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Design and Evaluation of a 16S-based Integrated Solution to Study Bacterial Diversity using the Ion Torrent™ PGM™ platform

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Abstract

Analysis of 16S sequences in microbial population gives a quick overview of the community diversity, and is usually performed by sequencing one or two hypervariable regions (V-regions), amplified as a single PCR fragment. We developed a novel approach that simultaneously surveys multiple V-regions in the 16S rRNA gene.

In the first design of PCR primer pools, V-regions 2, 3, 4, 6-7, 8 and 9 were amplified as individual ~200 bp fragments in one of two multiplex PCR reactions. The primer pools covered more than 80% of eubacterial sequences in the GreenGenes database with perfectly matched primer pairs for at least one V-region. In the second design the amplicons are longer, 300-400 bp products

Our data analysis module classified individual reads by mapping them to the reference libraries. With the fragment sizes ranging between 200-300 bp, we achieved genus and, in many cases species level taxonomic resolution, depending on which V-region was evaluated.

In an initial evaluation we tested the complete solution (PCR chemistry, workflow and software) on two mock community DNA samples from BEI resources: HM-276D - even community, with equal number of rDNA copies for each of 20 bacteria and HM-783D– staggered community, with variable number of rDNA copies. Several V-regions were amplified by our primer sets for every organism in the mock community. The number of classified reads in each V-region for every bacteria depended on both primer complementarity and ease of sequencing of the particular PCR fragment. Our approach of interrogating multiple V-regions and sequencing both amplicon strands improved system robustness against both PCR and sequencing biases.
The 314v2 chip achieved 1:100 sensitivity (detection of all organisms in the staggered mock community with 10^4-10^6 rDNA copies/PCR). Increased sequencing depth (316v2 and 318v2) increased sensitivity to 1:1000 (10^3-10^6 rDNA copies).

With human samples, we observed no PCR cross-reactivity with human DNA and were able to identify and determine characteristic V-signatures of several important species. The signatures not only help to increase the confidence in the organism presence and ID, but may potentially enable strain differentiation.

Survey of multiple V-regions is useful in monitoring changes in the microbial community composition

Materials and Methods

Materials for 16S Metagenomics workflow

16S Metagenomic – specific reagents

- Primers tube 248
- Primers tube 369
- *E.coli* gDNA control
- DNA dilution buffer
- NC water

*2x PCR Master mix

- AGENCOURT® AMPURE® XP - PCR Purification reagent
- Agilent High Sensitivity DNA Kit DNA kit for Bioanalyzer

Reagents for library preparation templating and sequencing

- Ion Plus Fragment Library kit PN 4471252;
- Ion Library quantitation kit PN 4468802;
- Ion PGM™ Template OT2 400 Kit PN 4479878
- Ion PGM™ Sequencing 400 Kit PN 4482002

*314 v2 chip, 316 v2 chip or 318v2 chip

Stage	Temperature	Time
Holding	95 °C	10 min
Cycling* 18 cycles	95 °C	30 sec
	58 °C	30 sec
	72 °C	20 sec
Holding	72 °C	7 min
Holding	4 °C	∞

Set up PCR with 2-4-8 and 3-6.7-9 primer sets. Recommended microbial DNA input is ~1-3 ng per 50 ul reaction for 18 cycles. Increase the number of cycles proportionally for lower DNA inputs. After PCR, combine equal volumes of 2-4-8 and 3-6,7-9 reaction products for library preparation as outlined in the Ion Plus Fragment Kit manual.

