APPENDIX

NEXT GENERATION SEQUENCING ON BACTERIAL 16S GENE

1. Extract DNA

UltraClean Microbial DNA Isolation Kit

Reagents	Storage	Volume/sample
MicroBead tubes	room	-
MicroBead solution	room	300µL
MD1	room	50µL
MD2	room	100µL
MD3	room	450µL
MD4	room	300µL
MD5	room	35µL

1. 300µL MicroBead solution is added to the MicroBead tubes.
2. Bacteria samples are collceted:
   Gram negatives: half of a 10µL loop
   Gram positives: a full 10µL loop
3. Bacteria samples are suspended in the MicroBead tubes.
4. 50µL **MD1** is added to the samples.
5. Samples are vortexed on a Vortex Adaptor at 5000Hz for 3x 30sec and spinned at max. rpm for 30 sec.

→ 5. Collection tubes are prepared with 100µL **MD2**.

6. Supernatant (~200µL) is transferred from the MicroBead tubes to the new collection tubes with the MD2 solution.
7. Samples are mixed gently.
8. Samples are put at 4 oC for 15-30 min.
9. Samples are spinned at max. rpm for 1 min.

→ 10. Collection tubes are prepared with 450µL **MD3**.

11. 200µL supernatant is transferred to the new collection tubes with the MD3 solution.
12. Samples are mixed gently.

→ 13. 650µL supernatant is transferred into the spin filters.

14. Samples are spinned at max. rpm for 30sec.

→ 15. Filters are transferred into a new collection tube.

16. 300µL **MD4** is dispensed on the filters.
17. Samples are spinned at max. rpm for 30sec.

→ 18. Filters are transferred into a new collection tube.

19. Samples are spinned at max. rpm for 1 min.

→ 20. Filters are transferred into a newcollection tube.

21. 35µL **MD5** is dispensed on the spin filters.
22. Samples are incubated at room temperature for 2 min.
23. Samples are spinned at max. rpm for 1 min.
24. Filter is removed.

→ 25. Supernatant is stored at -20 oC.

2. Measure DNA concentration

Nanodrop

Reagents	Storage	Volume/sample
Nuclease-free Water	room	2µL

Additional components	Volume
DNA	1µL

1. Contact point is cleaned with a tissue.
2. "Measure nucleic acid" option is chosen for Nanodrop.
3. 2µL Nuclease-free Water is put on the contact point - to measure blank.
4. Contact point is cleaned with a tissue.
5. 1µL of the DNA are put on the contact point - to measure samples.

3. Amplify 16S

PCR | 1µL → 50µL

Ion16S Metagenomics Kit

Reagents	Color	Storage	Volume/sample
DNA dilution buffer	transparent	2 ~ 8	18µL
16S Primer Set V2-4-8		-25 ~ -15	5µL
16S Primer Set V3-6-7-9		-25 ~ -15	5µL
Negative Control Water	white	2 ~ -8	19µL
E.coli DNA control		-25 ~ -15	2µL

Additional components	Color	Storage	Volume/sample
Amplitaq Gold		-25 ~ -15	25µL
DNA: 2,5-5ng/µL	-	-	1µL

→ 1. DNA is diluted with Nuclease-free water to the concentration of 2,5-5ng/µL.
→ 2. **E.coli DNA contol** is diluted 1:10 - 2µL E.coli DNA control is dissolved in 18µL **DNA Dilution Buffer**.
→ 3. For each sample 2 PCR tubes are prepeared: one tube for the 16S Primer Set V2-4-8 and an other tube for the 16S Primer Set V3-6-7-9 reagents:

Component	Volume
Negative Control Water	19µL
16S Primer Set (V2-4-8 / V3-6-7-9)	5µL
Amplitaq Gold	25µL
DNA (dilutedsample/diluted control)	1µL
	Σ50µL

4. Tubes are placed in the PCR and the following program is runned:

	Stage	Temperature	Time
	Holding	95 oC	10 min
18 cycles	Cycling	95 oC	30 sec
		58 oC	30 sec
		72 oC	20 sec
	Holding	72 oC	7 min
	Holding	4 oC	∞
			Σ 41 min

4. Purification for 16S

Magnetic rack | 2x50µL → 20µL

Reagents	Storage	Volume/sample
Agencourt AM Pure XP*	fridge	72µL
Ethanol**	fridge	600µL
Nuclease-free Water	room	15µL

*Agencourt AM Pure XP reagent is warmed up for room temperature before the purification process.
**70% Ethanol is prepared: 600µL/sample.

