16S & 18S rRNA Amplicon Sequencing Protocols

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16S rDNA amplification

- Use primers same as Earth microbiome project
  - (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards)

For 16S rRNA

PCR reagent mixture

Reagents	Volume (µl)
PCR Master Mix (2X)	12.5
Forward primer (10µM)	0.75
Reverse primer (10µM)	0.75
DNA template	1*
dH_2O	10*
Total reaction volume	**25**

*depend on DNA template conc. (ng)

PCR

Thermocyler conditions

Temp.	Time	Repeat
94 °C	3 min	
94 °C	45 s	≤28
50 °C	60 s	≤28
72 °C	90 s	≤28
72 °C	10 min	
4 °C	hold	

Primers

- For PCR (16S)

515FB forward primer, barcoded

Field descriptions (space-delimited):

1. 5′ Illumina adapter
2. Golay barcode
3. Forward primer pad
4. Forward primer linker
5. Forward primer (515FB)

AATGATACGGCGACCACCGAGATCTACACGCT XXXXXXXXXXXX TATGGTAATT GT GTGYCAGCMGCCGCGGTAA

806RB reverse primer

Field descriptions (space-delimited):

1. Reverse complement of 3′ Illumina adapter
2. Reverse primer pad
3. Reverse primer linker
4. Reverse primer (806RB)

CAAGCAGAAGACGGCATACGAGAT AGTCAGCCAG CC GGACTACNVGGGTWTCTAAT

http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/

18S rDNA amplification

- Use primers same as Earth microbiome project
  - (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards)

For 18S rRNA (without Mammal blocking primer)

PCR reagent mixture

Reagents	Volume (µl)
PCR Master Mix (2X)	12.5
Forward primer (10µM)	0.75
Reverse primer (10µM)	0.75
DNA template	1*
dH_2O	10*
Total reaction volume	**25**

*depend on DNA template conc. (ng)

Thermocyler conditions

Temp.	Time	Repeat
94 °C	3 min	
94 °C	45 s	≤28
57 °C	60 s	≤28
72 °C	90 s	≤28
72 °C	10 min	
4 °C	hold	

Primers

- For PCR (18S)

Illumina_Euk_1391f forward primer

Field descriptions (space-delimited):

1. 5′ Illumina adapter
2. Forward primer pad
3. Forward primer linker
4. Forward primer (1391f)

AATGATACGGCGACCACCGAGATCTACAC TATCGCCGTT CG GTACACACCGCCCGTC

Illumina_EukBr reverse primer, barcoded

1. Reverse complement of 3′ Illumina adapter
2. Golay barcode
3. Reverse primer pad
4. Reverse primer linker
5. Reverse primer (EukBr)

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG CA TGATCCTTCTGCAGGTTCACCTAC

http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/18s/

18S rDNA amplification

- Use primers same as Earth microbiome project
  - (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards)

For 18S rRNA (with Mammal blocking primer)

PCR reagent mixture

Reagents	Volume (µl)
PCR Master Mix (2X)	12.5
Forward primer (10µM)	0.75
Reverse primer (10µM)	0.75
DNA template	1*
Blocking primer (10µM)	4
dH_2O	6*
Total reaction volume	**25**

*depend on DNA template conc. (ng)

Thermocyler conditions

Temp.	Time	Repeat
94 °C	3 min	
94 °C	45 s	≤28
65 °C	15 s	≤28
57 °C	30 s	≤28
72 °C	90 s	≤28
72 °C	10 min	
4 °C	hold	

Primer

Mammal_block_l-short_1391f mammal blocking primer

The mammal blocking primer is to be used when there is a high probability of picking up host genomic DNA. The C3 spacer (/3SpC3/) is a chemical modification that prevents extension during the PCR. Please note that the use of blocking primer reduces the number of host sequences detected but does not completely eliminate them. Thus remaining host sequences should also be filtered out during the analysis phase. We have found blocking primers to be particularly useful for host-associated samples with a low biomass of eukaryotic DNA. Note: Sequence is formatted for ordering from IDT.

