Multi-Omics Microbiome Study: Pregnancy Initiative (MOMS-PI)

Protocols for 1st Proof-of-Principle Data Snapshot

<u>Marker Paper:</u>

The Integrative HMP (iHMP) Research Network Consortium. The Integrative Human Microbiome Project: Dynamic Analysis of Microbiome-Host Omics Profiles during Periods of Human Health and Disease. *Cell and Host Microbe*. (2014).

http://dx.doi.org/10.1016/j.chom.2014.08.014

<u>Data Use Policy:</u>

http://hmp2.org/datapolicy.html

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New OB: Sample kit

Sampling Kit Contents

Forms
- Consent
- Initial Health History Questionnaire (Tablet)
- Dietary Assessment
- RAMS Registry contact form
- Initial visit brochure
- RAMS Registry brochure
- Sample collection form

Other
- 1 blue sample collection bag
- Flip top 50 ml conical (for saline)
- 15 ml Sterile saline
- 1 clear bag with vaginal pH applicator and pen (to circle vaginal pH results)
- 1 clear bag with 2 purple blood vials and a clear biohazard bag

Swabs
- 1-2 double tipped cheek
- 3-4 double tipped cheek
- 5-6 double tipped nostril
- 7-8 double tipped inside elbow
- 9-10 double tipped vaginal
- 11-12 double tipped vaginal
- 13-14 double tipped vaginal
- 15-16 double tipped vaginal
- 17 single tipped cervical
- 18 single tipped cervical
- 19-20 double tipped rectal

Pre-processing Kit Contents

MN = Mom New OB

Cytokine buffer: 250 ul 100 mM Tris-HCL pH 7.5

MN CHEEK 2	MN VAGINAL 14
MCKY	MVAY

Lipid buffer: 500 ul 0.01% Butyl Hydroxy Toluene/PBS

MN VAGINAL 13
MVAL

RNA buffer: 500 ul RNA░░░░

MN VAGINAL 10	MN VAGINAL 12
MV1A	MV2A

Culture media: 1 ml BHS supplemented with 1% yeast extract, 2% gelatin, 0.1% starch and 1% glucose

MN CHEEK 3	MN NOSTRIL 6	MN ELBOW 8	MN VAGINAL 15	MN CERVICAL 18	MN RECTAL 20
MCKU	MNSU	MIEU	MVAU	MCVU	MRCN

DNA: 750 ul Powersoil Bead Solution

MN CHEEK 1	MN NOSTRIL 5	MN ELBOW 7	MN VAGINAL 9	MN VAGINAL 11	MN CERVICAL 17	MN RECTAL 19
MCKN	MNSN	MIEN	MV1N	MV2N	MCVN	MRCN

Extra: 500 ul 100 mM Tris-HCL pH 7.5

MN CHEEK 4	MN VAGINAL 16
MCKX	MVAX

TUBES IN CARDBOARD BOX

Blood vials: 1 clear bag with 4 empty tubes for blood processing.

MN BLOOD 21	MN BLOOD 22	MN BLOOD 23	MN BLOOD 24	MN BLOOD 25	MN BLOOD 26	MN BLOOD 27	MN BLOOD 28
MW1P	MW2P	MP1P	MP2P	MP3P	MP4L	MB1P	MB2P
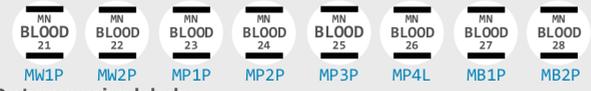
Post-processing labels
Blue sample collection bag
**Red biohazard bag**

New OB visit: Sampling instructions

Cheek
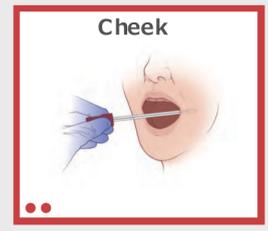
- Spin swab for 5 sec.
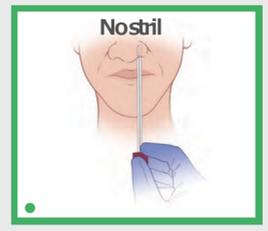
Nostril

- Dip swab in saline.
- Insert 1/2" in left nostril.
- Spin swab for 5 sec.
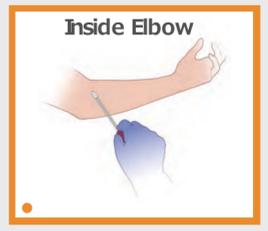
Inside Elbow

- Dip swab in saline.
- Press on inside elbow.
- Spin swab for 5 sec.