Results

Fig 1. Detection of 16S sequences in GreenGreens database by a V-region primer sets (number of perfect matches only)

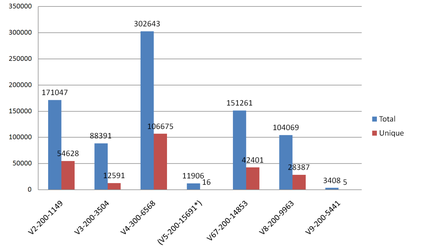

Fig 2. 16S Metagenomics primer sets simultaneously amplify several hypervariable regions of bacterial 16S rDNA gene

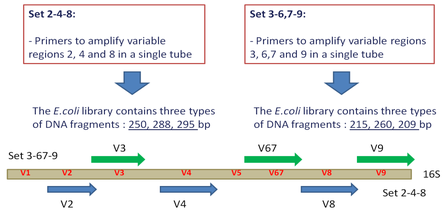

Table 1. Taxonomic resolution for the Mock Community B Even Sample with 16S Metagenomics kit and IR 16S Metagenomics software

Species	V2	V3	V4	V67	V8	V9	Profile code
Actinomyces odontolyticus		34	290	504	234		V4V67V8
Bacillus cereus	158/2330	2923	2025	3070	180		V2V3V4V67
Bacteroides vulgatus	2954	3676	2508	5437			V2V3V4V67
Clostridium beijerinckii	1616/1448	3344	326/2242	917/4187			V2V3V4V67
Deinococcus radiodurans	1827	139	1810	172			V2V4
Escherichia coli	1473	1714	1116	256/1513	115/851	2443/28	V2V3V4V67V8V9
Enterococcus faecalis	1885	2675	1345	2362	497		V2V3V4V67
Helicobacter pylori	1456/1158	3142	1652	2034	83		V2V3V4V67
Lactobacillus gasseri	3727	1827	2236	4208			V2V3V4V67
Listeria monocytogenes	1343	3064	1753	5024	943		V2V3V4V67V8
Acinetobacter baumannii	2277	2582	1291	64	1409	2079	V2V3V4V8V9
Neisseria meningitidis	1755	2237	1714		1093		V2V3V4V8
Propionibacterium acnes	448	35	97	697	930		V2V67V8
Pseudomonas aeruginosa	1447	2863	1376	1552	1005		V2V3V4V67V8
Rhodobacter sphaeroides	242	1407	754	586	897		V2V3V4V67V8
Staphylococcus aureus	1800	3640		497/2627			V2V3V4V67
Staphylococcus epidermidis	2498	624/3453	4592	465/2584	36/286		V2V3V4V67
Streptococcus mutans	1058	3726	2792	2995	1925		V2V3V4V67V8
Streptococcus agalactiae	770	2018	2839	2628	2172		V2V3V4V67V8
Streptococcus pneumoniae	369	936	852	1735	932		V2V3V4V67V8

species level ID
Inclusive Slash ID
species+Inclusive Slash ID
Inclusive+Exclusive Slash ID
species level (+non-target species) ID

IR Workflow parameters: Both Ends, 90% minimum hit coverage, 97% genus cut-off, 99% species cut-off, 0.2% Slash call reporting, Curated MicroSEQ(R) 16S Reference Library v2013.1 only

All 20 bacteria in the community are detected by at least one primer set. Seventeen out of 20 organisms were identified to a species level by at least on V-region.

Fig 3. Monitoring growth-conditions-induced changes in water microbial community

DNA , extracted directly from 1 ml of water sample or from the community cultured two days on R2A media on the 0.45 um PALL filter , was used as PCR template for analysis using 16S Metagenomics workflow.
Changes in community are easily seen: the entire group of microbes, amplified by V6,7 primer set is missing in cultured sample.

