Mar 30, 2020

High-Throughput Stool Metaproteome Extraction

mSystems

DOI
https://dx.doi.org/10.17504/protocols.io.9gph3vn

Carlos Gonzalez[1], Josh Elias[2]

[1]Stanford University; [2]Chan Zuckerberg Biohub

Carlos Gonzalez

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.9gph3vn

External link: https://doi.org/10.1128/mSystems.00200-20

Protocol Citation: Carlos Gonzalez, Josh Elias 2020. High-Throughput Stool Metaproteome Extraction. **protocols.io** https://dx.doi.org/10.17504/protocols.io.9gph3vn

Manuscript citation:
Gonzalez CG, Wastyk HC, Topf M, Gardner CD, Sonnenburg JL, Elias JE, High-Throughput Stool Metaproteomics: Method and Application to Human Specimens. mSystems 5(3). doi: 10.1128/mSystems.00200-20

Protocol status: Working
We use this protocol and it's working

Created: November 18, 2019

Last Modified: March 30, 2020

Protocol Integer ID: 29935

Keywords: Proteome, metaproteome, stool, mass spectrometry, throughput stool metaproteome extraction the gut microbiome, throughput stool metaproteome extraction, stool protein, gut microbiome, proteomic response, microbiome, mass spectrometer downtime, common multiplexing labeling technology, compatible with common multiplexing labeling technology

Abstract

The gut microbiome is strongly associated various disease related periods. In order to better understand how the host and microbiome interact during these periods, it is necessary to survey the proteomic response of both the host and microbes in a large portion of a population. However to date, techniques used to isolate stool proteins have been low-throughput and due to their lack of purity can foul columns and increase mass spectrometer downtime. To address these shortcomings, this protocol was developed. It allows for the processing of 96 samples in parallel and is compatible with common multiplexing labeling technology. It has thus far been used to process anywhere between 10 and 400 samples, with very reproducible results.

Guidelines

1. The optimal place to stop first part of protocol is after elution of digested peptides from S-trap column/plate
2. Small Nitrogen tanks (R-tanks) run out really quickly, order at least 3.

Materials

MATERIALS

- 96-well Sample Collection Plate 2 mL Square well 50/pk **Waters** **Catalog #**186002482
- 2 ML DEEP WELL PLATES PRE-FILLED WITH 0.1 MM CERAMIC BEADS – 10 PACK **Omni International** **Catalog #**27-6007
- Protifi S-trap 96-well plate **Catalog #**C02-96well-1
- Abgene™ 96 Well 1.2mL Polypropylene Deepwell Storage Plate **Thermo Fisher Scientific** **Catalog #**AB0787
- Eppendorf twin.tec® PCR plate 96 LoBind skirted 150 µL PCR clean colorless 25 plates **Eppendorf** **Catalog #**30129512
- Agilent AssayMap Bravo RPS Cartridges **Agilent Technologies** **Catalog #**G549660033 {RPSM07}
- Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7408-100ML
- Methanol Optima™ LC/MS Grade Fisher Chemical **Fisher Scientific** **Catalog #**A456-4
- Benzonase **Merck Millipore (EMD Millipore)** **Catalog #**101656
- Urea **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**U4884
- Acetonitrile Optima™ LC/MS Grade Fisher Chemical **Fisher Scientific** **Catalog #**A955-500
- TMT multiplex **Thermo Fisher Scientific** **Catalog #**varies-by-kit
- DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**DTT-RO
- Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I1149
- Waters 96 well sealing mat **Waters** **Catalog #**186006335
- Axygen™ AxyMats™ Sealing Mat for 96 Well PCR Microplates **Fisher Scientific** **Catalog #**14-222-024
- Promega trypsin **Promega** **Catalog #**V5113
- HEPES-Na **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H7006-100G
- Hydroxylamine solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**438227

STEP MATERIALS

- 2 ML DEEP WELL PLATES PRE-FILLED WITH 0.1 MM CERAMIC BEADS – 10 PACK **Omni International** **Catalog #**27-6007
- 96-well Sample Collection Plate 2 mL Square well 50/pk **Waters** **Catalog #**186002482
- Protifi S-trap 96-well plate **Catalog #**C02-96well-1
- DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**DTT-RO
- Abgene™ 96 Well 1.2mL Polypropylene Deepwell Storage Plate **Thermo Fisher Scientific** **Catalog #**AB0787
- Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7408-100ML
- Promega trypsin **Promega** **Catalog #**V5113