Vagina

No Speculum
- Insert swab 2" into vagina.
- Press against vaginal wall.
- Rotate swab for 5 sec.

Rectum

- Insert swab 1" into rectum.
- Rotate swab.
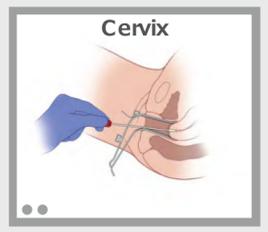
Cervix

Speculum
- Insert single tipped swab into the endocervix to the depth of 1/2 the swab tip.
- Rotate swab for 10 sec.

Chest

Dominant Palm
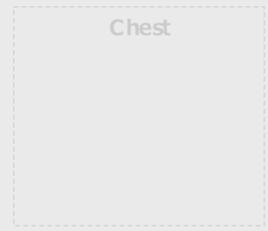
[X] **Vaginal pH**

[X] **Blood**

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Vaginal Cytokine Analysis Protocol

Materials:
- BIO-RAD Bio-Plex Pro™ Human Cytokine 27-Plex Assay, cat. no. M50-0KCAF0Y
- BIO-RAD Bio-Plex Pro Wash Station
- BIO-RAD Bio-Plex MAGPIX or Luminex MAGPIX System
- BIO-RAD Quick Start Bradford Protein Assay Kit 1, cat. no. 500-0201EDU
- Synergy HT Biotek Microplate Reader
- Vaginal swab samples suspended in 500μl 100 mM Tris pH 7.5 and frozen at -80ºC until use

Methods:

A. Cytokine Assay.
1. Thaw samples on ice and centrifuge at 10,000 g for 10 min at 4ºC prior to use
2. Prepare sample dilutions (1:4 in 100 mM Tris pH 7.5)
3. Reconstitute each standard in 500μl standard diluent
4. Prepare serial dilutions of standards, as recommended by the manufacturer
5. Vortex 10x magnetic beads for 30 sec, then dilute to 1x in assay buffer
6. Vortex diluted beads for 20 sec. Add 50μl to each well of assay plate
7. Wash plate with 100μl wash buffer 2 times
8. Add 50μl of each standard or sample in duplicate to assay plate
9. Cover with plate seal and wrap with aluminum foil. Shake on orbital shaker (850 ± 50 rpm) at room temperature for 30 min
10. With 10 min left in incubation, vortex 10x detection antibodies, then dilute to 1x in antibody diluent
11. Wash plate 3 times with 100μl wash buffer
12. Vortex diluted antibodies and add 25μl to each well
13. Repeat step 9. Incubate on shaker for 30 min
14. With 10 min left in incubation, vortex 100x streptavidin-PE, then dilute to 1x in assay buffer
15. Wash plate with 100μl wash buffer 3 times
16. Vortex 1x streptavidin-PE and add 50μl to each well
17. Repeat step 9. Incubate on shaker for 10 min
18. Wash plate with 100μl wash buffer 3 times
19. Add 125μl assay buffer to each well. Seal and cover, then shake for 30 sec
20. Read plate with default Bio-Plex MAGPIX settings
21. Analyze values using the five parameter logistic (5-PL) non-linear regression curve model. Apply the same acceptable recovery range to all assays (suggested range: 70-130%)

B. Bradford Assay.

1. Reconstitute BSA standard with 20 ml of H_2O for a final concentration of 1.418 mg/ml. Freeze $500\mu l$ aliquots at -20°C
2. Dilute dye 1:5 in H_2O and filter
3. Prepare 2-fold serial dilutions of standard for the standard curve to obtain the following final concentrations: 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/ml
4. Add $10\mu l$ of each undiluted sample or standard in duplicate to the assay plate
5. Add $200\mu l$ of diluted dye to each well of the plate
6. Shake for 5 min
7. Read absorbance at 595 nm with Synergy HT Biotek Microplate Reader
8. Calculate total protein concentration: fit standard curve to sample absorbance values using linear regression. For this data set, the linear regression analysis was performed using the KC4^{TM} data reduction software that is bundled with the plate reader

