It is critical that the HF DNA Polymerase is the last component added to the PCR mixture.

2. Dispense the PCR2 mix in each well of the 5R-PLEX index-plate containing the dried single unique index-adapter reverse primer.

   Note: The indexes are aligned from RDB1 at position A1, horizontally (RDB12 at A12). The directionality of the indexing plate is indicated with arrows.



3. Add 2-5 µL of the PCR1 product into each well tube.
   Note: For ultra-sensitive assay it is recommended to add 5 µL PCR1 product as template.
4. Pipette up and down 10 times to mix, and spin down the 96-well plate.
   Note: when using a smaller batch of less than 96 samples, transfer the reaction volume to a new PCR plate and store the 5R-PLEX index plate at –20 °C for further use.
5. Perform PCR2 on a thermal cycler using the following program.

Step	Temperature	Time	# Cycles
1	98 °C	2 minutes	1
2	98 °C	10 seconds	
3	64 °C	15 seconds	X6
4	72 °C	25 seconds	
5	72 °C	5 minutes	1

PCR2 Clean-up Bead Purification

Reagents

- Magnetic Beads*
- Elution Buffer (EB), microbial DNA-free
- Ethanol for molecular biology
- Water for molecular biology

*__Note__: The beads must be equilibrated to room temperature 30 minutes before use.

1. Vortex the beads thoroughly for 30 seconds to make sure that the beads are evenly dispersed. The beads must be homogenous.
2. Add 42.5 µL (0.85X) beads to 50 µL DNA (PCR2 product from previous page).
3. Mix up and down 10 times, seal the tubes and incubate at room temperature for 5 minutes.
4. Place the tubes on a magnetic stand for 2-5 minutes or until the supernatant has cleared.
5. Remove and discard the supernatant.
6. With the tubes on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
   a. Add 200 µL of freshly prepared 80% ethanol.
   b. Incubate the tubes on the magnetic stand for 30 seconds.
   c. Carefully remove and discard the supernatant.
7. With the tubes on the magnetic stand, perform a second ethanol wash as described in step 6 (above).
   Important: Completely remove all traces of the ethanol wash after the second wash. To do this, briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol first with a 200 µL pipette, and then use a 10 µL pipette to remove any residual ethanol.
8. With the tubes still on the magnetic stand, allow the beads to air-dry for 3-5 minutes (time can change according to humidity and temperature in the lab).
   Note: When completely dry, the beads should have a "cracked" appearance.
   Do not over-dry the beads.
9. Remove the tubes from the magnetic stand. Add 20 µL of EB to each tube.
10. Gently mix up and down 10 times until beads are fully resuspended.

12. Incubate at room temperature for 2 minutes.
13. Place the tubes on the magnetic stand for 2 minutes or until the supernatant has cleared.
14. Carefully transfer 18 µL of the supernatant (purified DNA) into a new tube. To avoid bead carryover, up to 2 µL of eluate can be left behind.

SAFE STOPPING POINT – Store at -15 °C to -25 °C

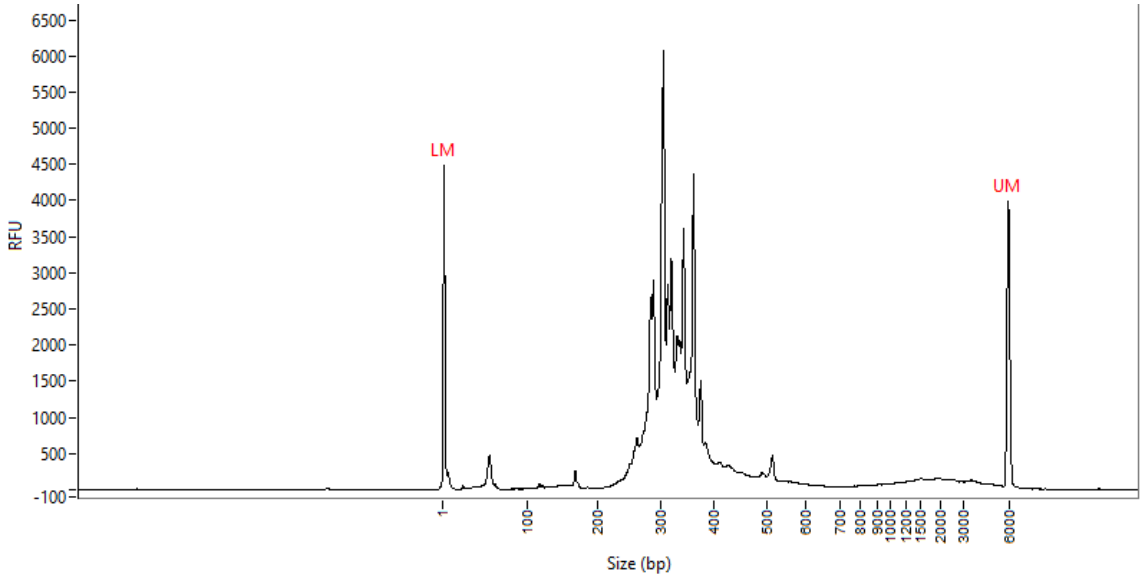
QC for sample library

Reagents

Multiplexed Capillary Electrophoresis (CE) HS reagents Elution Buffer (EB), microbial DNA-free

1. Run all libraries on a Multiplexed Capillary Electrophoresis (CE) instrument to confirm that there are traces of amplicons in the size range of 250-400 bp (the theoretical fragment sizes are: 287, 324, 342, 364 and 370 bp) See graph below.

Example of the purified library of the 5R-PLEX positive control on a Fragment Analyzer instrument



2. The number of amplicons, their sizes and relative amplitude may vary due to differential bacterial composition of the samples.

   Note: For tumor samples, if no library traces are visible repeat the library prep with lower DNA input.
3. Additional peaks at ~ 450-480 bp might appear (two amplicons joined).
4. Primers and primer dimers might appear at < 30-180 bp.

Quantify & Pool

Reagents

Qubit™ 1X dsDNA HS Assay Kit or Fluorometric quantification method that uses dsDNA binding dyes

1. Measure the DNA concentration of each purified sample library with Qubit™ dsDNA HS assay kit.

   Note: FFPE samples are expected to have a broad range in yield, down to the limit of detection.
2. Pool equal amount of each sample into a single tube (use the maximum amount possible).
3. Transfer a volume that is equivalent to a maximum of 10 µg DNA of the pooled library in a new tube and continue to library purification.

Library Purification I

Reagents

GenElute™ PCR Clean-Up Kit (NA1020)

Go to the GenElute™ PCR Clean-Up Kit product page at SigmaAldrich.com. Follow the technical bulletin and preparation instructions or watch the video.

1. Insert a GenElute™ plasmid mini spin column (with a blue o-ring) into a provided collection tube, if not already assembled. Add 0.5 mL of the Column Preparation Solution to each mini spin column and centrifuge at 12,000 x g for 30 seconds to 1 minute. Discard the elute.

   Note: This step maximizes binding of the DNA to the membrane and result in more consistent yields.
2. Add 5 volumes of Binding Solution to 1 volume of the pooled library (up to 10 µg) and mix. For example, add 500 µL of Binding Solution to 100 µL DNA sample. Transfer the solution into the binding column. Centrifuge the column at maximum speed (12,000-16,000 x *g*) for 1 minute. Discard the elute but retain the collection tube.

   Note: if the sample volume (of PCR + Binding Solution) exceeds the column capacity (500 µL), load partial volume, and repeat this step for the whole sample volume with the same column.
3. Apply 0.5 mL of dilution Wash Solution to the column and centrifuge at maximum speed for 1 minute. Discard the elute but retain the collection tube.

   Note: Be sure to add ethanol to the Wash Solution concentrate prior to first time use.
4. Replace the column into the collection tube. Centrifuge the column at maximum speed for 2 minutes, without any additional wash solution, to remove excess ethanol. Discard any residual elute as well as the collection tube.
5. Transfer the column to a fresh 2 mL collection tube. Apply 50 µL of Elution Solution. Incubate at room temperature for 1 minute.
6. To elute DNA, centrifuge the column at maximum speed for 1 minute. The PCR amplification products pool is now present in the elute and is ready for immediate use or storage at –20 °C.

