DNA & RNA Extraction from Stool via PowerFecal Qiagen Kit (Automated)

Protocol by the Broad Institute Genomics Platform (https://www.broadinstitute.org/genomics)
Version from October 28, 2020.

Process Overview

The purpose of this document is to serve as a standard operating procedure to isolate DNA & RNA from no more than 93 fresh or frozen stool samples. This will leave room in the plate for 2 control samples which are as follows: a negative control and a Microbial Omics Core (MOC) stool control. This will also leave space for an additional in process control for downstream Microbial Genome Sequencing.

Safety Precautions:

1. The SOP does not cover detailed safety procedures for handling Human Biological Materials (HBMs) and it is recommended that personnel follow institutional BL2 safety guidelines and always observe BL2 universal precautions.
2. In the event of any liquid spilling on the skin or eyes immediately flush with water and contact assistance.
3. Appropriate PPE such as lab coat and gloves must be worn at all times while conducting this protocol.
4. β-mercaptoethanol will be used in the protocol and should be exposed only in an active hood.
5. Ethanol based buffers must be disposed of in the properly labeled waste container.
6. Solution PM1 contains guanidinium thiocyanate and therefore should never be mixed with bleach during cleaning. In addition to this hazardous decomposition products will form under fire conditions. Keep away from oxidizing agents, and acidic or alkaline products.
7. Buffers RW1, AW1, and C4 in this kit contain guanidine hydrochloride and therefore should never be mixed with bleach during cleaning. In addition to this hazardous decomposition products will form under fire conditions. Keep away from oxidizing agents, and acidic or alkaline products.

Materials:

Equipment:

Item	Vendor	Model
Bravo	Agilent	96LT
TissueLyzer II	Qiagen	N/A
TissueLyser Adapter Set	Qiagen	69984
70°C Incubator	Varies	Varies
55°C Incubator	Varies	Varies

Supplies:

Item	Vendor	Catalog Number
RAPID Slit Seal	BioChromato	RSS-S96-80122
Anti-Static Polypropylene Weighing Funnels	MoBio	23302-50
50mL conical tube	VWR International, LLC	21008-775
150mL bottle	VWR International, LLC	29443-026
S-Blocks	QIAGEN INC	102655
Deepwell Plate with white label	QIAGEN INC	89085-622
Plate, 96w Twintec Blue (25/bx)	VWR International, LLC	47744-122
Plate, 96w Twintec Red (25/bx)	VWR International, LLC	47744-124

Reagents:

Item	Vendor	Catalog Number
AllPrep Powerfecal Kit	QIAGEN INC	1114341
2-Mercaptoethanol, ß-ME	Sigma	63689
Disinfectant for ALL labs, 22oz	WW GRAINGER CO	3VDL4
Wipe, RNase Zap Ambion (100/PK)	Life Technologies, Inc.	AM9786
DNA ZAP!, Degradation Solution	Life Technologies, Inc.	AM9890

Procedure:

Pre-Procedure

1. Clean all work surfaces with appropriate Citrus II, EtoH, RNAse, or DNAse zap before starting any work for the day.
2. Locate the samples needed for this extraction using cold chain best practices.
3. If the sample does not appear to be frozen in the tube or has liquid surrounding the bottom - **stop processing** and refreeze the samples. Identify what the material is and properly remove it before adding the sample to the input plate.
4. Obtain a Microbial Omics Core (MOC) stool control that can thaw on the bench top before adding to input plate.
5. Spin down Microbial Omics Core (MOC) stool control for 5 min @ ≥10,000 x G and pipette off the supernatant. Let sit on dry ice before adding to input plate. Stool should be solid consistency. If liquid is not removed on first spin, preform centrifugation again.
6. Add ethanol to all buffer concentrates (RPE, AW1, AW2); the amount shown indicated on each. Check, sign, and date each reagent.
    1. RPE Wash Buffer Concentrate 55mL needs **220mL of 100% ethanol**
    2. AW1 Wash Buffer Concentrate 98mL needs **130mL of 100% ethanol**
    3. AW2 Wash Buffer Concentrate 66mL needs **160mL of 100% ethanol**
7. Buffer PM1 (First step of Protocol) should be heated to 55°C for 10 to 30 minutes. β-mercaptoethanol should be added to the PM1 in a 1:100 ratio immediately before addition to frozen sample. **For a full plate use 68mL of PM1 with 680µL of β-ME.**
8. Buffer EB (Last step of Protocol) should be heated to 70°C prior to use in DNA elution. Do not allow prolonged exposure to heat as bubbles will form in the wells and cause uneven pipetting.
9. Prep all s-blocks needed. Label S-Blocks and fill with proper reagents according to chart:

S-Block Label	Volume/Well
IRS Lysate	150uL
C4 Lysate	600uL
100% EtOH	800uL
RW1	600uL
RPE	1200uL
AW1	600uL
AW2	600uL

10. Label one collection tube plate as "RNA Flow Through"
11. Label the following two empty S-Blocks that are fitted for filters as RNA waste and one as DNA waste.
12. Label 2 PCR plates for the elution of nucleic acids. A red plate can be used with 150uL of water in each well for RNA elution. A blue plate can be used with 150uL of EB for DNA elution.
13. Label 2 Elution Capture plates, one for RNA and one for DNA.
14. Centrifuge PowerBead plate (0.1mm/0.3mm bead mix) prior to opening for 1 minute @ 1000 x g. (This is to help keep beads towards the bottom of the block for better sealing).

15. Procedure - Manual Preparation

1. Transfer samples to PowerBead plate while tracking each sample as it is added to the plate. Update the mass used if needed. Do not transfer more than 250 mg of stool into the plate. **It is recommended to use 75-100mg of stool to avoid overloading the plate.**
2. Maintain cold chain, premature thawing will compromise the integrity of the nucleic acid. Do not allow PowerBead block to stay on dry ice for an extended period of time with the shipment mat attached to the top as this will prevent proper sealing. If the block needs to be stored at -80C overnight use a temporary freezer safe seal and keep the shipment mat in a sealed bag at room temperature.
3. Prime heated buffer PM1 with 1:100 of Beta-Mercaptoethanol (10 ul BME per 1 mL of PM1) before adding to samples. Do this by aliquoting out the appropriate amount of PM1 for the number of extraction samples, as B-ME is not stable for long term storage when mixed with PM1. Vortex thoroughly before using.
    1. **Full plate: 68mL of heated PM1 with 680uL B-ME added right before use**
    2. For partial plates use formula: 660uL x (1.2 x Number of Samples) = Total buffer needed
4. Take PowerBead plate off of dry ice immediately before adding 660 ul Buffer PM1/BME to each well. Stool should be frozen inside plate before lysis buffer addition. Reseal the plate with PowerBead Mat. Ensure to press down on PowerBead Mat firmly. **Check thoroughly that each well is pressed down securely before continuing.**
5. Place PowerBead Plate into TissueLyzer II bead beater using 2 room temperature plate attachments to secure the PowerBead plate with an equal balance. The flat top 96 adaptor plate (no black on inside) should be on the mat-side of the plate. The 24 well plate adaptor should be on the bottom. Secure the assembly by turning until difficult to tighten and turn click-knob to lock in place. Adequate sealing of bead plate mat and secure pressure from adapter blocks is critical in preventing sample cross-contamination and spilling.
6. Shake plate at 20 Hz for 5 minutes. If there is excessive foaming in the wells, centrifuge plate at 4500 x g for 3 min. Once complete, repeat step after rotating samples 180 degrees. (Samples on the close side to the shaking axis should now be on the far side; and vice versa. You can mark the first “outward facing” side as a reference to ensure position change. Seal the mat again to ensure that it will not leak. After the lysis ensure adequate mixing has occurred before moving forward.
7. Centrifuge PowerBead Plate for 6 minutes at 4,500 x g.
8. Carefully transfer 400uL of supernatant from the PowerBead Plate into a new S-block, labeled “IRS” that has been preloaded with 150uL of IRS buffer.
9. Seal plate and place on BioShake for 3 min @ 1200 RPM.
10. Centrifuge at room temperature for 6 minutes at 4,500 x g. Remove and discard seal after.

