Rapid, High-Throughput Isolation and Cultivation of Diverse and Rare Bacteria from Human Gut Microbiome (HGM) Samples using the Isolation Bio Prospector® System

Importance of assembling isolate libraries

Metagenomic analysis of the microbiome has been used widely to determine what species and phyla are present in a given HGM sample and in what proportions. However, understanding how gut bacteria exert influence on their hosts and on each other will require access to living strains that can be used for functional characterization studies. In addition, access to live isolates will need to scale such that large libraries of 100s or even 1000s of bacterial strains as monocultures are available for functional analysis1. While the classic techniques of microbiology traditionally used to construct such libraries are tedious and extremely labor intensive, these Petri dish-based approaches have remained essentially unchanged over many decades.

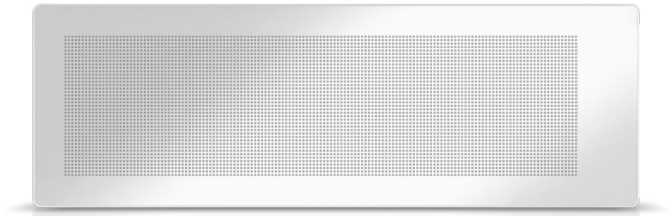
The Prospector® system from Isolation Bio provides a means of delivering hundreds of monoclonal cultures from HGM samples ready for identification by 16S rRNA sequencing, mass spectrometry, or other techniques in less than a week and with significant reductions in the amount of hands-on time that would be required to achieve this outcome by traditional Petri plating. More information on the workflow can be found in Isolation Bio's Technical Note AN-4-2019-1122.

Importance of rare species representation in libraries

One key aspect of assembling isolate libraries is ensuring that they are representative of the microbial communities from which they are derived. Rare and/or slow-growing species can be missed during cultivation using petri dishes. Although they may comprise only a small percentage of the overall mix of microbes, rare and/or slow-growing species can be key players in maintaining the overall equilibrium of a community and may even be keystone species3. Rare species can be missed if they are not well adapted to the medium being used, or if they inherently grow slowly, or if they are outcompeted by strains that are abundant and grow quickly.

The Prospector system

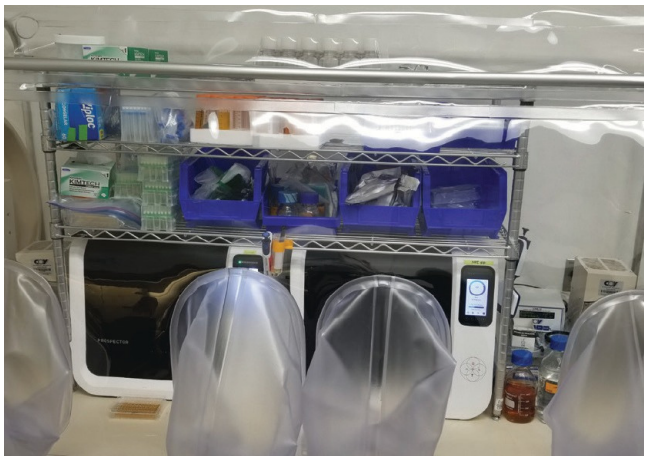
The Prospector system is able to address the capture of rare/ slow-growing species in two ways. First, the application of an appropriately diluted complex sample to the array results in over 1000 of the nanowells on the array containing only a single bacterium, so there will be no direct growth competition between strains. Second, because of the ease and speed with which multiple parallel experiments can be conducted with Prospector, it is practical to increase library diversity by strategically testing several different media.



Prospector microbial isolation and cultivation array

Anaerobic culturing for HGM

An issue in culturing HGM is the need to constantly maintain anaerobic conditions as are found in the gut. This is an area where the decreased hands-on time required for Prospector can beneficial. The Prospector instrument readily fits into an anaerobic chamber (Coy Lab Products and Anaerobe Systems) such that all steps–from array loading and sealing, incubation of the arrays, imaging of arrays for monitoring culture growth, transfer and sealing of metabolically active cultures in 96 well plates for scale up, and the incubation of these 96-well plates–can all be accomplished without samples ever leaving the chamber and without needing to manage dozens or hundreds of Petri dishes.