Additional components	Storage	Volume/sample
Nuclease-free Water	-25 ~ -15	15µL
DNA	-	20+20µL

→ 1.The primer sets of each sample are combined in a 1,5mL tube: 20µL 16S Primer Set V2-4-8 and 20µL 16S Primer Set V3-6-7-9.
2. 72µL (1.8x of the sample volume) **Agencourt AM Pure XP Reagent** is added.
3. Samples are vortexed and spinned briefly.
4. Samples are incubated at room temperature for 5 min.
5. Samples are placed at a magnetic rack for 3 min.
6. Supernatant is removed by a 200µL pipette without disturbing the magnetic beads.

Repeat
7. 300µL **70% ethanol** is added to the samples - tubes are turned on the magnetic rack.
8. Supernatant is removed by a 1000µL pipette without disturbing the magnetic beads.

9. samples are spinned, placed back on the magnetic rack and the residual supernatant is removed by a 20µL pipette.
10. Samples are air-dried for 4 min.
11. Samples are removed from the magnetic rack and 15µL **Nuclease-free Water** is added.
12. Samples are vortexed for 5-10 sec, spinned briefly and placed back to the magnetic rack for 1 min.
→ 13. Supernatant is removed by 20µL pipette without taking any magnetic beads and placed in a 1,5 mL tube.

5. Measure DNA concentration

Qubit 2.0 Fluorometer | 20µL → 18µL

Qubit dsDNA HS Assay Kits

Reagents	Storage	Volume/sample
Qubit dsDNA HS Reagent	-25 ~30	1µL
Qubit dsDNA HS Buffer	-25 ~30	199µL
Qubit dsDNA HS Standard 1	-25 ~8	10µL
Qubit dsDNA HS Standard 2	-25 ~8	10µL

Additional components	Storage	Volume/sample
DNA	-	2µL

→ 1. Qubit working solution is prepared by diluting **Qubit dsDNA HS Reagent** with **Qubit dsDNA Buffer** in a ratio of 1(reagent) : 200(buffer) - 200µL working soultion/sample.
→ 2. Qubit tubes are prepared for 2 standards and for the samples:
Standards: 190µL Qubit working solution + 10µL Qubit dsDNA HS Standard1/2
Samples: 198µL Qubit working solution + 2µL DNA sample.
3. Tubes are vortexed and spinned briefly.
4. Tubes are incubated at room temperature for 2 min.
5. Tubes are placed in the Qubit 2.0 Fluorometer in the following order: Standard 1 - Standard 2 - Samples.
6. Concentration of the original samples are calculated since here diluted samples were measured - results are multiplied by 100.

6. End repair

2-7µL → 100µL

Ion Plus Fragment Library Kit

Reagents	Color	Storage	Volume/sample
5X End Repair Buffer		-30 ~ -10	20µL
End Repair Enzyme		-30 ~ -10	1µL

Additional components	Storage	Volume/sample
Nuclease-free Water	room	to 79µL
DNA: 10-100ng	-	~2-7µL

→ 1. Dilute the DNA with Nuclease-free Water in a way that the solution's volume is 79µL and contains 10-100 ng DNA.
2. The following components are added to the diluted DNA:

Component	Volume
5X End Repair Buffer	20µL
End Repair Enzyme	1µL
	Σ 100µL

3. Sample is mixed and spinned briefly.
4. Sample is incubated at room temperature for 20 min.

7. Purification for 16S

Magnetic rack

2x50µL → 20µL

Reagents	Storage	Volume/sample
Agencourt AM Pure XP*	5	72µL
Ethanol**	5	1000µL
Nuclease-free Water	room	15µL

*Agencourt AM Pure XP reagent is warmed up for room temperature before the purification process.
**70% Ethanol is prepared: 1000µL/sample.

Additional components	Color	Storage	Volume/sample
Low TE*	transparent	-30 ~ -10	25µL
DNA	-	-	20+20µL

* From Ion Plus Fragment Library Kit.

1. 180µL (1,8 X of the sample volume) **Agencourt AM Pure XP Reagent** is added to the samples.
2. Samples are vortexed and spinned briefly.
3. Samples are incubated at room temperature for 5 min.
4. Samples are placed at a magnetic rack for 3 min.
5. Supernatant is removed by a 1000µL pipette without disturbing the magnetic beads.