16S Metagenomics PCR sets shows no cross-reactivity with human DNA

Table 2.a Example of data summary table for a sample containing human DNA

Database	Curated MicroSEQ(R) 16S Reference Library v2013.1										
Number of copies needed	10										
Primers detected	Both ends										
BP cutoff	140 - NOT in effect when using full coverage										
Total number of reads	59028										
Number of valid reads	35303										
Number of reads ignored	10131 (due to low number of copies <10)										
Mapped reads in sample	25222										
Un-Mapped reads in sample	0										
Analysis date	2/26/2014 13:41										
Primer name	# of mapped reads	# of valid reads	# of low copy number reads	# of un-mapped reads	# of forward reads	# of forward full found	# of forward short	# of forward valid reads	# of reverse found	# of reverse coverage	# of reverse full found	# of reverse short	# of reverse valid reads
V2	3356	5031	1675	0	4896	3501	0	3501	4649	1530	0	0	1530
V3	5894	7549	1655	0	4805	3663	0	3663	5620	3886	0	0	3886
V4	3920	5674	1754	0	4037	2056	0	2056	4631	3618	0	0	3618
V67	10127	13892	3765	0	10973	8854	0	8854	9062	5038	0	0	5038
V8	1925	3207	1282	0	2516	1550	0	1550	2371	1657	0	0	1657
V9	0	0	0	0	127	0	0	0	8	0	0	0	0

All valid reads are mapped to microbial database

Table 2.b Identification of *Streptococcus* in four samples

Streptococcus (genus level ID only)	*Streptococcus* (genus level ID only)	*Streptococcus* (genus level ID only)	*Streptococcus* (genus level ID only)
V2 **43** (blank) V3 **19** (blank) V4 **1037** (blank) V8 **90** (blank) ***intermedius*** V2 **709** 99.11 - 100 ***intermedius*** V3 **3585** 99.44 - 100 ***intermedius*** V4 **1239** 99.18 - 99.59 ***intermedius*** V67 **1078** 99.1 - 100 ***Streptococcus constellatus / Streptococcus constellatus subsp. vibergensis / Streptococcus intermedius*** V8 **1316** 99.21 - 100 **Samp1(8)**	***intermedius*** V2 **580** 99.11 - 100 ***intermedius*** V3 **765** 99.44 - 100 ***intermedius*** V4 **1337** 99.18 - 100 ***intermedius*** V67 **175** 99.55 - 100 ***Streptococcus constellatus / Streptococcus constellatus subsp. vibergensis / Streptococcus intermedius*** V8 **2652** 99.21 - 100 **Sample2(1)**	V2 **34** (blank) V3 **23** (blank) V4 **39** (blank) V8 **83** (blank) ***intermedius*** V2 **504** 99.11 - 100 ***intermedius*** V3 **3291** 99.44 - 100 ***intermedius*** V4 **2346** 99.19 - 100 ***intermedius*** V67 **1709** 99.1 - 100 ***Streptococcus constellatus / Streptococcus constellatus subsp. vibergensis / Streptococcus intermedius*** V8 **1280** 99.21 - 100 **Sample3(3)**	V2 **41** (blank) V3 **88** (blank) V67 **140** (blank) V8 **91** (blank) ***anginosus*** V2 **169** 99.11 - 99.12 ***intermedius*** V3 **1862** 99.44 - 100 ***anginosus*** V4 **483** 99.19 - 100 ***Streptococcus anginosus / Streptococcus equinus / Streptococcus troglodytidis*** V67 **130** 99.11 - 99.11 ***Streptococcus anginosus / Streptococcus troglodytidis*** V8 **1984** 99.1 - 99.55 ***Streptococcus anginosus / Streptococcus constellatus / Streptococcus constellatus subsp. vibergensis / Streptococcus hyointestinalis / Streptococcus intermedius / Streptococcus troglodytidis*** V8 **588** 99.21 - 99.8 **Sample4(8)**

Combination of multiple V-regions gives better differentiation in this case. If only V4 region was used for analysis, the ID for Sample 4 was *S. intermedius, where the correct ID is S.anginosus.*

Conclusion

16S Metagenomics solution on the Ion Torrent™ PGM™platform provides fast, easy to use, complete research workflow, which requires no expert knowledge in data analysis. Multi-V-region primer design help resolve samples to the species level ID, and quickly compares communities in multiple dimensions.

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