16. Procedure - Bravo Protocol (Automated)

Bravo Script 1
Set Up Bravo Protocol: **1) Qiagen AllPrep Initial Filter**

Deck 2: Sample "IRS Lysate"
Deck 3: AllPrep Filter Plate on top of Flow through collection tube plate.
Deck 4: Empty
Deck 5: "C4" S-Block with 600uL of C4 reagent*
Deck 7: Tips
Deck 8: Empty
Deck 9: Empty

*C4 reagent should be mixed vigorously and loaded into the plate immediately before use to prevent settling.

Run Program: 1) Qiagen Allprep Initial Filter

Bravo does the following:

1. Transfers 450ul of sample supernatant from 2 (IRS) to 5 (600uL of C4) mix 15 x with pipette.
2. Incubate for 1 min at room temperature.
3. Transfers 1000ul of sample from 5 (C4 plate) to 3 (Allprep DNA Filter Plate)
4. Msg: **Centrifuge Allprep kit at 4,500 x g for 3 min. Remember to save RNA flow through and ensure all sample has passed through the filter plate.**
5. Use plastic seal on Allprep DNA Filter Plate and place atop new 2ml S-block labeled "DNA wash waste".
6. **Msg: Store filter plate at 4°C until after RNA processing.**

Bravo Script 2
Set Up Bravo Protocol: **2) Qiagen Allprep RNA Isolation Washes**

Deck 2: RNA Flow Through Collection Tube Plate
Deck 3: RNeasy Filter Plate atop "RNA Waste"
Deck 4: 100% EtOH (900uL)
Deck 5: RW1 (600uL)
Deck 6: RPE (1200uL)
Deck 7: Tips
Deck 8: Tips
Deck 9: Tips

Note: The amount of volume that will pass through the filter plate may exceed the capacity of the waste trough inside the vacuum. **Be sure to check the levels of waste during use and dispose of properly with other B-ME waste to prevent overflow.**

Run Program: 2) Qiagen Allprep RNA Isolation Washes

Bravo does the following:

1. Transfers 1000ul of RNA Flow Through from 2 (RNA FT) to 4 (100% EtOH) and mix 15x
2. Msg: **Check RNA flow through mix; Ensure flow through and Ethanol have been properly mixed together**
3. *Incubate for 3 min at room temp*
4. Transfers 900ul of sample from 2 (RNA FT) to 3 (RNeasy FP)
5. Msg: **Vacuum Plate; Place on Vacuum plate for 1 min.**
    1. **If flow through does not pass then spin filter plate with wash S-block at 4500 x g for 4 minutes and then place both plates back on deck at position 3.**
6. Transfers 900ul of sample from 2 (RNA FT) to 3 (RNeasy FP)
7. Msg: **Vacuum Plate; Place on Vacuum plate for 1 min.**
    1. **If flow through does not pass then spin filter plate with wash S-block at 4500 x g for 4 minutes and then place both plates back on deck at position 3.**
8. Transfers 500ul of RW1 from 5 (RW1) to 3 (RNeasy FP)
9. Msg: **Vacuum Plate; Place on Vacuum plate for 1 min.**
    1. **If flow through does not pass then spin filter plate with wash S-block at 4500 x g for 4 minutes and then place both plates back on deck at position 3.**
10. Transfers 500ul of RPE from 6 (RPE) to 3 (RNeasy FP)
11. Msg: **Vacuum Plate; Place on Vacuum plate for 1 min.**
    1. **If flow through does not pass then spin filter plate with wash S-block at 4500 x g for 4 minutes and then place both plates back on deck at position 3.**
12. Transfers 500ul of RPE from 6 (RPE) to 3 (RNeasy FP)
13. Msg:
    Centrifuge plate at 4,500 x g for 10 minutes

Bravo Script 3
Set Up Bravo Protocol: **3) Qiagen Allprep RNA Final Elution**

Deck 1: 96 Matrix 0.75 ml tubes.
Deck 2: Elution capture plate.
Deck 3: RNeasy Filter Plate on elution plate.
Deck 5: RNAse-free water eppendorf plate.
Deck 7: Tips.