Repeat

6. 500µL **70% ethanol** is added to the samples - tubes are turned on the magnetic rack.
7. Supernatant is removed by a 1000µL pipette without disturbing the magnetic beads.

8. Samples are spinned, placed back on the magnetic rack and the residual supernatant is removed by a 20µL pipette.
9. Samples are air-dried for 4 min.
10. Samples are removed from the magnetic rack and 25µL **Low TE** is added.
11. Samples are vortexed for 5-10 sec, spinned briefly and placed back to the magnetic rack for 1 min.
→ 12. Supernatant (23-25µL) is removed by a 100µL pipette without taking any magnetic beads and placed in a 200µL PCR tube.

8. Ligation / Library preparation

PCR

25µL → 100µL

Ion Xpress Barcode Adapters 1-16 Kit

Reagents	Color	Storage	Volume/sample
Ion P1 Adapter		-30 ~ -10	2µL
Ion Xpress Barcode*	white	-30 ~ -10	2µL

* For each sample different barcode.

Ion Plus Fragment Library Kit

Reagents	Color	Storage	Volume/sample
10X Ligase buffer		-30 ~ -10	10µL
dNTP Mix		-30 ~ -10	2µL
DNA ligase		-30 ~ -10	2µL
Nick Repair Polymerase	transparent	-30 ~ -10	8µL

Additional components	Color	Storage	Volume/sample
Nuclease-free Water	white	-16	49µL
DNA	-	-	25µL

1.Nuclease-free water is added to the DNA to have a volume of 25µL.
2. The reagents are added to the DNA in the following order:

Component	Volume
10X Ligase buffer	10µL
Ion P1 Adapter	2µL
Ion Xpress Barcodes	2µL
dNTP Mix	2µL
Nuclease-free Water	49µL
DNA Ligase	2µL
Nick Repair Polymerase	8µL
	Σ 100µL

3. Samples are vortexed and spinned briefly.
4. Tubes are placed in the PCR and the following program is runned:

Stage	Temperature	Time
Hold	25 oC	15 min
Hold	72 oC	5 min
Hold	4 oC	∞
		Σ 20 min

→ 5. Samples are transferred into a 1,5 mL tube

9. Purification fof the library

Magnetic rack | 100µL → 20µL

Reagents	Storage	Volume/sample
Agencourt AM Pure XP*	8	140µL
Ethanol**	5	1000µL
Nuclease-free Water	room	15µL

*Agencourt AM Pure XP reagent is warmed up for room temperature before the purification process.
**70% Ethanol is prepared: 1000µL/sample.

Additional components	Color	Storage	Volume/sample
Low TE*	transparent	-30 ~ -10	20µL
DNA	-	-	100µL

* From Ion Plus Fragment Library Kit.

1. 140µL (1.4x of the sample volume) **Agencourt AM Pure XP Reagent** is added to the samples.
2. Samples are vortexed and spinned briefly.
3. Samples are incubated at room temperature for 5 min.
4. Samples are placed at a magnetic rack for 3 min.
5. Supernatant is removed by a 1000µL pipette without disturbing the magnetic beads.

Repeat

6. 500µL **70% ethanol** is added to the samples - tubes are turned on the magnetic rack.
7. Supernatant is removed by a 1000µL pipette without disturbing the magnetic beads.

8. Samples are spinned, placed back on the magnetic rack and the residual supernatant is removed by a 20µL pipette.
9. Samples are air-dried for 4 min.
10. Samples are removed from the magnetic rack and 20µL **Low TE** is added.
11. Samples are vortexed for 5-10 sec, spinned briefly and placed back to the magnetic rack for 1 min.
12. → Supernatant is removed by a 20µL pipette and transferred into a 1,5 mL tube.

10. Determination of Library concentration

qPCR | 20µL → 18µL

Ion Universal Library Quantitation Kit

Reagents	Color	Storage	Volume/standard
E.coli DH10B Control Library		-15 ~ -25 oC	5µL
TaqMan Fast Universal PCR Master Mix		2-8 oC	10µL
Ion Library TaqMan Quantitation Assay, 20X		-15 ~-25 oC	1µL

Additional components	Storage	Volume/sample
Nuclease-free Water	room	45/18µL
DNA	-	2µL

Serial dilution of E.coli Control Library

1. **E.coli DH10B Control Library** is vortexed and spinned briefly.
2. Serial dilutions are prepared in 4 dilutions for 2 standard series:

	DNA			
Dilutions	Standard - E.coli	Nuclease-free Water	Dilution factor	Concentration
1*	5µL from Control L.	45µL	1:10	6,8 pM
2*	5µL Dilution 1	45µL	1:100	0,68 pM
3*	5µL Dilution 2	45µL	1:1000	0,068 pM
4*	5µL Dilution 3	45µL	1:10000	0,0068 pM

*Mixed before aliquots are taken for the next dilution.