- TMT multiplex **Thermo Fisher Scientific** **Catalog #**varies-by-kit
- Eppendorf twin.tec® PCR plate 96 LoBind skirted 150 µL PCR clean colorless 25 plates **Eppendorf** **Catalog #**30129512
- HEPES-Na **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H7006-100G
- Axygen™ AxyMats™ Sealing Mat for 96 Well PCR Microplates **Fisher Scientific** **Catalog #**14-222-024
- Benzonase **Merck Millipore (EMD Millipore)** **Catalog #**101656
- Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I1149
- Abgene™ 96 Well 1.2mL Polypropylene Deepwell Storage Plate **Thermo Fisher Scientific** **Catalog #**AB0787
- Methanol Optima™ LC/MS Grade Fisher Chemical **Fisher Scientific** **Catalog #**A456-4
- Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7408-100ML
- 2 ML DEEP WELL PLATES PRE-FILLED WITH 0.1 MM CERAMIC BEADS – 10 PACK **Omni International** **Catalog #**27-6007

Protocol materials

- 2 ML DEEP WELL PLATES PRE-FILLED WITH 0.1 MM CERAMIC BEADS – 10 PACK **Omni International** **Catalog #**27-6007
- Protifi S-trap 96-well plate **Catalog #**C02-96well-1
- Abgene™ 96 Well 1.2mL Polypropylene Deepwell Storage Plate **Thermo Fisher Scientific** **Catalog #**AB0787
- Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7408-100ML
- Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I1149
- Acetonitrile Optima™ LC/MS Grade Fisher Chemical **Fisher Scientific** **Catalog #**A955-500
- DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**DTT-RO
- TMT multiplex **Thermo Fisher Scientific** **Catalog #**varies-by-kit
- Waters 96 well sealing mat **Waters** **Catalog #**186006335
- Promega trypsin **Promega** **Catalog #**V5113
- HEPES-Na **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H7006-100G
- Axygen™ AxyMats™ Sealing Mat for 96 Well PCR Microplates **Fisher Scientific** **Catalog #**14-222-024
- Urea **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**U4884
- Hydroxylamine solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**438227
- 96-well Sample Collection Plate 2 mL Square well 50/pk **Waters** **Catalog #**186002482
- Eppendorf twin.tec® PCR plate 96 LoBind skirted 150 µL PCR clean colorless 25 plates **Eppendorf** **Catalog #**30129512
- Methanol Optima™ LC/MS Grade Fisher Chemical **Fisher Scientific** **Catalog #**A456-4
- Agilent AssayMap Bravo RPS Cartridges **Agilent Technologies** **Catalog #**G549660033 {RPSM07}
- Benzonase **Merck Millipore (EMD Millipore)** **Catalog #**101656

Before start

- Make all buffers prior to starting protocol. It's also highly suggested that you plate all samples the day prior to starting the protocol. Keep them in -80.

Lysis and solubilization

1 Spin down bead beater plates for 1 minute to pull beads into well 00:01:00 at 1000 x g . Aliquot approximately 200 mg of stool into 96 well beads plate.
This protocol will work with less stool at the risk of increasing preparative replicate bias. The samples should be arrayed in a logical way, especially if the end goal is to use multiplexing reagents. Make sure samples are pushed to the bottom of the plate or the lysis buffer may overflow into neighboring wells.

2 ML DEEP WELL PLATES PRE-FILLED WITH 0.1 MM CERAMIC BEADS – 10 PACK **Omni International Catalog #**27-6007

2 To each sample, add 750 µL 750 uL of lysis buffer:
Urea [M] 6 Mass Percent
Tris-Base [M] 50 millimolar (mM)
SDS [M] 5 % volume
pH 08.1
Use a multichannel pipette or get carpal tunnel syndrome instantly.

3 Seal plate with provided top. Plate the sealed plate into 96-well bead beater. 20 Hz for 00:10:00 . Spin down samples in plate centrifuge at 3000 x g for 00:10:00 at 4 °C .