Prospector System in a Coy Lab Products Anaerobic Chamber

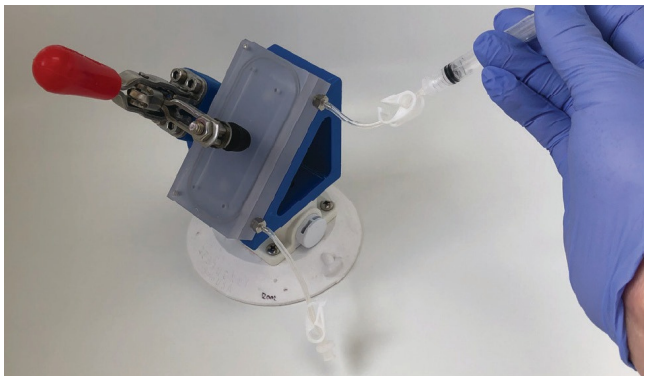
Use of resorufin as an indicator of anaerobic metabolism

The Prospector workflow to culture HGM sample uses resorufin as an indicator of anaerobic metabolism. The biological reduction of resorufin to dihydroresorufin by metabolic byproducts of anaerobic fermentation occurs more rapidly than does the abiotic reduction of resorufin by H2, allowing empty nanowells to be discriminated from those containing cultures. This discrimination under anaerobic conditions is accomplished by looking at the change in signal for each nanowell from time zero to any later timepoint. Nanowells showing a greater decrease in fluorescence than the abiotic background signal change shown by empty wells are designated culture positive.

In this application note, we show the use of the Prospector system to anaerobically culture clonal populations from human fecal samples in arrays and recover isolates that were present in less than 1% of the microbial population.

Methods

Five human fecal samples (The BioCollective, Colorado) for which there were paired metagenomic data (CosmosID) plus one sample with no paired metagenomic data were cultured on the Prospector system. Preliminary experiments on all samples were performed to determine appropriate cell densities for loading onto the arrays in order to achieve an optimal Poisson distribution4, i.e., a maximum number of nanowells containing a single bacterium with a minimum number of nanowells containing more than one bacterium. A target of 0.3 cells per well typically results in an optimal number of singly occupied wells5. Arrays and all plastic consumables and equipment were acclimated to anaerobic conditions by degassing overnight within an anaerobic chamber. All parts of the loading apparatus were sterilized by autoclaving before use. Cell dilutions were prepared in the various media to be tested, mixed with the growth indicator resorufin to a final concentration of 50 µM, and volumes of 3.0 mL were loaded onto each Prospector array inside the anaerobic chamber, then sealed. Arrays were next scanned to provide a time-zero reading of green fluorescence from resorufin and incubated anaerobically for 16 to 65 hours at 37°C with daily imaging on the Prospector to identify nanowells containing active cultures. Isolates from positive growth nanowells were then transferred from the arrays into 96 well plates and incubated anaerobically for 5 to 10 days. Isolates were identified with partial 16S rRNA Sanger sequencing; those with insufficient resolution at each taxa level were removed from further analysis.



Loading the Prospector array

The media evaluated in this application note were Gifu anaerobic medium (GAM), Brain heart infusion (BHI), Brucella blood agar (BRU), Peptone yeast-extract glucose broth (PYGB), Anaerobic enrichment broth (MTGE), and Yeast casitone fatty acids agar with carbohydrates (YCFAC).

Results

Six unique human fecal samples were cultured on the Prospector system and yielded 4242 isolates. Sanger sequencing of isolates with taxonomic resolution indicated 5 phyla, 10 classes, 15 orders, 26 families, 41 genera, and 75 species; 36 species for which at least six isolates were recovered are shown in a bar graph **(Figure 1)**.

