Metro South Health

SECTION: Clinical   **Procedure No.** 01137/v1/06/2018
PROCEDURE TITLE: Measuring bacterial density on biopsy samples using qPCR

Review Officer: Consultant, Department of Gastroenterology and Hepatology
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PURPOSE

In specific clinical situations (e.g. for patients with unexplained gastrointestinal symptoms or postprandial distress syndrome) and as a part of diagnostic work up, it may be required to determine the bacterial density in patient duodenal mucosal samples. The aim is to obtain an estimate of the copy number of the bacterial 16S rRNA gene by qPCR using the standard curve method. This Procedure provides guidance to perform the required assay.

OUTCOME

The bacterial load is obtained (as expressed through the ratio of 16S RNA and beta actin) in the proximal small bowel. This is a surrogate marker for the density of bacterial colonisation and can be used to diagnose small intestinal bacterial overgrowth (SIBO).

AUTHORISED TO UNDERTAKE THE PROCEDURE

Clinical and laboratory staff of the Department of Gastroenterology and Hepatology at PAH

INDICATIONS OR CONTRAINDICATIONS

Patients with otherwise unexplained gastrointestinal symptoms (in particular meal related dyspepsia symptoms)

RISKS AND PRECAUTIONS

N/A

STEPS OF THE PROCEDURE

Samples from the 2nd part of the duodenum are taken using the patented Brisbane Aseptic Biopsy Device (1) and will be stored in RNA later. All samples will be stored at -80 °C prior to analysis. Genomic DNA (gDNA) will be extracted from all duodenal samples using established methods (2)**.** Duodenal MAM density and bacterial load will be determined using the following protocol.

Primers

Bacterial
Bacterial 16S rRNA gene: 1114F   CGGCAACGAGCGCAACCC
                                1221R   CCATTGTAGCACGTGTGTAGCC
Human
Human β-actin gene: β-actin-gDNA-For   TCCGCAAAGACCTGTACGC
                                β-actin-gDNA-Rev   CAGTGAGGACCCTGGATGTG

Standards (for standard curves)

The standards are made from PCR products generated for 16S and β-actin (see Appendix A for details).

- Measure the concentration of the cleaned PCR product to be used as the standard
- Calculate the number of copies of either the 16S gene or β-actin gene per ng DNA in your PCR product as per instructions in Appendix B
- Dilute the PCR product such that you have a solution containing 10^{10} copies of the gene per 1 µL DNA (Appendix B)
- Aliquot this solution and store at -30°C, use as needed (avoid freeze-thawing)
- To create your standard curve for an assay - perform a 10-fold serial dilution, starting with the 10^{10} sample (e.g. 4 µL of 10^{10} PCR product and 36 µL water [mix well] → gives 10^9)
- Continue dilution series until you reach 10^2 copies
- Use the dilutions as template in your assay, a typical standard curve would consist of the standards 10^8 through 10^2

Reaction (Standards)
The standards are made from PCR products generated for 16S and β-actin (see Appendix A for details)

	Volume (μL)
Power SYBR green PCR master mix	10
Forward primer (10 μM)	0.2
Reverse primer (10 μM)	0.2
DNA template (16S or β-actin standard)	1
Water (DNase-RNase free PCR grade)	8.6
Total	**20**

All standards, controls and samples are to be performed in triplicate

Reaction (Samples)
The amount of template gDNA to add will vary depending on your sample. This has been optimised for intestinal mucosal biopsies (which have a large proportion of human DNA and a small amount of bacterial DNA). Adding too much DNA template will cause inhibition and low efficiency

	Amount
Power SYBR green PCR master mix	10 μL
Forward primer (10 μM)	0.2 μL
Reverse primer (10 μM)	0.2 μL
DNA template (gDNA extracted from biopsy)	15 ng
Water (DNase-RNase free PCR grade)	(up to 9.6 μL including DNA template)
Total	**20 μL**

Remember to include a no-template (water only) control for each primer set
All standards, controls and samples are to be performed in triplicate

Cycle Conditions
**Use the default conditions on the Viia7 machine*:*
Hold:	95°C 10 min
Cycle (40x):	95°C 15 sec
	60°C 60 sec
Melt curve:	95°C – 60°C – 95°C

Setting up the run
(On the bench)
- Prepare your standard curve dilution series
- Prepare your triplicate reactions for the standard curve (i.e. 4 μL of standard and 34.4 μL water will give you enough to do the three required wells, use 9.6 μL per well)
- Prepare your DNA samples to be used as template
  e.g. DNA sample: concentration of 60 ng/μL → dilute this 1/10
  For each well, now 2.5 μL is required per well (for 15 ng), along with 7.1 μL water
  Therefore, for triplicate, add 10 μL of diluted DNA sample, and 28.4 μL water
  Use 9.6 μL of this per well
- Store on ice until needed
(In the PCR hood)
- Prepare master mix of Power SYBR green and primers
- Add 10.4 μL of this master mix per well
(On the bench)
- Add 9.6 μL of prepared standards, templates and controls to each well as appropriate
- Seal plate with a qPCR plate seal (i.e. an optical seal)
- Spin down plate in bench top centrifuge for 1 min at 2000 rpm
- Place on ice in the dark until ready to run on qPCR machine

