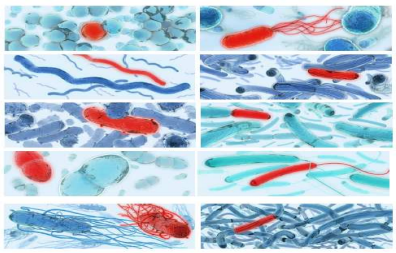
Sample & Assay Technologies

Microbiome: From Identification to Characterization



Ivorlyne Greene Ph.D.
QIAGEN Inc.

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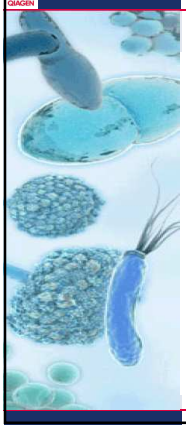
Agenda



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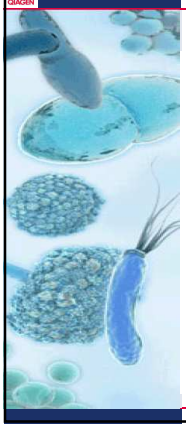

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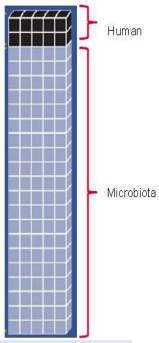
Humans or Superorganisms?

Cellular composition of the organism



Estimations of the number of microbial cells that live in and on the human body, human cells are outnumbered by a factor of 10.

Nomenclature:

Microbiota are the microbes that live in a specific location, e.g. the human body, the gut, soil, etc.

Metagenomics is the study of the collection of genomes derived from a specific sample or community.

Microbes are microscopic organisms that can be either single or multicellular.

For the purposes of this webinar, we will use **microbiome** to describe the collective genomes of the microbiota that inhabit a specific location.

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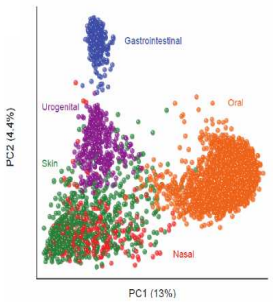
Microbiota composition

Microorganisms cluster by body site



Cataloguing efforts by the NIH Human microbiome project suggest:

~10,000 organisms live with us

~ 8 $\times 10^6$ genes in this "second genome"

Identifying microbiota in healthy individuals revealed:

Different body sites have unique communities

Race, Age, Gender, Weight or Ethnicity have an effect

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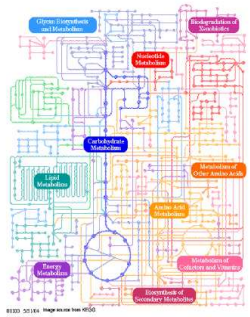
Complexity and function of genomic content

Function of microbiome enables individual survival



Each organism has developed genetic content for its own survival in a specific environment

Metabolism tuned to local nutrient sources

Virulence factors for stable colonization

Antibiotic resistance genes to metabolize toxins

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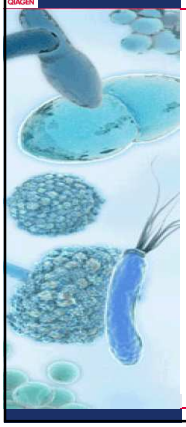

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Current Methods for microbial analysis

Identifying microbes and their genes

- Culture
  - Nutrients
  - Antibiotics
- 16S rRNA gene clone library construction
  - Pan 16S rRNA PCR amplification → cloned → sequenced
- Microarray
- Next generation sequencing
  - 16S rRNA sequencing
  - Whole genome sequencing
- MALDI
- qPCR
  - Target dependent (16S rRNA or gene)

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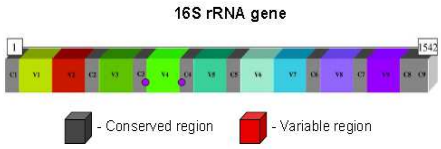
16S rRNA gene as a phylogenetic marker for bacterial ID



- Classification from the variable sequences
  - 16s rRNA sequence similarity

95% genus level, **97% species level**, 99% strain level

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How did we get started?