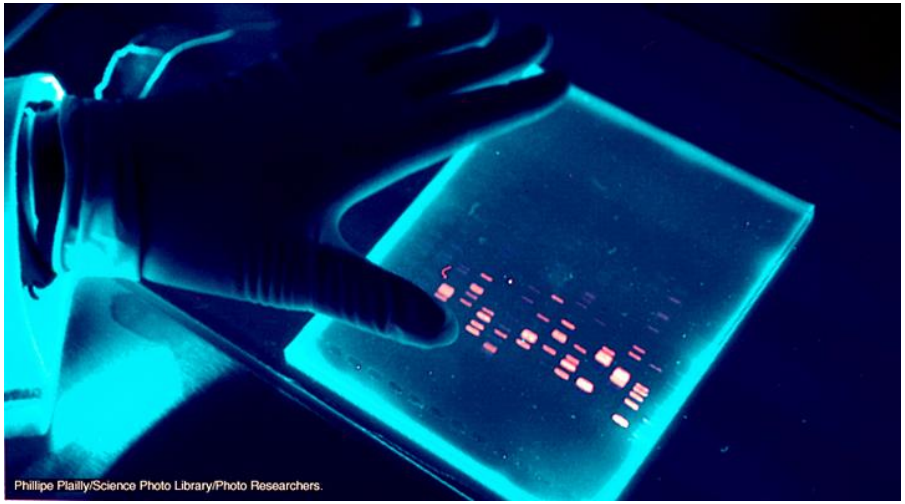
GCCCGTCGCTACTACCGATTGG/ideoxyI//ideoxyI//ideoxyI//ideoxyI//ideoxyI/TTAGTGAGGCCCT/3SpC3/

http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/18s/

Amplicon extraction

- Run agarose gel electrophoresis and cut proper-size DNA fragment
  - for 16S at ~381bp
  - for 18S at ~260bp

put a gel slice into 1.5 ml sterile tube, keep at -20°C and avoid from light (Do not keep it more than 1 week)



http://dna-researches.blogspot.com/2013/07/gel-electrophoresis.html

Amplicon (gel) purification

- Use GenepHlow™ Gel Extraction Kit or any available
  - Followed manufacturer's protocols

http://www.geneaid.com/products/gel-extraction/gel-extraction-kit-genephlow

Gel purification

Quick Protocol Diagram

QG Buffer (pH≤7.5, yellow color, premixed with pH indicator) reaction of gel slice

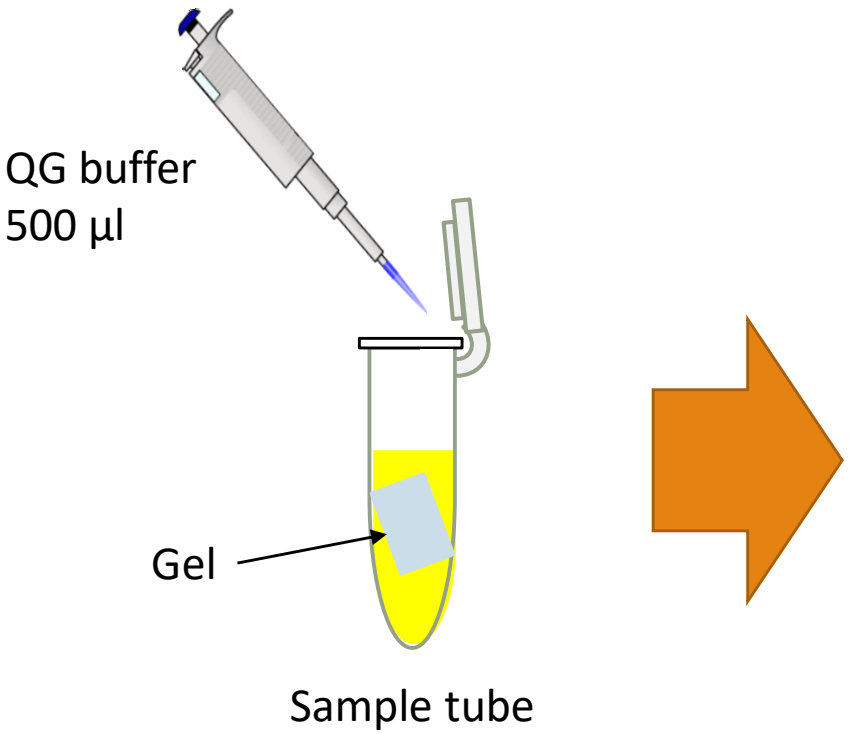
DNA binding to membrane while contaminants remain suspended