SAFE STOPPING POINT – Store at -15 °C to -25 °C

Library Purification II

Bead Purification

Reagents

- Magnetic beads*
- Elution Buffer (EB), microbial DNA-free
- Ethanol for molecular biology
- Water for molecular biology

*__Note__: The beads must be equilibrated to room temperature 30 minutes before use.

1. Vortex the beads thoroughly for 30 seconds to make sure that the beads are evenly dispersed. The beads must be homogenous.
2. Add 42.5 µL (0.85X) beads to 50 µL of the purified pooled library from the previous section.
3. Pipette up and down 10 times, seal the tube and incubate at room temperature for 5 minutes.
4. Place the tube on a magnetic stand for 2-5 minutes or until the supernatant has cleared.
5. Remove and discard the supernatant.

6. With the tube on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
    d. Add 200 µL of freshly prepared 80% ethanol.
    e. Incubate the tube on the magnetic stand for 30 seconds.
    f. Carefully remove and discard the supernatant.
7. With the tube on the magnetic stand, perform a second ethanol wash as described in step 6.

   Important: Completely remove all traces of the ethanol wash after the second wash. To do this, briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol first with a 200 µL pipette, and then use a 10 µL pipette to remove any residual ethanol.
8. With the tube still on the magnetic stand, allow the beads to air-dry for 3-5 minutes (time can change according to humidity and temperature in the lab).

   Note: When completely dry, the beads should have a "cracked" appearance. Do not over-dry the beads.
9. Remove the tube from the magnetic stand. Add 20 µL of EB.
10. Gently pipette the mix up and down 10 times until beads are fully resuspended.
11. Incubate at room temperature for 2 minutes.
12. Place the tube on the magnetic stand for 2 minutes or until the supernatant has cleared.
13. Carefully transfer 18 µL of the supernatant (purified pooled library) into a new tube. To avoid bead carryover, up to 2 µL of eluate can be left behind.

SAFE STOPPING POINT – Store at -15 °C to -25 °C

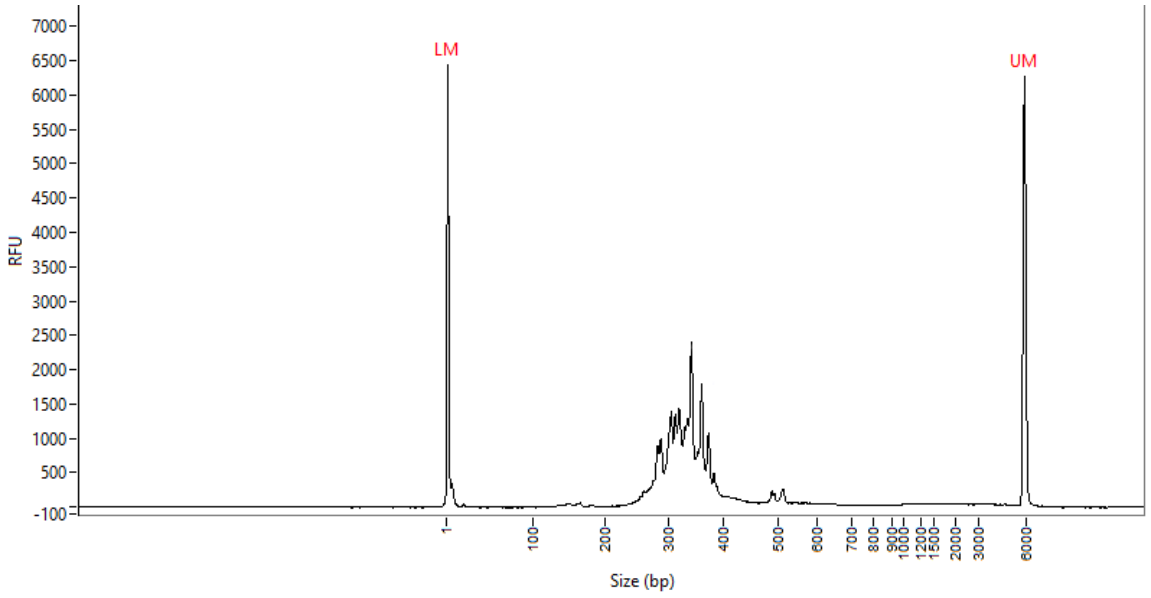
Pooled Library QC & Quantification

Reagents

- Multiplexed Capillary Electrophoresis (CE) HS reagents
- KAPA Library Quantification Kit Platforms

1. Run the purified pooled library on a multiplexed capillary electrophoresis (CE) instrument to confirm that there are traces of amplicons in the size range of 250-400 bp (the theoretical fragment sizes are: 287, 324, 342, 364 and 370 bp). See graph below.

Example of the purified pooled library on Fragment Analyzer instrument



2. The number of amplicons, their sizes and relative amplitude may vary due to differential bacterial composition of the samples.
3. Additional peaks at ~450-480 bp might appear (two amplicons joined).

   Important Note: If primer dimers are observed (~160-180 bp), repeat beads purification in Library Purification II.
4. Measure the DNA concentration of the purified pooled library. It is highly recommended to measure the purified pooled library concentration using the KAPA Library Quantification Kit (KK4873, ROCHE), a qPCR-based quantification of flanked by the P5 and P7 flow cell oligo sequences.
5. If Qubit™ 1X dsDNA HS Assay Kit was used for library quantification, calculate the DNA concentration in nM, based on an average fragment size of the library as determined by a Multiplexed capillary electrophoresis (CE) instrument (~337bp):

$$\frac{\text{(Concentration in ng/µL)}}{\text{(660 g/mol x average library size in bp)}} \times 106 = \text{Concentration in nM}$$

Adapt the 5R-Plex Libraries for Sequencing on the Singular Genomics G4

G4™ Sequencing Platform

The G4 Sequencing Platform is a versatile benchtop sequencer designed to meet the demands of advanced research applications. It utilizes an innovative 4-color Rapid Sequencing by Synthesis (SBS) chemistry to provide highly accurate reads in single or paired-read formats, with optional index reads, all within a single day. For added flexibility, the G4 Platform supports simultaneous loading of up to four flow cells, each featuring four independently fluidic lanes.

A single 5R-PLEX library or a pool of libraries can be adapted for sequencing on the G4 using the following protocol: "Adapting Libraries for G4™—Retaining Original Indices guide" (Document #600025 Rev. 1, July 2023) by Singular Genomics Systems, Inc.[6]

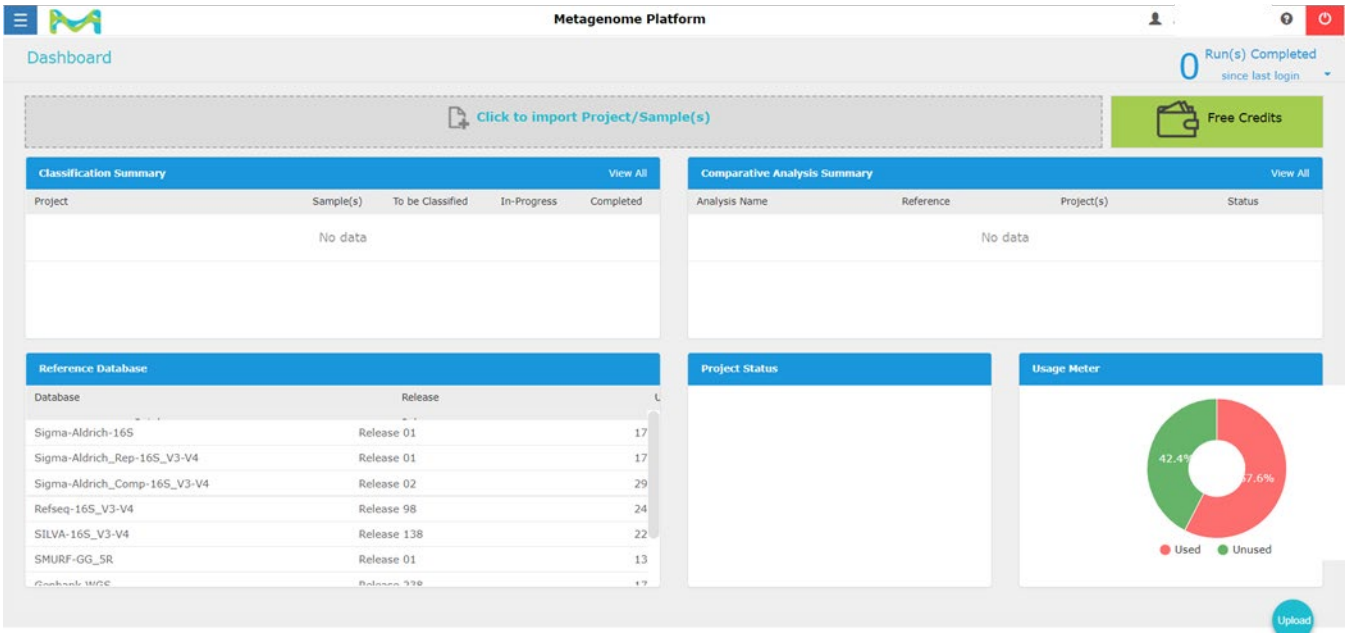
In short, perform a PCR using the provided SG Compatibility PCR Primer Mix from the SG Library Compatibility Kit. The resulting G4-compatible library retains sequences present within the PP1 and PP2 sequences of the indexed third-party library, including the indices, insert, and SP1 and SP2 sites. After this, clean up the product with a PCR purification kit and follow the regular workflow for clustering on the G4 Sequencing Platform.