First report of the composition of human body site

Science VOL 312 2 JUNE 2006

Metagenomic Analysis of the Human Distal Gut Microbiome

Steven R. Gill,[1*†] Mihai Pop,[1†] Robert T. DeBoy,[1] Paul B. Eckburg,[2,3,4] Peter J. Turnbaugh,[5] Buck S. Samuel,[5] Jeffrey I. Gordon,[5] David A. Relman,[2,3,4] Claire M. Fraser-Liggett,[1,6] Karen E. Nelson[1]

If we can do one….can we do them all….

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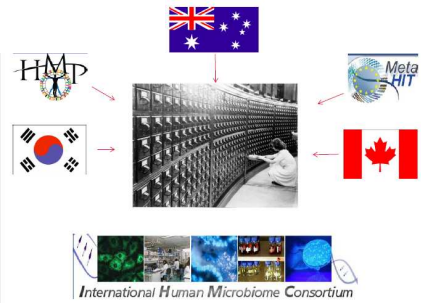
International efforts to catalog the microbiome



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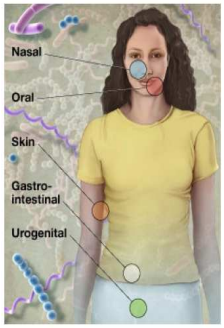
Catalog by five primary body sites

NIH Human Microbiome Project



Profiling 5 body sites
- Nasal
- Mouth
- Skin
- Gastrointestinal system
- Urogential

Compare between individuals:
- Healthy vs. Disease
- Treated vs. Untreated
- Twin studies
- Diet
- …

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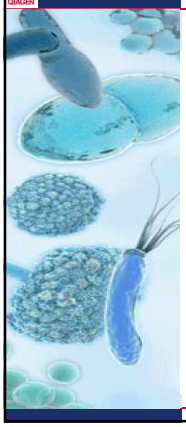

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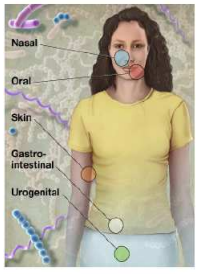
Association of the human microbiota and disease

- **Gut**
  - Intestinal infections
  - Obesity
  - Inflammatory Bowel Disease
- **Airway**
  - Pneumonia and other respiratory infections
  - Chronic Obstructive Pulmonary Disease
  - Cystic Fibrosis
- **Urogenital**
  - Bacterial Vaginosis
  - Urinary Tract Infections
  - Sexually Transmitted Disease
- **Blood**
  - Sepsis/Blood-stream infections
- **Cancer**
- **Heart disease**
- **Neurological disorders**
- **Oral**
  - Periodontitis
  - Gingivitis



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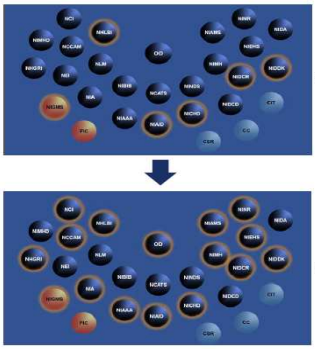
Physiological associations lead to new funding

NIH funding across institutes for microbiome-related studies



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Focused Metagenomics Applications

Examples from the next wave of microbiome experiments

Screening for microbial genes in a specific metagenomic sample
- Experiment 1: Antibiotic resistance genes in human gut

Relationships between microorganisms that permit cololnization
- Experiment 2: Profile changes in vaginal flora during bacterial vaginosis

Surveillance of samples for specific targets
- Experiments 3: Screen samples for specific pathogens, resistance genes or virulence factors