An additional 39 species were identified in the six samples for which there were five or fewer isolates **(Table 1)**. Not only were these isolates relatively rare in terms of their capture in culture, but in nearly every case, the five samples with paired metagenomics data had predicted a prevalence of ≤0.2% for these species or genera to be found in the samples.

Comparison of Sanger 16S rRNA sequencing data for identification at the species level with predicted prevalence from metagenomic data highlights the strength of Prospector in capturing rare members of a microbial community. The 34 species isolated from sample HGM-6 represent five phyla, 10 classes, 12 orders, 19 families, and 29 genera **(Table 2)**. Of these 34 species, 18 were predicted to be present at <1.0% abundance (Table 3), demonstrating the ability of the system to isolate rare strains. Prospector was also able to identify eight species not identified in the same sample by metagenomic analysis **(Table 3)**.

With the reduction in hands-on time and lab resources afforded by Prospector for performing culture isolation experiments, it becomes practical to evaluate multiple media in parallel to tease out the presence of difficult-to-culture strains that may be present in a sample **(Figure 2)**.

Summary

The advent of metagenomics has greatly advanced microbiome research, increasing our knowledge of the importance of these microbial communities to human health. High-level information on the phyla and species that comprise the HGM and their relative proportions is key. In order to discover the molecular mechanisms by which these microbes interact with each other and interact with their hosts, the establishment of isolate libraries consisting of hundreds of individual monocultures to support experimentation with living cells is needed. The traditional culture methods (e.g. Petri plating) are tedious, labor intensive, and time consuming. These methods are made still more onerous by the necessity of maintaining anaerobic culture conditions for the strains that comprise the HGM. The Prospector system relieves much of the tedium associated with classic methods while significantly reducing hands-on time required to generate isolates. And because the entire system can be placed into an anaerobic chamber, the difficulties associated with manipulating hundreds of Petri plates anaerobically is eliminated.

A key aspect of studying microbial communities of any type is the detection of rare, slow-growing species. Although easily missed by both metagenomic and culture approaches, rare strains can play crucial roles in maintaining the overall health and functionality of a microbiome. Data from the current study matched to metagenomic results demonstrates the use of the Prospector in culturing rare strains. An additional advantage for Prospector in identifying rare strains is the relative ease with which samples can be cultivated using multiple media.

Figure 1: Species isolated from six fecal samples.

TAXA N≤5	
N=1	Streptococcus salivarius
Gordonibacter faecihominis	Paraprevotella clara
Bacteroides sp.	Alistipes indistinctus
Alistipes onderdonkii	Streptococcus salivarius
Staphylococcus epidermidis	Christensenella minuta
Lactobacillus gasseri	Clostridium baratii
Streptococcus vestibularis	Anaerococcus lactolyticus
Clostridium tertium	Clostridium hylemonae
Anaerococcus tetradius	Clostridium nexile
Finegoldia magna	Flavonifractor plautii
Lactonifactor longoviformis	Subdoligranulum variabile
Blautia coccoides	Escherichia/Shigella fergusonii
Clostridium scindens	N=3
Coprococcus comes	Asaccharobacter celatus
Dorea longicatena	Eggerthella sinensis
Lactonifactor longoviformis	Lactobacillus paracasei
Dialister sp.	Clostridium citroniae
Bilophila wadsworthia	Hungatella effluvii
N=2	Akkermansia muciniphila
Propionibacterium acnes	N=4
Bifidobacterium breve	Streptococcus gallolyticus
Bacteroides massiliensis	N=5
Paraprevotella clara	Collinsella intestinalis
Alistipes indistinctus	Bacteroides xylanisolvens

Table 1: Species for which ≤5 isolates were recovered.