- See the 5R-PLEX Single Index List.
- In the example, we used a 0.85X ratio for cleanup.

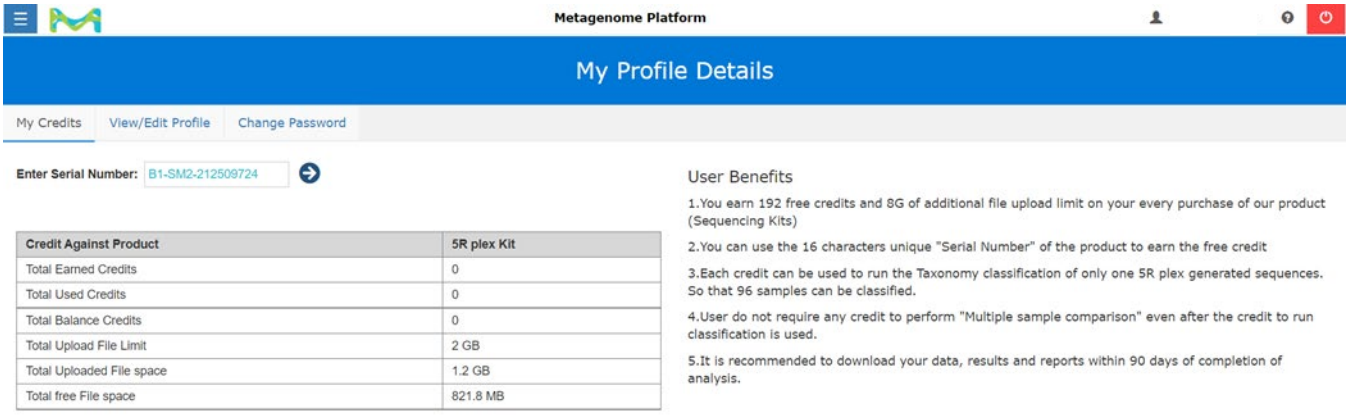
Upload Sequencing Files to the M-CAMP™ Platform

Sign in or create a new account: https://m-camp.info/microbiome

1. Visit the M-CAMP™ web platform using the link and navigate to the "Metagenome" module. Click on the "Free Credits" from Dashboard or the Application Menu.



2. After clicking the "Free credits" link, the user will be navigated to the "My Credits" tab where the user can enter the 16-digit unique key-code found on the quick card to earn the credits.



Customer Instructions for Unzipping and Re-zipping G4 Sequencing Platform files for analysis

The FASTQ output of the G4 Sequencing Platform and G4 Demultiplex Software are compressed using bgzip rather than gzip. Although bgzip is directly compatible with gzip, some applications (such as M-CAMP™) that try to directly open the bgzip compressed files will encounter an error. The solution is to uncompress the file, and then recompress using gzip. Suppose you have sample file (Sample_S0_L001_R1_001.fastq.gz, you can uncompress and recompress using the following command:

```

gunzip Sample_S0_L001_R1_001.fastq.gz

gzip Sample_S0_L001_R1_001.fastq

```Conversely, you can uncompress all fastq.gz files and then recompress all resulting fastq files by running the following commands:

```

gunzip *.fastq.gz

gzip *.fastq

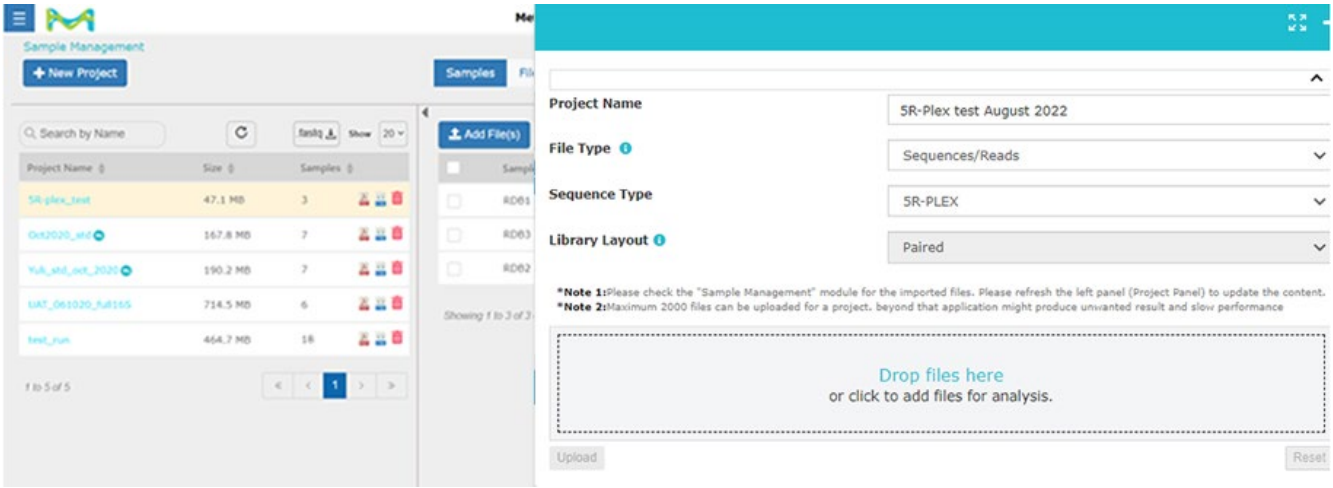
```

3. Navigate back to the Dashboard of the Metagenome Module. You can upload the fastq files from here (press "Click to import Project Samples"). Define Project name, Sequence type (5R-PLEX), Library Layout (Paired). Press Upload.