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Antibiotic resistance gene reservoirs in the body

Screening of the gut for presence of antibiotic genes

Gene	Resistance classification	1	2	3	4	5
aacC2	Aminoglycoside resistance					+
ermB	Macrolide lincosamide streptogramin B	+	+	+	+	+
mefA	Macrolide lincosamide streptogramin B	+	+	+	+	+
tetA	Tetracycline efflux pump	+	+	+		

500 ng of genomic DNA from stool samples originating from five healthy adults were analyzed for presence of antibiotic resistance genes. 87 unique antibiotic resistance genes were tested for by qPCR. Positive (+) / negative (blank) result for each antibiotic resistance gene was determined using a threshold cycle cutoff.

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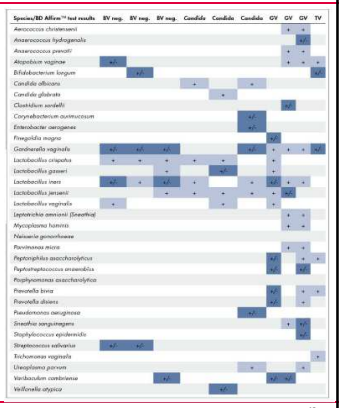
Identification of microbes in bacterial Vaginosis

BV neg. – bacterial vaginosis negative
Candida – *Candida* spp.
GV – *Gardnerella vaginalis*
TV – *Trichomonas vaginalis*



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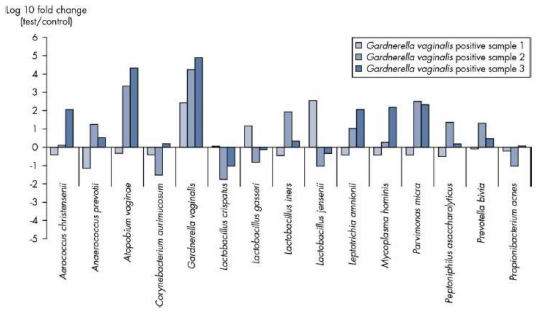
Cervical flora: Gardnerella vaginalis positive vs. BV negative



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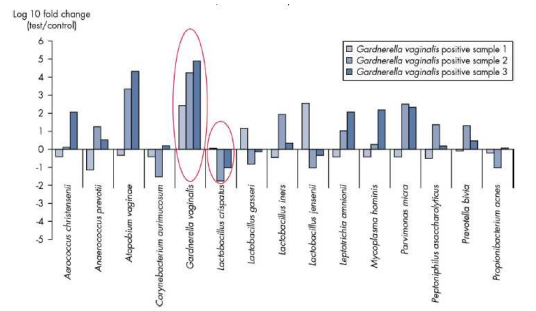
Cervical flora: Gardnerella vaginalis positive vs. BV negative

In samples with high *Gardnerella vaginalis* abundance, there was an increase in co-occurrence of BV-associated microorganisms and decrease in abundance of the normal flora, *Lactobacillus crispatus*.



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Antibiotic resistance genes in our food supply?

Genes	Resistance classification	Sewage
aacC1	Aminoglycoside resistance	+
aadA1	Aminoglycoside resistance	+
GES	Class A beta-lactamase	+
SHV	Class A beta-lactamase	+/-
SHV (156G)	Class A beta-lactamase	+/-
SHV (238G240E)	Class A beta-lactamase	+/-
TLA-1	Class A beta-lactamase	+
VEB	Class A beta-lactamase	+
ACT-1 group	Class C beta-lactamase	+/-
LAT	Class C beta-lactamase	+/-
MIR	Class C beta-lactamase	+/-
MOX	Class C beta-lactamase	+
OXA-10 group	Class D beta-lactamase	+
OXA-2 group	Class D beta-lactamase	+
AAC(6)-Ib-cr	Aminoglycoside resistance	+
QnrB-5 group	Fluoroquinolone resistance	+
QnrS	Fluoroquinolone resistance	+
ermB	Macrolide lincosamide streptogramin B	+
mefA	Macrolide lincosamide streptogramin B	+
tetA	Tetracycline efflux pump	+

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Common themes in these experiments

How best to carry out these focused metagenomic studies?