Wash (removal of contaminants while DNA remains bound to membrane)

Elution of pure DNA which is ready for subsequent reactions

Gel purification

1. Gel dissociation
   - Add 500 µl QG buffer to samples then mix by vortex

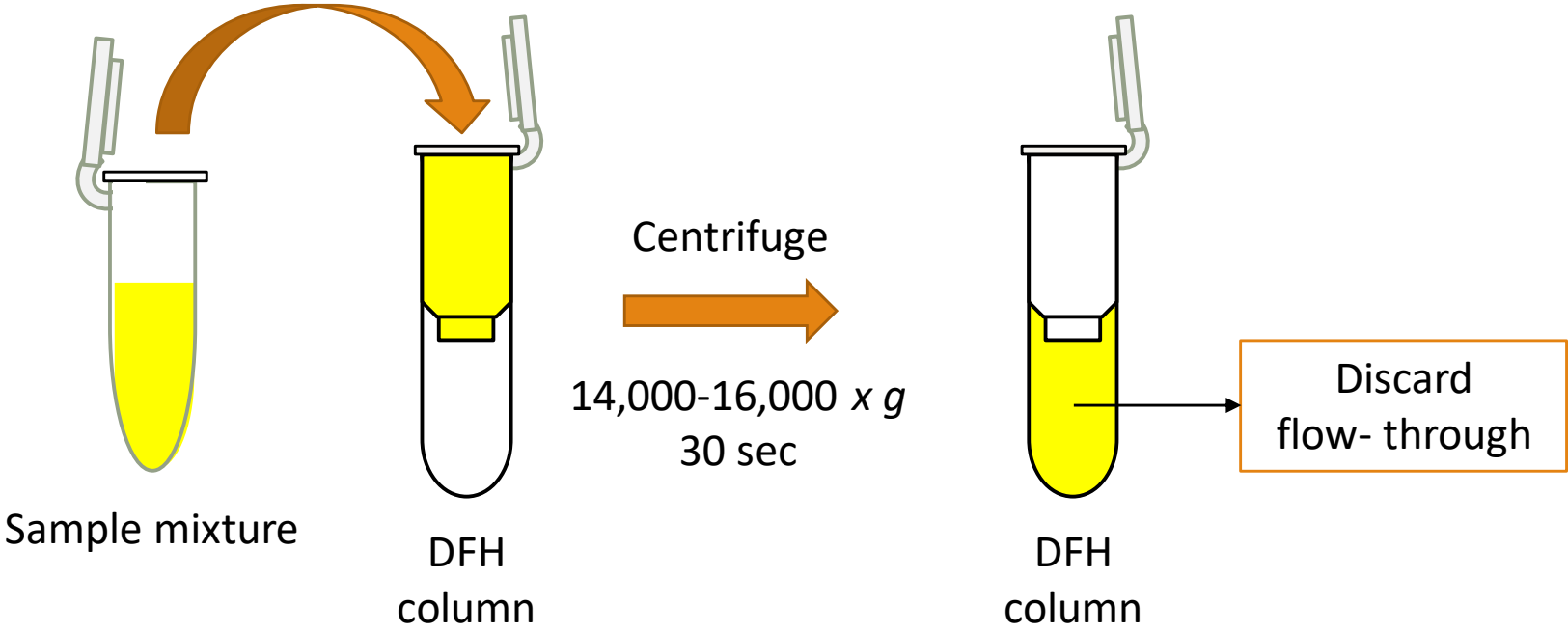


Incubate at
55-60°C for 10-15 minutes
or until the gel slice is
completely dissolved