   **Note**: The 5R-PLEX pipeline accepts only demultiplexed, paired-end, single-lane reads.

   - If the sequencing files are saved in multiple separated folders, combine all fastq files to the same folder (this can be done with the "find" search options, to access all fastq files within all subfolders, copy and pasting all files to the same folder).
   - For multiple lane fastq files, use an external source to merge all lanes for each sample (For example, https://github.com/merenlab/illumina-utils/blob/master/README.md#demultip). Save the merged fastq files in a separate folder.

```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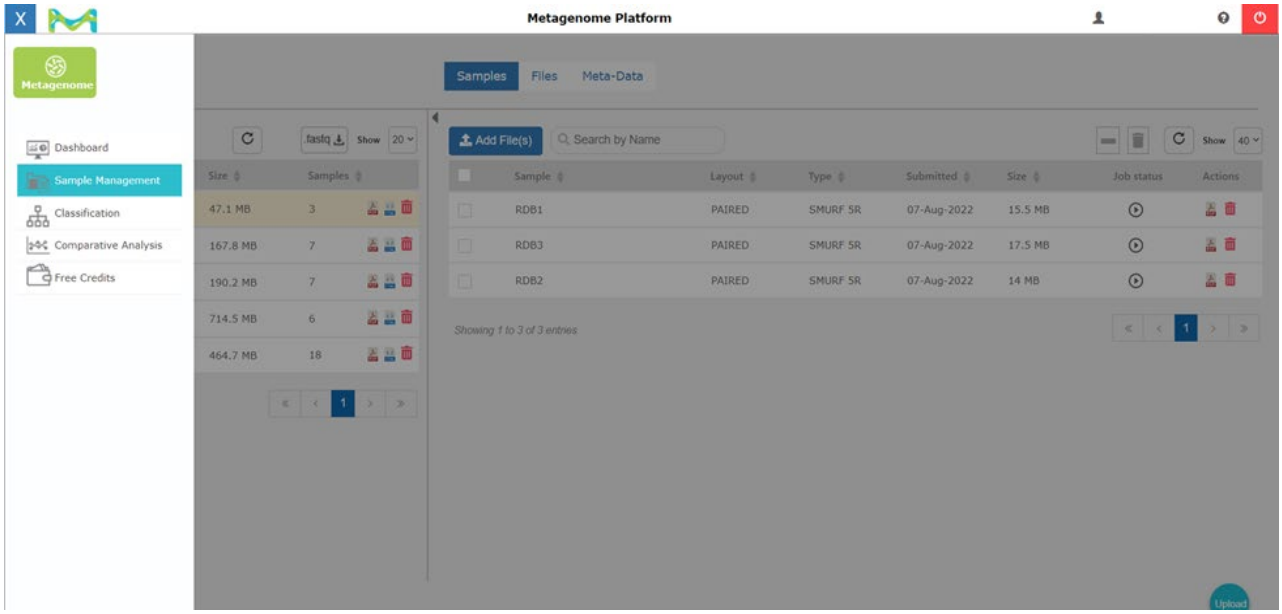
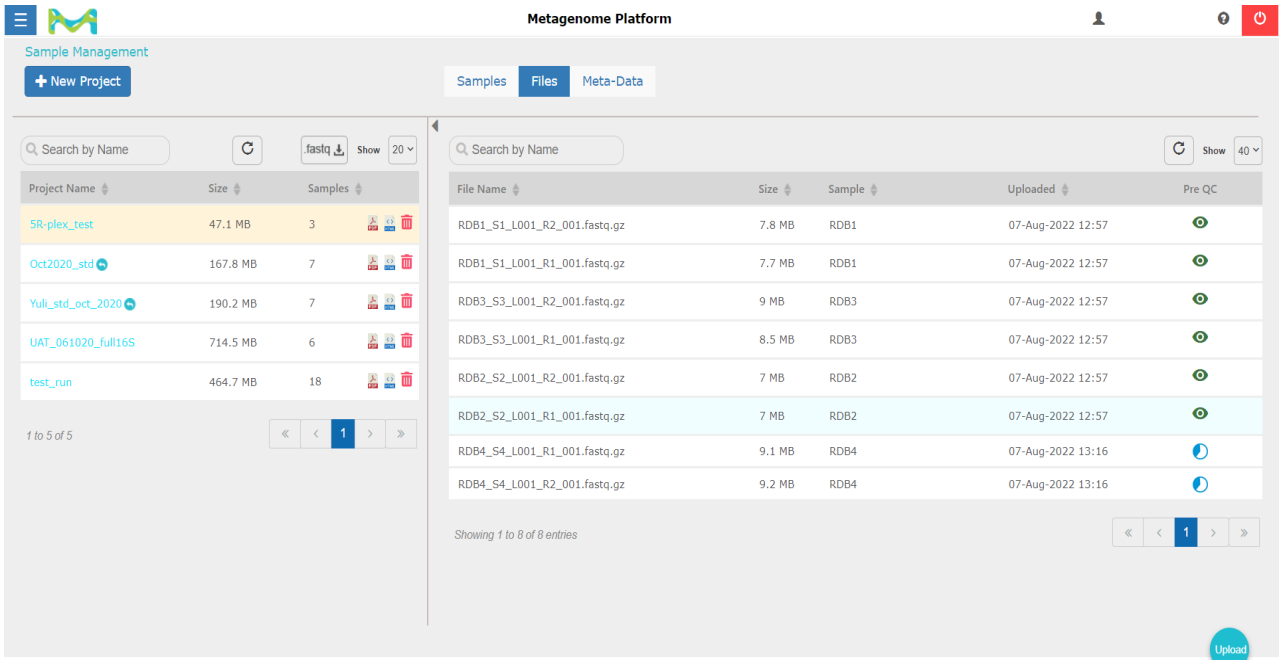
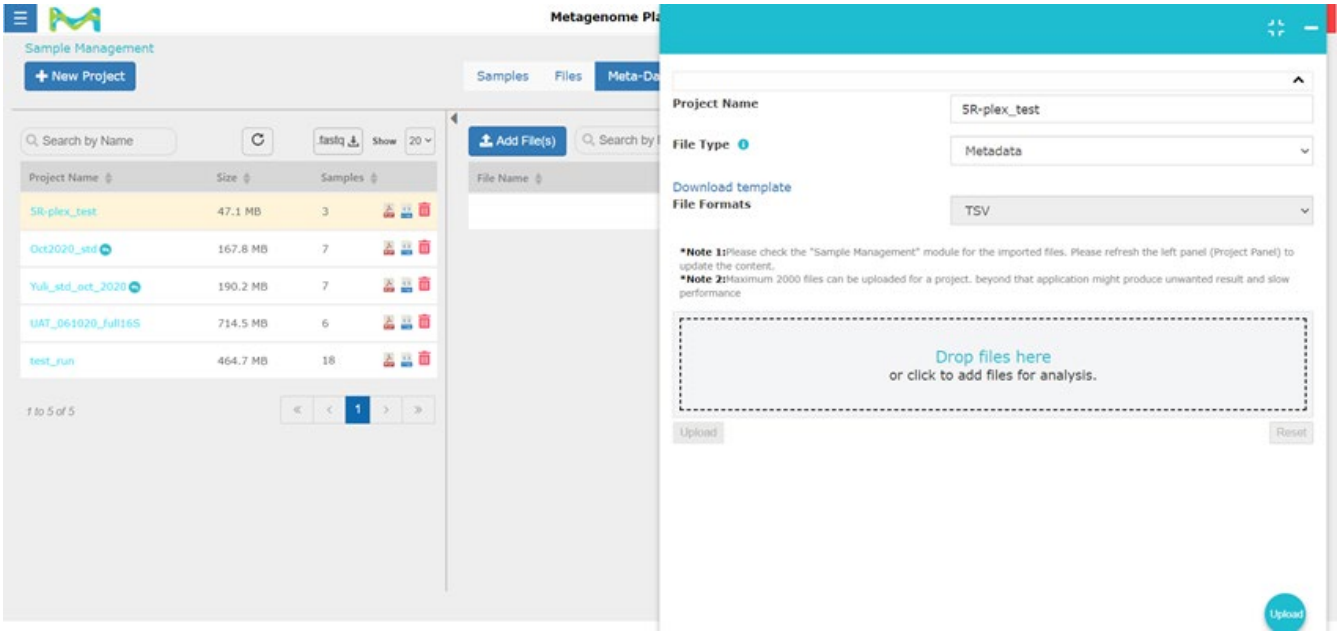
4. Once the upload is completed, go to “Sample management” in the main menu (upper left corner).



5. Pre-QC step can be monitored at Files section of the Project.



6. Supporting projects' metadata can be uploaded at Meta-data section of the Project.



Metadata Format Information

Please upload the sample information as tab delimited format (.txt) or comma separated values (.csv) only. Your metadata file must contain header with first column name as either in case in-sensitive ('id', 'sample-id', 'sampleid', 'sample-id') or case-sensitive ('#SampleID', '#Sample ID') followed by the other categories. The type of data should be defined as 'categorical'.

Example of metadata file content:

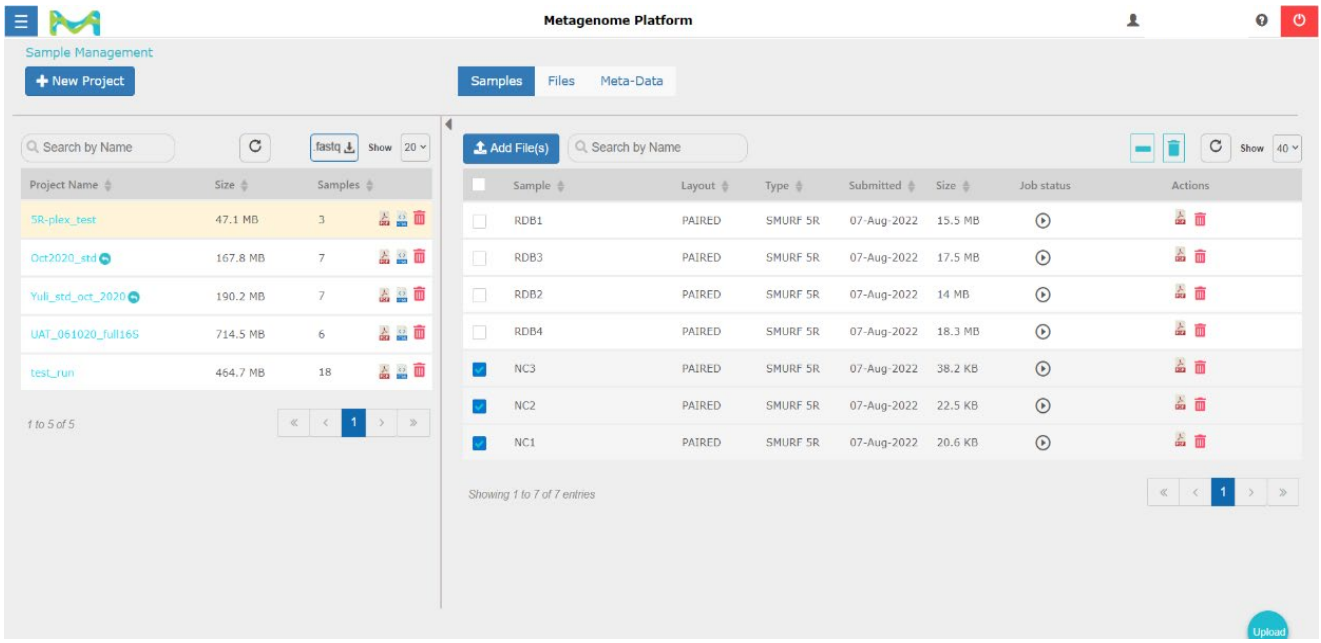
#SampleID #q2:types	Storage Categorical	Kit Categorical	Mouse Categorical	Cage Categorical	Date Categorical
1_S1_L001	Fresh Sigma	1	Cage 1	1	12.11.18
2_S2_L001	Fresh Sigma	1	Cage 1	1	12.11.18
3_S3_L001	Frozen Sigma	3	Cage 2	2	13.11.18
4_S4_L001	Frozen Sigma	3	Cage 2	2	13.11.18