Serial dilution of the samples

1. Serial dilutions are prepared from the samples:

These dilutions will be used for the qPCR

	DNA		
Dilutions	Sample	N-free Water	Dilution factor
1*	2µL	18µL	1:10
2*	5µL Dilution 1	45µL	1:100
3*	5µL Dilution 2	45µL	1:1000
4*	5µL Dilution 3	45µL	1:10000

*Mixed before aliquots are taken for the next dilution.

Preparation of 96 well tubes for qPCR

1. 15 µL/sampel Reaction mix is prepared in a DNA-free chamber:

Component	Volume/sample or standard
TaqMan Fast Universal PCR Master Mix	10µL
Ion Library TaqMan Quantitation Assay, 20X	1µL
Nuclease-free Water	4µL
	Σ 15µL

2. Reaction mix is briefly vortexed and spinned.
3. 15µL reaction mix is pipetted to the 96 well plates - for the reaction of the standards (8) for the blanks (2) and for the samples.
4. The following are added to the 96 well plate:
   - 5µL of Nuclease-free Water is added to the blank tubes
   - 5µL of the standard dilutions (4 dilutions/standard) to the satndard tubes
   - 5µL of the sample dilution (2 dilutions/sample) to the sample tubes

   Standards and the sampes are placed in the following way into the 96 well plate:

	A	B	C	D	E	F
1	Blank -a	Blank -b		Sample 1 (Dilution 3)	Sample 2 (Dilution 3)	.....
2	Standard 1 -a	Standard 1 -b		Sample 1 (Dilution 4)	Sample 2 (Dilution 4)	.....
3	Standard 2 -a	Standard 2 -b				
4	Standard 3 -a	Standard 3 -b				
5	Standard 4 -a	Standard 4 -b				

5. 96 plate is sealed and spinned.

qPCR

1. qPCR is programmed in the following way:
   E.coli library sandard's concentrations are entered: 6.8 pM, 0.68 pM, 0.068 pM, 0.0068 pM.
   Passive reference dye: ROX Reference Dye
   TaqMan probe reporter/quencher: FAM dye/MGB
   Reaction volume: 20µL
2. The following program is runned:

	Stage	Temperature	Time
	Hold optional	50 oC	2 min
	Hold optional	95 oC	20 sec
40 cycles	Cycle	95 oC	1sec
		60 oC	20 sec
	Hold optional	50 oC	2 min
	Hold optional	95 oC	20 sec
40 cycles	Cycle	95 oC	3 sec
		60 oC	30 sec

Calculation of library concentration

1. From the Ct values and the standards' concentrations the undiluted samples concentration is calculated.
2. Dilution factor of thew samples is calculated for 10 pM/L.

11. Addition of ISPs — Ion One Touch 2

Ion PGM Template OT2 **Supplies** 400

Instrument	Storage	Amount per run
Reagent Tubes	room	2
Recovery Router	room	2
Recovery Tubes	room	1
Amplification Plate	room	1
Sipper Tubes	room	2
Cleaning Adaptor	room	1

Ion PGM Template OT2 **Solutions** 400

Reagents	Storage	Volume needed	Placed on
Ion One Touch Oil (450 mL size)	room	1/2 of Reagent Tube	room
Recovery Solution	room	1/4 of Reagent tube	room
PCR Reagent B	room	285 µL	room
Reaction Oil (27-mL size)	room	500 µL	room
Wash Solution	room	500 µL	room

Ion PGM Template OT2 **Reagents** 400

Reagents	Color	Storage	Volume needed	Placed on
Reagent Mix		-30 ~ -10 oC	500 µL	room
Enzyme Mix		-30 ~ -10 oC	50 µL	ice
Ion Sphere Particles		-30 ~ -10 oC	100 µL	room
Reagent X	white	-30 ~ -10 oC	40 µL	ice

