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A Single-Tube, Multiplexed Microbial Identification Assay Using 16S rRNA V1-V9 regions, Fungal ITS 1 and 2 Genes, and Antimicrobial Resistance Genes

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Introduction

Metagenomic shotgun sequencing has transformed microbial diagnostics and ecology, but direct sequencing of multiple complex samples in parallel has relatively low sensitivity compared to polymerase chain reaction (PCR). Targeted sequencing can be employed to enrich for multiple microbial targets of interest in parallel. Using Accel-Amplicon, a single-pool multiplex PCR assay was developed that enriches for multiple variable regions of the 16S rRNA gene, Nuclear ribosomal internal transcribed spacer (ITS) regions, and 35 antibiotic resistance genes from multiple bacterial threats listed by the CDC. The two-hour protocol, which generates target enriched libraries for Illumina platforms from gDNA, was validated using gDNA from a mock community of 20 bacterial strains. Specificity, sensitivity, and quantification were further examined using extracts from clinical isolates, swine manure, and waste water. The single-tube target enrichment workflow accurately and reproducibly identified genomic content from reference strains with as low as six genomic copies per reaction spiked into complex samples. The assay performed for a wide input range, from picograms to nanograms. Enrichment of all variable regions of the 16S rRNA gene detected greater diversity and differing microbial profiles compared to a single conventionally used primer set targeting a subset of the V3-V4 regions. Simultaneous analysis of both the 16S rRNA gene and resistance genes in parallel provided greater differentiation of harmful bacterial pathogens and overall structure of the bacterial functional repertoire. Targeted analysis also influenced taxonomic assignment compared to shotgun metagenomics. This study highlights the power of targeted enrichment via single-tube amplification for NGS-based microbial diagnostics and ecology.

Swift Amplicon Workflow

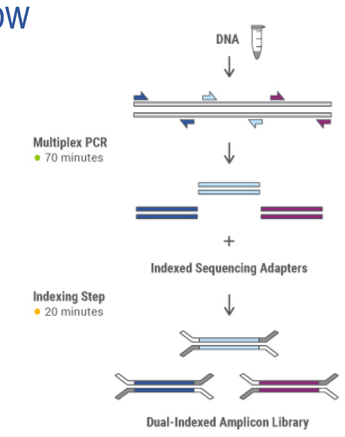


Figure 1. The standard Swift Amplicon workflow includes a multiplex PCR where overlapping primer pairs can be combined into a single tube for contiguous target coverage. This is followed by an indexed adapter ligation step that adds Illumina-compatible adapters with combinatorial dual indexes. These panels are available as pre-designed Panels (16S+ITS) or with custom content (see swiftbiosci.com)

16S+ITS Features and Assay Details

Feature	Swift Amplicon 16S+ITS
Input DNA	1 ng (10 pg - 50 ng)
Amplicons	5 (16S for bacteria and archaea) 2 (fungal ITS)
Genes Covered	Bacterial 16S rRNA (V1-V9) Fungal ITS1+ITS2
Assay Format	Single tube multiplex PCR; 2 hours DNA-to-Library
Components Provided	Target specific primer pool, PCR and library preparation reagents, including indexed adapters
Depth Recommendations	100-300K reads per sample
Multiplexing Capability	96 libraries on Illumina® MiSeq® v2 Standard
Compatible Platforms	Illumina MiSeq, MiniSeq

Table 1. The features and capabilities of the Swift Amplicon 16S+ITS amplicon panel.

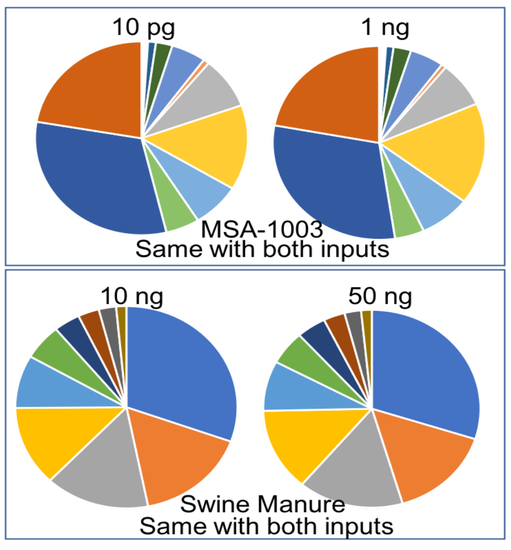
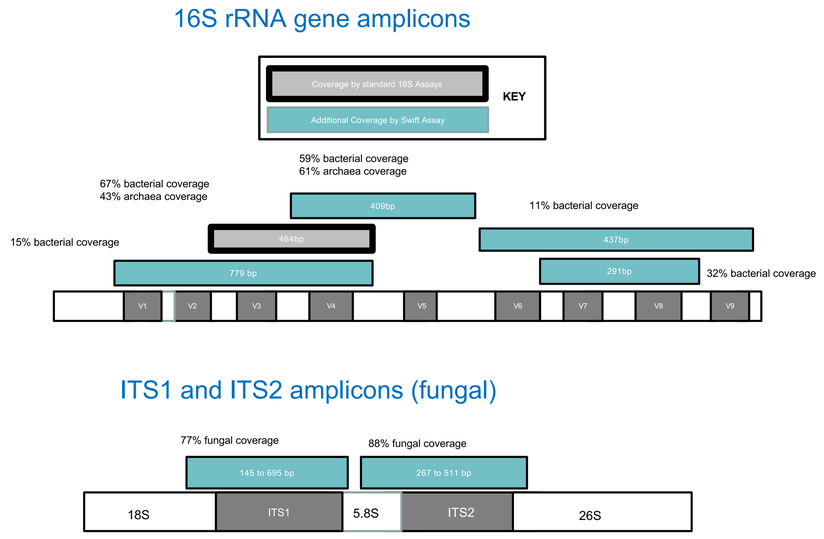