The comparative analysis module currently supports only categorical metadata columns with non-unique groups. It is recommended to exclude the missing values from the metadata file and the last column in the metadata file must not have any missing values. It is also recommended to have metadata file format pre-validated to avoid any run time issue (User can use any bioinformatics metadata file format validators like 'Keemei').

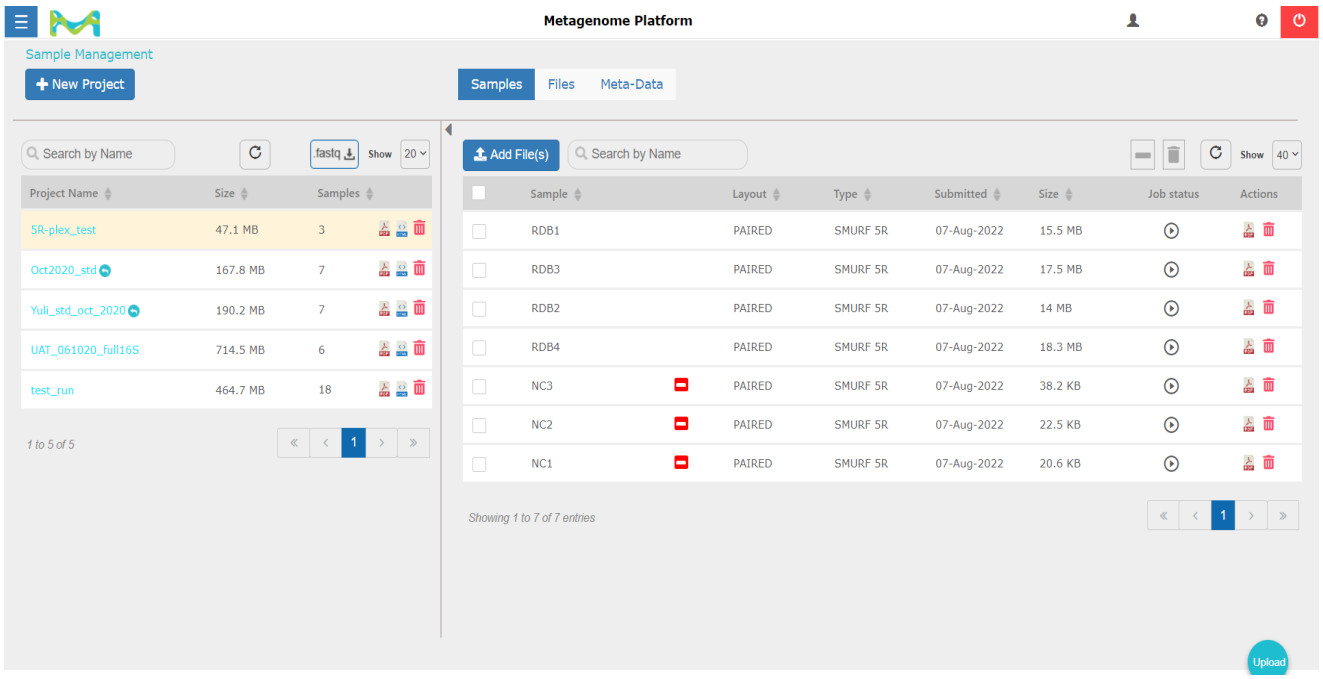
Removal of Contamination

The M-CAMP™ Platform has the option of bioinformatic removal of contamination, prevalence in low biomass samples. To use this feature on the App, at least 2 negative controls must be sequenced per batch. Decontam is based on Prevalence method (presence/absence across samples): prevalence of each OTU in true positive samples is compared to the prevalence in negative controls to identify contaminants. This method is recommended for low-biomass samples (2).

1. At "Sample Management" module, select your negative control samples and press decontam symbol (upper right corner, toggles negative control for sample)



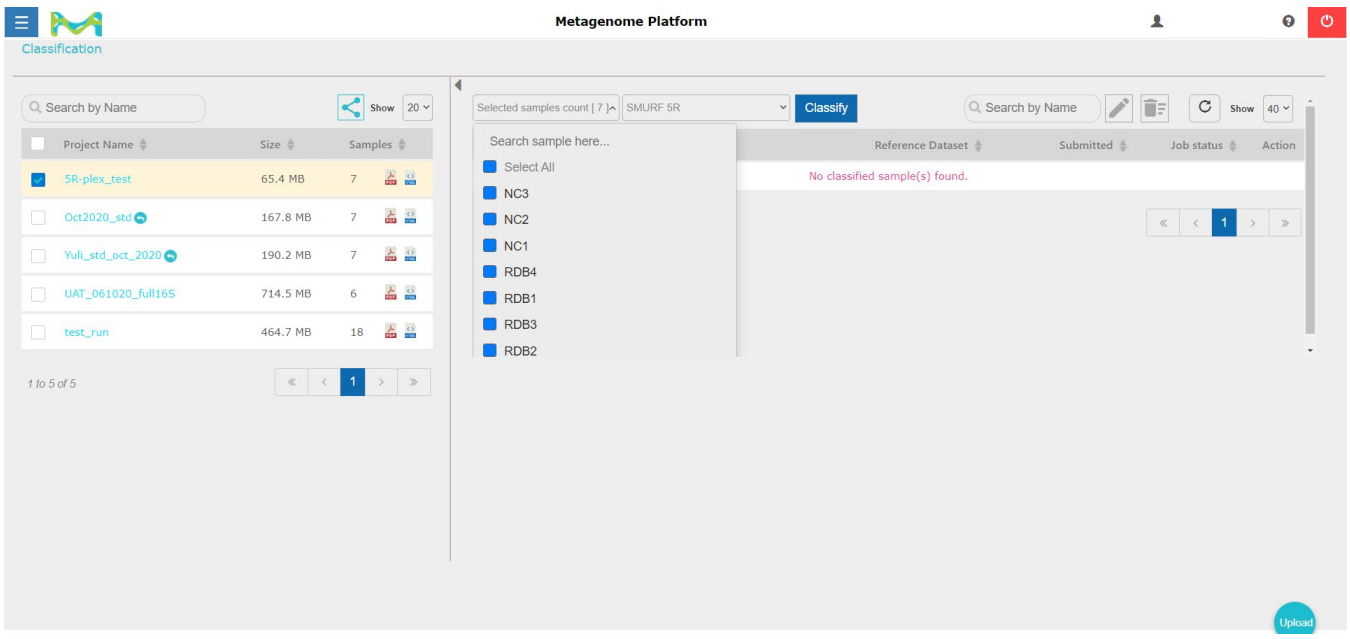
2. Your negative controls will be tagged as Negative controls.



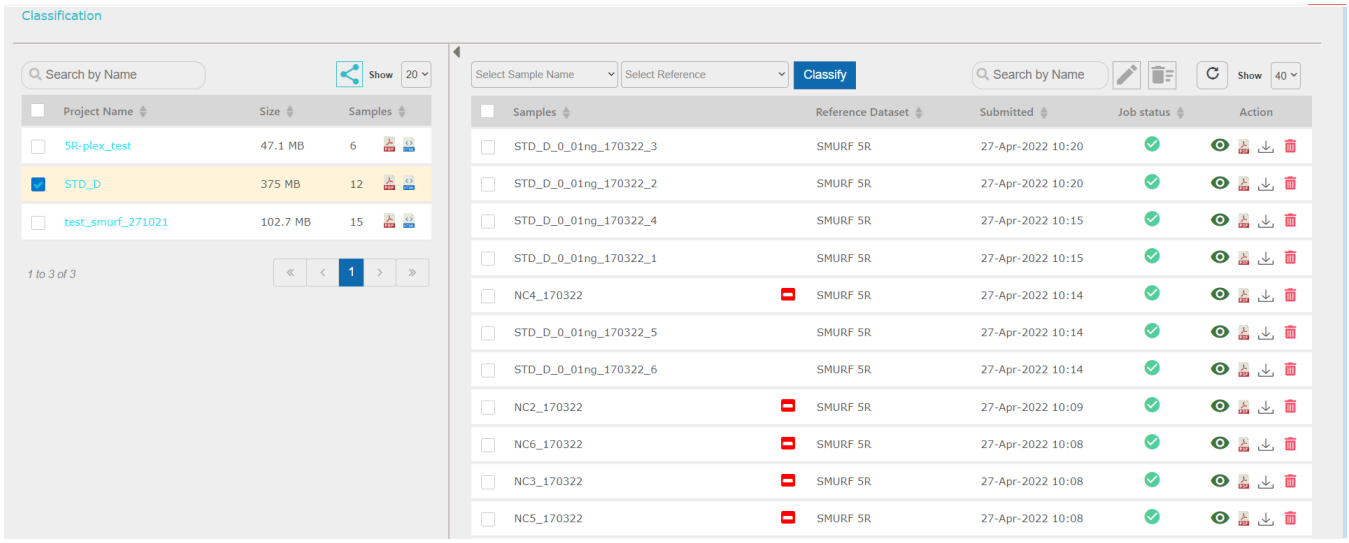
3. After assigning all negative control samples, proceed and run "Classification". Assigning different sample(s) as a negative control for a chosen project, requires re-performing Classification for all samples in the project de novo.

Classification and Analysis

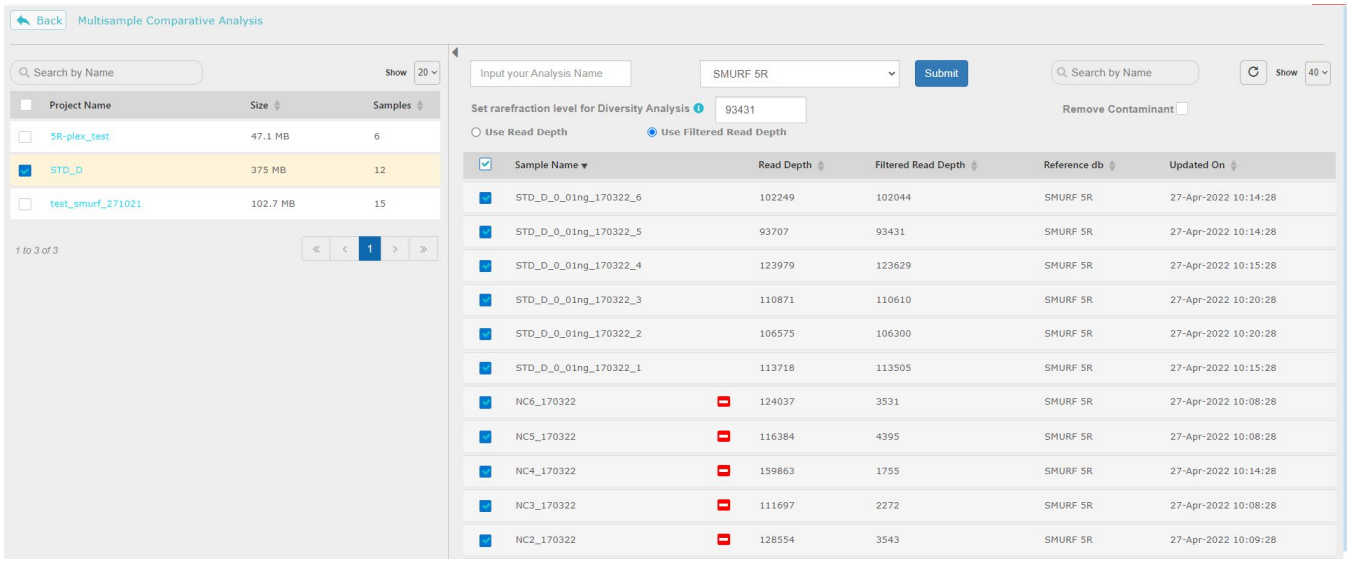
1. Navigate to "Classification" module. Select your samples and chose the reference database (5R-PLEX). Chose all samples from your project (including negative controls).
To start classification press "classify".



2. Upon completion of classification, samples will get a completion sign (green).

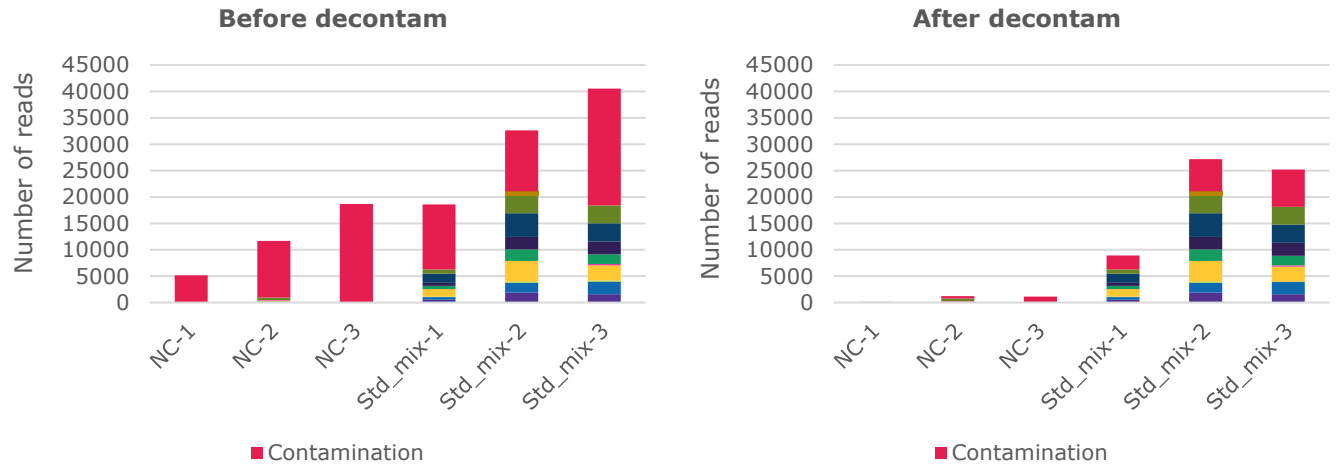


3. Navigate to "Comparative Analysis" module. Select all samples within the project. Name your comparative analysis.
4. Additionally, rarefraction level can be determined for Diversity analysis. Samples with fewer reads than the determined rarefraction level will be excluded from the analysis. By default, the lowest read depth of true sample (not negative control) is assigned as a rarefraction level.