The total protein concentration of each sample is used to normalize the cytokine concentration:

Normalized Cytokine Concentration (pg cytokine/mg protein) = $\dfrac{\text{\textit{Cytokine Concentration (pg/ml)}}}{\text{\textit{Total Protein Concentration (mg/ml)}}}$

Microbiome 16S rDNA Data Analysis Protocol for Vaginal Samples

MiSeq
Paired-End Sequencing
R1 - forward read
R2 - reverse read

↓ Merging Reads

CASPER

Overlapping Reads | Non-Overlapping Reads Merged inserting 15 *Ns*

↓

Merged Read

↓ Quality Filtering
1.0% MEE cutoff

HQ Read

Taxonomy
Classification

STIRRUPS   RDP
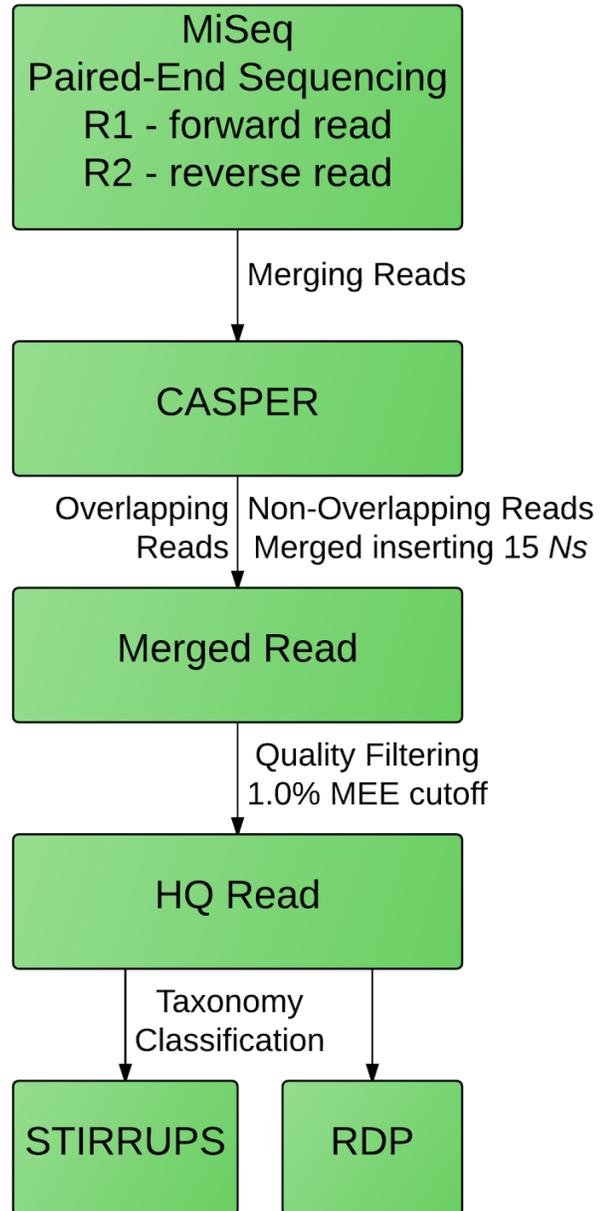
A. CASPER. Context aware scheme for paired-end reads.

A *k-mer* based approach to merge overlapping paired-end reads

Input parameters –

Parameter	Description	Value Used
K	k-mer size (minimum size of k-mer in bp used to represent contexts around mismatching bases)	19
Ω	Minimum overlap length (minimum length in bp of the overlap between forward and reverse reads)	10
Ƴ	Mismatch ratio (CASPER abandons merging if the mismatch ratio in the overlap is greater than Ƴ)	0.27
δ	Quality-score difference threshold (Context-based mismatch resolution starts if quality scores differ less than δ)	19

Ref - http://www.biomedcentral.com/1471-2105/15/S9/S10/

B. Quality Filtering. Filtering based on MEE (maximum expected error, not Q-score)
Traditionally, read quality filters use average-Q score. This is not an accurate measure of number of errors predicted by individual Q-scores
Example –
Two reads of length 150nt

Q scores in read	Avg. Q	Expected number of errors
140 x Q35 + 10 x Q2	33	**6.4 !**
150 x Q25	25	0.5

Read1 has a higher average Q-score. However, with 10 Q2 (P=0.5) bases, the maximum expected error in the read is at least 5 bases. This is much greater than that in Read2, in spite of a lower average Q-score.
Further reading - http://www.drive5.com/usearch/manual/

Filtering criteria used –
MEE threshold – 1% of read length, i.e. for a 500bp long read, the MEE cutoff for a high-quality read will be 5.