Instrument	Amount per run
Reaction Filter Assembly	1

Initialization of the machine

1. Install 2 Reagent tube: for **Oil** - 1/2 full tube, for **Recovery Solution** - 1/4 full tube.
2. New recovery tubes and the recovery router is installed.
3. New amplification plate is inserted - syringe is guided into the centrifuge's lid.
4. Sample preparation
   1. Samples are diluted with Nuclease-free water for 10 pM concentration.
   2. Equal amount of samples are put together into an Eppendorf LoBind tube - the final volume is 25 µL.
   → 3. Amplification solution is prepared in a 1,5 mL Eppendorf LoBind tube in the following order:

Component	Volume	Preparation
1. **Reagent mix**	500 µL	30 sec vortex, 2 sec spin
2. **PCR Reagent B**	285 µL	1 min vortex, 2 sec spin
3. **Enzyme Mix**	50 µL	2 sec spin
4. **Reagent X**	40 µL	2 sec spin
5. Diluted library	25 µL	

   4. Amlification solution is vortexed and spinned briefly.
   5. **Ion Sphere Particles** are prepared: 1 min vortex, 2 sec spin
   6. 100 µL Ion Sphere Particles are added to the amplification solution.
   7. Amplification solution is vortexed briefly.

   The amplification run in the machine has to start within 15 min!

   8. Amplification solution is pipetted up and down and pipettetted by a 1000 µL tip into the Reaction Filter Assembly.
   9. 500 µL **Reaction Oil** is pipetted into the Reaction Filter Assembly.
5. Reaction Filter Assembly is placed on the machine.
6. The machine is set for the "Run"mode, the right Kit and "Assisted" mode is chosen.
7. Enrichment of the library ~ 8 hours.

Samples have to be removed within 16 hours after the run started!

8. Samples are spinned in the machine: "Options" → "Final spin" ~10 min.
9. Recovery tubes are placed on a rack and the supernatant is removed in a way that 100 µL stays in the tubes and the ISP pellett is not disturbed.
10. 500 µL **Wash soulution** is added to each Recovery tube, then the ISPs are dissoved by pipetting up and down th solution.
11. The content of the Recovery tubes is transferred into a 1,5 mL Eppendorf LoBind tube.

ISPs can be stored at 2-8 oC max. for 3 days!

12. ISPs are heated at 50 oC for 2 min. And spinned at max speed for 2,5 min.
13. Supernatant is removed in a way that 102 µL stays in the tubes and the ISP pellett is not disturbed.

Cleaning of the machine

1. The machine is set to "clean" mode - Instruction om the machine's display:
   - There is >20 mL oil in the reagent tube.
   - Amplification plate stays in the machine, but the attached syringe is removed from the lid and guided into a waste tube.
   - Reaction Filter Assembly is removed and a new claning adapter is installed.
2. Cleaning run ~ 14 min.
3. Amplification Plate is removed, the heatblock left open.
4. Cleaning adaptor stays on the machine between runs.
5. The oil in the centrifuge chamber is wiped with a tissue.

12. Quality control of ISPs

Qubit 2.0 Fluorometer

Ion Sphere Quality Control Kit	Reagent	Storage	Amount / sample
	Alexa Fluor 488	?	
	Alexa Fluor 647	?	
	Ion Probes	?	1 µL
	Annealing Buffer	?	19 µL
	Quality Control Wash Buffer	?	800 µL

Ion PGM Template OT2 Solutions 400	Reagent	Storage	Amount / sample
	Ion OneTouch Wash Solution	?	up to 100 µL

1. **Alexa Fluor 488 & 647** are vortexed and spinned briefly.
2. Quibit → Ion → Alexa Fluor 488 : reagent is placed in Qubit - RFU value is recorded.
   → Alexa Fluor 647 : reagent is placed in Qubit - RFU value is recorded.
3. Sample volume is adjusted to 100 µL with **Ion OneTouch Wash Solution.**

→ 4. The followings are added to a 200 µL PCR tube:

Sample	2 µL
Annealing Buffer	19 µL
Ion Probes	1 µL
	Σ 22 µL

5. The content of the PCR tube is mixed, pipetted up and down.
6. The following PCR program is runned:

Stage	Temperature	Time
Hold	95 oC	2 min
Hold	37 oC	2 min
		Σ 4 min

Repeat 2X
7. 200 µL of **Quality Control Wash Buffer** is added to the PCR tube.
8. PCR tube is vortexed and centrifuged at 15,500 × g for 1.5 minutes.
9. Supernatant is removed in a way that the ISP pellet is not disturbed.