5. If you wish to perform decontam on your set of samples, click on "Remove Contaminant" (upper right corner).
6. The stringency of contamination removal can be determined by assigning a probability threshold. It is recommendable to try different thresholds, from 0.1 to 1. After you choose the threshold (for example 0.4), decontam contamination removal will be activated. The resulting table will include two columns for the number of reads: Read Depth (actual read depth) and Filtered Read Depth (after decontamination). Select "Filtered Read Depth" if you chose to run Comparative analysis on samples that were filtered with decontam.

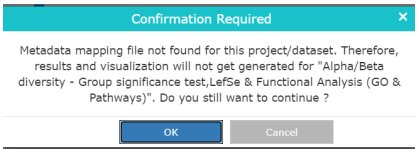


Include the 5R-PLEX positive control sample in each study project, to optimize the stringency of the decontam threshold. Bioinformatic contamination removal can be challenging if you have cross-contamination between actual samples and negative controls.

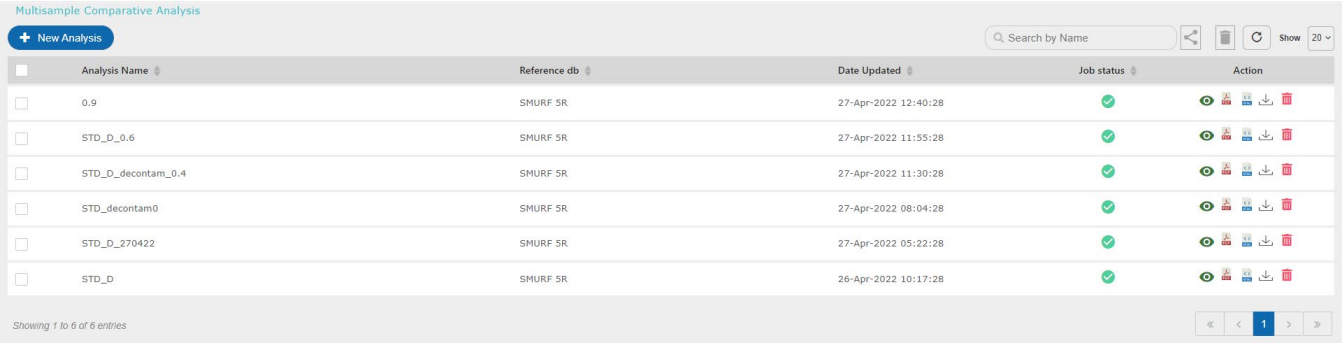
7. Example of decontamination application: The 5R-PLEX Positive Control and negative controls (NC) samples reads were filtered with decontam. Contaminating OTUs are marked in red.



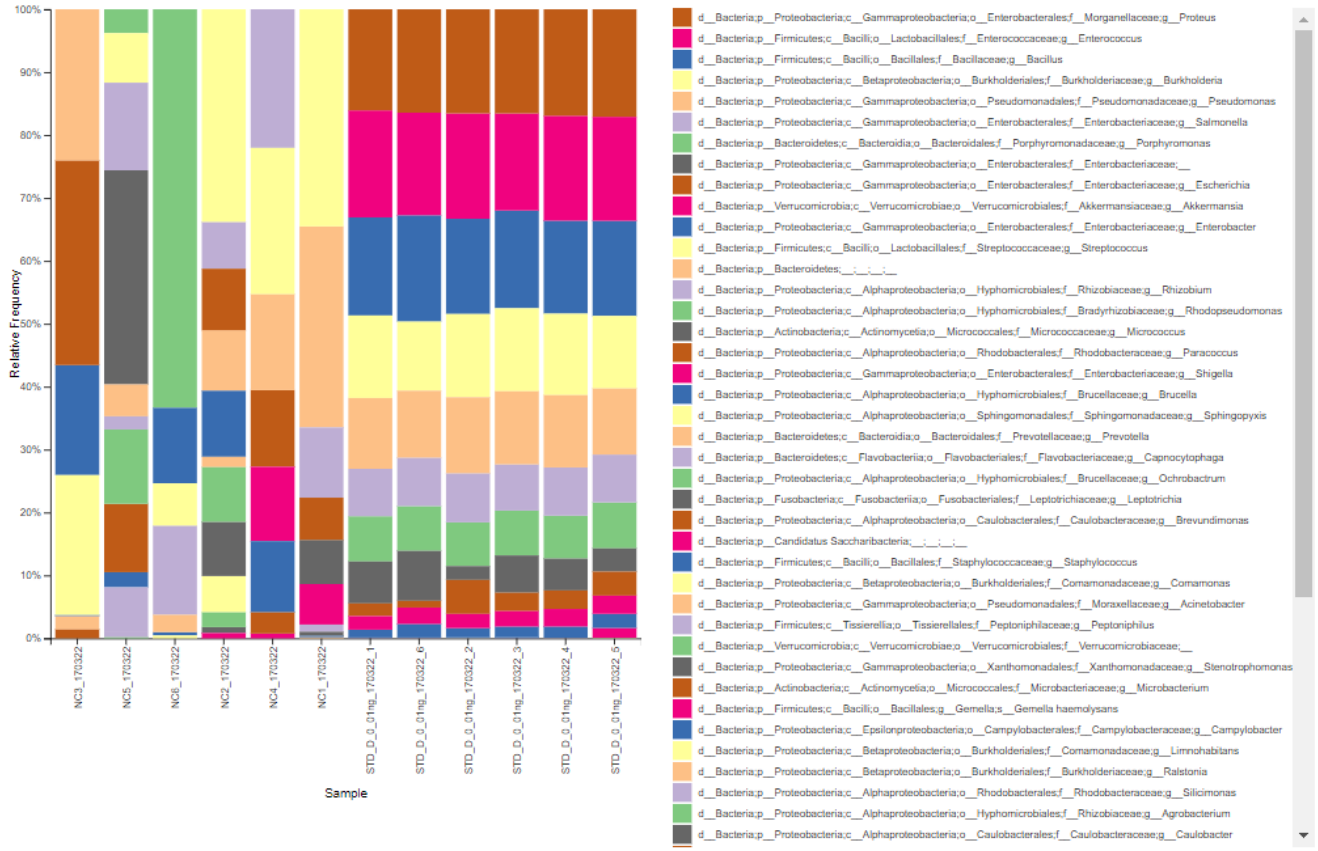
8. If a metadata file is not uploaded via "Sample Management" module, a notification will appear when submitting a comparative analysis. For projects without metadata, experimental categories in alpha- and beta-diversity will not be selected, and the statistical analysis will not be performed.



9. After completion of the Comparative Analysis, the results can be inspected online by clicking on the green eye icon. Also, all the results can be downloaded as pdf or html files. The comparative analysis online report can also be shared with another user with an App account (a share option can be found on the upper left corner).