2 ML DEEP WELL PLATES PRE-FILLED WITH 0.1 MM CERAMIC BEADS – 10 PACK **Omni International Catalog #**27-6007

4 Observe the samples and make sure they are sufficiently homogenated/solubilized. Aliquot 500 µL of the homogenated sample into a new Waters 2 mL deep well plate. Spin the new plate for 00:10:00 at 3000 x g and take supernatant into new 2 mL Waters plate, this will be the sample stock.

96-well Sample Collection Plate 2 mL Square well 50/pk **Waters Catalog #**186002482

5 Add 3 3 µL benzonase to each sample and place in incubator at 37 °C for 00:10:00

Benzonase **Merck Millipore (EMD Millipore) Catalog #**101656

6 To each well, add 10 uL [M] 500 millimolar (mM) DTT. Reseal with same mat. Place samples in incubator at 47 °C for 00:30:00

DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**DTT-RO

7 Remove plates from incubator and let cool to room temperature. Spin plates down for 00:00:30 at 1000 x g After plate has cooled, remove sealing mat and add 30 µL of [M] 500 millimolar (mM) iodoacetamide. Place into totally dark environment (e.g. closed drawer) for 01:00:00

Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I1149

Abgene™ 96 Well 1.2mL Polypropylene Deepwell Storage Plate **Thermo Fisher Scientific Catalog #**AB0787

Protifi Plate Digestion

8 Aliquot 50 µL of sample into new Thermo 1.2 mL plate (Waters plate works as well but is harder to pipette from). Add 5 µL of [M] 12 % volume phosphoric acid. Add 300 µL Protifi Sample Binding Buffer:

[M] 90 % volume methanol

[M] 10 % volume triethylammonium bicarbonate (TEAB)

Methanol Optima™ LC/MS Grade Fisher Chemical **Fisher Scientific Catalog #**A456-4

pH 7.1

Mix each sample by pipetting up and down for 00:00:10 .

Expected result

You will often see a cloudy colloidal suspension after adding the binding buffer.

Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T7408-100ML

9 Add the mixture from step 7 to the Protifi S-trap 96-well plate. Place S-trap on top of Waters 96 well 2 mL plate. This will be waste plate. Use the positive pressure machine to press sample through. Our experience suggests that 6 psi on the main dial and 15 on the secondary control dial will push through a majority of samples. There will be clogging and this can be corrected by gently scraping the top of the well with a pipette tip.

Protifi S-trap 96-well plate **Catalog #**C02-96well-1

Equipment

NAME	BRAND	SKU	LINK
Waters 96-well Positive Pressure Processor	Waters	NA	https://www.waters.com/waters/en_US/Waters-Positive-Pressure-96-Processor/nav.htm?cid=10161512&locale=en_US

10 Wash samples with 500 µL fresh Protifi Binding Buffer 5 times. You may have a few clogs at this point as well, clear them as before. Be sure to empty waste plate as needed. After washes are done, place S-trap plate on fresh 1.2 mL Thermo plate.

Abgene™ 96 Well 1.2mL Polypropylene Deepwell Storage Plate **Thermo Fisher Scientific Catalog #**AB0787

11 To each well, add 5 µg of trypsin dissolved in 125 µL of [M] 50 millimolar (mM) TEAB. Place Protifi S-trap lid back on plate (should be loose fitting). Place in incubator for 03:00:00 . After 02:00:00 have passed, check that digestion mixutre has been taken up by wells. If it is still sitting on top, place 96 well plate and it's collection plate into the positive pressure machine for approximately 00:00:05 and check if liquid drained into collection plate. If any liquid came out (it is normal that it does), place back into appropriate well and continue with digestion.

Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T7408-100ML

Promega trypsin **Promega Catalog #**V5113

12 After digestion, elute peptides using positive pressure. Once initial digestion volume has been eluted, add 100 µL of TEAB and use positive pressure to wash directly into previously eluted peptides. Repeat this with 100 µL of [M] 0.2 % volume formic acid (FA), then 100 µL of a mixture of [M] 50 % volume acetonitrile and [M] 0.2 % volume FA. This should be approximately 425 µL of digested peptides and wash. Speedvac to dryness.

RPS or C18 cleanup

13 Resuspend the dry peptides in 150 µL of [M] 0.2 % volume FA. Pipetting up and down as well as washing well walls is critical in getting maximum peptide recovery. Spin plate at 3000 x g for 00:10:00 . Carefully avoiding any debris left in the well, move peptide suspension into fresh plate.