*Invert the tube every 2-3 minutes

Gel purification

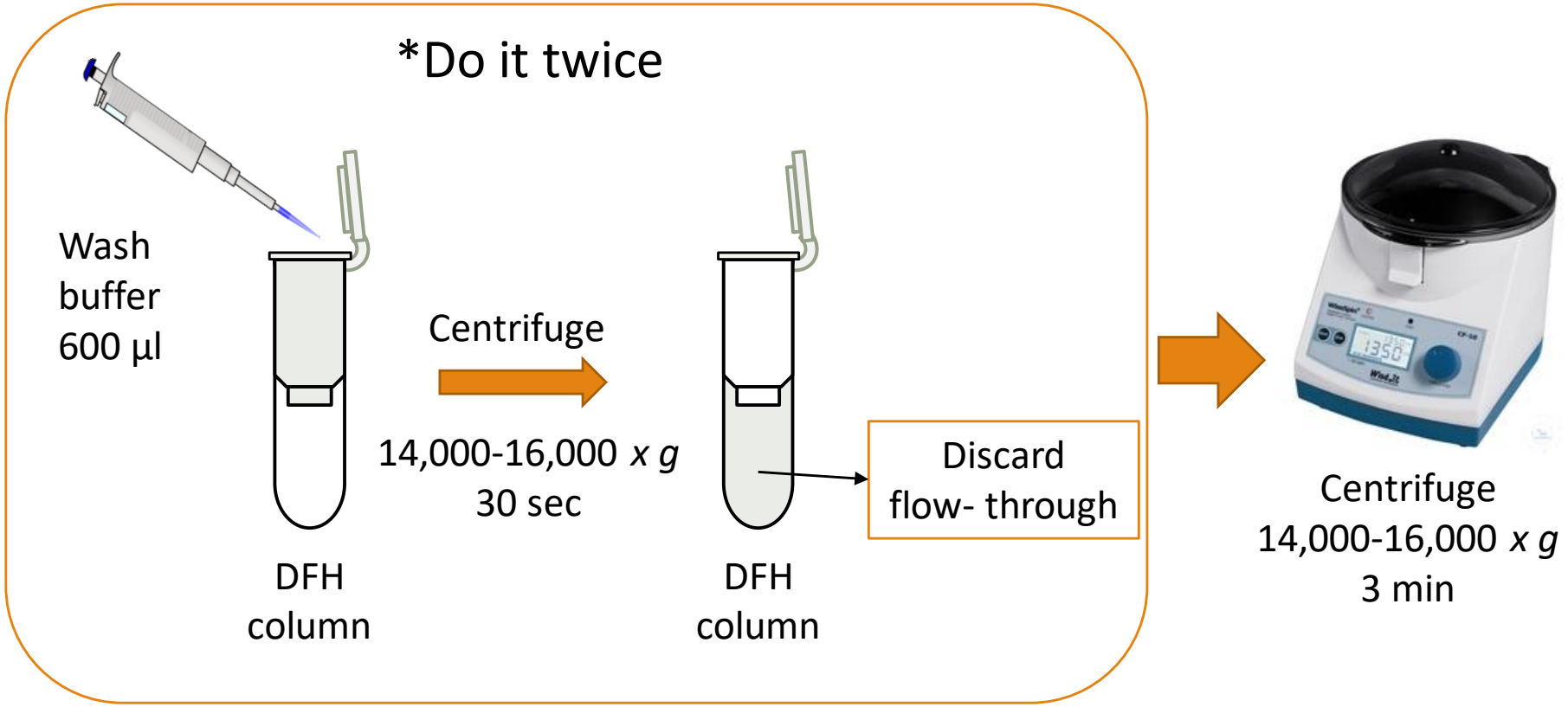
2. DNA Binding
   - Transfer 800 µl of the sample mixture to the DFH Column