→ 10. The remaining 10 µL sample is transferred into a Qubit tube.
11. 190 µL of Quality Control Wash Buffer is added to the sample and pipetted up and down.
→ 12. Negative control tube is prepared: 200 µL of Quality Control Wash Buffer is added to a Qubit tube.
13. Negative control and the sample is read by Qubit: Quibit " Ion " Alexa Fluor 488/647 - RFU values are recorded.
The Alexa Fluor 488 value must be >100 counts.
14. RFU values are filled in the Qubit 2.0 Easy Calculator Microsoft Excel Spreadsheet. (Calibration factor = 0,48)
15. Quality of the sample is read as the followings:

Percent Templated ISPs	Description
<10%	Sample contains an insufficient number of templated ISPs to achieve optimal loading density on the Ion Chip.
10–30%	Optimal amount of library.
>30%	Sample will yield multi-templated ISPs (mixed reads).

Qubit® 2.0 Fluorometer observation	Ion PGM™ System observation	Possible cause	Recommended action
<10% Templated ISPs	• Lower loading • Lower % enriched • Lower key signal • Lower throughput	Too little library input into template preparation	• Increase library input to target 20–25% templated ISPs. *or* • Continue with sequencing; expect lower throughput.
>30% Templated ISPs, but <70%	Increased number of filtered reads	Too much library input into template preparation	• Decrease library input to target 20–25% templated ISPs. *or* • Continue with sequencing; expect lower throughput.
>70% Templated ISPs	• Increased % primer dimer filtered reads • Lower throughput	Adapter dimer contaminating library, more likely in short amplicon, Ion AmpliSeq™ or miRNA libraries	• Check Bioanalyzer® traces for adapter dimer peak (Amplicon library or Ion AmpliSeq™ library peak around 70 bp; miRNA library peak around 60bp). • Re-purify Agencourt® library using AMPure® XP Kit clean-up steps as outlined in the appropriate user guides.
	• Low loading • Low % enriched • Lower throughput • High % filtered reads	Ion OneTouch™ 2 Instrument underperformance	Troubleshoot with Technical Support or a Field Application Scientist.

13. Enrichment of ISPs — Ion One Touch ES

Ion PGM™ Template OT2 Supplies 400

Instrument	Amount per run
8-well strip	1
Eppendorf LoRetention Dualfilter Tips	1

Ion PGM™ Enrichment Beads

Reagents	Color	Storage	Volume needed
Dynabeads MyOne Streptavidin C1 Beads		2-8 oC	13 µL

Ion PGM Template OT2 Solutions 400

Reagents	Color	Storage	Volume needed
Ion OneTouch Wash Solution	-	room	900 µL
Neutralization solution		room	
Tween Solution	-	room	280 µL
MyOne Beads Wash Solution		room	130 µL

Additional components	Volume
1M NaOH*	40 µL

* Freshly prepared with Nuclease-free water.

Residual volume test

1. New pipette tip is installed.
2. 80 µL water is loaded into Well 2 - the 8Well strip is placed then in the tray to the right side (square edge is on the left).
3. Run is started: when Well 2 is empty, the 8 Well strip is pushed to the left side.
4. When the solution is loaded into Well 4 the run is stopped.
5. The residue in Well 2 has to be <2 µL.

Preparation of the 8 Well strip →

→ 1. Melt off solution is prepared in the following order:

Tween Solution 280 µL
1M NaOH 40 µL

→ 2. **Dynabeads MyOne Streptavidin C1 Beads** are vortexed for 30 sec and spinned for 2 sec.
3. Dynabeads MyOne Streptavidin C1 Beads are pipetted up and down and 13 µL of it is put into a 1,5 mL Eppendorf LoBind tube.
4. The tube is placed on a magnetic rack for 2 min, then the supernatant is removed.
5. 130 µL of **MyOne Beads Wash Solution** is added to the tube.
6. Tube is vortexed for 30 sec and spinned for 2 sec.
7. the 8 Well strip is filled in the following way:

Well 1: 100 µL of template-positive ISP sample.
Well 2: 130 µL of Beads in the Wash Solution.
Well 3: 300 µL **Ion OneTouch Wash Solution**
Well 4: 300 µL Ion OneTouch Wash Solution
Well 5: 300 µL Ion OneTouch Wash Solution
Well 6: -
Well 7: 300 µL of Melt-off solution
Well 8: -

Enrichment of ISPs

1. A new pipette tip and a 200 µL PCR tube are installed.
2. 10 µL of Neutralization solution is added to the PCR tube.
3. 8 Well stip is placed dto the right side (square edge is on the left) and the magnetic beads are pipetted up and down before the run.
4. Start the run: ~ 35 min.