- Each case has the user profiling a distinct set of genes or species
- Experiments easily translate to large multi-factor experiments
- Range of sample types
- Quantitative and Qualitative data generation

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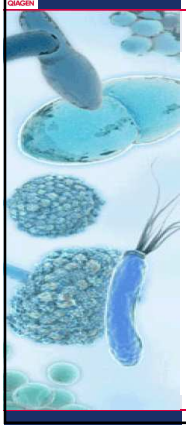

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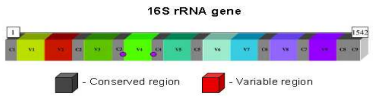
16S rRNA gene as a phylogenetic marker for bacterial ID

Sequencing or Real-time PCR (qPCR)



- Classification from the variable sequences
  - 16s rRNA sequence similarity
  - 95% genus level, **97% species level**, 99% strain level
- Assay design approach
- use only sequences with taxonomy classified by the GreenGenes taxonomy
  - fairly specific probe + fairly specific primer pair = specific assay

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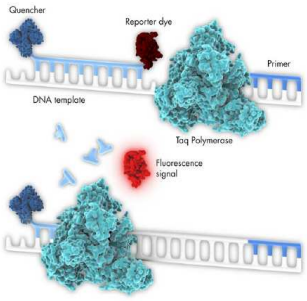
How do hydrolysis probe assays work?



Exonuclease activity of Taq polymerase disrupts FRET by separating reporter from quencher

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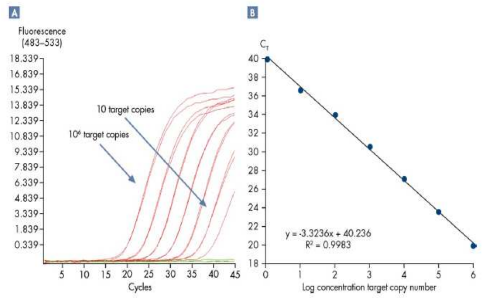
Performance testing of each assay

Dilution series testing for PCR efficiency and sensitivity



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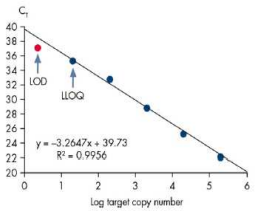
LLOQ versus LOD

Determine sensitivity of a microbial assay



LOD, limit of detection, is the lowest amount of analyte (DNA molecule) in a sample that is able to be distinguished from a sample that contains no analyte.

Often reported as colony forming units.

LLOQ, lower limit of quantitation, is the lowest amount of analyte that can be distinguished from a sample with another amount of analyte.

Often reported as gene copies, since colonies may contain multiple copies of a gene.

LLOQ is especially useful for quantitation because it states the limit at which two samples can be quantified as opposed to simple qualification.

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QIAGEN's Microbial DNA qPCR assay pipeline

Develop an assay pipeline to support microbiome research

- Over 500 assays that target species-specific or gene-specific microbial DNA
- >300 Bacteria identification assays
- 8 Fungi identification assays
- 1 Protist identification assay
- 87 Antibiotic resistance genes
- 87 Virulence factor genes

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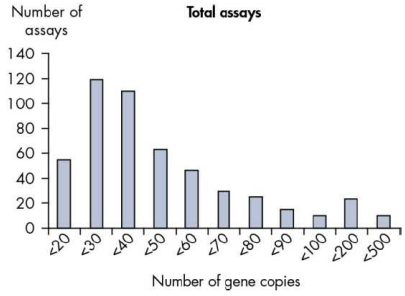
Sensitivity of the Microbial DNA qPCR assays