Run Program: **Qiagen Allprep RNA Final Elution**

Bravo does the following:

1. Transfers 60ul of water from 4 (Epp. Plate) to 3 (RNeasy FP)
2. *Incubate for 2 min at room temp*
3. Msg: **First Centrifuge Step; Incubate for 2 min at RT. Spin filter plate and elution collection plate at 4500 x g for 4 minutes then place both plates back on deck at position 3.**
4. Transfers 60ul of water from 4 (Epp. Plate) to 3 (RNeasy FP)
5. *Incubate for 2 min at room temp*
6. Msg: **Second Centrifuge Step; Incubate for 2 min at RT. Spin filter plate and elution collection plate at 4500 x g for 4 minutes. After spin, place ONLY the collection plate at position 3 and hit continue.**
7. Transfers 95ul of sample from 3 (Elution Plate) to 1 (96 Matrix)
8. Cap samples and store at -80°C.

Bravo Script 4
Set up Bravo Protocol: **4) Qiagen Allprep DNA Isolation Washes**

Deck 3: DNA AllPrep Filter Plate.
Deck 4: AW1 (600uL)
Deck 5: AW2 (600uL)
Deck 7: Tips.
Deck 8: Tips.

Remove from storage and unseal the Allprep 96 DNA plate atop 2ml "DNA wash waste" block.

Run Program: **4) Qiagen Allprep DNA Isolation Washes**

Bravo does the following:

1. Transfers 500ul AW1 from 4 (AW1) to 3 (DNA FP)
2. Msg: **Run DNA Allprep Plate on Vacuum Filtration; Please place DNA Allprep labware on vacuum and draw liquid through filter.**
    1. **If flow through does not pass then spin filter plate with wash S-block at 4500 x g for 4 minutes and then place both plates back on deck at position 3.**
3. Transfers 500ul AW2 from 5 (AW2) to 3 (DNA FP)
4. Msg: **Run DNA Allprep Plate on Vacuum Filtration; Please place DNA Allprep labware on vacuum and draw liquid through filter.**
    1. **If flow through does not pass then spin filter plate with wash S-block at 4500 x g for 4 minutes and then place both plates back on deck at position 3.**
5. Msg:
   Centrifuge plate at 4,500 x g for 10 minutes

Bravo Script 5
Set up Bravo Protocol: **5) Qiagen Allprep DNA Final Elution**

Deck 1: 96 Matrix 0.75 ml tubes.
Deck 2: Elution capture plate.
Deck 3: DNA Filter Plate.
Deck 4: EB warmed to 70°C and spun down
Deck 7: Tips.

Run Program: **5) Qiagen Allprep DNA Final Elution**

Bravo does the following:

1. Transfers 60ul of EB from 4 (Epp. Plate) to 3 (DNA FP)
2. Incubate for 5 min at room temp
3. Msg: **First Centrifuge Step; Incubate for 5 min at RT. Spin filter plate and elution collection plate at 4500 x g for 4 minutes then place both plates back on deck at position 3**
4. Transfers 60ul of EB from 4 (Epp. Plate) to 3 (DNA FP)
5. Incubate for 5 min at room temp
6. Msg: **Second Centrifuge Step; Incubate for 5 min at RT. Spin filter plate and elution collection plate at 4500 x g for 4 minutes. After spin, place ONLY the elution capture plate at position 3 and hit continue.**
7. Transfers 95ul of sample from 3 (Elution Capture Plate) to 1 (96 Matrix)
8. Cap samples and store at +4°C for short term storage or -80°C for long term storage.
9. Clean up area and make sure all waste is placed in appropriate disposal areas.
10. Empty waste collection trough inside vacuum and clean with Citrus II. Wipe down the QiaVac top plate and gasket with water. Ensure it is clean and dry before leaving the lab. The rubber gasket is highly susceptible to cracking when exposed to ethanol.
11. Clean all work surfaces with appropriate Citrus II, EtoH, RNAse, or DNAse zap before leaving the lab for the day.