Typical plate plan

Setting up the Viia7

- Log in to the computer
- Turn on the qPCR machine using the touch screen on the front
- Check which block is inserted in the machine (i.e. 96-well or 384-well) → change the block if required (ask for help if you don't know how to do this)
- Open the Quant Studio software
- Select new experiment
- Set up run:
  - 384-well plate, standard curve, SYBR green, Standard (not fast)
  - Define targets: type primer name under target (create multiple if you are using several primer sets on the same plate)
  - Assign: Create a standard curve using the "Define and set up standards" button; assign the remaining wells of your plate according to your plate plan
  - Run method: Make sure the correct volume per well is entered. Check the default cycle conditions match what you want
  - Save experiment set up
- Using the eject button on the touchscreen, open the plate holder
- Load your plate, then close using the eject button again
- On the computer, go to run – click the green Run experiment button, and then click on the number that appears underneath, to start the cycle running (the plate must already be in the machine)
- The block will heat and then the time remaining will appear on the screen (Around 1 h 45 min)
- At the end of the run, save your results, export figures and excel sheet, remove your plate and log off computer

Data analysis

- Check the quality of the run by looking at the curves, ensure negative control is negative, etc.
- Average all your triplicate Ct values (exclude any outliers)
- Create a standard curve using the template (excel sheet)
- Generate the equation that matches your standard curve
- Check the efficiency of your standard curve: this should be as close to 1 as possible (a range 0.9 to 1.1 is ok)
- Substitute your Ct values into the equation generated from the standard curve to calculate the copy number of 16S and/or β-actin in your DNA samples

- Take the ratio between 16S copies and β-actin copies

Appendix A – Standards
The PCR products used as standards in the above assay were produced as follows:
1. Using a mixture of gDNA from various biopsy samples, the 16S and β-actin PCRs were performed (using the primers above)
2. The PCR product was cloned into the pUC19 plasmid vector (blunt cloning); correct insertion was confirmed by Sanger sequencing
3. The plasmids pUC19-16S and pUC19- β-actin were grown and purified from E. coli
4. A PCR was performed using the M13 forward and M13 reverse primers
5. This PCR product was purified via gel extraction and is used as the template for the qPCR.

PCR product sizes (using M13 to amplify from plasmid)
β-actin: 245 bp
16S: 308 bp
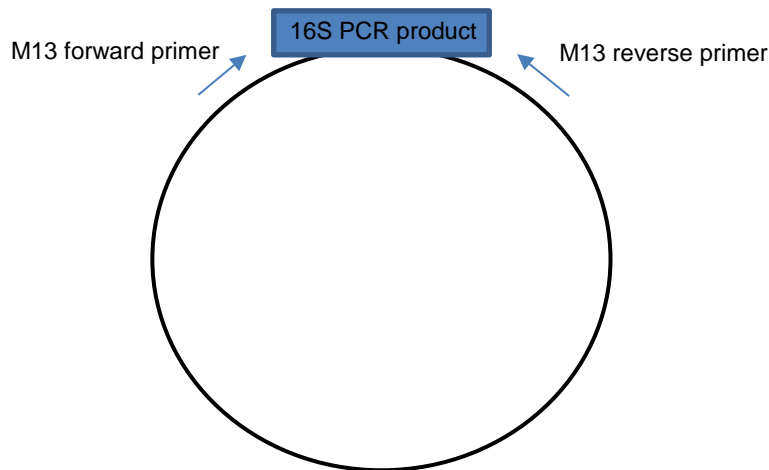
Appendix B – Calculating Copy Number
Go to: http://www.thermoscientificbio.com/webtools/copynumber/
Select custom fragment and enter the fragment length (e.g. PCR product 245 bp)
Enter the concentration of your DNA solution
You will be given copies/ng and copies/µl of your stock solution. You can also use this site to calculate dilutions of stock DNA to a particular copy number

e.g. PCR product with your gene of interest

PCR product size: 245 bp

Stock concentration: 10 ng/µL

Copies: 37814756670/µL

To make the 10^{10} stock (i.e. 10000000000 copies per µL):
10000000000/37814756670 = 0.26 µL of PCR product plus 0.74 µL water

To make 100 µL of this stock:
26.44 µL PCR product
73.56 µL water

EVALUATION METHOD
The method is based on setting up standard curves in a 384-well plate, using the Power SYBR Green PCR Master Mix (Thermo Fisher), and the Viia7 qPCR machine.Reactions will be prepared using Power SYBR Green PCR Reaction Mix (ThermoFisher) and 16S rRNA primers (forward: 5' CGGCAACGAGCGCAACCC 3'; reverse 5' CCATTGTAGCACGTGTGTAGCC 3' reverse; *E. coli* positions 1114F-1221-R). Variation in biopsy size will be controlled for through inclusion of the human β-actin gene; using primers 5' CACCCACACTGTGCCCATCT 3' (forward) and 5' CAGCGGAAACCGCYCAYYGCCA 3' (reverse).
gDNA from biopsy samples will be used as template in the reactions, which will be performed on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). For both the 16S and β-actin genes, a

standard will be constructed using PCR products amplified from gDNA sourced from a mixture of duodenal biopsies. This PCR product will be cloned into the pUC19 plasmid. Standard curves will be established with serial dilutions of the plasmids and used to estimate the copy number of 16S rRNA and β-actin in biopsy samples. Finally, bacterial load will be expressed as the ratio of copies of 16S rRNA to β-actin.

SUPPORTING DOCUMENTS
gDNA extraction protocol, available from the TRI Gastro laboratory files: TRI Protocols-Illumina sequencing – DNA extraction – gDNA – Modified Morrison protocol

REFERENCES
1. Shanahan E R, Zhong L, Talley N J, Morrison M and Holtmann G 2016 Characterisation of the gastrointestinal mucosa-associated microbiota: a novel technique to prevent cross-contamination during endoscopic procedures *Alimentary pharmacology & therapeutics* **43** 1186-96
2. Cuiv P, Aguirre de Carcer D, Jones M, Klaassens E S, Worthley D L, Whitehall V L, Kang S, McSweeney C S, Leggett B A and Morrison M 2011 The effects from DNA extraction methods on the evaluation of microbial diversity associated with human colonic tissue *Microb Ecol* **61** 353-62