Lower Limit of Quantitation for all Microbial DNA qPCR assays



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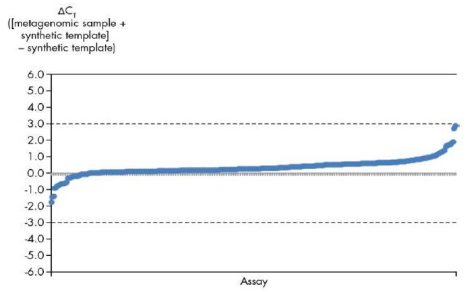
Assay performance in metagenomic background

Spike-in experiments test for specificity in a complex background



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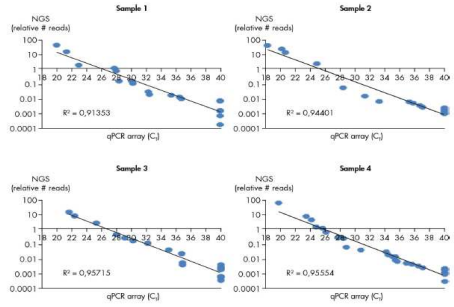
Correlate qPCR assay performance with NGS results

Profiles of vaginal flora by qPCR and whole genome sequencing



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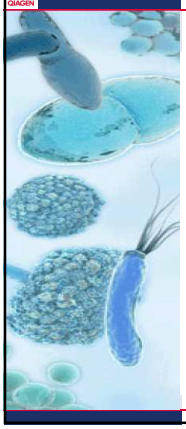

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Overview of Experimental Protocol

Focused metagenomic experiments

Test for one gene or organism → DNA Isolation → Detection by qPCR → Data Analysis

Microbial DNA qPCR Assays
Microbial DNA qPCR Assay Kits

Test for 2-6 genes or species → DNA Isolation → Detection by qPCR → Data Analysis

Microbial DNA qPCR Multi-Assay Kits

Test for 24-96 genes or species → DNA Isolation → Detection by qPCR → Data Analysis

Microbial DNA qPCR Arrays
Custom Microbial DNA qPCR Arrays

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Isolation of microbial DNA from metagenomic samples

Specialized kits and protocols to match samples

DNA Isolation → Detection by qPCR → Data Analysis

Sample Material	DNA Purification Kit	Catalog Number
Stool	QIAamp DNA Stool Mini Kit	51504
Blood	QIAamp UCP PurePathogen Blood Kit	50112
Cervical swab in transport media	QIAamp MinElute Media Kit	57414
Blood culture, bronchoalveolar lavage, carious dentine, cervical swab, isolated bacterial colony, sputum, saliva, swabs	QIAamp UCP Pathogen Mini Kit	50214

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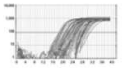
Assay User Protocol

DNA Isolation → Detection by qPCR → Data Analysis

Set up 4 PCR reactions per sample that include positive and negative controls.

Run real-time PCR to obtain raw Ct values for each assay.



Use analysis software to identify which species/gene are present.

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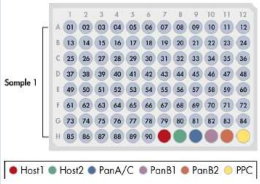
Microbial DNA qPCR Array

Pre-printed assays profile up to 90 different species/genes

DNA Isolation → Detection by qPCR → Data Analysis



PCR plates (either 96-well or 384-well) are pre-printed with primers and probes.

Each numbered well is a separate assay that tests the same sample.

Integrated control assays:

Host assays detect genomic DNA to test sample collection

Pan A/C is a pan-fungal assay that detects the presence of fungal rRNA

PanB1 and PanB2 detect bacterial 16S rRNA to determine bacterial load in the sample

PPC is a positive PCR control reaction that tests if the PCR reactions failed from PCR inhibitors from the sample, etc.