Figure 2. Consistent performance with varying biomass and sample types. Input quantities from 10 pg to 50 ng with MSA-1003 (top) and swine manure (bottom), gave consistent and expected sequencing results in terms of sensitivity and relative abundance. Varying input quantity within this range does not require changes to protocol or thermal cycling.

Covering All Variable Regions of the 16S rRNA gene and ITS1 and ITS2 genes

Figure 3. 16S rRNA gene and ITS genes for Illumina® sequencing
- Single pool of 7 amplicons covering V1-V9 + ITS1 and ITS2 (fungal)
- Non-polar chemistry generates complex libraries and avoids requirement for PhiX
- Customization possible (e.g. add virulence genes, biocide resistance genes)



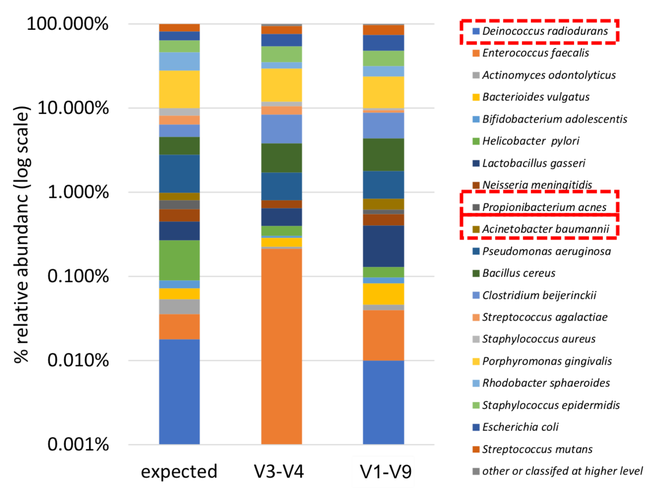


Figure 4. A mix of 20 bacterial species (ATCC MSA-1003) tested with Swift Amplicon 16S+ITS Panel, sequenced with Illumina® MiSeq® V3 (2x300bp reads). Strains were present from 0.02% to 18%. The panel covering all variable regions (V1-V9) provides an accurate representation of all species in the sample. When using a single amplicon covering variable regions 3 and 4 (V3-V4), 3 bacterial species were underepresented (indicated by the red box.), while all 20 species were observed using V1-V9. No PhiX was used during sequencing.

	V3-V4 only			V1-V9		
	Simpson Index	Shannon Index	Number of Species Identified	Simpson Index	Shannon Index	Number of Species Identified
Sample 1	2.84E-06	1.92	414	4.26E-06	2.728	523
Sample 2	3.78E-06	1.862	387	1.73E-06	2.869	486
Sample 3	2.73E-06	2.023	427	1.53E-06	2.716	603
Sample 4	2.53E-06	1.962	369	1.49E-06	2.703	500
Sample 5	4.14E-06	2.023	393	2.67E-06	2.905	580
Sample 6	8.11E-06	2.037	367	3.77E-06	2.872	546
Sample 7	8.11E-06	1.914	353	1.38E-06	2.726	561
Sample 8	8.11E-06	1.914	350	1.27E-06	2.859	526

Table 3. Greater diversity (1.4x to 1.7x) was observed with amplicons targeting all regions compared to V3-V4 alone. Sequencing was completed with 2x150 read lengths.

Performs with Flexible Read Lengths

	V1-V9, 2x150 PE sequencing		V1-V9, 2x300 PE sequencing	
Sample	Shannon Species Diversity	% Reads PF Classified to Genus	Shannon Species Diversity	% Reads PF Classified to Genus
Manure 1	2.84	94.1%	2.84	92.5%
Manure 2	2.87	94.6%	2.85	92.8%
Manure 3	2.71	94.6%	2.69	92.6%
Manure 4	2.74	95.0%	2.70	93.6%

Table 4. Longer read length does not increase diversity with the manure samples tested. We do not observe an increase in Shannon diversity score with 2x300 read lengths compared to 2x150 read lengths. This is due to the staggered arrangement of overlapping amplicons. This may be sample-dependent and further validation is recommended.