11. Example of Stacked Bar Chart of the 5R-PLEX Positive Control (Genus Level): technical repeats of 5R-PLEX assay were done with 0.01 ng.



5R-PLEX Single Index List

5R-PLEX Index-Plate Position	Index_ID	Index_Sequence
A1	RDB1	TTGGTGCA
A2	RDB2	ACAAGCTC
A3	RDB3	CGGAGTAT
A4	RDB4	TCAGACAC
A5	RDB5	GAGTAGAG
A6	RDB6	AACCTACG
A7	RDB7	TCTTACGG
A8	RDB8	GGCATTCT
A9	RDB9	GGTACGAA
A10	RDB10	AGCCAACT
A11	RDB11	TTCGAAGC
A12	RDB12	ATCCACGA
B1	RDB13	ATCTGACC
B2	RDB14	ATTAGCCG
B3	RDB15	CTCAGAAG
B4	RDB16	GAGCAATC
B5	RDB17	ACTTGGCT
B6	RDB18	ACTCCTAC
B7	RDB19	AACACCAC
B8	RDB20	ACTCTCCA
B9	RDB21	ATAGAGCG
B10	RDB22	GGCTCAAT
B11	RDB23	TATGCGGT
B12	RDB24	TGCGATAG
C1	RDB25	CCTATTGG
C2	RDB26	CTCTTGTC
C3	RDB27	GAGTGTGT
C4	RDB28	GCATCCTA
C5	RDB29	GGATTCAC
C6	RDB30	GTTGGCAT
C7	RDB31	TACGGTCT
C8	RDB32	TTGCAACG
C9	RDB33	CGATTGGA
C10	RDB34	CTATCCAC
C11	RDB35	GTGGTATG
C12	RDB36	TAACGTCG
D1	RDB37	TAGAACGC
D2	RDB38	TATGACCG
D3	RDB39	ACGCAGTA
D4	RDB40	CGGCATTA
D5	RDB41	GAAGACTG
D6	RDB42	GGAAGAGA
D7	RDB43	TGTCAGTG
D8	RDB44	ACGGACTT
D9	RDB45	CAACCTCT
D10	RDB46	GATCTTGC
D11	RDB47	TACTAGCG
D12	RDB48	AATGACGC
E1	RDB49	AGAGGATG
E2	RDB50	GTCGTTAC
E3	RDB51	CAAGCCAA
E4	RDB52	CATGTGTG
E5	RDB53	CATTGACG
E6	RDB54	GGTTGGTA
E7	RDB55	GTATTCCG
E8	RDB56	TGGTATCC
E9	RDB57	CCGTAACT
E10	RDB58	GATACCTG
E11	RDB59	GCCTTCTT
E12	RDB60	GGCGAATA
F1	RDB61	GTATCGAG
F2	RDB62	GTTCTTCG
F3	RDB63	TCCGATCA
F4	RDB64	TTCCTCCT
F5	RDB65	GAATGGCA
F6	RDB66	GACACAGT
F7	RDB67	TACATCGG
F8	RDB68	TGGATGGT
F9	RDB69	TTGCTTGG
F10	RDB70	AGACATGC
F11	RDB71	ATGACAGG
F12	RDB72	CACTGTAG
G1	RDB73	CTGGTCAT
G2	RDB74	GGAATGTC
G3	RDB75	GGTTAGCT
G4	RDB76	TCACGATG
G5	RDB77	TCTAGGAG
G6	RDB78	TGATCACG

5R-PLEX Index-Plate Position	Index_ID	Index_Sequence
G7	RDB79	CTCAAGCT
G8	RDB80	CTTACAGC
G9	RDB81	GAACGGTT
G10	RDB82	GGTAACGT
G11	RDB83	TAGTGCCA
G12	RDB84	TGACCGTT
H1	RDB85	AAGCCTGA
H2	RDB86	AAGTCCTC
H3	RDB87	ACGAGAAC

5R-PLEX Index-Plate Position	Index_ID	Index_Sequence
H4	RDB88	AGATTGCG
H5	RDB89	CACGATTC
H6	RDB90	CCGATGTA
H7	RDB91	TCGAGAGT
H8	RDB92	GCGTATCA
H9	RDB93	TGTTCCGT