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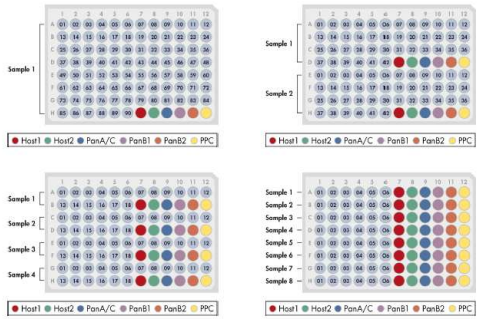
Layout of a Microbial DNA qPCR Array

Different arrays have different number of assays and samples



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Microbial DNA qPCR Arrays

Profile or Identify the presence of microbial DNA with any array

Identification experiment answers the following question:

Are any of these microbes or genes present in the sample?

Must be compared against a known negative sample.

Answers are Yes or No.

Profiling experiment answers the following question:

Have the amounts of any of these microbes or genes changed?

Must be compared against a reference sample.

Answers are fold change.

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Microbial DNA qPCR Array Content

14 Arrays

- Antibiotic Resistance Genes
- Bacterial Vaginosis
- Biodefense
- Food testing: Dairy
- Food testing: Meat
- Food testing: Poultry
- Food testing: Seafood
- Food testing: Vegetable
- Intestinal infections
- Respiratory Infections
- Sepsis
- Urinary Tract Infections
- Vaginal Flora
- Water Analysis

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Microbial DNA qPCR Arrays Protocol

Identification or Profiling

Isolate microbial genomic DNA using QiaAMP kit dependent upon sample.

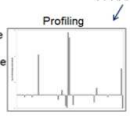
Mix sample DNA with Microbial qPCR mastermix and aliquot into each well of plate. If performing for identification analysis, then run No Template Control sample.

Run real-time PCR to obtain raw Ct values for each assay.

Profiling

Identification

Use analysis software to generate species profile or species/gene Identification.



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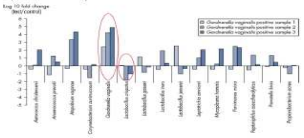
Application examples performed using these arrays

Focused metagenomic studies to identify and profile

Vaginal Flora qPCR Array

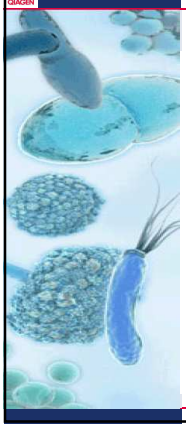


Antibiotic Resistance Gene qPCR Array

Gene	Resistance classification	1	2	3	4	5
aacC2	Aminoglycoside resistance					+
ermB	Macrolide lincosamide streptogramin B	+	+	+	+	+
mefA	Macrolide lincosamide streptogramin B	+	+	+	+	+
tetA	Tetracycline efflux pump	+	+	+		

Sample & Assay Technologies

Agenda



- Humans or Super-organisms?
  - Introduction to the microbiome
- Cataloging our "Second Genome"
  - Technologies that unlocked metagenomics
- Microbiota: Biomarkers or Effectors?
  - Population Studies and disease associations
- Identify and profile relevant targets
  - How to design assays for the microbiome
- Integrating focused metagenomics into my research
  - Solutions for identification of profiling
- Summary
  - Identification to Characterization
- Questions

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Summary

Defined the microbiome and the key role it plays in human health

Discussed the focused metagenomic studies

Introduced the Microbial DNA qPCR assay pipeline

- Largest collection of Microbial ID assays available
- Available in various product formats to simplify experiments

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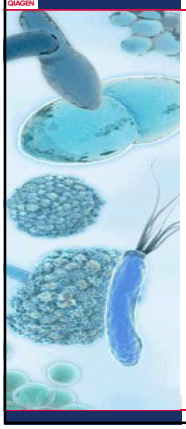

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Questions?

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