*If you have more than one duplicates you can load into the same column

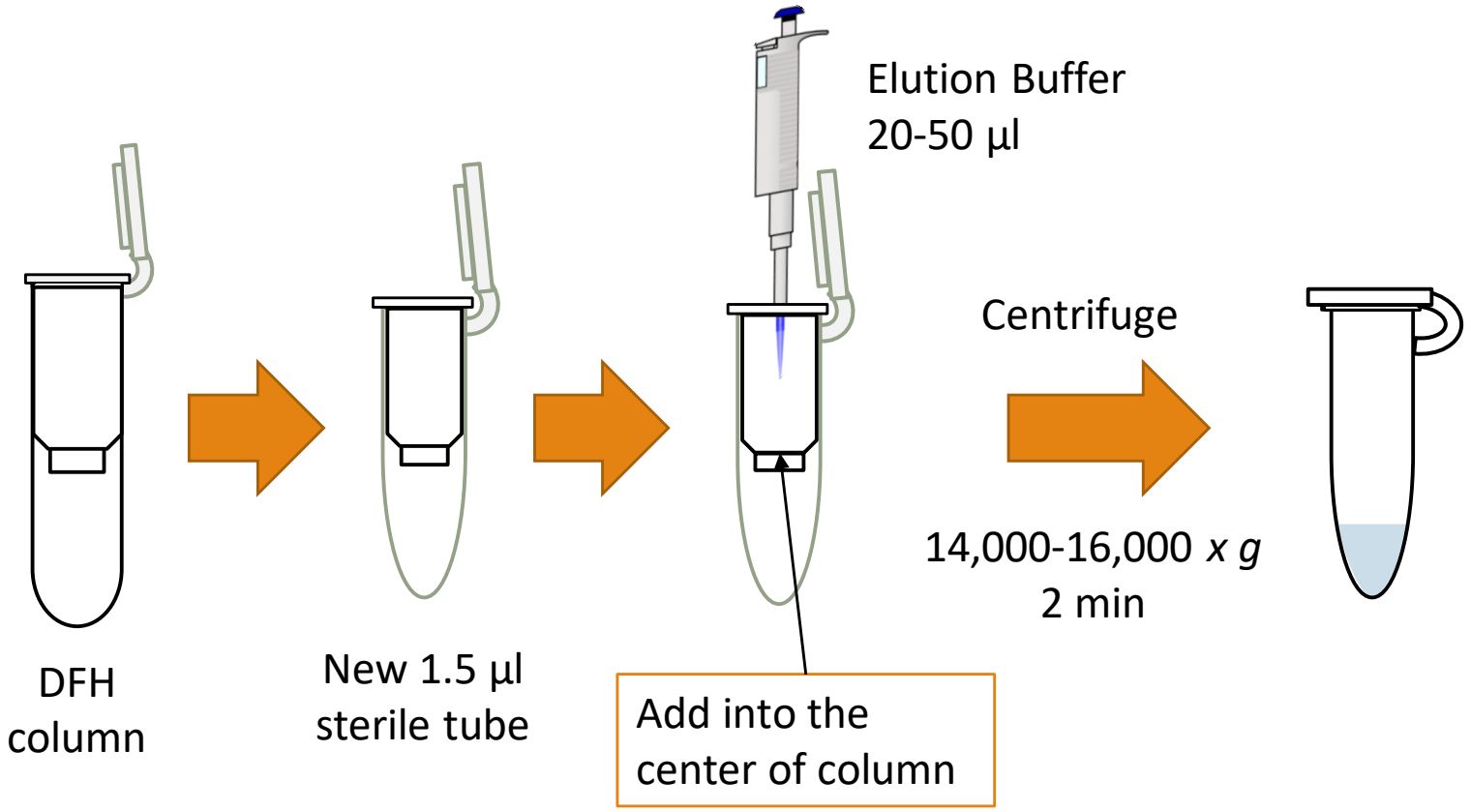
Gel purification

3. Wash
   - Add 600 µl of Wash Buffer (make sure absolute ethanol was added) into the DFH column



Gel purification

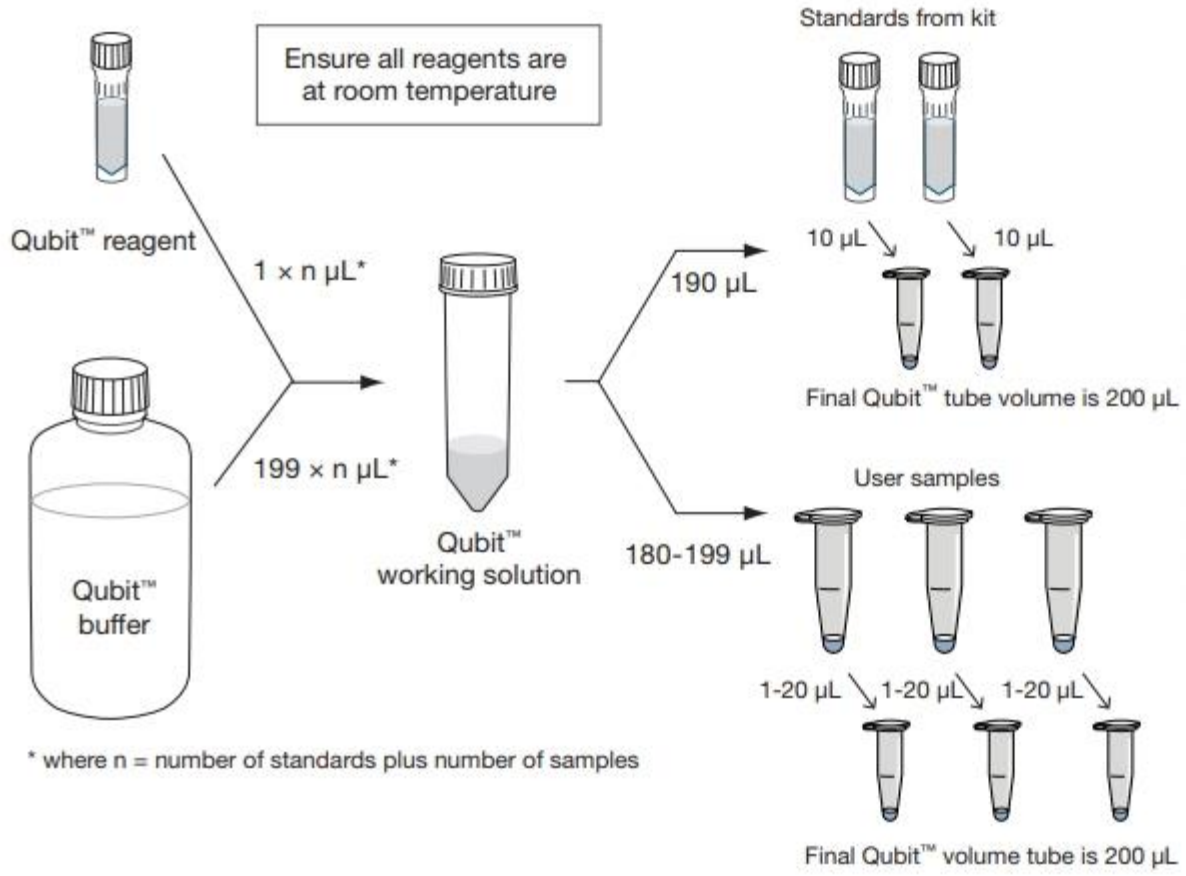
4. Elution
   - Transfer the dried DFH Column to a new 1.5 ml microcentrifuge tube



Pool sample and sequencing

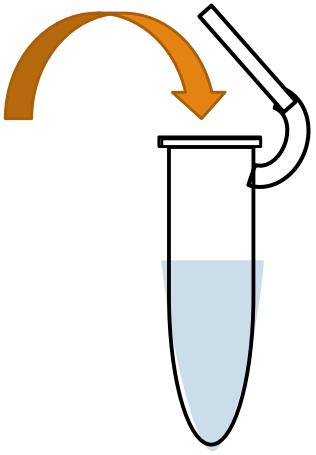
- Measure DNA concentration using Qubit fluorometer
  - DNA concentration should be equal to 240ng each

Qubit fluorometer flow chart

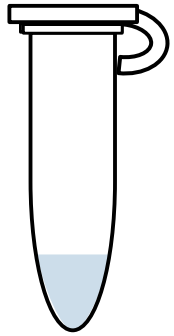


Pool samples and sequencing

- Pool all samples into one tube and Illumina sequencing with sequencing and index primers follow Caporaso *et al.*, 2012
  - Each sample was pooled at DNA concentration equal to 240 ng



Pool samples



Qubit check final concentration

Make excel sheet and Illumina sequencing

Primers

- For Sequencing

16S sequencing primers
Read 1 sequencing primer

Field descriptions (space-delimited):

1. Forward primer pad
2. Forward primer linker
3. Forward primer

TATGGTAATT GT GTGYCAGCMGCCGCGGTAA

Read 2 sequencing primer

Field descriptions (space-delimited):

1. Reverse primer pad
2. Reverse primer linker
3. Reverse primer

AGTCAGCCAG CC GGACTACNVGGGTWTCTAAT

Index sequencing primer

AATGATACGGCGACCACCGAGATCTACACGCT

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