ACTINOBACTERIA	N
Actinobacteria_unclassified	3
Propionibacterium acnes	2
Bifidobacterium adolescentis	6
Bifidobacterium animalis	39
Bifidobacterium bifidum	134
Bifidobacterium breve	2
Bifidobacterium longum	12
Bifidobacterium pseudocatenulatum	8
Bifidobacterium_unclassified	47
Bifidobacteriaceae_unclassified	1
Asaccharobacter celatus	3
Collinsella aerofaciens	7
Collinsella intestinalis	5
Collinsella_unclassified	2
Eggerthella lenta	432
Eggerthella sinensis	3
Eggerthella_unclassified	7
Gordonibacter faecihominis	1
Gordonibacter pamelaeae	26
Gordonibacter urolithinfaciens	8
Gordonibacter_unclassified	11
Coriobacteriaceae_unclassified	27

BACTEROIDES	N
Bacteroides caccae	43
Bacteroides cellulosilyticus	7
Bacteroides dorei	631
Bacteroides faecis	311
Bacteroides finegoldii	6
Bacteroides fragilis	134
Bacteroides intestinalis	121
Bacteroides massiliensis	2
Bacteroides ovatus	321
Bacteroides sp.	1
Bacteroides stercoris	53
Bacteroides uniformis	77
Bacteroides vulgatus	299
Bacteroides xylanisolvens	5
Bacteroides_unclassified	188
Odoribacter_unclassified	5
Parabacteroides distasonis	41
Parabacteroides goldsteinii	21
Parabacteroides merdae	118
Parabacteroides_unclassified	9
Paraprevotella clara	2
Alistipes indistinctus	2
Alistipes onderdonkii	1
Bacteroidales_unclassified	24
Bacteroidetes_unclassified	27

FIRMICUTES	N
Bacillus circulans	6
Bacillus_unclassified	3
Staphylococcus epidermidis	1
Staphylococcus_unclassified	5
Bacillales_unclassified	5
Enterococcus durans	11
Enterococcus_unclassified	59
Lactobacillus animalis	7
Lactobacillus gasseri	1
Lactobacillus paracasei	3
Lactobacillus_unclassified	4
Streptococcus gallolyticus	4
Streptococcus salivarius	2
Streptococcus vestibularis	1
Streptococcus_unclassified	4
Lactobacillales_unclassified	2
Christensenella minuta	2
Clostridium baratii	2
Clostridium tertium	1
Clostridium_sensu_stricto_unclassified	8
Anaerococcus lactolyticus	2
Anaerococcus prevotii	8
Anaerococcus tetradius	1
Anaerococcus vaginalis	6
Anaerococcus_unclassified	348
Finegoldia magna	1
Blautia coccoides	1
Clostridium aldenense	6
Clostridium bolteae	7
Clostridium citroniae	3
Clostridium hylemonae	2
Clostridium nexile	2
Clostridium scindens	1
Clostridium_XlVa_unclassified	13
Coprococcus comes	16
Coprococcus eutactus	1
Dorea longicatena	3
Hungatella effluvii	1
Lactonifactor longoviformis	21
Lachnospiraceae_unclassified	16
Clostridium leptum	2
Flavonifractor plautii	92
Ruminococcus bromii	2
Subdoligranulum variabile	151
Ruminococcaceae_unclassified	24
Clostridiales_unclassified	2
Faecalicoccus_unclassified	1
Erysipelotrichaceae_unclassifie	1
d Dialister sp.	17
Phascolarctobacterium	3
faecium Veillonella_unclassified	3
Selenomonadales_unclassified	4
Firmicutes_unclassified	

PROTEOBACTERIA	N
Sutterella wadsworthensis	8
Bilophila wadsworthia	1
Desulfovibrio_unclassified	12
Escherichia/Shigella fergusonii	29
Escherichia/Shigella_unclassified	26
Klebsiella oxytoca	1
Klebsiella_unclassified	4
Serratia_unclassified	14
Enterobacteriaceae_unclassified	1
Moraxellaceae_unclassified	3
Xanthomonadaceae_unclassified	15
Gammaproteobacteria_unclassified	16
Proteobacteria_unclassified	

VERRUCOMICROBIA	N
Akkermansia muciniphila	3

Table 2: Isolates recovered from 6 samples, grouped by phylum.

PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES	# ISOLATES	% MTG
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	Bifidobacterium longum	2	0.88%
		Coriobacteriales	Coriobacteriaceae	Eggerthella	Eggerthella lenta	7	0.03 %
				Gordonibacter	Gordonibacter faecihominis	1	NA
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides caccae	40	0.6 8 %
					Bacteroides dorei	630	6.15%
					Bacteroides faecis	182	0.0 1 %
					Bacteroides fragilis	54	0.13%
					Bacteroides intestinalis	121	4.86%
					Bacteroides ovatus	110	1.31%
					Bacteroides uniformis	66	2.88%
					Bacteroides vulgatus	30	3.89%
					Bacteroides xylanisolvens	5	0.40%
			Porphyromonadaceae	Parabacteroides	Parabacteroides distasonis	39	0.62%
					Parabacteroides goldsteinii	21	0.05%
					Parabacteroides merdae	118	2.54%
			Prevotellaceae	Paraprevotella	Paraprevotella clara	2	0.41%
			Rikenellaceae	Alistipes	Alistipes indistinctus	2	<0.01%
					Alistipes onderdonkii	1	0.25%
Firmicutes	Bacilli	Bacillales	Bacillaceae_1	Bacillus	Bacillus circulans	6	NA
			Staphylococcaceae	Staphylococcus	Staphylococcus epidermidis	1	NA
		Lactobacillales	Enterococcaceae	Enterococcus	Enterococcus durans	5	NA
			Lactobacillaceae	Lactobacillus	Lactobacillus paracasei	2	NA
	Clostridia	Clostridiales	Christensenellaceae	Christensenella	Christensenella minuta	1	0.14%
			Lachnospiraceae	Clostridium	Clostridium aldenense	6	NA
					Clostridium bolteae	1	0.0 2 %
				Coprococcus	Coprococcus comes	1	1.16%
					Coprococcus eutactus	6	0.36%
				Dorea	Dorea longicatena	1	0.76%
				Lactonifactor	Lactonifactor longoviformis	1	NA
			Ruminococcaceae	Ruminococcus	Ruminococcus bromii	1	3.09%
	Negativicute	Selenomonadales	Acidaminococcaceae	Phascolarctobacterium	Phascolarctobacterium faecium	17	NA
Proteobacteria	s Sutterella wadsworthensis	Burkholderiales	Sutterellaceae	Sutterella	Sutterella wadsworthensis	8	0.39%
	Bilophila wadsworthia	Desulfovibrionales	Desulfovibrionaceae	Bilophila	Bilophila wadsworthia	1	0.07 %
Verrucomicrobia	Akkermansia muciniphila	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	Akkermansia muciniphila	1	0.0 2 %

Table 3: Taxa at species level for recovered isolates from a single sample (HGM-6) with percent relative abundance by metagenomics (MTG).