14 Consult ownder of Assaymap Bravo to use Peptide Cleanup V2 app using either RPS or C18 tips. These tips have a capacity of approximately 100 µg but you will likely lose quite a bit of it during this step. Dry down samples in speedvac

15 Resuspend dried down peptides in 30 µL of [M] 0.2 % volume FA.

16 If samples will not be labeled (eg TMT, see below) use nanodrop to adjust concentration that is appropriate for your mass spectrometer (usually 0.5 µg to 1 µg is sufficient for nano-flow LC systems.

OPTIONAL - TMT labeling

17 From step 15, nanodrop samples and record concentration. If everything was properly done and you started with enough sample, each well should have upwards of 40 µg of unlabeled peptide. We will use 20 µg of this for TMT labeling. Resuspend each well at a concentration of 0.5 µg per 1 µL . Transfer 20 µg of peptide from each well into a new plate. Dry down both plates. Leave your original plate dryand store in -20 °C .

18 From the concentrations obtained in go to step #17 , calculate the ratio of TMT reagent to peptide. Thermo suggests that you have at least 8:1 TMT to peptide, however YMMV. For the example below our ratio was approximately 6.5:1 TMT to peptide.

Note

Kuster paper showing TMT ratio as low as 1:1 if concentrations are kept at least 4ug/uL.: https://www.mcponline.org/content/early/2019/04/09/mcp.TIR119.001385

19 Resuspend 20 µg plate in 50 µL of [M] 50 millimolar (mM) Na-HEPES at pH 08.5 (should be around that pH already). Move peptide suspension into fresh Eppendorf 150 uL plate

Eppendorf twin.tec® PCR plate 96 LoBind skirted 150 µL PCR clean colorless 25 plates **Eppendorf Catalog #**30129512

HEPES-Na **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H7006-100G

20 Take TMT kit out of freezer and let warm to room temperature for approximately 00:10:00 , do not open lids to avoid condensation . Spin down each reagent tube to collect residue on top on benchtop micro centrifuge. For each sample to be labeled with a specific channel, add 20 µL of acetonitrile (ACN). EXAMPLE: if you have a single 96 well plate and a TMT-11plex, and every sample in column 1 will be labeled with channel 126, add (n+1)*20 uL of ACN, where n is the number of samples in column 1. After each reagent has been resuspended, close lid and quickly vortex then wait 00:05:00 for reagent to completely dissolve, with another quick vortex 00:02:30 into 5 minute period . After reagent is dissolved, spin down and aliquot into fresh 96 well plate first row (up to TMT-11), you will use a multichannel in next step to redistribute into each sample.

TMT multiplex **Thermo Fisher Scientific Catalog #**varies-by-kit

21 With a multichannel pipette, carefully redistribute 20 µL of the appropriate TMT reagent into each well. Cover loosely with Axygen silicone cover to avoid ACN loss. Incubate for 01:00:00

Axygen™ AxyMats™ Sealing Mat for 96 Well PCR Microplates **Fisher Scientific Catalog #**14-222-024

22 Quench reaction with 10 µL of [M] 5 % volume hydroxylamine solution in [M] 50 millimolar (mM) HEPES-Na. Incubate for 15 minutes at room temperature

23 Acidify each well down to approximately pH 2 with [M] 25 % volume formic acid. This should be done on a dummy sample with no peptides in it. In general this may be up to 15 µL .

24 You now need to construct your ratio check samples. Each ratio check should have a total of 10 µg peptide. This means that each channel needs to contribute equally to that amount so the total taken from each well will depend on what TMT kit is used. For example if a TMT-11 kit is used, each sample/channel would contribute 10 ug/11 = 0.9 ug. Transfer appropriate amount from each sample/channel into its ratio check tube

If you are doing a bridge channel, each bridge will need a total of 20 ug so make sure you calculate that too.

25 Resuspend sample in 10 µL [M] 0.2 % volume FA and check concentration on nanodrop. Dilute to 0.5 ug/uL. Bottle up samples and shoot on mass spec. Quant ratios

26 Run resulting raw files through Proteome Discoverer 2.2. Calculate ratios from each channel using PSM quant values. Use these ratio values to adjust each channel on original plate for a 1:1 ratio. Compile new sample and reshoot.