C. Classifiers.

C.1. STIRRUPS.

All high-quality reads, both overlapping and non-overlapping, are used as input into the STIRRUPS pipeline.
Ref - http://www.biomedcentral.com/1471-2164/13/S8/S17

C.2. RDP Classifier.

All high-quality reads, both overlapping and non-overlapping, are used as input for RDP Classifier – a Bayesian classifier for taxonomic profiling for rRNA sequence data
RDP profiles – assignment confidence cutoff – 0.8
Ref - https://github.com/rdpstaff/classifier

Targeted lipidomic analysis was undertaken for each patient sample with each sample being investigated for changes in >150 lipids. These lipids consisted primarily of eicosanoids and sphingolipids. In this regards, UPLC ESI-MS/MS was utilized in the data acquisition. The exact analytical methods are detailed below.

Figure 1. General biosynthetic pathways for eicosanoids and EPA-derived lipid mediators derived from the activation of a PLA_2.
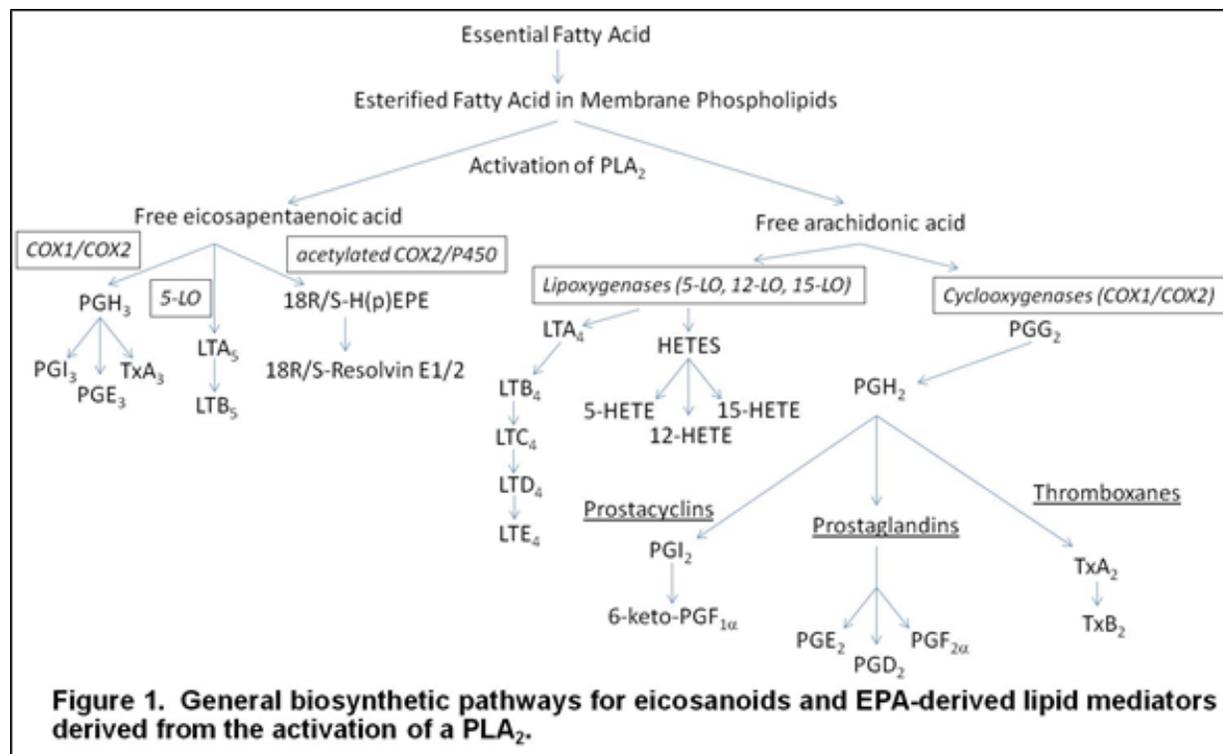
A. Quantitative analysis of eicosanoids.

