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High-Throughput Stool Metaproteome Extraction

Carlos Gonzalez¹, Josh Elias²

¹Stanford University, ²Chan Zuckerberg Biohub





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

NAME ~	CATALOG #	VENDOR ~
96-well Sample Collection Plate 2 mL Square well 50/pk	186002482	Waters
2 ML DEEP WELL PLATES PRE-FILLED WITH 0.1 MM CERAMIC BEADS – 10 PACK	27-6007	Omni International
Protifi S-trap 96-well plate	C02-96well-1	
Abgene™ 96 Well 1.2mL Polypropylene Deepwell Storage Plate	AB0787	Thermo Fisher Scientific
Eppendorf twin.tec® PCR plate 96 LoBind skirted 150 μL PCR clean colorless 25 plates	30129512	Eppendorf
Agilent AssayMap Bravo RPS Cartridges	G549660033 {RPSM07}	Agilent Technologies
Triethylammonium bicarbonate buffer	T7408-100ML	Millipore Sigma
Methanol Optima™ LC/MS Grade Fisher Chemical	A456-4	Fisher Scientific
Benzonase	101656	Emd Millipore
Urea	U4884	Millipore Sigma
Acetonitrile Optima™ LC/MS Grade Fisher Chemical	A955-500	Fisher Scientific
TMT multiplex	varies-by-kit	Thermo Fisher Scientific
DTT	DTT-RO	Millipore Sigma
lodoacetamide	l1149	Millipore Sigma
Waters 96 well sealing mat	186006335	Waters
Axygen™ AxyMats™ Sealing Mat for 96 Well PCR Microplates	14-222-024	Fisher Scientific

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NAME	CATALOG #	ALIADOK
Promega trypsin	V5113	Promega
HEPES-Na	H7006-100G	Millipore Sigma
Hydroxylamine solution	438227	Millipore Sigma
STEPS MATERIALS		
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CATALOG #

VENDOR

BEFORE STARTING

NAME >

 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

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 To each sample, add **3750 μl** 750 uL of lysis buffer:

Urea [M]6 Molarity (M)

Tris-Base [M] 50 Milimolar (mM)

SDS [M] 5 % volume

pH**08.1**

Use a multichannel pipette or get carpal tunnel syndrome instantly.

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 .



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



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



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



Remove plates from incubator and let cool to room temperature. Spin plates down for ③ 00:00:30 at ③ 1000 x g After plate has cooled, remove sealing mat and add □30 μl of [M]500 Milimolar (mM) iodoacetamide. Place into totally dark environment (e.g. closed drawer) for ⑤ 01:00:00



Abgene™ 96 Well 1.2mL Polypropylene
Deepwell Storage Plate
by 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)



рН**7.1**

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

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

Triethylammonium bicarbonate buffer by Millipore Sigma
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.

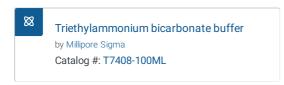




 10 Wash samples with **300 μl** fresh Protifi Binding Buffer 5 times. You may have a few clogs at this point as well, clear them as before. Be sure to empty waste plate as needed. After washes are done, place S-trap plate on fresh 1.2 mL Thermo plate.



To each well, add 5 μg of trypsin dissolved in 125 μl of [M]50 Milimolar (mM) TEAB. Place Protifi S-trap lid back on plate (should be loose fitting). Place in incubator for 3:00:00 . After 30:00:00 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 30:00:05 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.





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

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.

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- 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
- Resuspend dried down peptides in $\square 30 \, \mu l$ of [M] 0.2 % volume FA.
- If samples will not be labeled (eg TMT, see below) use nanodrop to adjust concentration that is appropriate for your mass spectrometer (usually **10.5** µg to **11** µg is sufficient for nano-flow LC systems.

OPTIONAL - TMT labeling

- 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 δ -20 °C.
- From the concentrations obtained in **o 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.

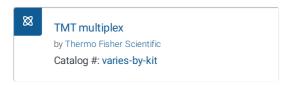


19 Resuspend 20 μg plate in 50 μl of [M]50 Milimolar (mM) Na-HEPES at pH08.5 (should be around that pH already). Move peptide suspension into fresh Eppendorf 150 uL plate





 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.



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 **301:00:00**



- 22 Quench reaction with **10 μl** of [M]**5 % volume** hydroxylamine solution in [M]**50 Milimolar (mM)** HEPES-Na. Incubate for 15 minutes at room temperature
- Acidify each well down to approximately μΗ2 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.
- 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

 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.

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