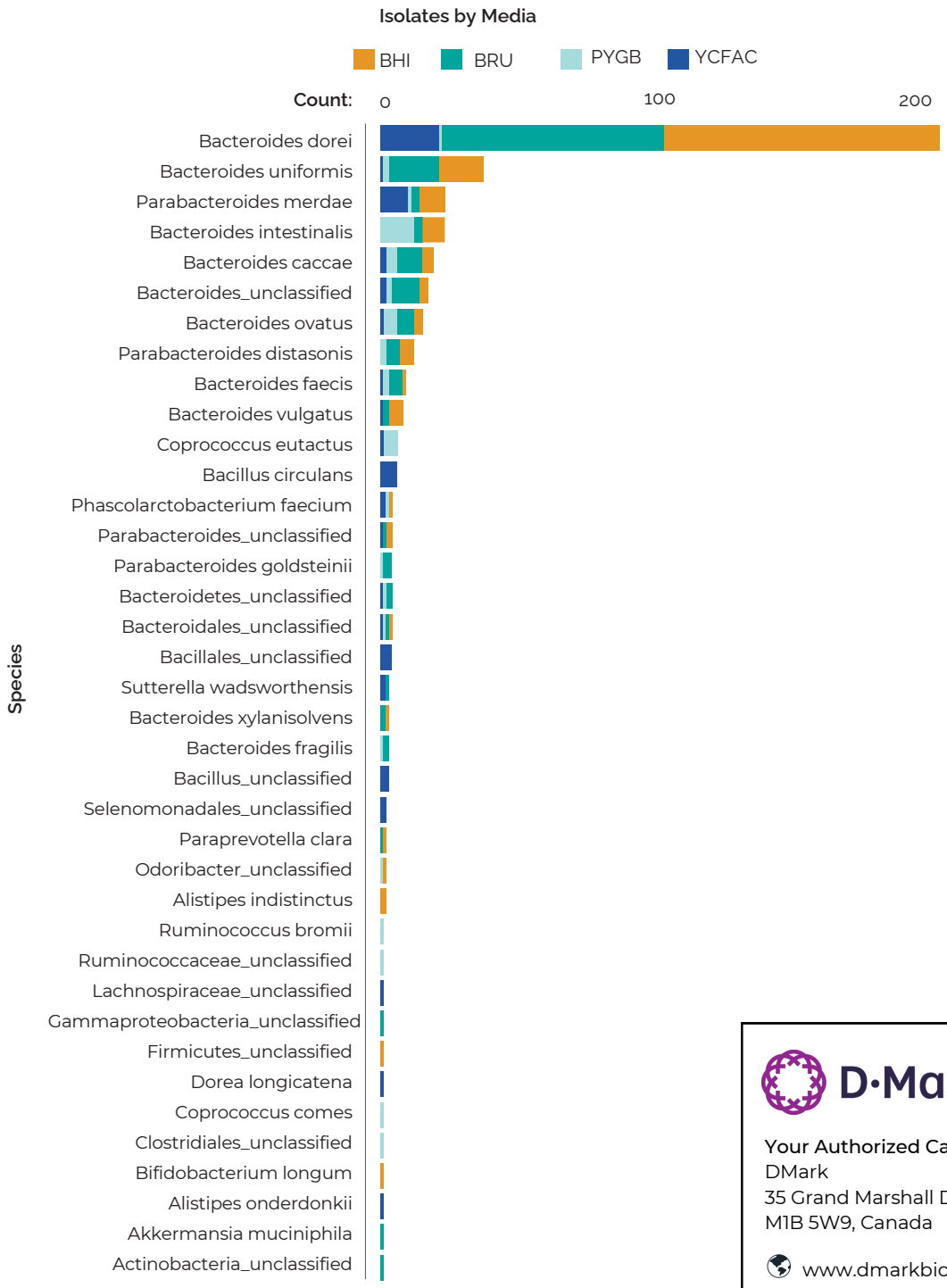


Figure 2: Strains captured at genus from a single sample with parallel media.

References

1. Zou Y, Xue W, Luo G, et al. 1,520 reference genomes from cultivated human gut bacteria enable functional microbiome analyses. Nat Biotechnol. 2019 Feb; 37(2):179-185.
2. Isolation Bio Application Note "Cultivation of Bacteria from a Soil Sample
3. Jousset A, Bienhold C, Chatzinotas A, et al. Where less may be more: how the rare biosphere pulls ecosystems strings. ISME J. 2017 Apr; 11(4):853–
4. 862. Isolation Bio Technical Note "Determination of Cell Dilution Factor for Loading a Prospector® Array"
5. Isolation Bio Technical Note "Poisson Distribution of Cells in the Microwells of the Prospector® System Arrays"