To 50 µl of a clarified vaginal swab contents dispersed in PBS, 50µl ethanol was added containing 10 ng each of the following internal standards. The specific internal standards consisted of 30 deuterated analytes including, (d_4) 6-keto $PGF_{1\alpha}$, (d_4) $PGF_{2\alpha}$, (d_4) PGE_2, (d_4) PGD_2, (d_4) LTB_4, (d_4) TXB_2, (d_4) LTC_4, (d_5) LTD_4, (d_5) LTE_4, (d_8) 5-hydroxyeicosatetranoic acid (5-HETE), (d_8) 15-hydroxyeicosatetranoic acid (15-HETE), (d_8) 14,15 epoxyeicosatrienoic acid, (d_8) arachidonic acid, and (d_5) eicosapentaenoic acid. The resultant mixture was centrifuged at 12,000 x g for 20 minutes and the supernatant was transferred into a 100 µl autosampler vial. Eicosanoid quantitation was carried out via UPLC ESI-MS/MS analysis. A 14 minute reversed-

phase LC method utilizing a Kinetex C18 column (100 x 2.1mm, 1.7μm) and a Shimadzu Nexera UPLC was used to separate the eicosanoids at a flow rate of 500 μL/min at 50°C. The column was first equilibrated with 100% Solvent A [acetonitrile:water:formic acid (20:80:0.02, v/v/v)] for two minutes and then 10 μL of sample was injected. 100% Solvent A was used for the first two minutes of elution. Solvent B [acetonitrile:isopropanol (20:80, v/v)] was increased in a linear gradient to 25% Solvent B to 3 minutes, to 30% by 6 minutes, to 55% by 6.1 minutes, to 70% by 10 minutes, and to 100% by 10.1 minutes. 100% Solvent B was held until 13 minutes, then was decreased to 0% by 13.1 minutes and held at 0% until 14 minutes. The eluting eicosanoids were analyzed using a hybrid triple quadrupole linear ion trap mass analyzer (AB SCIEX 6500 QTRAP®,) via multiple-reaction monitoring (MRM) in negative-ionization mode. Eicosanoids were monitored using species specific precursor → product MRM pairs. The mass spectrometer parameters used were: curtain gas: 30; CAD: High; ion spray voltage: -3500V; temperature: 500°C; Gas 1: 40; Gas 2: 60; declustering potential, collision energy, and cell exit potential were optimized per transition.

B. Quantitative analysis of Sphingolipids.

To 50 μl of a clarified vaginal swab contents dispersed in PBS, 50μl methanol was added containing 50 pmols of each of the following internal standards. The specific internal standards consisted of d_{17} sphinogsine, sphinganine, sphingosine-1-phosphate, sphinganine-1-phosphate and $d_{18:1/12:0}$ ceramide-1-phosphate, sphingomyelin, ceramide, and monohexosylceramide (Avanti). The mixture was vortexed and then centrifuged at 12,000 x g for 20 minutes. The resultant supernatant was transferred into a 100 μl autosampler vial. The mixture thus obtained was used in the analysis of sphingolipids The lipids were separated using a Kinetix C18 column (50 x 2.1 mm, 2.6μ) (Phenomenex) on a Prominence high performance liquid chromatography system (Shimadzu) and eluted using a linear gradient (solvent A, 58:41:1 CH_3OH/water/HCOOH 5 mm ammonium formate; solvent B, 99:1 CH_3OH/HCOOH 5 mm ammonium formate, 20–

100% B in 3.5 min and at 100% B for 4.5 min at a flow rate of 0.4 ml/min at 60 °C). The eluting sphingolipids were analyzed using a hybrid triple quadrupole linear ion trap mass analyzer (AB SCIEX 6500 QTRAP®,) via multiple-reaction monitoring (MRM) in positive-ionization mode.

C. Data Analysis.

All mass spectrometry data were analyzed using MultiQuant software (AB SCIEX) and quantitation was carried out via comparison against known quantities of internal standards and a 7-point dilution curve.