High Specificity Observed with Clinical Isolates

Name identified by culture or from ATCC	Source	Correctly identified reads
Methicillin-resistant Staphylococcus aureus	Hospital culture	99.80%
Staphylococcus aureus	Hospital culture	99.79%
Group A streptococcus (Streptococcus pyogenes)	Hospital culture	99.91%
Group B streptococcus (Streptococcus agalactiae)	Hospital culture	99.90%
Escherichia coli	Hospital culture	99.33%
Staphylococcus epidermidis	Hospital culture	99.82%
Proteus mirabilis	Hospital culture	99.88%
Klebsiella pneumoniae	Hospital culture	99.81%
Enterococcus faecalis	ATCC 19433	98.47%
Pseudomonas aeruginosa	ATCC 10145	99.79%
Enterococcus faecium	ATCC 51559	98.47%
Candida albicans	Hospital culture	99.38%
Citrobacter koseri	Hospital culture	99.38%
Acinetobacter haumannii	Hospital culture	99.93%
Campylobacter jejuni	ATCC 33291	99.86%
Klebsiella oxytoca	Hospital culture	99.21%
Stenotrophomonas maltophilia	Hospital culture	99.83%
Serratia marcescens	Hospital culture	99.88%
Enterobacter cloacae	ATCC 23355	99.73%
Haemophilus infuenzae	ATCC 49766	99.84%
Morganella morganii	Hospital culture	99.86%
Citrobacter freundii	Hospital culture	99.13%
Bacteroides fragilis	Hospital culture	99.84%
Enterobacter aerogenes	ATCC 13048	99.69%
Legionella pneumophila	ATCC 33152	99.72%

Fungal Sample Characterization

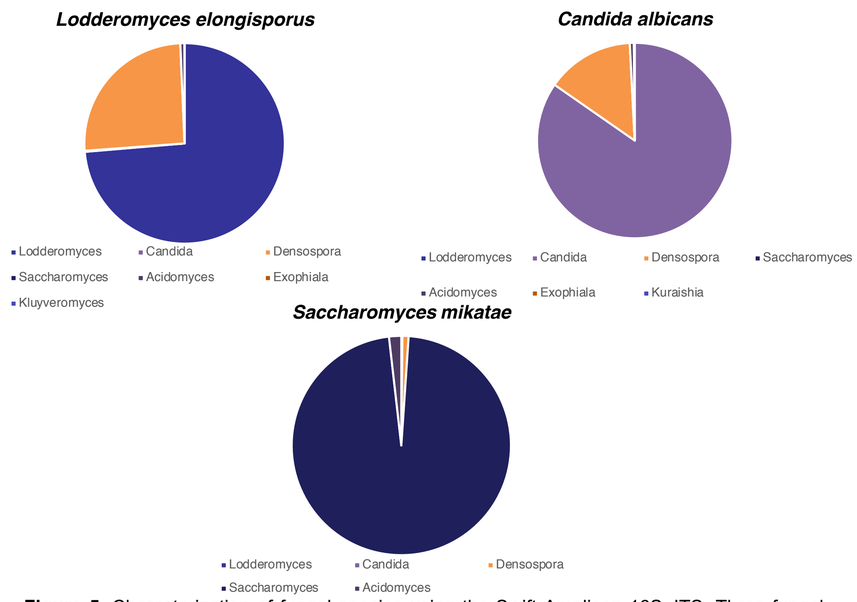
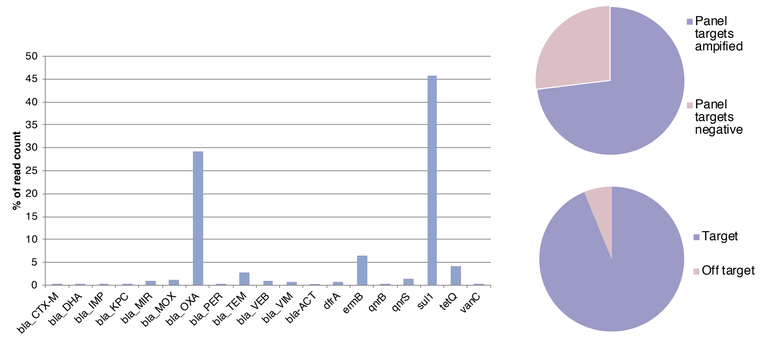


Figure 5. Characterization of fungal species using the Swift Amplicon 16S+ITS. Three fungal samples- Lodderomyces, Candida, and Saccharomyces- were successfully identified from ATCC stocks.

Antimicrobial Resistance Genes

Figure 6. Detection of targeted ARGs from 10ng of DNA extracted from a waste water sample. Primers to detect 38 common ARGs were used with a waste water sample. ARGs were found as expected (using single-plex PCR and verified via gel electrophoreses).



Conclusions

- Swift has developed an amplicon based panel that can easily characterize the 16S rRNA and ITS DNA present in a wide variety of samples
- Coverage of V1-V9 improves sensitivity compared to coverage of V3-V4 alone
- This assay can discern low level species from 10pg to 50ng
- These libraries can be sequenced with 2x150 bp reads and no PhiX, saving significant costs and improving results
- Important antimicrobial genes can be sensed from environmental sample using a 38 amplicon panel, leading to the exciting possibility of expanded amplicon based sensing of microbial function and structure.

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