H10	RDB94	ATACTGGC
H11	RDB95	CTGCCATA
H12	RDB96	GGTGTACA

References

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2. Davis, N.M. et al. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6:226 (2018).
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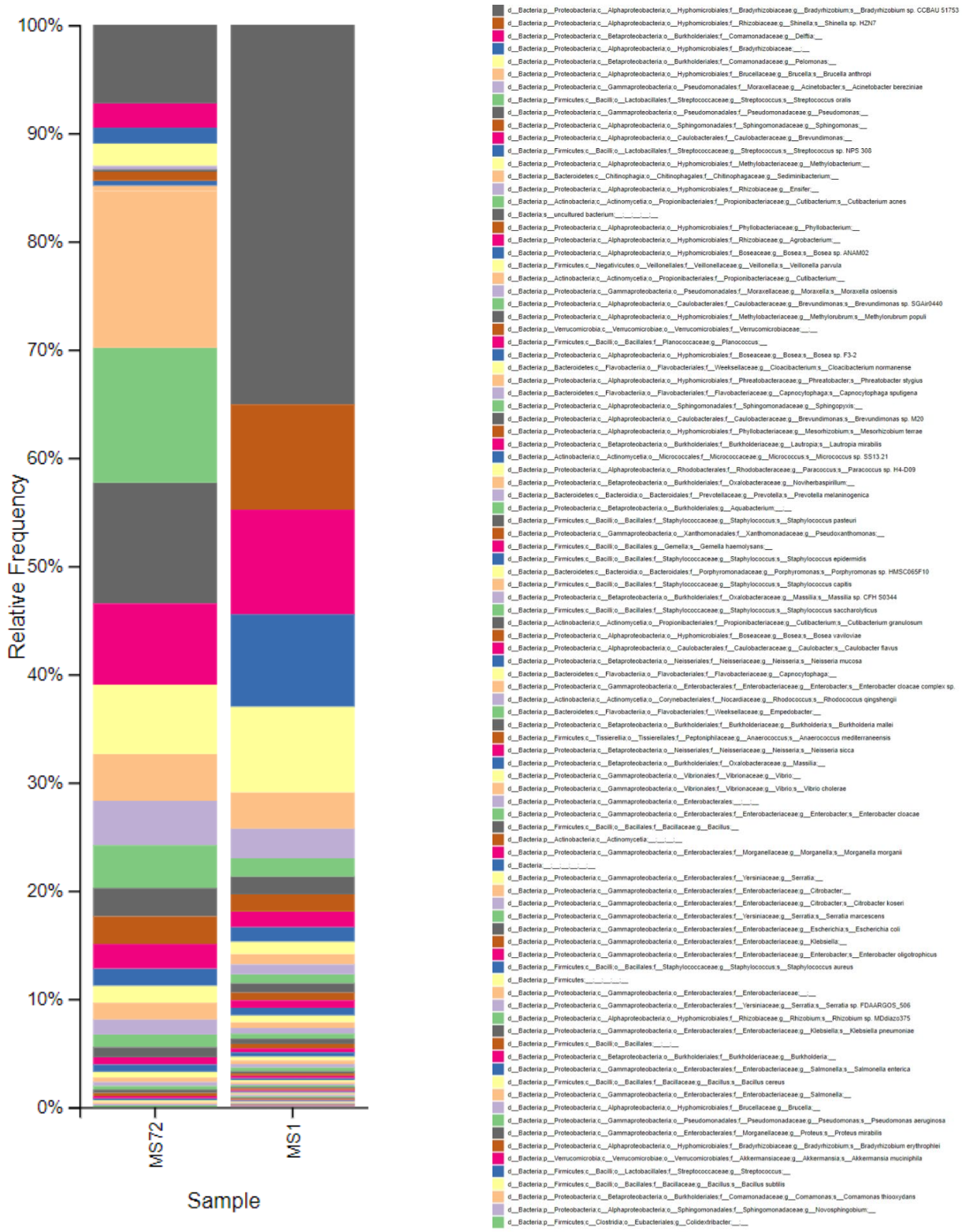
5. Nejman D, et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. Science. 368:973-980 (2020).
6. Singular Genomics Systems, Inc. Adapting Libraries for the G4™—Retaining Original Indices guide (Document #600025 Rev. 1 (July 2023). Web document. Accessed: https://techwriting.singulargenomics.com/Library-Prep/Adapting-Library-Retain-Index-600025.pdf

Application Example for Singular G4 Sequencing

G4 sequencing was performed on a library prepared with the 5R-Plex kit, with a final concentration of 54.4ng/ul. The library contained 96 samples indexed with kit's indices and was previously run on a Nextseq™ platform.

After following the protocol for Adapting Libraries for the G4™,[6] and using a 0.85X ratio for the library cleanup, the library was quantified with Qubit and QC was performed on Tapestation®. Libraries were then prepared for sequencing and run on the G4 platform using 2X150 bp, paired end sequencing with 8 bp Index 2 read, aiming for at least 200K reads per sample. The sequencing run generated 423 M reads using an F3, 300 cycle kit with Q30s between 91.9-93.9%.

For demonstration purposes, a high and low abundance sample was chosen and processed on the M-CAMP™ platform. The combination of sequencing depth that is achievable with G4, and the 5R-Plex method enables very high-resolution data. As an example, see the level 7 (species level) relative frequency of the two samples (Figure generated by the M-CAMP™ platform).



Level 7 (equivalent to species level) relative frequency of a high- and a low abundance sample sequenced on the Singular Genomics Platform and the M-CAMP™ bioinformatics algorithm.

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