14. Sequencing — Ion Torrent PGM

Ion PGM Sequencing Supplies 400

Reagents	Storage
Wash Bottle Sipper Tubes	room
Reagent Bottle Sipper Tubes	room
Reagent Bottles (50 mL)	room
Wash 1 Bottle (250 mL)	room
Wash 2 Bottle (2 L)	room
Wash 3 Bottle (250 mL)	room

Ion PGM Sequencing Reagents 400

Reagents	Color	Storage	Volume needed
Ion PGM Sequencing 400 dGTP		-30 ~-10 oC	20 µL
Ion PGM Sequencing 400 dCTP		-30 ~-10 oC	20 µL
Ion PGM Sequencing 400 dATP		-30 ~-10 oC	20 µL
Ion PGM Sequencing 400 dTTP		-30 ~-10 oC	20 µL
Ion PGM Sequencing 400 Polymerase		-30 ~-10 oC	1 µL
Sequencing Primer	white	-30 ~-10 oC	3 µL
Control Ion Sphere Particles	transparent	-30 ~-10 oC	5 µL

Ion PGM Sequencing Solutions 400

Reagents	Storage	Volume needed
Ion PGM Sequencing 400 W2 Solution	2-8 oC	whole bottle
Ion PGM Sequencing 400 1X W3 Solution	2-8 oC	50mL
Ion PGM Cleaning Tablet	2-8 oC	1 tablet
Annealing Buffer		100 µL

Ion 314™ Chip Kit v2

Instrument	Amount per run
Ion 314 Chip v2	1

Ion PGM™ Controls Kit

Reagents	Color	Storage	Volume needed
Ion Sphere Test Fragments			5 µL

Additional components	Storage	Volume/sample
1 M NaOH*	room	1 mL
100 mM NaOH*	room	420 µL

* Freshly prepared with Nuclease free / 18 MΩ water

Clean with:	Schedule:
18 MΩ water	• Daily, when instrument is in use (e.g., not necessary on weekends) • After ≤1000 flows (e.g., a single 400-base-read run or 2 × 200-base-read runs) • If more than 27 hours but less than 48 hours have elapsed between the last cleaning/initialization and the start of a run • If you cleaned with chlorite a week ago and have not used the instrument since then
Chlorite solution	• Once a week, unless the instrument has not been used since the last chlorite cleaning (in which case, clean with 18 MΩ water before using) • If the instrument has been left with reagents for more than 48 hours (for example, over the weekend)

Cleaning with chlorine

1. All tubes and bottles are removed from the machine.
2. An old chip (for chlorine cleaning) is in the machine.
3. Washing bottles are rinsed twice.
4. A glass bottle is filled with 1 L of 18 Ω water and an **Ion PGM CleaningTablet** (chlorite tablet) is added to the bottle - 10 min until it dissolves completely.
5. 1 mL of **1 M NaOH** is added to the bottle.
6. The solution is filtered through a 0.22-µm or 0.45-µm filter.

Chlorite solution has to be used within 2–3 hours!

7. 250 mL of filtered chlorite solution is added to the W1 bottle.
8. The sipper in W1 position is cleaned with 18 Ω water.
9.. 3 washing bottles are attached to the machine: W1: 250 mL chlorine solution, W2 and W3 are empty.
10. Collection trays are placed below the sipper tubes at A,T,G,C.

"Clean" mode is chosen and the following instructions are followed.
Cleaning takes ~ 14 min.

Cleaning with 18 Ω water

1. All tubes and bottles are removed from the machine.
2. An old chip is in the machine.
3. Washing bottles are rinsed twice.
4. 250 mL water is added to W1 bottle.
5. The sipper in W1 position is cleaned with 18 Ω water.
6. 3 washing bottles are attached to the machine: W1: 250 mL water, W2 and W3 are empty.
7. Collection trays are placed below the sipper tubes at A,T,G,C.

"Clean" mode is chosen and the following instructions are followed.
Cleaning takes ~ 14 min.

Preparation of Wash 2 bottle

1. Wash 2 bottle is rinsed three times.
2. The bottle is filled with ~ 2L of 18 Ω water unill the mold line.
3. The entire content of an **Ion PGM Sequencing 400 W2 Solution** is added to the bottle - the barcode is stored.
4. 70 µL of freshly prepared **100 mM NaOH** solution is added.
5. The content of the bottle is mixed and stored with closed cap.

Preparation of Wash 1 and 3 bottles

1. Bottles are rinsed with water 3X.
2. The following compounds are added to the bottles:
   Wash 1: 350 µL of freshly prepared **100 mM NaOH**
   Wash 3: 50 mL **Ion PGM Sequencing 400 1X W3 Solution**
3. Bottles are stored with closed caps.

Cleaning wash bottles are removed from the machine and new sippers are installed for the washing bottles.
Wash 1,2 and 3 bottles are attached to the machine.
The same chip is in the machine as it was during cleaning.
Machine is set to "Initialize" mode - ~ 30 min.

Plan run

1. Homepage: 145.39.105.108

2. Plan - Plan template run - 16S target sequencing - Settings: Plan run
- Run parameters: name, sample names and barcodes
- Applications: Metagenomics, 16S targeted
- Kits: Barcode set - Ion Xpress
- Output: 16S

Preparation of dNTP

Change gloves!

1. **dNTP** solutions are vortexed and spinned briefly.
2. 20 µL of dNTP solution is added to each Reagent bottle.
3. New sippers are installed - after one is installed the related Reagent bottle is attached.

Preparation of ISPs

→ 1. Half of the volume of the ISPs with the library is tranferred into a 200 µL PCR tube - one will be sequenced the other stored.
ISPs can be store at 2-8 oC up to 1 week!
2. **Contol Ion Sphere Particles** are vortexed and spinned briefly.
3. 5 µL Control Ion Sphere Particles are added to the half volume of ISPs - then is's pipetted up and down and spinned for 2 sec.
4. Supernatant is removed in a way that the ISP pellet is not disturbed and 3 µL liquid stays in the tube.
5. 3 µL **Sequencing Primer** is added - then it's pipetted up and down to disperse the pellet.
6. Sample is put on the following PCR program:

Stage	Temperature	Time
Hold	95 oC	2 min
Hold	37 oC	2 min
		Σ 4 min

Ion 314 Chip v2

No glowes!

1. A new chip is removed from its package - barcode is stored.
2. Chip is placed on the grounding plate - the cleaning chip stays in the machine and the machine is set for the "Run"mode.
3. The chip is replaced when it's indicated on the machine's screen - touching the groundig plate with fingers is necessary before.
4. Barcode is scanned when it's indicated on the machine's screen.
5. "Chip check"option on the sreen is chosen.
6. If the check is succesfull, the new chip is placed on the grounding plate and the old one is put back into the machine.
7. Waste container is emptied.
8. PCR tube is removed from the PCR and 1 µL **Ion PGM Sequencing Polymerase** is added.
9. Sample is pipetted up and down and incubated at room temperature for 5 min.
10. The liquid from the chip is removed by a 20 µL pipette.
11. Chip is spinned for 5 sec upside down (tab poiting in) - then it's placed on a desk but stays in the adapter bucket
12. Chip is loaded with the 7 µL sample - 0,5 µL stays in the tip to avoid air bubbles.
13. Chip is spinned for 30 sec in normal position (tab pointing in).
14. Sample is mixed: 5 µL volume is pipetted up and down.
15. Chip is spinned for 30 sec in normal position (tab pointing out).
16. Sample is mixed: 5 µL volume is pipetted up and down.
17. Chip is spinned for 30 sec in normal position (tab pointing in).
18. Sample is mixed: thw whole volume is pipetted up and down - then removed.
19. Chip is spinned for 5 sec in normal position (tab pointing out).
20. Chip is gently knocked to a desk to empty the remaining liquid, then wiped with a tissue.

Sequencing

1. "Planned run"option is selected.
2. Chip is placed on the groundig plate and put in the machine when it's indicated on the screen.
Run takes ~ 4 hours.

Cleaning with 18 Ω water

1. All tubes and bottles are removed from the machine.
2. Chip stays in the machine.
3. Washing bottles are rinsed twice.
4. 250 mL water is added to W1 bottle.
5. 3 washing bottles are attached to the machine: W1: 250 mL water, W2 and W3 are empty.
6. Collection trays are placed below the sipper tubes at A,T,G,C.
"Clean" mode is chosen and the following instructions are followed:
At the end the wash bottles and sippers stays attached to the machine.